Characterisation of calmodulin binding to cyclic nucleotide-gated ion channels from *Arabidopsis thaliana*

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Abstract The recently identified cyclic nucleotide-gated ion channels (AtCNGCs) from *Arabidopsis thaliana* have the ability to bind calmodulin. Using two different methods, we mapped the binding site of AtCNGC1 to the last predicted α helix of the cyclic nucleotide binding domain. This is in contrast to CNGCs from animals, where the calmodulin binding site is located in the N-terminus, implying that different mechanisms for CNGC modulation have evolved in animals and plants. Furthermore, we demonstrate that AtCNGC1 and AtCNGC2 have different calmodulin binding affinities and we provide evidence for target specificities among calmodulin isoforms.

Key words: Cyclic nucleotide-gated ion channel; Calmodulin binding site; Non-radioactive calmodulin overlay assay; Calmodulin isoform; Two-hybrid assay; *Arabidopsis thaliana*

1. Introduction

Recently, ion channels displaying characteristic features of cyclic nucleotide-gated ion channels (CNGCs) from animals have been described in plants [1–4]. They possess a *Shaker*-like structure with six membrane spanning domains, contain a cyclic nucleotide binding domain located in the C-terminus and a pore region with some characteristics of a K^+ channel pore but without the highly conserved signature sequence determining K^+ selectivity. The CNGCs from *Arabidopsis* comprise a large gene family. So far, six members have been described (*AtCNGC* 1–6) and several homologous sequences can be found in public databases [3]. In animals CNGCs are best characterised in photoreceptors and olfactory neurones, where they are involved in signalling pathways activated by light and odorants, respectively [5]. Animal CNGCs are permeable for monovalent cations and Ca^{2+} [6]. Electrophysiological characterisations of one member of the CNGCs from *Arabidopsis thaliana* (AtCNGC2) indicate that these plant homologues are permeable for K^+ and Ca^{2+} as well [7].

In animals Ca^{2+}/calmodulin attenuates the activity of CNGCs by increasing their apparent affinity for cyclic nucleotides [8,9]. For CNGCs of olfactory neurones (olfactory channels) it was demonstrated that calmodulin interferes with the interaction between the N- and C-terminal regions and thereby exerts its influence on channel activity [10]. In heteromeric CNGCs of retinal rod photoreceptors (rod channels) only the β subunit has the ability to bind calmodulin. This subunit, which has no functional channel characteristics per se, can modulate the activity of the α subunit in a Ca^{2+}-dependent manner [8,11–13].

In *Arabidopsis* multiple calmodulin isoforms have been identified (AtCaM1–6). This is in contrast to the situation in animals, where a single form of calmodulin is produced by a multigene family [14]. Among the highly conserved AtCaM isoforms only six different amino acid residues can be found [15,16]. Recently two novel, more divergent isoforms have been submitted to the public database (AtCaM8 and AtCaM9). Using yeast two-hybrid analysis we have previously demonstrated that AtCNGC1 and AtCNGC2 have the ability to bind to AtCaM2 and AtCaM4 [3]. Interestingly, the calmodulin binding site seemed to be located in the C-terminus, whereas in animal CNGCs the calmodulin binding site is localised in the N-terminus.

In this study we determined the location of the calmodulin binding site of AtCNGC1 in detail. In addition to the yeast two-hybrid analysis, we utilised a new technique, combining affinity purification of overexpressed calmodulins and affinity detection on far-Western blots, allowing a fast and non-radioactive detection of calmodulin binding proteins. Furthermore, we analysed whether different calmodulin isoforms from *Arabidopsis* have distinct binding properties for the C-termini of AtCNGC1 and AtCNGC2. To this end we performed quantitative yeast two-hybrid analysis and included highly divergent calmodulin isoforms in this investigation.

2. Materials and methods

2.1. Protein purification

The C-terminal 236 amino acids of AtCNGC1 were inserted into the vector pQE 30 (Qiagen) yielding an in-frame fusion with six histidine residues. The deletion of the calmodulin binding site (F602–Y617) was made by PCR introducing a unique *Sph*I site and the product was confirmed by sequencing. The histidine-tagged fusion proteins were purified under denaturing conditions using a Ni-NTA column as described by the manufacturer (Qiagen).

