

Regulation of CAX1, an Arabidopsis Ca²⁺/H⁺ Antiporter. Identification of an N-Terminal Autoinhibitory Domain¹

Jon K. Pittman and Kendal D. Hirschi*

Plant Physiology Group, U.S. Department of Agriculture/Agricultural Research Service Children's Nutrition Research Center, Baylor College of Medicine, 1100 Bates Street, Houston, Texas 77030 (J.K.P., K.D.H.); and Vegetable and Fruit Improvement Center, Texas A&M University, College Station, Texas 77845 (K.D.H.)

Regulation of Ca²⁺ transport determines the duration of a Ca²⁺ signal, and hence, the nature of the biological response. Ca²⁺/H⁺ antiporters such as CAX1 (cation exchanger 1), play a key role in determining cytosolic Ca²⁺ levels. Analysis of a full-length CAX1 clone suggested that the CAX1 open reading frame contains an additional 36 amino acids at the N terminus that were not found in the original clone identified by suppression of yeast (*Saccharomyces cerevisiae*) vacuolar Ca²⁺ transport mutants. The long CAX1 (lCAX1) could not suppress the yeast Ca²⁺ transport defects despite localization to the yeast vacuole. Calmodulin could not stimulate lCAX1 Ca²⁺/H⁺ transport in yeast; however, minor alterations in the 36-amino acid region restored Ca²⁺/H⁺ transport. Sequence analysis suggests that a 36-amino acid N-terminal regulatory domain may be present in all Arabidopsis CAX-like genes. Together, these results suggest a structural feature involved in regulation of Ca²⁺/H⁺ antiport.

Calcium (Ca²⁺) levels in the cytosol fluctuate in response to growth, development, and environmental perturbations (Sanders et al., 1999; Curran et al., 2000). The degree and duration of the cytosolic Ca²⁺ "spike" determines the plant's biological response. Like all eukaryotes, plants utilize transporter systems to meticulously control Ca²⁺ concentrations in different cellular compartments (Bush, 1995; Harper et al., 1998; Sze et al., 2000). Influx of Ca²⁺ to the cytosol occurs as a "downhill" transport through Ca²⁺ channels (Chung et al., 2000). Ca²⁺/H⁺ antiporters along with Ca²⁺-ATPases regulate the active Ca²⁺ efflux from the cytosol (Ueoka-Nakanishi et al., 1999; Chung et al., 2000; Hirschi, 2001). The mechanisms of Ca²⁺-ATPase regulation have begun to emerge recently (Curran et al., 2000; Hwang et al., 2000b); however, little is known about regulation of other plant Ca²⁺ transporters (Sze et al., 2000).

Ca²⁺/H⁺ exchange helps to establish the concentration gradient of Ca²⁺ across the tonoplast (vacuolar membrane; Schumaker and Sze, 1985; Blumwald and Poole, 1986). Two Arabidopsis genes, CAX1 (cation exchanger 1), and CAX2 were identified by their ability to sequester Ca²⁺ into vacuoles in *Saccharomyces cerevisiae* mutants deleted in vacuolar Ca²⁺ transport (Hirschi et al., 1996; Mäser et al., 2001). In Arabidopsis, the high-affinity, high-capacity Ca²⁺/H⁺

transporter CAX1, and a closely related gene, CAX3 (HCX1), are both highly expressed in response to exogenous Ca²⁺, whereas the low-affinity Ca²⁺/H⁺ transporter CAX2 is not induced by exogenous Ca²⁺ (Hirschi, 1999; Hirschi et al., 2000; Shigaki and Hirschi, 2000). Aside from these findings, nothing is known regarding the regulation of these Ca²⁺/H⁺ transporters.

The plant Ca²⁺-ATPases may serve as a useful prototype for potential regulatory mechanisms that may be utilized among plant Ca²⁺ transporters. The Arabidopsis endoplasmic reticulum Ca²⁺-ATPase, ACA2, can be activated or repressed by regulatory molecules binding to the N terminus (Harper et al., 1998; Hwang et al., 2000a). Calmodulin binding to the N terminus causes activation, whereas the pump can be inhibited by a Ca²⁺-dependent protein kinase (CDPK) phosphorylation at Ser-45 (Hwang et al., 2000b). Thus, the pump can be activated or repressed by different sensors that are responding to alterations in cytosolic Ca²⁺. In addition, N-terminal truncations of ACA2, the plasma membrane (PM) Ca²⁺-ATPase SCA1 (soybean; *Glycine max*), and the vacuolar Ca²⁺-ATPases ACA4 (Arabidopsis) and BCA1 (cauliflower; *Brassica oleracea*) are required for these gene products to suppress yeast mutants defective in vacuolar Ca²⁺ transport (Harper et al., 1998; Chung et al., 2000; Geisler et al., 2000; Malmström et al., 2000). These studies suggest that Ca²⁺ pumps in plants contain important regulatory domains at the N terminus.

To investigate the potential N-terminal regulatory domains within CAX1, we obtained a full-length cDNA clone of CAX1. This long CAX1 (lCAX1) clone contained additional coding sequences at the N terminus that were not present in the clone character-

¹ This work was supported by the U.S. Department of Agriculture/Agricultural Research Service (under Cooperative Agreement No. 58-6250-6001) and by the National Institutes of Health (grant nos. CHRC 5 P30 and 1R01 GM57427).

* Corresponding author; e-mail kendalh@bcm.tmc.edu; fax 713-798-7078.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.010409.

ized by function in yeast (Hirschi et al., 1996). We expressed, localized, and determined the transport properties of ICAX1 when expressed in yeast. We then modified the N terminus of ICAX1 in an attempt to modify transport activity in yeast. Sequence analysis suggests that the Arabidopsis CAX transporters contain N-terminal amino acids not found in the previously cloned CAX1 and CAX2. These findings offer insights into the regulation of $\text{Ca}^{2+}/\text{H}^{+}$ antiporter.

RESULTS

Identification of ICAX1 cDNA

A BLAST search of the GenBank sequence database using the CAX1 cDNA sequence (Hirschi et al., 1996) identified an expressed sequence tag (EST) clone (accession no. BE038949) that had a high level of similarity with the CAX1 sequence. As shown in Figure 1, the EST codes for a predicted open reading frame identical to all 427 amino acids of CAX1 except for an additional 36 amino acids at the N terminus. This EST was obtained and fully sequenced to verify the fidelity of the open reading frame. We have named this cDNA long CAX1 (ICAX1) in order to differentiate it from the shorter CAX1 (sCAX1) previously cloned. The ICAX1 sequence is consistent with the predicted open reading frame of CAX1 from the genomic database (accession no. AC003028).

ICAX1 Cannot Suppress a Yeast Mutant Defective in Vacuolar Ca^{2+} Transport

To test the function of ICAX1 in yeast, the ICAX1 cDNA was inserted into a high-copy yeast expression plasmid under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter (Nathan et al., 1999) and expressed in the yeast mutant K667 (Cunningham and Fink, 1996). This strain lacks the endogenous vacuolar Ca^{2+} -ATPase PMC1 and vacuolar $\text{Ca}^{2+}/\text{H}^{+}$ antiporter VCX1 and thus is defective in vacuolar Ca^{2+} transport, making it unable to grow on high- Ca^{2+} media (Cunningham and Fink, 1996). This

mutation can be suppressed by VCX1, Arabidopsis sCAX1 and CAX2, and mung bean (*Vigna radiata*) VCAX1 (Cunningham and Fink, 1996; Hirschi et al., 1996; Ueoka-Nakanishi et al., 2000). We examined the growth of K667 yeast cells expressing sCAX1, ICAX1, or the vector alone in liquid medium containing a range of CaCl_2 concentrations from 100 to 200 mM. The ICAX1-expressing strain was unable to suppress the Ca^{2+} sensitivity of K667, whereas the sCAX1-expressing strain successfully rescued the growth defect of K667 (Fig. 2A). The growth of ICAX1-expressing strains was indistinguishable from that of the vector control-transformed yeast (Fig. 2A). In a similar manner, when growth of these yeast strains was compared on solid media containing 200 mM CaCl_2 , no growth was visible for the ICAX1-expressing strain, whereas sCAX1 suppressed the growth defect (Fig. 2B). We tested the possibility that ICAX1 was not being expressed in the transformed K667 yeast strain. Although a specific antibody to ICAX1 was unavailable, the transcription of ICAX1 in yeast was demonstrated by reverse transcriptase (RT)-PCR using specific primers designed against the ICAX1 sequence. The presence of ICAX1-specific transcripts in ICAX1-transformed yeast, but not from vector-alone or sCAX1-expressing yeast (data not shown), confirmed that the ICAX1 mRNA was transcribed in K667.

