PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2009, 36(7): 890~896
www.pibb.ac.cn

Site-directed Mutagenesis of *Arabidopsis* Calmodulin Isoform 2 and Its Application in Detecting Calcium-independent Calmodulin-binding Proteins*

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Abstract Not only calmodulin (CaM) with Ca^{2+} regulates the activity of many enzymes and proteins, but also free-CaM (no Ca^{2+} bound) and Ca^{2+}-independent CaM-binding proteins play roles in plant and animal cells. There is no *in vivo* method to identify the interaction between free-CaM and Ca^{2+}-independent CaM-binding protein (CaMBP). Using site-directed mutagenesis by polymerase chain reaction (PCR), 5 mutant *Arabidopsis* calmodulin isoform 2 (*AtCaM2*) genes, $mCaM2_1$, $mCaM2_{12}$, $mCaM2_{123}$, $mCaM2_{124}$ and $mCaM2_{1234}$ were obtained. The mutant *mCaM2* encoded glutamine in place of glutamate (E32Q; E68Q; E105Q; E141Q) in one or more EF-hand Ca^{2+}-binding motifs of AtCaM2. The recombinant mCaM2 proteins were produced in *Escherichia coli*, and subsequently separated on SDS-PAGE in the presence of Ca^{2+} or EGTA, their electrophoresis mobilities were related with that of mutant EF-hand motifs. $^{45}Ca^{2+}$ overlay analysis indicated that the more glutamate replaced by glutamine, the lower affinity with Ca^{2+} in the mCaM2 proteins. The $mCaM2_{1234}$ mutant protein (E32Q; E68Q; E105Q;E141Q) was unable to bind Ca^{2+}. Using yeast two-hybrid technique with $mCaM2_{1234}$ as bait, it was possible to see interaction in *Arabidopsis* of AtCaM2 with IQD26, a calcium-independent CaM-binding protein. Site-directed mutation of AtCaM2 will aid the research of Ca^{2+}, CaM and Ca^{2+}-independent CaMBPs in plant biological processes.

Key words site-directed mutagenesis, *Arabidopsis*, calmodulin, calcium-independent, calmodulin-binding protein
DOI: 10.3724/SP.J.1206.2008.00786

In plants, calcium ion (Ca^{2+}) has important roles in regulating cellular responses to extensive stimuli of both biotic and abiotic stresses[1, 2]. Calmodulin (CaM), which is a highly conserved and heat-stable protein with four EF-hand motifs, is a vital Ca^{2+} sensor. The CaM existence form is different between animal and plant: the genomes of vertebrates contain multiple CaM genes that only encode a single CaM isoform, and the genomes of yeasts and filamentous fungi contain single genes encoding one CaM isoform[3~5]. But in higher plants encode and express a variety of CaM isoforms[6]. In the *Arabidopsis* genome, there are 11 CaM genes that encode at least seven isoforms[7]. This suggests that plants have a more-complex regulatory mechanism for Ca^{2+} -signal *via* Ca^{2+} -CaM than animals do.

In most cases, activated CaM (i.e. Ca^{2+}-CaM) mediates the activity of many CaM-binding proteins (CaMBP), such as protein kinases[8], transcription factors[6], nuclear proteins[9], metabolic enzymes[10], cytoskeleton proteins[11], ion transporters, and channels[12], and this kind of Ca^{2+}-dependent CaMBPs have been well characterized. But there is another CaM existing form, Ca^{2+}-free form (apo-CaM)[13, 14], which can bind another kind of Ca^{2+}-independent CaMBPs. At first Ca^{2+}-independent CaMBPs were studied in animals, including structural proteins, and signaling proteins involved in neurotransmitter production and release, nerve growth, muscle

*This work was supported by grants from Program for New Century Excellent Talents (NCET-06-0256) and National Basic Research Program of China (2006CB910600).
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Received: November 15, 2008 Accepted: January 9, 2009

relaxation, and intracellular movement of organelles along actin filaments[15~17], but in plants they have not receive a great deal of attention. Most of the Ca^{2+}-independent CaMBPs are much less well characterized and its functions are insufficient understood. It was proposed that these Ca^{2+}-independent CaMBPs alter the Ca^{2+}-binding dynamics of free CaM and activated CaM [18]. The Ca^{2+}-independent CaMBPs influence the formation of activated CaM by accelerating the rates of association and dissociation of Ca^{2+} from the free CaM. However the Ca^{2+}-dependent CaMBPs depend on the activation of activated CaM. In this way the Ca^{2+}-independent CaMBPs indirectly regulate the biological function of the Ca^{2+}-dependent CaMBPs[18~20]. But, until now, there was no evidence of an *in vivo* interaction between CaM and Ca^{2+}-independent CaMBPs, which restrict us to elucidate the activities of Ca^{2+}-independent CaMBPs regulated by free-CaM.

