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Isolation and Characterization of a Calmodulin-binding Ca^{2+}-ATPase 2 (SCA2) in Soybean

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We previously reported the isolation and characterization of a gene, *SCA1* (for soybean Ca^{2+}-ATPase 1), encoding a calmodulin-regulated Ca^{2+}-ATPase that is located in the plasma membrane in soybean. Here, a Ca^{2+}-ATPase designated as SCA2 was isolated from soybean. The two Ca^{2+}-ATPases, SCA1 and SCA2, share a remarkably high degree of similarity (78%). Ten transmemebrane domains were predicted by hydropathy analysis. Using gel overlay assays, CaM was found to bind to SCA2 in a Ca^{2+}-dependent manner. Southern blot analysis revealed the presence of two copies of the Ca^{2+}-ATPase gene in the soybean genome. An N-terminal truncation mutant that deletes sequence through the putative calmodulin binding site was able to complement a yeast mutant (*K616*) that was deficient in two endogenous Ca^{2+} pumps. Our results indicate that SCA2 is structurally highly conserved with type IIB Ca^{2+} pumps in plants.

Key words : Calcium, Ca^{2+}-ATPase, Calmodulin, plasma membrane, soybean (*Glycine max*)

Introduction

The calcium ion (Ca^{2+}) is intimately involved as a secondary messenger in numerous signal transduction processes. The transient increase of the cytoplasmic calcium levels leads to a variety of physiological responses in plants [27]. Various stimuli such as abiotic stresses, hormones, wounds, and plant pathogens can trigger an influx of Ca^{2+} into the cytoplasm. Calcium signaling is well known to play a role in plant defense signaling pathways [18]. The transient Ca^{2+} signals are translated into a physiological response by a variety of Ca^{2+}-sensing proteins, such as calmodulin (CaM), calcineurin B-like protein, and calcium-dependent protein kinase [10]. CaM is known to couple Ca^{2+} signals to changes in the activity of downstream target proteins via direct interaction [10,28]. Unlike animals, which have only one form of the CaM protein, multiple CaM proteins occur in higher plant species [28,29]. For example, the model plant *Arabidopsis thaliana*, harbors nine CaM genes (AtCaM1-9) coding seven different CaMs [29,30]. Likewise, the plant *Glycine max* (soybean) contains not less than five CaM genes (GmCaM1-5) coding for four distinct CaMs [20], whereas *Oryza sativa* (rice) also has at least five CaMs [6]. Several studies have demonstrated that each CaM isoform is utilized to control different enzymes that are involved in specific physiological reactions [19,21].

The cytosolic Ca^{2+} concentration is restored immediately after a stimulus to a low resting level by removing Ca^{2+} across the plasma membrane or by sequestering it into intercellular organelles, such as the vacuole, the endoplasmic reticulum, or the Golgi apparatus. Plant Ca^{2+}-pumps, which are responsible for this ATP-driven transport process, belong to the superfamily of P-type Ca^{2+} pumping ATPases [14]. According to their ability to bind CaM, plant Ca^{2+}-ATPases have been classified into two groups: type IIA and type IIB. Among these, type IIB Ca^{2+}-ATPases are characterized by an extended cytosolic regulatory domain whose auto-inhibitory action can be suppressed by binding of calmodulin (CaM) [1,11] or of acidic phospholipids [5,25]. Localization of this terminal regulatory domain is the main structural difference between plant and animal members of the type IIB group of Ca^{2+}-ATPases. Plant type IIB Ca^{2+}-ATPases possess an N-terminal extension that harbors a CaM binding domain (CaMBD). In contrast, their closest animal homologs, the plasma membrane Ca^{2+}-ATPases, have an extension at the C-terminal end that encompasses their CaMBD. Despite this structural difference, animal and plant type IIB Ca^{2+}-ATPases share a number of catalytic and regulatory fea-

