

# Characterization of CAX4, an Arabidopsis H<sup>+</sup>/Cation Antiporter<sup>1</sup>

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Ion compartmentalization is essential for plant growth and development. The Arabidopsis open reading frames for CAX1, CAX2, and CAX3 (cation exchangers 1, 2, and 3) were previously identified as transporters that may modulate ion fluxes across the vacuolar membrane. To understand the diversity and role of H<sup>+</sup>/cation transporters in controlling plant ion levels, another homolog of the CAX genes, CAX4, was cloned from an Arabidopsis cDNA library. CAX4 is 53% identical to CAX1 at the amino acid level, 42% identical to CAX2, and 54% identical to CAX3. CAX4 transcripts appeared to be expressed at low levels in all tissues and levels of CAX4 RNA increased after Mn<sup>2+</sup>, Na<sup>+</sup>, and Ni<sup>2+</sup> treatment. An N-terminal CAX4-hemagglutinin fusion appeared to localize to both yeast and plant vacuolar membranes. When expressed in yeast, CAX4, like CAX3, failed to suppress the Ca<sup>2+</sup> sensitivity of yeast strains deficient in vacuolar Ca<sup>2+</sup> transport. Several modifications to CAX4 allowed the protein to transport Ca<sup>2+</sup>. Addition of amino acids to the N terminus of CAX4 and CAX3 caused both transporters to suppress the sensitivity of yeast strains deficient in vacuolar Ca<sup>2+</sup> transport. These findings suggest that CAX transporters may modulate their ion transport properties through alterations at the N terminus.

Plants have multiple mechanisms to maintain appropriate intracellular levels of various ions (Fox and Guerinot, 1998). Uptake, transport of ions across the plasma membrane, and intracellular sequestration, all regulate the levels of nutrients and toxins in the cytosol. The plant vacuole plays a major role in the intracellular sequestration of various compounds (Marschner, 1995; Marty, 1999). Vacuolar transporters may provide an important mechanism for ion sequestration (Salt and Wagner, 1993; Shaul et al., 1999). In fact, a concentration gradient of Na<sup>+</sup>, Ca<sup>2+</sup>, Cd<sup>2+</sup>, and Mn<sup>2+</sup> is established across the vacuolar membrane (tonoplast) by H<sup>+</sup>/Na<sup>+</sup>, H<sup>+</sup>/Ca<sup>2+</sup>, H<sup>+</sup>/Cd<sup>2+</sup>, and H<sup>+</sup>/Mn<sup>2+</sup> exchange activities (Schumaker and Sze, 1985; Salt and Wagner, 1993; Apse et al., 1999; Gonzalez et al., 1999); however, the precise number of genes encoding these biochemical activities and the capacity of the individual transporters to transport various ions remain largely unknown.

Several plant vacuolar cation transporters have been isolated (Hirschi et al., 1996; Shaul et al., 1999). The Arabidopsis transporters, CAX1, CAX2, and CAX3 (cation exchangers 1, 2, and 3), may play a central role in Ca<sup>2+</sup> and metal sequestration into the

vacuole. CAX1 and CAX2 were identified (Hirschi et al., 1996) by their ability to sequester Ca<sup>2+</sup> into yeast vacuoles in *Saccharomyces cerevisiae* mutants deleted for the vacuolar high-affinity Ca<sup>2+</sup>-ATPase (*PMCI*) and low-affinity H<sup>+</sup>/Ca<sup>2+</sup> antiporter (*VCX1*). Biochemical activities of CAX1 in yeast vacuoles correlate well with those described for the vacuolar H<sup>+</sup>/Ca<sup>2+</sup> antiport activities from plant sources (Hirschi et al., 1996; Ueoka-Nakanishi et al., 1999). CAX2 appears to be a plant vacuolar metal transporter (Hirschi et al., 2000). CAX3 cannot suppress the Ca<sup>2+</sup> sensitive phenotype of yeast mutants defective in vacuolar Ca<sup>2+</sup> transport and the precise function of this transporter remains unknown (Shigaki and Hirschi, 2000). The Arabidopsis genome also contains several other CAX-like open reading frames (Maser et al., 2001) that may contribute to ion homeostasis.

Using yeast as an experimental tool, two domains have been identified that modulate CAX1 activity (Pittman and Hirschi, 2001; Shigaki et al., 2001). The first domain has been termed the Ca<sup>2+</sup> domain, located between amino acids 87 to 95 in CAX1 (Shigaki et al., 2001). CAX3 can suppress the Ca<sup>2+</sup> sensitivity of yeast vacuolar Ca<sup>2+</sup> transport mutants if this nine-amino acid region of CAX1 is inserted into CAX3 (CAX3-9). Exchanging this nine-amino acid region of CAX1 into CAX2 greatly increased yeast vacuolar Ca<sup>2+</sup> transport (CAX2-9). The second domain that regulates CAX function has been termed the regulatory or autoinhibitory domain (Pittman and Hirschi, 2001). The CAX1 open reading frame contains an additional 36 amino acids at the N terminus that

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were not found in the original "shorter" clone (sCAX1) identified by suppression of yeast vacuolar  $\text{Ca}^{2+}$  transport mutants. This "longer" version of CAX1 does not suppress the yeast  $\text{Ca}^{2+}$  transport defects, despite localization to the yeast vacuole. Minor alterations in the 36-amino acid region restore  $\text{H}^+/\text{Ca}^{2+}$  transport (Pittman and Hirschi, 2001). Sequence analysis suggests that a 36-amino acid N-terminal regulatory domain may be present in all Arabidopsis CAX-like transporters. These findings suggest structural features involved in regulation of  $\text{H}^+$ /cation antiport; however, the extent to which these regulatory motifs modulate other CAX-like genes has not been addressed.

To investigate the diversity of CAX gene function and the ubiquity of CAX gene regulation, we have cloned an additional CAX homolog, termed CAX4. We monitored the expression of CAX4 in Arabidopsis and monitored localization in yeast and plants using an N-terminal hemagglutinin (HA) tag. We then modified the N terminus and  $\text{Ca}^{2+}$  domain of CAX4 in an attempt to modify transport activity in yeast. We made similar modifications to CAX3 and CAX1 and assayed their functions in yeast. These findings offer insights into the modulation of  $\text{H}^+$ /cation antiport in plants.

