Cloning and Characterization of CXIP1, a Novel PICOT Domain-containing Arabidopsis Protein That Associates with CAX1*

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Regulation of Ca2+ transporters is a vital component of signaling. The Arabidopsis H+/Ca2+ exchanger CAX1 contains an N-terminal autoinhibitory domain that prevents Ca2+ transport when CAX1 is heterologously expressed in yeast. Using a yeast screen, we have identified three different proteins that activate CAX1. One of these, CXIP1 (CXI-interacting protein-1; 19.3 kDa) has amino acid similarity to the C terminus of PICOT (protein kinase C-interacting cousin of thioredoxin) proteins. Although PICOT proteins are found in a variety of organisms, a function has not been previously ascribed to a plant PICOT protein. We demonstrate that CXIP1 activated the CAX1 homolog CAX4, but not CAX2 or CAX3. An Arabidopsis homolog of CXIP1 (CXIP2) weakly activated CAX4, but not CAX1. In a yeast two-hybrid assay, CXIP1 interacted with the N terminus of CAX1. In competition analysis, CXIP1 and a CAX1 N-terminal peptide appeared to bind to similar N-terminal domains of CAX1. Chimeric CAX3 constructs containing the N terminus of CAX1 were activated by CXIP1. In Arabidopsis, CXIP1 transcripts, like CAX1, accumulated in response to different metal conditions. This work thus characterizes a new class of signaling molecules in plants that may regulate CAX transporters in vivo.

Ca2+ signal transduction requires the judicious control of cytosolic Ca2+ levels. Cytosolic “Ca2+ spikes” are either directly or indirectly translated into biological responses that govern all aspects of growth and development (1, 2). Endomembrane Ca2+ transporters are believed to play an important role in specifying the duration and amplitude of these cytosolic Ca2+ fluctuations (2, 3). Understanding the regulatory mechanisms of these Ca2+ transporters is a fundamental component in dissecting signaling specificity.

Expression in yeast has facilitated the cloning of numerous plant genes, including plant endomembrane Ca2+ transporters (4, 5). Initially, plant H+/Ca2+ antiporter genes were cloned by their ability to suppress the Ca2+-hypersensitive phenotype of a Saccharomyces cerevisiae mutant (4, 6). These genes are termed CAX for cation exchangers, and CAX1 from Arabidopsis thaliana is a high capacity Ca2+ transporter. Arabidopsis appears to have up to 11 other putative cation/H+ antiporters (CAX2–11 and MHX) (7). CAX1, CAX2, CAX4, and MHX have been shown to localize to the plant vacuole (8–11). The activity of CAX1 appears to be regulated by an N-terminal autoinhibitory domain that was absent in the initial clone characterized by heterologous expression in yeast (12, 13). Ectopic expression of deregulated CAX1 missing the N-terminal autoinhibitor (sCAX1) in tobacco increases Ca2+ levels in the plants and causes numerous stress-sensitive phenotypes often associated with Ca2+ deficiencies (14, 15). Thus, a wide range of environmental responses appear to require regulation of CAX1 transport activity; however, the mechanism by which CAX1 transport becomes activated is currently not understood.

From recent studies, it has become clear that the N termini of particular plant Ca2+-ATPases act as points of convergence between Ca2+-signaling molecules that can both positively and negatively regulate Ca2+ transport (3, 5). A Ca2+-dependent protein kinase competes with calmodulin for binding to the N termini of these Ca2+-ATPases to negatively regulate pump activity (16). Interestingly, the N terminus of CAX1 does not contain a calmodulin-binding site, and biochemical studies show that CAX1 cannot be activated by exogenous calmodulin (12). Moreover, expression of an activated Ca2+-dependent protein kinase in yeast strains harboring CAX1 does not alter activity.1 These findings strongly suggest that a unique set of signal transduction molecules physically interact with the N terminus of CAX1 to modulate Ca2+ transport.