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tures [1,11]. On the other hand, the activities of plant type IIA Ca^{2+}-ATPases are CaM-independent. Although several type IIB Ca^{2+}-ATPases have been identified in different plant species, the best studied organism is *A. thaliana* which contains 10 different type IIB Ca^{2+}-ATPases (ACAs), which have different subcellular localizations [3]. For example, ACA2 and ACA4 are located at the endoplasmic reticulum and the prevacuolar membrane, respectively, whereas ACA8 and ACA9 are located on the plasma membrane [4]. Previously, we have reported that the SCA1 protein is a type IIB Ca^{2+}-ATPase and located on the plasma membrane in soybean [8]. Calmodulin binding overlay assays demonstrated that SCA1 contains two separable Ca^{2+}-dependent calmodulin binding sites in its N-terminal domain. The Ca^{2+}-ATPase activity of the SCA1 was increased by calmodulin. Furthermore, the expression of SCA1 was highly and rapidly induced by salt (NaCl) stress and a fungal elicitor but not by osmotic stress.

Here we isolated a type IIB Ca^{2+}-ATPase, SCA2, from a soybean cDNA library. Using gel overlay assays, CaM binds to SCA2 in a Ca^{2+}-dependent manner. An N-terminal truncation mutant that deletes sequence through the putative calmodulin binding site was able to complement a yeast mutant (*K616*) that was deficient in two endogenous Ca^{2+} pumps. Our results indicate that SCA2 is structurally conserved with plant type IIB Ca^{2+} pumps.

Materials and Methods

Plant material and yeast and *Escherichia coli* strains

Soybean (*Glycine max*) and soybean suspension cells were used for plant materials [15]. *Saccharomyces cerevisiae* strains used for complementation studies, protein expression, and ATPase assays were *W303-1A* (*MAT*a, *leu2, his3, ade2, ura3*) and *K616* (*MAT*a *pmr1*::*HIS3 pmc1*::*TRP1 cnb1*::*LEU2, ura3*) [9]. For DNA cloning, we used the *E. coli* stains *XL1-Blue* and *DH 5α* (Stratagene, La Jolla, CA, USA). The expression of fusion protein was performed in *E. coli* BL21 (pLysS) DE3.

Construction of *SCA2* cDNA

The full-length *SCA2* cDNA was amplified by PCR with a forward (5′) primer containing a *Xho*I site (5′-CTCGAGATGGAGATTTACCTGAGTGAG-3′) and a reverse (3′) primer containing a *Spe*I site (5′-ACTAGTTACTAG GGACACCCTAGACAG-3′). The PCR product was cloned in *pGEM-T Easy* Vector (Promega, Madison, WI, USA) and sequenced to verify the correct DNA sequence. The construct was subcloned into *pGEX-5X* expression vector using *Xho*I and *Spe*I enzyme sites.

pYES2-SCA2 encodes the SCA2 full-length protein and was subcloned from a *Xho*I-*Spe*I fragment of SCA2-wt into the *Xho*I-*Spe*I site of *pYES2* (Invitrogen, Carlsbad, CA, USA). *pYES2-SCA2 nt83* encodes the SCA2 nt83 protein and was generated by PCR using the following forward (F) and reverse (R) primers that contained at their 5′-ends *Xho*I (5′-CTCGAG) and *Spe*I (5′-ACTAGT) restriction sites, respectively: F-(5′-CTCGAGATGGAGTACACTGTACCAGAA GAA-3′), and R-(5′-ACTAGTTACTAGGGACACCCTAGA CAG-3′). PCR was performed with *pGEX-SCA2* as a template and the *Xho*I-*Spe*I fragment was subcloned into the *Xho*I-*Spe*I sites of *pYES2*.

