Manganese Specificity Determinants in the Arabidopsis Metal/H\(^{+}\) Antiporter CAX2*

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In plants and fungi, vacuolar transporters help remove potentially toxic cations from the cytosol. Metal/H\(^{+}\) antiporters are involved in metal sequestration into the vacuole. However, the specific transport properties and the ability to manipulate these transporters to alter substrate specificity are poorly understood. The Arabidopsis thaliana cation exchangers, CAX1 and CAX2, can both transport Ca\(^{2+}\) into the vacuole. There are 11 CAX-like transporters in Arabidopsis; however, CAX2 was the only characterized CAX transporter capable of vacuolar Mn\(^{2+}\) transport when expressed in yeast. To determine the domains within CAX2 that mediate Mn\(^{2+}\) specificity, six CAX2 mutants were constructed that contained different regions of the CAX1 transporter. One class displayed no alterations in Mn\(^{2+}\) or Ca\(^{2+}\) transport, the second class showed a reduction in Ca\(^{2+}\) transport and no measurable Mn\(^{2+}\) transport, and the third class, which contained a 10-amino acid domain from CAX1 (CAX2-C), showed no reduction in Ca\(^{2+}\) transport and a complete loss of Mn\(^{2+}\) transport. The subdomain analysis of CAX2-C identified a 3-amino acid region that is responsible for Mn\(^{2+}\) specificity of CAX2. This study provides evidence for the feasibility of altering substrate specificity in a metal/H\(^{+}\) antiporter, an important family of transporters found in a variety of organisms.

The differential partitioning of cations is crucial for life processes, and transporters play a critical role in maintaining the proper concentrations of these ions in various cellular compartments (1). The inability of plants to actively avoid toxic concentrations of particular cations in the environment places a particular importance on cation transporters. One mechanism employed by plants and fungi to avoid cation toxicity is the sequestration of cations into large vacuoles (2). A fundamental question that arises is whether there are separate vacuolar transporters for each cation, and if not, what is the metal specificity of a given transporter?

Several types of active transport mechanisms exist in plants to drive cations out of the cytosol against a steep concentration gradient (3, 4). One important class of transporters is the H\(^{+}\)-coupled cation antiporters, which have been identified at the vacuolar (tonoplast) membrane and are driven by a proton electrochemical gradient (5–8). These transporters have numerous functions including resetting cytosolic levels of Ca\(^{2+}\) post signaling, vacuolar sequestration of potentially toxic concentrations of Cd\(^{2+}\), Zn\(^{2+}\), Mn\(^{2+}\) and other metals, and vacuolar storage of essential micronutrients such as Zn\(^{2+}\), Mg\(^{2+}\), and Mn\(^{2+}\). Despite numerous descriptive reports in whole plants (6, 9, 10) and the recent cloning of several of these transporters (11–13), there is a dearth of information available regarding H\(^{+}\)-coupled ion selectivity, and much less for the residues that define specific cation transport (14).

Two Arabidopsis cation exchanger (CAX)\(^{1}\) genes, CAX1 and CAX2, were identified that could suppress mutants of Saccharomyces cerevisiae defective in vacuolar Ca\(^{2+}\) transport (11). Experiments using vacuolar membranes from yeast cells expressing CAX1 (11) demonstrate that this protein has biochemical properties similar to those of native plant vacuolar H\(^{+}\)/Ca\(^{2+}\) exchangers (5, 15). In similar experiments in yeast, CAX2 appears to have a higher \(K_{m}\) for Ca\(^{2+}\) than CAX1 and a lower capacity for Ca\(^{2+}\) transport (11). CAX2 localizes to the plant vacuole, and when expressed at high levels in transgenic plants, increases vacuolar metal transport and causes the plants to accumulate more Ca\(^{2+}\), Mn\(^{2+}\) and Cd\(^{2+}\) (16). Furthermore these transgenic plants were more tolerant to Mn\(^{2+}\) in the growth media. These transport properties of CAX2 suggest the potential for broad substrate specificity among the 11 CAX-like transporters found in the Arabidopsis genome (17).