In our research we first isolated five mutated *Arabidopsis* calmodulin isoform 2 (*AtCaM2*) genes. Of these, mutant $CaM2_{1234}$ ($mCaM2_{1234}$) (E32Q; E68Q; E105Q; E141Q) could not bind with Ca^{2+} in the presence of 1×10^{-7} mol/L Ca^{2+}, which is similar to the cytosolic Ca^{2+} concentration in plant cells [7]. We use $mCaM2_{1234}$ in yeast to test the interaction between CaMBPs and CaM2 depending on Ca^{2+} or not. We also can use it as bait in yeast two-hybird system to detect the novel Ca^{2+}-independent CaMBPs in *Arabidopsis*. Using $mCaM2_{1234}$ as bait in yeast two-hybrid, we provided *in vivo* evidence of interactions between CaM and AtIQD26 (At3g16490), one Ca^{2+}-independent CaMBP in *Arabidopsis*[21, 22].

1 Materials and methods

1.1 Materials

Synthetic oligonucleotide primers for polymerase chain reaction (PCR) and PCR-based site-directed mutagenesis were obtained from Sangon (China, http://www.sangon.com). Taq DNA polymerase, restriction enzymes and T4-DNA ligase were obtained from TaKaRa (Japan, http://www.takara.com.cn). The Muta-direct™ site-directed mutagenesis kit used in our study was a product of Saibaisheng (SBS) Genetech Company (China, http://www.sbsbio.com).

Escherichia coli BL21 and the plasmid pET28b were from Novagen (Germany, http://www.novagen.com). Plasmid vectors (pGADT7 and pGBKT7) and AH109 yeast cells were bought from Clontech (Japan, http://www.clontech.com). pET5a-*AtCaM2* (GenBank accession number NM_179766) was a gift from the Zielinski laboratory (Ray Zielinski's Laboratory at the University of Illinois, http://www.life.uiuc.edu/zielinski).

1.2 Gene cloning and plasmid construction

Full-length cDNA for *AtCaM2* was amplified by PCR from the plasmid pET5a-*AtCaM2* using the forward primer *CaM2*f (5′ GGAATTCCATATGGCA-GATCAGCTCAC CG 3′) and reverse primer *CaM2*r (5′ CGGAATTCTCACTTTGCCATCATAACTTTG 3′). The PCR product, which was cut by the restriction endonucleases enzymes *Nde* Ⅰ and *Eco*R Ⅰ, was ligated into plasmids to form the plasmid pET28b-*CaM2*. pET28b-*CaM2* was then used to produce the CaM2 recombination protein and the yeast two-hybrid bait vector pGBKT-*CaM2*.

IQD26 from RNA extracted from the pollen of *Arabidopsis thaliana* (Col-0) seedling was amplified by reverse transcriptase PCR (RT-PCR) using the *IQD26*f (5′ GGAATTCATGGGAAGAGCTGCGAGATGGT-TCA 3′) and *IQD26*r (5′ CGGGATCCCTAATTAT-GAATCTAAATCAGTCT 3′) primers. The RT-PCR product, cut with *Eco*R Ⅰ and *Bam*H Ⅰ enzymes, was ligated into plasmids to produce a pET28b-*IQD26* plasmid. This was, in turn, used to produce the IQD26 recombination protein and pGADT7-*IQD26*, which was used to test the pGBKT7-*CaM2* interaction in yeast.

1.3 Site-directed mutagenesis by PCR

The point mutations of *AtCaM2* were made by PCR using synthetic oligonucleotide primers (Table 1) containing the desired mutation, with pET28b-*CaM2* as amplified template. The method was performed using Muta-Direct™ Enzyme DNA polymerase and a temperature cycle (step Ⅰ: 95℃ for 30 s for one cycle; step Ⅱ: 95℃ for 30 s, then 55℃ for 1 min; finally, 72℃ for 1 min for 15 cycles) according to the Muta-direct™ site-directed mutagenesis kit manual. The PCR product was treated with Mutazyme™ Enzyme to digest the parental DNA template. The mutation-containing synthesized DNA was then transformed into DH5α supercompetent cells. DNA sequencing was performed to determine the changes in base pairing.

