FUNCTIONAL AND STRUCTURAL CHARACTERIZATION OF CATION/H⁺ ANTIPORTERS

A Dissertation

by

MURLI MANOHAR

Submitted to the Office of Graduate Studies of Texas A&M University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2012

Major Subject: Molecular and Environmental Plant Sciences
Functional and Structural Characterization
of Cation/H⁺ Antiporters

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May 2012

Major Subject: Molecular and Environmental Plant Sciences
ABSTRACT

Functional and Structural Characterization

of Cation/H\(^+\) Antiporters. (May 2012)

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Co-Chairs of Advisory Committee: Dr. Kendal Hirschi
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Inorganic cations play decisive roles in many cellular and physiological processes and are essential components of plant nutrition. Therefore, the uptake of cations and their redistribution must be precisely controlled. Vacuolar antiporters are important elements in mediating the intracellular sequestration of these cations. CAXs (for CAtion eXchanger) are members of a multigene family and appear to predominately reside on vacuoles. Defining CAX regulation and substrate specificity have been aided by utilizing yeast as an experimental tool. Studies in plants suggest CAXs regulate apoplastic Ca\(^{2+}\) levels in order to optimize cell wall expansion, photosynthesis, transpiration and plant productivity. CAX studies provide the basis for making designer transporters that have been used to develop nutrient enhanced crops and plants for remediating toxic soils.

In my second study, I have characterized and defined autoinhibitory domain of *Arabidopsis* CAX3. Several CAX transporters, including CAX1, appear to contain an approximately 40 amino acid N-terminal regulatory regions (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. 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 of the NRR. Experiments on endomembrane-enriched vesicles isolated from yeast expressing activated CAX3 demonstrate that the gene encodes Ca$^{2+}$/H$^+$ exchange with properties distinct from CAX1. These studies demonstrate shared and unique aspects of CAX1 and CAX3 transport and regulation.

My third study is to express and purify CAX proteins for X-ray crystallographic analysis. In this study, I initiated crystallization of vacuolar membrane localized CAX protein from eukaryotes. Membrane proteins continue to be challenging targets for structural biology because of their hydrophobic nature. We have demonstrated here that eukaryotic Ca$^{2+}$/H$^+$ exchanger can be successfully expressed in *E. coli* based expression system. Collectively, our findings suggest that CAX protein can be successfully expressed, detergent solublized and purified from *E. coli* with a yield sufficient for functional and structural studies.
DEDICATION

To my parents
ACKNOWLEDGEMENTS

In the first place I would like to express profound gratitude to my committee co-chair, Dr. Kendal Hirschi, for his invaluable support, encouragement, supervision, critique, and guidance throughout my research work. I am deeply indebted to my committee co-chair, Dr. Bhimanagouda Patil, who is always patient, ready to provide his support and continuous guidance that enabled me to complete my work successfully. Without his support, my research project would not have gone this far. I also gratefully thank my committee members, Dr. Hisashi Koiwa, and Dr. Wayne Versaw for their support, advice, knowledge, and guidance throughout the duration of my research.

I would like to thank Dr. Toshiro Shigaki, Dr. Hui Mei, and Dr. Jay Morris, for all their tremendous help with my various experimental projects and valuable advice in scientific discussions, Dr. Lei Zheng and his lab members for help with the protein crystallization project, and Dr. Jean Gould for the help with plant tissue culture.

I would like to thank my wife, Anshu Kumari, for supporting and encouraging me to pursue this degree. Without her support, I would not have finished the degree in timely manner.

I wish to thank my parents, Jai. V. Sharma and Rekha Sharma, for their support and love throughout my life. To them I dedicate this work.
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<td>TMHMM</td>
<td>Transmembrane Hidden Markov Model</td>
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<td>CAX</td>
<td>Cation/H⁺ Exchangers</td>
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<td>TM</td>
<td>Transmembrane Domain</td>
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<td>NRR</td>
<td>N-terminal Regulatory Region</td>
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<td>CaMV</td>
<td>Cauliflower Mosaic Virus</td>
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<td>ICP-AES</td>
<td>Inductively Coupled Plasma Atomic Emission Spectroscopy</td>
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I. INTRODUCTION: REVIEW OF LITERATURE*

Transporters play one of the most fundamental roles in life, namely, selective import or removal of molecules across biological membranes. In *Arabidopsis* approximately 3% of the genome appears to code for transporters (The *Arabidopsis* Genome Initiative, 2000). A vast majority of these transporters are secondary transporters which are energized by the proton gradient and membrane potential. The plant cation/H\(^+\) exchangers (CAXs) are part of the ensemble of transporters that coordinate the redistribution of various cations including calcium (Ca\(^{2+}\)) in exchange for the protons generated by H\(^+\)-pumps. Elucidating CAX functions is challenging, requiring transport studies and molecular and genetic assays that can distinguish CAX functions among the multitude of transporters that may have similar functions.

CAXs are members of a multigene family and appear to predominantly reside on the vacuole (Martinoia et al. 2007; Shigaki et al. 2006). The *Arabidopsis thaliana* genome appears to contain six CAX open reading frames, and genetic studies suggest CAXs play overlapping roles in many cell functions.