We propose that one possible mechanism of activation of CAX1 is through a protein cofactor that directly interacts with the antiporter. In this work, we have utilized autoinhibited CAX1 in a yeast screen to identify and characterize plant gene products that activate CAX1-mediated H+/Ca2+ transport. We have characterized the specificity of this activation and the regions of CAX1 involved in the physical interaction between CAX1 and one of these activators. This study details a new class of signaling molecules in plants that may be involved in activation of H+/Ca2+ transporters in plants.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plant Materials—S. cerevisiae strain K667 (MATa cnb1::LEU1 pmc1::TRP1 vcx1Δ ade2-1 can1-100 his3-11,15 leu2-3,112 trpl-1 ura3-1) (17) was used in all yeast experiments involving the expression of CAX genes with or without CXIP1 (CXI-interacting protein) genes. Yeast strain Y190 (MATa gal4 gal80 his3 trpl-1 ura3-112 ade2-101 ura3-52 leu2-3,112 + UR3::GAL → lacZ, LY52::GAL(UAS) → HIS3 cyh2) was used in the yeast two-hybrid assay (18). The A. thaliana Columbia ecotype was used in this study.

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1 R. J. Pittman and K. D. Hirschi, unpublished observations.

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Yeast Transformation and Screening of a cDNA Library—Yeast strain K667 expressing CAX1, which is hypersensitive to high concentrations of Ca\(^{2+}\), was transformed with an A. thaliana (ecotype Landsberg) cDNA library constructed in the episomal yeast-Ascherichia coli shuttle vector pFL61 (19). Stable transformants were selected on synthetic complete medium lacking His and Ura (20). Of these, \(-2 \times 10^6\) colonies were replicated onto yeast extract/peptone/dextrose (YPD) medium supplemented with 200 mM CaCl\(_2\) to identify Ca\(^{2+}\)-tolerant transformants. cDNA clones of interest were isolated, and plasmid DNA was retransformed into the CAX1-expressing K667 yeast cells to confirm that the activation of CAX1 Ca\(^{2+}\) transport activity was attributable to the Arabidopsis gene DNA inserts. All cDNA inserts were subcloned into pBluescript and completely sequenced.

\footnotesize{\textsuperscript{a} A homolog of CXIP1 that was cloned from an Arabidopsis gene expression library using PCR amplification.} 
\footnotesize{\textsuperscript{b} Deduced using the protein analysis program ProtParam Tool (available at www.expasy.ch/cgi-bin/protparam).} 
\footnotesize{\textsuperscript{c} Determined using the BLAST program.}

### RESULTS

**Cloning and Sequencing of Arabidopsis cDNAs That Activate CAX1**—A pmc1vxc1cnb1 yeast strain (K667) lacking the vacuolar Ca\(^{2+}\) transporters Pmc1 and Vcx1 and calcineurin (Cnb1) is hypersensitive to high levels of Ca\(^{2+}\) (17). CAX1-expressing K667 cells grow well in standard medium, but do not grow in medium containing high levels of Ca\(^{2+}\) (12). Only expression of deregulated CAX1 (containing mutations or truncations of the N terminus) can suppress the yeast vacuolar Ca\(^{2+}\) transport defect (4, 13). Using a yeast expression Arabidopsis cDNA library (19), we have isolated Arabidopsis cDNAs that allow full-length CAX1-expressing K667 strains to grow in medium containing high levels of Ca\(^{2+}\). The cDNAs were isolated from the yeast and then retransformed into K667 strains with or without CAX1 to verify that they do not suppress the Ca\(^{2+}\) sensitivity of K667 by themselves and to confirm that the clones are indeed required for CAX1 to suppress the Ca\(^{2+}\) sensitivity of K667. We have used the term CXIP (CAX-interacting protein) for the protein products of these cDNAs.

