A Novel Family of Calmodulin-binding Transcription Activators in Multicellular Organisms

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SUMMARY

Screening of cDNA expression libraries derived from plants exposed to stress, with \[^{35}\text{S}\]-labelled recombinant calmodulin as a probe, revealed a new family of proteins containing a transcription activation domain and two types of DNA-binding domains designated CG-1 and TIG, ankyrin repeats, and a varying number of IQ calmodulin-binding motifs. Based on domain organisation and amino acid sequence comparisons, similar proteins, with the same domain organisation, were identified in the genomes of other multicellular organisms including human, Drosophila, and Caenorhabditis, whereas none were found in the complete genomes of single-cell eukaryotes and prokaryotes. This family of proteins was designated CAMTAs (for calmodulin-binding transcription activators). Arabidopsis thaliana contains 6 CAMTA genes (AtCAMTA1 – AtCAMTA6). The transcription activation domain of AtCAMTA1 was mapped by testing a series of protein fusions with the DNA-binding domain of the bacterial LexA transcription factor, and two reporter genes fused to LexA recognition sequences in yeast cells. Two human proteins designated HsCAMTA1 and HsCAMTA2 were also shown to activate transcription in yeast using the same reporter system. Subcellular fractionation of Arabidopsis tissues revealed the presence of CAMTAs predominantly in the nucleus. Calmodulin binding assays identified a region of 25 amino acids capable of binding calmodulin with high affinity (\(K_d = 1.2\) nM) in the presence of calcium. We suggest that CAMTAs comprise a conserved family of transcription factors in a wide range of multicellular eukaryotes, which possibly respond to calcium signalling by direct binding of calmodulin.
INTRODUCTION

In spite of the completion or near completion of the genome sequence of several prokaryotes and eukaryotes including human, fly, nematode and higher plants, the function of a large proportion of the genes remains unknown. Transcription factors play a crucial role in regulating every aspect of the organism’s life cycle and are fit to respond to signals originating from within and without the organism. Not surprisingly, a high proportion of eukaryote genomes encode transcription factors, estimated to be approximately 2,000 in humans (roughly 5% of the genome) where the gene expression machinery seems to be particularly complex (1). In Arabidopsis a remarkable estimate of 3,000 genes (11.8% of the genome) were suggested to be involved in different aspects of transcription regulation (2). These include many new factors whose role in gene expression are unknown.

In mammalian cells, Ca"++ and the Ca"++ receptor calmodulin are involved in regulating gene transcription. For example, expression of the c-fos gene is mediated by Ca"++ signals through two DNA regulatory elements, the CRE (cyclic-AMP-response element) and the SRE (serum-response element). Increase in nuclear Ca"++ concentration stimulates CRE-dependent gene expression, whereas elevation of cytosolic Ca"++activates transcription via SRE (3). Thus, nuclear and cytoplasmic Ca"++ control transcription by distinct mechanisms. Certain transcription factors are selectively activated in response to distinct Ca"++-signal duration and amplitude. NF-κB and JNK are activated by a large transient cytoplasmic Ca"++ rise, whereas NFAT is
activated by a low, sustained plateau (4). Therefore, different types of oscillating Ca" signals can modulate downstream transcription factor activity. Ca" can also directly bind and regulate transcription factors. For example, the DREAM protein contains four EF-hand motifs and represses transcription (5) as DREAM affinity for DNA is reduced upon binding to Ca".

Calmodulin modulates the nuclear activity of various proteins, like the mammalian family of nuclear Ca"/calmodulin-dependent protein kinases (6). When activated by Ca"/calmodulin, calmodulin-kinase II can specifically decode the frequency of Ca" spikes into distinct levels of kinase activity (7) and phosphorylates a large number of target proteins. In plants, recent advances revealed that post-translational modifications of CaM53, a novel petunia calmodulin isoform, could modify the sub-cellular localisation of the protein and direct it to the nucleus or the plasma membrane (8).

In addition, certain transcription factors of the basic helix-loop-helix family were shown to bind calmodulin, thus inhibiting their DNA-binding properties by masking the DNA-binding domain (9-11). Therefore, interaction of calmodulin with transcription factors is a mechanism by which transcriptional activity may be regulated in response to Ca" signals originating from a variety of stimuli.

We used protein-protein interaction for library screenings to identify plant calcium/calmodulin-binding proteins. One family of calmodulin-binding proteins, designated CAMTA (for calmodulin-binding transcription activator), which has been identified in the course of this study, resembles a group of putative transcription activators recently identified in the human genome (12). These were reported to contain a novel DNA-binding domain termed CG-1, a TIG-like DNA-binding domain and ankyrin repeats. However, the properties
of these proteins as transcription activators have never been tested
and the extent of their distribution in eukaryotes has not been
investigated. Here we investigated the properties of members of this
family of putative transcription factors from Arabidopsis and human,
demonstrating the ability of both to activate transcription in yeast
cells. We also expanded the bioinformatic analysis of this protein
family to reveal their occurrence and domain organisation in
multicellular organisms.

EXPERIMENTAL PROCEDURES

Expression library screenings for calmodulin-binding proteins
A Brassica napus library from leaves of drought-stressed plants (13) was
kindly provided by J. Giraudat (Institut des Sciences Végétales, CNRS, Gif-
sur-Yvette, France). Expression library screening was performed with [35S]-
labelled recombinant calmodulin from petunia (CaM81; GenBank accession
number: S70768) as a probe (14).

Electronic-databases information and in silico analysis
Several databases were used to retrieve and compare sequences: the GenBank
database (NCBI server; www.ncbi.nlm.nih.gov), the Arabidopsis Genome
Initiative database (TAIR server; www.arabidopsis.org) and the Berkeley fly
database (BDGP server; www.fruitfly.org). Domains identification and
comparisons were done with the InterPro database (www.ebi.ac.uk/interpro;
15). Multiple sequences alignment were assembled with the ClustalX program
(16). Intron/exon junctions were predicted with the NetGene2 program in A.
thaliana (17) and NNSPLICE0.9 program in D. melanogaster (18).
ESTs corresponding to complete cDNA clones were obtained from the
Arabidopsis Biological Resource Center (ABRC at TAIR server) for AtCAMTA1
(clone# H9D3T7) and AtCAMTA5 (clone# 4G3T7P), the Kazusa DNA Research
Institute for AtCAMTA2 (clone# AV528637), HsCAMTA1 (clone# KIAA0833) and HsCAMTA2 (clone# KIAA0909).

CAMTAs expression in Sf9 insect cells

The Sf9 cell line of Spodoptera frugiperda (19) was maintained as a monolayer culture at 27°C in Grace medium (19) supplemented with 10% foetal calf serum (Biological Industries, Israel). Cells were recultured every 4 days to maintain a density ranging from ~5 x 10^5 to 2.5 x 10^6 cells per ml.