Two domains have been described that modulate CAX1 activity (14, 18, 19). The first domain has been termed the Ca\(^{2+}\)-domain (CaD), located between amino acids 87 and 95 in CAX1 (14). This domain appears to be necessary for Ca\(^{2+}\) transport by CAX1. Exchanging this 9-amino acid region of CAX1 into CAX2 (giving the construct CAX2–9) greatly increases its Ca\(^{2+}\) transport activity but does not appear to alter transport of other metals (14). The second domain that regulates CAX function has been termed the regulatory or autoinhibitory domain (18, 19). Sequence analysis suggests that an N-terminal regulatory domain may be present in all plant CAX-like transporters (20). The CAX1 and CAX2 open reading frames contain additional amino acids at the N terminus that were not found in the original “shorter” N-terminally truncated CAX (sCAX) clones (sCAX1 and sCAX2) identified by suppression of yeast

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1 The abbreviations used are: CAX, cation exchanger; CaD, Ca\(^{2+}\)-domain; CDF, cation diffusion facilitator; MES, 4-morpholineethanesulfonic acid; sCAX, N-terminally truncated CAX; YPD, yeast extract-peptone-dextrose medium; TMD, transmembrane domain.

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vacular Ca\(^{2+}\) transport mutants (20, 21). These findings suggest structural features involved in regulation and Ca\(^{2+}\) transport but do not identify domains that may confer metal specificity among these CAX transporters. The manipulation of CAX transporters, through alteration in expression and substrate specificity, is an essential component in developing plants with increased tolerance to metals or removing toxic levels of metals from soil (phytoremediation) (22). For a successful phytoremediation strategy, it is important to understand what determines the specificity of broad range metal transporters such as CAX2.

In this report, we have characterized the transport properties of the CAX2 transporter. We identify specific domains within CAX2 that mediate Mn\(^{2+}\) substrate specificity and alter these domains to abolish the Mn\(^{2+}\) transport capabilities of CAX2, thus increasing its metal specificity. These findings serve as a framework for engineering metal specificity among the various H\(^+/\)cation exchangers found in bacteria, fungi, and plants.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Vectors, and DNA Manipulations**—K667 (cnb1::LEU2 pmc1::TRP1 vcx1A (23)) was the starting strain used to express wild type and mutant genes. The wild type or mutant genes were propagated in pBluescript II SK (+) (Strategene, La Jolla, CA), and inserts were transferred to the shuttle vector pHGpd (24) for the expression in the yeast. The plasmids were introduced into yeast by the lithium acetate/single-stranded DNA/polycethylene glycol transformation method (25). Standard techniques were used to manipulate the DNA used in this study (26). All of the CAX1 and CAX2 clones used in this study were identical to the N-terminally truncated clones originally identified (11). Thus the proteins encoded by sCAX1 and sCAX2 lacked the first 36 and 42 amino acids, respectively.

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed by the Class III restriction enzyme-mediated method (27), using sCAX2 in pBluescript as the template DNA. BsmBI was the Class III restriction enzyme used throughout this study. CAX2-9 was constructed previously (14). The primers used were as follows: for CAX2-A, 5'-GAA TTC CCT CTC GTA ATC ACC AAC AAG TCA TCG ATG CA-3' (forward); for CAX2-B, 5'-GAA TTC CCT CTC CTT TGA CAA ACA AAG TGG CAG TCC AAT ATT CG-3' (reverse); for CAX2-C, 5'-GAA TTC CCT CTC CTT TGA CAA ACA AAG TGG CAG TCC AAT ATT CG-3' (reverse); for CAX2-D, 5'-GAA TTC CCT CTC CTT TGA CAA ACA AAG TGG CAG TCC AAT ATT CG-3' (reverse); for CAX2-E, 5'-GAA TTC CCT CTC CTT TGA CAA ACA AAG TGG CAG TCC AAT ATT CG-3' (reverse). The bold letters in the primers represent the BamHI restriction enzyme site at the 3'-end of the CAX2 gene (26).

**Construction of CAX1 Utilizing chimeric Transcripts**—To create the chimeric transcript used to express wild type and mutant genes, pmc1 was introduced into the yeast by the lithium acetate/single-stranded DNA/polycethylene glycol transformation method (25). Standard techniques were used to manipulate the DNA used in this study (26). All of the CAX1 and CAX2 clones used in this study were identical to the N-terminally truncated clones originally identified (11). Thus the proteins encoded by sCAX1 and sCAX2 lacked the first 36 and 42 amino acids, respectively.

**Preparation of Membrane Vesicles for Ca\(^{2+}\) Transport Assays**—Yeast membrane microsomes were prepared as described previously (28).