Six different CXIP cDNA clones have been identified using this yeast activation approach (Table I). One of the cDNA clones, CXIP1, which is the focus of this study, encodes a novel Arabidopsis protein with sequence homology at the C terminus to PICOT proteins (24–26). We cloned an Arabidopsis homolog of CXIP1 that we have termed CXIP2, which encodes a protein of 293 amino acids (32 kDa). The yeast activation approach also identified CXIP3, which encodes an FKBP protein (\(A. \)thaliana FKBP15-2) (27). Four of the six cDNA clones identified in the yeast activation screen are identical and encode a protein whose function has not been previously characterized. We have termed this gene CXIP4.

K667 yeast cells expressing CXIP1 or CAX1 alone were unable to grow on 200 mM CaCl\(_2\), whereas coexpression of CXIP1 with CAX1 significantly enhanced the growth of K667 (Fig. 1A). However, growth of the (CAX1 + CXIP1)-expressing strain was not as strong as growth of K667 cells expressing N-terminally truncated sCAX1. The ability to suppress the Ca\(^{2+}\) sensitivity of the yeast mutants inferred activation of autoinhibited CAX1; however, it was important to demonstrate directly through biochemical studies that the combination of full-length CAX1 and CXIP1 mediated H\(^+\)/Ca\(^{2+}\) antiport activity in yeast. Therefore, we measured \(\Delta pH\)-dependent 10 \(\mu\)M 45CaCl\(_2\) uptake into microsomal vesicles isolated from K667 yeast strains expressing both Arabidopsis cDNAs. H\(^+\)/Ca\(^{2+}\) antiport activity was observed in membrane vesicles from a (CAX1 + CXIP1)-expressing strain and an sCAX1-expressing strain (Fig. 1B).

### Table I

**Summary of CXIPs**

| GeneBank™/EBI accession no. | Location in Arabidopsis genome | mRNA (bp) | Amino acids | Molecular mass (kDa) |
|-----------------------------|--------------------------------|-----------|-------------|---------------------|
| AY157988                    | Chromosome 2                   | 882       | 293         | 32                  |
| AY157989                    | Chromosome 3                   | 662       | 15          | 173                 |
| U52047                      | Chromosome 5                   | 999       | 32          | 93                  |
| AY163162                    | Chromosome 2                   | 332       | 173         | 522                 |

\footnotesize{\textsuperscript{d} GeneMass (kDa) and mRNA (bp) are taken from GenBank.}

### Notes

1. The abbreviations used are: HD, homology domain; Trx, thioredoxin.
However, no H\(^+\)/Ca\(^{2+}\) antiport activity was detectable in vesicles from full-length CAX1-expressing yeast strains (Fig. 1B and data not shown). The H\(^+\)/Ca\(^{2+}\) antiport activity measured in CAX1 + CXIP1 vesicles was consistently −10% of the activity measured in sCAX1 vesicles.

The CXIP1 cDNA consists of 522 base pairs (GenBank\textsuperscript{TM}/EBI accession number AY157988). The amino acid sequence consists of 173 amino acids (with a predicted molecular mass of 19.3 kDa) and has extensive homology to PICOT proteins found in a broad taxonomic distribution, including mammals, yeast, and bacteria (28). In other organisms, these proteins appear to play a negative regulatory role in cellular stress responses (24,
The primary amino acid sequence of CXIP1 (Fig. 2) is 26% identical (38% similar) to the 171-amino acid GLP-1 PICOT protein from Plasmodium falciparum (PfGLP-1) and 33% identical (45% similar) to the 335-amino acid human PICOT (HsPICOT) protein. The PICOT proteins contain a modular unit of 84 amino acid located in the C terminus that is highly conserved (PICOT-HD) (Fig. 2). The human PICOT protein has two tandem PICOT-HD repeats, whereas both CXIP1, CXIP2, and P. falciparum GLP-1 have a single PICOT-HD in their C termini. Within the PICOT-HD, several stretches of amino acids appear to be highly conserved, for example, -CGFS- and -SNWPT- (amino acids 97–100 and 133–137, respectively) (Fig. 2).

