Plant Cell Rep (2010) 29:1297–1304
DOI 10.1007/s00299-010-0916-7

ORIGINAL PAPER

AtCML8, a calmodulin-like protein, differentially activating CaM-dependent enzymes in *Arabidopsis thaliana*

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Received: 10 May 2010 / Revised: 21 July 2010 / Accepted: 17 August 2010 / Published online: 4 September 2010
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Abstract Plants express many calmodulins (CaMs) and calmodulin-like (CML) proteins that sense and transduce different Ca^{2+} signals. Previously, we reported divergent soybean (*Glycine max*) CaM isoforms (GmCaM4/5) with differential abilities to activate CaM-dependent enzymes. To elucidate biological functions of divergent CaM proteins, we isolated a cDNA encoding a CML protein, AtCML8, from *Arabidopsis*. AtCML8 shows highest identity with GmCaM4 at the protein sequence level. Expression of *AtCML8* was high in roots, leaves, and flowers but low in stems. In addition, the expression of *AtCML8* was induced by exposure to salicylic acid or NaCl. AtCML8 showed typical characteristics of CaM such as Ca^{2+}-dependent electrophoretic mobility shift and Ca^{2+} binding ability. In immunoblot analyses, AtCML8 was recognized only by antiserum against GmCaM4 but not by GmCaM1 antibodies. Interestingly, AtCML8 was able to activate phosphodiesterase (PDE) but did not activate NAD kinase. These results suggest that AtCML8 acts as a CML protein in *Arabidopsis* with characteristics similar to soybean divergent GmCaM4 at the biochemical levels.

Keywords *Arabidopsis* · Calcium · Calmodulin (CaM) · Isoform · Calmodulin-like protein (CML) · Phosphodiesterase (PDE) · NAD kinase

Communicated by J. R. Liu.

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Introduction

In plants, intracellular Ca^{2+} concentration is one of most important second messengers in the regulation of cellular events such as developmental cues and environmental biotic and abiotic stimuli (Sanders et al. 2002). Calcium is decoded by many calcium-binding proteins in calcium mediated signaling (DeFalco et al. 2010). Calmodulins (CaMs), representative Ca^{2+} binding proteins, are highly conserved and ubiquitous proteins in plants (McCormack et al. 2005). CaM also mediates Ca^{2+} signals in dependence on developmental and environmental cues and transmits signals to various target enzymes and signaling proteins, including metabolic enzymes, transcription factors, ion channels, protein kinases/phosphatases and structural proteins (Snedden and Fromm 2001; Bouché et al. 2005; Kim et al. 2009).

In addition to CaMs, plants also possess a large repertoire of CaM-like proteins (CMLs) that encode potential calcium sensors and exhibit significant structural divergence from the typical CaM (McCormack and Braam 2003; McCormack et al. 2005; Ranty et al. 2006; Boonburapong and Buaboocha 2007). Based on sequence analysis of CML proteins, amino acids have been substituted in the EF-hand loop motif, which binds a single Ca^{2+} ion. Recent data indicated that CaM and CML proteins differ in their Ca^{2+} affinity and target-binding activities (Hua et al. 2003;

Lee et al. 2000; Popescu et al. 2007). However, the biochemical and physiological functions of CMLs are still largely unknown in plants.

Previously, we cloned five genes specifying *CaM* isoforms from soybean designated as GmCaM1 to GmCaM5, and divided them into two groups, the conserved group (GmCaM1 through 3) and a divergent group (GmCaM4 and 5) on the basis of primary structural diversity (Lee et al. 1995). In biochemical analyses, we reported that a divergent GmCaM isoform (GmCaM4) showed a different CaM-dependent enzyme regulation pattern. Although a conserved CaM isoform (GmCaM1) activated both PDE and NAD kinase, which are well-characterized CaM-dependent enzymes, the divergent CaM isoform (GmCaM4) activated PDE but not NAD kinase (Lee et al. 1995, 1997). In addition, CaM isoforms reciprocally regulated NOS and calcineurin. GmCaM4 activated mammalian NOS, while GmCaM1 serves as a competitive antagonist of this activation. In contrast, GmCaM1 activated the mammalian protein phosphatase calcineurin (CaN), while GmCaM4 competitively antagonized its activation (Cho et al. 1998; Lee et al. 2000).