The full-length AtCAMTA1 was excised from the EST clone H9D3T7 (ABRC) with SalI and XbaI, and inserted in a pFastBac1 donor plasmid (Invitrogen; 20) downstream of the promoter of the viral polyhedrin gene. Similarly, the BnCAMTA cDNA sequence, coding for a partial BnCAMTA protein (Ile^1-Lys^688), was amplified by PCR with a Pfu DNA polymerase (Promega) and cloned into the EcoRI and SalI sites of pFastBac1. Plasmids were then transformed into DH10BAC E. coli cells (Invitrogen) for transposition into the Bacmid. The screening and isolation of recombinant Bacmid DNA were done according to the manufacturer's instructions. Sf9 cells were transfected with recombinant Bacmid DNA using CellFECTIN (Invitrogen). Recombinant baculoviruses were harvested 72 h after the start of transfection. Sf9 cells were layered at a density of 5 x 10^6 cells per 90-mm plate and infected with high-titer recombinant baculoviruses. After 3 days of incubation at 27°C, cells were harvested by centrifugation at 500 g for 10 min, washed once with Phosphate-Buffered Saline (PBS) centrifuged for 10 min at 500 g and resuspended (1 ml/plate) in extraction buffer containing 100 mM Tris-HCl pH 7.5, 10% glycerol, 1 mM EDTA and 1 mM PMSF. Cells were broken in liquid nitrogen, or by addition of 0.5% NP40. Cell lysates were centrifuged at 4°C, 14,000 g for 15 min and the supernatant was collected. Protein concentrations were determined with a Bradford reagent (BioRad).
Preparation and purification of polyclonal antibodies

To prepare polyclonal antibodies against the N-terminal part of AtCAMTA1, the CG-1 domain of AtCAMTA1 (Val$^2$-Lys$^{148}$) was fused in frame to the GST coding sequence in the BamHI and EcoRI sites of the pGEX-3X vector (Pharmacia). To prepare antibodies against the ANK-repeat region, the corresponding sequence from BnCAMTA (Gln$^{588}$-Gly$^{687}$) was amplified as described above, and subcloned in the NdeI and SalI sites of a pET12c vector (Novagen, Inc.). These constructs were introduced in E. coli strain BL21(DE3)pLysS to produce the recombinant proteins as described (14).

Inclusion bodies from the insoluble fraction of the bacterial cells, containing most of the recombinant proteins, were purified, solubilized in sample buffer (21) and proteins were separated by SDS-PAGE. An acrylamide band containing the recombinant protein (either the ANK repeat region or the N-terminal part of AtCAMTA1 fused to GST) was excised from the gel, crushed, and mixed (1:1) with complete Freund’s adjuvant (Sigma). Three ml of the mixture containing 100 µg of recombinant protein were injected into two rabbits. Each rabbit was given four booster injections about two weeks apart. The rabbits were bled about 10 days after each injection. The serum containing anti-CG-1 antibodies was depleted from the antibodies against GST by passing it on a GST column (Pierce).

Double stranded DNA and heparin affinity chromatography

Double stranded calf-thymus DNA-cellulose (Sigma) and heparin-sepharose CL-6B (Pharmacia) were pre-equilibrated with the following buffer: 25 mM HEPES-KOH pH 7.5, 40 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT and 1 mM PMSF. Soluble fraction proteins obtained from Sf9 insect cells were dialysed against this buffer at 4°C using VSWP-25 filters (Millipore) and loaded on either column. After washing with 10-column volumes of buffer, proteins were eluted with the same buffer containing KCl as indicated.
Subcellular fractioning and nuclei isolation

Aerial parts from 4 weeks old Arabidopsis thaliana Columbia ecotype plants grown in vitro under the following conditions: photoperiod 16 h day (100-150 μE/m²/s) / 8 h night; temperature 20°C day / 15°C night; humidity 70%, were frozen and ground in liquid nitrogen to a fine powder with a mortar and pestle. All subsequent steps were carried out at 4°C. Part of this powder was homogenized with plant extraction buffer (100 mM Tris-HCl pH 7.5, 10 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin and 2 μg/ml aprotinin). This extract was filtered through two layers of Miracloth and centrifuged at 10,000 g for 15 min. The insoluble and soluble fractions were collected. The rest of the powder was mixed with nuclei isolation buffer (22): 1 M hexylene glycol, 10 mM PIPES-KOH pH 7, 10 mM MgCl₂, 0.2% Triton X-100, 5 mM β-mercaptoethanol and 1 mM PMSF, and filtered through two layers of Miracloth and one 100 μm nylon mesh. The extract was centrifuged at 2,000 g for 10 min. The pellet was resuspended in nuclei wash buffer (0.5 M hexylene glycol, 10 mM PIPES-KOH pH 7, 10 mM MgCl₂, 0.2% Triton X-100, 5 mM β-mercaptoethanol and 1 mM PMSF) and centrifuged again at 3,000 g for 10 min. The pellet was then washed two more times, and finally resuspended in 5 ml of nuclei wash buffer. Nuclei were further purified in a discontinuous Percoll gradient (23). The gradient contained 5 ml layers of 40%, 60% and 80% (v/v) Percoll on a 5 ml layer of 2 M sucrose cushion. The Percoll contained 0.5 M hexylene glycol, 10 mM PIPES-KOH pH 7, 10 mM MgCl₂ and 0.2% Triton X-100. The gradient was centrifuged at 4,000 g in a Sorvall HB4 swinging bucket rotor for 30 min. Most of the nuclei banded in the 80% Percoll, just above the sucrose cushion. They were removed with a Pasteur pipette, washed twice with 15 ml of nuclei wash buffer to remove Percoll, and centrifuged again at 3,000 g for 10 min. This nuclei-enriched fraction was resuspended in protein sample buffer (21) to be loaded onto SDS-PAGE gels for western blot analysis.
Expression of CAMTAs in yeast

The complete AtCAMTA1, HsCAMTA1 and HsCAMTA2 cDNA, plus 12 different AtCAMTA1 regions (corresponding to residues 1-147, 1-230, 1-680, 1-820, 148-1008, 231-1008, 681-1008, 821-1008, 231-680, 231-397, 398-566, and 567-680 for constructs 2-13, respectively), were fused in frame with the coding sequence of the DNA-binding domain of LexA in the pEG202 vector (OriGene) carrying the HIS3 selectable marker. These plasmids were then introduced in EGY48 yeast strain (MATa trpl ura3 his3 LEU2::plexAop6-LEU2; 24) by lithium acetate transformation, together with the pSH18.34 vector (OriGene) carrying the URA3 selectable marker and the LacZ reporter gene fused to eight LexA operators. Yeast transformants were selected on plates containing complete minimal (CM) dropout medium without uracil (Ura) and histidine (His), but with glucose (Glc) as a unique carbon source. Individual yeast colonies were then transferred to liquid medium (CM dropout +Glc, -His, -Ura) and grown to late log phase. These single-colony derived cultures were tested for the production of β-Gal with the chromogenic substrate o-nitrophenyl-β-D-galactoside (ONPG) or for their ability to grow on plates containing CM dropout medium without uracil, histidine and leucine. To study the interaction between calmodulin and the calmodulin-binding domain of AtCAMTA1 in vivo, a petunia calmodulin (CaM81, GenBank accession: S70768) was fused in frame to the B42 transcription activator domain in the pJG4-5 vector (Origene) that carries the TRP1 selectable marker. This construct was introduced into a EGY48 yeast strain, and transformants were selected as previously described, except that tryptophan (Trp) was omitted from the medium.