## RESULTS

### Identification of CAX4

To further understand the diversity of  $\text{H}^+$ /cation antiporters, we were interested in cloning and characterizing other CAX-like genes from Arabidopsis. The sequence of the Arabidopsis genome suggests that several other putative  $\text{H}^+$ /cation antiporters exist that may function in ion homeostasis (Maser et al., 2001). The open reading frame most similar to the previously characterized CAX genes is located on chromosome 5 and has been designated CAX4. Specific primers were synthesized according to genomic sequence from an Arabidopsis BAC clone F7A7\_10 and template DNA was derived from an Arabidopsis Landsberg *erecta* cDNA library (Minet et al., 1992) to clone CAX4 by a PCR-based approach. The cloned CAX4 open reading frame contained 1,341 nucleotides, which could encode 446 amino acids and produce a putative 49-kD protein (Fig. 1A). The CAX4 open reading frame has many of the signature elements of  $\text{H}^+/\text{Ca}^{2+}$  antiporters characterized from bacteria, fungi and plants (Mäser et al., 2001). CAX4 has approximately the same  $M_r$  as many microbial  $\text{H}^+/\text{Ca}^{2+}$  antiporters, and computer-assisted hydrophobicity analyses generated profiles for CAX4 that are similar to CAX1, CAX2, and CAX3 (Fig. 1B). Like these open reading frames, CAX4 contains a central hydrophilic motif rich in acidic amino acid residues (the acidic motif) that bisects the polypeptide into two groups of approximately equal length. CAX4 is 53% identical (67% similar) to CAX1, 54% identical to

CAX3 (69% similar), and 42% identical to CAX2 (52% similar). CAX4 contains an N-terminal hydrophilic region that is similar to the CAX1 autoinhibitory domain (Pittman and Hirschi, 2001). The putative  $\text{Ca}^{2+}$  domain of CAX4 does not resemble the  $\text{Ca}^{2+}$  domains of the previously characterized CAX genes (Shigaki et al., 2001).

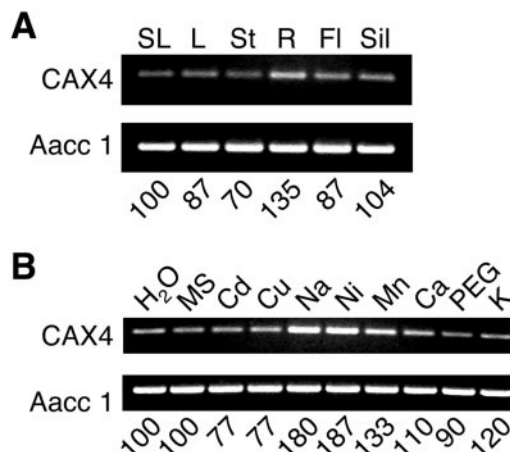
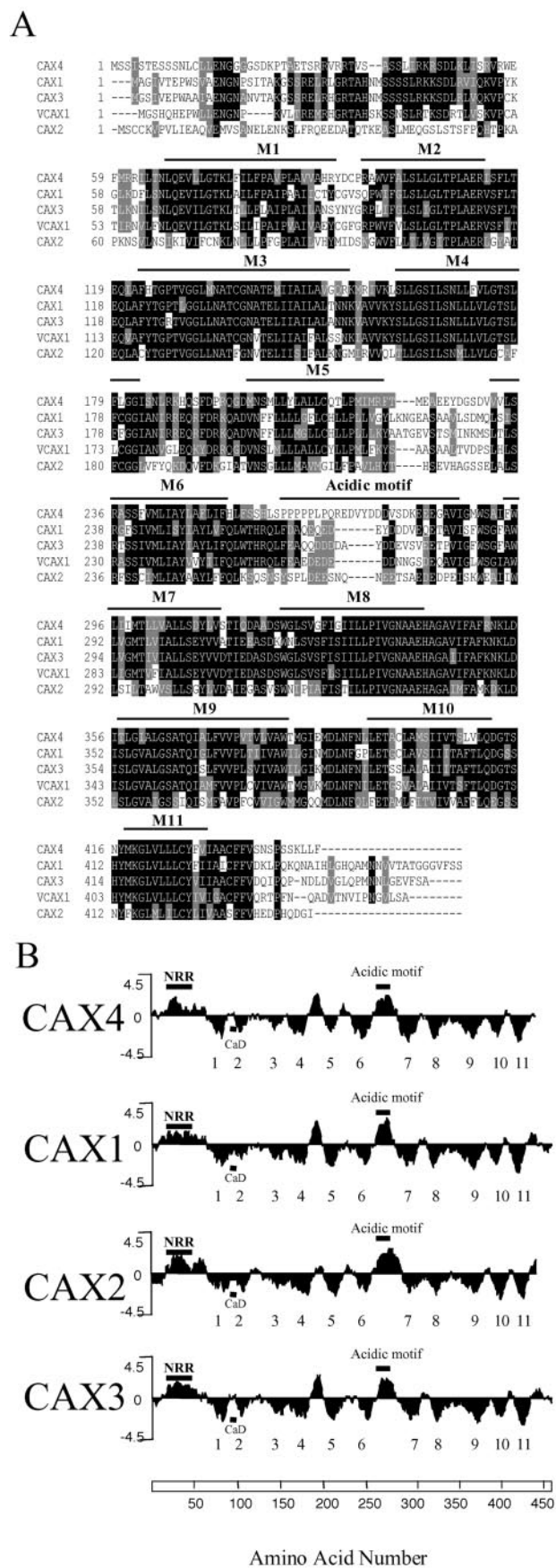
### Expression of CAX4 in Arabidopsis

The expression of CAX1 and CAX2 correlates with the transport properties of each transporter in yeast (Hirschi, 1999; Hirschi et al., 2000). That is, the high-affinity, high-capacity  $\text{H}^+/\text{Ca}^{2+}$  transporter CAX1 is highly expressed in response to exogenous  $\text{Ca}^{2+}$  in plants, whereas the low-affinity  $\text{H}^+/\text{Ca}^{2+}$  transporter CAX2 is not induced by exogenous  $\text{Ca}^{2+}$  in plants. To test the expression of CAX4, standard northern analysis using total RNA was done using the entire CAX4 coding sequence. Using a variety of experimental conditions, no CAX4 transcripts could be detected (data not shown); however, using a more sensitive reverse transcription (RT)-PCR approach, we were able to amplify CAX4 specific cDNA in all tissues analyzed (Fig. 2A). This semi-quantitative approach suggests that CAX4 RNA levels increased slightly in response to  $\text{Mn}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Na}^+$ , and were not induced by other ions such as  $\text{Ca}^{2+}$  (Fig. 2B).