Among the various PICOT proteins, several include a single thioredoxin (Trx) HD in their N termini. The human PICOT protein is involved in the interaction with protein kinase C through this Trx-HD and negatively regulates the c-Jun N-terminal kinase/AP-1 and NF-κB pathway (24). CXIP2 is 30% identical (43% similar) to CXIP1 overall and is >54% identical (71% similar) to CXIP1 within the PICOT-HD. CXIP2 contains a Trx-HD in the N terminus like that of the human PICOT protein (Fig. 2).

Specificity of CXIP1—Using the yeast Ca²⁺ suppression assay, we were interested in determining whether CXIP1 and CXIP2 can activate various CAX transporters. Three close homologs of CAX1 (CAX2–4) also appear to be N-terminally regulated, and neither full-length CAX3 nor CAX4 is able to suppress the Ca²⁺ sensitivity of K667 (10, 30). Unlike CXIP1, CXIP2 could not activate CAX1 (Fig. 3A). CXIP1 and CXIP2 could both weakly activate full-length CAX4 (Fig. 3B); however, both CXIP1 and CXIP2 failed to activate CAX2 and CAX3 (data not shown).

Properties of CXIP1—As an initial step toward deducing the mechanism of CAX1 activation by CXIP1, we tested the ability of CXIP1 to activate various chimeric CAX constructs. CAX3 could not suppress the K667 Ca²⁺-sensitive phenotype. Three close homologs of CAX1 (CAX2–4) also appear to be N-terminally regulated, and neither full-length CAX3 nor CAX4 is able to suppress the Ca²⁺ sensitivity of K667 (10, 30). Unlike CXIP1, CXIP2 could not activate CAX1 (Fig. 3A). CXIP1 and CXIP2 could both weakly activate full-length CAX4 (Fig. 3B); however, both CXIP1 and CXIP2 failed to activate CAX2 and CAX3 (data not shown).
subtraction of the gramicidin background values and as a percentage of N-terminal regulatory region peptide. The results are shown following measured at a 10-min time point with various concentrations of CAX1–73aa) and CAX1-N(1–65aa) in pAS2 were grown on synthetic complete medium lacking His, Trp, and –73aa) and CAX1-N(1–65aa) in pAS2 were grown on synthetic complete medium lacking His, Trp, and Leu and assayed for LacZ expression. Shown are the results from β-galactosidase assays on a filter. B, effect of the CAX1-N-terminal regulatory region peptide on ΔpH-dependent 10 μM 45Ca2+ transport by sCAX1 (●) and sCAX1 + CXIP1 (○) into yeast endomembrane vesicles measured at a 10-min time point with various concentrations of CAX1-N-terminal regulatory region peptide. The results are shown following subtraction of the gramicidin background values and as a percentage of Ca2+ uptake of the control sample in the absence of peptide. All results shown here are the means of three independent experiments, and the bars indicate S.E.

strains in which CXIP1 was coexpressed with chimeric sCAX3-CAX1 constructs containing the central region or C terminus of CAX1 were unable to suppress the Ca2+-sensitive phenotype (Fig. 4, B and C). However, when a chimeric construct (called sCAX3-α1) was used that contained 37 amino acids of the N-terminal region of CAX1 (Met37–Leu73) fused to CAX3, CXIP1 could activate this chimeric construct (Fig. 4C). We have previously shown that a 9-amino acid domain within CAX1, which we termed the “Ca2+-domain,” is required for Ca2+ transport (21). Furthermore, when this 9-amino acid domain from CAX1 is swapped into an N-terminally truncated CAX3 construct (to give sCAX3-α1), this “activated” CAX3 mutant can transport Ca2+ (21). The activation of CXIP3 (sCAX3-α1) in the presence of CXIP1 demonstrated in Fig. 4C did not require the CAX1 9-amino acid Ca2+-domain. The chimera sCAX3-α1, when coexpressed with CXIP1 in K667 yeast strains, demonstrated ΔpH-dependent 10 μM 45Ca2+ uptake into microsomal vesicles (Fig. 4D). This H+/Ca2+ antiport activity was comparable to that measured for sCAX1 (data not shown). The sCAX1-CAX3 chimera sCAX1-9 (21), with the 9-amino acid Ca2+-domain removed and replaced with the equivalent 9-amino acid region of CAX3, was not able to strongly suppress the Ca2+ sensitivity of K667; however, expression of CXIP1 + sCAX1-9 strongly suppressed the Ca2+ toxicity (Fig. 4C).