**Measurement of \(^{45}\)Ca\(^{2+}\) and \(^{54}\)Mn\(^{2+}\) Uptake**—For the measurement of \(^{45}\)Ca\(^{2+}\) and \(^{54}\)Mn\(^{2+}\) uptake, membrane vesicles (yeast spheroplasts) were incubated in buffer containing 0.3 m sorbitol, 5 mM Tris-MES (pH 7.6), 25 mM KCl, 0.1 mM sodium azide, and 0.2 mM sodium orthovanadate. Vacuolar H\(^{+}\)-translocating ATPase-catalyzed H\(^+\) translocation was initiated by the addition of 1 mM MgSO\(_4\) and 1 mM ATP. The vesicles were allowed to reach steady state with respect to pH gradient for 5 min at 25 °C before the addition of \(^{45}\)Ca\(^{2+}\) (5 mCi/ml; American Radiolabeled Chemicals, St. Louis, MO) or \(^{54}\)Mn\(^{2+}\) (6.5 mCi/ml; PerkinElmer Life Sciences). The final concentration of Ca\(^{2+}\) and Mn\(^{2+}\) in the reaction mixture was 10 μM and 1 mM, respectively. At the indicated times, aliquots (70 μl) of the reaction mix were removed and filtered through premoistened 0.45-μm pore size cellulose acetate GS-type filters (Milipore, Bedford, MA) after a 1-ml wash with ice-cold wash buffer (0.5 mM sorbitol, 5 mM Tris-MES, pH 7.6, 25 mM KCl, and 0.1 mM CaCl\(_2\) or MnCl\(_2\) as appropriate), the filters were air-dried, and radioactivity was determined by liquid scintillation counting. For metal competition experiments, ΔPf-dependent 10 μM \(^{45}\)Ca\(^{2+}\) uptake was measured at a 10-min time point in the presence of 100 μM or 1 mM nonradioactive metals.

**RESULTS**

**CAX2, but not CAX1, CAX3, or CAX4, Suppresses the Mn\(^{2+}\) Sensitivity of a Yeast Mutant**—Yeast strains lacking functional calcineurin (for example, cnb strains) display increased Mn\(^{2+}\) sensitivity due, in part, to decreased activity of the Golgi Ca\(^{2+}\)/Mn\(^{2+}\)-ATPase (29, 30). This indicates that VCX1 may transport Mn\(^{2+}\) in addition to Ca\(^{2+}\). Yeast mutants deleted in VCX1 and the vacuolar Ca\(^{2+}\)-Mn\(^{2+}\)-ATPase PMC1 are sensitive to high concentrations of Ca\(^{2+}\) in the media (23). Expression of VCX1 and N-terminally truncated CAX2 can suppress both the Mn\(^{2+}\) and Ca\(^{2+}\) sensitivity of a yeast mutant (K667, pmc1 vcx1 cnb1) defective in calcineurin and the vacuolar Ca\(^{2+}\) transporters (16). As shown previously, N-terminal truncations of CAX1 can strongly suppress the Ca\(^{2+}\) sensitivity of these strains, whereas N-terminal truncations of CAX4 only weakly suppress the Ca\(^{2+}\) phenotype, and N-terminal truncations of CAX3 are unable to suppress the Ca\(^{2+}\) phenotype (11, 31, 32). We were interested in testing whether these characterized CAX transporters could suppress the Mn\(^{2+}\) sensitivity of this yeast strain. As shown in Fig. 1, these CAX transporters cannot suppress the Mn\(^{2+}\) sensitivity of this yeast strain.
porter characterized to date with the ability to suppress the Mn\(^{2+}\) sensitivity of calcineurin-deficient yeast mutants (Fig. 1), a similar series of chimeric CAX constructs utilizing CAX2 should delineate the Mn\(^{2+}\) specificity domain(s) of this transporter. We chose to make chimeric constructs between CAX1 and CAX2 because each construct should maintain the ability to transport Ca\(^{2+}\). Thus, the fidelity of each construct could be rapidly assessed through suppression of Ca\(^{2+}\) sensitivity, and we could then test for alterations in Mn\(^{2+}\) suppression. Initially we planned a systematic approach to identify CAX2 specificity domains, similar to that used to identify the CaD of CAX1 (14). We chose to divide CAX1 and CAX2 into four segments of approximately equal size and exchange each segment to create eight different chimeric constructs (33). Although the construction of the chimeric clones was successful, Ca\(^{2+}\) antiport activity was abolished for some of the constructs, indicating that protein stability was affected (data not shown). Therefore, an alternative approach was used.