### Expression of CAX4 in Yeast

To test the properties of CAX4, we expressed the open reading frame in various yeast strains. The CAX4 cDNA was placed initially under the transcriptional control of the yeast promoter glyceraldehyde phosphate dehydrogenase (GPD) on a high-copy-number yeast plasmid (Nathan et al., 1999). This plasmid was then introduced into yeast strains that contained defects in vacuolar  $\text{Ca}^{2+}$  transport to determine whether CAX4 could suppress yeast vacuolar  $\text{Ca}^{2+}$  transport mutants in a manner similar to the N-terminal truncated CAX1 (sCAX1) and CAX2 (Hirschi et al., 1996). As shown in Figure 3A, sCAX1 suppresses this growth defect. Under these assay conditions, CAX2 suppresses the  $\text{Ca}^{2+}$  sensitivity in a manner indistinguishable from sCAX1 (Hirschi et al., 1996; data not shown); however, like CAX3 (Shigaki and Hirschi, 2000), CAX4 was not able to suppress the  $\text{Ca}^{2+}$  sensitivity of yeast strains deficient in vacuolar  $\text{Ca}^{2+}$  transport.

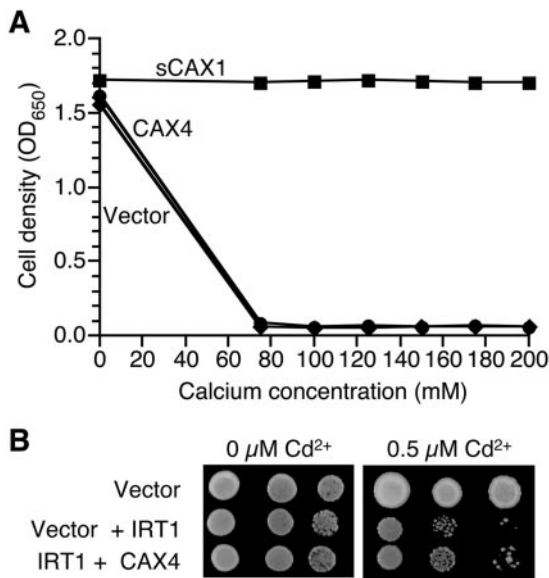
We also tested the possibility that CAX4 transports other metals, thus making the host yeast strains tolerant (or hypersensitive to) to these metals. Expression of CAX4 in a  $\text{Mg}^{2+}$  requiring strain (CM66) did not suppress the ion sensitivity of this strain (MacDiarmid and Gardner, 1998). A range of metals, such as  $\text{Al}^{3+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Na}^+$ , and  $\text{Zn}^{2+}$ , was tested with a K661 yeast strain expressing CAX4. For such



**Figure 2.** Expression of CAX4 in Arabidopsis. A, CAX4 transcripts were detected in total RNA from different tissues by RT-PCR. SL, Stem leaf; L, rosette leaf; St, stem; R, root; Fl, flower; Sil, silique. B, CAX4 transcripts were examined by RT-PCR in total RNA from seedlings treated for 12 h with the following solutions: water (as a control), Murashige and Skoog (nutrient media), 0.5 mM CdCl<sub>2</sub>, 0.1 mM CuSO<sub>4</sub>, 80 mM NaCl, 0.1 mM NiSO<sub>4</sub>, 2 mM MnCl<sub>2</sub>, 100 mM CaCl<sub>2</sub>, 10% (w/v) polyethylene glycol 3350 solution (118 mOsm/kg), and 80 mM KCl, respectively. Top panel in A and B, a 414-bp CAX4-specific fragment was amplified by RT-PCR. Bottom panel in A and B, a 457-bp actin aac1 gene fragment was amplified by RT-PCR as an internal control. The numbers indicate the relative intensities of the PCR bands that are given in percentages as compared with control (SL as a control in A, water-treated as a control in B).

metal sensitivity assays, this yeast strain is indistinguishable from wild-type strains in our assay conditions (Shigaki and Hirschi, 2000). CAX4 expression did not alter the yeast growth on any media except Cd<sup>2+</sup> (data not shown). An Arabidopsis plasma membrane ion transporter, IRT1, can transport Cd<sup>2+</sup> into the cytosol, and when expressed in wild-type yeast strains (DY1457), it causes the strain to be more sensitive to Cd<sup>2+</sup> (Rogers et al., 2000; Fig. 3B). When CAX4 was expressed in the yeast strain harboring IRT1, CAX4 was able to partially rescue the Cd<sup>2+</sup> sensitivity of the IRT1-expressing strain (Fig. 3B).

**Figure 1.** A, Alignment of deduced amino acid sequences of polypeptides encoded by Arabidopsis CAX 1–4 genes and mung bean VCAX1. Alignments were performed using the ClustalW 1.8 program (Baylor College of Medicine; Thompson et al., 1994). Consensus amino acid residues are boxed in black (identical) or gray (similar). Gaps introduced to maximize the alignments are denoted by hyphens. The 11 putative transmembrane spans (M1–11) predicted for CAX and the central hydrophilic motif rich in acidic residues are overlined. B, Hydropathy profile of Arabidopsis CAXs are predicted over a running window of 15 amino acid residues according to Kyte and Doolittle (1982). Putative transmembrane spans are numbered. The N-terminal regulatory region (NRR), Ca<sup>2+</sup> domain (CaD), and acidic motif are indicated. The accession numbers for CAX 1 to -4 and VCAX1 are AF461691, AF424628, AF256229, AF409107, and AB012932, respectively.



**Figure 3.** A,  $\text{Ca}^{2+}$  sensitivity assay of yeast K667 strains expressing vector, sCAX1 and CAX4, respectively. Cells were diluted 10-fold into fresh media containing a range of  $\text{CaCl}_2$  concentrations and incubated for 1 d at  $30^\circ\text{C}$  in flat-bottom 24-well dishes (1.0 mL/well). Optical density at 650 nm was measured for each resuspended culture. B,  $\text{Cd}^{2+}$  sensitivity assay of IRT1 expressing yeast strains. The yeast strains, DY1457/FL61 (wt) and DY1457/IRT1, were transformed with vector and CAX4. The indicated yeast strains were grown in liquid media and then a series of dilutions were spotted on to the control and  $\text{Cd}^{2+}$ -containing media.