Quantitative assay of β-galactosidase activity in liquid cultures of yeast

3 ml of selective medium were inoculated with 15 to 30 µl of saturated culture grown to late-log phase. Yeast cells were grown overnight at 28°C, under agitation. Cells were then centrifuged 5 min at 2,500 rpm and resuspended in 3 ml of Z buffer (Na2HPO4·7H2O 60 mM; NaH2PO4·H2O 40 mM; KCl
10 mM; MgSO₄·7H₂O 1 mM; β-mercaptoethanol 50 mM final; adjusted to pH 7.0), then placed on ice. OD₆₀₀ was determined for each sample and the following two reaction tubes were set up (1 ml each), by mixing: (a) 100 µl cells with 900 µl Z buffer and (b) 50 µl cells with 950 µl Z buffer. To break the cells, one drop of 0.1% SDS and 2 drops chloroform were added to each sample which were then vortexed 10 to 15 sec and incubated 15 min in a 30°C water bath. 0.2 ml of 4 mg/ml ONPG were added and samples were vortexed 5 sec and place in a 30°C water bath. Timing was beginning. When a medium-yellow colour had developed, the reaction was stopped by adding 0.5 ml of 1 M Na₂CO₃ and the time was noted. Cells were centrifuged 5 min at 2,500 rpm and OD₄₂₀ plus OD₅₅₀ of the supernatant were determined. To calculate β-Gal units the following equation was applied:

$$U = \frac{1000 \times (OD_{420} - (1.75 \times OD_{550}))}{(t \times v \times OD_{600})}$$

where $t$ is the time of reaction (min), $v$ is the volume of culture used in the assay (ml), $OD_{600}$ represents the cell density at the start of the assay; $OD_{420}$ is a combination of absorbency by o-nitrophenol and light scattering by cell debris and $OD_{550}$ is the light scattering by cell debris. All the measurements were done in triplicate and the β-Gal units counted results from an average number. Controls used are encoded by plasmids pSH17.4 (positive control) and pRFHM1 (negative control) commercialised by OriGene.

To confirm that proteins were expressed at the same level in yeast, cells were disrupted with glass beads (Sigma), total proteins were extracted and separated on SDS-PAGE. LexA DNA-binding domain fusions were detected by Western blots using a monoclonal antibody raised against LexA (Clontech).

**Mapping AtCAMTA1 calmodulin-binding domain**

DNA fragments derived from the AtCAMTA1 cDNA from residues 682-1007, 682-897, 682-869, 863-1007, 913-1007, 823-897, 823-869, 863-897 (constructs 1-8, respectively) were fused in frame to the coding sequence of GST in a pGEX-3X vector (Pharmacia). Fusion proteins were expressed in E. coli XL1-Blue strain. After induction of the expression, total proteins were extracted, separated by SDS-PAGE and electrotransferred to nitrocellulose
membranes. [\textsuperscript{35}S]-calmodulin was prepared and used as described (25). Following autoradiography of blots, immunodetection of proteins by anti-GST antibodies on the same blots was performed as described (25).

**Analysis of Calmodulin/Peptide interaction**

For nondenaturing PAGE, samples containing 120 pmoles of bovine brain calmodulin (Sigma) and different quantities of HPLC-purified synthetic peptides in 100 mM Tris-HCl (pH 7.2), and 0.1 mM CaCl\textsubscript{2}, making a total volume of 30 \( \mu l \), were incubated for 1 h at room temperature. Samples were analyzed by nondenaturing gel electrophoresis as previously described (25). For fluorescence measurements of peptide interactions with dansyl-calmodulin, dansylated bovine calmodulin (400 nM; Sigma) was incubated with different concentrations of synthetic peptide in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.5 mM CaCl\textsubscript{2}. After each addition of peptide, the calmodulin/peptide solution was mixed and incubated for 5 min at 23°C. Emission fluorescence at 480 nm was then measured using a SLM AMINCO 8000 fluorimeter (SLM Instruments); excitation wavelength was at 340 nm. Each measurement was the average of 3 readings. The apparent dissociation constant (\( K_d \)) was determined as described (26).

**RESULTS**

Molecular cloning of a novel gene family containing transcription factor motifs and calmodulin-binding domains

Plant adaptation to environmental stresses is mediated by Ca\textsuperscript{2+} signaling (27) and Ca\textsuperscript{2+} responsive proteins, among them calmodulin and calmodulin-related proteins (28). To isolate cDNAs encoding calmodulin-binding proteins with possible role in plant response to
abiotic stress, we used $^{35}$S-recombinant calmodulin as a probe to screen cDNA expression libraries derived from plants exposed to various stress conditions. In particular, screening of a cDNA library from Brassica napus leaves (Experimental procedures) resulted in the isolation of one clone that contained putative domains with DNA-binding properties, and a domain that proved to function as a transcription activator, as will be shown. This clone was designated BnCAMTA (for Brassica napus calmodulin-binding transcription activator).

Based on domain organisation and amino acid sequence of BnCAMTA, we identified members of the CAMTA family in various eukaryotes (Fig. 1A): in nematode, fly, human and in other plants, including Arabidopsis. The latter’s genome has six highly similar CAMTA genes designated AtCAMTA1 – AtCAMTA6. In human, two homologous cDNA clones have been identified, designated HsCAMTA1 (GenBank accession: XM_042323) and HsCAMTA2 (GenBank accession: XM_053753). These were isolated from a population of size-fractionated human brain mRNAs (29). Gene expression profiles revealed that they are expressed in all human organs tested but highly expressed in the brain (29). Interestingly, only one CAMTA gene was identified in the complete genomes of both C. elegans and D. melanogaster. In contrast, no members of the CAMTA family have been found in the complete genomes of S. cerevisiae and prokaryotes. Importantly, to date, no function has been attributed to any of the CAMTA genes identified in any organism, although based on domain organisation their relationship to transcription factors has been suggested (12).

Alignment of the amino acid sequences of CAMTAs using the ClustalX program (16), and comparisons with protein domain databases (InterPro database; 15) revealed different types of conserved
regions in all CAMTAs (Fig. 1A). The conserved domains include: (a) a bipartite nuclear localisation signal (NLS) in the N-terminal part of all CAMTA proteins; (b) a TIG domain (transcription factor immunoglobulin) reported to be involved in non-specific DNA contacts in various transcription factors, like those of the Rel/NF-κB family or NFAT (30); (c) ankyrin (ANK) repeats, known to be involved in protein-protein interactions (31,32), and present in a large number of functionally diverse proteins; and (d) IQ motifs, known as calmodulin-binding sites (33,34), localised in the C-terminal part of CAMTAs. These vary in number from zero (CeCAMTA) to five (AtCAMTA6). Although spacing is highly variable, overall domain organisation is conserved in all proteins.