Expression of recombinant proteins in *E. coli* and CaM binding assay

All clones were individually introduced into *E. coli* BL21 (pLysS) DE3 and expressed. Expression of GST-fusion proteins was induced by application of 1 mM IPTG for 5 hr at 25°C. Cells were harvested, resuspended in lysis buffer (50 mM Tris-HCl [pH 7.5], 2 mM PMSF, 1 mM DTT, 100 μg/ml lysozyme), and incubated on ice for 20 min. The mixture was sonicated for 1 min using a 50% pulse and then centrifuged at 6,000× *g* for 10 min to remove cell debris. Recombinant proteins were purified on a glutathione-sepharose 4B column (Amersham Pharmacia Biotech, Uppsala, Sweden). From each clone, 1 μg of purified protein was separated on 10% SDS-polyacrylamide gel and transferred onto Immobilon-P membrane (polyvinylidene difluoride) after which expressed GST-fusion proteins were detected using a polyclonal GST-specific antiserum. To examine the CaM binding ability of recombinant proteins, a duplicate blot was probed with GmCaM1::HRP conjugate in the presence of 1 mM $CaCl_2$ or 5 mM EGTA. The CaM:HRP overlay assay was carried out as described previously [8]. Bound CaM was visualized using an ECL detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Southern blot analysis

Southern blot hybridization was carried out as described previously [17]. Genomic DNA from the leaf of soybean was digested with *Hin*dIII, *Bam*HI, *Eco*RI, and *Xba*I, separated by electrophoresis on a 0.8% agarose gel and transferred onto a nylon membrane (Amersham Pharmacia, Piscataway, NJ,

USA). ^{32}P-labeled gene specific probes were made from a 559-bp *Eco*RI-*Xho*I fragment of *SCA2* corresponding to the 3′ region of cDNA by random primer labeling method [13]. The membrane was hybridized and washed in 0.2× SSC, 0.1% SDS at 60°C.

Yeast transformation

For yeast transformations, *W303-1A* and *K616* yeast strains were grown in standard yeast extract peptone dextrose (1% [w/w] yeast extract, 2% [w/w] peptone, 2% [w/w] dextrose) (YPD) media supplemented with 10 mM $CaCl_2$. Yeast was transformed with the *pYES2* vector (Invitrogen, Carlsbad, CA, USA), *pYES2-SCA2* and *pYES2-SCA2 nt83* by using the lithium acetate/polyethylene glycol methods, respectively [12]. Transformants were selected for uracil prototrophy by plating on synthetic complete medium minus uracil (SC-URA) supplemented with 2% (w/v) glucose as a carbon source and 2% (w/v) agar. For complementation studies, colonies were streaked on SC-URA agar plates containing 2% (w/v) glucose or galactose and 10 mM EGTA, pH 6.0, and incubated for 4 days at 30°C.