### Localization of CAX4 in Yeast

The inability of CAX4 to suppress the  $\text{Ca}^{2+}$  sensitivity of yeast mutants defective in vacuolar  $\text{Ca}^{2+}$  transport could be due to targeting of the protein to a different membrane. To identify the cellular location of CAX4 in yeast, an epitope-tagged variant was generated by the fusion of a triple copy of HA to the N terminus (HA:CAX4). An N-terminal HA epitope tag has previously been used to identify the cellular location of VCX1, CAX1, and CAX3 in yeast (Cunningham and Fink, 1996; Pittman and Hirschi, 2001; Shigaki et al., 2001). As shown in Figure 4, western-blot analysis of yeast membranes fractionated on Suc gradients showed that CAX4 cofractionated with vacuolar membranes. The distribution of HA:CAX4 corresponded with that of HA:sCAX1 and the yeast vacuolar membrane marker, alkaline phosphatase.

### Localization of CAX4 in Transgenic Tobacco

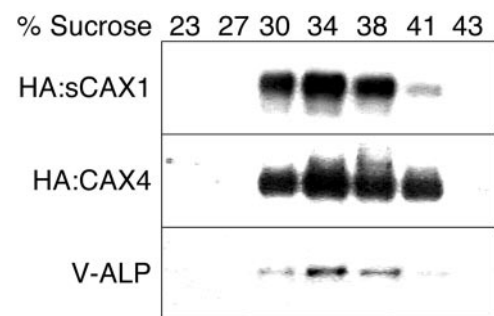
The localization of HA:CAX4 on the vacuolar membrane of plants was shown by the heterologous expression of the CAX4 fusion protein in a suspension of tobacco cells. The fusion protein under the control of the cauliflower mosaic virus 35S promoter was expressed in tobacco BY-2 cells. In the case of BY-2 cells, many vacuoles are generally observed in a single cell (Kost et al., 1998). As shown in Figure

5, A and B, confocal images of red fluorescent signals stained by Texas Red conjugated antibody revealed that HA:CAX4 is localized in the vacuolar membrane.

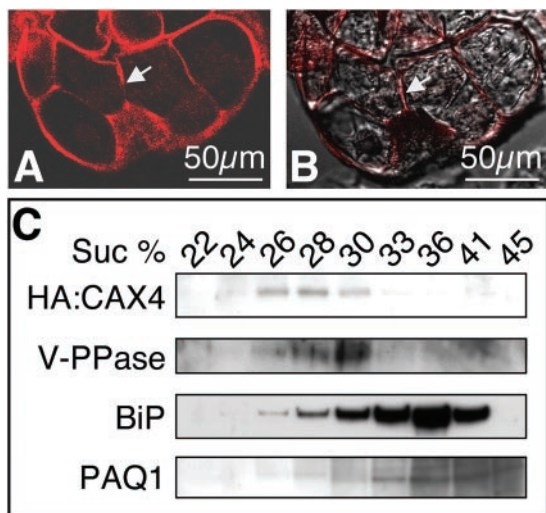
To confirm vacuolar membrane localization of the fusion protein, the crude membrane fraction of tobacco cells was subjected to Suc gradient centrifugation and immunoblotting with the anti-HA antibody. The distribution of the fusion protein (fractions from 26% to 33%) was paralleled by that of a vacuolar marker (mung bean vacuolar pyrophosphatase) and not that of an endoplasmic reticulum marker (plant endoplasmic reticulum luminal protein) and a plant plasma membrane marker (radish plasma membrane aquaporin; Fig. 5C).

### Alterations in CAX4 That Facilitate Yeast Vacuolar $\text{Ca}^{2+}$ Transport

Given that the N terminus appears to act as an autoinhibitory domain for CAX1  $\text{Ca}^{2+}$  transport in yeast, we were interested in testing whether the N terminus of CAX4 acts as an autoinhibitory domain. Truncated and, thus, possibly active, versions of CAX4 (sCAX4) should not be present in cDNA libraries because the CAX4 open reading frame, unlike the CAX1 and CAX2 open reading frames, does not contain a Met residue after the putative autoinhibitory domain. We, thus, created a truncated version of CAX4 by deleting the first 37 amino acids of CAX4 and replacing the Ser residue at position 38 with a Met residue. Yeast strains expressing sCAX1 grow well in the  $\text{Ca}^{2+}$ -containing media, whereas the sCAX4 expressing cells grow poorly (Fig. 6A). The growth of several sCAX4 expressing cells is meaningful when compared with the complete absence of



**Figure 4.** Subcellular localization of CAX4 in yeast. *Saccharomyces cerevisiae* K667 strains were transformed with plasmid DNA containing HA:sCAX1 and the HA:CAX4 fusion. Microsomal membranes were extracted from HA:sCAX1- and HA:CAX4-expressing yeast cells and fractionated in a linear Suc gradient. Ten micrograms of total proteins from the numbered fractions were separated on 12% (w/v) SDS-PAGE and transferred onto nitrocellulose membrane. Western blotting analyses were performed using the following primary antibodies: monoclonal mouse IgG against HA epitope and monoclonal mouse IgG against yeast vacuolar alkaline phosphatase (V-ALP), which is used as an indicator of yeast vacuolar membranes. The numbers indicate the Suc concentration of each fraction.



**Figure 5.** Expression of HA:CAX4 in tobacco BY-2 cells. A and B, Immunostaining of HA:CAX4 in BY-2 cell. A, HA:CAX4 was detected on vacuolar membrane by HA antibody and Texas Red conjugated secondary antibody (indicated by arrow). B, A superimposed image of red channel and transmitted light demonstrated only vacuolar membranes were stained (indicated by arrow). Bars = 50  $\mu\text{m}$ . C, Immunoblotting of HA:CAX4 in tobacco BY-2 cells. Western blot was performed as described in "Materials and Methods." Monoclonal antibodies against HA and a plant endoplasmic reticulum luminal protein (BiP), an indicator of endoplasmic reticulum, were used at dilutions of 1:1,000 and 1:1,500, respectively. Polyclonal antibodies against mung bean (*Vigna radiata*) vacuolar pyrophosphatase (V-PPase) and radish plasma membrane aquaporin (PAQ1) were used at 1:1,000 dilutions. The numbers indicate the concentration of the Suc fraction.

growth for CAX4 or vector expressing yeast cells (Fig. 6A).

