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Characterization of *Arabidopsis* $\text{Ca}^{2+}/\text{H}^+$ Exchanger CAX3

Murli Manohar,^{†,‡} Toshiro Shigaki,[§] Hui Mei,[†] Sunghun Park,^{||} Joy Marshall,[⊥] Jonathan Aguilar,^{†,▽} and Kendal D. Hirsch^{*,†,‡}

[†]United States Department of Agriculture/Agricultural Research Service Children's Nutrition Research Center, Baylor College of Medicine, 1100 Bates Street, Houston, Texas 77030, United States

[‡]Vegetable and Fruit Improvement Center, Texas A&M University, College Station, Texas 77845, United States

[§]Papua New Guinea National Agricultural Research Institute, Bubia, P.O. Box 4415, Lae, Papua New Guinea

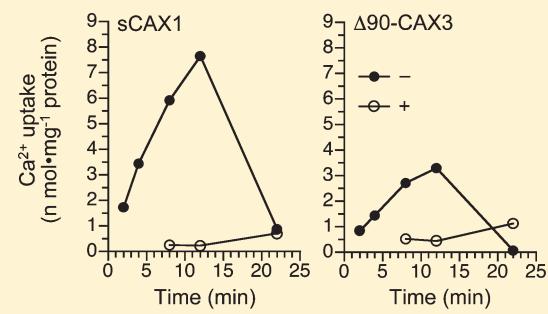
^{||}Department of Horticulture, Forestry and Recreation Resources, Kansas State University, Manhattan, Kansas 66506, United States

[⊥]Prairie View A&M University, FM 1098 Road and University Drive, Prairie View, Texas 77446, United States

[▽]Human Genome Sequencing Center-Pre-Graduate Education Training Program, Baylor College of Medicine, 1100 Bates Street, Houston, Texas 77030, United States

Supporting Information

ABSTRACT: Plant calcium (Ca^{2+}) gradients, millimolar levels in the vacuole and micromolar levels in the cytoplasm, are regulated in part by high-capacity vacuolar cation/ H^+ exchangers (CAXs). Several CAX transporters, including CAX1, appear to contain an approximately 40-amino acid N-terminal regulatory region (NRR) that modulates transport through N-terminal autoinhibition. Deletion of the NRR from several CAXs (sCAX) enhances function in plant and yeast expression assays; however, to date, there are no functional assays for CAX3 (or sCAX3), which is 77% identical and 91% similar in sequence to CAX1. In this report, we create a series of truncations in the CAX3 NRR and demonstrate activation of CAX3 in both yeast and plants by truncating a large portion (up to 90 amino acids) of the NRR. Experiments with endomembrane-enriched vesicles isolated from yeast expressing activated CAX3 demonstrate that the gene encodes $\text{Ca}^{2+}/\text{H}^+$ exchange with properties distinct from those of CAX1. The phenotypes produced by activated CAX3-expressing in transgenic tobacco lines are also distinct from those produced by sCAX1-expressing plants. These studies demonstrate shared and unique aspects of CAX1 and CAX3 transport and regulation.



Calcium (Ca^{2+}) fluxes within the cytosol are an important determinant in many plant responses.¹ Therefore, plants must maintain Ca^{2+} homeostasis to achieve normal growth, development, and environmental adaptations. $\text{Ca}^{2+}/\text{H}^+$ exchangers help control the efflux of Ca^{2+} from the cytosol. In *Arabidopsis*, six cation/ H^+ exchangers termed CAXs (for cation exchangers) are involved in ion homeostasis.¹ CAXs are localized predominantly to the tonoplast and sequester Ca^{2+} and other cations into the vacuole utilizing the H^+ gradient.^{2,3} CAX3 is phylogenetically closely related to CAX1, and both are classified as type IA CAXs.³ Furthermore, CAX3 and CAX1 are thought to play similar physiological roles in *Arabidopsis*.^{4,5}

Plant $\text{Ca}^{2+}/\text{H}^+$ exchangers were cloned because of the ability of N-terminally truncated versions of the proteins to function in *Saccharomyces cerevisiae* mutants defective in vacuolar Ca^{2+} transport.^{1,6,7} The originally identified CAX1 and CAX2 transporters are in fact products of partial-length cDNAs and contain an N-terminal truncation that facilitates activity in yeast (now termed sCAX1 and sCAX2). *In planta*, CAX1 contains a 36-amino acid region at the N-terminus that is not present in sCAX1. In yeast, CAX1 acts as a weak vacuolar $\text{Ca}^{2+}/\text{H}^+$ antiporter, as

transport activity is severely reduced when compared to that in sCAX1.⁴ Interestingly, in the initial yeast assays, the presence of a methionine codon at the 37th and 43rd amino acid residues in CAX1 and CAX2, respectively, allows translational initiation from the truncated cDNAs.^{8,9} A notable exception to these apparent ~40-amino acid N-terminal regulatory regions is CAX3, the closest homologue of CAX1. CAX3, like CAX1, is unable to suppress the Ca^{2+} sensitivity in yeast expression assays.¹⁰ However, when the first 36 amino acids of CAX3 are removed (sCAX3) to allow translation to start from an engineered Met₃₇, the putative transporter appears to remain inactive in yeast expression assays.¹ The inability to obtain CAX3-mediated phenotypes in yeast expression assays is enigmatic and hampered its characterization.