This dissertation follows the style of *Plant Biology*.
For example, plants which have lost a single CAX transport function are almost indistinguishable from the wild type plants under normal growth conditions (Shigaki et al. 2006). However, when some double CAX deletions are made, the plants display severe growth defects, implicating their importance and their compensatory functions (Cheng et al. 2003; Conn et al. 2011).

Understanding the mechanism of cellular transport requires precise analysis of how the substrates are recognized by the “gate” of the transporter, how they are carried through the pore, and how the energy required for the transport is transmitted to the substrate. To date, structure function studies have centered on creating modified CAXs, and measuring transport properties of these CAX derivatives.

The past several years has focused on the physiological roles, biochemistry, and molecular biology of CAXs. One significant finding was, even though CAXs were identified by suppression of Ca$^{2+}$ sensitivity in yeast, their substrate range is broad (Shigaki & Hirschi 2006). CAXs are now recognized to be involved in a number of important aspects of plant growth and development (Conn et al. 2011) and may be excellent tools for nutritional enhancement of crops and mitigating pollutants in soils. We will summarize in this review the knowledge gained over the last decade.

**Diversity of CAXs**

To date, there are over 200 full-length CAX cDNAs, or predicted open reading frames (ORFs) in the GenBank database (Manohar et al. 2010). CAXs are one of the five closely related families of transporters that together form a large phylogenetic clade termed the Ca$^{2+}$/cation antiporter (CaCA) superfamily (Shigaki & Hirschi 2006). These
CAXs have been classified into three major categories based on phylogenetic analysis: Type I (CAXs similar to A. thaliana CAX1), II (CAXs similar to S. cerevisiae VNX1) and III (CAXs similar to E. coli ChaA). (Shigaki et al. 2006). Several Type I and III CAXs have been well characterized across species but, to date, only two Type II CAXs, the yeast Vnx1p and zebrafish DrCax1 have been characterized. Type II CAXs have a unique N-terminal secondary structure with an additional two or more transmembrane domains which are absent from both Type I and Type III CAXs (Cagnac et al. 2007, 2010; Manohar et al. 2010). CAXs are reported to be present in a wide range of taxa, including plants, most animals, fungi, and bacteria. CAXs do not appear to present in the genomes of mammals, insects, C. elegans, and archaebacteria. The reason for the apparent absence in various taxa is an interesting subject and may lead to revelations regarding CAX function.

In yeast there have been extensive studies regarding the vacuolar Ca\(^{2+}\)/H\(^{+}\) antiporter mediated by VCX1. Cytosolic Ca\(^{2+}\) spikes caused by hypertonic shock are reset by the vacuolar Vcx1p (Denis & Cyert 2002), but other environmental changes such as hypotonic shock also elicit a cytosolic Ca\(^{2+}\) spike. However this spike is not dissipated by Vcx1p. This suggests that Vcx1p Ca\(^{2+}\) transport is important in a particular subset of Ca\(^{2+}\) signal transduction events. In higher organisms, some of these CAXs vary in their tissue and substrate specificity; however, these CAX transporters also appear to differ in their transcriptional and post-translational regulation (Shigaki et al. 2010). One explanation for differential regulation of these transporters is that they may modulate particular environmental or developmental events.
Plant CAXs are classified under Type I CAXs and further divided into two phylogenetically distinct groups (Type I-A and Type I-B; Shigaki et al. 2006). This distinction suggests functional differentiation between the two groups. *Arabidopsis* and rice have six and five CAX open reading frames, respectively, with approximately the same numbers belonging to each of the two phylogenetic groups (Kamiya et al. 2005, 2006; Qi et al. 2005). However, no clear functional differences have been so far identified.

Plant CAXs are characterized by 11 transmembrane (TM) helices predicted by computer algorithms such as TMHMM (Fig. 1.1A; Shigaki & Hirschi 2006). These TMs are further divided into three components, namely TM1, TM2-6, and TM7-11. TM2-6 and TM7-11 are weakly homologous elements and thought to be the result of an ancient gene duplication event (Zhao et al. 2009b). TM1 appears to be a dispensable element for cation transport function in some CAXs (Schaaf et al. 2002).

The primary amino acid sequences in the TM regions are highly conserved in all plant CAXs, which may indicate the structural importance of the membrane helices (Shigaki et al. 2006). The majority of the sequence diversity of CAXs exists in the loop and tail regions. These hydrophilic elements are thought to define the differential substrate specificities and intramolecular regulation that exist among different CAXs. A highly variant 9 amino acid region between TM1 and TM2 has been shown to control Ca\(^{2+}\) transport in CAX1 and it is a target for genetic manipulation to alter CAX transporter specificity (Shigaki & Hirschi 2006).
Fig. 1.1. Membrane Topology and Phenotypes of Yeast Mutants Expressing CAX Transporters

A. The putative transmembrane (TM) helices 1-11 of CAX transporters are depicted along with the N-terminal autoinhibitory domain (depicted in thick black line). The N-terminal hydrophilic region may interact with several signaling proteins such as the kinase Salt overly sensitive 2 (SOS2) and CAX Interacting protein 1 (CXIP1) to modulate CAX function. CAXs can be divided into two weakly homologous halves at the short negatively charged loop between transmembrane (TM) 6 and TM7, termed the “acidic motif”. For example, here the N- and C-terminal halves of CAX are termed the N-terminus (TM2-6) and C-terminus (TM6-11).

