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Calmodulin Isoforms Differentially Enhance the Binding of Cauliflower Nuclear Proteins and Recombinant TGA3 to a Region Derived from the Arabidopsis *Cam-3* Promoter

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Many stimuli increase cytoplasmic Ca^{2+} concentrations as an early signal transduction event and alter the patterns of nuclear gene transcription, but the mechanisms by which Ca^{2+} signals are transduced to the nucleus are not known. This article shows that at least four DNA binding proteins from cauliflower nuclear extracts are also calmodulin (CaM) binding proteins. CaM enhances the binding of these proteins to a C/G-box sequence element in the Arabidopsis *Cam-3* promoter. Binding to the C/G-box is enhanced preferentially by the CaM isoform encoded by *Cam-3*. However, it is not clear whether the effect is mediated directly by CaM or indirectly through the activity of a CaM-regulated protein phosphatase. CaM also binds recombinant TGA3 and enhances its binding to the same *Cam-3* promoter element. These results are consistent with the idea that a Ca^{2+}-mediated signaling pathway eliciting some changes in gene expression may consist of CaM, or a structurally related Ca^{2+} binding protein, and transcription factors.

INTRODUCTION

One of the earliest events in the response of plant cells to a wide variety of stimuli is an increase in their intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, which is used as a second messenger to trigger a range of changes in biochemical activities. The primary receptors for second messenger Ca^{2+} are a group of structurally related Ca^{2+} binding proteins, the EF hand proteins. Although numerous EF hand proteins from animals have been characterized biochemically and genetically (Kawasaki and Kretsinger, 1994), only two members of this family, calmodulin (CaM) and the calmodulin-like domain protein kinase, have been studied extensively in plant systems (Roberts and Harmon, 1992). CaM acts by binding to and modifying the activities of cytoskeletal, enzymatic, and membrane transport proteins. However, one of the major shortcomings in understanding Ca^{2+}-mediated signal transduction in plant cells is the lack of information on the identities of the protein targets regulated by CaM.

Although most of the well-characterized components and events associated with Ca^{2+}-based signaling are localized in the cytoplasm, the same external signals that cause changes in $[Ca^{2+}]_i$ also elicit changes in the patterns of nuclear gene expression. These stimuli include mechanical perturbation (Braam and Davis, 1990; Knight et al., 1992), light (Shacklock et al., 1992; Millar et al., 1994), and treatment with phytohormones (Guilfoyle et al., 1993; Bush, 1995). Of particular interest are two lines of evidence implicating Ca^{2+} and CaM as components of at least one signaling pathway acting to transduce photomorphogenic signals into the nucleus. Treatment of dark-adapted photoautotrophic soybean cell cultures with a Ca^{2+} ionophore resulted in the transient accumulation of *Cab* mRNA in the absence of a light signal (Lam et al., 1989); microinjecting cells of a phytochrome A mutant of tomato with Ca^{2+} and CaM induced the expression of a ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit, light-harvesting complex of photosystem II, photosystem I, and ATP synthase gene products (Neuhaus et al., 1993; Bowler et al., 1994). A critical question arising from these studies is: What are the molecular components involved in transducing Ca^{2+} signals, which originate in the cytoplasm, to specific genes in the nucleus?

One potential group of components involved in transducing signals between the cytoplasm and the nucleus is the EF hand family of Ca^{2+} binding proteins and their protein targets; a number of these molecules have been shown to reside in the nucleus (Hiraga et al., 1993; Bachs et al., 1994). Most recently, some transcription factors in the basic helix-loop-helix (bHLH) family have been shown to bind CaM (Corneliussen et al., 1994) and the structurally related S100a protein (Baudier et al., 1995). In these cases, transcription factor interaction with CaM prevented the proteins from forming homodimers and binding DNA (Corneliussen et al., 1994), or it prevented the factors from being phosphorylated by a protein kinase (Baudier et al., 1995). In both cases, CaM binding altered the pattern of factor

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dimerization. Consistent with the decreased in vitro DNA binding ability of these bHLH protein–CaM complexes, expression of reporter gene constructs with promoters bearing *cis*-acting binding sites for these transcription factors decreased in cells treated with a Ca^{2+} ionophore (Corneliussen et al., 1994). Thus, transcription factor interaction with CaM or a related Ca^{2+} binding protein may represent a mechanism that can exert a broad influence on the pattern of transcriptional activity in the nucleus.

In this report, the binding of CaM to plant nuclear proteins is examined, and it is demonstrated that several CaM binding proteins also bind a region of the *Cam-3* promoter. Among the plant nuclear proteins interacting with CaM is TGA3, a member of a family of basic leucine zipper (bZIP) transcription factors for which competitive binding has been shown to play a role in controlling the relative levels of expression of different genes (Neuhaus et al., 1994). In contrast to the reduced binding displayed by mammalian bHLH proteins that interact with CaM, CaM binding enhanced the interaction of both plant nuclear proteins and recombinant TGA3 with the *Cam-3* promoter.

