Characterization of CXIP4, a novel *Arabidopsis* protein that activates the H^+/Ca^{2+} antiporter, CAX1

Ning-Hui Cheng[a,*], Jian-Zhong Liu[b], Richard S. Nelson[b], Kendal D. Hirschi[a,c]

[a] *United States Department of Agriculture/Agricultural Research Service Children's Nutrition Research Center, Baylor College of Medicine, 1100 Bates Street, Houston, TX 77030, USA*
[b] *Plant Biology Division, Samuel Roberts Noble Foundation, Inc., 2510 Sam Noble Parkway, Ardmore, OK 73402, USA*
[c] *Department of Human and Molecular Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA*

Received 7 November 2003; revised 3 January 2004; accepted 3 January 2004

First published online 26 January 2004

Edited by Stuart Ferguson

Abstract **Precise regulation of calcium transporters is essential for modulating the Ca^{2+} signaling network that is involved in the growth and adaptation of all organisms. The *Arabidopsis* H^+/Ca^{2+} antiporter, CAX1, is a high capacity and low affinity Ca^{2+} transporter and several CAX1-like transporters are found in *Arabidopsis*. When heterologously expressed in yeast, CAX1 is unable to suppress the Ca^{2+} hypersensitivity of yeast vacuolar Ca^{2+} transporter mutants due to an N-terminal autoinhibition mechanism that prevents Ca^{2+} transport. Using a yeast screen, we have identified CAX interacting protein 4 (CXIP4) that activated full-length CAX1, but not full-length CAX2, CAX3 or CAX4. *CXIP4* encodes a novel plant protein with no bacterial, fungal, animal, or mammalian homologs. Expression of a GFP-CXIP4 fusion in yeast and plant cells suggests that CXIP4 is targeted predominantly to the nucleus. Using a yeast growth assay, CXIP4 activated a chimeric CAX construct that contained specific portions of the N-terminus of CAX1. Together with other recent studies, these results suggest that CAX1 is regulated by several signaling molecules that converge on the N-terminus of CAX1 to regulate H^+/Ca^{2+} antiport.**

Key words: H^+/Ca^{2+} antiporter; Calcium signaling; CAX1; Activation; *Arabidopsis*

1. Introduction

Cytosolic Ca^{2+} acts as a second messenger that is involved in an array of biological signaling networks [1,2]. To translate the generic signals to specific events, the concentration of cytosolic Ca^{2+} is finely regulated, in turn, by influx systems, such as Ca^{2+} channels, and efflux systems, such as Ca^{2+} pumps and antiporters [3]. It has been hypothesized that the endomembrane Ca^{2+} transporters, such as those present at the endoplasmic reticulum and vacuolar membrane, may play an important role in specifying the duration and amplitude of specific cytosolic Ca^{2+} fluctuations [3,4]. Therefore, understanding the regulatory mechanisms of these Ca^{2+} transporters may be fundamental to dissecting Ca^{2+} signaling specificity.

The first plant H^+/Ca^{2+} antiporters were cloned by their ability to suppress the Ca^{2+}-hypersensitive phenotype of a *Saccharomyces cerevisiae* mutant [5,6]. These genes have been termed cation exchangers (CAX) [5]. CAX1 from *Arabidopsis thaliana* is a high capacity and low affinity Ca^{2+} transporter. CAX1 has been shown to localize to the plant vacuole [7] and its activity appears to be regulated by an N-terminal autoinhibitory domain [8,9]. *Arabidopsis* has up to 12 putative H^+/cation antiporters (CAX1–11 and MHX) [10]. Three close homologs of CAX1, termed CAX2, CAX3 and CAX4, also appear to be N-terminally regulated and neither full-length CAX2, CAX3 nor CAX4 is able to suppress the Ca^{2+} sensitivity of K667 ([11–13]; Pittman et al., unpublished). In addition, ectopic expression of the N-terminally truncated, and thus activated, CAX1 (sCAX1) in tobacco increases Ca^{2+} levels in the plants and causes numerous stress sensitivity phenotypes often associated with Ca^{2+} deficiencies [14]. However, recent biochemical and genetic studies indicate that in *Arabidopsis* the native CAX1 does not appear to be truncated in plants [7]. Thus, the major question raised is how does CAX1 become activated in planta?

Post-translational regulation of Ca^{2+} transporters is evident in yeasts, higher plants and mammals [15,16]. For example, genetic analysis suggests that the yeast H^+/Ca^{2+} exchanger, VCX1, is negatively regulated by calcineurin [17]. In plants, a type IIB Ca-ATPase, ACA2, contains an N-terminal autoinhibitor, and is activated by calmodulin [18]. From numerous studies, it has become clear that the N-termini of particular plant Ca^{2+}-ATPases act as points of convergence between Ca^{2+} signaling molecules that can both positively and negatively regulate Ca^{2+} transport [19]. Our previous studies suggest that CAX1 also has an N-terminal autoinhibitor [8]. However, CAX1 is apparently not regulated by binding calmodulin at its N-terminus or phosphorylation through a Ca^{2+}-dependent protein kinase [8]. This finding strongly suggests that a unique set of regulators interact with the N-terminus of CAX1 to modulate Ca^{2+} transport.

In this present report, we have utilized a functional yeast screen to identify a novel *Arabidopsis* protein, CXIP4, which has no homologs in non-plant organisms. We demonstrate that CXIP4 can activate CAX1 in a yeast growth assay. We further characterize the subcellular localization of the protein by making a fusion of the plant gene product with the green fluorescence protein (GFP). We have also characterized the

*Corresponding author. Fax: (1)-713-798 7078.
E-mail address: ncheng@bcm.tmc.edu (N.-H. Cheng).

Abbreviations: CAX, cation exchanger; sCAX, deregulated CAX missing the N-terminal autoinhibitor; YPD, yeast extract/peptone/dextrose medium; CXIP, CAX interacting protein

0014-5793/04/$30.00
doi:10.1016/S0014-5793(04)00036-5

specificity of this activation and the region of CAX1 which is involved in the association between CAX1 and CXIP4. These findings along with other recent studies [20,21] suggest an array of proteins can associate with the N-terminus of CAX1 to specifically modify this Ca^{2+} transporter.