ICAX1 Is Localized at the Vacuolar Membrane in Yeast

The inability of ICAX1 to suppress yeast mutants defective in vacuolar Ca^{2+} transport could be due to localization of the protein to a different membrane. The extended N terminus may encode a signal peptide, although none were identified using various motif prediction programs (data not shown). To identify the cellular location of ICAX1 in yeast, an epitope-tagged variant was generated by the fusion of a triple copy of hemagglutinin (HA) to the N terminus (HA:ICAX1). An N-terminal HA epitope tag has been used previously to identify the cellular location of VCX1 in yeast and transgenic Arabidopsis

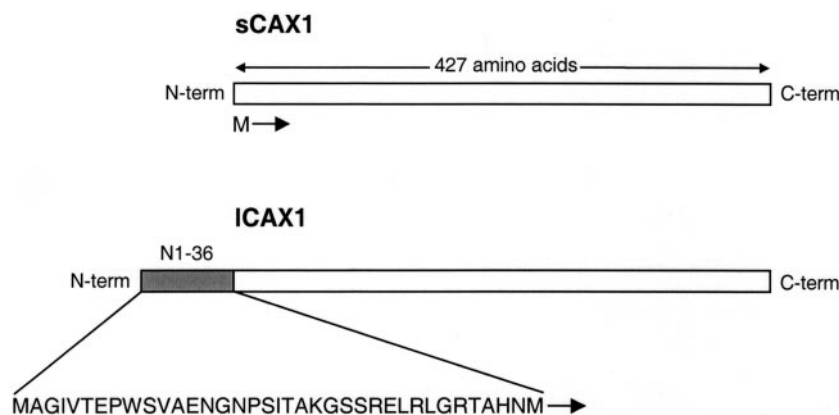


Figure 1. Structure of ICAX1 compared with that of sCAX1. ICAX1 contains an additional 36 amino acids at the N terminus. This domain (shaded) has been named N1-36. The sequence of the first 37 amino acids of ICAX1 is shown. The start Met for sCAX1 is also shown. Following the identification of a sequencing error, the original nucleotide sequence of sCAX1 deposited in the GenBank database was recently amended, as previously described (Shigaki and Hirschi, 2000), thereby changing the length of the sCAX1 open reading frame from 459 amino acids to 427 amino acids. This altered open reading frame is identical to amino acids 37 to 463 of ICAX1.

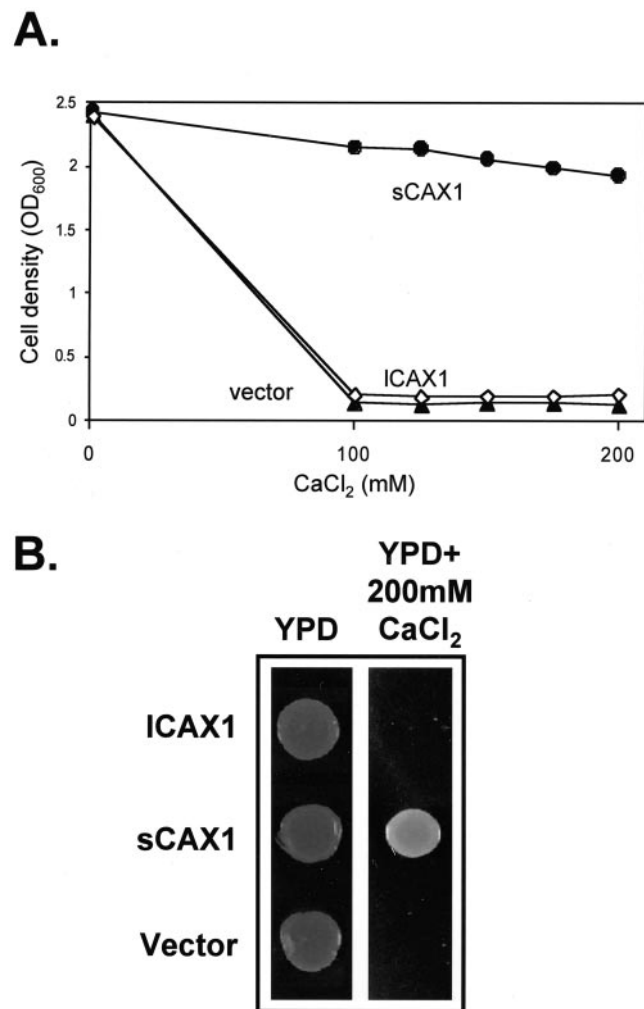


Figure 2. A, Ca²⁺ tolerance assay of K667 mutant yeast-expressing vector alone (\blacktriangle), sCAX1 (\bullet), or ICAX1 (\diamond). Yeast strains were grown in selection media overnight at 30°C and diluted to an optical density at 600 nm (OD₆₀₀) of 1.0, then inoculated into fresh yeast peptone dextrose (YPD) media containing a range of CaCl₂ concentrations from 100 to 200 mM. Yeast cells were grown for 16 h at 30°C in flat-bottomed 24-well dishes. Cell density was determined by measurements at OD₆₀₀. B, K667 yeast strains expressing vector alone, sCAX1, and ICAX1 were grown in selection media overnight at 30°C and diluted to an OD₆₀₀ of 1.5, then spotted onto YPD media alone and YPD media containing 200 mM CaCl₂. Yeast growth on YPD alone and YPD with 200 mM CaCl₂ was photographed after 1 and 3 d, respectively.

(Cunningham and Fink, 1996; Hirschi et al., 2001), and to confirm the localization of sCAX1 to the vacuolar membrane of yeast (T. Shigaki and K.D. Hirschi, unpublished data). The HA epitope tag did not appear to disrupt antiport function because both HA:VCX1 and HA:sCAX1 had the ability to suppress the Ca²⁺ sensitive phenotype of K667 (Cunningham and Fink, 1996; T. Shigaki and K.D. Hirschi, unpublished data). In addition, an N-terminal green fluorescence protein tag has successfully been used to identify the localization of VCAX1 in transgenic to-

bacco (*Nicotiana tabacum*; Ueoka-Nakanishi et al., 2000). As shown in Figure 3, western-blot analysis of yeast membranes fractionated on Suc gradients showed that sCAX1 cofractionated with vacuolar membranes. HA:ICAX1 was detected only in the 28% to 36% (w/w) Suc fractions and its distribution corresponded with that of HA:sCAX1 and the yeast vacuolar membrane marker ALP.

Inability of ICAX1 to Transport ⁴⁵Ca in Yeast

It is presumable that ICAX1 failed to suppress yeast strains deficient in vacuolar Ca²⁺ transport due to this transporter's inability to drive Ca²⁺ transport into the yeast vacuole. To directly test Ca²⁺/H⁺ antiport activity, we isolated yeast endomembrane vesicles purified from sCAX1, and ICAX1-expressing K667 cells. The capacity for ΔpH-dependent Ca²⁺ uptake was measured by a filtration assay. As shown in Figure 4A, Ca²⁺/H⁺ transport by sCAX1 measured using 10 μM ⁴⁵CaCl₂ was consistent with previous results (Hirschi et al., 1996), but no Ca²⁺ transport was demonstrated for ICAX1 when assayed with a range of Ca²⁺ concentrations from 1 to 100 μM (Fig. 4B; data not shown). The presence of the Ca²⁺-binding protein calmodulin also had no effect on ICAX1-mediated Ca²⁺ transport (data not shown).