B. Suppression of Ca\(^{2+}\) sensitivity in yeast mutant cells that are defective in 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 media. This picture was taken after 3 days of incubation at 30°C.
CAXs are known to have a broad range of cation specificity, and the specificity depends on the amino acid sequence diversity among different CAXs (Shigaki & Hirschi 2006; Shigaki et al. 2005). Therefore, exploration of natural diversity of CAXs may prove to be a good strategy to design CAXs with desired transport properties. Recently, noncannonical CAXs with an additional TM inserted between TM4 and TM5 were reported from plants in the Asteraceae family (Jain et al. 2009). Characterization of these CAXs may show novel properties that can be useful for engineering.

**CAX Variants**

Calcium transporters and their regulation have been intensively studied in yeast (Mei et al. 2008). Because the Ca\(^{2+}\) signaling and transport pathways are similar among organisms, heterologous expression in yeast strains that are defective in Ca\(^{2+}\) transport is a powerful tool to accelerate research in the identification and characterization of Ca\(^{2+}\) transporters- particularly the plant CAXs. Specifically, a mutant yeast strain lacking vacuolar Ca\(^{2+}/H^+\) transport and Ca\(^{2+}\)-ATPase activity is sensitive to high Ca\(^{2+}\) and has been used to identify and study plant CAX-type exchangers (Fig. 1.1B; Fig. 1.2; Cunningham & Fink 1994, 1996, Pittman et al. 2004a). Plant cation/H\(^+\) exchangers were initially cloned by the ability of N-terminal truncated versions of the proteins to function in these yeast mutants defective in vacuolar Ca\(^{2+}\) transport (Shigaki & Hirschi 2006; Ueoka-Nakanishi et al. 2000). These assays characterized the initial CAX transporters and the presence of N-terminal autoinhibitory domains among a range of plant CAXs (Fig. 1.1A; Fig. 1.2B; Mei et al. 2007). Deletions of the N-terminus increase activity in
both yeast (now termed sCAX1 and sCAX2) and plants (Fig. 1.1B, Shigaki & Hirschi 2006; Mei et al. 2007). *In planta*, CAX1 contains 36 amino acids at the N-terminus which is not present in sCAX1 that was initially identified in yeast assays (Shigaki & Hirschi 2006; Mei et al. 2007). In yeast, CAX1 acts as a weak vacuolar \( \text{Ca}^{2+}/\text{H}^+ \) antiporter as transport activity is severely reduced compared to sCAX1 (Fig. 1.1B; Fig. 1.2; Cheng et al. 2005; Shigaki et al. 2010). This autoinhibition is caused by the N-terminus physically interacting with a neighboring N-terminal region (residues 56-62) (Fig. 1.1A; Shigaki & Hirschi 2006). The N-terminal regulatory region may interact with regulatory proteins such as SOS2 (for salt overly sensitive 2) and CXIP1 (CAX Interacting protein 1) to activate CAXs *in planta* (Fig. 1.1A; Shigaki & Hirschi 2006). These findings suggest that multiple forms of a cDNA should be used when conducting heterologous expression studies. For instance, in yeast assays the 3’-untranslated region (UTR) of CAX1 appears to negatively influence RNA and protein expression levels (Shigaki et al. 2010). In fact, the identification of the N-terminal autoinhibition of CAX1 was facilitated by the serendipitous inclusion of the 3’-UTR. It will now be interesting to reexamine various CAX1-expressing yeast phenotypes using the CAX1 ORF. Furthermore, emerging works with the CAX transporters suggest that it is difficult to know a priori where regulatory domains may reside. For example, using yeast assays it is difficult establishing functional assays for CAX3 (or sCAX3) which is most similar to CAX1 (Shigaki & Hirschi 2006).

Aside from the NRR, several other distinct cation domains have been characterized using yeast expression assays (Shigaki et al. 2003; Shigaki & Hirschi
2006). Furthermore, putative regulatory elements have been identified which activate CAX1 (Fig. 1.1A; Cheng et al. 2004a,b). Future work will be directed at determining if these regulatory proteins function in planta to modulate CAX activity.

**Physiological Functions**

Tonoplast-localized proton-coupled Ca\(^{2+}\) transporters encoded by CAX genes play a critical role in sequestering Ca\(^{2+}\) into the vacuole (Pittman et al. 2011; Shigaki & Hirschi 2006). These transporters may function in coordination with Ca\(^{2+}\) release channels, to shape stimulus-induced cytosolic Ca\(^{2+}\) elevations. Recent analysis of *Arabidopsis* CAX knockout mutants, particularly *cax1* and *cax3*, identify a variety of phenotypes including sensitivity to abiotic stresses, which indicate that these transporters might play a role in mediating the plants stress response (Cheng et al. 2003, 2005; Zhao et al. 2008). Mutants in CAX1 are tolerant to serpentine soils (soils with a low Ca\(^{2+}\):Mg\(^{2+}\) ratio). This illustrates how modulating CAX activity may be important in improving tolerance to toxic metals (Bradshaw 2005; Visscher et al. 2010). On the other hand, expression of a deregulated *Arabidopsis* CAX1 in tobacco causes tip burning and other Ca\(^{2+}\) deficiency-like symptoms while expression of deregulated CAX2 confers some Mn\(^{2+}\) tolerance (Fig. 1.3A; Hirschi 2001).