2. Materials and methods

2.1. Yeast strains and plant materials

S. cerevisiae strain K667 (*MATa cnb1::LEU1 pmc1::TRP1 vcx1Δ ade2-1*, *can1-100*, *his3-11*, *15leu2-3*, *112trp1-1* and *ura3-1*) [17] was used in all yeast experiments involving the expression of *CAX* genes with or without *CXIP4*. *A. thaliana* Columbia ecotype and *Nicotiana tabacum* 'Xanthi' and BY-2 cell line [22] were used in this study.

2.2. Yeast transformation and Ca^{2+} tolerance assay

Yeast cells expressing CAX transporters (CAX1, CAX2, CAX3, CAX4, chimeric CAX constructs or CAX1 mutants) were transformed with CXIP4 as described previously [20]. Stable transformants were assayed on YPD (yeast extract/peptone/dextrose) medium supplemented with 200 mM $CaCl_2$ [8,23]. Some yeast strain colonies had different colors when grown on Ca^{2+}-containing media and YPD medium. We found this in all our yeast growth assays. However, we have never documented that these yeast cells with different colors have changed their calcium transport properties (or other phenotypes).

2.3. DNA constructs and site-directed mutagenesis

CAX chimeric constructs (sCAX3-β, sCAX3-γ, sCAX3-δ, sCAX1-α and sCAX3-α1) and CAX1 variants were previously described [9,23].

2.4. Preparation of microsomal membrane-enriched vesicles and Ca^{2+} transport assay

Microsomal membrane-enriched vesicles were isolated from yeast as described previously [9]. Time-dependent H^+/Ca^{2+} transport into endomembrane vesicles was examined using the direct filtration method [8].

2.5. RNA gel blotting analysis

RNA sample preparation and Northern blot analysis were performed as previously described [7,20].

2.6. Construction of eGFP-CXIP4 fusion and expression in yeast and plant cells

To fuse the GFP to the N-terminus of CXIP4, the eGFP (Clontech, Palo Alto, CA, USA) gene was amplified by polymerase chain reaction (PCR) using the following primer sets. Forward primer: 5′-CGG CTC GAG CCA TGG TGA GCA AGG GCG-3′; reverse primer: 5′-AGG ACT GGA TCC GCC CTT GTA CAG CTC GTC CAT-3′. *Xho*I and *Bam*HI sites (underlined) were introduced for subsequent cloning sites. The eGFP gene fragment without stop codon was cloned into *Xho*I/*Bam*HI sites of pBluescript KS (Stratagene, La Jolla, CA, USA), resulting in pKS-eGFP. The *CXIP4* gene was amplified by PCR using the following primer sets. Forward primer: 5′-GCC GGA TCC ATG CCG CGC ACA GCA GGA AGA-3′; reverse primer: 5′-GGG AGC TCT CGA GTC GAC CTA CTC TCG CTC TTT CCT GTG-3′. *Bam*HI and *Sac*I sites (underlined) were introduced for subsequent cloning sites. The *CXIP4* gene fragment was cloned into the *Bam*HI/*Sac*I sites of pKS-eGFP, resulting in pKS-GFP-CXIP4. The fusion construct was completely sequenced to confirm no PCR errors. The fusion protein product would have three amino acid residues added between GFP and CXIP4 due to the introduction of the *Bam*HI site. To express the fusion protein in the yeast cells and plants, the GFP-CXIP4 was subcloned into yeast expression vector piUGpd [24] and the plant transient expression vector pRTL2 [25]. The plasmid DNA of piUGpd-GFP-CXIP4 was used to transform the yeast strain K667 and selected on −Ura medium. Stable transformants were grown in the same selection medium and the expression of the fusion protein was examined by confocal microscopy [12]. The plasmid DNA of pRTL2-GFP-CXIP4 was delivered into tobacco leaves or BY-2 cells by Biolistic Particle Delivery System (model PDS-1000, Dupont, DE, USA) as previously described [26]. For visualization of nuclei or mitochondria, the bombarded BY-2 cells were briefly fixed with 2% (v/v) formaldehyde in PME (50 mM PIPES, 4 mM $MgSO_4$, and 10 mM EGTA) buffer at 8–12 h post bombardment for 1–5 min and then stained with 0.1% propidium iodide (Sigma, St. Louis, MO, USA) or Mito-Tracker (Molecular Probes, Eugene, OR, USA) for 5 min. The transient expression and subcellular localization of the fusion protein and nuclei or mitochondria were observed by confocal microscopy (model 1024ES, Bio-Rad, Hercules, CA, USA). The green channel (excitation at 488 nm, emission at 522 nm, and barrier filter at 522–535 nm) and red channel (excitation at 568 nm, emission at 598 nm, and barrier filter at 605–632 nm) were scanned separately to avoid cross-contamination and then merged using Lasersharp 3.1 software provided by the manufacturer (Bio-Rad). The confocal images were processed using Adobe Photoshop software (version 6.0; Adobe Systems, San Jose, CA, USA).