At the amino acid level, CAX2 is 43% identical (56% similar) to the CAX1 open reading frame. A sequence comparison among CAX1, CAX2, CAX3, and CAX4 identified five short domains, each consisting of 9–15 amino acids, which had very low sequence similarity between CAX1 and CAX2 (Fig. 2). We have designated these domains A to E. The A domain (amino acids 65–73 of CAX1) is present at the start of TMD 1, the B domain (amino acids 150–160 of CAX1) is present between TMD 3 and 4, the C domain (amino acids 175–184 of CAX1) is present in TMD 4, the D domain (amino acids 219–233 of CAX1) is present between TMD 5 and 6, and the E domain (amino acids 257–265 of CAX1) is present between TMD 6 and the acidic motif. There is very little variation among CAX1, CAX3, CAX4, and the mung bean VCA1 in domains A, B, C, and E, whereas domain D and the region corresponding to the CAX1 9-amino acid CaD is very divergent among all CAX sequences (Fig. 2). We created five mutants in CAX2 that contain the corresponding CAX1 A, B, C, D, or E domain. These mutants were designated CAX2-A, CAX2-B, CAX2-C, CAX2-D, and CAX2-E, respectively. For these studies we have also used the previously constructed CAX2-9 construct, which contains the CAX1 9-amino acid CaD (14). All of the mutants were N-terminally truncated, i.e. constructed without the first 42 amino acids of CAX2. Because we had previously found that some CAX1/CAX2 chimeric clones had altered protein stability, it was very important to verify that each construct was expressed at approximately equal levels in yeast, and so we tagged each chimeric construct with a C-terminal c-Myc epitope and analyzed protein expression (Fig. 3). Each chimera was expressed similarly to CAX1 and CAX2.

**Altered Ca\(^{2+}\)- and Mn\(^{2+}\)-sensitive Yeast Growth by CAX2 Mutants**—As we anticipated, each of these constructs could suppress the Ca\(^{2+}\) sensitivity of the yeast mutants relatively equally when grown on 200 mM CaCl\(_2\) (Fig. 4). However, when the yeast strains expressing these constructs were grown on higher levels of Ca\(^{2+}\) (250 mM CaCl\(_2\)), we observed growth differences. Although the CAX2-C-, CAX2-D-, CAX2-E-, and CAX2-9-expressing strains grew in a manner similar to CAX2 strains, the CAX2-A- and CAX2-B-expressing strains were significantly reduced in their growth under these media conditions (Fig. 4). In media containing 10 mM MnCl\(_2\), yeast strains harboring the CAX2, CAX2–9, CAX2-D, and CAX2-E constructs all grew (Fig. 5). Like strains expressing CAX1, those expressing CAX2-A, CAX2-B, and CAX2-C completely failed to suppress Mn\(^{2+}\) sensitivity. Even at lower MnCl\(_2\) concentrations (5 mM), the CAX2-A, CAX2-B, and CAX2-C strains were unable to grow (Fig. 5).

**Ca\(^{2+}\) and Mn\(^{2+}\) Transport**—To examine the Ca\(^{2+}\) transport properties of these three Mn\(^{2+}\)-negative mutants directly, \(\Delta\text{pH}\)-dependent 10 \(\mu\text{M Ca}^{2+}\) uptake into yeast membrane vesicles was measured (Fig. 6A). Ca\(^{2+}\) antiport activity mediated by CAX2-A and CAX2-B was significantly lower than for CAX2 (57.4 and 33.4% of CAX2 activity, respectively), whereas Ca\(^{2+}\) antiport activity mediated by CAX2-C was not significantly different from CAX2. The ability to suppress the Mn\(^{2+}\) sensitivity of the yeast mutants infers Mn\(^{2+}\) transport capability. However, it is important to demonstrate directly that these CAX2 constructs mediate Mn\(^{2+}/H^+\) antiport activity in yeast. Therefore, we measured \(\Delta\text{pH}\)-dependent 1 mM \(54\text{MnCl}_2\) uptake into microsomal vesicles isolated from K667 yeast strains expressing various CAX transporters. Mn\(^{2+}/H^+\) transport activity was observed in membrane vesicles from CAX2- and CAX2-E-expressing strains (Fig. 6B), but no Mn\(^{2+}/H^+\) antiport activity was detectable in vesicles from CAX1- and CAX2-C-expressing yeast (data not shown). The Mn\(^{2+}/H^+\) antiport activity measured from CAX2-E vesicles was modestly greater than the activity of CAX2 (Fig. 6B), confirming the slight increase in yeast growth of CAX2-E strains compared with CAX2 strains on high Mn\(^{2+}\) media (Fig. 5).