Insertion of the CAX1  $\text{Ca}^{2+}$  domain into CAX3 allows yeast cells expressing this chimeric construct to suppress vacuolar  $\text{Ca}^{2+}$  transport defects (Shigaki et al., 2001). Given that this region of CAX4 is unlike CAX1, CAX2, or CAX3, we proceeded to change the 11 amino acids in this region to the amino acids found in the CAX1  $\text{Ca}^{2+}$  domain. Although we have altered 11 amino acids, we have termed this construct CAX4-9 so that the nomenclature will be consistent with similar modifications made in CAX2 and CAX3 (Shigaki et al., 2001). Yeast cells expressing CAX4-9 grew weakly in  $\text{Ca}^{2+}$  containing media at a rate comparable with sCAX4 expressing cells (Fig. 6A).

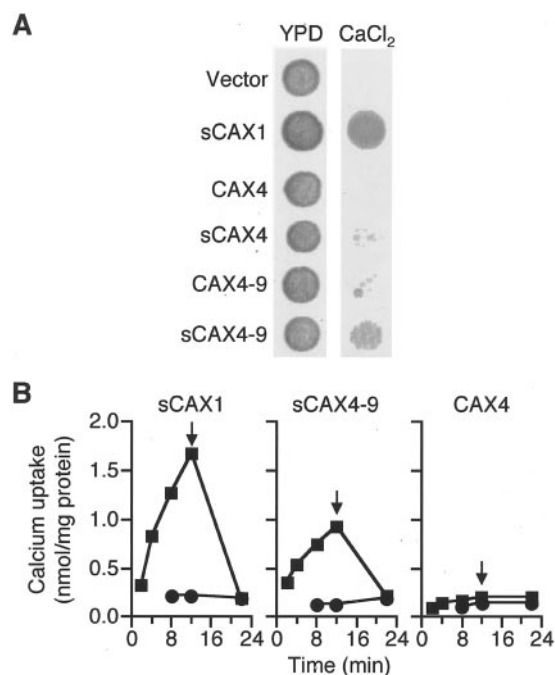
We then created chimeric CAX4 constructs containing both the N-terminal truncation and the CAX1  $\text{Ca}^{2+}$  domain (sCAX4-9). Yeast cells expressing sCAX4-9 suppressed the vacuolar  $\text{Ca}^{2+}$  transport defects in a manner indistinguishable from CAX1 variants, which lack the N-terminal autoinhibitory domain (Fig. 6A).

To confirm that the  $\text{Ca}^{2+}$  tolerance observed for the sCAX4-9 expressing yeast strain was due to a restoration of  $\text{Ca}^{2+}$  transport activity,  $\text{H}^+$ / $^{45}\text{Ca}^{2+}$  transport was measured from endomembrane vesicles obtained from sCAX4-9-expressing yeast cells.  $\text{H}^+$ /

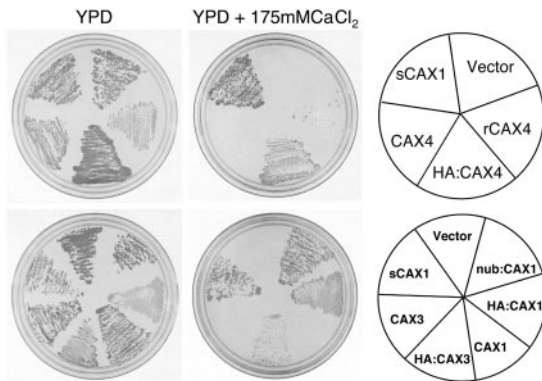
$\text{Ca}^{2+}$  transport by sCAX4-9 was measured using 10  $\mu\text{M}$   $^{45}\text{CaCl}_2$ , and was comparable with the activity observed for sCAX1-containing vesicles, whereas transport was not found in vesicles harboring CAX4 (Fig. 6B).

#### N-Terminal Additions Facilitate CAX-Mediated $\text{Ca}^{2+}$ Transport

Alterations in the N terminus can alter the function of many proteins, including the soluble insulin-like growth factor-I. Even additional N-terminal residues can alter the activity of this growth regulator (Tomas et al., 1997). We were interested in determining whether N-terminal additions could also alter the activity of CAX4 or the other CAX transporters. Due to the localization studies, we already had a series of HA-tags fused to the N terminus of the CAX genes. As shown in Figure 7, addition of HA allows CAX4-expressing yeast cells to suppress vacuolar  $\text{Ca}^{2+}$  transport mutations in a manner indistinguishable from sCAX1 expression.



**Figure 6.** Suppression of  $\text{Ca}^{2+}$  hypersensitivity and  $\text{Ca}^{2+}$  uptake assay. A, Yeast assay for suppression of  $\text{Ca}^{2+}$  hypersensitivity by various CAX4 proteins. Yeast K667 strain was transformed with plasmid DNA containing vector, sCAX1, CAX4, sCAX4, CAX4-9, and sCAX4-9. Yeast strains were grown to  $\text{OD}_{650}$  of 1.0 in selection media at 30°C. Cells were diluted 5-fold and 5  $\mu\text{L}$  of each dilution was spotted on yeast peptone dextrose (YPD) plate supplemented with 175 mM  $\text{CaCl}_2$  and incubated for 2 d at 30°C. B,  $\text{H}^+$ / $\text{Ca}^{2+}$  transport of sealed enriched vesicles purified from yeast expressing CAX4, sCAX1, and sCAX4-9.  $\text{Ca}^{2+}$  uptake was examined with 10  $\mu\text{M}$  total  $\text{CaCl}_2$  containing  $^{45}\text{Ca}$  in the presence (●) and absence (■) of 5  $\mu\text{M}$  gramicidin. The  $\text{Ca}^{2+}$  ionophore A23187 was added at 12 min to a concentration of 5  $\mu\text{M}$  (indicated by arrow).



**Figure 7.** Yeast assay for suppression of  $\text{Ca}^{2+}$  hypersensitivity by CAX proteins with N-terminal additions. K667 yeast strain was transformed with vector, sCAX1, CAX1, HA:CAX1, nub:CAX1, CAX3, HA:CAX3, CAX4, HA:CAX4, and rCAX4. A single colony of each transformant was streaked on YPD plate supplemented with 175 mM  $\text{CaCl}_2$  and incubated at 30°C for 2 d.