Truncations of the ICAX1 N-Terminal Region Restore Activity

To determine which amino acid residues were involved in inhibition of ICAX1 activity, a number of ICAX1 mutants were generated by PCR-based site-

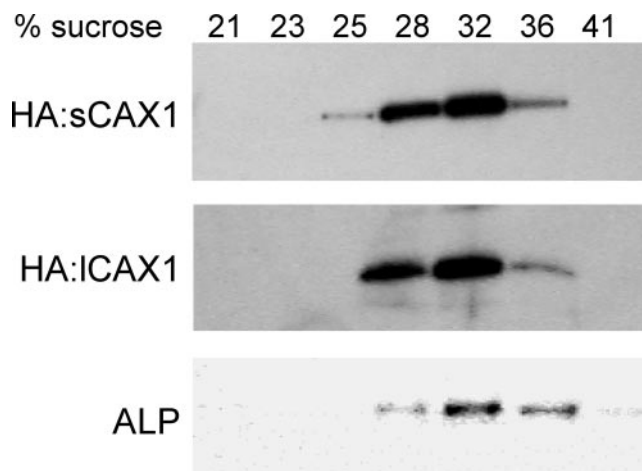


Figure 3. Intracellular localization of HA:sCAX1 and HA:ICAX1 in K667 mutant yeast. Yeast microsomal membranes were extracted and fractionated through a 15% to 50% (w/w) Suc gradient and 1-mL fractions were collected. Approximately 2 μg of protein from each of the seven fractions from 21% to 41% (w/w) Suc were separated by SDS-PAGE, blotted, then subjected to western-blot analyses using the anti-HA monoclonal antibody (HA:sCAX1 and HA:ICAX1) and an antibody against a yeast vacuolar membrane marker alkaline phosphatase (ALP).

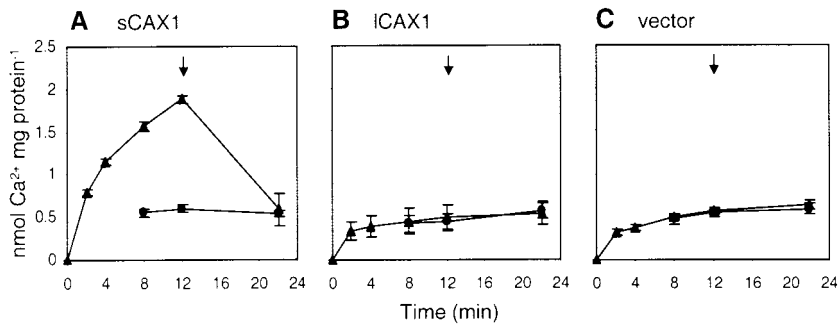


Figure 4. Time course of ΔpH -dependent $10\text{-}\mu\text{M}$ $^{45}\text{Ca}^{2+}$ transport into endomembrane-enriched vesicles prepared from K667 mutant yeast expressing either A, sCAX1; B, ICAX1; or C, vector alone. Ca^{2+} transport was determined in the absence (\blacktriangle) or presence (\bullet) of $5\ \mu\text{M}$ gramicidin. Ca^{2+} transport in the presence of gramicidin was not determined for the first two time points. All time course experiments were performed in the presence of $0.1\ \text{mM}$ NaN_3 , $0.2\ \text{mM}$ Na orthovanadate, and $1\ \text{mM}$ ATP. The Ca^{2+} ionophore A23187 ($5\ \mu\text{M}$) was added at the times indicated (arrow). Results are the average ($\pm\text{SE}$) of three independent experiments.

directed mutagenesis (Fig. 5A). The introduction of a point mutation converting Met-37 to Ile-37 (ICAX1-M37I) did not restore growth of K667 on $200\ \text{mM}$ CaCl_2 (Fig. 5B), indicating that the lack of ICAX1 activity was not due to the presence of two Met residues in close proximity (Met-1 and Met-37). A truncated variant of ICAX1 lacking the first 36 residues was generated by converting Met-1 into Ile (ICAX1-M1I), so that translation would be initiated at Met-37, thereby converting ICAX1 to sCAX1. Removal of all 36 residues restored the activity of ICAX1, as demonstrated by suppression of the K667 phenotype on $200\ \text{mM}$ CaCl_2 , making it comparable to the growth of the sCAX1-expressing cells (Fig. 5B). In an attempt to delineate any potential regulatory domain in N1-36, various N-terminal truncated variants of ICAX1 were generated, lacking either the first 10, 20, 30, or 32 amino acid residues ($\Delta 10$ -ICAX1, $\Delta 20$ -ICAX1, $\Delta 30$ -ICAX1, and $\Delta 32$ -ICAX1; Fig. 5A). All of the truncated forms of ICAX1 were able to suppress the K667 mutant, and growth was found to be indistinguishable from sCAX1 and ICAX1-M1I (Fig. 5B; data not shown).

To confirm that the Ca^{2+} tolerance observed for the $\Delta 10$ -ICAX1-expressing yeast strain was due to a restoration of Ca^{2+} transport activity, $^{45}\text{Ca}^{2+}/\text{H}^{+}$ transport was measured from endomembrane vesicles obtained from $\Delta 10$ -ICAX1-expressing yeast cells. $\text{Ca}^{2+}/\text{H}^{+}$ transport by $\Delta 10$ -ICAX1 was measured using $10\ \mu\text{M}$ $^{45}\text{CaCl}_2$, and was comparable to the activity observed for sCAX1-expressing cells, whereas transport was absent for ICAX1 (Fig. 6).

Other CAX-Like Genes Contain Similar N-Terminal Domains

The N-terminal 36-amino acid sequence of ICAX1 (N1-36) was compared with the sequences of other CAX-like genes. This analysis found that VCAX1 of mung bean (Ueoka-Nakanishi et al., 1999) and Arabidopsis CAX3 (Shigaki and Hirschi, 2000) also possess similar N-terminal sequences (Fig. 7A). In addition, the genomic database suggests that the en-

dogenous CAX2 (accession no. AB024034) may contain a 42-amino acid extension not found in the clone that suppressed the yeast vacuolar Ca^{2+} transport mutant (Hirschi et al., 1996). However, the sequence of VCX1 from *S. cerevisiae* does not appear to contain an extended N terminus (Fig. 7A). The N1-36 domain of ICAX1 shares significant sequence similarity with the extended N termini of most of these genes, with the highest similarity found between ICAX1 and CAX3 (Fig. 7A). This gene has previously been shown to share 77% identity at the amino acid level with the entire sCAX1 sequence (Shigaki and Hirschi, 2000). As shown in Figure 7A, 24 of the 36 amino acids in the N1-36 domain are shared between ICAX1 and CAX3.