The specific function of CAX3 has been difficult to determine (Cheng et al. 2003, 2005; Conn et al. 2011). Shared phenotypes among *cax1* and *cax3* include an increased sensitivity to both abscisic acid (ABA) and sugar during germination and an increased tolerance to ethylene during early seedling development (Cheng et al. 2005; Zhao et al. 2008). Phenotypes unique to *cax3* lines include salt, lithium and low pH
sensitivities. In planta, transport measurements ascribe these cax3 sensitivities to a reduction in vacuolar Ca$^{2+}$/H$^+$ transport during salt stress (Zhao et al. 2008). However, as mentioned previously, yeast assays to establish activity assays for variants of CAX3 have proved problematic. Arabidopsis preferentially accumulates Ca$^{2+}$ in the vacuoles of mesophyll cells. A cell-specific microarray comparing Arabidopsis thaliana epidermal (‘Ca$^{2+}$-poor’) and mesophyll (‘Ca$^{2+}$-rich’) transcriptomes suggests AtCAX1 is the most abundant and differentially expressed Ca$^{2+}$-transporter between epidermal and mesophyll cells (Conn et al. 2011). Analysis of loss-of-function mutants demonstrated that cax1/cax3 which lacked expression of both AtCAX1 and AtCAX3 (a gene ectopically expressed in mesophyll cells upon abolishment of AtCAX1), has reduced mesophyll [Ca$^{2+}$] (Conn et al. 2011). Reduced capacity for mesophyll Ca$^{2+}$ accumulation results in reduced cell wall extensibility, stomatal aperture, gas exchange, leaf growth rate and altered expression of cell-wall modifying proteins (Conn et al. 2011). These phenotypes result from altered apoplastic free [Ca$^{2+}$] which is 3-fold greater in cax1/cax3 than in wild type plants. These findings suggest that CAXs function as key regulators of apoplastic [Ca$^{2+}$] through compartmentation into mesophyll vacuoles; a mechanism essential for optimal plant function and productivity (Conn et al. 2011).

A common feature emerging among these CAX mutants is the perturbation of H$^+$-ATPase activity at both the tonoplast and the plasma membrane, suggesting a tight interplay between the Ca$^{2+}$/H$^+$ exchangers and proton pumps (Fig.1.4; Cheng et al. 2003, 2005; Zhao et al. 2008). For example, CAX1 mutation causes decrease in V-type ATPase activity and a increase in P-type ATPase activity while deletion of CAX3
appears to cause decrease in both V- and P-type ATPase activity (Cheng et al. 2003, 2005; Zhao et al. 2008)). We speculate that indirect regulation of proton flux across the membrane by the exchangers may be as important as the direct regulation of Ca\(^{2+}\) flux. A reoccurring observation in CAX genetic studies is the plethora of compensatory responses in transporters seen when alterations are made to Ca\(^{2+}/H^+\) antiport.

Recent work has focused on the function of CAX transporters that are less highly expressed. For example, AtCAX4 is expressed in the root apex and lateral root primordial (Mei et al. 2009). Among the *Arabidopsis* CAXs, this root expression pattern appears to be unique; however, when expressed at high levels in plants, its biochemical properties resembled other CAXs. A cax4 loss-of-function mutant and CAX4 RNA interference lines display altered root growth in response to Cd\(^{2+}\), Mn\(^{2+}\), and auxin (Mei et al. 2009). These findings suggest that CAX4 is a cation/H\(^+\) antiporter that plays an important function in root growth under heavy metal stress conditions (Mei et al. 2009).

CAX proteins have been characterized from several plants. In rice, the CAXs have been extensively studied and appear to help protect cells from Ca\(^{2+}\) toxicity and function to transport Ca\(^{2+}\) into vacuoles as a mineral nutrient and second messenger (Kamiya et al. 2005, 2006). Cereal monocots appear to accumulate Ca\(^{2+}\) in epidermal cells and not within mesophyll cells (reviewed in Conn & Gilliham 2010). Further studies using mutant plants or RNA interference (RNAi)-mediated suppression of monocot CAXs will certainly be illuminating.

To assess the conservation of the characteristics among CAX2-like transporters in higher plants, AtCAX2 orthologues from *Arabidopsis* (AtCAX5), tomato (LeCAX2)
and barley (HvCAX2) have been characterized (Edmond et al. 2009). Each of these
CAXs can transport Ca\(^{2+}\) and Mn\(^{2+}\) into the yeast vacuole with different cation transport
kinetics (Edmond et al. 2009; Williams et al. 2010). Expression of AtCAX2 is not
modulated by metal stress (Shigaki & Hirschi, 2006); however, AtCAX5 and HvCAX2
are transcriptionally up-regulated by high Mn\(^{2+}\) treatment, and by Ca\(^{2+}\) and Na\(^{+}\) stress
respectively (Edmond et al. 2009). These observations and additional transport studies
suggest that despite the high sequence identity between plant CAX2 orthologues, there is
diversity in their functional characteristics.