We also identified a highly conserved uncharacterised domain of about 130 amino-acid residues designated CG-1, containing the predicted bipartite NLS (Fig. 1B). The CG-1 domain is named after a partial cDNA clone isolated from parsley (Petroselinum crispum L.) encoding a sequence-specific DNA-binding protein (35). Bioinformatic studies have recently revealed CG-1 domains in the human proteins HsCAMTA1 and HsCAMTA2 (12). To test the possible occurrence of similar domains in other proteins, we compared the CG-1 amino acid sequence against the nucleotide databases translated in all six reading frames (NCBI tblastn). When comparisons were done on the completely sequenced 66 microbial genomes (eg. Escherichia coli; Yersinia pestis; Pseudomonas aeruginosa), the lowest blast Expect value (E) observed was not significant (eq. 0.0059) indicating that prokaryotes do not contain CG-1 domains. In contrast, CG-1 domains were found in the Expressed Sequence Tag (EST) database from various multicellular organisms including Mus musculus, Medicago truncatula, Oryza sativa, Sorghum propinquum, Solanum tuberosum, Gossypium
arboreum, Hordeum vulgare (Fig. 1B). A phylogenetic tree was drawn by the neighbour-joining method (36) comparing all the CG-1 sequences identified so far (Fig. 1C), suggesting that this domain was present in a common ancestor of multicellular organisms, but absent in prokaryotes and unicellular eukaryotes (eg. yeast).

Transcription activators typically share the following properties: (1) they interact with DNA (or with a DNA-binding protein); (2) they are targeted to the nucleus, and (3) they activate transcription. Here we demonstrate that CAMTA proteins possess all these functional properties.

**CAMTAs bind DNA and are predominantly localized in the nucleus**

TIG domains appear in a variety of functionally distinct proteins. In transcription factors these domains were shown to interact with DNA and to be involved in protein dimerization (37,38). However, in these proteins, the sequence-binding specificity is typically provided by a different type of adjacent DNA-binding domain. In contrast, the first CG-1 domain to be identified was shown to bind DNA in a sequence-specific manner. It preferentially bound to DNA containing the sequence motif CGCG (35).

To confirm that other plant CAMTAs are DNA-binding proteins, a recombinant form of BnCAMTA (Ile\textsuperscript{1}-Lys\textsuperscript{688}) lacking the C-terminal part of the protein with the IQ motifs, but containing the CG-1 and TIG putative DNA-binding domains and the ANK repeats was expressed in Sf9 insect cells (Experimental procedures) and tested for its ability to bind to DNA and heparin. Soluble proteins extracted from
Sf9 insect cells were loaded on a column of calf-thymus dsDNA-cellulose. The column was washed with excess of loading buffer, and proteins were eluted by stepwise increasing of the KCl concentration (Fig. 2A). Western blot analysis of the collected fractions using a polyclonal antibody raised against the ANK repeats region, showed that most of the recombinant protein fraction was retained on the column with KCl up to 100 mM, and completely eluted at 300 mM KCl (Fig. 2A). In a similar approach, we verified that BnCAMTA1 binds heparin, a glycosaminoglycan known to interact with DNA-binding proteins (39). In this case, the protein was retained with KCl up to 300 mM and was completely eluted at a concentration of 500 mM KCl (Fig. 2B). No proteins were immunodetected when extracts were taken from Sf9 insect cells transformed with the vector alone (not shown). These results indicate that the BnCAMTA protein interacts with dsDNA and heparin, and that the region of the protein responsible for this interaction is within the first 688 amino acids containing the CG-1 and TIG domains. These results are consistent with the former report on the parsley CG-1 protein, but further analysis is required to determine CAMTAs sequence-binding specificity.

To study the subcellular localisation of CAMTA proteins, fractionation of aerial tissues of four-week-old Arabidopsis plants was performed. Insoluble and soluble fractions were prepared, and nuclei were isolated on a discontinuous Percoll gradient (Experimental procedures; 23). The fractionated extracts were analysed by western blot using antibodies against AtCAMTA1, and control antibodies against various proteins of known subcellular localisation. Polyclonal antibodies raised against the CG-1 domain of AtCAMTA1 reacted with a single protein band in the nuclear enriched extract, of electrophoretic mobility similar to that of the
full-length recombinant protein expressed in Sf9 insect cells (Fig. 3). An antibody against MSI1, a known plant nuclear protein (40), also reacted with a single band corresponding to the expected MSI1 gel mobility in the same nuclear-enriched fraction. The mitochondrial protein prohibitin (41) was detected in the insoluble fraction, whereas GAD2 (42), a cytosolic protein, was mostly detected in the soluble extra-nuclear fraction. These results suggest that AtCAMTA1 is present predominantly in the nuclei of Arabidopsis cells.

**CAMTAs activate transcription in yeast**

We further investigated the possible presence of a transcription activation domain in CAMTAs. First, we attempted to map the region within AtCAMTA1 that might be involved in transcription activation, in yeast cells. The complete AtCAMTA1 cDNA, plus 12 different AtCAMTA1 regions were fused, separately, in frame with the coding sequence of the DNA-binding domain of LexA, a bacterial transcription factor (Fig. 4A, constructs 1 to 13). The chimeric plasmids were introduced into the EGY48 yeast strain together with a vector carrying the LacZ reporter gene fused to eight LexA operators. Yeast transformants were transferred to liquid medium and grown to late-log phase. These single-colony-derived cultures were tested for the production of β-Gal with the chromogenic substrate o-nitrophenyl-β-D-galactoside (ONPG). The complete AtCAMTA1 protein (Fig. 4A, construct 1) activated transcription to levels similar to those of the positive control (a fusion protein between LexA DNA-binding domain and the yeast GAL4
activator protein). Analysis of the different recombinant fusion proteins (Fig. 4A, constructs 2 to 13), revealed a region between Asp^{233-}Gly^{398} (Fig. 4A, construct 11) that can activate transcription in the absence of any other AtCAMTA1 sequences. No other region of AtCAMTA1 on its own was capable of activating transcription of LacZ, including the CG-1 domain (Fig. 4A, construct 2), the TIG domain (Fig. 4A, construct 12), the ANK repeats (Fig. 4A, construct 13) and the calmodulin-binding domain with the C-terminal part of the protein (Fig. 4A, construct 9). All of these constructs resulted in β-Gal levels similar to those produced by the negative control. To exclude the possibility that the activation results were biased by different levels of recombinant protein expression in yeast, we tested the levels, in yeast cells, of recombinant proteins encoded by constructs 2, 3, 8, 10, 12 and 13 on Western blots using a monoclonal antibody raised against LexA. All proteins were expressed at similar levels (not shown), thus validating the occurrence of a transcription activation domain in AtCAMTA1.