DISCUSSION

Regulation of Ca^{2+} signals is contingent upon the precise control of transporters and channels that modulate the amount of Ca^{2+} in the cytosol (McAinsh and Hetherington, 1998). $\text{Ca}^{2+}/\text{H}^{+}$ antiporters are part of the ensemble of transporters that help modulate the duration of these Ca^{2+} signaling events (Ueoka-Nakanishi et al., 2000). However, the mechanisms by which the plant $\text{Ca}^{2+}/\text{H}^{+}$ antiporters are regulated are unknown (Sze et al., 2000; Hirschi, 2001). The Arabidopsis CAX1 gene was identified previously as the putative vacuolar $\text{Ca}^{2+}/\text{H}^{+}$ antiporter due to the gene product's ability to suppress yeast mutants defective in vacuolar Ca^{2+} transport (Hirschi et al., 1996). Ectopic expression of this CAX1 gene product in tobacco causes alterations in Ca^{2+} homeostasis and stress sensitivities, which implies that regulated expression of $\text{Ca}^{2+}/\text{H}^{+}$ antiporter activity is a vital component of plant responses to the environment (Hirschi, 1999). Analysis of the Arabidopsis genome and ESTs suggested that the endogenous CAX1 may contain 36 amino acids at the N terminus not present in the initial clone of CAX1. For the sake of clarity in this report, we have termed the original clone short CAX1 (sCAX1), and the CAX1

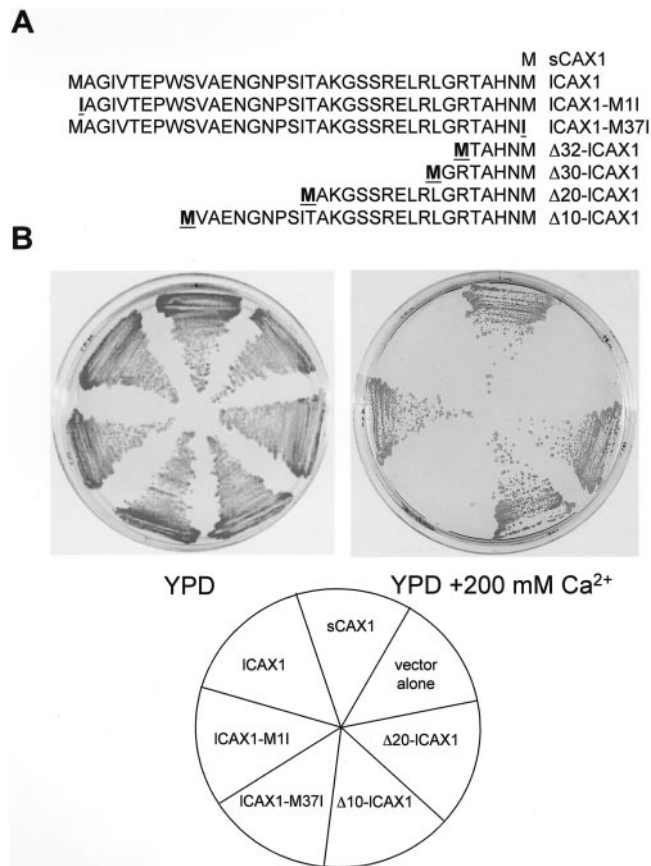
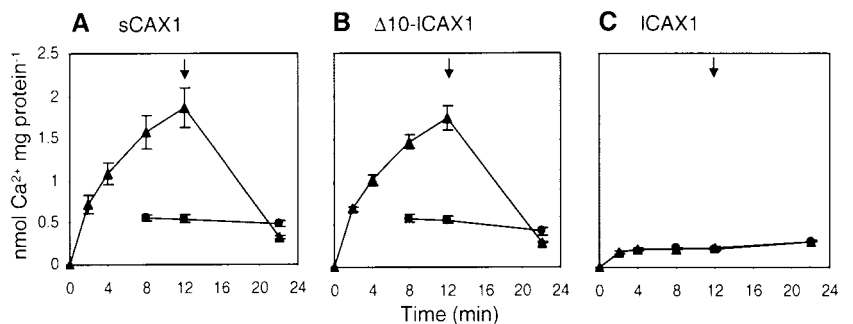


Figure 5. The ability of ICAX1 mutants with structural alterations at the N-terminal tail to suppress the Ca^{2+} -sensitive growth phenotype of K667 mutant yeast. A, Schematic representation of the first 37 amino acids of ICAX1 summarizing the point mutations and truncations that were generated. Highlighted Ile residues indicate a substitution from Met. Highlighted Met residues indicate the addition of a Met that was created to initiate translation following truncation. B, Growth analysis of K667 mutant yeast expressing sCAX1, ICAX1, ICAX1-M11, ICAX1-M37I, Δ 20-ICAX1, Δ 10-ICAX1, and vector alone. The yeast strains were streaked onto either plates containing YPD alone or YPD supplemented with 200 mM CaCl_2 , then grown at 30°C for 2 d.

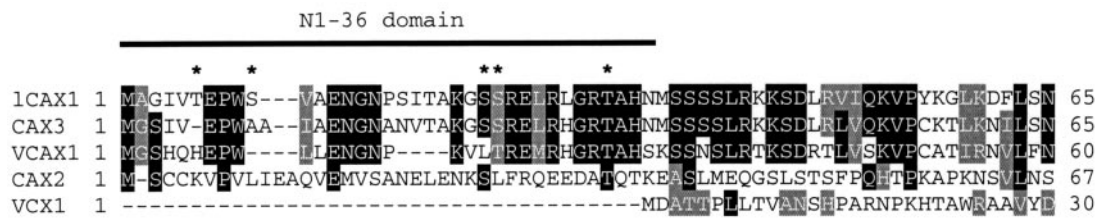
Figure 6. Time course of ΔpH -dependent $10\text{-}\mu\text{M}$ $^{45}\text{Ca}^{2+}$ transport into endomembrane-enriched vesicles prepared from K667 mutant yeast expressing either A, sCAX1; B, Δ 10-ICAX1; or C, ICAX1. Ca^{2+} transport was determined in the absence (\blacktriangle) or presence (\bullet) of $5\text{-}\mu\text{M}$ gramicidin. Ca^{2+} transport in the presence of gramicidin was not determined for the first two time points. All time course experiments were performed in the presence of 0.1 mM NaN_3 , 0.2 mM Na orthovanadate, and 1 mM ATP. The Ca^{2+} ionophore A23187 ($5\text{-}\mu\text{M}$) was added at the times indicated (arrow). Results are the average (\pm SE) of two independent experiments.



cDNA clone containing the 36-amino acid N-terminal region long CAX1 (ICAX1). This 36-amino acid region has been termed N1-36 or the regulatory region. In the future, we will refer to ICAX1 as CAX1 and sCAX1 will become the constitutively activated form of CAX1.

The ICAX1 clone was unable to suppress yeast mutants defective in vacuolar Ca^{2+} transport (Fig. 2, A and B). Using RT-PCR, we demonstrated that ICAX1 was transcribed in yeast (data not shown). Thus, the failure to suppress the yeast mutations was not due to a failure to transcribe the Arabidopsis gene. Furthermore, HA-tagged sCAX1 and ICAX1 both colocalize to the yeast vacuolar membrane (Fig. 3). This suggests that the failure of ICAX1 to suppress these mutations was not due to altered localization of the protein in yeast. This also indicates that the N terminus is not necessary for vacuolar membrane localization. We then isolated endomembrane vesicles from yeast cells expressing ICAX1. The failure to suppress the yeast mutants was not due to lower activity of $\text{Ca}^{2+}/\text{H}^{+}$ transport, but rather it appeared to be due to the complete absence of endomembrane $\text{Ca}^{2+}/\text{H}^{+}$ activity. The level of transport activity in ICAX1 cells was comparable to levels found in yeast membranes expressing vector controls (Fig. 4).