In physiological analyses, two divergent GmCaM isoforms (GmCaM4 and GmCaM5) were involved in plant disease resistance responses in a salicylic acid independent manner in tobacco and *Arabidopsis* (Heo et al. 1999; Park et al. 2004). These CaM isoforms are believed to serve as mediators of intracellular Ca^{2+} signals that are transiently increased by pathogen attack in plants. In addition, transgenic plants over-expressing GmCaM4 showed enhanced resistance against salt stress in *Arabidopsis* through the up-regulation of a MYB transcription factor that regulated salt and dehydration responsive genes (Yoo et al. 2005). The biochemical and physiological differences distinguishing the two CaM sub-families may be due to altered primary structures between the divergent CaM isoforms and the highly conserved CaM isoforms.

The presence of GmCaM4 homologs at the protein level has previously been predicted by immunoblot analysis in *Arabidopsis* total protein extracts with GmCaM4 specific antibody (Lee et al. 1995). Recently, it was reported that divergent CaMs from *Arabidopsis,* including AtCaM8 (AtCML8), were able to functionally complemented a yeast calmodulin null mutant (Zielinski 2002). In addition, AtCaM7 in this divergent sub-family is a transcriptional regulator that directly interacts with the promoters of light-inducible genes and promotes photomorphogenesis (Kushwaha et al. 2008; Kim et al. 2009). It has also been reported that AtCML9 is responsive to diverse stimuli, and that the loss of function of AtCML9 results in altered responses to abiotic stress and ABA (Magnan et al. 2008). However, the biochemical activity of the CML proteins in *Arabidopsis* has not yet been determined. In order to understand the biochemical role of the CML proteins, we isolated one member of this group, AtCML8, which is most closely aligned with GmCaM4/5 in the model species *Arabidopsis*. Here, we describe the biochemical properties of AtCML8 which resemble those of a soybean divergent CaM (GmCaM4) isoform with respect to antigenicity and CaM-dependent enzyme activation.

Materials and methods

Phylogenetic analysis of AtCML8

For phylogenetic analysis of AtCML8, 13 CaMs and AtCML8 were aligned with CLUSTAL-W program (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalW/clustalw.html). GenBank accession numbers for the analysis are as follows: AtCaM1, NM123137; AtCaM2, NM180013; AtCaM3, NM115539; AtCaM4, NM105313; AtCaM5, NM179766; AtCaM6, NM180529; AtCaM7, NM114249; AtCML8, NM117546; GmCaM1, L01430; GmCaM2, L01431; Gm CaM3, L01432; GmCaM4, L01433; GmCaM5, L19359 and Human CaM, AAH08437, respectively.

Production of recombinant AtCML8 protein in *E. coli*

A T7 expression vector, *pET-3d*, was used for production of AtCML8 protein in *E. coli* BL21 (DE3) pLysS. The full-length *AtCML8* cDNA clone was amplified by PCR with a forward (5′) primer containing a *Nco*I site (5′-CCATGGA AGAAACAGCACTGAC-3′) and a reverse (3′) primer containing a *Bam*HI site (5′-GGATCCTCAGTCAATGTT GATCATCAT-3′). The PCR product was cloned in *pGEM-T Easy* Vector (Promega, Madison, WI) and sequenced to verify no PCR mistake. The cDNA was subcloned *Nco*I and *Bam*HI site of the *pET-3d* expression vector (NOVAGEN). Recombinant AtCML8 protein was expressed in *E. coli* and purified by phenyl-Sepharose column chromatography (Amersham Bioscience) as described (Harmon et al. 1984). Protein concentration was determined using Protein assay kit (Bio-Rad) with bovine serum albumin and bovine calmodulin (σ) as standards.

Reverse transcription (RT)-PCR analysis

RT–PCR analysis was carried out as previously described (Hwang et al. 2009). cDNA was prepared from each tissue; roots, rosette leaves, stem, cauline leaves, and flowers. Primers for the PCR were: 5′-ATGGAAGAAACAGCAC TGACAAAA-3′ and 5′-TCAGTCAATGTTGATCATCAT CTT-3′ for *AtCML8*, and 5′-CCAACAACGTGAAATCG

ACAG-3′ and 5′-TCTTGGTATTGCTGGTACTCT-3′ for the internal standard *tubulin2* (GenBank accession number, M84700). The sizes predicted from amplified products were 456 bp for *AtCML8* and 243 bp for internal standard *tubulin2*. PCR amplifications were carried out in conditions of a 1 min denaturation at 94°C, followed by 25 cycles of 94°C for 40 s, 55°C for 30 s, and 72°C for 40 s. After PCR, 10 μl of each reaction was removed and electrophoresed in 1% agarose gel.