Results and Discussion

Primary structure of SCA2

We previously reported the isolation and characterization

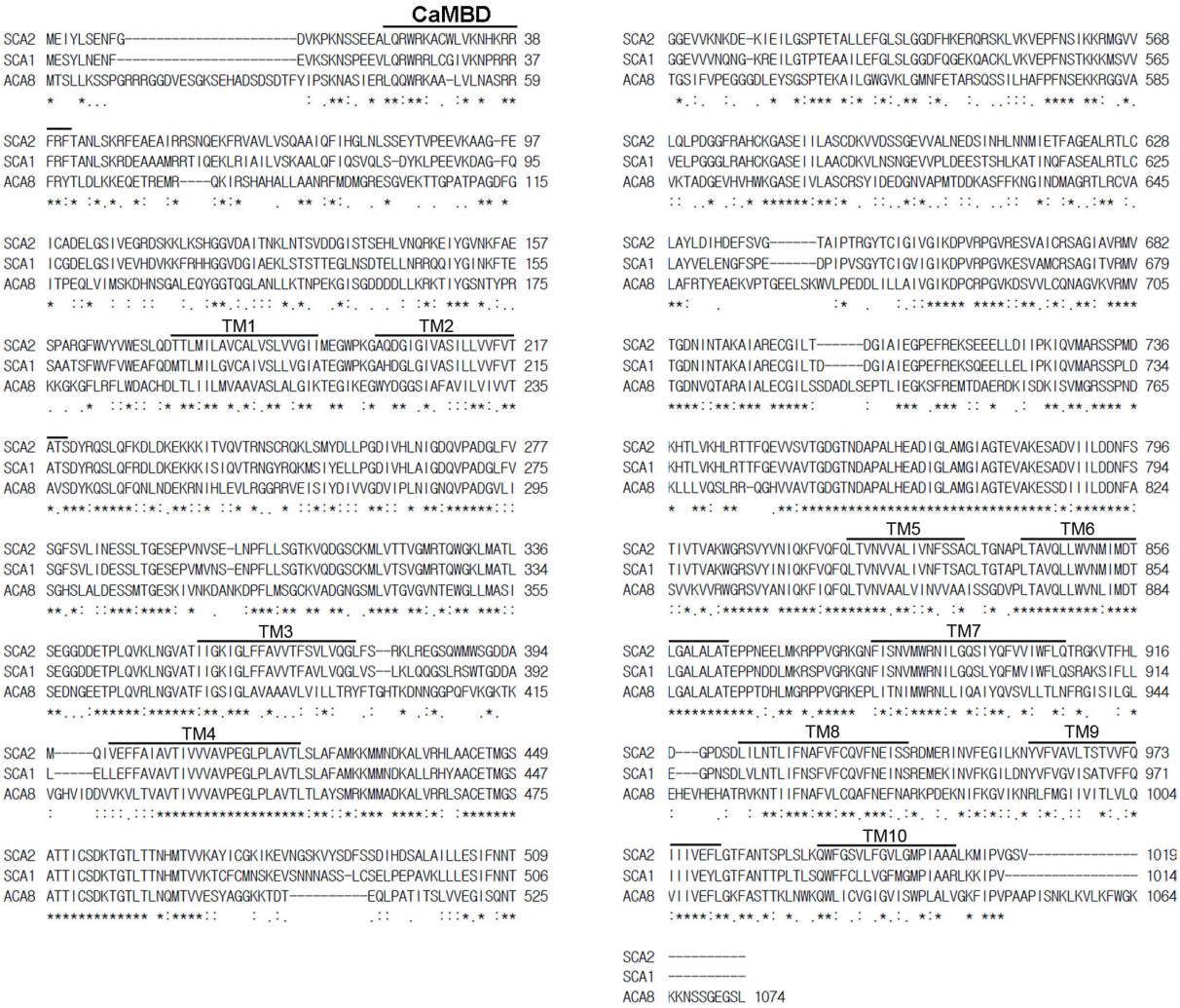

Fig. 1. Amino acid sequence alignment showing a high degree of identity between SCA2, SCA1, and ACA8. Accession numbers used in building the alignment: SCA2 (AF195029), SCA1 (AF195028), and ACA2 (AF025842). Identical residues are indicated with asterisk. Ten transmembrane domains (TM1 to TM10) were predicted by a transmembrane prediction program (ExPASy Web server; http://www.expasy.ch/tools/) based on hydropathy plot and are indicated by lines above the sequences. The putative calmodulin binding sequences are indicated by lines above the sequences and marked as CaMBD.

of a gene, *SCA1* (for soybean Ca^{2+}-ATPase 1), encoding a calmodulin-regulated Ca^{2+}-ATPase that is located at the plasma membrane in soybean [8]. Database search revealed that a isoform of SCA1 existed in soybean. To isolate the full-length cDNA sequence, we conducted PCR from soybean cDNA and obtained full-length cDNA clone, named as *SCA2*. The deduced amino acid sequences consists of 1019 aa (GenBank Accession No. AF195029) with a molecular mass of 111.4 kDa. SCA2 showed the greatest sequence identity to the plant type IIB Ca^{2+}-ATPases SCA1 (78%) and ACA8 (50%) (Fig. 1). In contrast, SCA2 showed only 35% identity to a typical mammalian plasma membrane-type Ca^{2+}-ATPase and even less identity (<20%) to typical ER-type (type IIA) Ca^{2+}-ATPases, such as ECA1p from *Arabidopsis* [23] or SERCA1p from rabbit [7]. Ten transmembrane domains were predicted by hydropathy analysis and comparisons with other type IIB Ca^{2+}-ATPase (Fig. 2). This predicted topology suggested that SCA2 has a long N-terminal regulatory domain similar to other type IIB pumps in plants.