The cDNAs of *AtCaM8* (accession number: AF178074), *AtCaM2* and *AtCaM4* [16] were amplified by PCR from a Matchmaker cDNA library (Clontech). The cDNA of *AtCaM9* (accession number: AF178075) was amplified by RT-PCR using inflorescence RNA from *Arabidopsis* as template. The PCR fragments were inserted into the vector pASK-IBA3 (Institute for Bioanalytic GmbH, Germany) producing a C-terminal fusion with the *Strep*-tag II. Purification of native proteins was performed using a *Strep*-Tactin column according to the instructions of the manufacturer (Institute for Bioanalytic GmbH).

2.2. Strep-tagged calmodulin overlay

Proteins were resolved on a 12% SDS–PAGE and electrophoretically transferred onto Immobilon membranes. Membranes were blocked with 0.1 M NaCl, 50 mM Tris–HCl, pH 7.5, 1 mg/ml bovine serum albumin, 0.05% Tween 20 in the presence of either 5 mM $CaCl_2$

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or 5 mM EGTA for 30 min at room temperature. *Strep*-tagged calmodulin was added at a final concentration of 0.1 μg/ml in the above buffer and was incubated for 30 min. After washing with the above buffer, the membranes were incubated in a 1:4000 dilution of streptavidin conjugated with horseradish peroxidase (Institute for Bioanalytic GmbH) in the presence of either 5 mM $CaCl_2$ or 5 mM EGTA. The membranes were washed in the above buffer and then detected with the ECL detection kit (Amersham).

2.3. Two-hybrid interaction tests and ONPG assays

Constructs pGBT9-AtCNGC1 and pGBT9-AtCNGC2 were as described [3]. The cDNAs for AtCaM2, AtCaM4, AtCaM8 and AtCaM9 were inserted into the vector pGAD424 (Clontech). Interaction tests between different constructs were performed by co-transformation into yeast strain HF7c (Clontech). His^+ colonies were assayed for β-galactosidase activity using the substrate ONPG as described by the manufacturer (Clontech).

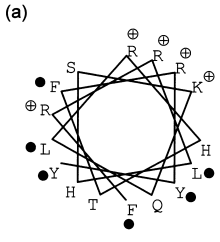
3. Results

3.1. Mapping of the CaM binding site of AtCNGC1

Using yeast two-hybrid analysis we have previously shown that the C-terminus of AtCNGC1 has the ability to bind calmodulin [3]. This location was unexpected as in CNGCs from animals the binding site for calmodulin is localised in the N-terminus. Calmodulin binding sites encompass segments of 14 amino acid residues that are characterised by long hydrophobic or aromatic residues at positions 1 and 14 and the ability to adopt the conformation of a basic amphiphilic α helix [17]. As the exact localisation of the calmodulin binding site may give important insights into the regulation of the AtCNGCs in plants we searched for motifs which conform to features of calmodulin binding sites. One motif was identified (F602–Y617) that shows a potential basic amphiphilic structure (Fig. 1a). However, this motif contains an arginine (R615) at relative position 14, which is atypical of a conventional calmodulin binding motif.

Sequence comparison of the C-terminal region of AtCNGC1 with the catabolite gene activator protein from *Escherichia coli* showed that the identified calmodulin binding motif is localised within the last predicted α helix of the putative cyclic nucleotide binding domain [3]. Putative calmodulin binding sites are present at the corresponding positions in different members of the AtCNGC gene family [3] as well as in homologous proteins from barley (HvCBT1) [1] and tobacco (NtCBT4) [4] (Fig. 1b).

Consequently, we tested whether this motif is indeed responsible for calmodulin binding. For this purpose we deleted residues F602–Y617 and investigated the ability of calmodulins AtCaM2 and AtCaM4 to bind to the modified C-terminus of AtCNGC1 (AtCNGC1*) using quantitative two-hybrid



(b)

AtCNGC1 602- FR-RLHSKQLRHTFRYY -617
AtCNGC2 645- FRYKFANERLKRTARYY -661
AtCNGC3 591- YR-RLHSKQLRHMFRFY -606
AtCNGC4 610- FRYTFVNEKVKRSARYY -626
HvCBT1 589- FR-RLHSKQLRHTFRFY -604
NtCBP4 595- FR-RLHSKQLRHTFRFY -610

Fig. 1. a: Helical wheel projection of the calmodulin binding site of AtCNGC1, showing the segregation of basic (+) and hydrophobic (●) residues to opposite sides of the helix. b: Comparison of the calmodulin binding sites of AtCNGC1–4 [3], HvCBT1 [1] and NtCBT4 [4]. Basic residues are highlighted in bold.