3. Results

3.1. CXIP4 encodes a novel polypeptide that specifically activates CAX1

In a previous study, using a yeast expression *Arabidopsis* cDNA library [27], we identified and isolated several *Arabidopsis* cDNAs that allow the full-length CAX1-expressing K667 (*pmc1 cnb1 vcx1*) yeast strain, which is hypersensitive to high Ca^{2+}, to grow in media containing high levels of Ca^{2+} [20,21]. We named the protein products of these cDNAs CAX interacting proteins (CXIP). In this screen, four out of the six cDNA clones isolated were identical and encode a protein whose function has not been previously elucidated in any organism. We have termed this gene *CXIP4*. The *CXIP4* cDNA consists of 999 base pairs (GenBank™ accession number AY163162). Basic Local Alignment Search Tool (BLAST) searches against genome and EST databases revealed that *CXIP4* is a novel plant gene with no homologs in bacteria, fungi, animals and mammals. *CXIP4* has homologs in other plant species, including rice, wheat, barley, tomato, grape, sunflower, and *Medicago truncatula*, suggesting that the *CXIP4* gene is specifically expressed in plants (Fig. 1). *CXIP4* encodes a protein that consists of 332 amino acids with a predicted molecular mass of 37.8 kDa. Analysis of the amino acid composition of the CXIP4 polypeptide found that CXIP4 is rich in the following residues: Arg (15.1%), Lys (11.1%) Glu (11.7%) and Ser (15.4%). A search for specific functional motifs by using the MotifScan program revealed that there is a single zinc finger CCHC motif at the N-terminal region and also an arginine-rich region located at the C-terminus of CXIP4 (Fig. 1). A PSORT program to predict the organelle-targeting signals revealed that CXIP4 has multiple nuclear-targeting signatures, which are located in the C-terminus. In addition, the TargetP program [28] assigned a score of 0.83 for mitochondrial localization, suggesting that CXIP4 might target to mitochondria in vivo.

To verify the ability of CXIP4 to activate CAX1 Ca^{2+} transport activity, a yeast growth assay was performed as previously described [20]. Expression of CXIP4 or full-length CAX1 alone did not suppress the Ca^{2+} hypersensitivity of the yeast vacuolar Ca^{2+} transport defect mutant (K667), whereas K667 coexpressing CXIP4+CAX1 could grow on 200 mM $CaCl_2$ in a similar manner to the yeast cells expressing N-terminally truncated CAX1 (sCAX1, Fig. 2A). This result supports the hypothesis that activation of CAX1 by CXIP4 mediated H^+/Ca^{2+} antiport activity in yeast. To directly test this hypothesis, we measured ΔpH-dependent 10 μM $^{45}CaCl_2$ uptake into microsomal vesicles isolated from K667 yeast strains expressing sCAX1, CAX1+CXIP4, CAX1 or CXIP4 alone. H^+/Ca^{2+} transport activity was observed in membrane

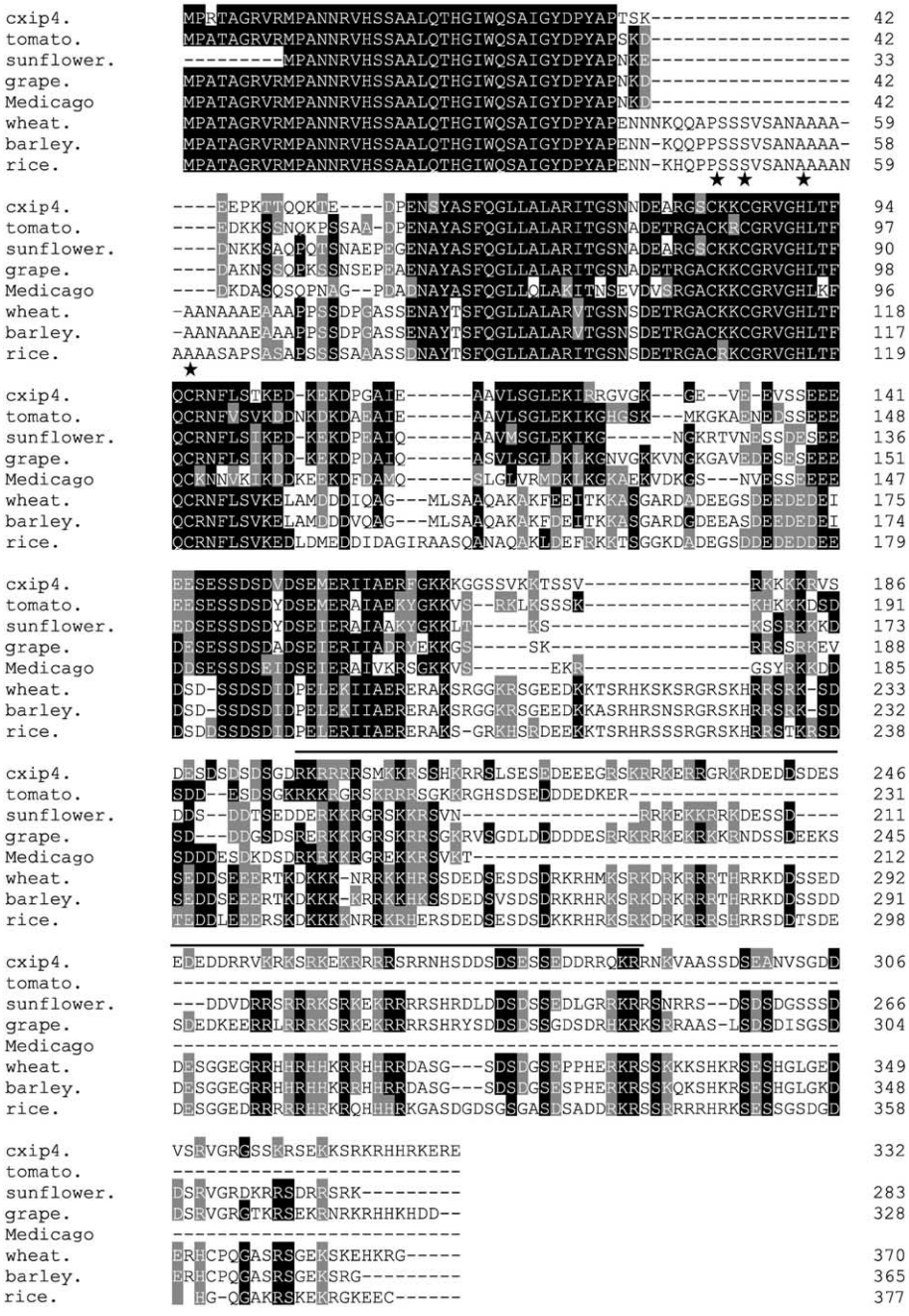

Fig. 1. Amino acid sequence alignment of *Arabidopsis* CXIP4 with its homologs from other plant species. Alignments were performed using the Clustal W 1.8 program (Baylor College of Medicine [34]). Consensus amino acid residues are boxed in black (identical) or gray (similar). Gaps introduced to maximize the alignments are denoted by hyphens. The asterisks indicate a zinc finger CCHC type motif. An arginine-rich region located at the C-terminus is overlined. The EST cDNA accession numbers for tomato, sunflower, grape, medicago, wheat, barley and rice are TC118856, TC12511, TC19312, TC83616, TC123418, TC149427 and TC126252, respectively. All EST cDNA sequences shown are truncated at the carboxy-terminus.