Ca\(^{2+}\) disorders, likely involving altered CAX activity, may be responsible for
losses throughout production agriculture (Ho & White 2005). Recent work suggests
altered CAX activity may modify defense related signaling in barley (Zhang et al. 2009).
Blossom-end rot (BER) in tomato and bitter pit in apples may also be linked to changes
in CAX activity (Freitas et al. 2010; Park et al. 2005). To explain the primary causes of
BER, two hypotheses have been considered, 1) Ca\(^{2+}\) deficiency and 2) aberrant Ca\(^{2+}\)
homeostasis. Interestingly, fruits of transgenic tomato plants expressing a deregulated
vacuolar Ca\(^{2+}/H^+\) antiporter have 2-3 fold higher Ca\(^{2+}\) concentrations than that of wild-
type plants and show a remarkable increase in the incidence of BER (Park et al. 2005;
Chung et al. 2010). These CAX expressing lines may provide a tool to unravel the
mechanistic link between altered Ca\(^{2+}\) homeostasis and BER incidence.

CAXs may have functions beyond the vacuole. In soybean, a plasma membrane
CAXs appear to be involved in removing toxic metals from the cell (Luo et al. 2005).
Other work in cucumber suggests root plasma membrane antiporter systems are also
present that are stimulated by exogenous heavy metals (Migocka & Klobus 2007). Presence of Ca$^{2+}$/H$^+$ antiport has also been reported in the plasma membrane of rice roots (Shen 2005). Studies may now be focused on CAX mediated efflux across the plasma membrane.

**Structure-Function and Biochemistry**

The topology of Vcx1p has been determined using multiple experimental approaches. The protein has an odd number of transmembrane domains and its termini are located on opposite sides of the membrane, with the N-terminus in the cytoplasm (Segarra & Thomas 2008). These studies and previous structure function studies focusing on domains which are important in substrate specificity provide insights into the structure and function of CAX transporters (Shigaki & Hirschi 2006).

The identification and analysis of *Chlamydomonas reinhardtii* CAX (CrCAX1) facilitates further comparison between the CAX transporters, particularly with those that have been studied in detail (Pittman et al. 2009). Despite having greater sequence similarity to ScVCX1 than AtCAX1, CrCAX1 has a broader substrate specificity than ScVCX1, showing both Ca$^{2+}$/H$^+$ and Na$^+$/H$^+$ exchange activity, and possibly the ability to transport Co$^{2+}$ and Cd$^{2+}$. A particularly intriguing feature of CrCAX1 is the structural similarities with the higher plant CAX transporters with regard to the extended hydrophilic N-terminal tail.

Numerous CAXs from plants and microorganisms have been functionally characterized whereas only a few Type II CAXs have been characterized (Cagnac et al. 2007, 2010; Manohar et al. 2010). The yeast Type II CAX, Vnx1p may localize on
various membranes but Vnx1p appears to readily transport Na\(^+\) and K\(^+\) into the vacuole (Cagnac et al. 2007). Indeed, a vacuolar enriched fraction of \(\text{vnx1}\Delta\) cells is totally devoid of this exchange activity. Furthermore, Vnx1p transports Ca\(^{2+}\) under particular conditions. The contribution of Vnx1p to Ca\(^{2+}\) transport is most clearly observed when other transporters are missing (Manohar et al. 2010). The elevated activity of Vnx1p in these mutants may occur due to altered distribution or post-translational modification of Vnx1p, as these mutants may cause defects in protein sorting and processing (Rudolph et al. 1989; Antebi & Fink 1992). While a large phylogenetic distance exists between Vnx1p and a zebrafish CAX (DrCax1), several findings indicate that functional overlap still exists between animal and fungal Type II CAX proteins (Manohar et al. 2010). Future work will determine if animal Type II CAXs also encode Na\(^+\) and K\(^+\) antiporter activity.

Given that higher plants do not appear to contain Type II CAXs, it will be interesting to determine if under particular conditions plant CAXs can function to bind and efflux both Ca\(^{2+}\) and Na\(^+\). Previous analysis of higher plant CAX transporters has identified two highly conserved sequence repeats that have been suggested to function as cation selectivity filters (Shigaki et al. 2006). These regions are similar and show sequence conservation with regions required for cation binding in mammalian Na\(^+\)/Ca\(^{2+}\) exchangers (Philipson & Nicoll 2000). Specific residues within these domains have been shown to be required for plant CAX mediated Ca\(^{2+}\) transport (Shigaki et al. 2005), and these residues are conserved in the CrCAX1. It is unknown whether these domains will also determine Na\(^+\) binding. A comparison of CrCAX1 with other CAX transporters,
none of which has yet been shown to transport Na\(^+\), suggests residues in CrCAX1 that differ from the consensus sequence.

In animals, Ca\(^{2+}/H^+\) efflux activity in mitochondria has been described (Gunter et al. 2000). However, the molecular identity of this carrier has remained elusive (Villa et al. 1998), and only recently has the gene been identified (Jiang et al. 2009). A putative mitochondrial Ca\(^{2+}/H^+\) transporter from the human malaria parasite *Plasmodium falciparum*, has also been characterized (Rotmann et al. 2010) and while being functionally similar, lacks appreciable homologies to the previously characterized mitochondrial Ca\(^{2+}/H^+\) antiporters from humans and drosophila, suggesting that there might be distinct classes of mitochondrial Ca\(^{2+}/H^+\) antiporters. Delineating structures of CAXs will certainly accelerate future structure function studies. Unfortunately, membrane structural biology is lagging some 20-30 years behind the study of soluble proteins (Baker 2010). However, the field is catching up as more robust ways to make, purify and crystallize membrane proteins have emerged. These approaches will certainly spurn efforts to obtain CAX crystal structures.