For quantitative real-time PCR, total RNAs were isolated from 10-day-old *Arabidopsis* plants treated with 0.5 mM SA (salicylic acid) and 100 mM NaCl. RNA samples were treated with DNase I at 37°C for 30 min. cDNA synthesis was performed using the SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The following primers were used for the quantitative real-time PCR: *AtCML8*: 5′-CAAC AGTGATCCGTTCGTTG-3′ and 5′-TCTTCCTCCGCAT CACTTTC-3′; *tubulin2*: 5′-TGGCATCAACTTTCATTGG A-3′ and 5′-ATGTTGCTCTCCGCTTCTGT-3′. The *tubulin2* was used as a control to normalize the expression data. Quantitative real-time RCRs were performed in triplicate with a Bio-Rad CFX96^{TM} Real-time system (http://www.bio-rad.com/). Data were analyzed with Bio-Rad CFX MANAGER software ($2^{-\Delta\Delta Ct}$ method).

$^{45}Ca^{2+}$ binding assay

GST and CaMs purified by phenyl-Sepharose column chromatography were electrophoresed on two independent SDS-denaturing gels. One gel was stained and the second gel was blotted onto PVDF membrane (Millipore). The blot was incubated in binding buffer (60 mM KCl, 5 mM $MgCl_2$, and 10 mM imidazole–HCl, pH 6.8) containing 1 μCi $^{45}Ca^{2+}$ (Maruyama et al. 1984). After washing, the blot was exposed and visualized by X-ray film.

Immunoblot analysis

To determine antibody cross-reactions among AtCML8, GmCaM1, GmCaM4 and bovine brain CaM, purified anti-GmCaM1 and anti-GmCaM4 antibodies were used in immunoblot experiments. Purified AtCML8 protein, GmCaM proteins, and bovine brain CaM were electrophoresed on 13.5% SDS–polyacrylamide gels in the presence of either 5 mM EGTA or 5 mM $CaCl_2$ in the SDS sample buffer. Proteins were transferred onto a PVDF membrane (Millipore) and incubated either with anti-GmCaM1 or with anti-GmCaM4 antibody. Protein bands were detected using the ECL system (Amersham Pharmacia Biotech) after incubating with horseradish peroxidase-conjugated mouse anti-goat IgG antibody (Pierce).

Calmodulin-dependent enzyme assays

Phosphodiesterase (PDE) activity was assayed using commercially available bovine heart CaM-deficient phosphodiesterase (Roche Applied Science) as described (Schechtele and Marme 1988). NAD kinase was partially purified from pea seedlings by successive protamine sulfate precipitation, polyethylene glycol precipitation, and DEAE-Sephacel column chromatography as previously described (Muto and Miyachi 1977). Effluents from the DEAE-Sephacel column were used for NAD kinase assays without further purification. NAD kinase assay was done as described (Harmon et al. 1984) with varying amount of activator CaMs. As controls, NAD kinase activation was examined with reaction mixtures either in the presence of 5 mM EGTA or absence of exogenous activator CaM to verify that our NAD kinase preparation was free of endogenous CaM contamination and that the activation of NAD kinase was a calcium-dependent process. To determine activation parameters, curve fittings were done by the aid of the GraFit software through a non-linear regression analysis with a modified Hill equation (Lee et al. 1995).

$$v = V_{\max}[\text{CaM}]^n / \{(K_{act})^n + [\text{CaM}]^n\}$$

where v is the observed rate, $V_{\max}$ is the maximal activity, [CaM] is the concentration of added CaM, K_{act} is the concentration of CaM required for half-maximal activity, and n is the Hill coefficient.