**Analysis of the CAX2 C-Domain**—The CAX2-C-expressing strains have a specific defect in Mn\(^{2+}\) tolerance, as the growth of these strains is largely indistinguishable from CAX2- on Ca\(^{2+}\)-containing media (Fig. 4) despite the lack of growth on Mn\(^{2+}\)-containing media (Fig. 5). The inability of the CAX2-A and CAX2-B-expressing strains to suppress Mn\(^{2+}\) sensitivity also implicates these domains as being involved in Mn\(^{2+}\) transport (Fig. 5). However, CAX2-A and CAX2-B-expressing strains also exhibited diminished growth on Ca\(^{2+}\)-containing media and decreased Ca\(^{2+}/H^+\) antiport activity (Figs. 4 and 6A), suggesting a nonspecific reduction in transport capabilities. To examine which amino acids were involved in determining the Mn\(^{2+}\) specificity of CAX2-C, we divided the CAX2 C domain into two regions, each containing 3 different amino acids than those present in the CAX1 C domain. The CAX2-C chimera contains the 3 amino acids TSL from CAX1, replacing the amino acids CAF of CAX2, and the CAX2-C2 construct contains the 3 amino acids IAN from CAX1, replacing the amino acids LVF of CAX2 (Fig. 2). Yeast strains expressing the CAX2-C2 construct were indistinguishable from CAX2, as these strains could suppress both the Ca\(^{2+}\) and Mn\(^{2+}\) sensitivity of K667 yeast (Fig. 7A). Yeast strains expressing CAX2-C1 were able to strongly suppress the Ca\(^{2+}\) sensitivity phenotype, but there was no growth of these strains on Mn\(^{2+}\)-containing media.
When the transport properties of CAX2-C1 were determined by direct pH-dependent \(^{45}\)Ca\(^{2+}\) and \(^{54}\)Mn\(^{2+}\) uptake measurements into membrane vesicles prepared from CAX2-C1-expressing yeast, CAX2-C1 was found to have significant Ca\(^{2+}\) antiport activity, which was indistinguishable from that of CAX2, but no Mn\(^{2+}\) antiport activity (Fig. 7B).

**Cation Selectivity Comparisons of CAX2 Mutants**—To further analyze the altered transport properties of the CAX2 mutants, competition experiments were performed. This approach allowed us to determine the effect of the domain swapping between CAX1 and CAX2 on cation selectivity. \(\Delta pH\)-dependent 10 \(\mu\)M \(^{45}\)Ca\(^{2+}\) uptake was measured at a single 10-min time point into yeast microsomal vesicles isolated from strains expressing CAX1, CAX2, and the six CAX2 mutants. Ca\(^{2+}\) uptake determined in the absence of excess nonradioactive metal (control) was compared with Ca\(^{2+}\) uptake determined in the presence of two concentrations (10\(\times\) and 100\(\times\)) of various nonradioactive metals (Fig. 8). Inhibition of Ca\(^{2+}\) uptake by nonradioactive Ca\(^{2+}\) was used as an internal control, and as expected, Ca\(^{2+}\) uptake by each CAX transporter was strongly inhibited by excess Ca\(^{2+}\), particularly at the higher Ca\(^{2+}\) concentrations, did not completely inhibit Ca\(^{2+}\) uptake, further highlighting the low Ca\(^{2+}\) affinity of the CAX transporters. We have previously demonstrated that tobacco plants ectopically expressing CAX2 have significantly increased vacuolar transport of Cd\(^{2+}\) (16). Ca\(^{2+}\) uptake by CAX1 and CAX2 were both strongly inhibited by Cd\(^{2+}\), and this Cd\(^{2+}\) inhibition was consistently observed in all of the six CAX2 mutants. However, this inhibition was only significant for all CAX constructs at the higher Cd\(^{2+}\) concentration. In agreement with the yeast suppression and \(^{54}\)Mn\(^{2+}\) antiport data, excess nonradioactive Mn\(^{2+}\) inhibited Ca\(^{2+}\) transport by CAX2 but not by CAX1. Of the CAX2 mutants, Mn\(^{2+}\) inhibition was only observed for CAX2-D, CAX2-E, and CAX2–9, but not for CAX2-A, CAX2-B and CAX2-C, confirming the yeast suppression data. Two other divalent cations, Zn\(^{2+}\) and Ni\(^{2+}\), were also tested. No significant Ca\(^{2+}\) uptake inhibition by Zn\(^{2+}\) was
observed for CAX1, whereas for CAX2, the degree of Ca\(^{2+}\) uptake inhibition by Zn\(^{2+}\) was similar to that observed for Mn\(^{2+}\). These results indicate that CAX2 may also be able to transport Zn\(^{2+}\). To test this theory further, CAX2 was expressed in a highly Zn\(^{2+}\)-sensitive yeast double mutant (zrc1 cot1) lacking the vacuolar cation diffusion facilitator (CDF) transporters ZRC1 and COT1 (34). The results were inconsistent, and it was concluded that CAX2 was unable to significantly suppress the Zn\(^{2+}\) sensitivity of the double mutant (data not shown). The highest concentration of excess Zn\(^{2+}\) was able to inhibit Ca\(^{2+}\) uptake to varying degrees by every CAX2 mutant except CAX2-C. However, except for CAX2-9, no inhibition of Ca\(^{2+}\) uptake was seen by the lower Zn\(^{2+}\) concentration for any of the CAX2 mutants. Ni\(^{2+}\) inhibited Ca\(^{2+}\) uptake by CAX1 only when added at the higher concentration, but no significant inhibition by Ni\(^{2+}\) was observed for CAX2 or any CAX2 mutant.