**Inter/Intra Molecular Interactions**

Functional association between CAX1 and CAX3 has been suggested through genetic studies and yeast expression assays (Cheng et al. 2005; Zhao et al. 2008, 2009a,b). Coincident expression of *Arabidopsis* CAX1 and CAX3 occurs during various phases of plant growth (Zhao et al. 2009a). In planta coimmunoprecipitation suggest that a protein-protein interaction may also occur between CAX1 and CAX3 (Zhao et al. 2009a). In yeast expression assays, co-expressing both CAX1 and CAX3 demonstrate
phenotypes that cannot be recapitulated by expression of deregulated versions of either transporter (Zhao et al. 2009b).

Many transporter proteins are made up of two or more similar modules that may be products of ancient gene duplication events. Indeed, CAX transporters appear to consist of two weakly homologous modules (Fig. 1.1A; Shigaki & Hirschi 2006; Zhao et al. 2009b). Interestingly, the N-terminal and C-terminal halves of sCAX1 and sCAX3 can be expressed in yeast and function (Fig. 1.2A, B; Zhao et al. 2009b). Further studies have shown a physical interaction between these halves both in yeast and plant cells. The finding indicates: (1) the half protein modules can fold properly; (2) both half proteins are localized to the same endomembrane; and (3) there is sufficient interaction to facilitate function.

The ability to split the Ca\(^{2+}\)/H\(^+\) exchanger into two domains is similar to the situation found with the cardiac Na\(^+\)/Ca\(^{2+}\) exchanger (Ottolia et al. 2001). This transporter can also be divided into two domains that reassemble in the membrane of *Xenopus* oocytes. Likewise, it has been shown that separately expressed halves of the potato sucrose/H\(^+\) transporter SUT1 can interact and reconstitute a functional transporter (Reinders et al. 2002a,b).

This “split protein” approach expands our potential repertoire of functional transporters. For example, we could engineer novel transporters by combining CAXs with unique substrate affinities. We have shown that expressing a C-terminal CAX1 variant containing a single amino acid mutation can modify transport activity of CAX1
and N-sCAX1 (Zhao et al. 2009b). A large body of work has established that sCAX2 has
different biochemical properties than sCAX1 (Pittman et al. 2004b; Shigaki & Hirschi
2006). We may now be able to generate novel transporters by selecting heteromeric
combinations of CAX1/CAX2. Similarly, site-directed mutagenesis has created a series
of sCAX1 and sCAX2 variants with different transport properties that could now be
combined. Alternatively, we may be able to create dominant negative forms of the half
proteins to down regulate CAX activity. Harnessing this technology should allow further
manipulation of the nutritional status of plants.

**CAX Mediated Biofortification and Phytoremediation**

The possibilities associated with transgenic approaches keep plant biologists
optimistic despite the current political and economic landscape that is not completely
receptive to this technology (Freese & Schubert 2004; Weil 2005; Johnson et al. 2007;
Powell et al. 2007). Even with these limitations, the potential for genetic modifications
to alleviate hunger and nutrient deficiencies warrants advocacy of this technology.
Increased bioavailable mineral content in agriculturally important crops can be
engineered through manipulating plant mineral transporters (Morris et al. 2008; Dayod
et al. 2010; White & Brown 2010). We hypothesized that an engineered version of a
plant Ca$^{2+}$/H$^+$ antiporter could be used for biofortification by increasing Ca$^{2+}$ levels
within edible roots, such as carrots.
Fig. 1.2. CAXs Halves can Functionally Assemble in Yeast

A. The N-terminal half of CAX1 expressed in yeast cells along with the C-terminal half of CAX1 or CAX3 (shown in blue) can suppress the Ca\textsuperscript{2+} sensitivity phenotype (shown as a happy face) but expression of the N- or C-halves of the transporters alone are not functional (shown as an unhappy face).

B. Suppression of Li\textsuperscript{+} and Ca\textsuperscript{2+} sensitivity of yeast strains by co-expressing N-CAX1 with C-CAX1 or C-CAX3 indicate functional assembly of CAX halves and the reassembled transporter may have distinct transport properties. The activated CAX1-expressing yeast strain (sCAX1) is shown as a control for Ca\textsuperscript{2+} tolerance.