We envision three scenarios to explain the inability to observe any Ca^{2+} transport phenotypes when CAX3 (or sCAX3) is expressed in yeast. First, CAX3 may be an inactive transporter, or a weak Ca^{2+} transporter, with activity that may be

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undetectable in conventional assays. Second, CAX3 may transport other cations. Third, CAX3 may be an autoinhibited Ca^{2+} transporter, but its transport is regulated by a distinct N-terminal region. Given its high degree of homology to CAX1, we favored this third possibility. In this study, we identify deletions in the N-terminus of CAX3 that activate Ca^{2+} transport. We then utilize these variants to demonstrate the properties of CAX3 using both yeast and plant expression assays.

MATERIALS AND METHODS

Construction of CAX3 Truncations. The truncated CAX3 DNAs were made by polymerase chain reaction (PCR) amplification using a primer set raised at the appropriate regions of the CAX3 open reading frame, with *Xba*I (5'-end) and *Sst*I (3'-end) restriction site sequence tags. A codon for methionine was added by incorporating it into the primer at the 5'-end of each construct. The amplified products were cloned into the pCR-II-TOPO vector (Invitrogen), and the sequences were verified for the absence of errors. The inserts were subcloned into the piUGpd shuttle vector¹¹ between *Xba*I and *Sst*I restriction sites. For stable integration into the yeast genome, CAX3 variants with the GPD promoter were dropped from piUGpd shuttle vector by *Kpn*I and *Sst*I and ligated into the pRS306 integration vector.¹² Integration was confirmed by PCR using a CAX3 specific primer and a yeast-based Ca^{2+} suppression assay.

Yeast Transformation, Growth, and Assays. *S. cerevisiae* strain K667 (*vcx1::hisG cnb1::LEU2 pmc1::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*)¹³ was used as the host yeast strain to express CAX1 and CAX3 constructs. Yeast cells were transformed using the standard lithium acetate method and selected on synthetic complete medium minus uracil (SC-Ura) medium.¹⁴ We performed Ca^{2+} tolerance assays by growing yeast at 30 °C for 3 days on solid YPD medium supplemented with the appropriate amount of CaCl_2 . For liquid Ca^{2+} tolerance assays, yeast strains were grown to saturation in SC-Ura medium at 30 °C and then inoculated into YPD medium supplemented with the appropriate amount of CaCl_2 to a final optical density (OD) A_{650} of 0.01. The cultures were grown at 30 °C in a 24-well tissue culture plate with shaking at 200 rpm for 40 h before measurement.

Vacuolar-Enriched Membrane Fractionation. Transformants were inoculated into 1200 mL of YEP supplemented with 4% dextrose and grown to an OD₆₀₀ of ~1.5. The cells were pelleted by centrifugation at 4000g for 5 min and then washed with 50 mL of water and spheroplast buffer [100 mM potassium phosphate buffer (pH 7.0) and 1.2 M sorbitol]. Yeast cells were then resuspended in 5 × pellet volume spheroplast buffer supplemented with 10 mM dithiothreitol (DTT) and 1% dextrose. One unit of Zymolyase/ A_{600} per unit of cells was added and incubated at 30 °C for 1–2 h to generate spheroplasts. The spheroplasts were washed twice with 30 mL of ice-cold spheroplast buffer. The spheroplasts were then resuspended in 5 mL of ice-cold buffer A [10 mM MES-Tris (pH 6.9), 0.1 mM MgCl_2 , and 12% Ficoll PM 400] (Sigma Aldrich, St. Louis, MO) and homogenized. The lysate was then centrifuged at 3000 rpm for 10 min, and the supernatant was collected. For vacuole fractionation, 5 mL of ice-cold buffer A was layered over the supernatant and centrifuged at 60000g for 30 min. A thin white floating wafer of vacuoles was collected and resuspended in 5 mL of fresh buffer A and overlaid with 5 mL of buffer B [10 mM MES-Tris (pH 6.9), 0.5 mM MgCl_2 , and 8% Ficoll 400]. It was then centrifuged at 60000g for 30 min, and the floating white wafer was collected and resuspended again in 5 mL of ice-cold buffer