These findings suggested that the N-terminal region of ICAX1 acts as an autoinhibitory domain for $\text{Ca}^{2+}/\text{H}^{+}$ transport activity in yeast. The Arabidopsis Ca^{2+} -ATPase, ACA2, has been shown previously to have regulatory elements at the N-terminal autoinhibitory region (Harper et al., 1998; Hwang et al., 2000a). Only ACA2 cDNA clones with truncations at the N terminus are able to suppress yeast mutants defective in endomembrane Ca^{2+} transport. A calmodulin-binding sequence is present within the first 36 residues of the N-terminal domain of ACA2, and the full-length gene product demonstrates calmodulin-stimulated Ca^{2+} transport in yeast (Harper et al., 1998; Hwang et al., 2000a). This indicates that the N terminus of ACA2 acts as a calmodulin-regulated autoinhibitory domain. A CDPK-binding site is present in the N terminus of ACA2 that phosphorylates a Ser residue near the calmodulin-binding site. This CDPK activity inhibits ACA2 activity (Hwang et al., 2000b). Thus, ACA2 is

A**B**

136

MAGIVTEPWSVAENGNPSITAKGSSRELRLGRTAHN

Figure 7. A, Partial amino acid sequence alignment of the N-terminal tail region of various CAX-like genes from Arabidopsis (ICAX1, CAX2, and CAX3), mung bean (VCAX1), and *S. cerevisiae* (VCX1). The aligned sequences correspond to the entire N-terminal tails up until the first predicted transmembrane domain. The N1-36 region of ICAX1 is underlined. Alignments were performed using ClustalW 1.8 (Baylor College of Medicine Software Programs). Identical residues are shaded in black and similar residues are shaded in gray. Gaps introduced to maximize the alignment are denoted by hyphens. An asterisk denotes a putative phosphorylated residue (see B). The deduced amino acid sequence of CAX2 used here was derived from the extracted sequence of the genomic clone (accession no. AB024034). B, Amino acid sequence of the N1-36 domain of ICAX1 highlighting putative phosphorylation sites. Putative phosphorylation sites were determined using the prediction software NetPhos (Blom et al., 1999) and from analyzing known binding sites of CDPKs.

regulated between calmodulin stimulation and CDPK inhibition. In a manner analogous to the Ca²⁺-ATPases, much evidence suggests that plant PM H⁺-ATPases are regulated by a C-terminal autoinhibitory domain (for review, see Palmgren, 2001). For example, the Arabidopsis H⁺-ATPase AHA2 is activated by the binding of a 14-3-3 protein to the autoinhibitory domain and this binding is induced by a protein kinase-mediated phosphorylation of a specific Thr residue in this domain (Palmgren, 2001).

Like ACA2, ICAX1 was derepressed by alterations in the N terminus. Deletions of the first 10, 20, 30, or 32 amino acids at the N terminus caused the gene product to suppress yeast mutants defective in vacuolar Ca²⁺ transport (Figs. 5 and 6; data not shown). Because Δ 10-ICAX1 was able to suppress the mutant yeast phenotype (Fig. 5) and transport Ca²⁺ as efficiently as sCAX1 (Fig. 6), it might be concluded that the first 10 amino acids of ICAX1 are critical for its activity and that the putative autoinhibitory domain is present within this region. In an alternate manner, deletion of the first 10 amino acids may significantly alter the structure of the N-terminal tail and therefore perturb its regulatory activity. Therefore, further experiments are required to determine exactly which of the first 36 amino acids are required for autoinhibition. Work on the Ca²⁺-ATPases suggests that the N-terminal deletions are more active than the calmodulin-stimulated full-length clones (Hwang et al., 2000a). If this is true of CAX1, then sCAX1 may be

more active than any modified form of the endogenous protein. We are currently testing the hypothesis that the altered Ca²⁺ homeostasis exhibited in transgenic tobacco plants expressing sCAX1 is also the fortuitous consequence of expressing a constitutively activated form of the transporter (Hirschi, 1999).

Alterations in the N terminus can alter the function of many proteins. For example, these results with CAX1 are analogous to work done in mammalian systems with the soluble insulin-like growth factor-I (IGF-1). In these studies, the addition or subtraction of N-terminal residues conferred increased activity onto the growth regulator (Tomas et al., 1997). With plant Ca²⁺ transporters, there has been some evidence suggesting that they can be modulated by protein cleavage (Askerlund, 1996). Trypsin treatment resulted in cleavage of the calmodulin-binding domain from the N terminus of the cauliflower Ca²⁺-ATPase BCA1 and subsequently activated this protein (Askerlund, 1996; Malmström et al., 2000). It is conceivable that CAX1 may be regulated through such events.

Unlike ACA2, the ICAX1 gene product was not activated by addition of exogenous calmodulin (data not shown). Sequence analysis of the 36-amino acid regulatory region did not identify a calmodulin-binding site; however, there are several putative CDPK-binding sites (Fig. 7B). These results suggest that CAX1 is not up-regulated by calmodulin, and that the transporter may be activated by CDPKs or

other regulatory molecules. There are numerous examples in animal studies where protein kinases directly activate Ca^{2+} transport function (Enyedi et al., 1996).

This study suggests that sCAX1 suppression of the vacuolar transport mutants is due to a truncated cDNA and a second Met codon fortuitously found in CAX1. It is unclear whether the truncated sCAX1 is an artifact of the original yeast suppression screen and is therefore a partial-length cDNA. In an alternate manner, there may be splice variants of CAX1 in Arabidopsis; therefore, sCAX1 and lCAX1 may be products of alternative splicing. An informative example of this type of regulation occurs in mammalian PM-type Ca^{2+} -ATPases, which exhibit alternative splicing that alters the presence of the calmodulin regulatory domains (Penniston and Enyedi, 1998). We have shown previously that the CAX1 RNA levels in Arabidopsis increase in response to exogenous Ca^{2+} in the media (Hirschi, 1999). Using primers specific for lCAX1, we have performed RT-PCR experiments on RNA from exogenous metal-treated Arabidopsis tissues and demonstrated that the CAX1 RNA corresponds to the lCAX1 sequence (data not shown). These preliminary studies suggest that lCAX1 is the predominant form of CAX1 found in Arabidopsis.

It is interesting that sequence data demonstrate that CAX2 may contain an additional N-terminal sequence that codes for amino acids not found in the cDNA that suppresses yeast vacuolar Ca^{2+} transport mutants (Fig. 7A). Based on this sequence analysis, CAX3, a homolog of CAX1, also contains a putative N-terminal regulatory region very similar to that present on CAX1. The mung bean $\text{Ca}^{2+}/\text{H}^{+}$ antiporter, VCAX1, also contains a putative N-terminal regulatory domain, but this region does not repress Ca^{2+} transport activity in yeast (Fig. 7A; Ueoka-Nakanishi et al., 2000). This finding suggests that mung bean may regulate $\text{Ca}^{2+}/\text{H}^{+}$ transport in a different manner than Arabidopsis. Comparisons between lCAX1 and VCAX1 may offer further insight into the important sequences required for lCAX1 autoinhibition (Fig. 7A). Yeast VCX1, however, does not possess an extended N terminus. In yeast, the function of VCX1 is inhibited by the Ca^{2+} /calmodulin-dependent protein phosphatase, calcineurin. Calcineurin is believed to regulate VCX1 activity at the posttranslational level (Cunningham and Fink, 1996) and it is likely that this involves a direct protein-protein interaction, although the possible site of calcineurin binding to VCX1 is unknown.

Some of the regulatory mechanisms of other cation/ H^{+} antiporters from various species have begun to be elucidated. The mammalian $\text{Na}^{+}/\text{H}^{+}$ exchanger, NHE1, contains a calmodulin-binding autoinhibitory domain that reduces the affinity of this transporter for H^{+} (Wakabayashi et al., 1997). In addition, protein kinase-dependent regulation has

been observed for both $\text{Na}^{+}/\text{H}^{+}$ and $\text{Na}^{+}/\text{Ca}^{2+}$ exchangers in animal cells (Iwamoto et al., 1998; Haworth et al., 1999). A long hydrophilic C-terminal tail has recently been shown to be important for the activity of a $\text{Na}^{+}/\text{H}^{+}$ antiporter from *Synechocystis* sp. PCC 6803 as truncation of this tail significantly reduced antiporter activity (Hamada et al., 2001). The vacuolar $\text{Na}^{+}/\text{H}^{+}$ antiporter from *S. cerevisiae*, NHX1, contains a putative N-terminal regulatory domain. This region does not act as an autoinhibitor but has been suggested to be a cleavable signal peptide and is absent from the Arabidopsis homologue AtNHX1 (Darley et al., 2000). The regulation of plant $\text{Ca}^{2+}/\text{H}^{+}$ antiporters by N-terminal autoinhibition as proposed in this study appears to be a novel mechanism of regulation for cation/ H^{+} antiporters.