RESULTS

Identification of a Class of DNA Binding Proteins That Are Also CaM Binding Proteins

A genomic clone harboring sequences encoding the calmodulin 3 gene (*Cam-3*) from Arabidopsis was isolated and characterized previously (Perera and Zielinski, 1992). Figure 1A shows that within the −282 to −243 region of the *Cam-3* promoter, several DNA sequence elements containing the motif TGAC were identified. This motif has been shown to play an important role in the expression driven by a number of gene promoters, including the cauliflower mosaic virus 35S promoter (Benfey et al., 1989) and the auxin-regulated *GH3* promoter (Liu et al., 1994). We termed this region of the promoter the TGAC repeat domain (TRD). However, although deletion of the TRD greatly reduces basal transcription driven by the *Cam-3* promoter in transgenic tobacco (D.B. Szymanski, M.J. Timme, and R.E. Zielinski, unpublished data), the contribution of this domain to the control of *Cam-3* transcription by Ca^{2+} is not known.

One of the sequences within the TRD was of particular interest because it contains an ACGT core, which is characteristic of binding sites for plant bZIP transcription factors (Izawa et al., 1993). In accordance with the nomenclature for bZIP binding sites adopted by Izawa et al. (1993), this imperfect palindromic site is referred to as a C/G-box. Its sequence is T**G**ACGT**G**A, whereas a C-box octamer is T**G**ACGT**C**A and a G-box is C**C**ACGT**G**G. The DNase I footprint experiments shown in Figure 1B confirmed the binding of cauliflower nuclear proteins to this region of the *Cam-3* promoter and revealed a protected domain of 25 to 28 bp, which is similar in length

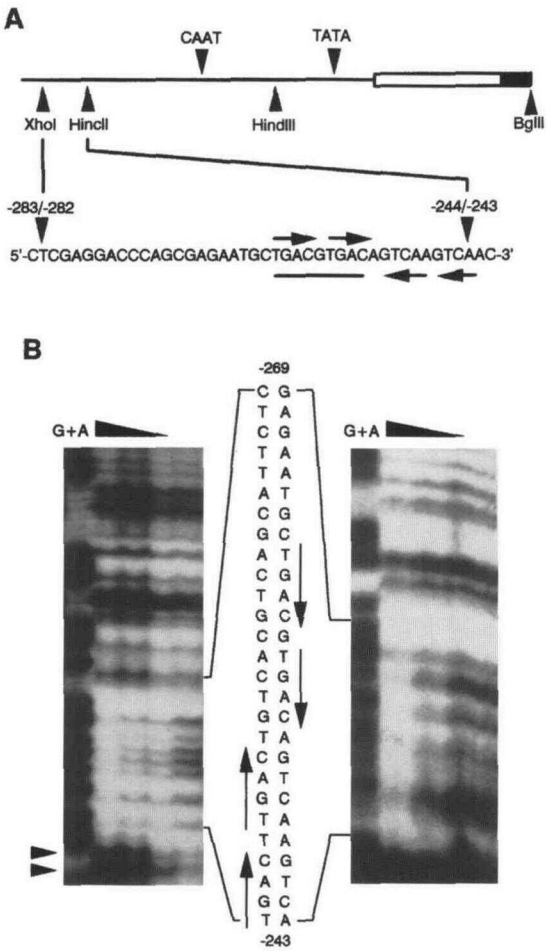

Figure 1. The TRD of the *Cam-3* Promoter Contains Four TGAC DNA Sequence Motifs That Are Bound by Nuclear Proteins.

(A) DNA sequence of the TRD and its location within the *Cam-3* 5′ flanking sequences relative to the TATA- and CAAT-box sequences. The locations of individual TGAC sequence motifs are indicated with arrows. The underlined sequence motif is the C/G-box.

(B) DNase I footprint assays of cauliflower nuclear proteins bound using ^{32}P-labeled top (right) and bottom (left) strands of the TRD region as probes. Lanes designated G+A are Maxam-Gilbert G+A sequencing reactions (Maxam and Gilbert, 1980) of the same DNA probes used as markers. The slanted symbols indicate decreasing amounts of nuclear proteins added to protect the DNA probes. The locations of TGAC sequence motifs are indicated with arrows. Arrowheads indicate DNase hypersensitive sites appearing downstream from the protected region.

to the 25-bp protected domain associated with an ACGT core sequence in the nopaline synthase promoter (Lam et al., 1990). Cauliflower was chosen as the source of nuclear proteins for these assays, based on the close evolutionary relationship of cauliflower and Arabidopsis and the ease with which large amounts of cauliflower nuclear proteins can be isolated. In ad-

dition, *Cam-3* mRNA is expressed in the rapidly growing apical regions and floral tissues of Arabidopsis (Perera and Zielinski, 1992). Nuclear protein–dependent DNase I hypersensitive sites were also detected in these experiments at positions +14 and +15 relative to the C/G-box on the template stand of the *Cam-3* promoter.