Results

Comparison of the AtCML8 amino acid sequence

To understand the biological significance and molecular genetic characteristics of CaM-like protein, we cloned a CaM-like gene cDNA from *Arabidopsis* by polymerase chain reaction. Recently, the cDNA clone had been renamed as *AtCML8* (McCormack and Braam 2003). The *AtCML8* cDNA encodes 150 amino acids, whereas other conserved CaMs have 148 amino acid residues. AtCML8 has been shown to exhibit high amino acid sequence identity of 85.4% with a CaM-like protein, AtCML11 from *Arabidopsis* (McCormack and Braam 2003), and amino acid identity of 81% with GmCaM4 from soybean (Lee et al. 1995). Because the biochemical activity of AtCML11 has not been characterized as CaMs, the AtCML8 protein sequence was aligned with those of well-characterized CaMs such as AtCaM2, GmCaM1, and GmCaM4 (Fig. 1a). In order to visualize the primary sequence relationship of AtCML8 with other known CaMs, a phylogenetic analysis with 13 other CaMs was performed (Fig. 1b). The phylogenetic tree shows a close relationship of

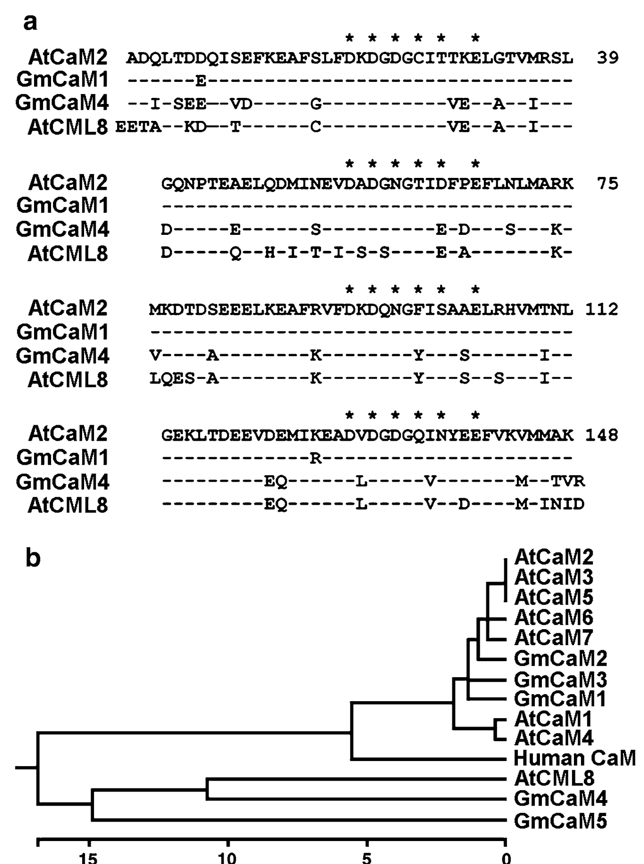

Fig. 1 Comparison of the deduced amino acid sequences of AtCML8 and other CaMs. **a** Sequence alignment of AtCML8 with other divergent soybean CaMs (GmCaM4/5) and conserved *Arabidopsis* CaM (AtCaM2). Sequences are arranged to illustrate the relationships among the four Ca^{2+}-binding domains. Identical amino acids are indicated by a dash (–). Residues marked with asterisks (*) correspond to ones that act as Ca^{2+}-binding motifs, EF-hands, in conserved CaM proteins. **b** Phylogenetic relationships of AtCML8 and CaM proteins. Thirteen CaMs and CML8 amino acid sequences from *Arabidopsis*, soybean, and human were compared to construct a phylogenetic tree by neighbor joining method