1.4 Recombinant protein expression and purification

BL21 *E. coli* bacteria, with ahead construct pET28b, were used to produce recombinant proteins CaM2-His, mutant CaM2-His. The single-clone

bacteria were grown overnight in 5 ml Lysogeny broth containing 50 mg/L Kanamycin (LB-Kan_{50}) at 37℃. 5 ml of the culture was inoculated with 250 ml of fresh LB-Kan_{50} and grown at 37℃ until the cells reached a density of $A_{600}=0.6$. Isoporopylthio-β-D-galactoside (IPTG) was then added to a final concentration of 1 mmol/L and the culture was allowed to grow for a further 3 h. Finally, the cells were collected and centrifuged at 6 000 *g* at 4℃ for 10 min. The cell pellet was suspended in buffer Ⅰ (50 mmol/L Tris pH 7.5; 0.5 mmol/L DTT) on ice for 30 min, and sonicated on ice for 10 min. The lysate was centrifuged at 40 000 *g* at 4℃ for 45 min, and the resultant supernatant was loaded onto a His-selected Nickel Affinity Gel from Sigma (United States, http://www.sigmaaldrich.com) according to their manuals, to obtain the purified recombinant proteins CaM2-His and mutant CaM2-His.

1.5 SDS-PAGE mobility shift

2 μl of BL21 harboring the expression plasmids pET28b-*CaM2*, pET28b-$CaM2_1$, pET28b-$CaM2_{12}$, pET28b-$CaM2_{123}$, pET28b-$CaM2_{124}$, pET28b-$CaM2_{1234}$ were dissolved in SDS sample buffer (0.1 mol/L Tris-HCl, pH 6.8, 30% glycerol, and 2% SDS) in the presence of either 5 mmol/L $CaCl_2$ or 5 mmol/L EGTA, then heated at 95～100℃ for 5 min. The samples were centrifuged at 13 000 *g* at 4℃ for 5 min prior to loading on gels. 12% SDS-PAGE was used to separate the proteins (i.e. the recombinant CaM and the various mutant CaM proteins). The mobility ratio was calculated as the distance migrated by the molecule to that migrated by bromophenol blue. The data processed in GraphPad Prism 4 (http://www.graphpad.com).

1.6 $^{45}Ca^{2+}$ overlay

2 μg of recombinant CaM2 and mutated-CaM2 purified by His-Select Nicked Affinity Gel were run on a 12% SDS-PAGE gel and then transferred to a Polyvinylidene-Fluoride (PVDF) membrane. The PVDF membranes were incubated in $^{45}Ca^{2+}$ buffer (60 mmol/L KCl, 5 mmol/L $MgCl_2$, 10 mmol/L imidazole-HCl pH 6.8) with 2.96×10^{12} μBq/L $^{45}Ca^{2+}$ (7.4×10^{10} Bq/L, 95.3 mg/L, Amersham Biosciences from Sweden, http:// www.amersham.com) at 23℃ for 30 min with shaking. After rinsing three times with 50% ethanol for 5 min, the membranes were dried at room temperature for 3 h. Finally, they were exposed to a storage phosphor screen for 12 h. Images were scanned using a Typhoon 9210 imager (Amersham Biosciences) and analyzed using Quantity One software and the data processed in GraphPad Prism 4 (http://www.graphpad.com).

1.7 Yeast two-hybrid

The coding regions of CaM2, mutated-CaM2 or IQD26 were constructed by PCR and confirmed by DNA sequencing. The constructs were then introduced into the *Nco* Ⅰ/*Bam*H Ⅰ site of pGBKT7 and pGADT7, respectively. Yeast cells (AH109 strain) were co-transformed with different pGADT7 and pGBKT7 constructs. The transformants were streaked onto media lacking Tryptophan and Leucine (media lacking Trp and Leu), and cultured at 30℃ for 3 days. The yeast cells were then streaked onto media lacking Adenine, Histidine, Trp and Leu (media lacking Ade, His, Trp and Leu), and incubated at 30℃ for 3 days. pGADT7-RecT/pGBKT7-p53 and pGADT7-RecT/pGBKT7-Lam served as positive and negative controls. LacZ activity was detected in the yeast cells using X-gal as a substrate.