In characterizing the binding of nuclear proteins to the *Cam-3* promoter C/G-box, we tested the idea that CaM itself might serve as a transduction link between increases in cytosolic Ca^{2+} concentration and altered patterns of gene expression by modulating DNA–protein interactions. In the initial experiments, cauliflower nuclear extracts were subjected to affinity chromatography on columns of CaM–Sepharose. These affinity columns were constructed by immobilizing a mixture of recombinant Arabidopsis CaM isoforms and bovine brain CaM to facilitate the isolation of a broad range of protein substrates that interact with CaM. Proteins that bound to the column in the presence of Ca^{2+} and eluted from the column when the ion was chelated with EGTA were tested for their ability to bind the C/G-box on protein gel blots probed with DNA and in gel mobility shift assays.

Figure 2A shows that the crude nuclear protein fraction contained four major proteins with apparent masses of 85, 60, 58, and 36 kD that recognized and bound a ^{32}P-labeled TRD DNA probe. Following CaM–Sepharose affinity chromatography, all but the 85-kD DNA binding protein were recovered in the protein fraction that bound to the column in the presence of Ca^{2+} and eluted from the matrix in the presence of EGTA, which is characteristic of CaM binding proteins. Consistent with these results, Figure 2B indicates that when the proteins from the CaM–Sepharose fractions displaying DNA binding activity were examined by gel shift assays using the same TRD DNA probe, the CaM binding protein fraction formed DNA–protein complexes having electrophoretic mobilities similar to those formed by cauliflower nuclear extracts that had not been subjected to affinity chromatography. On the other hand, an equivalent proportion of the CaM–Sepharose wash fraction, which contained only the 85-kD DNA binding protein, yielded only very low levels of DNA–protein complex, using the TRD probe.

CaM Binding Affects DNA–Protein Complex Formation

The data in Figure 2 demonstrate that nuclear fractions containing CaM binding proteins also possess DNA binding activity. To determine the effect of CaM on the DNA binding activity of these proteins, gel shift assays were conducted in which nuclear extracts were supplemented with Ca^{2+} and recombinant Arabidopsis CaM. Figure 3A shows that proteins from cauliflower nuclear extracts bound to a ^{32}P-labeled TRD DNA probe to form three DNA–protein complexes with slightly different mobilities. These are similar to the complexes shown in Figure 2B. Neither 100 μM Ca^{2+} nor CaM alone significantly altered the apparent size distribution of the DNA–protein complexes or the apparent affinity of the nuclear proteins for the TRD probe. However, in the presence of Ca^{2+}, CaM enhanced the binding of cauliflower nuclear proteins to the *Cam-3* TRD probe. The enhancement resulted in both a higher recovery of DNA–protein complex compared with the untreated reactions and an increase in the apparent size distribution of the DNA–protein complexes. Initially, an equimolar mixture of three recombinant CaM isoforms was added to the gel shift reaction. But, when the proteins were tested individually for their abilities to enhance nuclear protein binding, the CaM-2 isoform

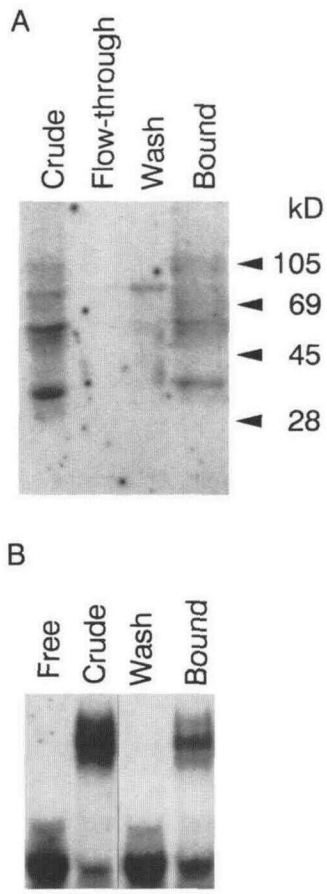

Figure 2. *Nuclear CaM Binding Proteins Recovered from CaM–Sepharose Affinity Columns Also Bind the Cam-3 Promoter TRD Sequences.*

Cauliflower nuclear proteins were fractionated on a column of CaM–Sepharose. Equal proportions of proteins from the crude, flow-through, wash, and bound fractions collected from the column were tested for their ability to bind the *Cam-3* TRD probe.

(A) Protein gel blot probed with a ^{32}P-labeled *Cam-3* TRD probe. The positions of the molecular mass markers are shown at right in kilodaltons.

(B) Gel mobility shift assay using the same protein fractions and DNA probe. The lane designated Free shows the mobility of the DNA probe in the absence of protein.

was found to be more effective in enhancing DNA–protein complex formation than were either the CaM-4 or CaM-6 isoforms. However, because of the complexity of the gel shift assays, it could not be determined whether the proteins whose binding to DNA is enhanced by CaM-2 are identical to the proteins whose binding is enhanced by CaM-4 or CaM-6. The increase in DNA binding we observed with CaM-4 and CaM-6, therefore, could have been a reflection of enhanced binding by proteins whose abundance in the nuclear extracts is lower than that of proteins whose binding is enhanced by CaM-2.