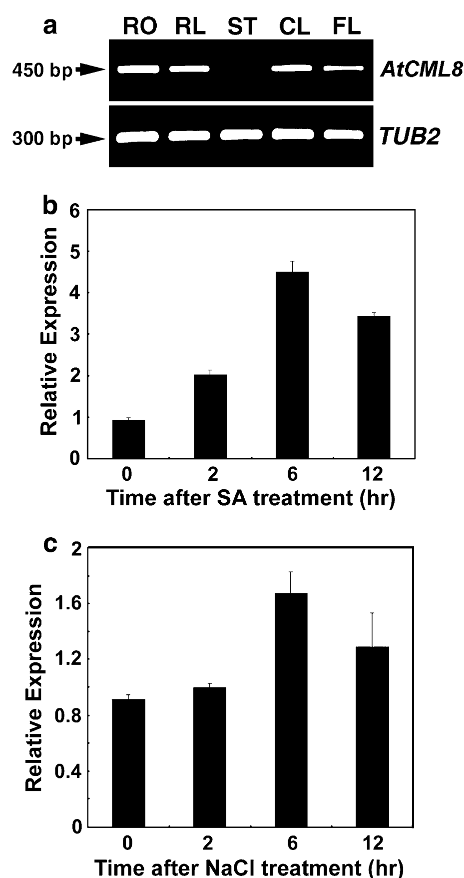

Fig. 2 Expression analysis of *AtCML8* gene in *Arabidopsis* tissues and in response to environmental stresses. **a** Expression patterns of *AtCML8* gene in *Arabidopsis* tissues. The cDNAs were synthesized from total RNAs of each tissue, *RO* root, *RL* rosette leaves, *ST* stem, *CL* cauline leaves, and *FL* flower of 3 weeks grown plants. Subsequently, polymerase chain reactions (PCRs) were performed using gene-specific oligonucleotides primers. Amplified products were separated on a 1% agarose gel. Amplified products of the *tubulin2* were used for quantitative controls. The sizes of PCR products are shown on the left. **b** qRT-PCR analysis of *AtCML8* gene expression during 0.5 mM salicylic acid treatment for 0, 2, 6, and 12 h. **c** qRT-PCR analysis of *AtCML8* gene expression during 100 mM NaCl treatment for 0, 2, 6, and 12 h. In **b** and **c**, *tubulin2* was used as an internal control to normalize the expression data. Data represent the average of three independent experiments ± SD

AtCML8 with GmCaM4 and GmCaM5 among the plant CaMs.

Gene expression of *AtCML8* in various tissues and in response to environmental stresses

Several reports have explored the expression patterns of CaMs and CaM-like proteins in different tissues and in the response to environmental stresses (Takezawa et al. 1995; Yang et al. 1998; Lee et al. 1999; McCormack et al. 2005). In order to observe the relative expression level of *AtCML8*, RT–PCR analysis was performed. The amplified products by RT–PCR were 450 bp for *AtCML8* and 300 bp for *Tubulin2*, respectively. As shown in Fig. 2a, the *AtCML8* gene was highly expressed in root (RO), leaves (RL and CL) and flower (FL). In stems (ST), however, the transcripts of *AtCML8* were detected only at very low level. In order to investigate whether expression of the *AtCML8* gene could be induced in response to biotic or abiotic stimuli, we examined the expression patterns in salicylic acid (SA, inducer of PR proteins) and NaCl-treated *Arabidopsis* plants by quantitative RT–PCR analysis. As shown in Fig. 2b and c, treatments of the plants with SA or NaCl resulted in increased transcripts of the *tCML8* gene. Especially, SA treatment showed high increase in relative mRNA levels (Fig. 2b). Therefore, these results imply that AtCML8 may be involved in response to pathogen attack and NaCl stress, as it is known for GmCaM4/5 in soybean.

Basic characteristics of AtCML8 as a calmodulin-like protein

In order to determine the characteristics of an *Arabidopsis* CML8, we expressed AtCML8 in *E. coli,* and purified the protein to homogeneity by Ca^{2+}-dependent hydrophobic interaction chromatography. During the purification, the AtCML8 protein behaved in a fashion similar to that observed with other CaM isoforms with respect to heat stability and elution profiles on a phenyl-Sepharose column. To test the characteristic Ca^{2+}-binding pattern of the calmodulin-like protein, we investigated the electrophoretic mobility shift of the AtCML8 protein upon Ca^{2+} binding. Purified proteins were electrophoretically separated in SDS gels in the presence of either 5 mM CaCl2 or 5 mM EGTA in sample buffer (Fig. 3a). AtCML8 protein showed a Ca^{2+}-dependent electrophoretic mobility shift, although the extent of this shift was different among calmodulin isoforms. In order to investigate further the Ca^{2+}-binding ability of AtCML8, $^{45}Ca^{2+}$-binding assays were carried out (Fig. 3b). Purified proteins were electrophoresed on an SDS gels and stained or blotted onto membranes. The membranes were overlaid with a buffer containing $^{45}Ca^{2+}$ and subsequently washed and exposed to X-ray film. As a result, calcium was bound to AtCML8 and GmCaM1 but not bound to GST.