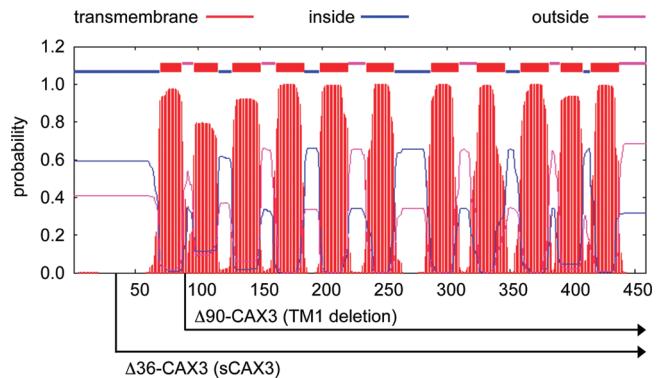


Figure 1. Predicted secondary structure of CAX3. The probability of transmembrane locations predicted by the TMHMM algorithm (Sonnhammer et al., 1998; online program located at <http://www.cbs.dtu.dk/services/TMHMM/>) is indicated by red vertical bars. The blue line and the magenta line indicate inside (cytosol) and outside (vacuolar lumen) locations, respectively. The positions of truncation for $\Delta 36$ and $\Delta 90$ are also indicated.

C [10 mM MES-Tris (pH 6.9), 5 mM MgCl_2 , and 25 mM KCl]. Afterward, it was spun at 60000g for 30 min, and the pellet was finally resuspended in buffer C supplemented with 10% glycerol and stored at –80 °C until use. Protein concentrations were determined using the Bio-Rad (Hercules, CA) protein assay. Time-dependent $^{45}\text{Ca}^{2+}/\text{H}^+$ transport into endomembrane vesicles was later measured using the filtration method as described previously.⁹ The K_m measurement was performed by using Graphpad Prism 5, version 5.4 (Graphpad, La Jolla, CA).

Reverse Transcriptase PCR (RT-PCR) and Western Blotting Analyses. RT-PCR and Western blotting analysis were performed as previously described.^{9,15} RT-PCR was conducted using a primer set designed to amplify $\Delta 90$ -CAX3. For Western blot analysis, the monoclonal antibody against HA (Berkeley Antibody Co., Richmond, CA) was used at a 1:1000 dilution.

Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) Analysis of Yeast Ca^{2+} Content. Yeast cultures were processed using the method described in a previous report.^{16,17} Briefly, yeast cultures were inoculated in 5 mL of YPD and 1/100 volume of 100× mineral supplement stock with additional 10 mM CaCl_2 . The cells were grown at 30 °C to the stationary phase. A 2.5 mL aliquot of each culture was harvested by vacuum filtration with isopore membrane filters (1.2 μm pore size) (Fisher). Cells were washed three times with 1 mL of a 1 μM ethylenediaminetetraacetic acid disodium salt solution (for EDTA) (pH 8.0) by vacuum filtration and then with 1 mL of deionized water three times. The filters were dried at 70 °C in an oven for the 48 h ICP-AES analysis.¹⁸

Plant Materials, Transformation, and Growth Conditions. CAX3, sCAX3, and $\Delta 63$ -CAX3 cDNAs were subcloned into plant expression vector pBin19 under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The plasmids were transformed into *Agrobacterium tumefaciens* strain LBA4404. Tobacco (*Nicotiana tabacum*) plants (cv. KY14) were transformed as previously described.¹⁹

RESULTS

Yeast Mutant Cells Expressing N-Terminal Truncated Variants of CAX3 Are Tolerant to High Calcium Concentrations. Sequence analysis of CAX3 suggests that the protein

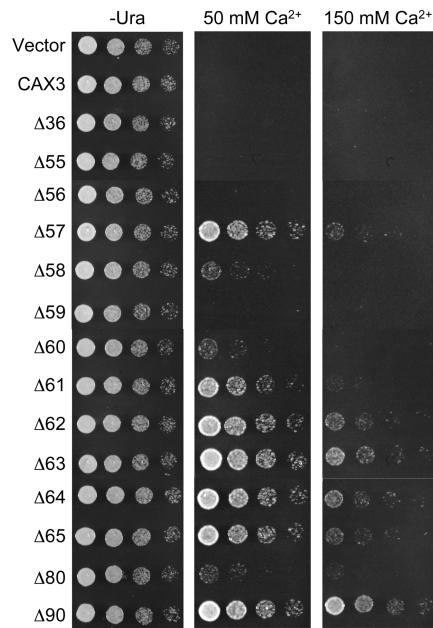


Figure 2. Suppression of Ca^{2+} sensitivity of the yeast mutant by CAX3 deletion constructs. Saturated liquid cultures of *pmc1 vcx1 cnb1* yeast strains (sensitive to high concentrations of Ca^{2+}) containing the indicated truncated CAX3 constructs were serially diluted and spotted onto medium permissive for growth (-Ura) or medium that selects for the presence of plasmid-borne vacuolar Ca^{2+} transport (YPD containing 50 mM CaCl_2 or 150 CaCl_2). The picture was taken after incubation at 30 °C for 48 h.