**DISCUSSION**

Previously, CAX2 has been shown to suppress yeast mutants sensitive to high Mn\(^{2+}\) concentrations, localize to the plant vacuolar membrane, and increase Ca\(^{2+}\), Cd\(^{2+}\), and Mn\(^{2+}\) accumulation and vacuolar transport when expressed ectopically in tobacco (16). In this study we confirm by direct transport measurements that CAX2 is a Mn\(^{2+}/H^+\) antiporter. Although we have not determined the \(K_m\) for Mn\(^{2+}\) of CAX2, experimental observations indicate that it has a low affinity for Mn\(^{2+}\). CAX2 Ca\(^{2+}\) transport activity could be measured using 10 \(\mu M\) \(^{45}\)CaCl\(_2\). However, to measure Mn\(^{2+}\) transport activity by CAX2, 1 \(mM\) \(^{54}\)MnCl\(_2\) had to be used (Fig. 6), as activity was very weak when measured using 100 \(\mu M\) \(^{54}\)MnCl\(_2\) and could not be detected using 10 \(\mu M\) \(^{54}\)MnCl\(_2\). Furthermore, a 100-fold excess of nonradioactive CaCl\(_2\) was able to inhibit 50% of 10 \(\mu M\) \(^{45}\)Ca\(^{2+}\) transport activity mediated by CAX2, whereas only a 100-fold excess of nonradioactive MnCl\(_2\) was able to inhibit 50% of activity (Fig. 8). Similarly, measurements of vacuolar cation/H\(^+\) antiporter activity from oat root indicate that H\(^+\)-dependent transport of Mn\(^{2+}\) is less efficient than that of Ca\(^{2+}\), Cd\(^{2+}\), or Zn\(^{2+}\) (9). However, CAX2-expressing plants were slightly more tolerant to Mn\(^{2+}\) in the growth media (16), demonstrating that despite being a low affinity antiporter, CAX2 can be an important component in providing Mn\(^{2+}\) tolerance. Here we demonstrate that CAX1, CAX3, and CAX4 cannot suppress the Mn\(^{2+}\) sensitivity of a yeast mutant (K667; pmc1 vcx1 cnb1), which suggests that ectopic expression of these transporters in plants will cause altered accumulation, transport, and possibly
H+ transport. We have identified two conserved Asp residues and a Glu residue within a transmembrane span that are essential for metal resistance and antiport activity, but no alteration in metal specificity was observed (39). Recently it has been described that the C-terminal tail of a cyanobacterial Na+/H+ antiporter has a role in determining ion specificity (40). Exchange of the C-terminal tail between similar Na+/H+ antiporters from *Aphanthece halophytica* and *Synechocystis* sp. PCC 6803 greatly affected ion specificity, particularly with respect to Li+/H+ antiport activity (40). However, this may have little significance to CAX2, as these antiporters have very little sequence similarity with the CAX-like antiporters and are predominantly alkali metal transporters rather than divalent heavy metal transporters.