To investigate antigenic characteristics of AtCML8, we performed western blot analysis by using anti-GmCaM1 and anti-GmCaM4 antibodies. Previously, we had shown antigenic differences between conserved type CaM (GmCaM1 and bovine CaM) and divergent type CaM (GmCaM4) by using specific goat antisera raised against GmCaM1 and GmCaM4 (Lee et al. 1995). As shown in Fig. 4, anti-GmCaM4 antiserum recognized GmCaM-4 and AtCML8 as expected, but not GmCaM1 and bovine CaM. In contrast, anti-GmCaM1 antiserum recognized GmCaM1 and bovine CaM, but not GmCaM4 and AtCML8. These results suggest that AtCML8 has similar antigenic characteristic as the divergent CaM isoform, GmCaM4 in *Arabidopsis.*

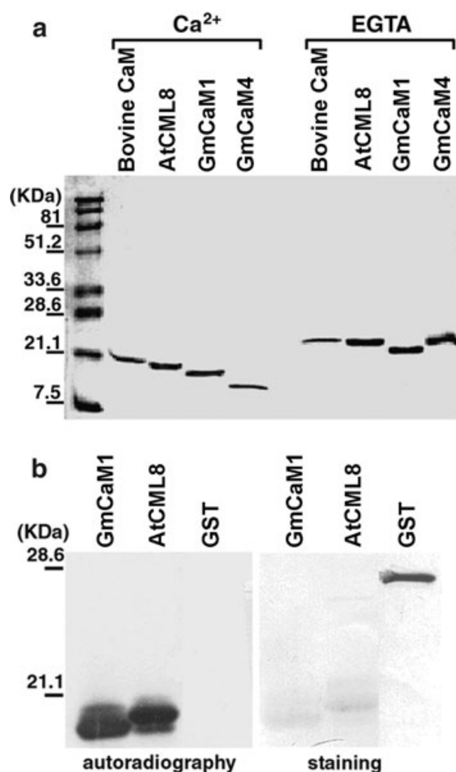

Fig. 3 Ca^{2+}-binding ability of AtCML8. **a** Ca^{2+}-dependent electrophoretic mobility shifts of AtCML8. AtCML8, GmCaM1, and four proteins were produced in *E. coli* using a T7 expression vector system and purified to homogeneity using phenyl-Sepharose column chromatography as described under "Materials and methods." 2 μg of purified CaMs, CML protein and bovine brain calmodulin (σ) was electrophoresed on a 13.5% SDS–PAGE either in the presence of 5 mM $CaCl_2$ or 5 mM EGTA in sample buffers. Protein bands were visualized by Coomassie Brilliant Blue staining. **b** $^{45}Ca^{2+}$ binding assay. Protein samples were separated by SDS–PAGE, and either stained with Coomassie Brilliant Blue (*right panel*) or overlaid and autoradiographed with $^{45}Ca^{2+}$ (*left panel*). GmCaM-1 and GST are a positive control and negative control for $^{45}Ca^{2+}$ binding assay, respectively

Differential activation of phosphodiesterase and NAD kinase by AtCML8

To assess the biochemical ability of the AtCML8 to activate CaM-dependent enzymes, two representative

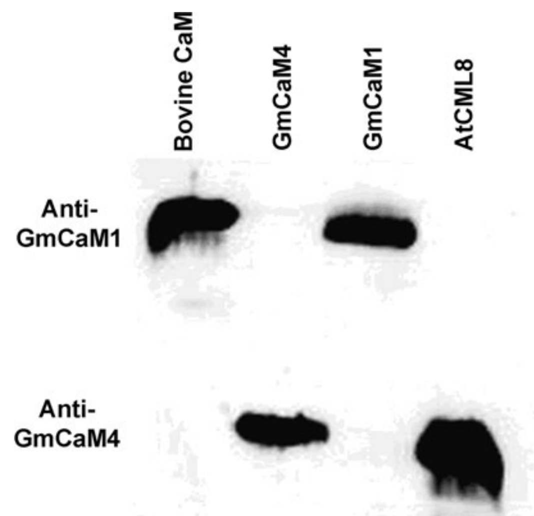

Fig. 4 Immunoblot analysis of purified AtCML8 protein with antibodies raised against GmCaM1 or GmCaM4 proteins. Equal amount of GmCaM1/4 proteins, bovine CaM, and AtCML8 (25 ng per lane) was electrophoresed on a 13.5% SDS–PAGE. Fractionated proteins were blotted onto a PVDF membrane and incubated with purified either anti-GmCaM1 or anti-GmCaM4 antisera. The immune complexes were visualized by using the ECL system after incubating with horseradish peroxidase-conjugated anti-goat IgG