contains a long hydrophilic N-terminus. The transmembrane-hidden Markov models (TMHMM) algorithm predicts that the first transmembrane (TM) domain of CAX3 starts with the 70th residue and ends with the 87th residue (Figure 1). Considering the sequence conservation among all CAXs, we speculate that the secondary structure of CAX1 is similar to that of CAX3 (Figure S1 of the Supporting Information). However, this assumption does not preclude the possibility that the N-terminal regulatory regions (NRRs) of CAX1 and CAX3 are distinct. For example, CAX3 might be regulated by a longer NRR. To test this hypothesis, we made a series of truncations in the N-terminal tail of CAX3. After the removal of 57 amino acids, some CAX3 truncations (N-terminally truncated variants) when expressed in yeast strains deficient in vacuolar Ca^{2+} transport were able to suppress the Ca^{2+} sensitivity of the yeast mutant cells (Figure 2). Strong growth was observed when yeast mutant cells expressed CAX3 variants that lacked the first 63 or 64 amino acids. Interestingly, yeast mutant cells expressing a CAX3 variant missing the entire NRR and TM1 (deletion of 90 amino acids, $\Delta 90\text{-CAX3}$) demonstrated suppression of the Ca^{2+} sensitivity of the yeast cells (Figure 2). The results of the assay of yeast growth on high- Ca^{2+} medium were similar to the results of the experiment in which the cells were grown in liquid medium containing high Ca^{2+} concentrations (data not shown). However, these assays do not allow us to make precise comparisons of $\text{Ca}^{2+}/\text{H}^+$ antiport activity.

To monitor expression levels in yeast, we measured both CAX3 RNA and protein levels. RT-PCR analysis established differences in CAX expression levels in yeast cells expressing CAX3, sCAX3, $\Delta 63\text{-CAX3}$, $\Delta 64\text{-CAX3}$ and $\Delta 90\text{-CAX3}$ (Figure 3A). Variation in CAX3 RNA abundance among these cells was also confirmed by Northern blot using a CAX3 specific

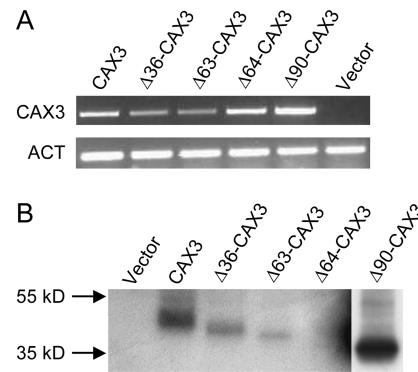


Figure 3. RT-PCR and Western blot showing relative levels of CAX3, sCAX3, $\Delta 63\text{-CAX3}$, $\Delta 64\text{-CAX3}$, and $\Delta 90\text{-CAX3}$. (A) RT-PCR was performed on total RNA extracted from the yeast strains expressing CAX3, sCAX3, $\Delta 63\text{-CAX3}$, $\Delta 64\text{-CAX3}$, and $\Delta 90\text{-CAX3}$ using specific primers against $\Delta 90\text{-CAX3}$. Actin primers were used as a control. (B) Western blot analysis was performed on vacuolar-enriched protein extracted from C-terminally triple-HA-tagged full-length CAX3, sCAX3, $\Delta 63\text{-CAX3}$, $\Delta 64\text{-CAX3}$, or $\Delta 90\text{-CAX3}$. Twenty micrograms of microsomal samples was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, blotted, and subjected to Western blot analysis using a monoclonal antibody reactive to hemagglutinin. The exposure time used for $\Delta 90\text{-CAX3}$ expression is 5-fold faster than that used for the other CAX variants.

probe (Figure S2 of the Supporting Information). Protein levels were determined in strains expressing these CAX3 constructs that contained triple hemagglutinin (HA) epitopes at the C-terminus. These tagged CAX3 constructs did not alter their activity in the yeast expression assays described previously for CAX1.⁹ The protein levels were different among the strains expressing the different constructs (Figure 3B) but did not correlate with activity levels as high-level expression was seen in yeast cells expressing CAX3 (inactive) and $\Delta 90\text{-CAX3}$ (active).

Yeast Cells Expressing CAX3 Variants Accumulate More Ca^{2+} . We sought to confirm that the suppression of Ca^{2+} sensitivity of the yeast mutant cells was due to the sequestration of Ca^{2+} . The total metal content in yeast cells increases upon expression of active CAXs and can be measured by ICP-AES.^{16,17} ICP data may be correlated with the data from yeast tolerance assays and the uptake of $^{45}\text{Ca}^{2+}$ into vacuolar-enriched microsomes.²⁰ When yeast mutant cells were grown in medium supplemented with Ca^{2+} , the expression of CAX3 variants caused the Ca^{2+} content to increase more than 4-fold when compared to yeast cells expressing the vector, CAX3, or sCAX3 (Figure 4). Of note, $\Delta 63\text{-CAX3}$ accumulates more Ca^{2+} than $\Delta 90\text{-CAX3}$ during ICP-AES analysis. In all cases, there were no significant differences among the strains in the content of other cations measured under these growth conditions (data not shown).