The experiment shown in Figure 3A was performed with saturating levels of CaM (5 μM). Imaging the gel used in the titration experiment shown in Figure 3B with a PhosphorImager, however, revealed a small enhancement of DNA binding at CaM-2 concentrations as low as 500 nM; enhanced binding was more clearly observed with additions of 800 nM CaM-2. Figure 3B also shows more clearly the change in apparent size of the DNA–protein complexes as a function of CaM concentration. The yield of complex c, which had the smallest apparent mass, gradually declined as CaM was added to the gel shift reaction mixtures. Because of the complexity of the nuclear protein fractions used in this experiment, however, the precise relationships among the DNA–protein complexes designated a, b, and c in Figure 3B could not be determined. Thus, it is not clear whether the increased yield of DNA–protein complexes mediated by exogenous CaM was a consequence of a precursor–product relationship between complex c and complexes a and b, or whether CaM mediated the formation of additional DNA–protein complexes with mobilities similar to those of complexes a and b.

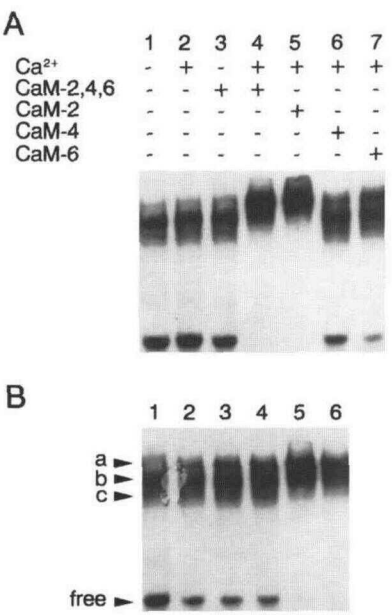

Figure 3. CaM/Ca^{2+} Enhances the Binding of Cauliflower Nuclear Proteins to the C/G-Box Derived from the *Cam-3* Promoter.

(A) Equivalent amounts of the *Cam-3* TRD probe and cauliflower nuclear extract were incubated in standard gel shift assay buffer in the presence (+) or absence (–) of 100 μM $CaCl_2$ and 5 μM CaM. Lane 1, no additions; lane 2, Ca^{2+} only; lane 3, an equimolar mixture of three recombinant CaM isoforms (CaM-2,4,6) only; lane 4, Ca^{2+} and the recombinant CaM mixture; lane 5, Ca^{2+} and CaM-2; lane 6, Ca^{2+} and CaM-4; lane 7, Ca^{2+} and CaM-6.

(B) Concentration dependence of CaM-2–enhanced DNA–protein complex formation. Gel shift reactions were supplemented with 100 μM $CaCl_2$ and 0, 0.5, 0.8, 1, 3, or 5 μM CaM-2 in lanes 1 to 6, respectively. Three distinct DNA–protein complexes, designated a, b, and c, are indicated in the figure. The mobility of the free DNA probe is indicated.

TGA3 Binding to the *Cam-3* Promoter Is Enhanced by CaM

The previous experiments revealed the presence of a class of plant nuclear proteins that bind both CaM and DNA. However, the complexity of the cauliflower nuclear extract precluded unambiguous identification of any of the proteins. Furthermore, CaM-regulated phosphatases or protein kinases could also have been included in the crude nuclear or CaM–Sepharose fractions and could have indirectly influenced the binding of proteins to the TRD DNA probe. To resolve this ambiguity, the binding of a recombinant transcription factor to the TRD probe was examined. Because of the similarity of the C/G-box sequence contained within the *Cam-3* TRD to the consensus C-box binding site preferred by TGA transcription factors (Izawa et al., 1993), we hypothesized that members of the TGA protein family are likely to bind the *Cam-3* promoter sequence element.

Figure 4 shows an experiment in which the ability of proteins derived from bacteria harboring an expression plasmid encoding TGA3 (Miao et al., 1994) to bind the TRD probe was compared with a similar protein fraction derived from uninduced bacteria harboring the same plasmid. Protein from the induced bacteria bound the TRD probe and formed a detectable DNA–protein complex in the gel mobility shift assay, whereas proteins derived from uninduced bacteria did not. Figure 4 also shows that, in the absence of CaM, when the TRD probe was challenged with saturating levels of cauliflower nuclear protein, the qualitative pattern of DNA–protein complexes was changed slightly compared with the pattern at subsaturating levels of protein. However, this change was not as dramatic as the qualitative change observed when CaM-2 was added to the binding reactions (cf. Figures 3A and 4). Also, quantitative formation of DNA–protein complexes required five times the input of nuclear protein in the absence of CaM than it did in the presence of CaM.

When extracts containing recombinant TGA3 were supplemented with CaM, as shown in Figure 5, TGA3 binding to the CaM-3 TRD probe was enhanced. Unlike the results with crude nuclear proteins, the enhanced binding of TGA3 was clearly CaM isoform specific. But in contrast to the cauliflower

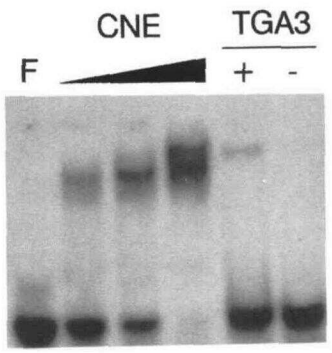

Figure 4. Recombinant TGA3 Binds the CaM-3 TRD Promoter Sequences.