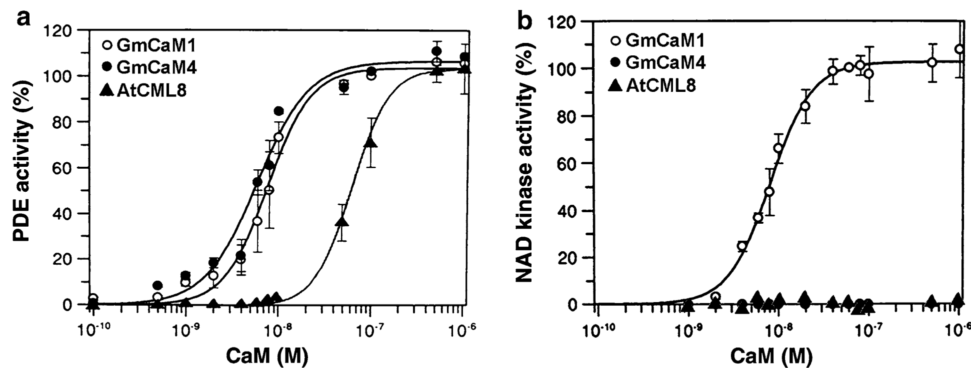

Fig. 5 Activation of calmodulin-dependent enzymes by CaMs and AtCML8. **a** Activation of phosphodiesterase (PDE) by CaMs and AtCML8. Dose/response curves of PDE are shown. Data points represent means of three independent assays for PDE, and error bars represent standard deviations. Fitted curves are drawn from the Hill equation as described under "Materials and methods." PDE assay was done using CaM-deficient bovine heart PDE and CaMs or CML (GmCaM1/4 and AtCML8) as activators. Activity of PDE was monitored with varying amounts of CaMs or AtCML8 and expressed as a relative activity to that of PDE in the presence of 100 nM activator CaMs or CML (GmCaM1/4 and AtCML8). **b** Differential activation of NAD kinase by CaMs or AtCML8. Dose/Response curves of NAD kinase are shown. Data points represent means of three independent assays for NAD kinase, and error bars represent standard deviations. The activity of pea NAD kinase is expressed as the activity relative to that of NAD kinase in the presence of 80 nM of isolated CaMs or CML (GmCaM1/4, and AtCML8)

CaM-dependent enzymes, phosphodiesterase (PDE) and NAD kinase, were tested. In PDE enzyme assay, not only GmCaM1 and GmCaM4 but also AtCML8 activated bovine brain PDE well (Fig. 5a). The half-maximal activation value (K_{act}) of AtCML8 was 90.01 nM, which is higher than those of GmCaM1 and 4. However, the maximal activation value (V_{max}) of two GmCaM isoforms and AtCML8 for PDE were not significantly different from each other. In the presence of EGTA, PDE enzyme did not show activity regardless of the presence of activator, AtCML8 (data not shown). This result indicates that the activation of the enzyme by AtCML8 is a calcium-dependent process.

In NAD kinase assays, we observed GmCaM1-activated NAD kinase with a K_{act} (8.07 nM) and Hill coefficient (1.86) in the presence of Ca^{2+} but not in the absence of Ca^{2+}. However, the divergent AtCML8 did not activate NAD kinase, similar to the behavior of GmCaM4, even at 500-fold higher concentration than that of GmCaM1 for a maximal activation of NAD kinase (Fig. 5b). The results strongly indicated that AtCML8 is a functional CML protein and may be an ortholog of GmCaM4 at the biochemical level in *Arabidopsis*.

Discussion

Calmodulins are well known as highly conserved calcium-binding proteins in eukaryotes (Chin and Means 2000; DeFalco et al. 2010). Many studies in plants revealed the presence of multiple calmodulin isoforms and CML proteins (McCormack et al. 2005). Although the Ca^{2+}/calmodulin mediated signal transduction mechanism of plants is very similar to that of animals, the presence of multiple CaMs and CMLs implicates that the plant proteins may have unique features (Snedden and Fromm 2001; Sanders et al. 2002; McCormack and Braam 2003; Bouché et al. 2005). One group of conserved plant CaMs shows identities of 95% or higher when compared with animal CaMs. However, a set of divergent plant CaMs shows less than 75% identity with the conserved CaMs in both animals and plants. In this study, the *AtCML8* cDNA was shown to encode a CML protein in *Arabidopsis* when it was isolated and characterized at the biochemical level.