As AtCAMTA1 was clearly shown to be able to activate transcription, we hypothesised that other CAMTA proteins could share the same function. In a similar approach, the cDNAs encoding two complete human CAMTAs (HsCAMTA1; clone KIAA0833 and HsCAMTA2; clone KIAA0909) were fused in frame to the LexA DNA-binding domain, and their ability to activate transcription in yeast was assessed. As shown in figure 4B, both HsCAMTA1 and HsCAMTA2 could activate the transcription of LacZ leading to the production of β-Gal to levels similar to those produced by the positive control in the case of HsCAMTA1 and lower in HsCAMTA2. The negative controls were unable to produce detectable levels of β-Gal activity in this experiment.
In a complementary experiment, we used the yeast strain EGY48 carrying a modified LEU2 gene (required in the biosynthetic pathway for leucine). In EGY48, the original LEU2 promoter was replaced by six LexA operators combined with a minimal promoter. Subsequently, a transcription factor fused in frame to the DNA-binding domain of LexA can bind to the LexA operators and activate the transcription of LEU2 allowing the strain to grow in the absence of leucine. When fused in frame to the DNA-binding domain of LexA, HsCAMTA1, HsCAMTA2 and AtCAMTA1 activated the transcription of LEU2 allowing the strain to grow in the absence of leucine (Fig. 4C, -Leu), while the vector and the negative control could not support growth in the absence of leucine. Yeast cells transformed with any of these constructs grew equally well in the presence of leucine (Fig. 4C, + Leu).

Altogether, these experiments suggest that CAMTAs, including the two human clones identified so far, activate transcription in yeast. Therefore, CAMTAs are nuclear DNA-binding proteins that contain a transcription activator domain, which we mapped in AtCAMTA1. We note that even though CAMTAs are not present in yeast, CAMTAs from plants and human interact with the yeast transcription machinery to promote transcription.

**CAMTA proteins bind calmodulin**

IQ motifs were detected in all CAMTAs except in that from C. elegans (Fig. 1A). These motifs often appear adjacent to other calmodulin-binding domains, and mediate complex regulatory properties in the presence and/or absence of Ca\(^{2+}\) (34). This raises the possibility that most CAMTAs are regulated through direct
binding of calmodulin. CAMTA proteins were previously shown to bind $[^{35}S]$-recombinant calmodulin in a calcium-dependent manner as demonstrated with partial NtCAMTA (ie. NtER1, GenBank accession: AAG39222; 43) and AtCAMTA (ie. EICBP, GenBank accession: AAD23613; 44) clones expressed in E. coli. In a preliminary analysis, a full-length AtCAMTA1 recombinant protein was expressed in Sf9 insect cells and shown to bind calmodulin (data not shown). We then mapped the calmodulin-binding domain of AtCAMTA1. The C-terminal part of AtCAMTA1 containing the two IQ motifs and smaller fragments of this region were fused in frame with the coding sequence of glutathione S-transferase (GST). The ability of the fusion proteins to bind $[^{35}S]$-recombinant calmodulin on a blot was tested. This revealed a minimal region of 35 amino acids (Gln$^863$-Ala$^897$) that was sufficient for calmodulin binding (Fig. 5A). This binding was Ca$^{2+}$-dependent as no binding was detected in the presence of 2 mM EGTA. Two GST fusion proteins containing the IQ motifs did not bind to $[^{35}S]$-recombinant calmodulin in this blot assay (Fig. 5A, constructs 3 and 7).

Calmodulin is known to bind to 12-30 amino acid-long peptides that tend to form amphipathic a-helices with one face of the helix positively charged (45,46). By delimiting the calmodulin-binding domain to only 35 amino acids, we were able to analyze it for potential calmodulin-binding sequences. This analysis revealed a 18-amino acid region with typical calmodulin-binding characteristics between amino acids Gly$^{872}$-Arg$^{889}$. When drawn in the form of a $\alpha$-helical wheel, it exhibits an amphipathic structure with a positively charged binding face and an opposite hydrophobic face (Fig. 5B). We then prepared a synthetic peptide of 25 amino acids corresponding to AtCAMTA1 amino acids Gly$^{872}$-Val$^{896}$ and tested its
ability to bind bovine calmodulin in a gel-shift assay on non-denaturing PAGE. In the absence of this peptide (Fig. 5C, lane 0), calmodulin appeared as a single band on the Coomassie-stained gel. When the peptide was added in the presence of 0.1 mM Ca"++, a second slower mobility band appeared, representing a peptide/Ca"+/calmodulin complex (Fig. 5C). At a peptide-to-calmodulin molar ratio of 2, only trace amounts of free calmodulin was detected. Importantly, no mobility shift of calmodulin was apparent in the absence of Ca"+ (Fig. 5C). Determination of the affinity of calmodulin-target interactions may be necessary for establishing the physiological relevance of these interactions. We investigated the affinity between the AtCAMTA1 calmodulin-binding peptide and Ca"+/calmodulin using fluorescence measurements of dansyl-calmodulin (47) with or without the AtCAMTA1 calmodulin-binding peptide, as we previously reported for another calmodulin-binding protein (26). Without the peptide, the photoexcited emission spectrum of dansyl-calmodulin (300 nM) peaked at 500 nm (Fig. 6A). At a peptide concentration of 450 nM, which should have converted all the calmodulin to the bound form, the fluorescence intensity of dansyl-calmodulin increased by 1.77 times, and the emission peak shifted to 482 nm (Fig. 6A). These observations suggest that the dansyl moiety occupied a more hydrophobic environment upon binding of the peptide to dansyl-calmodulin (48). With the dansyl-calmodulin concentration at 300 nM, the fraction of bound calmodulin increased linearly with total peptide concentration until the signal saturated at approximately 300 nM peptide (Fig. 6B). These data are consistent with the non-denaturing gel results (Fig. 5C), which suggest a one-to-one binding ratio between calmodulin and the AtCAMTA1 peptide. We calculated a $K_d$ of 1.23 ± 1.04 nM by fitting these data using a method described in
Experimental procedures. Altogether, these results suggest that AtCAMTA1 could bind Ca\(^{++}\)/calmodulin with an affinity which is physiologically relevant, and comparable to that for other calmodulin-binding proteins (28, 49). Moreover, conservation of the IQ motifs in the C-terminal part of almost all CAMTAs implies that regulation by calmodulin is a common feature of this new transcription factor family. The fact that in our assay AtCAMTA1 bound calmodulin in a region that does not include IQ motifs, raises the possibility that there is more than one calmodulin-binding site in CAMTAs, but some are functional only under specific physiological conditions.