To verify that HA:CAX4 suppression was due to the altered conformation of CAX4 and not expression of the HA, we made a CAX4 construct containing 10 arbitrary amino acids fused to the N terminus (rCAX4). Yeast cells expressing rCAX4 weakly suppressed the vacuolar  $\text{Ca}^{2+}$  transport mutations. This growth was subtle and like sCAX4 and CAX4–9 could be monitored by the growth of small individual yeast colonies on  $\text{Ca}^{2+}$  containing media (Fig. 7). This growth on  $\text{Ca}^{2+}$  containing media was significantly weaker than the HA:CAX4 growth but was meaningful compared with the complete lack of yeast growth by vector expressing cells.

Expression of HA:CAX1 and HA:CAX3 in yeast also suppressed the vacuolar  $\text{Ca}^{2+}$  transport defects. Furthermore, expression of the N-terminal one-half of ubiquitin fused to CAX1 at the N terminus (nub:CAX1) also suppressed the yeast mutations. Suppression with HA:CAX1 or nub:CAX1 was consistently stronger than HA:CAX3 suppression (Fig. 7).  $\text{H}^+$ / $\text{Ca}^{2+}$  transport activity was observed from HA:CAX1, HA:CAX3, HA:CAX4, and nub:CAX1 vesicle preparations (data not shown).

## DISCUSSION

$\text{H}^+$ /cation antiporter activity has been described in numerous plant species and on several membranes, including the vacuolar, plasma, and chloroplast thylakoid membranes (Schumaker and Sze, 1985; Blumwald and Poole, 1986; Kasai and Muto, 1990; Ettinger et al., 1999; Shaul et al., 1999). This study was carried out to investigate the diversity of the genes that may confer these activities in plants. This study reports the identification and partial characterization of an Arabidopsis CAX gene, CAX4, which has greater than 50% identity to the previously characterized  $\text{H}^+$ / $\text{Ca}^{2+}$  transporter CAX1. CAX4 has extensive sequence homology with all the previously character-

ized  $\text{H}^+$ / $\text{Ca}^{2+}$  antiporters (Ueoka-Nakanishi et al., 1999, 2000; Hirschi, 2001). The expression pattern of CAX4 in plants suggests that this gene is expressed at levels lower than CAX1, CAX2, or CAX3. Like the metal transporter CAX2, CAX4 RNA levels were not induced by exogenous  $\text{Ca}^{2+}$  (Hirschi et al., 2000). Like CAX3 and CAX1, CAX4 levels increase with some ion imbalances (Fig. 2;  $\text{Mn}^{2+}$ ,  $\text{Na}^+$ , and  $\text{Ni}^{2+}$  treatment). Although the induction of CAX4 after these stimuli is less than 2-fold, this infers that CAX4 may be capable of adjusting specific cytosolic ion concentrations.

CAX4 appeared to be highly expressed in yeast and N-terminal HA-tagged versions of CAX4 localized at the yeast vacuole (Fig. 4). Furthermore, the plant vacuolar membrane location of CAX4 was shown by N-terminal HA-tagged CAX4 in tobacco cells (Fig. 5). This is a useful system for morphologically identifying the localization of proteins, although it should be noted that, in this experiment, the HA:CAX4 fusion protein was expressed under artificial conditions. The HA-tag was observed predominantly in the vacuolar membrane and not in the plasma membrane or nuclear envelope. This distribution was confirmed in immunoblot experiments and is consistent with the expression of sGFP-VCAX1 in mung bean (Ueoka-Nakanishi et al., 2000).

Conceivably, the N terminus could target the CAX proteins to various membranes in yeast and plants, and the HA-tag or other modifications could perturb intracellular targeting and cause the transporters to localize by default to the vacuolar membrane. Several observations suggest that this is not the case. (a) Expression of chimeric constructs containing the N terminus of CAX3 fused to CAX1 suppress yeast mutants defective in vacuolar  $\text{Ca}^{2+}$  transport (J.K. Pittman and K.D. Hirschi, unpublished data). This strongly implies that the CAX3 N terminus is not localizing proteins to a membrane other than the vacuolar membrane. (b) Removal of the N terminus of CAX3 or CAX4 does not allow strong suppression of the vacuolar  $\text{Ca}^{2+}$  transport defects. This suggests that the N terminus is not directing CAX3 or CAX4 to a membrane other than the vacuole for  $\text{Ca}^{2+}$  transport. (c) Expression of the full-length vacuolar  $\text{H}^+$ / $\text{Ca}^{2+}$  transporter from mung bean, VCAX1, suppresses yeast mutants defective in vacuolar  $\text{Ca}^{2+}$  transport. Given that the N terminus of VCAX1 is similar to CAX1, CAX2, CAX3, and CAX4, it is unlikely that this sequence targets the mung bean transporter to the vacuolar membrane and the Arabidopsis proteins are being targeted to a different membrane.

The CAX4 gene product does not suppress yeast defects in vacuolar  $\text{Ca}^{2+}$  transport. However, CAX4 expression weakly suppressed the  $\text{Cd}^{2+}$  sensitivity of an IRT1 harboring strain. Although this suppression was not strong, it suggests that CAX4 functions in providing  $\text{Cd}^{2+}$  tolerance in this strain, indicating

that CAX4 may transport  $\text{Cd}^{2+}$ . The lack of  $\text{Ca}^{2+}$  activity of CAX4 in yeast resembles CAX3 (Shigaki and Hirschi, 2000). Expression of CAX4 N-terminal truncations or CAX4 chimeric constructs containing the CAX1  $\text{Ca}^{2+}$  domain both very weakly suppress the yeast vacuolar  $\text{Ca}^{2+}$  transport defects. This suppression consists of only a few isolated colonies growing on the  $\text{Ca}^{2+}$  containing media (Fig. 6A). When these two alterations are combined, expression of the chimeric CAX4 constructs strongly suppressed the yeast mutations. From these observations, it appears that removal of the N terminus or alterations of the  $\text{Ca}^{2+}$  domain alone are insufficient for high levels of CAX4 mediated  $\text{Ca}^{2+}$  transport. Juxtaposed with these observations was the finding that N-terminal amino acid additions alone were sufficient to mediate CAX4, CAX3, or CAX1  $\text{H}^+/\text{Ca}^{2+}$  transport in yeast (Fig. 7). This suggests that repositioning of the N terminus relative to the native  $\text{Ca}^{2+}$  domain of CAX3 and CAX4 is sufficient to mediate  $\text{H}^+/\text{Ca}^{2+}$  transport.