SCA2 binds to CaM in a Ca^{2+}-dependent manner

Comparison of the CaMBDs of many CaMBPs has shown that there are multiple sequence motifs for CaM complex formation [26]. Based on the structural characteristics of known CaMBDs, a putative CaM-binding motif was predicted in the N-terminus of SCA2, between Leu^{22} and Phe^{40} (CaMBD) (Fig. 3A). Within this 20-amino acid stretch, hydrophobic amino acids are present at positions 1 (Trp^{25}), 5 (Cys^{29}) and 8 (Val^{32}), and several basic residues (three lysine,

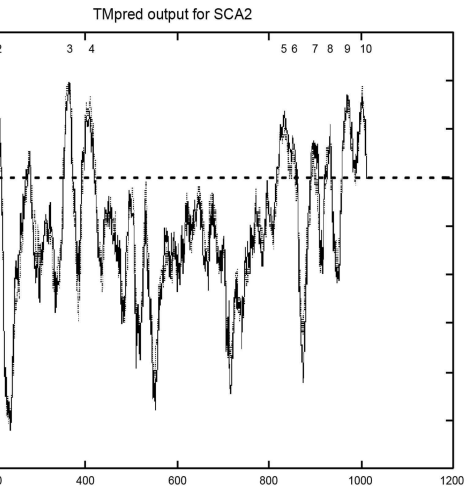

Fig. 2. Transmembrane (TM) region prediction of SCA2. Putative TM regions of SCA2 is predicted by using TM prediction program (http://www.expasy.ch/tools/) based on the hydropathy plot (Kite and Doolittle). Window size for this prediction was 17. Predicted TM domains are numbered 1-10.

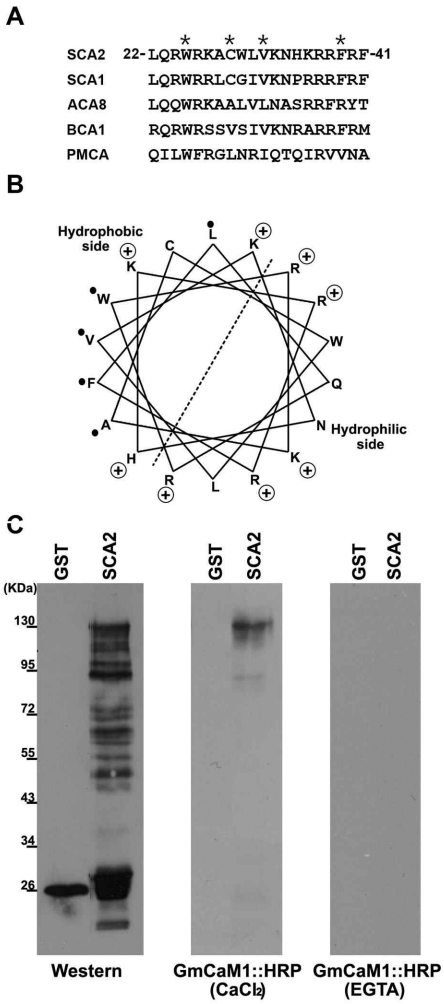

Fig. 3. The direct interaction of SCA2 with calmodulin. (A) Amino acid sequences of the calmodulin binding domain (CaMBD; 22 to 41 residues) were compared with other CaMBDs. Asterisks indicate the positions of SCA2 key residues Trp^{25}, Cys^{29}, Val^{32}, and Phe^{39}, which are believed to be important for binding to calmodulin. Conserved amino acid residues of CaMBD and other CaMBDs were aligned with these important positions. The other CaMBDs were SCA1 [8], ACA8 [2], BCA1 [24], and plsma membrane Ca^{2+} pump (PMCA) [16]. (B) Helical wheel projection of the putative CaMBD of SCA2. Hydrophobic and basic amino acid residues are marked with • and +, respectively. The dashed line indicates that the helix is amphipathic, one side being hydrophilic and the other side hydrophobic. (C) CaM binding analysis. GST and GST fusion protein of SCA2 were expressed in *E.coli* and analyzed by Western blotting with anti-GST antibody.