The apparent lack of CAMTA-like genes in the yeast genome could be related to some of the unique properties of the calcium/calmodulin messenger system in yeast. For example, yeast calmodulin has only three EF-hand calcium-binding sites, and it was shown to carry out functions in the absence of calcium (50). We decided to make further use of the yeast system to test the possible binding of a plant calmodulin to the plant AtCAMTA1 protein in vivo, in yeast cell nuclei. To this end, the full-length cDNA of a petunia calmodulin (CaM81) was fused in frame to the B42 transcription activator domain (51), which also contains a nuclear localization signal. As depicted in figure 7A, when this chimeric protein is expressed in yeast cells together with a fusion protein containing a calmodulin-binding domain and a LexA DNA-binding domain, the latter is expected to bind to the LexA operators and the former to the plant calmodulin, thus allowing the B42 domain to activate the LacZ gene. Using this experimental system, we tested several LexA-AtCAMTA1 fusions for their ability to bind calmodulin in vivo by monitoring β-Gal activity (Fig. 7B). Importantly, activation of the
reporter gene occurred only when the LexA-AtCAMTA1 chimeric protein carried the calmodulin-binding domain (Fig. 7B). When the full-length AtCAMTA1 protein was expressed, the β-Gal production observed was as high as for the activation experiments described previously (Fig. 4A-B). Because the expression of the CaM81-B42 chimeric protein was under the control of the GAL1 galactose-inducible promoter, we verified in control experiments that the results observed for the transcription domain mapping (cf. Fig. 4A) were the same when yeast were grown on galactose and glucose. Namely, only the full-length AtCAMTA1 can activate the reporter gene in the absence of the CaM81 fusion protein, whereas the truncated AtCAMTA1 proteins with the calmodulin-binding domain activate the reporter gene only in the presence of the CaM81-B42 fusion protein. Collectively, these results show that a plant calmodulin can interact with the calmodulin-binding domain of AtCAMTA1 in vivo in the yeast cell nucleus.

DISCUSSION

In the present study we describe a group of genes encoding nuclear calmodulin-binding proteins that can interact with DNA in vitro, and activate transcription in yeast. We show that CAMTAs are largely distributed among higher organisms as members of this protein family were identified in plants, Drosophila, Caenorhabditis and human. The protein’s primary structure contains five conserved regions arranged in the same co-linear order, including a new characterised domain designated CG-1, a TIG domain, two ANK repeats and a variable number of IQ motifs. Because we could not detect CG-1
domains in prokaryotes and yeast, and because all CAMTA proteins identified so far contain a highly conserved CG-1 domain (Fig. 1B), it is likely that CAMTAs are present exclusively in multicellular organisms. This may suggest their involvement in cell-cell communication and/or developmental processes that are unique to multicellular organisms.

We further demonstrated that a member of the CAMTA family, BnCAMTA, could bind to dsDNA (Fig. 2A) and heparin (Fig. 2B). These data are consistent with the presence of one or more DNA-binding domains in BnCAMTA. The parsley clone containing the first CG-1 domain to be identified (Fig. 1B, PcCG-1, GenBank accession: S48041) was shown to bind preferentially to DNA containing a CGCG motif (35). Because the parsley clone was incomplete (about 130 amino acids long) and contained essentially the CG-1 domain, CG-1 domains are most likely sequence-specific DNA-binding domains. Interestingly, when organisms for which complete genomes are available (Drosophila, Arabidopsis and Caenorhabditis) were screened for the presence of CG-1 domains, these were found only in CAMTA proteins including one in Drosophila, one in Caenorhabditis, and six in Arabidopsis. Only two were identified in humans. Therefore, CG-1 domains represent a new category of DNA-binding domain associated with CAMTA proteins. However, we cannot exclude the possibility that CG-1 domains identified in partial cDNA sequences (Fig. 1B) belong to proteins different from CAMTAs. The DNA binding specificity of different CG-1 domains remains to be elucidated.

A second type of DNA-binding domain, the TIG domain, was identified in all CAMTAs (Fig. 1A). TIG domains are characterised in a large number of proteins, including transcription factors (30) and cell surface receptors (52). In transcription factors such as NF-κB
(37,38) or Olf-1 (53), TIG domains are involved in DNA contact but also in dimerization, whereas a different domain is typically involved in providing the DNA-binding sequence specificity. CAMTAs might interact with DNA in a similar way. Namely, the TIG domain as a non-specific DNA-binding domain while the CG-1 domain provides sequence specificity. A database comparison (tblastn), using the TIG domains present in plant CAMTAs as queries, indicates that CAMTAs are the only Arabidopsis proteins to contain TIG domains, whereas functionally diverse proteins contain TIG domains in the genomes of Drosophila and Caenorhabditis (N. Bouché, unpublished observations).

AtCAMTAs are nuclear proteins as confirmed by cellular fractionation and immunodetection (Fig. 3). Proteins targeted to the nucleus usually contain a nuclear localisation signal (NLS) composed of basic amino acids arginine (R) and lysine (K), organised in groups (54). A putative NLS was detected in the CG-1 domain of all CAMTAs identified so far (Fig. 1A,B). Therefore, this region might constitute a signal that directs CAMTAs to the nucleoplasm.

We defined a region of 166 amino acids in AtCAMTA1 that is sufficient to activate transcription of two reporter genes (LacZ and LEU2) in yeast cells (Fig. 4A). We further demonstrated that full-length human CAMTAs could activate transcription in the same manner (Fig. 4B,C). Therefore, CAMTAs constitute a new family of transcription activators containing two conserved DNA-binding domains (CG-1 and TIG), and a transcription activator domain that do not overlap, as none of the putative DNA-binding domains of AtCAMTA1 was capable of activating transcription in yeast (Fig. 4A). However, we cannot rule out the possible interactions of the transcription activation domain with other domains. The transcription activation domain identified in AtCAMTA1 was compared to the same protein
region in all other CAMTA members, but no consensus sequence could be identified. Moreover, when this region was compared to the GenBank databases, no significant homology to known transcription activation domains could be detected. Families of transcription factors often share conserved DNA-binding domains, but have different activation domains as in the case of the Maize Dof1 and Dof2 proteins (55,56). Dof (for DNA binding with one finger) is a family of transcription factors present in higher plants. They share a highly conserved DNA-binding domain (for example, 91% identity between Dof1 and Dof2) but diverse activation domains. Further analysis of the transcription activation domains of CAMTAs is necessary to elucidate their mode of action.

Analysis of the primary structure of CAMTAs revealed the presence of two ANK (ankyrin) repeats (31,32) which are present as tandemly-repeated modules of about 33 amino acids in a large number of eukaryote proteins and viruses. ANK repeats, like other conserved domains with a specific secondary structure (eg. SH2- and SH3-domains) evolved as a universal module mediating protein-protein interactions. The primary structure of ANK motifs found in CAMTAs is similar to a consensus sequence (31) corresponding to a large number of ANK containing proteins (data not shown). CAMTAs might therefore interact with other proteins or form heteromeric (or homomeric) complexes by means of their ANK domains.

Interaction between AtCAMTA1 and calmodulin was demonstrated by locating the corresponding calmodulin-binding domain to the C-terminal part of the protein (Fig. 5) and defining a peptide capable of binding Ca\(^{2+}\)/calmodulin with high affinity, and an apparent \(K_d\) of 1.23 ± 1.04 nM (Fig. 6). This interaction is in the physiological range known for other calmodulin-binding proteins (28,49) and is
consistent with the data obtained for NtCAMTA protein (43; ie. NtER1, GenBank accession: AAG39222). The C-terminal part of the protein also contains two IQ motifs that consist of low complexity regions with the repetitive motif IQXXXRGXXXR. Peptides containing IQ motifs can bind calmodulin in the absence or presence of calcium (33,34), with some exceptions. None of the IQ motifs detected in AtCAMTA1 interacted with Ca\textsuperscript{2+}/calmodulin on blots, as two GST fusion proteins containing these sequences did not bind calmodulin (Fig. 5A, constructs 3 and 7). As IQ motifs were shown to bind calmodulin-like proteins and not exclusively calmodulin (33,57), we cannot exclude the possibility that CAMTAs are regulated by both calmodulin and other EF-hand proteins as demonstrated for other IQ-containing proteins (eg. Caldesmon; 58). Therefore, different types of EF-hand proteins might interact and compete for the same region in CAMTAs. The presence of more than one type of calmodulin-binding domain in the same region of AtCAMTA1, suggests a complex nature of regulation by calcium signaling.