A 9-amino acid domain located between putative membrane spanning domains 1 and 2 was identified recently as an important domain in mediating CAX1 specificity toward Ca2+. Inserting this CaD of CAX1 into CAX2 (CAX2–9) increases Ca2+ transport of this chimeric construct but does not appear to alter substrate specificity globally (14). As we demonstrate here, K667 yeast strains expressing CAX2–9 maintain CAX2-like suppression of the Mn2+ sensitivity phenotype, further implicating another domain in the mediation of CAX2 Mn2+ specificity.

Utilizing CAX2 chimeric constructs in a robust yeast assay for Mn2+ and Ca2+ tolerance, we identified a domain that appears to be involved specifically in Mn2+ transport. We have termed this domain, located in TMD 4 at amino acids 177–186 of CAX2, the C-domain (Fig. 2). When the CAX1 C-domain was
inserted into CAX2 (CAX2-C) and expressed in yeast, the strains strongly suppressed the yeast Ca\(^{2+}\) sensitivity in a manner similar to CAX2 (Fig. 4); however, these strains failed to suppress the Mn\(^{2+}\) sensitivity (CAX1-like phenotype). All of the yeast growth differences on both Ca\(^{2+}\) and Mn\(^{2+}\) media were confirmed in direct measurements of \(^{45}\)Ca\(^{2+}\) and \(^{55}\)Mn\(^{2+}\) transport and ion competition studies (Figs. 6, 7B, and 8). CAIX2 transported \(^{45}\)Mn\(^{2+}\), whereas CAX2-C and CAX1 did not. Furthermore, excess nonradioactive Mn\(^{2+}\) inhibited CAX2-mediated \(^{45}\)Ca\(^{2+}\) transport but did not inhibit CAIX1 or CAX2-C \(^{45}\)Ca\(^{2+}\) transport (Fig. 8). Strains expressing the CAX2-C1 mutant, which contains only the CAF to TSL change, had the same phenotype as the CAX2-C mutant. Moreover, direct \(^{45}\)Mn\(^{2+}\) transport measurements confirmed that the lack of growth on Mn\(^{2+}\)-containing media of yeast expressing CAX2-C1 was due to the loss of Mn\(^{2+}/H^+\) antiport activity by CAX2, resulting from the CAF to TSL change. Of all the domains studied in these experiments (domains A to E and the 9-amino acid domain), only the C domain is located within a TM domain. Because of this transmembrane localization, we tentatively suggest that these CAF residues within the CAX2 TMD4 may be part of a pore that confers Mn\(^{2+}\) specificity.

It is interesting to note that the CAX2 CAF residues are also found in the deduced amino acid sequences of the putative Arabidopsis CAX5 (Arabidopsis genome initiative number At1g55730) and CAX6 (At1g55720) transporters, as well as ZCA52 (GenBank\textsuperscript{TM} accession number AB044567) from Zea mays, suggesting that these CAX transporters may also transport Mn\(^{2+}\). Additionally, the yeast Ca\(^{2+}/H^+\) antiporter VCV1 contains LCF residues at this domain, hinting that this region is more similar to CAX2 than to CAIX1.

This putative Mn\(^{2+}\) domain may also be involved in Zn\(^{2+}\) transport. In competition studies, \(^{45}\)Ca\(^{2+}\) transport mediated by CAX2, CAX2-A, -B, -D, -E, and CAX2-9 was inhibited by 100× excess Zn\(^{2+}\), whereas CAX1- and CAX2-C-mediated \(^{45}\)Ca\(^{2+}\) transport was not (Fig. 8). The inability of CAX2 to suppress the Zn\(^{2+}\) sensitivity of the zrc1 cot1 S. cerevisiae mutant (data not shown) may infer that CAX2 does not transport Zn\(^{2+}\). However, another putative vacuolar Zn\(^{2+}\) transporter, ZAT1 of Arabidopsis, failed to suppress the Zn\(^{2+}\) sensitivity of this strain but was able to suppress a Schizosaccharomyces pombe Zn\(^{2+}\)-sensitive mutant (41). Future direct measurements of Zn\(^{2+}\) transport in yeast strains expressing CAX2, CAX2-C, CAX2-C1, and CAX2-C2 will need to be performed to confirm whether this observed inhibition of Ca\(^{2+}\) transport by Zn\(^{2+}\) was in fact due to Zn\(^{2+}/H^+\) antiport by CAX2, and if so, to distinguish the Mn\(^{2+}\) and Zn\(^{2+}\) domains of CAX2. The competition experiment also gave an indication that CAIX1 but not CAX2 may transport Ni\(^{2+}\). Furthermore, it appears that none of the domains analyzed are involved in Ni\(^{2+}\) transport.