2 Results and Discussion

2.1 Site-directed mutagenesis of *AtCaM2*

CaM has four Ca^{2+} binding EF-hand domains. The Ca^{2+} binding ability of CaM depends on the conserved amino acid in the EF-hand domain. The more conserved amino acid in the EF-hand domain was mutated, the weaker binding ability with Ca^{2+} [7]. Here we wanted to alter the Ca^{2+} binding ability of CaM2 by site-directed mutagenesis of the conserved amino acid in the EF-hand domain. *AtCaM2* was cloned into pET28b vector to obtain pET28b-*CaM2* expression plasmid. According to the Muta-direct™ site-directed mutagenesis kit manual, we got pET28b-$mCaM2_1$ using pET28b-*CaM2* as the PCR template with the $mCaM2_1$F, and $mCaM2_1$R primers (Table 1); we generated pET28b-$mCaM2_{12}$ using the PCR template pET28b-$mCaM2_1$ and the $mCaM2_2$F, and $mCaM2_2$R primers (Table 1); then with the similar method we got pET28b-$mCaM2_{123}$, pET28b-$mCaM2_{124}$ and pET28b-$mCaM2_{1234}$ one by one based on the former well being done. These expression plasmids were transformed into *E. Coli* BL21 strain to produce recombinant mCaM2 mutant proteins, with Glutamate (E) replaced by Glutamine (Q) in the EF-hand motifs of CaM2 (Table 2).

Table 1　The oligonucleotides primers used in site-directed mutagenesis of *AtCaM2*

Primer	Primer sequence (5′～ 3′)[1)]
mCaM2$_1$F	GGTTGCATCACAACGAAACAGCTAGGAACAGTGATGA
mCaM2$_1$R	TCATCACTGTTCCTAGCTGTTTCGTTGTGATGCAACC
mCaM2$_2$F	GGAACCATAGACTTCCCTCAGTTTCTGAACCTAATGG
mCaM2$_2$R	CCATTAGGTTCAGAAACTGAGGGAAGTCTATGGTTCC
mCaM2$_3$F	GGTTTCATCTCGGCAGCTCAGTTAAGACATGTAATGA
mCaM2$_3$R	TCATTACATGTCTTAACTGAGCTGCCGAGATGAAACC
mCaM2$_4$F	GGTCAGATCAATTATGAACAGTTTGTCAAAGTTATGATGGC
mCaM2$_4$R	GCCATCATAACTTTGACAAACTGTTCATAATTGATCTGACC

[1)] The mutated residues are underlined.

Table 2　Summary of CaM2 mutations

Mutant protein	Mutation amino acid	Mutant site in
mCaM2$_1$	E32Q	EF-hand 1
mCaM2$_{12}$	E32Q; E68Q	EF-hand 1,2
mCaM2$_{123}$	E32Q; E68Q; E105Q	EF-hand 1,2 and 3
mCaM2$_{124}$	E32Q; E68Q; E141Q	EF-hand 1,2 and 4
mCaM2$_{1234}$	E32Q; E68Q; E105Q; E141Q	EF-hand 1,2 and 3,4

E: Glutamate; Q: Glutamine.