CXIP1 Associates with the N Terminal of CAX1 in Yeast—To determine whether this activation of CAX1 by CXIP1 is caused by a physical interaction between CXIP1 and the CAX1 N terminus, a yeast two-hybrid experiment was performed. CXIP1 coexpressed with the N terminus of CAX1 (Met37–Leu73 or Met1–Asn65) caused the lacZ gene in the Y190 yeast strain to be activated, indicating that a direct interaction between CXIP1 and the CAX1 N terminus had occurred (Fig. 5A). No color reaction occurred in the absence of CXIP1 or in the absence of the CAX1 N-terminal fragments.

We have previously demonstrated that a synthetic peptide corresponding to the first 36 amino acids of the CAX1 N terminus is able to inhibit H+/Ca2+ transport mediated by N-terminally truncated CAX1 (sCAX1), but does not inhibit Ca2+ transport by other truncated H+/Ca2+ antiporters (13). The interaction of this N-terminal peptide with CAX1 was mapped to include residues 56–62 within CAX1 (13). We were interested in coexpressing CXIP1 and sCAX1 to determine whether the presence of CXIP1 would alter the amount of synthetic peptide needed to inhibit N-terminally truncated CAX1. A peptide concentration of 5 ± 1.5 μM was sufficient to inhibit 50% of 10 μM Ca2+ transport activity mediated by sCAX1 (Fig. 5B) (13), whereas 12 ± 2 μM peptide was required to inhibit 50% of sCAX1 transport in the presence of CXIP1 (Fig. 5B).

Disruption of the CXIP1 PICOT-HD—To determine whether the CXIP1 PICOT-HD is required for activation of full-length CAX1, we mutated two highly conserved regions within the CAX1 N terminus (Fig. 4A). We have shown previously that the conserved CGFS domain (amino acids 133–137) was changed to AAAAA. Growth of CXIP1 coexpressed with CAX1 was unable to activate full-length CAX1, but does not inhibit Ca2+ transport by other truncated H+/Ca2+ antiporters (13). The interaction of this N-terminal peptide with CAX1 was mapped to include residues 56–62 within CAX1 (13). We were interested in coexpressing CXIP1 and sCAX1 to determine whether the presence of CXIP1 would alter the amount of synthetic peptide needed to inhibit N-terminally truncated CAX1. A peptide concentration of 5 ± 1.5 μM was sufficient to inhibit 50% of 10 μM Ca2+ transport activity mediated by sCAX1 (Fig. 5B) (13), whereas 12 ± 2 μM peptide was required to inhibit 50% of sCAX1 transport in the presence of CXIP1 (Fig. 5B).

Expression of CXIP1—Northern blot analysis was used to assess the expression of CXIP1 mRNA in various Arabidopsis tissues. Hybridization with the CXIP1 probe revealed expression of CXIP1 in all tissues, with abundant expression in leaves and low level expression in roots and flowers, compared with almost equal expression of CAX1 in leaf, stem, and flower tissue and very low expression in roots (Fig. 6). When seedlings were exposed to different metals, there were alterations in the expression of CXIP1. CXIP1 levels were modestly increased when Ca2+ was added to the medium and decreased when Na+, Mn2+, and Ni2+ were added to the medium (Fig. 6).