The comparison of amino acid sequences of CaMs in *Arabidopsis* showed that all CaMs except AtCML8, AtCML9 (At3g51920) and other CMLs exhibit higher than 90% identity to each other. In contrast, AtCML8 shares less than 72% identity with the AtCaMs of the highly conserved type. When comparing AtCML8 and AtCaM2, one difference is that Tyr^{99} of AtCML8 was substituted by Phe^{99} of AtCaM2 in the third Ca^{2+}-binding EF-hand domain (Fig. 1a). This Tyr^{99} residue was only found in the plant divergent CaMs, GmCaM4, GmCaM5 and NtCaM13 (Yamakawa et al. 2001). Like bovine and human CaMs, this Tyr^{99} is thought to be important for the regulation of the calmodulin by phosphorylation. Other telltale substitutions were changed from Gly^{40} in AtCaM2 to Asp^{40} in AtCML8, and from Ser^{81} in AtCaM2 to Ala^{81} in AtCML8. These residues are known as essential residues promoting helix bending (Marmé and Dieter 1983). Although the amino acid sequences of AtCML8 diverge considerably from that of AtCaM2, the positions of strong hydrophobic residues in the protein are highly conserved (Zielinski 2002).

Zielinski (2002) reported that AtCaM-8 (AtCML8) was identified as a CML protein based on sequence comparisons, when expressed in yeast conferred reduced rates of growth in Ca^{2+}-depleted media to the yeast cells. In addition, AtCaM-8 (AtCML8) showed different target specificities when compared with those of highly conserved AtCaMs (Köhler and Neuhaus 2000; Charpenteau et al. 2004). However, no direct evidence was provided if AtCML8 constituted a functional CML protein. Here, the AtCML8 protein is identified as a genuine Ca^{2+}-binding sensor protein by providing evidence not only through the observation of the Ca^{2+}-dependent electrophoretic mobility shift assay but also by $^{45}Ca^{2+}$-binding assays (Fig. 3). Also, the 17 kDa protein in *Arabidopsis* was recognized immunologically by a GmCaM4 specific antibody (Lee et al. 1995). We show that AtCML8 is recognized by a GmCaM4 specific antibody but not by GmCaM1 specific antibody (Fig. 4).

CaMs and CMLs could have different functions based on target specificity, distinct subcellular localization, and different Ca^{2+} affinity (Hoeflich and Ikura 2002; McCormack and Braam 2003). The binding of Ca^{2+} to CaM is well known to evoke a conformational change of CaM that results in a more compact structure. The structural change of CaM by Ca^{2+} exposes its hydrophobic surface that serves to interact with and alter the activities of target proteins. We previously reported that CaM isoforms exhibited differences in the Ca^{2+} dependence of activation of target enzymes and activated differentially and/or reciprocally. Two soybean CaM isoforms (GmCaM1/4) exhibited the opposite activation and competitive inhibition of CaM target enzymes (Lee et al. 1995; Lee et al. 2000).

Recently, Magnan et al. (2008) reported that *AtCML9* gene was induced in the response to various environmental stimuli. Specifically, the protein was shown to participate in salt stress tolerance through its effects on ABA-mediated pathways. To identify the physiological function of *AtCML8,* we investigated not only AtCML8 T-DNA insertion mutants but also AtCML8 over-expressing transgenic plants. However, we could not detect any phenotype either during normal growth or under stressed conditions. These results imply that, in *Arabidopsis*, other CML isoforms are able to compensate the function of AtCML8 through gene redundancy, or that the AtCML8 protein is controlled by fine-tuning of its activation but not by its quantity.

Here, we report that the biochemical activity of AtCML8 from *Arabidopsis* is similar to that of GmCaM4, a known divergent CaM from soybean. The characterization of divergent CaM isoforms in *Arabidopsis* is a requisite for identifying its biological functions in plant Ca^{2+}-signaling pathways. The model character of *Arabidopsis thaliana* and the available transgenic and mutational tools will help in studying biological functions in calcium signaling of the CaMs that have evolved into a plant-specific function.

Acknowledgments We thank Dr. Hans J. Bohnert for critical reading and insightful comments. This work was supported by grants from World Class University Program (R32-10148) funded by MOEST and by BioGreen 21 Program (20080401034023) funded by RDA. KEK was supported by scholarship from the BK21 program of MOEST.

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