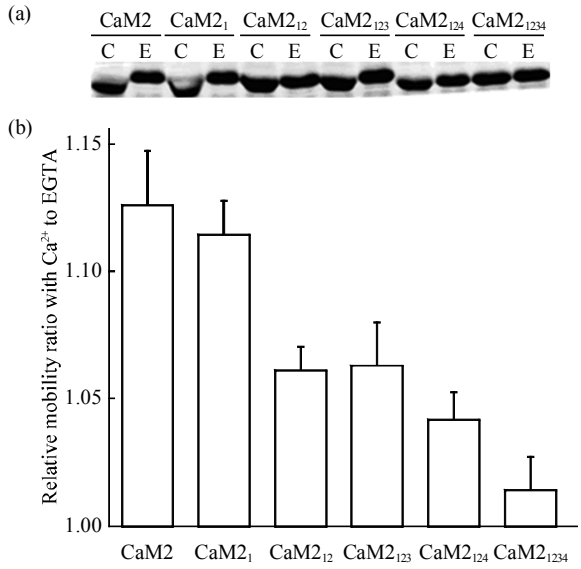

Fig. 1　The electrophoresis mobility shifts of mutated CaM2

(a) Proteins were extracted from induced BL21 harboring the expression plasmid pET28b-*CaM2* and pET28b-*mCaM2*. Electrophoresis mobility shift analyses were performed in the presence of 5 mmol/L Ca^{2+} (C) or 5 mmol/L EGTA (E), separated by electrophoresis in a 12% SDS-PAGE, and detected using Coomassie blue staining. (b) The relative mobility ratio of normal and mutant CaM2 with 5 mmol/L Ca^{2+} to 5 mmol/L EGTA. The data and error bars represent $\bar{x} \pm s$. The experiment was repeated twice independently with comparable results.

2.2　The electrophoresis mobility and Ca^{2+}-binding ability of mutant CaM

The same amount of induced BL21, harboring the expression plasmids pET28b-*CaM2*, pET28b-*mCaM2*$_1$, pET28b-*mCaM2*$_{12}$, pET28b-*mCaM2*$_{123}$, pET28b-*mCaM2*$_{124}$ or pET28b-*mCaM2*$_{1234}$ was directly dissolved in SDS buffer, and analyzed by SDS-PAGE in the presence of either 5 mmol/L $CaCl_2$ or 5 mmol/L EGTA(Figure 1a). All proteins except mCaM2$_{1234}$ exhibited the characteristic Ca^{2+}-dependent electrophoretic mobility shift, which is dependent on the number and location of the mutated EF-hand domain of CaM2. The mobility-shift difference of wild-type CaM2 in the presence of $CaCl_2$ or EGTA was larger than that of mutant CaM2 (Figure 1a). It is obviously that the relative mobility ratio of mCaM2$_{1234}$ with Ca^{2+} to EGTA was almost equal to 1 (Figure 1b). Thus, our results show that the speed of Ca^{2+}-dependent electrophoretic mobility is relative to the number of EF-hand motifs present. However, mCaM2$_{1234}$ is different because its mobility shift was identical in the presence of both $CaCl_2$ and EGTA (Figure 1a and 1b).

To identify the calcium-binding ability of the mutant CaM2 proteins, the more-sensitive method of $^{45}Ca^{2+}$ overlay was adopted (Figure 2). From the protein band stained by amino-black in the PVDF, all the lanes were with the approximately same amount of protein, but autoradiography film of $^{45}Ca^{2+}$ overlay showed different radiations of the protein bands, and the $^{45}Ca^{2+}$ radiation in wild-type CaM2 band was the strongest (Figure 2a). The mutation CaM2 binds Ca^{2+} ability is obviously weakened. The more EF-hand motifs were altered, the weaker binding with Ca^{2+} (Figure 2a). Ca^{2+}-binding ability was slightly reduced by the alteration of the EF-hand 1, of EF-hand 1 and 2, or of EF-hand 1, 2, and 3. However, it was markedly weakened by the alteration of EF-hand 1, 2, and 4. When all four EF-hand (1～4) were mutated, mCaM2$_{1234}$ displayed no Ca^{2+} binding at all (Figure 2a). We defined the relative $^{45}Ca^{2+}$-binding ability of the mutant CaM2 proteins using Quantity One software based on the results of $^{45}Ca^{2+}$ overlay assay. The $^{45}Ca^{2+}$-binding ability of normal CaM2 was defined as 100%. Then the relative $^{45}Ca^{2+}$-binding ability of mutant CaM2 were defined (Figure 2b). Bovine serum albumin (BSA)

could not bind Ca^{2+}, and its $^{45}Ca^{2+}$-binding ability was equal to background of PVDF. Obviously the relative Ca^{2+}-binding ability of $mCaM2_{1234}$ was similar to that of BSA, that was to say, the Ca^{2+}-binding ability of $mCaM2_{1234}$ was completely lost in our experiment conditions(Figure 2b). We used 2.96×10^{12} μBq/L $^{45}Ca^{2+}$ in the expreriment system, the final concentration of $^{45}Ca^{2+}$ was equal to 1×10^{-7} mol/L, which is equal to the resting cytosolic Ca^{2+} concentration in plant cells[7]. So the $mCaM2_{1234}$ couldn't bind Ca^{2+} at the resting cytosolic Ca^{2+} concentration in plant cells.