Certain transcription factors have already been described as calmodulin-binding proteins. In plants, the transcription factor TGA3, which belongs to the basic leucine zipper family, was shown to bind calmodulin in a Ca\textsuperscript{2+}-dependent manner (59). However, its calmodulin-binding domain has not been identified, and the affinity of the interaction remains to be determined. Other transcription factors were shown to bind calmodulin. Corneliussen et al. (11) reported that calmodulin binds certain transcription factors of the basic-helix-loop-helix (bHLH) family, thus preventing DNA binding (9-11). However, this interaction is rather unusual in being polar in nature (9) and occurs within the DNA-binding domain itself, which consequently becomes inaccessible to DNA. In contrast, CAMTAs bind
calmodulin at a distinct site, which is separate from the DNA-binding domain. Moreover, the amino acid sequence of the CAMTA-derived peptide that binds calmodulin (Fig. 5) suggests that the interaction with calmodulin is of a hydrophobic-polar nature, which is typical of most known calmodulin–protein interactions.

We currently do not know what the role of calmodulin binding to CAMTAs is. Binding may occur in the cytosol and/or in the nucleus and it could play roles such as control of transport of CAMTA to the nucleus, control of DNA binding, or direct control of transcription activation. Such regulatory functions may involve association of CAMTAs with other proteins (e.g., through the ankyrin-repeat domain) and post-translational modifications. Nevertheless, our experiments show that plant calmodulin binds plant CAMTA in the yeast cell nucleus and that Arabidopsis CAMTAs are detected exclusively in the plant cell nucleus (Fig. 3). These data suggest that calmodulin regulates CAMTAs within the cell nucleus of plants and animal cells. However, this does not exclude a possible role for calmodulin regulating CAMTA functions in the cytosol. The mechanism involved in regulation by calcium/calmodulin should be addressed in detail once the downstream gene targets of CAMTAs have been identified.

In summary, we report the characterisation of a new family of transcription factors in multicellular organisms. These proteins contain a distinct calmodulin-binding domain, two types of DNA-binding domains and a transcription activation domain. The physiological roles of this new family of transcription factor remain to be elucidated in each of the organisms containing these proteins. In plants, expression of two members of the CAMTA gene family was shown to be enhanced by ethylene, a gaseous hormone involved in many aspects of plant development (43, i.e. NtER1,
GenBank accession: AAG39222; 60, i.e. ER66, GenBank accession: AAD46410). Northern blot analysis indicated that NtCAMTA mRNA accumulated within 15 minutes after an ethylene treatment while no expression was observed when plants were pre-treated with an inhibitor of ethylene action (43). In similar expression studies, NtCAMTA was shown to be induced by senescence in leaves and petals (43). Microarray transcriptome analysis of an Arabidopsis line overexpressing the homeobox gene KNAT1 suggests that expression of AtCAMTA2 might be under the control of this gene (Arabidopsis Functional Genomics Consortium, web-posted results for EST clone# G8F8T7). Microarray analysis of an abscisic acid responsive Arabidopsis mutant suggests that AtCAMTA3 might be involved in an abscisic acid signaling pathway (AFGC, web-posted results for EST clone# 193M6T7). Therefore, there are indications that CAMTAs are involved in different regulatory pathway in plants. Additional studies of CAMTAs are needed to understand their mode of action, and to identify the downstream gene targets of these transcription factors in various eukaryotes.

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FOOTNOTES

1 The abbreviations used are: CAMTA, calmodulin-binding transcription activators; ANK, ankyrin; TIG, transcription factor immunoglobulin-like domain; dsDNA, double stranded DNA; NLS, nuclear localisation signal; Leu, leucine; EST, expressed sequence tag.
FIG. 1: CAMTA proteins are largely distributed among multicellular organisms and contain a conserved domain organisation.

(A) Schematic presentation of CAMTA proteins (drawn to scale). Domains were identified by alignment of CAMTA proteins with the ClustalX program (16) and comparisons with known protein domain (InterPro database, which comprise Pfam, PRINTS and Prosite databases; www.ebi.ac.uk/interpro/; 15). InterPro identification numbers are IPR001472 for the NLS, IPR002909 for the TIG domain, IPR002110 for ANK repeats and IPR00004 for IQ motifs. Similar domains were found in partial clones from tomato (60; ie. ER66, GenBank: AAD46410), and tobacco (43; ie. NtER1, GenBank accession: AAG39222).

(B) Comparison of CG-1 domain amino acid sequences. Shaded residues are identical (red residues) or similar (blue residues in small letters) in 80% of the sequences compared. Sequence of the putative Nuclear Localisation Signal (NLS), contained in CG-1 domains, is framed.

(C) Phylogenetic tree of CG-1 domains. The unrooted tree was produced by the neighbor-joining method (36) using the sequence comparison shown in (B).

The species abbreviations are: At, Arabidopsis thaliana; Bn, Brassica napus; Ce, Caenorhabditis elegans; Dm, Drosophila melanogaster; Ga, Gossypium arboreum; Hs, Homo sapiens; Hv, Hordeum vulgare; Le, Lycopersicon esculentum; Mm, Mus musculus; Mt, Medicago truncatula; Nt, Nicotiana tabacum; Os, Oryza sativa; Pc, Petroselinum crispum; Sp, Sorghum propinquum; St, Solanum tuberosum. The GenBank accession numbers are AtCAMTA1, CAC05467 (At5g09410); AtCAMTA2, BAB09853 (At5g64220); AtCAMTA3, AAD23613 (At2g22300);
AtCAMTA4, NP_176899 (At1g67310); AtCAMTA5, NM_117710 (At4g16150); AtCAMTA6, BAA94977 (At3g16940); BnCAMTA, submitted; HsCAMTA1, BAA74856; HsCAMTA2, BAA74932; CeCAMTA, AAA68394; DmCAMTA, AE003833; PcCG-1, S48041; MmCG-1, BB633827; MtCG-1, AW686473; OsCG-1, AU174776; SpCG-1, BG102820; StCG-1, BE341351; GaCG-1, BF278589; HvCG-1, AV835190.