Most CAX genes appear to be expressed in all tissues. The biological significance of the fact that truncated forms of CAX1 and CAX2 can suppress yeast mutants defective in vacuolar  $\text{Ca}^{2+}$  transport is not immediately clear. A truncated version of CAX3 cannot suppress this phenotype, whereas truncated CAX4 variants weakly suppress the phenotype; however, both CAX3 and CAX4 can suppress this phenotype if N-terminal extensions are present (Fig. 7). Thus, CAX3 and CAX4 contain the necessary components to drive yeast vacuolar  $\text{Ca}^{2+}$  transport; however, we must now address the questions of how and when these transporters function as vacuolar  $\text{Ca}^{2+}$  transporters in plants.

## MATERIALS AND METHODS

All novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material.

### Plant Materials and Yeast Strains

*Arabidopsis* ecotype Columbia was used in this study. Seeds were germinated in artificial soil (Metro-Mix 200, Scotts-Sierra, Maysville, OH) and grown and treated with different metal solutions as previously described (Hirschi, 1999).

*Saccharomyces cerevisiae* strains K661 *MATa vcx1<sup>Δ</sup>* and K667 (*cnb1::Leu1 pmc1::TRP1 vcx1*) were used. Those strains have the following additional mutations: *ade2-1*, *can1-100*, *his3-11*, *15leu2-3*, *112trp1-1* and *ura3-1* (Cunningham and Fink, 1996). The wild-type yeast strain, W301A, and the magnesium-requiring strain, CM66, were used for metal sensitivity assay (Wallis et al., 1989; MacDiarmid and Gardner, 1998). The yeast strain CM66 (*alr1::HIS3 alr2::TRP1 his3-200 ura3-52 leu2-1 lys2-202 trp1-63*) was ob-

tained from Dr. Richard Gardner (University of Auckland). The pFL61/IRT1 in DY1457 and pFL61 alone in DY1457 yeast strains were obtained from Dr. Erin Connolly (University of South Carolina, Columbia). Yeast cells were transformed using the lithium acetate method and selected on synthetic complete minus His (SC-His) media (Sherman et al., 1986). For  $\text{Ca}^{2+}$  tolerance assays, yeast were grown at 30°C for 1 to 3 d on solid YPD medium containing 2% (w/v) yeast extract (DIFCO Laboratories, Detroit), 1% (w/v) bacto-peptone, and 2% (w/v) dextrose, and supplemented with 175 or 200 mM  $\text{CaCl}_2$  (Hirschi et al., 1996). We used 175 mM  $\text{CaCl}_2$  in some experiments because the HA: CAX4 expressing strains grew poorly at 200 mM  $\text{CaCl}_2$ . The liquid  $\text{Ca}^{2+}$  tolerance assays were done as previously described (Pittman and Hirschi, 2001).

### Isolation of CAX4

CAX4 was identified on a BAC clone F7A7\_10 (GenBank accession no. AL161946). To clone the full-length cDNA of this gene, two oligonucleotide primers that are complementary to the 5' and 3' ends of the predicted CAX4 gene were generated: CAX4 forward primer, 5'-TCTAGAAGATGTCTCAATCAGTACGG-3' and CAX4 reverse primer: 5'-GGCGAGCTCTTATCAAAGAGAAGCTTACTTGA-3'. *Xba*I and *Sac*I sites (underlined) were introduced for sub-cloning. Two micrograms of an *Arabidopsis* Landsberg *erecta* cDNA library (Minet et al., 1992) was screened using the CAX4 specific primers. The conditions for amplification were 95°C for 2 min followed by 35 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 2 min, and 72°C for 10 min. The amplified fragments were gel-purified using QIAGEN MAX (Qiagen USA, Valencia, CA) and cloned into pGEM-T-easy vector (Promega, Madison, WI). Multiple clones were completely sequenced. CAX4 cDNAs were also amplified from cDNA pools that were extracted from *Arabidopsis* seedling (Col. Ecotype; see below). All clones identified were identical to the original clone isolated from the cDNA library.

### RNA Extraction and RT-PCR

RNA was isolated using the RNeasy Plant Kit (Qiagen USA), according to the manufacturer's instructions. RNA samples were treated with DNase to minimize any contamination of genomic DNA. Quantitative RT-PCR was performed to detect CAX4 mRNA transcript. The first strand of cDNA was synthesized using 0.2  $\mu\text{g}$  of total RNA as template in 20  $\mu\text{L}$  of reaction mixture, which included 0.5  $\mu\text{g}$  of oligo(dT)<sub>(12-18)</sub> primer and 200 units of Superscript II transcriptase (Invitrogen, Carlsbad, CA). One microliter of the first strand cDNA was used to amplify a CAX4 gene-specific fragment and an actin 1 fragment (Geisler et al., 2000). Sense and antisense primers for CAX4 and actin 1 were used at concentrations of 0.5  $\mu\text{M}$  each in amplification conditions identical to those used to isolate CAX4 (5'-TCTAGAAGATGTCTTCAATCAGTACGG-3' and 5'-CCACATGTGGCGTTCATTAAT-3' [CAX4] and 5'-GTGCCGACTCTGGAGATGGTGTG-3' and 5'-CGGCGATTC-

CAGGGAACATTGTGG-3' [actin 1]). PCR products were run on 2.5% (w/v) agarose gels and stained with ethidium bromide. The gels were photographed with a digital camera (Kodak, Rochester, NY), and the net intensity of individual PCR products was determined using Kodak ID 2.02 analysis software. The relative intensities in different lanes within each individual experiment were independent of the number of PCR cycles performed.

### Construction of Modified CAX Constructs

The truncated CAX4 gene was amplified with sCAX4 forward primer: 5'-GCTCTAGAGGATCCGAGATGCGTCTCGTTGATAAGGAAG-3' and CAX4 reverse primer as described above.

To fuse the triple HA epitope (YPYDVPDYA) to the N terminus of CAX4, HA fragment and CAX4 fragment were generated, respectively, using the following primer sets: HA-5: 5'-GAATTCTCTAGAATGGGCCGCATCTTTATCC-CATACGAT-3' and HA-3: 5'-CCGTACTGATTGAAGACATGCACTGAGCAGCGTAATC-3' for HA fragment; CAX4-5: 5'-AGATTACGCTGCTCAGTGCATGTCTTCA-ATCAGTACG-3' and CAX4 reverse primer as described above for CAX4. HA:CAX4 fusion fragment was amplified with HA-5 and CAX4 reverse primer.