vesicles from a CAX1+CXIP4-expressing strain and a sCAX1-expressing strain (Fig. 2B). However, no H^+/Ca^{2+} antiport activity was detectable in vesicles from full-length CAX1-expressing or CXIP4-expressing yeast strains (Fig. 2B, data not shown). The H^+/Ca^{2+} antiport activity measured from CXIP4+CAX1 vesicles was not high, about $10 \pm 1.5\%$ of the activity measured from sCAX1 vesicles, but significantly meaningful compared with CAX1 alone.

Given that expression of other full-length *Arabidopsis* CAX transporters does not suppress yeast mutants deficient in vacuolar Ca^{2+} transport ([11–13]; Pittman et al., unpublished), we were interested in the specificity of CXIP4+CAX activation. As shown in Fig. 2A, CXIP4 was unable to activate full-length CAX2, CAX3 and CAX4.

3.2. *CXIP4 activation requires the presence of the N-terminus of CAX1*

CXIP4 specifically activated CAX1. Given that CAX3 is 93% similar to CAX1 [11], we were interested in determining the domains or regions within CAX1 required for CXIP4

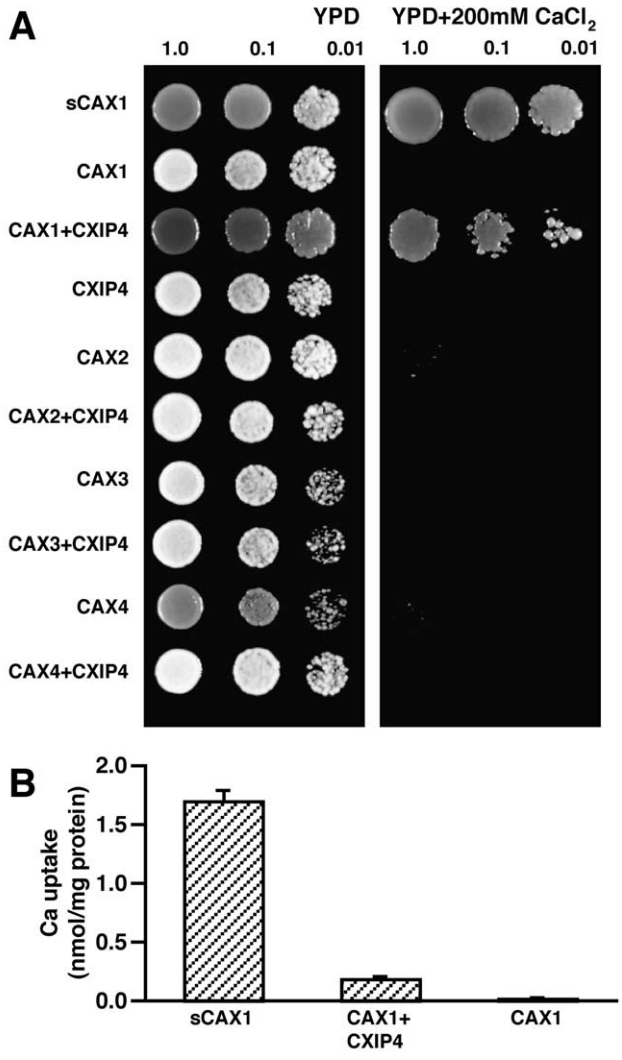

Fig. 2. CXIP4 specifically activates CAX1 Ca^{2+} transport activity. A: Suppression of the *pmc1 vcx1 cnb1* (K667) yeast Ca^{2+} sensitivity in cells expressing sCAX1 and full-length CAX1, CAX2, CAX3 and CAX4 with or without CXIP4. Yeast cells coexpressing various plasmids, as indicated, were grown to $A_{600} = 1.0$ in selection medium at 30°C. Five microliters of serial dilutions, as indicated, were spotted onto YPD medium and the same medium supplemented with 200 mM $CaCl_2$. Photographs were taken after 3 days. B: Uptake after 8 min of 10 μM $^{45}Ca^{2+}$ from microsomal vesicles prepared from *S. cerevisiae* strain K667 after transformation with sCAX1, CAX1+CXIP4 and CAX1. Uptake was estimated as the difference between parallel samples with and without the addition of gramicidin (5 μM). Data are the means of three independent experiments, and the bars indicate S.E.

activation. In a previous study, a series of CAX1/CAX3 chimeras were created to determine the structural determinant required for Ca^{2+} transport [23]. In those chimeras, both CAX1 and CAX3 lack the first 36 amino acids that appear to be required to negatively regulate antiport activity. An N-terminal truncation of CAX3 (sCAX3), like full-length CAX3, was still unable to suppress the vacuolar Ca^{2+} transport deficiency despite lacking its autoinhibitory domain ([23], Fig. 3). In addition, sCAX3 coexpressed with CXIP4 was unable to suppress the yeast Ca^{2+} sensitivity (Fig. 3). As shown in Fig. 3, yeast cells expressing numerous sCAX3/CAX1 proteins+CXIP4 were unable to suppress the Ca^{2+} sensitivity of K667 yeast. For example, K667 strains in which CXIP4 was coexpressed with sCAX3/CAX1 chimeric proteins that contained the central region or C-terminus of CAX1 were unable to suppress the Ca^{2+}-sensitive phenotype. However, when a chimeric protein (called sCAX3-α1) was used that contained 37 amino acids of the N-terminal region of CAX1 (Met^{37} to Leu^{73}) fused to CAX3, CXIP4 could activate this chimeric protein, suggesting that this region in CAX1 is required for CXIP4 to activate CAX1.