Growth, development, and adaptation require dynamic oscillations in cytosolic Ca^{2+} levels (Navazio et al., 2000; Hirschi, 2001). In this study, we demonstrate that the activity of $\text{Ca}^{2+}/\text{H}^{+}$ transporters may be modulated by changes at the N terminus of these proteins. This regulation may occur through RNA splicing, protein cleavage, or regulation by activators or suppressors binding to this regulatory domain. By further analysis of the mechanisms of CAX1 regulation, namely important components of Ca^{2+} ion homeostasis, the molecules that are directing the Ca^{2+} traffic may be identified.

MATERIALS AND METHODS

DNA Manipulation of lCAX1 cDNA

lCAX1 cDNA (EST clone, accession no. BE038949) in pBluescript (Stratagene, La Jolla, CA) was obtained from Dr. Hans Bohnert (University of Arizona, Tucson). The 5' and 3' ends were sequenced to confirm that the clone was full length. lCAX1 mutant variants (lCAX1-M1I and lCAX1-M37I) were produced using the type IIS restriction enzyme-based site-directed mutagenesis method (Shigaki and Hirschi, 2001). Specific point mutations were generated by PCR using the mutagenic primers lCAX1-M1I forward (5'-GAA TTC CGT CTC GAG AAA TAG CGG GAA TCG TGA CAG AG-3'), lCAX1-M1I reverse (5'-GAA TTC CGT CTC TTT CTC TAC TGA CTC AAA ACT TTG-3'), lCAX1-M37I forward (5'-GAA TTC CGT CTC ACA ACA TAT CTT CTT CTT CTT TGA GGA-3'), and lCAX1-M37I reverse (5'-GAA TTC CGT CTC TGT TGT GAG CGG TTC TTC CAA GTC-3'). All primers contained the type IIS restriction enzyme site *BsmBI* as underlined. Truncated variants of lCAX1 ($\Delta 10$ -lCAX1, $\Delta 20$ -lCAX1, $\Delta 30$ -lCAX1, and $\Delta 32$ -lCAX1) were generated by PCR using the forward primers $\Delta 10$ -lCAX1 (5'-CGC GGA TCC ATG GTA GCT GAG AAC GGA AAC CCA-3'), $\Delta 20$ -lCAX1 (5'-CGC GGA TCC ATG GCG AAA GGA TCG AGC AGA GAA-3'), $\Delta 30$ -lCAX1 (5'-CGC GGA TCC ATG GGA GAA ACC GCT CAC AAC ATG-3'), $\Delta 32$ -lCAX1 (5'-CGC GGA TCC ATG GCT CAC AAC ATG TCT TCT TC-3'), and the CAX1 reverse primer (5'-AAC GAG CTC TTA AGA TGA GAA AAC TCC TCC TCC TGT TGC A-3'). A *BamHI* site (un-

derlined) was generated into each forward primer and a *SacI* site (underlined) was generated into the reverse primer. Three tandem copies of the HA epitope (YPYDVP-DYA) were used to produce an in-frame fusion of HA to the 5' end of ICAX1 by PCR, generating HA:ICAX1. The forward primer (5'-GAT TAC GCT GCT CAG TGC ATG GCG GGA ATC GTG ACA-3') and reverse primer (5'-TGT CAC GAT TCC CGC CAT GCA CTG AGC AGC GTA ATC-3'), which both code for the last six amino acids of HA and the first six amino acids of ICAX1, were used to generate the fusion, then an HA-specific forward primer (5'-GAA TTC TCT AGA ATG GCG CGC ATC TTT TAC CCA TAC GAT-3') and the CAX1 reverse primer were used to amplify the entire construct. An *XbaI* site (underlined) was generated in the HA forward primer. All PCR amplifications were performed using the high-fidelity Expand polymerase kit (Roche, Mannheim, Germany). Amplification was performed by initially heating at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 60°C for 1 min, and 68°C for 4 min, followed by a final 10-min extension at 68°C. The mutant and epitope-tagged ICAX1 constructs were cloned into pGEM-T Easy (Promega, Madison, WI) for sequencing and propagation in *Escherichia coli* DH5 α . All mutant and epitope-tagged ICAX1 constructs were fully sequenced to confirm the presence of the expected sequence and to check for the presence of unwanted PCR-generated mismatches. The wild-type, mutant, and epitope-tagged ICAX1 constructs were subcloned into the yeast (*Saccharomyces cerevisiae*) expression vector piHGpd (Nathan et al., 1999) for the expression in yeast.

Yeast Transformation, Growth, and Manipulations

The *Saccharomyces cerevisiae* strain K667 (*MATa ade2-1 can1-100 his3-11, 15 leu2-3, and 112 trp1-1 ura3-1 cnb1::LEU2 pmc1::TRP1 vcx1 Δ* ; Cunningham and Fink, 1996) was used. Yeast cells were transformed using the lithium acetate method and selected on synthetic complete minus His (SC-His) media (Sherman et al., 1986). For Ca^{2+} tolerance assays, yeast were grown at 30°C for 1 to 3 d on solid YPD medium containing 2% (w/v) Difco yeast extract, 1% (w/v) bacto-peptone, and 2% (w/v) dextrose, and supplemented with 200 mM CaCl_2 (Hirschi et al., 1996). For liquid Ca^{2+} tolerance assays, yeast strains were grown to OD_{600} of 1.0 in SC-His medium at 30°C, then inoculated into YPD medium supplemented with 100 to 200 mM CaCl_2 , and finally grown for 16 h, shaking at 30°C, in 24-well flat-bottomed plates. Cell growth was then determined by OD_{600} measurements.

RT-PCR

Total RNA was extracted from yeast using the acid phenol extraction procedure as described by Ausubel et al. (1998). RT-PCR was performed by standard methods using a specific forward primer against ICAX1 (5'-TCT CAG AAT TTA CAA AGT TTT GAG TCA-3') and the CAX1 reverse primer (see above). First-strand cDNA was produced using the Superscript II reverse transcription kit

(Gibco-BRL, Gaithersburg, MD), then PCR was performed as described above.

Membrane Fractionation and Western Analysis of HA-Tagged ICAX1

Microsomal membranes were prepared from yeast expressing HA:ICAX1-piHGpd and HA:SCAX1-piHGpd, essentially as described by Hwang et al. (2000a), with a few modifications. Transformants were inoculated into 500 mL of SC-His and grown to stationary phase. The cells were pelleted by centrifugation at 4,000g for 5 min, then washed with 10 mL of ice-cold water, and finally resuspended in 10 mL of glass bead buffer {10% [w/v] Suc, 20 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.5, 2 mM EGTA, 1 mM MgSO_4 , and 1 mM dithiothreitol [DTT]}. The washed cells were resuspended in 2 mL of glass bead buffer with 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM benzamidine, 1 $\mu\text{g mL}^{-1}$ leupeptin, and 5 $\mu\text{g mL}^{-1}$ pepstatin. To break cells, an equal volume of dry glass beads was added to cells and vortexed for 3 min (30 s \times six times) at 4°C. The samples were centrifuged at 5,000g for 5 min and the supernatants were collected. The process was repeated three times and the supernatants were combined. For Suc gradient fractionation, 1 mL of supernatants from the broken yeast cells was layered onto a 15% to 50% (w/w) continuous Suc gradient and centrifuged at 25,000g at 4°C for 16 h. Membrane fractions were stored at -80°C . Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Immunoblots were performed and the HA epitope was detected essentially as described previously (Hirschi et al., 2001). Protein fractions were separated by SDS-PAGE on a 12% (w/v) gel and transferred to polyvinylidene fluoride membrane (Pall Gelman, Ann Arbor, MI). The blots were blocked in 5% (w/v) nonfat dried milk in phosphate-buffered saline with Tween (PBS-T; 10 mM $\text{NaH}_2\text{PO}_4/\text{NaOH}$, pH 7.2, and 150 mM NaCl with 0.1% [v/v] Tween 20) for 1 h, and then reacted with a 1:3,000 dilution of anti-HA monoclonal primary antibody (Berkeley Antibody Co., Richmond, CA) in PBS-T for 1 h at room temperature. The vacuolar membrane marker antibody ALP against the yeast vacuolar ALP (Molecular Probes, Eugene, OR) was used at a 1:250 dilution. The blots were washed in PBS-T before incubating for 1 h in PBS-T containing a 1:10,000 dilution of horseradish peroxidase-coupled anti-mouse secondary antibody (Amersham, Little Chalfont, UK). The blots were then washed in PBS-T. ECL Plus reagents (Amersham) were used to develop the blots, which were then exposed to Hyperfilm photographic film (Amersham).