**Discussion**

Using a yeast-based approach to activate full-length CAX1, we have identified an Arabidopsis cDNA termed CXIP1 gene, encoding a predicted protein containing a PICOT-HD domain (Figs. 1 and 2). This domain is highly conserved throughout evolution, and comparative sequence analysis indicates that the PICOT-HD is distinct from all protein domains (28). The physiological function of the PICOT-HD has not been identified; and until this study, no plant gene with this motif has been functionally characterized. In this work, we have demonstrated that, in a yeast expression system, CXIP1 interacted with the N terminus of CAX1 to modify vacuolar H+/Ca2+ antiport activity. This study thus ascribes, for the first time, a function to a plant gene containing a PICOT-HD. Like all PICOT-HD-containing proteins, CXIP1 appears to be a soluble protein. Two other non-transmembranous proteins that activated CAX1 Ca2+ transport in yeast were also identified in this study (Table I). CXIP3 is identical to a previously characterized protein, A. thaliana FKBP15-2, a member of the FKBP-type immunophilin family (27), whereas CXIP4 is a novel protein of un-
known function that appears to be unique to plants. We chose to
confine this study to further analysis of CXIP1.

Several proteins that contain a PICOT-HD also contain a
Trx-HD, including Arabidopsis CXIP2, which weakly activated
CAX4 Ca\(^{2+}\) transport (Fig. 3B). Proteins containing the
Trx-HD are important in a range of cellular process, including
controlling the redox state of the cell (31). In plant Ca\(^{2+}\) sig-
naling, there have been some reports linking the plasma mem-
brane redox state with release of Ca\(^{2+}\) from intracellular stores
(32); however, the mechanisms for transducing these signals
have not been elucidated.

The inability of CXIP2 to activate CAX1 suggests specificity
in the CXIP1-CAX1 interaction. Three different experimental
approaches suggest this specificity depends on the N terminus
of CAX1. Two-hybrid analysis demonstrated an interaction
between CXIP1 and the N terminus of CAX1 (Fig. 5A). This
interaction did not require the first 36 amino acids of CAX1, as
a construct containing Met\(^{37}\)–Leu\(^{73}\) interacted with CXIP1 in a
manner indistinguishable from the CAX1 Met\(^{1}\)–Asn\(^{65}\) con-
struct. The second approach utilized chimeric CAX3 constructs
to demonstrate the importance of the N terminus of CAX1.
Only the chimeric CAX3 construct containing Met\(^{37}\)–Leu\(^{73}\)
from CAX1, but lacking the first 36 amino acids of CAX1
(sCAX3-a1), was capable of being activated by CXIP1 (Fig. 4, C
and D). The third approach utilized a competition experiment
to suggest that CXIP1 binds to the N terminus of CAX1.
Our previous studies have demonstrated that a synthetic peptide
corresponding to amino acids 1–36 of CAX1 specifically inhibits
Ca\(^{2+}\) transport mediated by sCAX1 lacking these 36 N-termi-
nal amino acids (13). Furthermore, we showed that these 36
amino acids physically interact with amino acids 56–62 at the
N terminus of CAX1 to facilitate autoinhibition of CAX1 (13).
We hypothesized that if CXIP1 binds to this same region (ami-
no acids 56–62) of CAX1, more peptide should be required to
cause Ca\(^{2+}\) transport inhibition. Indeed, approximately double
the peptide concentration was needed to inhibit 50% of sCAX1
transport activity in the presence of CXIP1 (Fig. 5B).

The requirement for the CXIP1 PICOT-HD in this interaction
was inferred by the inability of mutant forms of CXIP1 to
activate CAX1 (data not shown). However, at this time, we
cannot rule out the possibility that these mutants perturb
protein expression or stability. Computer analysis suggests
that the PICOT-HD may form a globular topology, which in-
cludes three \(\alpha\)-helices and intervening \(\beta\)-strands (26). It has
been proposed that this structure can (i) form a transient
intermolecular interaction; (ii) bind to substrates, regulators,
or cofactors; or (iii) tether proteins to specific subcellular com-
partments. In the future, it will be interesting to further deline-
ate the mechanism of CXIP1-mediated CAX1 regulation.