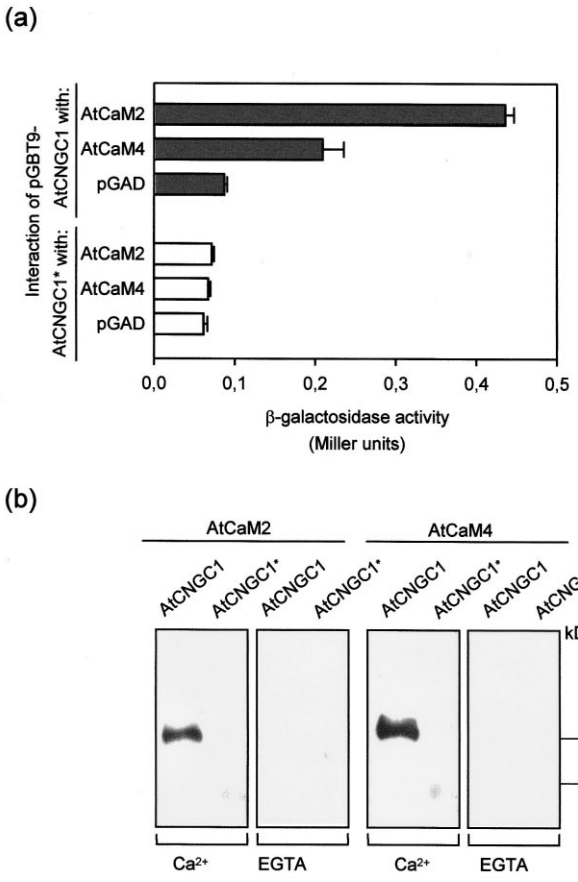


Fig. 2. Deletion of the calmodulin binding site of AtCNGC1 abolished binding of calmodulin. a: Quantitative two-hybrid analysis of interaction of AtCNGC1 C-terminal peptides with AtCaM2 and AtCaM4 in yeast. AtCNGC1* corresponds to the AtCNGC1 C-terminal peptide with the calmodulin binding site deleted. The graphs indicate the β-galactosidase reporter activity in yeast cells corresponding to the combinations of transformed constructs. For each combination at least six assays were performed. Error bars represent S.E.M. b: Calmodulin overlay analysis with *Strep*-tagged calmodulins and the purified C-terminal peptides of AtCNGC1 and AtCNGC1*. The calculated M_rs of the AtCNGC1 and AtCNGC1* C-terminal peptides are 29 and 26.8 kDa, respectively. Proteins (0.5 μg) were separated by SDS–PAGE, subjected to Western blot and incubated with *Strep*-tagged calmodulin in the presence of either 5 mM $CaCl_2$ or 5 mM EGTA.

analysis. The β-galactosidase activity mediated by co-expression of AtCNGC1* and either AtCaM2 or AtCaM4 was not significantly higher than the background activity established by the empty vector pGAD alone (Fig. 2a). In addition, we tested calmodulin binding in vitro. For this purpose we developed a novel system combining affinity purification of calmodulins with affinity detection on far-Western blots. The cDNAs of both calmodulins were fused with a *Strep*-tag, expressed in *E. coli* and purified to homogeneity using a *Strep*-tag affinity column. The *Strep*-tag can bind to streptavidin conjugated with horseradish peroxidase providing a sensitive and non-radioactive alternative for the detection of calmodulin binding proteins. The results from overlay experiments using these *Strep*-tagged AtCaM2 and AtCaM4 proteins showed that the calmodulin binding to the C-terminus of AtCNGC1 is Ca^{2+}-dependent and that deletion of residues F602–Y616 abolished this interaction, indicating that this site is indeed the binding site for calmodulin (Fig. 2b).