The resemblance of CAX3 to CAX1 and the finding that suppression of the *S. cerevisiae* mutant by expression of CAX3 variants imply that CAX3 is a bona fide cation exchanger. In the yeast-based Ca^{2+} suppression assays, $\Delta 90\text{-CAX3}$ -mediated phenotypes appear to be weaker than those mediated by sCAX1 expression (Figure 5A). To test this directly, endomembrane-enriched vesicles were purified from sCAX1- and $\Delta 90\text{-CAX3}$ -transformed K667 yeast cells, and their capacity for pH-dependent Ca^{2+} uptake was examined. Addition of MgCl_2 and ATP and establishment of a steady-state pH by the V-ATPase associated with the vacuolar membrane followed by the addition of $^{45}\text{Ca}^{2+}$

resulted in uptake into endomembrane vesicles from $\Delta 90$ -CAX3-expressing K667 vesicles. The $\Delta 90$ -CAX3-expressing K667 had approximately 50% of the uptake of sCAX1-expressing vesicles, which further suggests that this CAX3 variant has less $\text{Ca}^{2+}/\text{H}^+$ activity than sCAX1 (Figure 5B). In $\Delta 90$ -CAX3-expressing K667 vesicles, the inclusion of gramicidine (an uncoupler of the proton gradient) in the uptake medium decreased the rate of uptake of

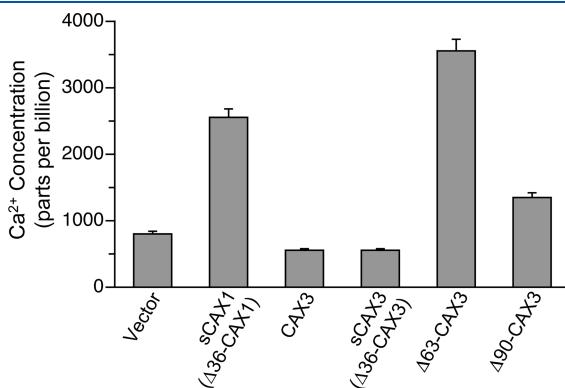


Figure 4. Concentration of Ca^{2+} (in parts per billion) in yeast-expressing vector, sCAX1, or various CAX3 constructs determined by ICP-AES. Values correspond to the mean \pm the standard error of data from three samples.

$^{45}\text{Ca}^{2+}$ to a level similar to that seen in the absence of MgCl_2 and ATP as described previously.⁶ As previously reported, the low rate of uptake found in vesicles from K667 cells transformed with control vector was not inhibited by gramicidin or by the V-ATPase inhibitor, baflomycin, as previously described.⁹ These results, together with the release of $^{45}\text{Ca}^{2+}$ observed with the addition of the Ca^{2+} ionophore A23187 to $\Delta 90$ -CAX3 vesicles, versus the small increase observed in $^{45}\text{Ca}^{2+}$ uptake with vector control vesicles, demonstrate that $\Delta 90$ -CAX3-generated uptake is concentrative (Figure 5B). To further analyze the transport of $\Delta 90$ -CAX3, Michaelis–Menten kinetic analysis was performed. pH-dependent $^{45}\text{Ca}^{2+}$ transport in yeast endomembranes expressing $\Delta 90$ -CAX3 over the range of 0–100 μM Ca^{2+} demonstrated a K_m value for the transporter of $14.01 \pm 2.8 \mu\text{M}$ (Figure 5C).

To analyze the substrate specificity of $\Delta 90$ -CAX3, we performed competition experiments with yeast strains expressing $\Delta 90$ -CAX3. This approach allowed us to compare and contrast sCAX1 and $\Delta 90$ -CAX3 transport; pH-dependent 10 μM $^{45}\text{Ca}^{2+}$ uptake was measured at a single 10 min time point in the absence of excess nonradioactive metal (control with 100% activity) and compared with $^{45}\text{Ca}^{2+}$ uptake determined in the presence of two concentrations (10 \times and 100 \times) of various nonradioactive metals (Figure 5D). Inhibition of uptake of $^{45}\text{Ca}^{2+}$ by nonradioactive Ca^{2+} was used as an internal control, and as expected, uptake of $^{45}\text{Ca}^{2+}$ was strongly inhibited by excess Ca^{2+} ; however,