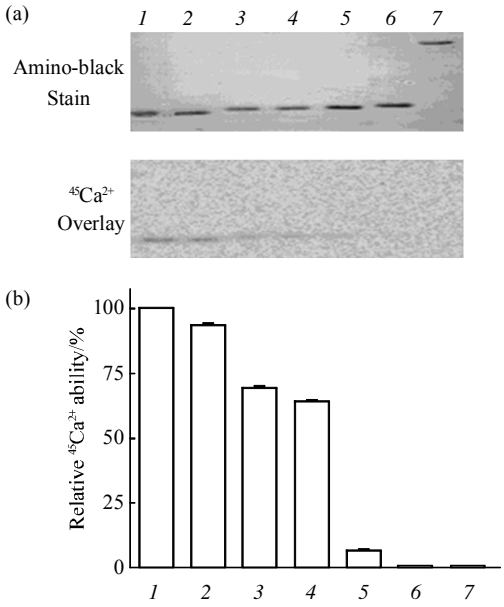

Fig. 2 Autoradiogram of $^{45}Ca^{2+}$ binding to CaM2 and mutated-CaM2

(a) 2 μg proteins purified by His-Select Nicked Affinity Gel were run on a 12% SDS-PAGE, then transferred to PVDF membranes. Stained by amino black (upper panel), and overlay with 2.96×10^{12} μBq/L $^{45}Ca^{2+}$ (lower panel), respectively. BSA served as a negative control. The experiment was repeated twice independently with comparable results. (b) The relative $^{45}Ca^{2+}$ binding ability. The data were generated by using Quantity One software and the error bars represent $\bar{x} \pm s$. *1*: CaM2; *2*: $CaM2_1$; *3*: $CaM2_{12}$; *4*: $CaM2_{123}$; *5*: $CaM2_{124}$; *6*: $CaM2_{1234}$; *7*: BSA.

To understand the structure and function of CaM in the activation of its target proteins, several mutant CaMs have been generated *in vitro* in plant and animal systems. To determine which domains are responsible for differential activation of target NAD kinase, a series of chimeric soybean CaMs were generated by exchanging functional domains between sCaM4 and sCaM1 [23]. And, to learn more about the roles of the individual Ca^{2+}-binding site in *Drosophila melanogaster* CaM, Maune *et al*.[24] changed the conserved glutamate into glutamine or lysine in the four EF-hand motifs. In our present study, we first changed the amino acids glutamate into glutamine in the EF-hand domains of *Arabidopsis* CaM2, which resulted in altered Ca^{2+}-binding ability. Especially the $mCaM2_{1234}$ displayed no Ca^{2+}-binding ability under the normal resting cytosolic Ca^{2+} concentration of plant cells (Figure 2).

2.3 Detecting Ca^{2+}-independent CaM-binding proteins in yeast using $mCaM2_{1234}$

CaM overlays *in vitro* have been used to determine whether the interaction of CaMBP with CaM was dependent on Ca^{2+} or not, but there is no valid method *in vivo* to identify the CaMBPs bind CaM depending on Ca^{2+} or not. Now we can use the $mCaM2_{1234}$ as bait in yeast two-hybird system to detect the novel Ca^{2+}-independent CaMBPs in *Arabidopsis*. To prove the feasibility we chose the AtIQD26 as a control *in vivo*, which is a putative Ca^{2+}-independent CaMBP[21, 22]. We had previously confirmed that IQD26 interacted with AtCaM2 in a Ca^{2+}-independent manner *in vitro* by Bio-CaM or ^{35}S-CaM [22]. In the present study, we used the mutant $CaM2_{1234}$ as bait to further identify the Ca^{2+}-independent CaM-binding characteristic of IQD26 in yeast two-hybrid system (Figure 3). Yeast co-transformed with pGBKT7-*mCaM2*$_{1234}$ and pGADT7-*IQD26* showed blue on X-gal filter assay and grew on media lacking Trp, Leu, His and Ade, while the system positive and negative controls were normal.