Equivalent amounts of the ^{32}P-labeled CaM-3 TRD probe were incubated in the presence of 1, 4, or 8 μg of cauliflower crude nuclear extract (CNE) or 2 μg of soluble proteins derived from induced (+) or uninduced (−) *E. coli* harboring a TGA3 expression plasmid. Lane F shows the mobility of the free TRD probe.

nuclear proteins, TGA3 binding was preferentially enhanced by the CaM-6 isoform. Analysis of the gel shown in Figure 5 and a duplicate experiment with a PhosphorImager indicated that 60% more DNA–protein complex was recovered from binding reactions supplemented with Ca^{2+} and CaM-6 than from DNA binding reactions lacking either Ca^{2+} or CaM. CaM-2 and CaM-4, in contrast, increased TGA3 binding by 20 and 30%, respectively. By comparison, we estimated that the yields of DNA–protein complexes vary by as much as 10% in replicate experiments.

TGA3 Is a CaM Binding Protein

To demonstrate directly that CaM is bound by TGA3, protein gel blot assays were performed using biotinylated, recombinant CaM as a probe. Figure 6 shows that many proteins present in crude cauliflower nuclear extracts, including proteins with apparent molecular masses of 60 and 58 kD, bound biotinylated CaM in a Ca^{2+}-dependent manner. This finding is consistent with the experiment shown in Figure 1. However, the 36-kD CaM binding DNA binding protein was obscured in this experiment by the presence of a presumptive biotin binding protein of the same apparent mass. Figure 6 also shows that soluble protein fractions derived from bacteria harboring a TGA3 expression plasmid and induced with isopropyl β-D-thiogalactopyranoside (IPTG) contained a CaM binding protein having the same apparent mass as that predicted for recombinant TGA3 (45 to 46 kD). This polypeptide bound biotinylated CaM in a Ca^{2+}-dependent manner, and it comigrated with a Coomassie Brilliant Blue R 250–stained protein whose expression was induced by IPTG (data not shown). Identically prepared protein fractions from uninduced bacteria, in contrast, lacked detectable levels of CaM binding protein of the same apparent molecular mass. In this experiment, a number of *Escherichia coli* polypeptides also bound the biotinylated CaM probe, but none of these proteins was of the size predicted for TGA3.

DISCUSSION

In recent years, increasing attention has focused on determining the identities and functions of CaM binding proteins found in the nucleus (reviewed in Bachs et al., 1994). Because the wide variety of external stimuli that triggers increases in $[Ca^{2+}]_i$ also elicits specific changes in the pattern of nuclear gene expression, identifying CaM-regulated nuclear proteins and ascertaining their functions should provide some important insights into the mechanisms by which nuclear and cytoplasmic activities are coordinated. The results presented here demonstrate that a recombinant plant bZIP transcription factor, TGA3, and several other unidentified DNA binding proteins in cauliflower nuclear extracts bind CaM. Their abilities to bind CaM are correlated with an apparent enhancement of their abilities to bind a sequence element derived from the *Cam-3* promoter or to interact with other nuclear proteins to form a DNA–protein complex.

The degree of enhancement of DNA–protein interaction observed in this work was dependent on the particular CaM isoform added exogenously to gel mobility shift assay reactions, an observation that supports the idea that sequence variants of CaM may have arisen during evolution to optimize

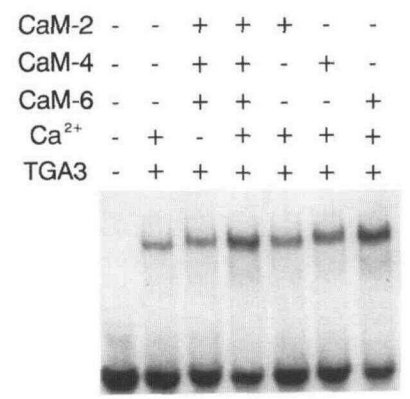

Figure 5. CaM-6 Preferentially Enhances the Binding of Recombinant TGA3 to C/G-Box Sequences.

Gel mobility shift assay mixtures were supplemented with 100 μM $CaCl_2$ or 5 μM CaM, as indicated by the symbols above each lane. The leftmost lane illustrates the migration of the DNA probe in the absence of TGA3-containing bacterial protein extract. The presence (+) or absence (−) of Ca^{2+} and an equimolar mixture of CaM isoforms or individual CaM isoform proteins are indicated.

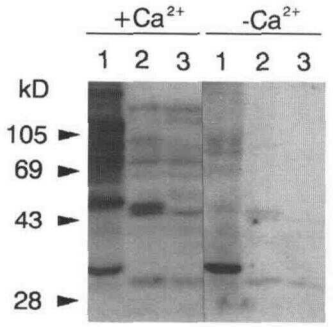

Figure 6. TGA3 Is a CaM Binding Protein.