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                                * * * * *  *       39
AtCaM2: MAD-QLTDDQISEFKEAFSLFDKDGDGCITTKELGTVMRSL
AtCaM4: --------E-------------------------------
AtCaM8: -EETA-K----T------C-----------VE--A--I---
AtCaM9: ----AF--E--Q--Y---C-I---S--F--KEK-TK--K-M
SCaM4 : --I---SEE--VD-----G-----------VE--A--I---
SCaM5 : --V---SEE----I----G-----------VD-FV--I---

                                * * * * *  *       75
AtCaM2:       GQNPTEAELQDMINEVDADGNGTIDFPEFLNLMARK
AtCaM4:       ----------------------------------K-
AtCaM8:       D-----Q--H-I-T-I-S-S----E-A-------K-
AtCaM9:       -K--KAEQ--Q-MSD--IF---G-T-DD--YI--QN
SCaM4 :       D-----E------S----------E-D---S---K-
SCaM5 :       V-----E----------------E-V-------K-

                                * * * * *  *      112
AtCaM2:      MKDTDSEEELKEAFRVFDKDQNGFISAAELRHVMTNL
AtCaM4:      -------------------------------------
AtCaM8:      LQES-A--------K--------Y---S--S---I--
AtCaM9:      TSQESASD--I-V-----R-GD-L--QL--GEG-KDM
SCaM4 :      V----A--------K--------Y---S------I--
SCaM5 :      --E--E--D-----K--------Y---S------I--

                                * * * * *  *      149
AtCaM2:      GEKLTDEEVDEMIKEADVDGDGQINYEEFVKVMMAK
AtCaM4:      ---------E---R----------H----------
AtCaM8       ---------EQ------L-----V--D----M-INID
AtCaM9:      -M-I-A--AEH-VR---L----FLSFH--S-M-I-ASY
SCaM4 :      ---------EQ------L-----V-------M--TVR
SCaM5 :      ---------EQ--E---L-----V--D----M--TIG
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Fig. 3. Comparison of amino acid sequences of the conserved *Arabidopsis* calmodulins AtCaM2 and AtCaM4 with the diverged calmodulins AtCaM8 and AtCaM9 from *Arabidopsis* and SCaM4 and SCaM5 from soybean. The Ca^{2+} binding ligands are denoted by asterisks, dashes indicate amino acids identical to those of AtCaM2. GenBank accession numbers: AtCaM2 (M38380), AtCaM4 (Z12022), AtCaM8 (AF178074), AtCaM9 (AF178075), SCaM4 (L01433), SCaM5 (L19359).

3.2. Two diverged calmodulins (AtCaM8 and AtCaM9) have no ability to bind to AtCNGC1 and AtCNGC2

In the previously performed two-hybrid screen we have identified AtCaM2 and AtCaM4 as interacting partners for AtCNGC1 and AtCNGC2 [3]. This screen was not saturating, covering about one fifth of independent clones present in the library. Therefore, we tested whether other AtCaM isoforms have the ability to interact with AtCNGC1 and AtCNGC2 as well. For this investigation we chose two novel calmodulin isoforms (AtCaM8 and AtCaM9), which differ considerably from the conserved *Arabidopsis* calmodulins, exhibiting only ~73% and ~53% sequence identity, respectively. Both isoforms are more similar to the divergent isoforms from soybean SCaM4 and SCaM5 [18] than to the conserved isoforms from *Arabidopsis*. A sequence comparison of AtCaM8 and AtCaM9 with the conserved isoforms from *Arabidopsis* AtCaM2 and AtCaM4 and with SCaM4 and SCaM5 is shown in Fig. 3. Because AtCaM8 and AtCaM9 have not yet been biochemically characterised, we investigated the electrophoretic mobility shift of both isoforms upon Ca^{2+} binding that is a typical characteristic of calmodulins [19]. Purified proteins of AtCaM8 and AtCaM9 as well as of AtCaM2 and AtCaM4 showed Ca^{2+}-dependent electrophoretic mobility shifts (Fig. 4a). Interestingly, the extent of the mobility shift was similar among the isoforms, but the mobility of the isoforms itself differed. The molecular weights and isoelectric points of AtCaM2 (16.82; 4.07) and AtCaM4 (16.86; 4.14) as well as AtCaM8 (17.16; 3.97) and AtCaM9 (17.04; 4.15) are nearly identical, indicating that the residual conformation of AtCaM4 and AtCaM9 in sample buffer is possibly more compact, allowing a faster migration in SDS–PAGE.