Preparation of Endomembrane Vesicles

Endomembrane vesicles were prepared as previously described (Nakanishi et al., 2001), with a few modifications. Transformants were grown in 50 mL of SC-His media at 30°C for 2 d, then inoculated into 1 L of YPD medium. The cells were pelleted by centrifugation at 4,000g for 5

min, then washed with 0.1 M Tris-HCl, pH 9.4, 50 mM 2-mercaptoethanol, and 0.1 M Glc at 30°C for 10 min. Spheroplasts were produced by incubating the cells at 30°C for 1 h in 0.05% (w/v) Zymolyase 20T (Seikagaku Kogyo, Tokyo), 0.9 M sorbitol, 0.1 M Glc, 50 mM Tris-MES [2-(N-morpholino)-ethanesulfonic acid], pH 7.6, 5 mM DTT, 0.043% (w/v) YPD, and 0.25× dropout mix (Sherman et al., 1986). The suspension was centrifuged at 3,000g for 10 min and washed with 1 M sorbitol. Spheroplasts were resuspended in 50 mM Tris-ascorbate, pH 7.6, 1.1 M glycerol, 1.5% (w/v) polyvinylpyrrolidone 40,000, 5 mM EGTA, 1 mM DTT, 0.2% (w/v) bovine serum albumin, 1 mM PMSF, and 1 mg L⁻¹ leupeptin and homogenized with a glass homogenizer (Wheaton Science Products, Millville, NJ). The homogenate was centrifugated at 2,000g for 10 min and the supernatant was then centrifuged at 120,000g for 30 min. The microsomal pellet was resuspended in 15% (w/w) Suc solution (containing 10 mM Tris-MES, pH 7.6, 1 mM EGTA, 2 mM DTT, 25 mM KCl, 1.1 M glycerol, 0.2% [w/v] bovine serum albumin, 1 mM PMSF, and 1 mg L⁻¹ leupeptin) and layered onto a 35% (w/w) Suc solution, then centrifuged at 150,000g for 30 min. Endomembrane-enriched vesicles were collected at the interface and diluted in 5 mM Tris-MES, pH 7.6, 0.3 M sorbitol, 1 mM DTT, 1 mM EGTA, 0.1 M KCl, 1 mM PMSF, 1 mg L⁻¹ leupeptin, and 5 mM MgCl₂. The membranes were centrifuged at 150,000g for 30 min and resuspended in 5 mM Tris-MES, pH 7.6, 0.3 M sorbitol, 1 mM DTT, 1 mM PMSF, and 1 mg L⁻¹ leupeptin. The membrane vesicles were stored at -80°C until use.

Ca²⁺ Transport Assay

Time-dependent ⁴⁵Ca²⁺/H⁺ transport into endomembrane vesicles was measured using the filtration method (Hwang et al., 1997). Membrane vesicles (30–40 μg mL⁻¹) were incubated in a reaction mixture containing 200 mM Suc, 25 mM HEPES-bis(tris[hydroxymethyl]methylamino) propane (pH 7.5), 50 mM KCl, 0.1 mM NaN₃, and 0.2 mM Na orthovanadate. Vacuolar H⁺-translocating ATPase-catalyzed H⁺ transport was initiated by the addition of 1 mM MgSO₄ and 1 mM ATP. The vesicles were allowed to reach steady state with respect to the pH gradient for 5 min at 25°C. ⁴⁵Ca²⁺ uptake was initiated by the addition of 1 to 100 μM ⁴⁵Ca²⁺ (6 mCi mL⁻¹; American Radiolabeled Chemicals, St. Louis). At the times indicated, 70-μL aliquots of the reaction mix were removed and filtered through premoistened 0.45 μm pore-size cellulose acetate GS type filters (Millipore, Bedford, MA) and washed with 2 mL of ice-cold wash buffer (250 mM Suc, 2.5 mM HEPES-bis(tris[hydroxymethyl]methylamino) propane, pH 7.5, and 0.2 mM CaCl₂). The filters were air dried and radioactivity was determined by liquid scintillation counting. The ΔpH-dependent component of ⁴⁵Ca²⁺ uptake was determined in the presence of 5 μM gramicidin. For some experiments, ⁴⁵Ca²⁺/H⁺ transport was measured in the presence of 0.5 to 5 μM bovine brain calmodulin (Sigma, St. Louis).

ACKNOWLEDGMENTS

We are grateful to Ning-hui Cheng, Toshiro Shigaki, and Coimbatore Sreevidya for critical reading of this manuscript. We are thankful to Heven Sze and Zhongyi Wu for helping us establish the Ca²⁺ transport assay in our lab and to Heven Sze for useful comments. We are also very grateful to Toshiro Shigaki for the HA:sCAX1-piHGpd plasmid.

Received May 1, 2001; returned for revision June 28, 2001; accepted July 11, 2001.

LITERATURE CITED

- Askerlund P** (1996) Modulation of an intracellular calmodulin-stimulated Ca²⁺-pumping ATPase in cauliflower by trypsin: the use of Calcium Green-5N to measure Ca²⁺ transport in membrane vesicles. *Plant Physiol* **110**: 913–922
- Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K** (1998) *Current Protocols in Molecular Biology*. Greene Publishing Associates/Wiley Interscience, New York
- Blom N, Gammeltoft S, Brunak S** (1999) Sequence- and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol* **294**: 1351–1362
- Blumwald E, Poole RJ** (1986) Kinetics of Ca²⁺/H⁺ antiport in isolated tonoplast vesicles from storage tissue of *Beta vulgaris* L. *Plant Physiol* **80**: 727–731
- Bush DS** (1995) Calcium regulation in plant cells and its role in signaling. *Annu Rev Plant Physiol Plant Mol Biol* **46**: 95–122
- Chung WS, Lee SH, Kim JC, Heo WD, Kim MC, Park CY, Park HC, Lim CO, Kim WB, Harper JF et al.** (2000) Identification of a calmodulin-regulated soybean Ca²⁺-ATPase (SCA1) that is located in the plasma membrane. *Plant Cell* **12**: 1393–1407
- Cunningham KW, Fink GR** (1996) Calcineurin inhibits VCX1-dependent H⁺/Ca²⁺ exchange and induces Ca²⁺ ATPases in *Saccharomyces cerevisiae*. *Mol Cell Biol* **16**: 2226–2237
- Curran AC, Hwang I, Corbin J, Matinez S, Rayle D, Sze H, Harper JF** (2000) Autoinhibition of a calmodulin-dependent calcium pump involves a structure in the stalk that connects the transmembrane domain to the ATPase catalytic domain. *J Biol Chem* **275**: 30301–30308
- Darley CP, van Wuytswinkel OCM, van der Woude K, Mager WH, De Boer AH** (2000) *Arabidopsis thaliana* and *Saccharomyces cerevisiae* NHX1 genes encode amiloride sensitive electroneutral Na⁺/H⁺ exchangers. *Biochem J* **351**: 241–249
- Enyedi A, Verma AK, Filoteo AG, Penniston JT** (1996) Protein kinase C activates the plasma membrane Ca²⁺ pump isoform 4b by phosphorylation of an inhibitory region downstream of the calmodulin-binding domain. *J Biol Chem* **271**: 32461–32467
- Geisler M, Frangne N, Gomes E, Martinoia E, Palmgren MG** (2000) The ACA4 gene of *Arabidopsis* encodes a vacuolar membrane calcium pump that improves salt tolerance in yeast. *Plant Physiol* **124**: 1814–1827