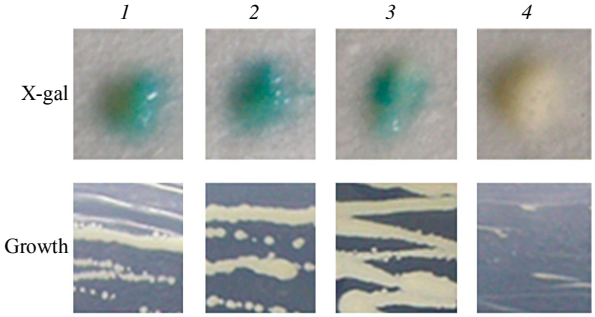

Fig. 3 Mutant $CaM2_{1234}$ interacted with IQD26 in yeast two-hybrid

The indicated combinations of plasmids were cotransformed into the yeast reporter strain (AH109), and the interactions were assessed by growth on media lacking Trp, Leu, His and Ade. LacZ reporter activity was detected using X-gal as a substrate. pGADT7-RecT/pGBKT7-p53 and pGADT7-RecT/pGBKT7-Lam served as positive and negative controls. *1*: pGADT7-*IQD26*/pGBKT7-*CaM2*; *2*: pGADT7-*IQD26*/pGBKT7-*mCaM2*$_{1234}$; *3*: pGADT7-RecT/pGBKT7-53; *4*: pGADT7-RecT/pGBKT7-Lam.

Recently, several Ca^{2+}-independent CaMBPs have been found in plant and animal, and their

Ca^{2+}-independent CaM-target interactions are extensive, involving transcription factor OsCBT from rice [25], unconventional myosins [20], Ca^{2+} channels and PEP-19[18], GAP-43[26, 27] which exist in neuronal tissues. All of these suggest that Ca^{2+}-independent CaMBPs play important roles in growth and development of plant and animal. But until now only few of Ca^{2+}-independent CaMBPs in the plant cells have been found and their functions have not been well charactered. To investigate the biological roles of Ca^{2+}-independent CaMBPs, the first step is to isolate them. Using the mutant $CaM2_{1234}$ as bait in yeast two-hybrid system, we will identify more Ca^{2+}-independent CaMBPs in plant cells. Depending on this method we have proved that AtIQD26 interacted with CaM2 in a Ca^{2+}-independent manner (Figure 3); or mutant $CaM2_{1234}$ gene was transformed into plant to compete with free-CaM, breaking the balance of Ca^{2+}, free-CaM and Ca^{2+}-CaM, and then we could observe the phenotype for exploring the functions of free-CaM and Ca^{2+}-independent CaMBPs in plant development and responses to environmental stimuli.

Acknowledgement We thank Ray Zielinski for presenting pET5a-*AtCaM2* plasmid.

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拟南芥钙调素定点突变基因分离及其在钙不依赖钙调素结合蛋白检测中的应用 *

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摘要 动植物系统研究表明，钙调素不仅在结合钙离子时调节多种靶酶或靶蛋白的活性，而且没有钙离子结合时，还可以通过结合钙不依赖的钙调素结合蛋白，发挥多种生物学作用．然而，目前却没有体内分析钙调素与钙不依赖钙调素结合蛋白相互作用的方法．首先，采用定点突变的方式，得到了拟南芥钙调素亚型 2 的多个突变基因 *mCaM2*，随后，大肠杆菌重组表达突变蛋白的电泳迁移率及 $^{45}Ca^{2+}$ 覆盖分析表明，得到了编码失去钙结合能力的钙调素的突变基因 $mCaM2_{1234}$, $mCaM2_{1234}$ 突变钙调素中所有 4 个钙结合 EF-hand 结构域中的关键氨基酸谷氨酸均突变为谷氨酰胺．在酵母双杂交体系中，作为诱饵蛋白的突变钙调素 $mCaM2_{1234}$ 与我们前期体外方法报道的钙不依赖性钙调素结合蛋白 AtIQD26 存在相互作用．这将为钙不依赖性钙调素结合蛋白提供有用的体内研究工具，有利于我们全面认识钙 - 钙调素 - 钙调素结合蛋白信号途径．

关键词 定点突变，拟南芥，钙调素，钙不依赖，钙调素结合蛋白

学科分类号 Q291 **DOI:** 10.3724/SP.J.1206.2008.00786

* 教育部新世纪优秀人才支持计划(NCET-06-0256)和国家重点基础研究发展计划(973)(2006CB910600)部分资助项目.

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收稿日期：2008-11-15，接受日期：2009-01-09