Cauliflower nuclear proteins and soluble proteins extracted from bacteria harboring a TGA3 expression plasmid were fractionated by SDS–gel electrophoresis, transferred to a nylon filter, and probed with biotinylated CaM-6 in the presence (+) or absence (–) of Ca^{2+}, as indicated. CaM–protein complexes were detected by using streptavidin–alkaline phosphatase conjugates and the chemiluminescent substrate CSPD. Lanes 1 contain cauliflower nuclear proteins; lanes 2, total proteins from *E. coli* BL21(DE3)pLysS cells harboring a TGA3 expression plasmid 2 hr after induction with 1 mM IPTG; lanes 3, total proteins from uninduced BL21(DE3)pLysS cells harboring the same TGA3 expression plasmid. The positions of the molecular mass markers are shown at left in kilodaltons.

the interaction between CaM and specific target proteins. In these experiments, the CaM-2 isoform, whose amino acid sequence is identical to that of the protein encoded by *Cam-3* (Perera and Zielinski, 1992), produced the maximal enhancement of protein binding to the *Cam-3* promoter element. This finding is consistent with the idea that autoregulation may be among the controls exerted over *Cam-3* expression. It also lends support to the idea that some specificity in Ca^{2+}-mediated signaling may be generated by differential expression of individual Ca^{2+} receptor proteins, which in turn preferentially interact with distinct groups of target proteins. Differential stimulation of NAD kinase activity by the Arabidopsis CaM isoforms was reported recently (Liao et al., 1996). However, in the case of the NAD kinase, the differences in activity mediated by the CaM isoforms were not as dramatic as the apparent differences in their abilities to enhance the DNA–protein complex formation observed in this work.

The observation that binding of the TRD probe by cauliflower inflorescence nuclear proteins and recombinant TGA3 was enhanced by different CaM isoforms is likely to be a consequence of the fact that TGA3 is expressed maximally in root tissues in Arabidopsis (Miao et al., 1994) rather than a difference between the populations of nuclear proteins derived from the two plant species. CaM-4 and CaM-6 isoforms were less effective in enhancing DNA–protein interaction than was CaM-2 with the cauliflower nuclear protein extracts. However, the complexity of the gel shift patterns shown in Figure 3 prevented identification of the spectrum of proteins whose binding is enhanced by CaM. Thus, we cannot preclude the possibility that CaM-4 and CaM-6 enhanced the binding of a slightly different, less abundant set of nuclear proteins to the TRD probe than did CaM-2. This ambiguity will be resolved when methods for separating and purifying the cauliflower nuclear proteins are developed or when sequences encoding these proteins are cloned and used to produce recombinant proteins. In addition, more extensive studies in which the effect of Ca^{2+} and CaM on the DNA binding properties of nuclear proteins derived from all of the organs from a single plant species are required to determine what differences might exist in the Ca^{2+}-mediated signal transduction pathways in their nuclei and to assess their potential impact on gene expression.

It will be critical in future studies to determine whether CaM is the physiologically relevant Ca^{2+}-sensitizing component of the enhanced binding observed in the studies reported here. Members of the EF hand family of Ca^{2+} binding proteins frequently can substitute for one another in enzyme assays in vitro (Kawasaki and Kretsinger, 1994). The rather high levels of exogenous CaM required for increased DNA–protein interaction may indicate that a Ca^{2+} binding protein structurally related to, but distinct from, CaM modulates this interaction in vivo. Recent examinations of the effect of Ca^{2+} binding proteins on the ability of various bHLH proteins to interact with DNA (Corneliussen et al., 1994; Baudier et al., 1995) revealed a requirement for CaM in concentrations comparable to those employed in our experiments. However, S100a, an EF hand Ca^{2+} binding protein structurally related to CaM, bound some of these transcription factors with a higher apparent affinity in vitro than did CaM. The nuclear protein fractions used in our experiments were obtained by precipitation with $(NH_4)_2SO_4$, under conditions in which CaM and several other EF hand Ca^{2+} binding proteins would not have been recovered because of their high solubility in salt solutions near neutral pH. Future studies will be directed toward identifying the physiologically relevant protein(s) involved in mediating the Ca^{2+}-dependent interactions characterized in this work.