It was demonstrated that specific amino acids residues within the N-terminus of CAX1 are critical for autoinhibition [9]. For example, when serine-25 was changed to aspartic acid (D) and when the threonine-33 residue was changed to any other amino acid such as alanine (A) or glutamic acid (E), CAX1 was no longer autoinhibited and H^+/Ca^{2+} transport could occur. In contrast, other single or double point mutations within the CAX1 N-terminus did not alter CAX1 autoinhibition [9]. To test if CXIP4 could allow autoinhibited CAX1 mutants to suppress the Ca^{2+} sensitive phenotypes, we expressed CXIP4 in yeast strains expressing these CAX1 mutants. As shown in Fig. 4, some CAX1 mutants, such as CAX1-S25A, CAX1-S25T and CAX1-E7A/E13A, remained autoinhibited in the presence of CXIP4. However, other CAX1 single or double point mutants that retain autoinhibition were activated following coexpression with CXIP4. CXIP4 did not appear to alter the activity of any of the deregulated CAX1 constructs, such as the single point mu-

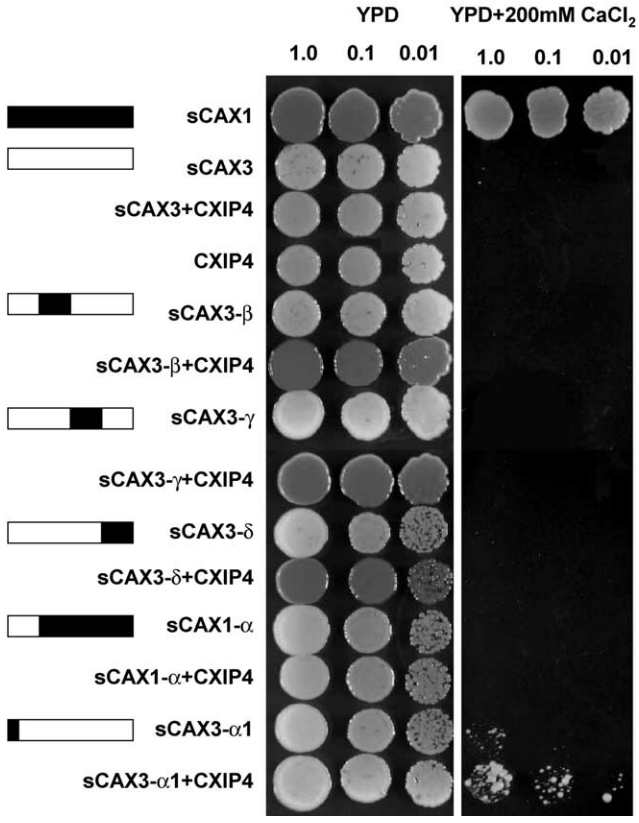

Fig. 3. Identification of regions in CAX1 which can activate CAX3. Suppression of the yeast vacuolar Ca^{2+} transport defect in cells expressing CAX chimeras+CXIP4. K667 yeast cells coexpressing various plasmids, as indicated, were grown as described in Fig. 1. The CAX1 open reading frame is shown as a solid bar while CAX3 is shown as an open bar.

tants CAX1-S25D, CAX1-T33A or the double point mutant CAX1-R29A/R32A, as these all remained active following CXIP4 expression (data not shown).

3.3. *Expression of* CXIP4

To examine expression of *CXIP4*, total RNA from various tissues from mature *Arabidopsis* plants was used for RNA gel blot analysis. As shown in Fig. 5A, *CXIP4* was expressed in all tissues with highly abundant expression found in leaves, stems, and roots, but slightly lower expression in flowering tissue. To determine how *CXIP4* is expressed in response to ion stresses, 3-week-old seedlings were treated with various metal ions. The *CXIP4* RNA transcript levels were modestly increased when Ca^{2+} was added to the medium, while the *CXIP4* RNA levels did not change when seedlings were exposed to Na^{+}, Mn^{2+} or Ni^{2+}, compared to control seedlings treated with H_2O or Murashige and Skoog (MS) medium. Interestingly, *CXIP4* expression was reduced upon $ZnCl_2$ treatment (Fig. 5B).

3.4. *Subcellular localization of CXIP4*

To investigate the subcellular localization of the CXIP4 protein, we constructed an N-terminal GFP fusion with