- Hamada A, Hibino T, Nakamura T, Takabe T** (2001) $\text{Na}^{+}/\text{H}^{+}$ antiporter from *Synechocystis* species PCC 6803, homologous to SOS1, contains an aspartic residue and long C-terminal tail important for the carrier activity. *Plant Physiol* **125**: 437–446
- Harper JF, Hong B, Hwang I, Guo HQ, Stoddard R, Huang JF, Palmgren MG, Sze H** (1998) A novel calmodulin-regulated Ca^{2+} -ATPase (ACA2) from *Arabidopsis* with an N-terminal autoinhibitory domain. *J Biol Chem* **273**: 1099–1106
- Haworth RS, Sinnott-Smith J, Rozengurt E, Avkiran M** (1999) Protein kinase D inhibits plasma membrane $\text{Na}^{+}/\text{H}^{+}$ exchanger activity. *Am J Physiol* **277**: C1202–C1209
- Hirschi KD** (1999) Expression of *Arabidopsis* CAX1 in tobacco: altered calcium homeostasis and increased stress sensitivity. *Plant Cell* **11**: 2113–2122
- Hirschi KD** (2001) Vacuolar $\text{H}^{+}/\text{Ca}^{2+}$ transport: who's directing the traffic? *Trends Plant Sci* **6**: 100–104
- Hirschi KD, Korenkov VD, Wilganowski NL, Wagner GJ** (2000) Expression of *Arabidopsis* CAX2 in tobacco: altered metal accumulation and increased manganese tolerance. *Plant Physiol* **124**: 125–134
- Hirschi KD, Miranda ML, Wilganowski NL** (2001) Phenotypic changes in *Arabidopsis* caused by expression of a yeast $\text{Ca}^{2+}/\text{H}^{+}$ antiporter. *Plant Mol Biol* **46**: 57–65
- Hirschi KD, Zhen R-G, Cunningham KW, Rea PA, Fink GR** (1996) CAX1, an $\text{H}^{+}/\text{Ca}^{2+}$ antiporter from *Arabidopsis*. *Proc Natl Acad Sci USA* **93**: 8782–8786
- Hwang I, Harper JF, Liang F, Sze H** (2000a) Calmodulin activation of an endoplasmic reticulum-located calcium pump involves an interaction with the N-terminal autoinhibitory domain. *Plant Physiol* **122**: 157–168
- Hwang I, Ratterman DM, Sze H** (1997) Distinction between endoplasmic reticulum-type and plasma membrane-type Ca^{2+} pumps. *Plant Physiol* **113**: 535–548
- Hwang I, Sze H, Harper JF** (2000b) A calcium-dependent protein kinase can inhibit a calmodulin-stimulated Ca^{2+} pump (ACA2) located in the endoplasmic reticulum of *Arabidopsis*. *Proc Natl Acad Sci USA* **97**: 6224–6229
- Iwamoto T, Pan Y, Nakamura TY, Wakabayashi S, Shigekawa M** (1998) Protein kinase C-dependent regulation of $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger isoforms NCX1 and NCX3 does not require their direct phosphorylation. *Biochemistry* **37**: 17230–17238
- McAinsh MR, Hetherington AM** (1998) Encoding specificity in Ca^{2+} signaling systems. *Trends Plant Sci* **3**: 32–36
- Malmström S, Åkerlund H-E, Askerlund P** (2000) Regulatory role of the N-terminus of the vacuolar Ca^{2+} -ATPase in cauliflower. *Plant Physiol* **122**: 517–526
- Mäser P, Thomine S, Schroeder JI, Ward JM, Hirschi KD, Sze H, Talke IN, Amtmann A, Maathuis FJM, Sanders D et al.** (2001) Phylogenetic relationships within cation-transporter families of *Arabidopsis thaliana*. *Plant Physiol* **126**: 1646–1667
- Nakanishi Y, Saijo T, Wada Y, Maeshima M** (2001) Mutagenic analysis of functional residues in putative substrate-binding site and acidic domains of vacuolar H^{+} -pyrophosphatase. *J Biol Chem* **276**: 7654–7660
- Nathan DF, Vos MH, Lindquist S** (1999) Identification of SSF1, and HCH1 as multicopy suppressors of a *Saccharomyces cerevisiae* Hsp90 loss-of-function mutation. *Proc Natl Acad Sci USA* **96**: 1409–1414
- Navazio L, Bewell MA, Siddiqua A, Dickinson GD, Galione A, Sanders D** (2000) Calcium release from the endoplasmic reticulum of higher plants elicited by the NADP metabolite nicotinic acid adenine dinucleotide phosphate. *Proc Natl Acad Sci USA* **97**: 8693–8698
- Palmgren MG** (2001) Plant plasma membrane H^{+} -ATPases: powerhouses for nutrient uptake. *Annu Rev Plant Physiol Plant Mol Biol* **52**: 817–845
- Penniston JT, Enyedi A** (1998) Modulation of the plasma membrane Ca^{2+} pump. *J Membr Biol* **165**: 101–109
- Sanders D, Brownlee C, Harper JF** (1999) Communicating with calcium. *Plant Cell* **11**: 691–706
- Schumaker KS, Sze H** (1985) $\text{Ca}^{2+}/\text{H}^{+}$ antiport system driven by the proton electrochemical gradient of a tonoplast H^{+} -ATPase from oat roots. *Plant Physiol* **79**: 1111–1117
- Sherman F, Fink GR, Hicks JB** (1986) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
- Shigaki T, Hirschi KD** (2000) Characterization of CAX-like genes in plants: implications for functional diversity. *Gene* **257**: 291–298
- Shigaki T, Hirschi KD** (2001) Use of class IIS restriction enzymes for site directed mutagenesis: variations on Phoenix mutagenesis. *Anal Biochem* (in press)
- Sze H, Liang F, Hwang I, Curran AC, Harper JF** (2000) Diversity and regulation of plant Ca^{2+} pumps: insights from expression in yeast. *Annu Rev Plant Physiol Plant Mol Biol* **51**: 433–462
- Tomas FM, Walton PE, Dunshea FR, Ballard FJ** (1997) IGF-I variants which bind poorly to IGF-binding proteins show more potent and prolonged hypoglycaemic action than native IGF-I in pigs and marmoset monkeys. *J Endocrinol* **155**: 377–386
- Ueoka-Nakanishi H, Nakanishi Y, Tanaka Y, Maeshima M** (1999) Properties and molecular cloning of a $\text{Ca}^{2+}/\text{H}^{+}$ antiporter in the vacuolar membrane of mung bean. *Eur J Biochem* **262**: 417–425
- Ueoka-Nakanishi H, Tsuchiya T, Sasaki M, Nakanishi Y, Cunningham KW, Maeshima M** (2000) Functional expression of mung bean $\text{Ca}^{2+}/\text{H}^{+}$ antiporter in yeast and its intracellular localization in the hypocotyl and tobacco cells. *Eur J Biochem* **267**: 3090–3098
- Wakabayashi S, Ikeda T, Iwamoto T, Pouyssegur J, Shigekawa M** (1997) Calmodulin-binding autoinhibitory domain controls “pH-sensing” in the $\text{Na}^{+}/\text{H}^{+}$ exchanger NHE1 through sequence-specific interaction. *Biochemistry* **36**: 12854–12861