Using the two-hybrid system we investigated the ability of AtCaM8 and AtCaM9 to interact with the C-termini of AtCNGC1 and AtCNGC2. Fig. 4b shows a growth assay on selective media. While AtCaM2 and AtCaM4 bound efficiently to AtCNGC1 and AtCNGC2, neither AtCaM8 nor AtCaM9 conferred comparable growth to yeast.

3.3. AtCNGC1 and AtCNGC2 have different affinities for AtCaM2 and AtCaM4

To investigate whether AtCaM2 and AtCaM4 have different affinities for the C-termini of AtCNGC1 and AtCNGC2 we analysed the interaction quantitatively by measuring β-galactosidase activity. Fig. 5 shows that the interaction of both isoforms with the C-terminus of AtCNGC2 was much stronger than with that of AtCNGC1 (28-fold for AtCaM2 and 78-fold for AtCaM4). Furthermore, AtCNGC1 and AtCNGC2 had different affinities for both isoforms. AtCNGC1 bound AtCaM2 with higher affinity than AtCaM4, whereas AtCNGC2 had a higher affinity for AtCaM4 than for AtCaM2. For AtCaM8 and AtCaM9 there was no significant

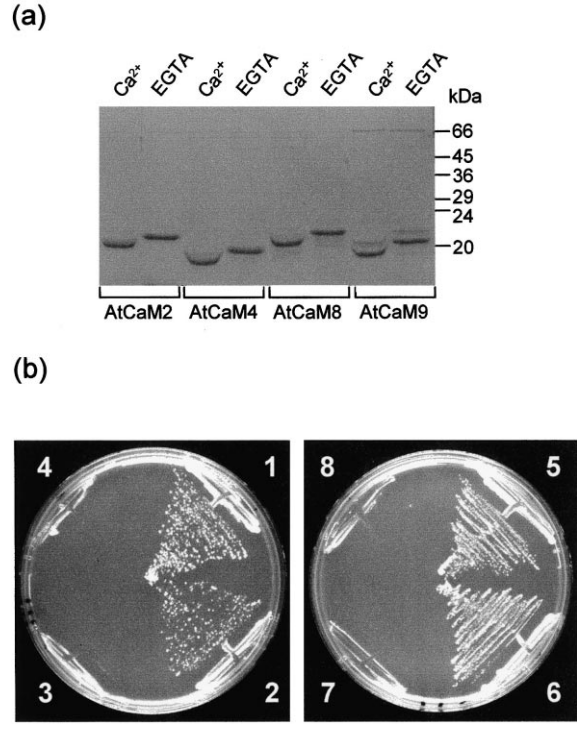

Fig. 4. Calmodulin isoforms AtCaM8 and AtCaM9 show a Ca^{2+}-dependent mobility shift (a), but have no ability to bind to AtCNGC1 and AtCNGC2 (b). a: Purified *Strep*-tagged calmodulin fusion proteins were produced in *E. coli* and purified to homogeneity. Purified calmodulins (1 μg) were separated on 15% SDS–PAGE in the presence of either 5 mM $CaCl_2$ or 5 mM EGTA in sample buffers. Proteins were visualised by Coomassie brilliant blue staining. b: Two-hybrid interaction of AtCNGC1 and AtCNGC2 C-terminal peptides with calmodulins AtCaM2, AtCaM4, AtCaM8 and AtCaM9. Numbers 1–4 indicate interaction of AtCNGC1 with AtCaM2 (1), AtCaM4 (2), AtCaM8 (3) and AtCaM9 (4); numbers 5–8 indicate interaction of AtCNGC2 with AtCaM2 (5), AtCaM4 (6), AtCaM8 (7) and AtCaM9 (8). Interaction tests between different constructs were performed by co-transformation into yeast cells and growth was scored on media lacking histidine.