**FIG. 2: CAMTAs are DNA-binding proteins**

The soluble fraction of extracts from *Sf9* insect cells expressing a truncated recombinant BnCAMTA protein (Ile<sup>1</sup>-Lys<sup>688</sup>) was subjected to ds-calf-thymus DNA-cellulose chromatography (A) or heparin-Sepharose chromatography (B). Proteins were eluted by stepwise increasing KCl concentration as indicated. Proteins were separated by SDS-PAGE, blotted and tested with anti-BnCAMTA antibodies raised against the ankyrin-repeat domain.

**FIG. 3: CAMTAs are targeted to the nucleus.**

Subcellular localisation of AtCAMTA1 in *Arabidopsis*. Total extracts from 4-weeks old *Arabidopsis* aerial tissue were centrifuged at 10,000 g for 15 min and the supernatant (Sol) and pellet (Insol) fractions were collected. A nuclear-enriched fraction (Nuc) was obtained using a Percoll gradient. Extracts were separated on SDS-PAGE stained with Coomassie blue or transferred to nitrocellulose membranes. The membranes were probed with the following antibodies: anti-AtCAMTA1 (raised against the CG-1 domain), anti-MSI1 (40), anti-Prohibitin (41) and anti-GAD2 (42). A sample of the full-length AtCAMTA1 expressed in insect cells was loaded (Rec) as a control.
FIG. 4: CAMTA proteins activate transcription in yeast.

(A) Determination of the transcriptional activation domain of AtCAMTA1 in yeast cells. Protein fusions between the DNA-binding domain of LexA and AtCAMTA1 were introduced in yeast strain EGY48 together with a plasmid carrying the LacZ reporter gene controlled by 8 LexA operators. Production of β-Galactosidase in the transformants was assayed with a chromogenic substrate (ONPG). The minimal domain that was found to activate transcription is marked by an arrow. Positive control: fusion between the DNA binding domain of LexA and the yeast activator protein GAL4 (carried by the plasmid pSH17-4). Negative control: fusion between the DNA-binding domain of LexA and the N-terminus of the Drosophila protein bicoid that is unable to activate transcription (carried by the plasmid pRFHM1). Vector: pEG202 empty vector. The different domains identified in CAMTA proteins are indicated (CG-1, TIG, ANK), the calmodulin-binding domain is represented by a hatched box and the transcription activation domain is represented by a black box.

(B) Human CAMTAs activate transcription in yeast. HsCAMTA1 and HsCAMTA2 cDNAs (29; GenBank accessions: BAA74856 and BAA74932) were fused in frame to the DNA binding domain of LexA and their ability to activate transcription of β-Galactosidase in yeast was assessed as in (A).

(C) Yeast transformants were tested for growth in the presence (+) or absence (−) of leucine (Leu). The yeast strain is carrying a modified LEU2 gene whose transcription is under the control of 6 LexA operators. Controls are the same as in (A).
FIG. 5: CAMTAs contain a distinct calmodulin-binding domain.

(A) Determination of the calmodulin-binding domain in AtCAMTA1. Different regions of the C-terminal part of AtCAMTA1 were fused in frame to the glutathione S-transferase (GST) coding sequence and expressed in E. coli. Total protein extracts were transferred to a nitrocellulose membrane and probed with Ca"/[35S]-calmodulin. The panel shows a schematic representation of the different GST fusions tested (1 to 9) and their ability to bind calmodulin is represented by a plus or a minus. The minimal AtCAMTA1 fragment that conferred calmodulin-binding is denoted by a grey box. The positions of the two IQ motifs are indicated.

(B) Predicted α-helical wheel formed by AtCAMTA1 amino acid residues Gly\textsuperscript{872}-Arg\textsuperscript{889} using the HelicalWheel program from the Wisconsin sequence analysis software package (GCG 9.1). Hydrophobic amino acids are in streaked circles and basic amino acids are in grey circles.

(C) A 25 amino acid synthetic peptide derived from AtCAMTA1 (872-GLLEKIIILRWRKGNGLRGFKRNAV-896) forms a stable complex with calmodulin. Complex formation between calmodulin and the purified peptide was assessed in the presence (upper panel) or absence (lower panel) of calcium. Various amounts of peptide (peptide/calmodulin molar ratios indicated) were incubated with 120 pmoles of bovine calmodulin and samples were separated by nondenaturing PAGE and stained with Coomassie. Incubations and electrophoresis were done in the presence of 4 M Urea. Arrows indicate the positions of free calmodulin and the calmodulin/peptide complex.
FIG. 6: Interaction of a AtCAMTA1-derived peptide with dansyl-calmodulin.

(A) Fluorescence emission spectra of dansyl-calmodulin and its complex with the AtCAMTA1-derived peptide Gly<sup>872</sup>-Val<sup>896</sup>. Fluorescence emission spectra of 300 nM dansyl-calmodulin without (squares) and with (circles) 450 nM peptide was measured at 23°C in 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 0.5 mM Ca<sup>2+</sup>, using an excitation wavelength of 345 nm with a band pass of 8 nm.

(B) Titration of the dansyl-calmodulin with AtCAMTA1-derived calmodulin-binding peptide monitored by fluorescence enhancement. The concentration of the dansyl-calmodulin was 300 nM. Emitted fluorescence was measured at 480 nm. The data were fitted according to Faiman (61) and as described by Arazi et al. (26).

FIG. 7: AtCAMTA1 binds plant calmodulin in vivo.

(A) Schematic presentation of the yeast two-hybrid system used to test the ability of plant calmodulin to bind the AtCAMTA1 calmodulin-binding domain in vivo. A petunia calmodulin (CaM81) was fused in frame to the activation domain of B42 containing a nuclear localization signal (NLS). Binding of CaM81 to its target results in the activation of the LacZ reporter gene.

(B) Mapping of the AtCAMTA1 calmodulin-binding domain in yeast. Protein fusions between the DNA-binding domain of LexA and several regions of AtCAMTA1 were investigated for their ability to activate the LacZ reporter gene when a CaM81-B42 chimeric protein was expressed. Domain indications are the same as for figure 4A. Vector: pJG4-5 empty vector. Activation is expressed in β-Galactosidase units.
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Figure 2
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Figure 3
(A) CG-1 domain  TIG  ANK  CaM-binding domain  \(\beta\)-Galactosidase (units)

1  2  3  4  5  6  7  8  9  10  11  12  13

Vector  Controls

(DNA-binding domain of lexA)

(B) \(\beta\)-Galactosidase (units)

HsCAMTA1  HsCAMTA2  At CAMTA1  Vector  Control +  Control -

0  200  400  600

(C) -Leu

Control -  Control +  HsCAMTA2  HsCAMTA1  At CAMTA1  +Leu

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Figure 4
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Figure 5
(A) Fluorescence (Arbitrary Units) vs. Wavelength (nm) for CaM + peptide and CaM only.

(B) Fraction Bound vs. Peptide Concentration (nM) showing CaM binding: Kd = 1.23 ± 1.04 nM.

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**Figure 7**

**A**

- B42 activation domain and NLS
- CaM81
- LexA DNA-BD
- LacZ reporter gene

**B**

- CG-1 domain
- Activation domain
- TIG
- ANK
- CaM-binding domain

β-Galactosidase (units)

0 400 800

- Vector (B42 protein)

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