Mutagenesis was performed to replace the 11 amino acid residues of the Ca<sup>2+</sup> domain of CAX4 with that of CAX1 (Shigaki and Hirschi, 2001) using primers: 5'-GAATTC-CGTCTCCCGCATTATTTGCACCTATTGTGGCGTCA-GTCAGCCTTGGTCTTTGCGTTGAGC-3' and 5'-GAATTC-CGTCTCCGGCGAGAGGAACGGCCGGGAAG-3'. The type IIS restriction enzyme *BsmBI* site is underlined. The bold letters indicate the introduced CAX1 11 amino acid residues. The italic letters indicated the CAX4 sequences.

To generate an in-frame fusion of 10 arbitrary amino acid residues to N terminus of CAX4, mutagenesis was performed as described above. The following primer was used to create the 10 arbitrary amino acid residues at the N terminus of CAX4 in piHGpd: 5'-GAA TTC CGT CTC CCG CCG ATG (AGC) NN (GAC) NN (CGA) NN (AGC) NN (GAC) NN (CGA) NN (GCA) NN (CAG) NN (ACG) NN (CGA) NN ATG TCT TCA ATC AGT ACG G-3'. The type IIS restriction enzyme *BsmBI* site is underlined. The bold letters indicate the introduced 10 arbitrary amino acid residues. The italic letters indicated the CAX4 sequences (Shigaki and Hirschi, 2001).

All PCR amplifications were performed using Expand High Fidelity PCR System (Roche Molecular Biochemicals, Indianapolis), and products were cloned into pGEM-T-easy vector (Promega, Madison, WI). Multiple clones of each construct were sequenced. The gene fragments of CAX4, CAX4 variants and HA:CAX4 were subcloned into the yeast expression vector piHGpd (Nathan et al., 1999) at *XbaI/SacI* sites for expression in yeast (Shigaki and Hirschi, 2000).

To generate an in-frame fusion of N-terminal ubiquitin (nub) to CAX1, the 37 amino acids of nub were amplified by PCR from plasmid pNubG-ALG5 (Stagljar et al., 1998; obtained from Dr. I. Stagljär, University of Zurich-Irchel

using the forward primer (5'-CGC GGA TCC ATG CAG ATT TTC GTC AAG ACT-3') and reverse primer (5'-AAT GCC ATG GAG GGA TAC CTT CCT TGT CTT G-3'). A *BamHI* site (underlined) was generated into the forward primer, and an *NcoI* site (underlined) was generated into the reverse primer. CAX1 was amplified by PCR from the original CAX1 cDNA clone in pBluescript using the forward primer (5'-CGC GGA TCC AAA ACC ATG GAT GGC GGG AAT CGT GAC AGA GCC GTG G-3') and reverse primer (5'-AAC GAG CTC TTA TCT AGA TGA GAA AAC TCC TCC TCC TGT TGC A-3'). A *BamHI* site (underlined) and an *NcoI* site (double underlined) were generated into the forward primer, and a *SacI* site (underlined) was generated into the reverse primer. CAX1 was cloned into the *BamHI-SacI* site of the yeast expression vector piHGpd. To fuse nub to the 5' end of CAX1, nub was subcloned into the *BamHI-NcoI* site of CAX1-piHGpd, generating nub:CAX1.

### Membrane Fractionation and Western Analysis

Microsomal membranes were prepared from yeast expressing HA:CAX1 and HA:CAX4, essentially as described by Hwang et al. (2000), with a few modifications (Pittman and Hirschi, 2001). Immunoblots were performed and the HA epitope was detected essentially as described previously (Hirschi et al., 1998; Pittman and Hirschi, 2001).

### Preparation of Microsomal Membrane-Enriched Vesicles from Yeast Cells

Microsomal membrane-enriched vesicles were isolated from the yeast cells expressing various constructs (Pittman and Hirschi, 2001; Shigaki et al., 2001), essentially as described by Nakanishi et al. (2001).

### Ca<sup>2+</sup> Uptake Assay

Time-dependent H<sup>+</sup>/Ca<sup>2+</sup> transport into endomembrane vesicles was examined using the direct filtration method (Hwang et al., 1997), with a minor modification as described previously (Salt and Wagner, 1993; Pittman and Hirschi, 2001; Shigaki et al., 2001). Cold CaCl<sub>2</sub> (10 μM) including <sup>45</sup>Ca<sup>2+</sup> (carrier free, American Radiolabeled Chemicals, St. Louis) was used in this examination.

### Expression of HA:CAX4 in Tobacco Cells

The coding region of HA:CAX4 was cloned into pBIN19 (CLONTECH, Palo Alto, CA), which contained the cauliflower mosaic virus 35S-promoter fragment and nos terminator (Hull et al., 2000). The recombinant plasmids, or vector controls, were transformed into tobacco (*Nicotiana tabacum* cv BY-2) cells mediated by *Agrobacterium tumefaciens* (strain LBA4404; Invitrogen) as previously described (Matsuoka and Nakamura, 1991). To determine the subcellular localization of CAX4 in plant cells, transformed BY-2 cells (HA:CAX4) were subcultured for 4 d and then fixed with 3.7% (w/v) formaldehyde in 50 mM Na<sub>2</sub>HPO<sub>4</sub> overnight at 4°C. The fixed cells were washed three times with



1× phosphate-buffered saline (pH 7.0) and dried on slides, and then treated with 0.5% (w/v) Triton X-100 for 5 min. Cells were blocked with 3% (w/v) bovine serum albumin and then applied with monoclonal antibody against HA at a 1:500 dilution. After treatment with primary antibody, cells were incubated with rabbit IgG against mouse IgG conjugated with Texas Red (Molecular Probes, Eugene, OR), washed four times with 1× phosphate-buffered saline, and then mounted with Aqua Poly/Mount (Polysciences Inc., Warrington, PA). The images were observed and captured by laser scanning biological microscope (Fluoview, Olympus America Inc., Melville, NY; Cheng et al., 2000). To determine the intracellular localization of the fusion protein by immunoblotting, the transformed cells subcultured for 4 d were homogenized and treated as previously described (Hirschi et al., 2000; Ueoka-Nakanishi et al., 2000). Detection of the marker proteins was performed as described (Hong et al., 1999; Shaul et al., 1999; Hirschi et al., 2001; Suga et al., 2001). Enhanced chemiluminescence was performed according to the instructions given by the manufacturer (Amersham, Buckinghamshire, UK). To ensure reproducibility of the results obtained from immunoblots, at least three independent experiments were performed at exposure times which varied from 2 to 30 min.

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