Given that both CAIX1 and CAX2 can strongly suppress the K667 yeast Ca\(^{2+}\) sensitive phenotype, we were surprised that the CAX2-A and CAX2-B constructs demonstrated weaker Ca\(^{2+}\) suppression (Fig. 4) and reduced Ca\(^{2+}/H^+\) antiport activity (Fig. 6) as well as the loss of Mn\(^{2+}/H^+\) antiport, as inferred from the lack of Mn\(^{2+}\) suppression (Fig. 5). Both proteins appeared to be expressed at high levels in yeast (Fig. 3), but perturbations in protein folding or membrane topology may not allow these proteins to be fully functional. These alterations in transport have also abolished the Mn\(^{2+}\) competition of CAX2-A and CAX2-B-mediated Ca\(^{2+}\) transport, and therefore we cannot completely rule out the possibility that these domains play some role in Mn\(^{2+}\) transport. Thus, using this chimeric gene approach, we cannot precisely assess the roles of these domains in metal specificity. However, the difference between these constructs and CAX2-C is noteworthy.

Many transition metal transporters, such as those of the N Bram and ZIP transporter families, appear to have broad substrate ranges (1, 42). However, there has been little research into the understanding of the molecular mechanisms determining substrate specificity and how it can be altered. Other techniques have been used to understand and alter transport specificity in different types of metal transporters. The Arabidopsis plasma membrane transporter, IRT1, transports Cd\(^{2+}\), Fe\(^{3+}\), Mn\(^{2+}\), and Zn\(^{2+}\) (42). Replacing Asp residues at either position 100 or 136 with an Ala eliminates transport of both Fe\(^{3+}\) and Mn\(^{2+}\) (43). Similarly, replacement of a Glu residue with Ala at position 103 eliminates the ability of Zn\(^{2+}\) transport by IRT1 but retains the transport of other metals. A number of other residues in or nearby transmembrane domains appear to be essential for IRT1 function. Mutagenesis of a charged residue within a membrane-spanning domain in the yeast Golgi Ca\(^{2+}/\)Mn\(^{2+}\)-ATPase PMR1 has normal Ca\(^{2+}\) transport but a 60-fold reduction in the apparent affinity for Mn\(^{2+}\) (44). Substitution of Ser with Ala at position 775 in a membrane-spanning region 5 in a Na\(^+/K^+\)-ATPase causes a 30-fold decrease in K\(^+\) but not Na\(^+\) affinity (45, 46). Increased Na\(^+\) affinity has also been reported by a point mutation in a wheat K\(^+\)-Na\(^+\) transporter (47). Future studies, focusing on altering other regions in CAX2, may delineate other domains involved in metal specificity.

In summary, we have shown that it is possible to generate a metal/H\(^+\) antiporter that is more specific to a particular metal, in this case Ca\(^{2+}\), by mutation of a discrete amino acid domain. The CAX2 constructs described here demonstrate the validity of altering the metal transport profile of a CAX family member. Examples of how such transporters could prove beneficial include making transgenic crop plants that express high levels of CAX2 variants that no longer transport Mn\(^{2+}\). Unlike CAIX1 ectopic expression, CAX2 expression is not deleterious to plant growth (48). CAX2 variants could be used to accumulate higher levels of a Ca\(^{2+}\) but not accumulate unwanted metals, thereby boosting the bioavailable Ca\(^{2+}\) in foods. In future studies we may be able to use a similar mutagenic approach to increasing the specificity toward other, more toxic metals. Alternatively, ectopic expression of CAX2 variants that have increased metal transport could be used to help remove toxic metals from soils. The first step toward these applications is expressing these CAX2 variants in plants and analyzing the changes in metal accumulation and vacuolar metal transport.

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