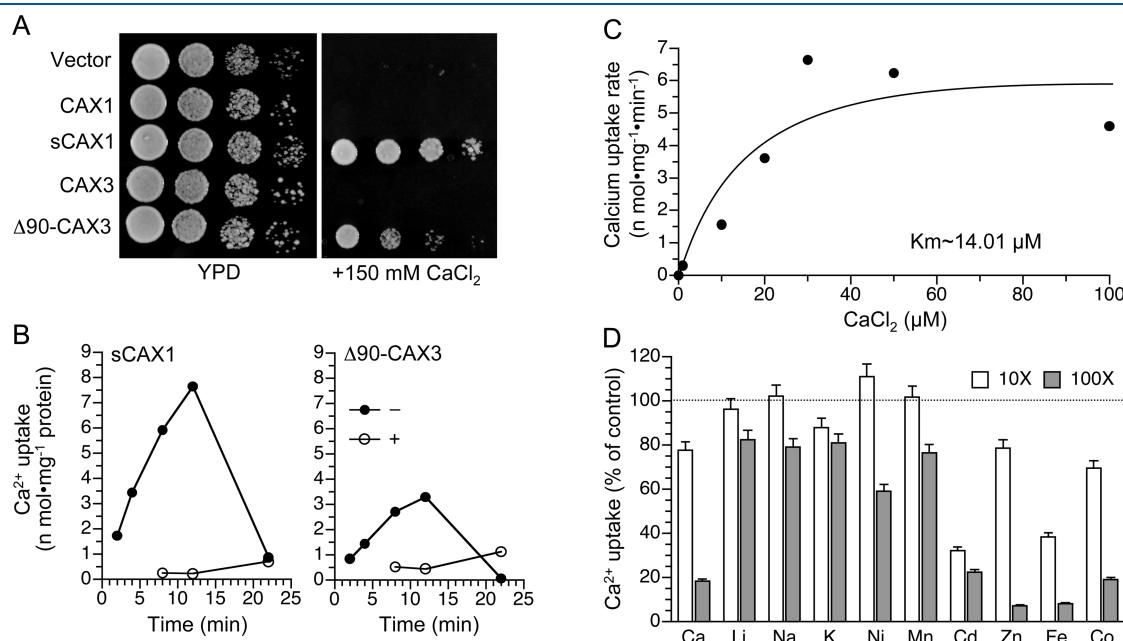


Figure 5. Phenotypes of yeast cells expressing CAX3 transporters. (A) Suppression of Ca^{2+} sensitivity in yeast mutant cells that are defective with respect to vacuolar Ca^{2+} transport. Suppression assays were performed by spotting dilutions of CAX-expressing yeast mutant strains and growing the cells on Ca^{2+} -containing medium. This picture was taken after incubation at 30 °C for 3 days. sCAX1 indicates a 36-amino acid truncation from the N-terminal half of CAX1. Similarly, $\Delta 90$ - indicates amino acid truncations from the N-terminal half of CAX3. (B) Time course of uptake of $^{45}\text{Ca}^{2+}$ into vacuolar vesicles prepared from yeast strain K667 expressing sCAX1 or $\Delta 90$ -CAX3. Results are shown in the absence and presence of the protonophore gramicidin. The Ca^{2+} ionophore, A23187 (5 μM), was added at 12 min, and uptake was measured at 22 min. These data are representative of three independent experiments. (C) Michaelis–Menten kinetic analysis of the initial rate of metal/ H^+ exchange. A preset steady-state pH gradient was generated in vacuolar-enriched vesicles from yeast cells expressing $\Delta 90$ -CAX3 by activation of the V-ATPase. Initial rates of H^+ -dependent Ca^{2+} uptake were calculated over a range of Ca^{2+} concentrations from 0 to 100 μM . The data are representative of three independent experiments. (D) Inhibition of uptake of Ca^{2+} by $\Delta 90$ -CAX3 into yeast vacuolar-enriched vesicles in the presence of other metals. Uncoupler sensitive (ΔpH -dependent) uptake of 10 μM $^{45}\text{Ca}^{2+}$ was assessed in the absence (control with 100% activity shown with a dashed line) or presence of 10 \times or 100 \times nonradioactive CaCl_2 , LiCl , NaCl , KCl , NiSO_4 , MnCl_2 , CdCl_2 , ZnCl_2 , FeCl_3 , or CoCl_2 after 10 min. The data are averages of at least three replications from two independent membrane preparations, and the bars indicate the standard error.