(This research was originally published in Journal of Biological Chemistry. Zhao, J., Connoroton, J. M., Guo, Y., Li, X., Shigaki, T., Hirschi, K. D., and Pittman, J. K. (2009) Functional studies of split Arabidopsis Ca\textsuperscript{2+}/H\textsuperscript{+} Exchangers. Journal of Biological Chemistry 284, 34075-34083 © the American Society for Biochemistry and Molecular Biology.)
We modified carrots expressing high levels of a deregulated *Arabidopsis* Ca$^{2+}$/H$^+$ antiporter (CAX1 with truncated N-terminus autoinhibitory domain) that accumulates almost two-fold more Ca$^{2+}$ in the edible part compared to control plants, without perturbing growth, development or fertility, under controlled lab conditions (Morris et al. 2008). Feeding trials using these labeled carrots demonstrated that the total amount of Ca$^{2+}$ absorbed was significantly increased, in both mice and humans, with diets containing the modified carrots (Morris et al. 2008). In the human feeding studies, Ca$^{2+}$ absorption efficiency was 42.6 ± 2.8% and 52.1 ± 3.2% (p<0.001) for the sCAX1 carrots and control carrots, respectively; however, total Ca$^{2+}$ absorption per 100 g of carrots was 41 ± 2% higher in CAX carrots compared to control carrots (26.50 vs. 15.34 mg Ca$^{2+}$/100g, p<0.001).

Interestingly, not all the increased Ca$^{2+}$ in the transporter-modified carrots was bioavailable. This may be due to a fraction of the extra Ca$^{2+}$ being bound to antinutrients within the carrot. This serves as a cautionary example for scientists who assume that all increases in nutrient content directly equate to increased bioavailability. However, the modified carrots are a better source of Ca$^{2+}$ because total Ca$^{2+}$ was higher (Reviewed in Hirschi 2008).

The same general strategy used for biofortification can be applied to remove environmental contaminations by CAX-expressing plants (Kotrba et al. 2009). Phytoremediation, the use of plants and their associated microbes for environmental cleanup (Doty 2008), has been gaining popularity because it is economically feasible and involves minimum disturbance of the surrounding environment (Raskin et al. 1997).
Various mechanisms confer Cd\(^{2+}\) tolerance and accumulation in plants (Song et al. 2003; Korenkov et al. 2007a,b; Verbruggen et al. 2009; Wojas et al. 2009). Among the transporters thought to be capable of moving Cd\(^{2+}\) into the vacuole, CAXs (cation/H\(^{+}\) exchangers) have been well characterized (Korenkov et al. 2007a,b). In earlier work using tobacco, expression of *Arabidopsis* CAX2 results in enhanced Cd\(^{2+}\) transport in root tonoplast vesicles (Shigaki & Hirschi 2006). In addition, expression of CAX2 and CAX4 in tobacco results in higher tonoplast Cd\(^{2+}\)/H\(^{+}\) antiporter activity, Cd\(^{2+}\) accumulation and Cd\(^{2+}\) tolerance (Korenkov et al. 2007a,b). The transgenic *Arabidopsis* plants overexpressing CAX4 also display increased accumulation and tolerance of Cd\(^{2+}\) which is presumably resulting from increased Cd\(^{2+}\) sequestration into the vacuole (Mei et al. 2009). Recently, Wu et al. (2011) reported that a CAX variant-expressed in petunia plants are tolerant to high concentrations of Cd\(^{2+}\) in the tissue culture media, and accumulate high amounts of the metal in the plants (Fig. 1.3B). This study demonstrates that CAXs may be a useful tool for phytoremediation. To be used *in situ*, future studies must be conducted with plants with a large root system so that toxic metal absorption is more efficient. In the petunia study, a strong constitutive promoter was used to drive the expression of the sCAX1 variant (Fig. 1.3B). However, a more tissue specific promoter to drive expression may be ideal.
Fig. 1.3. Phenotypes of Various CAX-Expressing Lines.

A. Expressing Arabidopsis CAXs cause various phenotypes in tobacco. Expression of Arabidopsis CAX1 causes unique leaf tip burning phenotype in tobacco (left). Expression of the Arabidopsis CAX2 makes plants tolerant to Mn$^{2+}$. The CAX2 expressing and control plants shown here were grown for one week in hydroponic solution supplemented with 0.5 mM MnCl$_2$ (right).


B. Expression of a CAX1 variant causes Cd$^{2+}$ tolerance in petunia. Petunia CAX-expressing lines can grow in 100 µM CdCl$_2$ while control lines are chlorotic after 11 weeks.

**Fig. 1.4. Schematic Representation of the Role of CAX1 and CAX3 in Regulating Calcium Levels in Arabidopsis Mesophyll Cells**

*Arabidopsis* CAX1 and CAX3 are tonoplast localized proteins that export cations, including Ca\(^{2+}\), out of the cytosol. CAXs are energized by the pH gradient established by the vacuolar H\(^+\)-ATPase (V-ATPase) and the H\(^+\)-pyrophosphatase (V-PPase). In Arabidopsis, AtCAX1 is the major Ca\(^{2+}\) transporter (transport depicted with a bold black arrow) in leaf mesophyll cells while AtCAX3 (transport depicted as a thin black arrow) is a weak Ca\(^{2+}\) transporter in these cells. Interplay between these CAXs (depicted with a red arrow) is shown genetically as disruption of CAX1 has no significant effect on mesophyll apoplastic Ca\(^{2+}\) levels while disruption of both CAX1 and CAX3 exhibit a significant alteration of apoplastic Ca\(^{2+}\) levels. Alteration of apoplastic Ca\(^{2+}\) levels in mesophyll cells regulates several vital biological responses. CAX transporters also alter the proton flux generated by the P-type Proton ATPase (P-ATPase), CAX1 appears to negatively regulate P-ATPase (shown in yellow), while CAX3 appears to positively regulate P-ATPase activity (arrow shown in blue). Additionally, preliminary data suggests CAX1 and CAX3 can also form a functional heterodimer (CAX1-CAX3 heterodimer depicted in violet-red color) with the complex exhibiting unique transport properties.
There are additional areas where engineering CAXs may prove lucrative. For example, CAX-expressing plants could extract precious metals (phytomining) from soils. CAXs could be engineered to transport precious metals. A moss, *Funaria hygrometrica* Hedw., may selectively accumulate a significant amount of gold (approximately 10% of its dry weight) in its body, although its mechanism is yet to be known (Itouga et al. 2010). It is interesting to speculate that CAX may mediate this metal uptake.