five arginine and one histidine) are interspersed between these hydrophobic residues (Fig. 3A, 3B). To confirm the presence of the putative CaMBD, we generated a GST-fused construct containing the full-length *SCA2* cDNA. Recombinant fusion protein was separated by SDS-PAGE and transferred to PVDF membranes for Western blotting and CaM overlay assays. Expression of the GST fusion protein was verified by probing the blot with an anti-GST antibody. As shown in Figure 3C, soybean calmodulin-1 conjugated with horseradish peroxidase (GmCaM1::HRP) [22] bound to SCA2 in a Ca^{2+}-dependent manner but did not bind to GST only (GST). In addition, the CaM bound to SCA2 in the presence of Ca^{2+} but not in the absence of Ca^{2+} (Fig. 3C). These results demonstrate that CaM binds to a CaMBD in the N-terminal domain of SCA2 in a Ca^{2+}-dependent manner.

SCA1 contains two separable Ca^{2+}-dependent calmodulin binding sites in its N-terminal domain [8]. The first calmodulin binding site is located in the first 40 residues and is probably a putative amphipathic basic helix between Leu^{21} and Phe^{40}. The second includes the residues between positions 52 and 71 is probably a modified IQ motif between Ile^{57} and Val^{67}. However, it is unclear that SCA2 contains the IQ motif. ACA8 also did not report the presence of a second CaMBD [2]. Whether two CaM binding sites are common to all of the plant type IIB pumps or instead endows specific isoforms with a different mode of regulation awaits further investigation.

Determination of copy number of *SCA2*

To estimate the copy number of *SCA2* gene in the soybean genome, Southern blot analysis was performed (Fig. 4). The specific probe corresponding to 3′ untranslated region of *SCA2* was hybridized to blot of soybean genomic DNA cleaved with four restriction endonucleases. There were two bands hybridized with the *SCA2* probe in all the lanes (Fig. 4). This result indicated that there might be two copies of *Ca^{2+}-ATPase* gene in soybean genome.

N-terminal-truncated mutant pump (SCA2 nt83) complements disruption of yeast Ca^{2+} pump

To determine if SCA2 functions as a Ca^{2+} pump *in vivo*, we expressed a wild-type (SCA2) and an N-terminal - truncated mutant (SCA2 nt83) protein in a yeast strain (*K616*) harboring a disruption of three genes encoding proteins involved in Ca^{2+} homeostasis: PMR1, PMC1, and CNB1 [9].

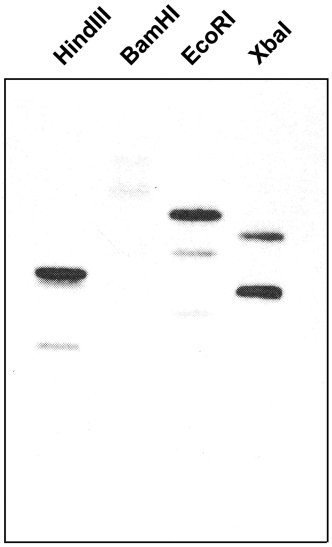

Fig. 4. Southern blot analysis of soybean genomic DNA for *SCA2*. Twenty micrograms of genomic DNA was digested with *Hin*dIII, *Eco*RI, *Bam*HI, or *Xba*I and loaded in each lane. The *Eco*RI-*Xho*I fragment corresponding to the 3′ untranslated region of SCA2 was used as a probe as described in Materials and methods.

PMR1 and *PMC1* encode Ca^{2+}-ATPases in the vacuole and Golgi, respectively. *CNB1* encodes the B subunit of calcineurin, protein phosphatase 2B, which regulates a H^+/Ca^{2+} exchanger. Because of the disruption of the yeast Ca^{2+}-ATPases, the *K616* strain shows poor growth on Ca^{2+}-depleted media (i.e., containing 10 mM EGTA). Both *SCA2* and *SCA2 nt83* cDNAs were cloned in a yeast expression vector, *pYES2* (Invitrogen, Carlsbad, CA, USA), to express proteins under the control of the *galactose* promoter. The constructs were introduced into *K616*, and the transformed yeasts were grown in Ca^{2+}-depleted media containing glucose or galactose. As controls, the growth patterns of the wild type and of *K616* transformed with the vector alone were compared. Only *K616* expressing SCA2 nt83 was able to grow on 10 mM EGTA-galactose medium (Fig. 5). This result suggests that SCA2 nt83, an N-terminal - truncation mutant of SCA2, functions as an active Ca^{2+}-ATPase in yeast.