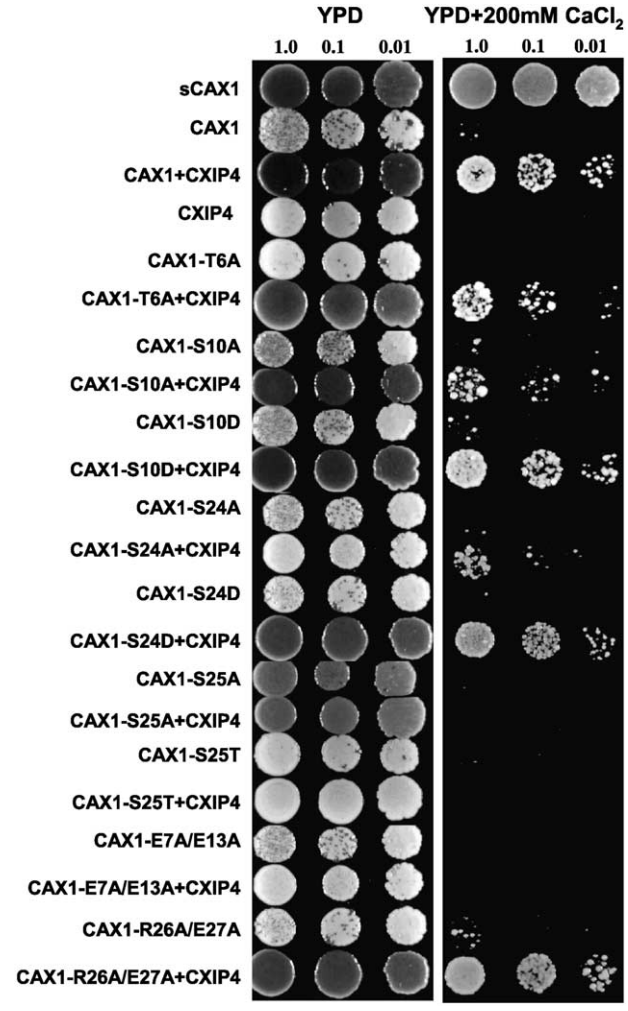

Fig. 4. CXIP4 activates CAX1 mutants. Suppression of the yeast vacuolar Ca^{2+} transport defect mutant by cells expressing CAX1 mutants+CXIP4. K667 yeast cells coexpressing various plasmids, as indicated, were grown and assayed as described in Fig. 2A.

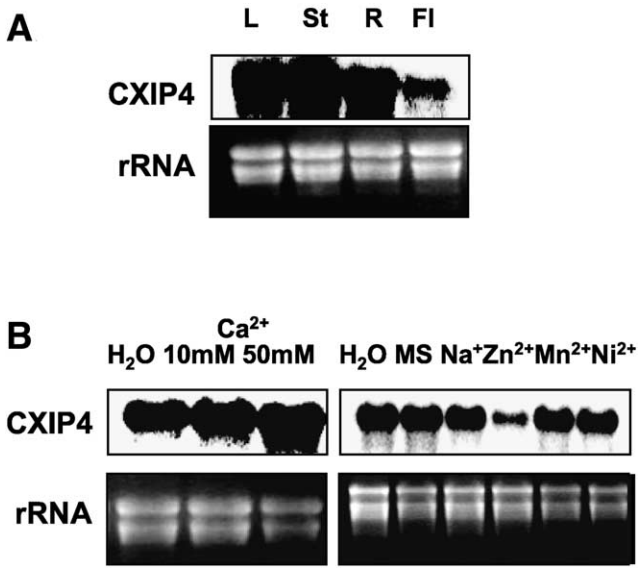

Fig. 5. Tissue distribution and induction of *CXIP4*. A: Total RNA samples were isolated from *Arabidopsis* rosette leaves (L), stems (St), roots (R) and flowers (Fl). B: Total RNA samples were extracted from 3-week-old *Arabidopsis* seedlings previously treated for 12 h with the following solutions: water (as a control), MS nutrient medium, 10 or 50 mM $CaCl_2$, 80 mM NaCl, 1 mM $ZnCl_2$, 2 mM $MnCl_2$ or 0.1 mM $NiSO_4$. Twenty micrograms of total RNA were hybridized with ^{32}P-labeled *CXIP4* cDNA. Ethidium bromide (EtBr)-stained rRNAs are shown as a loading control.

CXIP4 and expressed this in yeast cells under the control of the yeast glyceraldehyde-3-phosphate dehydrogenase promoter and in tobacco leaves or BY-2 cells under the control of a CaMV 35S promoter. When GFP-CXIP4 was coexpressed with CAX1 in the yeast mutant strain, K667, the yeast cells were able to suppress the Ca^{2+}-sensitive phenotype, suggesting that the fusion protein remained functional (data not shown). The localization of the fusion protein in these yeast cells was observed predominantly in the nucleus, but we also saw specific microdomains in the cytosol where the fusion protein is localized (Fig. 6A). Consistent with the result from yeast cells, the GFP-CXIP4 transiently expressed in tobacco BY-2 cells was predominately located in nuclei, which were visualized with propidium iodide staining (Fig. 6B). The GFP-CXIP4 was also transiently expressed in tobacco leaves and the fusion protein was again located in the nuclei of leaf epidermal, mesophyll and trichome cells (data not shown). Interestingly, in leaf epidermal cells, the GFP-CXIP4 was observed in both nuclei and the cytoplasm (Fig. 6C). Apparently, those green fluorescent fusion proteins are located in discrete areas of the cytoplasm; however, they are not colocalized with the mitochondria (Fig. 6D).

4. Discussion

It was previously demonstrated that the H^{+}/Ca^{2+} antiporter, CAX1, plays an important role in ion homeostasis [7,14]. In addition, biochemical studies in yeast indicate that full-length CAX1 contains an autoinhibition domain at its N-terminus that regulates Ca^{2+} transport [9]. In this work, we have used a yeast-based approach to characterize an *Arabidopsis* gene, *CXIP4*, that activates full-length CAX1 to modify microsomal membrane H^{+}/Ca^{2+} antiport activity in yeast (Figs.