Figure 6. Tobacco seedlings (cv. K14) ectopically expressing (A) CAX3, (B) sCAX3, or (C) Δ 63-CAX3 under the control of the CaMV 35S promoter. These plants are representative of at least eight independent transgenic lines.

the $10\times$ concentration did not completely inhibit $^{45}\text{Ca}^{2+}$ uptake, highlighting the low Ca^{2+} affinity of the CAX transporters. Like that of sCAX1, Δ 90-CAX3 $^{45}\text{Ca}^{2+}$ uptake was strongly inhibited by Cd^{2+} , Fe^{2+} , Co^{2+} , and Zn^{2+} ; however, unlike sCAX1-expressing yeast strains, Δ 90-CAX3 transport showed little inhibition by Mn^{2+} . Furthermore, Δ 90-CAX3 did not exhibit significant inhibition to any monovalent ion tested (Li^+ , Na^+ , and K^+).

Transgenic Tobacco Plants Expressing CAX3 Variants Exhibited Altered Growth. To demonstrate activity in planta, we expressed one of the CAX3 variants that displayed robust Ca^{2+} tolerance in our initial yeast assays (Δ 63) and compared this to CAX3 and sCAX3. If active, we envisioned phenotypes similar to those of sCAX1 expression, including stress sensitivities and Ca^{2+} deficiency-like symptoms.¹⁹ We generated at least eight independent lines of tobacco expressing the empty vector, CAX3, sCAX3, and Δ 63-CAX3, and DNA integration and gene expression were confirmed (Figure S3 of the Supporting Information). Ninety-two percent (34 of 37 plants) of the plants expressing empty vector, CAX3, or sCAX3 grew normally. However, all plants ($n = 8$) expressing Δ 63-CAX3 displayed stunted growth as seedlings (Figure 6), and none of these lines survived to produce flowers and seeds.

■ DISCUSSION

Regulatory elements must often be removed before protein activities can be measured.¹ In this report, we have made a series of N-terminal CAX3 deletions beyond the N-terminal \sim 40-amino acid NRR. Yeast strains expressing CAX3 variants lacking at least the first 57 amino acids displayed some apparent Ca^{2+} transport (Figure 2). Of note, deletions beyond the first 57 amino acids appeared to have increased CAX activity based on these yeast growth assays. An interesting result from this study was that TM1 of CAX3 was not required for Ca^{2+} transport (Figure 1). TM1 of CAXs has no apparent homology with other related transporters, such as NCX ($\text{Na}^+/\text{Ca}^{2+}$ antiporters) and NCKX (K^+ -dependent $\text{Na}^+/\text{Ca}^{2+}$ antiporters).²¹ TM1 is not part of the symmetrical arrangement of these transporters as symmetry is found between the TM2–TM6 region and the TM7–TM11 region (Figure 1). Recently, it was hypothesized that antiporters evolved through a duplication of half-sized progenitors that act as a dimer arranged in an antiparallel

topology.²² If CAXs have evolved in a similar manner, the TM2–TM11 core module performs the transport and TM1 may function as a regulatory module. Schaaf and colleagues²³ reported a CAX2 variant that lacks the first TM can still transport both Ca^{2+} and Mn^{2+} . In the future, it will be interesting to test the transport of other CAXs when TM1 has been removed.

The apparent mechanism of N-terminal regulation differs between CAX3 and CAX1. In yeast assays, small deletions in the CAX1 regulatory region abolish autoinhibition. In fact, deletions of as few as 10 amino acids in the N-terminus activate transport,⁹ whereas autoinhibition was maintained in CAX3 when the first 56 amino acids were removed. Proteins that can bind to the CAX1 NRR and activate CAX transport do not activate CAX2 or CAX4.^{24–26} The difference in the sequences and lengths of NRR may account for these differences in regulation. While CAX1 is predominately expressed in leaves, CAX3 is expressed mostly in roots.⁴ This work suggests that CAX3 may have its own unique activating proteins that specifically bind to the N-terminal regulatory region. *Arabidopsis* CAX1 and CAX3 overlap in their expression during particular stress conditions, in reproductive organs, and during early stages of development.^{4,27} The activity of CAX1 and CAX3 may be due to the presence or absence of N-terminal regulatory region-interacting proteins. It will be interesting to find CAX3 specific activating proteins and compare and contrast their expression with that of CAX1-interacting proteins.

Previously, we have shown that changing single or multiple amino acids of sCAX3 can confer some Ca^{2+} transport (less than 30% of that of CAX1).²⁸ Here we demonstrate that extensive deletions in the N-terminal regulatory region of CAX3 also appear to confer Ca^{2+} transport. In yeast competition studies, $^{45}\text{Ca}^{2+}$ transport mediated by Δ 90-CAX3 was inhibited by $100\times$ concentrations of Cd^{2+} , Zn^{2+} , Fe^{2+} , and Co^{2+} (Figure 5D). In fact, some studies in transgenic plants demonstrate CAXs can transport Cd^{2+} .^{29,30} The inability of monovalent cations to inhibit Ca^{2+} transport suggests that Δ 90-CAX3 does not transport these metals. Previous work in planta suggests CAX3 may transport Na^+ or Li^+ , but these results of yeast expression assays using the deregulated transporter do not recapitulate CAX3 activity in planta. Alternatively, the sensitivity of the *cax3* mutant plants to salt stress and acidic pH may be caused by its indirect effects on P-ATPase or V-ATPase activity.³¹

The role of transporters across the tonoplast in maintaining Ca^{2+} homeostasis depends on their kinetic properties. CAX1 is a high-capacity, low-affinity transporter with a K_m value between 10 and $15\ \mu\text{M}$.³² Our data here suggest that Δ 90-CAX3 has a K_m value of $\sim 14.01\ \mu\text{M}$. As mentioned previously, a lingering question from our studies is how well this variant of CAX3 represents activated CAX3 transport in planta.