Genetic complementation of yeast mutant *K616* by SCA2 nt83 confirmed that SCA2 can function as a Ca^{2+} pump *in vivo*. Both full-length and truncated mutants were expressed in yeast *K616*, in which not only its two endogenous Ca^{2+} pumps (PMR1 and PMC1) but also a Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin (CNB1), are disrupted [9]. An N-terminally truncated mutant (SCA2

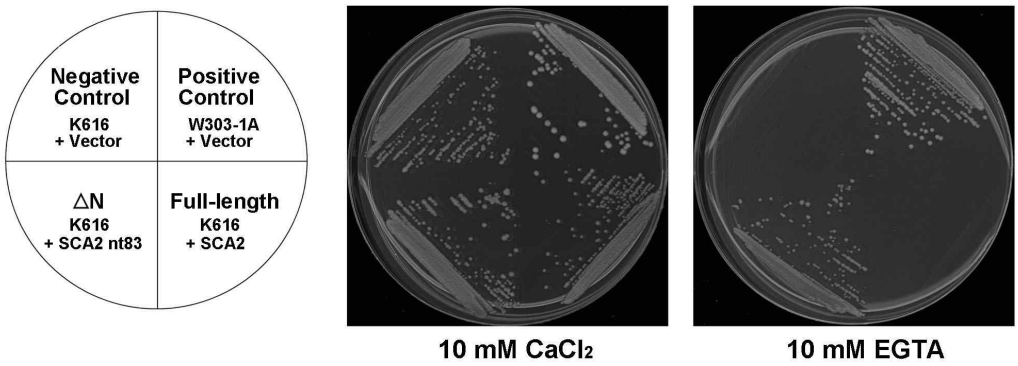

Fig. 5. Complementation of yeast mutant *K616* through expression of SCA2 nt83. Wild-type (*W303-1A*) and triple mutant (*K616*) cells were transformed with a vector (*pYES2*) alone, *pYES2-SCA2* and *pYES2-SCA2 nt83*, respectively. A diagram indicates yeast strains and transformed vectors. Full-length and ΔN indicate SCA2 and SCA2 nt83, respectively.

nt83) allowed the yeast *K616* to grow on Ca^{2+}-depleted media, whereas full-length SCA2 did not (Fig. 5). This ability of the truncated but not the full-length enzyme to provide complementation is probably the result of a higher constitutive Ca^{2+}-ATPase activity of the truncated enzyme.

Acknowledgments

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초록 : 칼모듈린에 결합하는 대두 Ca^{2+}-ATPase 2 (SCA2)의 분리 및 특성 분석

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대두의 세포막에 존재하는 SCA1은 칼모듈린에 의해서 조절된다는 내용을 이전에 보고하였다. 본 연구에서는 대두의 Ca^{2+}-ATPase인 SCA2에 관한 특성을 연구하였다. SCA2는 SCA1과 아미노산 서열 비교에서 78%로 높은 유사성을 나타내며, 10개의 transmembrane 도메인이 존재하는 것을 확인하였다. CaM overaly assay로부터, SCA2는 칼슘에 의존적인 방법으로 칼모듈린과 결합한다는 것을 보여주었으며, Southern blot 분석 결과, 대두의 genome에는 두 종류의 Ca^{2+}-ATPase가 존재하는 것으로 보인다. SCA2의 Ca^{2+}-ATPase 효소활성을 확인하고자 yeast mutant를 이용하여 complementation assay를 수행해 보면, SCA2가 Ca^{2+}-ATPase의 효소활성을 가지는 것을 보여 주었다. 이러한 결과들은 SCA2가 식물에 존재하는 type IIB Ca^{2+}-ATPase들과 구조적으로 높은 유사성을 가진다는 것을 시사한다.