The best-characterized CaM binding proteins contain short polypeptide regions that have no recognizable amino acid sequence motif but that form basic, amphiphilic α-helices when they are bound by CaM (O'Neil and DeGrado, 1990). Although detailed studies to map the CaM binding domain of TGA3 have not been completed, analysis of the amino acid sequence of TGA3 revealed that a region (residues 199 to 220) residing C-terminal to the bZIP domain meets the general criteria defining a CaM binding domain. This region is predicted to be α-helical, and the calculated hydrophobic moment and mean hydrophobicity of the peptide formed by this region are intermediate in value compared with surface-seeking helices and transmembrane helices, which is characteristic of most biochemically well-characterized CaM binding peptides (Erickson-Viitanen and DeGrado, 1987). Analyses of the sequences of other members of the TGA family of proteins (Kawata et al., 1992; Schindler et al., 1992) revealed domains with properties very similar to those of the putative CaM binding domain of TGA3. However, no such structural motifs were predicted within the sequences of Em binding protein-1 (Guiltinan et al., 1990) or the common plant regulatory factor proteins (Weisshaar

et al., 1991). An interesting correlation with this structural prediction is that members of the TGA transcription factor family possess leucine zipper dimerization regions that consist of only three repeated leucine residues (Miao et al., 1994), compared with the more conventional arrangement of five to seven that are observed in other plant and animal bZIP transcription factors (Busch and Sassone-Corsi, 1990; Weisshaar et al., 1991; Schindler et al., 1992). However, the significance of this correlation remains to be determined.

The interaction of CaM with proteins in crude cauliflower nuclear fractions and the *Cam-3* C/G-box promoter sequence seems likely to be more complex than the CaM–TGA3 interaction. In animal systems, CaM is well known to activate protein kinases and a protein phosphatase, whose substrates have been shown to include transcription factors (Hiraga et al., 1993; Enslen et al., 1994; Matthews et al., 1994). The gel shift results from using crude cauliflower nuclear proteins presented in Figure 3 were obtained in reactions from which ATP was absent; this condition eliminated the likelihood that Ca^{2+}/CaM–stimulated protein kinase activity was the biochemical link between Ca^{2+} and the enhanced DNA–protein complex formation. However, the possibility that enhanced DNA binding was, at least in part, the result of a Ca^{2+}/CaM–stimulated protein phosphatase activity cannot be ruled out in this experiment. An alternative explanation for the data presented here is that CaM may modulate the interaction between DNA binding transcription factors and non-DNA binding nuclear proteins directly by binding to and altering the conformation of the transcription factor either before or after it occupies its binding site on the C/G-box.

In conclusion, this work has demonstrated that protein–protein interactions involving CaM could potentially link second messenger Ca^{2+} signals with changes in gene expression. Enhanced binding of a recombinant transcription factor to DNA was shown directly, and a more complex enhanced binding of nuclear proteins to a DNA–protein complex, either directly or through a CaM-regulated protein phosphatase, was demonstrated. Future studies will be aimed at defining the roles of these interactions in regulating the expression of *Cam-3* and other genes in response to stimuli that elicit changes in $[Ca^{2+}]_i$.

METHODS

Nuclear Extract Preparation

Nuclear extracts were prepared from cauliflower with minor modifications of the methods of Foster et al. (1992). All steps were performed at 4°C. Cauliflower heads obtained from a local produce wholesaler were rinsed in distilled H_2O, and the florets were excised with razor blades. Florets (250 g) were suspended in 1 L of nuclear extraction buffer (0.5 M hexylene glycol, 10 mM Pipes-KOH, pH 7.0, 10 mM $MgCl_2$, 0.02% [w/v] Triton X-100, 5 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM benzamidine) and homogenized using a Polytron (Brinkmann Instruments, Westbury, NY), fitted with a PTA 20 S probe, at low speed for 60 sec, with high-speed bursts every 10 sec. The homogenate was filtered through four layers of cheesecloth and one 70-μm and two 20-μm nylon filters. The nuclei were collected by centrifugation at 2000*g* for 10 min. They were resuspended twice in nuclear wash buffer (20% [v/v] glycerol, 10 mM Pipes-KOH, pH 7.0, and 10 mM $MgCl_2$) and collected by centrifugation at 3000*g* for 5 min. Sedimented nuclei were resuspended in nuclear lysis buffer (110 mM KCl, 15 mM Hepes-KOH, pH 7.6, 5 mM $MgCl_2$, 1 mM DTT, 5 μg/mL leupeptin, and 5 mg/mL antipain), and the protein concentration of the suspension was adjusted to 5 mg/mL. One-tenth volume of 4 M $(NH_4)_2SO_4$ was added in three aliquots, and the suspension was rocked at 4°C for 30 min. Insoluble material was sedimented at 90,000*g* for 60 min. Finely ground $(NH_4)_2SO_4$ was added slowly to the supernatant (0.3 mg/mL) with stirring, and the mixture was rocked at 4°C for 15 min. The protein precipitate was collected by centrifugation at 10,000*g* for 15 min. The pellet was resuspended in 1 mL of nuclear protein extract buffer (20% [v/v] glycerol, 40 mM KCl, 25 mM Hepes-KOH, pH 7.6, 0.1 mM EDTA, 1 mM DTT, 5 μg/mL antipain, and 5 μg/mL leupeptin) and dialyzed against the same buffer without protease inhibitors. Protein concentration was adjusted to 2 to 3 mg/mL; the typical yield was ~10 mg/kg of cauliflower florets.