While Δ 63-CAX3 expression in yeast phenocopies aspects of sCAX1 expression, the phenotypes in planta suggest differences among the activated transporters. Ectopic expression of Δ 63-CAX3 in planta caused severe tip burning and stunting like sCAX1-expressing lines; however, these Δ 63-CAX3-expressing lines exhibited more dramatic alterations in growth and failed to make viable seeds (Figure 6). These phenotypes may cause altered compartmentalization of several different nutrients.⁵ To study CAX3 function in planta, other activated forms of CAX3 must be utilized. A series of CAX3 N-terminal truncations identified several clones that confer milder phenotypes in yeast (Figure 2). For example, Δ 57-CAX3-expressing cells exhibited some growth on Ca^{2+} -containing medium, and expression of this

variant *in planta* may cause less severe growth defects. This milder activated variant of CAX3 may aid in the future characterization of CAX3 *in planta*.

Our data here confirm that CAX1 and CAX3 have some overlaying functions particularly with regard to Ca^{2+} transport (Figure 5). Analysis of loss-of-function mutants demonstrates that *cax1/cax3*, which lacks expression of both *AtCAX1* and *AtCAX3* (ectopically expressed in mesophyll cells upon abolishment of *AtCAX1*), has reduced mesophyll Ca^{2+} levels.⁵ A reduced capacity for mesophyll Ca^{2+} accumulation results in reduced cell wall extensibility, stomatal aperture, gas exchange, and leaf growth. This suggests both CAX1 and CAX3 act as key regulators of apoplastic Ca^{2+} , a function essential for optimal plant function and productivity.⁵

Our findings here support the concept that the various CAX transporters also have different transport and regulatory properties that can be engineered to alter plant nutrient acquisition.³² The gradient of yeast suppression ability demonstrated by the series of deletions of CAX3 in our study suggests a fine-tuning of transport properties is possible by protein engineering. For example, sCAX1 has been used for boosting Ca^{2+} content in crop plants.³³ However, excessive sequestration of Ca^{2+} may produce undesirable phenotypes for agronomic applications,³⁴ and attenuated CAX1 activity may be important for tolerance to serpentine soil (soils with a low $\text{Ca}^{2+}:\text{Mg}^{2+}$ ratio).³⁵ Therefore, modulated transport is necessary under specific environmental conditions. Here we have demonstrated that N-terminal truncation of CAX3 generates both weak and strong Ca^{2+} transport variants. Additionally, our expression data suggest that N-terminal truncations of CAX3 may also have a role in protein expression or stability (Figure 3).

Removal of regulatory elements reveals the kinetic properties that are masked in the unmodified protein, and our approach here should prove useful for other types of transporters. Via removal of all the hydrophilic regions from heavy metal transporters, it may be possible to enhance the transport and/or create new substrate specificities. For example, the removal of a histidine-rich loop from *Arabidopsis* $\text{Zn}^{2+}/\text{H}^+$ exchanger AtMTP1 stimulates transport.³⁶ Alternatively, if one is looking to temper activity, a transporter may be modulated by inserting an appropriate loop between two TMs.

In summary, our study demonstrated that CAX1 and CAX3 have both shared and unique features. Of particular note is the distinct autoinhibitory domain of CAX3 suggesting the transporters are differentially regulated *in planta*.

■ ASSOCIATED CONTENT

S Supporting Information. Alignment of the predicted N-terminal regulatory region (NRR) of *AtCAX1* and *AtCAX3* (Figure S1), expression of different truncations of CAX3 in yeast (Figure S2), and genomic DNA integration and expression of different truncations of CAX3 in tobacco (Figure S3). This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Children's Nutrition Research Center, 1100 Bates St., Room 9016, Houston, TX 77030. Phone: (713) 798-7039. Fax: (713) 798-7171. E-mail: kendalh@bcm.edu.

Author Contributions

M.M. and T.S. contributed equally to this work.

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■ ABBREVIATIONS

CAX, cation exchanger; ICP-AES, inductively coupled plasma atomic emission spectroscopy; TMHMM, transmembrane hidden Markov models; NRR, N-terminal regulatory region; CaMV, cauliflower mosaic virus.

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