We suggest that CXIP1 may activate CAX1 through a direct
interaction that disrupts autoinhibition and thus alters the
confirmation of autoinhibited CAX1 to allow Ca\(^{2+}\) transport.
However, the Ca\(^{2+}\) transport mediated by CXIP1-activated
CAX1 is much less than that of deregulated sCAX1 (Fig. 1).
Evidence suggests that activation of CAX1 by N-terminal trunc-
ation (as with sCAX1) is artificial and does not occur in planta
(11), and we believe that the strong Ca\(^{2+}\) transport activity
mediated by sCAX1 may not be realized by fully activated
CAX1. For example, Ca\(^{2+}\) transport mediated by a version of
full-length CAX1 activated by point mutations within the N
terminus is less than that mediated by sCAX1.1 Similarly,
Ca\(^{2+}\) transport mediated by the calmodulin-activated Arabi-
dopsis Ca\(^{2+}\)-ATPase ACA2 is significantly less than that medi-
dated by an N-terminally truncated version of ACA2 lacking
its calmodulin-binding autoinhibitory domain (33). However,
the level of H\(^{+}\)/Ca\(^{2+}\) antiport activity measured for CXIP1-
activated CAX1 is still much less than the activity measured in
Arabidopsis tissue. It is possible that additional protein inter-
actions, such as with a multiple protein complex, are required
to fully activate CAX1. Other proteins may be required to
interact directly with CAX1 or cooperatively with CXIP1, or
CXIP1 itself may require additional regulation.

Interestingly, it appears that CXIP1 is able to strongly activate
some chimeric CAX constructs. The level of Ca\(^{2+}\) transport measured
in yeast vesicles expressing CXIP1-activated sCAX3-a1 was
equivalent to the level of activity in vesicles expressing sCAX1
(Fig. 4D). Our previous findings show that the sCAX1-CAX3
chimera sCAX1-9 is unable to transport Ca\(^{2+}\) (21). This con-
struct contains an N-terminal truncation, but also contains the
9-amino acid region from CAX3 (residues 87–95) that replaced
the equivalent 9-amino acid Ca\(^{2+}\) domain of CAX1 and was
thought to abolish function due to the inability of this CAX3
domain to allow Ca\(^{2+}\) transport. However, we show here that
sCAX1-9 could also be activated by coexpression of CXIP1 and
that this activity was equivalent to that of sCAX1 (Fig. 4C).

Previously, we demonstrated that truncations or additions to
the N terminus of CAX1 or CAX4 can activate these Ca\(^{2+}\)
transporters in yeast (10). Similarly, CAX3 can also be acti-
vated by the addition of amino acids to the N terminus (10).
These findings imply that any protein binding to the N-termi-
nal region could activate CAX1 transport in yeast by altering
the autoinhibitory region or the conformation of the trans-
porter. Thus, although CXIP1 clearly interacts with CAX1 at
the autoinhibitory region or the conformation of the trans-
porter, there have been some reports linking the plasma mem-
brane redox state with release of Ca\(^{2+}\) from intracellular stores
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We suggest that CXIP1 may activate CAX1 through a direct
interaction that disrupts autoinhibition and thus alters the
confirmation of autoinhibited CAX1 to allow Ca\(^{2+}\) transport.
ferent ions (data not shown); however, the expression levels and tissue distribution of these genes differed from those of CXIP1, suggesting that those CXIP genes might play different roles in the regulation of CAX1 activity in planta.

The use of yeast to reconstitute a plant response pathway has been achieved with genes involved in Na\(^+\) homeostasis (34). However, these genes were first identified using standard genetic approaches (35). Here we have demonstrated that CXIP1, but not its homolog CXIP2, can regulate the Ca\(^{2+}\)/H\(^{+}\) antiporter, CAX4. Indeed, we have previously proposed that each CAX transporter may be regulated by different pathways (30). We speculate that multiple components (or CXIPs) are required for regulation of CAX1 and other plant CAX transporters and that each CXIP may differ in its interaction with CAX1. Future work using reverse genetics in Arabidopsis will help clarify the contribution of CXIPs to CAX-mediated H\(^{+}\)/Ca\(^{2+}\) transport.

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