Recombinant Protein Production

Purifying recombinant Arabidopsis calmodulin (CaM) isoform proteins from induced *Escherichia coli* BL21(DE3), labeling recombinant CaM with biotin, and detecting CaM binding proteins by protein gel blot assays were performed as described previously (Liao and Zielinski, 1995; Liao et al., 1996). Concentrations of stock solutions of the proteins were estimated by Lowry assay and by titration with Ellman's reagent (Liao et al., 1996). CaM–Sepharose affinity columns were made and used to isolate CaM binding proteins, according to the methods of Klee and Krinks (1979), except that 2 mg of CaM per mL of CNBr-activated Sepharose was used to construct the affinity matrix. The protein used to construct this column consisted of equal proportions of the recombinant Arabidopsis CaM-2, -4, and -6 isoforms and bovine brain CaM. To improve the specificity of the CaM–Sepharose affinity columns, after crude nuclear proteins were bound to the matrix in the presence of Ca^{2+} and 50 mM NaCl, washes were performed in which the NaCl concentration was raised to 150 mM. After this wash, CaM binding proteins were eluted from the affinity column in the presence of 5 mM EGTA.

A cDNA clone encoding TGA3 (Miao et al., 1994) cloned in the T7-based expression vector pET23a was obtained from E. Lam (Rutgers University, Piscataway, NJ). Recombinant TGA3 production was induced by growing *E. coli* BL21(DE3)pLysS, harboring the expression plasmid, in Luria-Bertani medium at 37°C until the A_{600} of the culture was between 0.3 and 0.4, adding isopropyl β-D-thiogalactopyranoside to 1 mM, and incubating the culture for an additional 3 hr. The induced bacteria were collected by centrifugation and lysed by freezing in a dry ice–ethanol bath. The thawed pellet was resuspended in nuclear lysis buffer containing 0.1% (v/v) Triton X-100, rocked gently at 4°C for 30 min, and sonicated briefly. After clearing the extract by centrifugation at 10,000*g* for 20 min, 2 μg of protein in the supernatants of induced and uninduced cells was used in mobility shift assays.

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays that involved the binding of nuclear factors to the *Cam-3* promoter were conducted in either 8% (80:1,

acrylamide/bisacrylamide) or 5% (40:1, acrylamide/bisacrylamide) acrylamide gels in 7 mM Tris-acetate, pH 7.6, and 1 mM EDTA. Binding reaction buffer consisted of 25% (v/v) glycerol, 25 mM Hepes-KOH, pH 7.6, 1 mM $MgCl_2$, 0.5 mM DTT, and 0.01% (w/v) bromphenol blue. Binding reactions contained ~30 fmol of radioactively labeled DNA molecules, 400 ng poly(dI·dC) as a nonspecific competitor, and nuclear protein extract or bacterial soluble protein extract, which was added last. After a 10-min incubation at room temperature, the reactions were fractionated at 10 V/cm for 1 hr. The gels were dried onto DE-81 anion exchange paper and visualized by imaging with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or autoradiography.

DNase I Footprinting Assays

For DNase I footprint binding reactions, ^{32}P-labeled DNA and proteins were incubated in the same binding reaction buffer used for gel mobility shift experiments. The binding reactions were incubated for 1 min after the addition of equal volumes of 5 mM $CaCl_2$ and 10 mM $MgCl_2$, and then digested for 1 min with 0.013 units of DNase I (RQ1 DNase; Promega, Madison, WI). Reactions were stopped by adding an equal volume of 0.2 M NaCl, 30 mM EDTA, 1% (w/v) SDS, and 150 μg/mL yeast RNA, which had been extracted with phenol-chloroform and concentrated by ethanol precipitation. The products of the reaction were fractionated in 8% (w/v) acrylamide sequencing gels in parallel with a Maxam-Gilbert G+A sequencing reaction (Maxam and Gilbert, 1980) of the end-labeled probe as a standard.

Depending on the application, double-stranded DNA molecules were either end-labeled with T4 polynucleotide kinase or end-labeled by filling in with the Klenow fragment of DNA polymerase I. DNA molecules with 5′ protruding ends were radioactively labeled with the Klenow fragment and the appropriate α-^{32}P-deoxynucleotide triphosphate (dNTP). In 25-μL reactions, 10 pmol of DNA ends were incubated with 0.5 units of the Klenow fragment (Bethesda Research Laboratories), 125 μCi of α-^{32}P-dNTP (3000 Ci/mmol), and 1 mM unlabeled dNTPs (excluding the ^{32}P-labeled nucleotide) in 10 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, and 1 mM DTT. Reactions were incubated at room temperature for 12 min and stopped by the addition of 60 μL of 50 mM EDTA. Thirty-microliter labeling reactions included 10 pmol of dephosphorylated 5′ ends, 10 units of T4 polynucleotide kinase, 5 μCi of γ-^{32}P-ATP (3000 Ci/mmol) in 60 mM Tris-HCl, pH 7.6, 12 mM 2-mercaptoethanol, 10 mM $MgCl_2$, and 0.33 mM ATP. Reactions were incubated at 37°C for 30 min and stopped by the addition of 60 μL of 50 mM EDTA. Unincorporated nucleotides were removed by spin column chromatography over Bio-spin 30 (Bio-Rad, Richmond, CA) acrylamide beads.

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