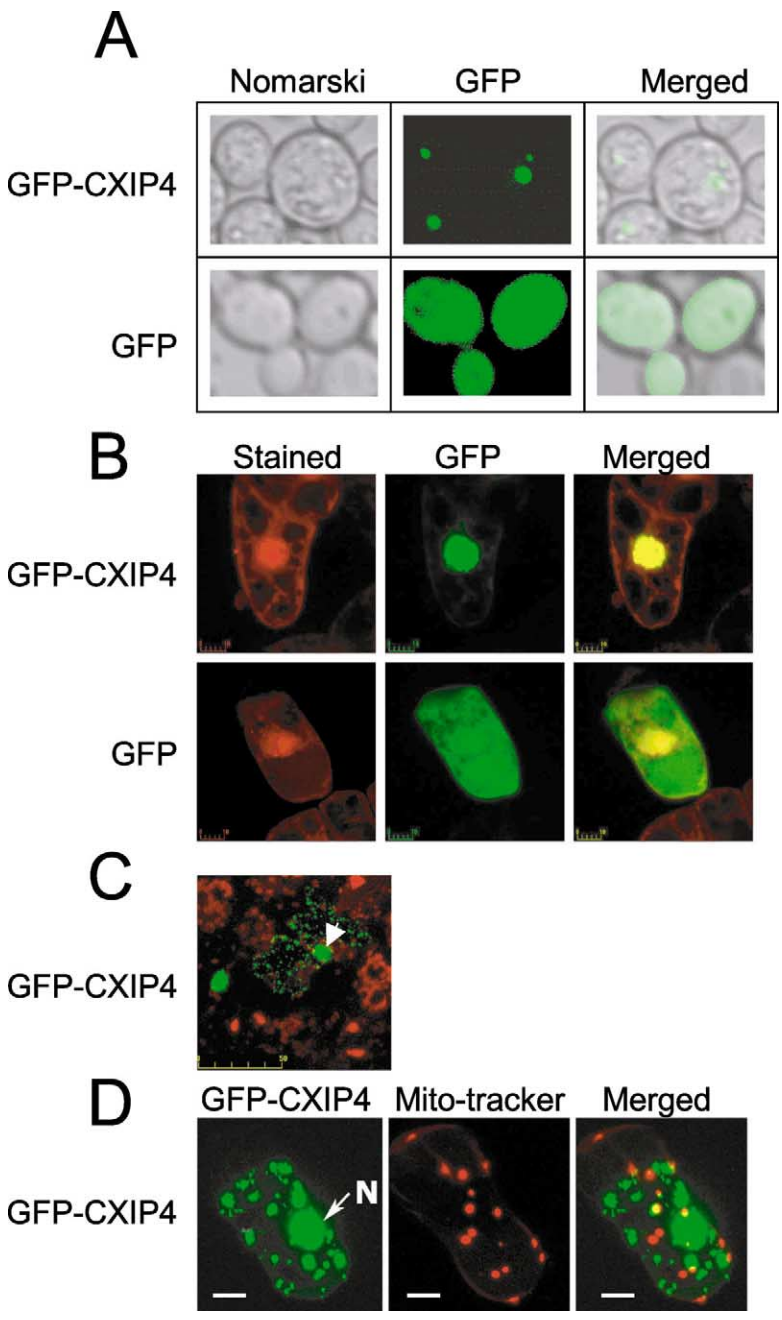

Fig. 6. Subcellular localization of GFP-CXIP4 in yeast and tobacco BY-2 and leaf cells. A: GFP-CXIP4 fusion is localized to nuclei in yeast cells. In the upper panels, the GFP-CXIP4 fusion was present in the nuclei of yeast cells. In the lower panels, the free GFP was present in entire yeast cells. The magnification for each image is 120×. B: GFP-CXIP4 fusion targets to the nuclei of tobacco BY-2 cells. The upper panels showed that the GFP-CXIP4 fusion was transiently expressed in nuclei of tobacco BY-2 cells. The lower panels showed that the free GFP was expressed in both nuclei and cytoplasm of tobacco BY-2 cells. The nuclei of tobacco cells were stained with 0.1% propidium iodide. Scale bars: 10 μm. C: The GFP-CXIP4 was transiently expressed in tobacco leaf epidermal cell. The arrowhead indicates the nucleus. Scale bar: 50 μm. D: Shown is that the GFP-CXIP4 fusion was transiently expressed in nuclei and cytoplasm of tobacco BY-2 cells. The mitochondria were stained by Mito-Tracker. The arrow indicates the nucleus. Scale bar: 50 μm.

2–4). *CXIP4* encodes a novel nuclear-targeting polypeptide (Fig. 6) and the function of *CXIP4* has not been previously reported from any organism.

Sequence analysis of *CXIP4* indicates that *CXIP4* is a plant-specific open reading frame (ORF, Fig. 1). The *Arabidopsis CXIP4* ORF and the *CXIP4*-like ORFs from other plant species are highly conserved at the amino-terminal half of the protein and less conserved in the carboxy-terminal half of the protein (Fig. 1). Computational analysis suggests that the nuclear-targeting motifs are predominately located at the C-terminal half of the putative proteins. In agreement, GFP fused to the N-terminus of CXIP4 did not appear to disrupt the localization and function of CXIP4 (Fig. 6; data not shown). Interestingly, within this conserved portion of the ORF, the *CXIP4*-like ORFs from monocotyledonous species have several extra stretches of sequences which are also conserved among monocots, suggesting that these domains might play a unique role in these plants. Future work will be focused

on the physiological function of these conserved regions of CXIP4.

The inability of CXIP4 to activate full-length CAX2, CAX3 and CAX4 suggests specificity in the CXIP4/CAX1 activation (Fig. 2A). To determine which regions of CAX1 were required for this activation, we utilized chimeric CAX3 constructs to demonstrate the importance of the N-terminus of CAX1 (Fig. 3). Apparently, the growth of the yeast strain expressing sCAX3-α1+CXIP4, which lacks the first 36 amino acids of CAX1, was not as strong as that expressing full-length CAX1+CXIP4 (Fig. 3), suggesting that either the entire N-terminus of CAX1 or other portions of the CAX1 ORF are also involved in CXIP4-mediated activation. When CXIP4 was coexpressed with several CAX1 N-terminal mutants which remain autoinhibited, these yeast strains were activated similar to the full-length CAX1+CXIP4 (Fig. 4). However, some CAX1 N-terminal mutants were not capable of being activated by CXIP4 (Fig. 4). In sum, these results imply that there are multiple regulatory mechanisms used to activate CAX1 Ca^{2+} transport and that multiple domains in the N-terminus of CAX1 are involved in this activation.