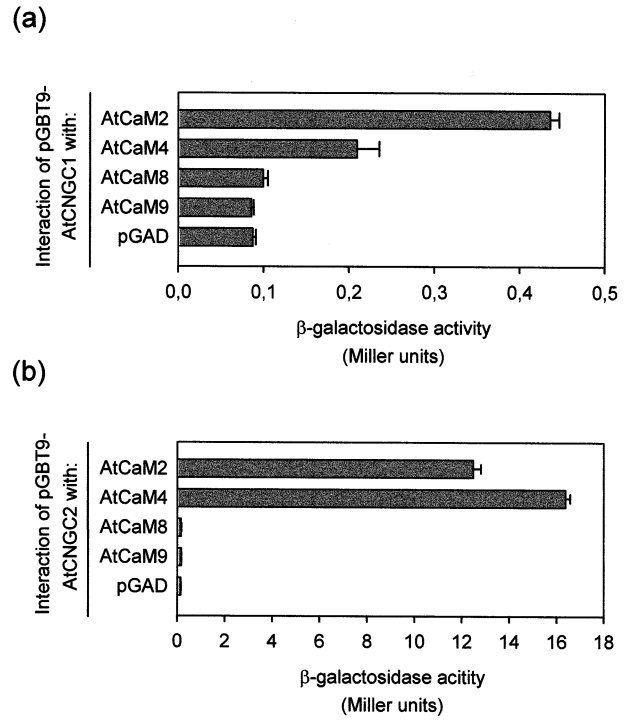

Fig. 5. Quantitative analysis of interactions of AtCNGC1 (a) and AtCNGC2 (b) C-terminal peptides with AtCaM2, AtCaM4, AtCaM8 and AtCaM9 in yeast. The graphs indicate the β-galactosidase reporter activity in yeast cells corresponding to the combinations of transformed constructs. For each combination at least six assays were performed. Error bars represent S.E.M.

increase in β-galactosidase activity detectable, confirming our previous results (Fig. 4b).

4. Discussion

In this study we demonstrate that the calmodulin binding site of AtCNGC1 is localised within the last predicted α helix of the putative cyclic nucleotide binding domain. This is in sharp contrast to CNGCs from animals, where the calmodulin binding site is located in the N-terminus. The overlap of the calmodulin binding site with the putative cyclic nucleotide binding domain suggests that interaction of calmodulin binding and cyclic nucleotide binding is conserved between animals and plants, but relies on different mechanisms. Negative regulation between cyclic nucleotides and calmodulin in olfactory channels depends on the interaction between N-terminus and C-terminus [10]. We did not find an interaction between N- and C-termini of AtCNGC1 in the two-hybrid system (data not shown), supporting the hypothesis that AtCNGC1 is regulated by cyclic nucleotides and calmodulin by a novel mechanism.

The existence of multiple calmodulin isoforms in plants is a further distinct difference with animals, where a single form of calmodulin is produced by a multigene family [14]. The occurrence of divergent calmodulins that are similar in *Arabidopsis* and soybean indicates that calmodulin gene diversification is not restricted to *Arabidopsis* or closely related species. This raises the question whether different calmodulin isoforms are functionally redundant, or whether there are differences in the target proteins for calmodulin in plants. Our investigation using four of the six calmodulin isoforms from *Arabidopsis* provides evidence for a functional divergence. All tested isoforms show a Ca^{2+}-dependent electrophoretic mobility shift, which is a typical characteristic of calmodulins, but AtCaM8 and AtCaM9 fail to bind to AtCNGC1 and AtCNGC2. Further support of functional non-redundancy arises from the observed differences in the binding affinity of AtCaM2 and AtCaM4 to AtCNGC1 or AtCNGC2.

The strong differences in the calmodulin binding affinity of AtCNGC1 and AtCNGC2 provide the first indication for a functional divergence of the AtCNGC family. It appears possible that AtCNGC2 is very sensitive to changes in the Ca^{2+} concentration in the cell, whereas the Ca^{2+} modulation of AtCNGC1 is only weak. The presence of multiple calmodulin isoforms with different target specificities and ion channels with different calmodulin affinities provides a fine-tunable system that allows the plant cell to react very specifically to changes in the Ca^{2+} concentration. Interestingly, different evolutionary strategies for Ca^{2+}/calmodulin signalling seem to have evolved in animals and plants.

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