Direct measurement of Ca^{2+} transport from the vesicles isolated from the yeast strains expressing CXIP4+CAX1 indicates that the H^+/Ca^{2+} antiport activity mediated by CXIP4-activated CAX1 is significantly less than that of the deregulated sCAX1 (Fig. 2B). We postulate that activation of CAX1 by N-terminal truncation (as with sCAX1) is artificial and does not occur in planta [7], and the strong Ca^{2+} transport activity mediated by sCAX1 may not be realized by the fully activated CAX1. Similarly, Ca^{2+} transport mediated by the calmodulin-activated *Arabidopsis* Ca^{2+}-ATPase ACA2 is significantly less than an N-terminally truncated version of ACA2 lacking its calmodulin-binding, autoinhibitory domain [18]. Considering the lower Ca^{2+} antiport activity in our measurement, a possible explanation might be that a large proportion of CXIP4 was lost when microsomal membrane vesicles were purified. Moreover, as mentioned above, there might be multiple regulatory mechanisms or a protein complex, which are involved in the activation of CAX1 in vivo. Expression of CXIP4+CAX1 might not be sufficient to fully activate Ca^{2+} transport activity in yeast.

CXIP4 RNA transcripts were highly abundant in all tissues of *Arabidopsis* plant (Fig. 5A). Consistent with this observation, four identical *CXIP4* cDNA clones were isolated from our initial yeast screen [20]. Similar to other *CXIP* genes, the expression of *CXIP4* is modestly induced by Ca^{2+} (Fig. 5B) [20]. However, the RNA level of *CXIP4* does not appear to be regulated by other ions except zinc (Fig. 5B). It is well documented that zinc can modulate expression of many genes, including proteins which contain zinc finger motifs [29,30]. For example, a yeast zinc finger gene, *Zap1*, is down-regulated by intracellular Zn^{2+}, and plays an important role in zinc homeostasis in yeast cells [31]. Given CXIP4 contains a zinc finger CCHC motif at its N-terminus, it is possible that *CXIP4* is regulated by Zn^{2+} in response to ion stresses.

GFP-CXIP4 appears to predominately localize to nuclei in yeast cells, as well as tobacco cells (Fig. 6; data not shown). Given that CAX1 appears to localize in both yeast and plant vacuoles and functions in vacuolar Ca^{2+} transport, the potential interaction between the nuclear localized CXIP4 and CAX1 is difficult to envision. In our yeast assays, when CXIP4 was coexpressed with sCAX1 or particular CAX1 mutants, CXIP4 did not alter CAX1 localization (Fig. 4; data not shown). Moreover, expression of CAX1+GFP-CXIP4 in yeast does not appear to alter the localization of GFP-CXIP4. Interestingly, during transient expression of the GFP-CXIP4 in tobacco leaf epidermal cells, which contains a large central vacuole, the fusion protein was also observed in the cytoplasm (Fig. 6C), which is similar to the localization observed in some yeast cells (Fig. 6A). This suggests that CXIP4 might be localized to different compartments in certain tissues or cell types. Because the GFP-CXIP4 fusion proteins were observed in discrete microdomains within the cytoplasm and not dispersed throughout the cytoplasm, there is a possibility that the fusion proteins are also targeting to a particular endomembrane, such as endoplasmic reticulum lumen or Golgi apparatus. Apparently these proteins are not colocalized with the mitochondria as shown in Fig. 6D.

There are many possible mechanisms by which CXIP4 may regulate CAX1. For example, CXIP4 could activate CAX1-mediated Ca^{2+} transport through changing the transporter conformation. As an example, it has been demonstrated that a regulatory protein, tescalcin, directly interacts with the C-terminus of a mammalian Na^+/H^+ exchanger and changes the transporter conformation, and thus modulates Na^+/H^+ exchange [32]. Alternatively, CXIP4 could associate with additional proteins to alter the subcellular localization of CAX1 and consequently mediate transport activity. Finally, CXIP4 may alter cytosolic Ca^{2+} levels and this environmental perturbation modulates the N-terminus of CAX1 to activate the transporter. Although we present no evidence to directly support any of these models, our previous results suggest that multiple components associate with CAX1 and they may be required in specific combinations for maximal transport activity.

CXIP4 is a novel protein whose function has not been previously characterized in any plant species (Fig. 1). Given the predominately nuclear localization of this protein, we speculate that this protein may have functions unrelated to regulation of CAX1 transport. For example, a β_{4c} subunit of a mammalian voltage-gated Ca^{2+} channel, which is localized to the plasma membrane, directly interacts with a nuclear protein and alters the function of this protein in the nucleus [33]. In addition, the single zinc finger motif in the N-terminal conserved region implies that this protein is modulated by the zinc status in the cells and may function as a transcription factor in regulating gene expression in response to ion stresses [31]. Future work perturbing the expression of CXIP4 within the plant will help clarify the various functions of this protein.

In this study, our efforts to test the direct interaction between CAX1 and CXIP4 were not successful. First, we performed yeast two-hybrid assay to test if CXIP4 could physically interact with the CAX1 N-terminus. This assay failed due to self-activation mediated by CXIP4. As an alternative approach, we cloned *CXIP4* into pGEX-TK2 to make a GST-CXIP4 fusion construct. However, the GST-CXIP4 was not expressed in this bacterial expression system (BL21 DE3 bacterial strain). Thus we are unable to directly test whether the activation of CAX1 by CXIP4 is due to the direct interaction between the CAX1 N-terminus and CXIP4. Continuing studies will determine whether the GFP fusion correctly reflects the localization of the native CXIP4 and also use different approaches to test the nature of CXIP4 activation of CAX1.

Our previous studies demonstrated that there might be mul-

tiple signaling molecules that are involved in regulating CAX1 Ca^{2+} transport [20,21]. Here, we show that a novel nuclear-targeting protein, CXIP4, also modulates CAX1. Taken together, we postulate that a macromolecular signaling network might be required for activating CAX1 to modify CAX1-mediated Ca^{2+} transport. Future work using reverse genetics in *Arabidopsis* will help clarify the contribution of individual CAX interacting proteins in CAX-mediated H^+/Ca^{2+} transport and dissect the mechanistic link between Ca^{2+} signaling mediated by CAX1 and CXIP-mediated signal transduction pathways.

Acknowledgements: This work was supported by the United States Department of Agriculture/Agricultural Research Service under Cooperative Agreement 58-6250-6001 and by the National Science Foundation Grant 020977. We thank Jon Pittman, Toshiro Shigaki and Chad Hall for their comments.

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