**Conclusions**

Studies have highlighted CAXs as drivers of $\text{Ca}^{2+}$ accumulation in plant tissues (Cheng et al. 2003), and some CAXs are implicated in increased freezing tolerance after cold acclimation (Catala et al. 2003), delayed germination on sucrose, increased sensitivity of germination to ABA, tolerance to ethylene with respect to germination, inhibition of hypocotyl elongation and delayed flowering (Cheng et al. 2003, 2005), tolerance to serpentine soil and mars-like levels of magnesium sulfate (Bradshaw 2005; Visscher et al. 2010) and more recent studies have implicated CAXs as being essential in plants to regulate apoplastic $\text{Ca}^{2+}$ levels in order to optimize cell wall expansion, photosynthesis, transpiration and plant productivity (Conn et al. 2011). CAXs are also an important genetic tool, thanks to their amenability for protein engineering to alter substrate specificity and heighten transport. Future efforts will be directed toward detailed structural analyses of CAXs to provide rational engineering strategies to
expedite CAX-mediated approaches to enhance nutrient uptake and mitigate environmental pollutants.
II. CHARACTERIZATION OF *ARABIDOPSIS* Ca\(^{2+}/H^+\) EXCHANGER CAX3*

**Introduction**

Calcium (Ca\(^{2+}\)) fluxes within the cytosol are an important determinant in many plant responses (Manohar et al. 2011). Therefore, plants must maintain Ca\(^{2+}\) homeostasis in order to achieve normal growth, development, and environmental adaptations. Ca\(^{2+}/H^+\) 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 (Manohar et al. 2011). CAXs are localized predominantly to the tonoplast and sequester Ca\(^{2+}\) and other cations into the vacuole utilizing the H\(^+\)-gradient (Martiona et al. 2007; shigaki et al. 2006). CAX3 is phylogenetically closely related to CAX1 and both are classified as Type IA CAXs (Shigaki et al. 2006). Furthermore, CAX3 and CAX1 are thought to play similar physiological roles in *Arabidopsis* (Cheng et al. 2005; Conn et al. 2011).

Plant Ca\(^{2+}/H^+\) exchangers were cloned by the ability of N-terminal truncated versions of the proteins to function in *Saccharomyces cerevisiae* mutants defective in vacuolar Ca\(^{2+}\) transport (Manohar et al. 2011; Hirschi et al. 1996; Ueoka-Nakanishi et al. 2000).

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In planta, CAX1 contains a 36 amino acid region at the N-terminus which is not present in sCAX1. In yeast, CAX1 acts as a weak vacuolar Ca\(^{2+}/H^+\) antiporter, as transport activity is severely reduced when compared to sCAX1 (Cheng et al. 2005). Interestingly, in the initial yeast assays, the presence of a methionine codon at the 37th and 43rd amino acid residue in CAX1 and CAX2, respectively, allows translational initiation from the truncated cDNAs (Pittman & Hirschi, 2001; Pittman et al. 2004). 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 (Shigaki & Hirschi, 2000). However, when the first 36 amino acids of CAX3 are removed (sCAX3) to allow translation to start from an engineered Met\(_{37}\), the putative transporter appears to remain inactive in yeast expression assays (Manohar et al. 2011). 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 undetectable by 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 similarity 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 PCR amplification using a primer set raised at the appropriate regions of the CAX3 open reading, with XbaI (5’-end) and SstI (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 pCRII-TOPO vector (Invitrogen) and the sequences were verified for the absence of errors. The inserts were subcloned into piUGpd shuttle vector (Nathan et al. 1999) between XbaI and SstI restriction sites. For stable integration into yeast genome, CAX3 variants with GPD promoter were dropped from piUGpd shuttle vector by KpnI and SstI and ligated into pRS306 integration vector (Sikorski & Hieter 1989). Integration was confirmed by PCR using CAX3 specific primer and yeast based Ca\(^{2+}\) suppression assay.

Yeast Transformation, Growth, and Assays. The *Saccharomyces cerevisiae* strain K667 (*vcx1::hisG cnb1::LEU2 pmc1::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*; Cunningham & Fink 1996) 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) media (Sherman et al. 1986). Ca\(^{2+}\) tolerance assays were performed 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 hours before measurement.

**Vacuolar Enriched Membrane Fractionation.** Transformants were inoculated into 1200 ml of YEP supplemented with 4% dextrose and grown to O.D$_{600}$ ~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, 1.2 M sorbitol). Yeast cells were then resuspended in 5X pellet volume spheroplast buffer supplemented with 10 mM dithiothreitol (DTT) and 1% dextrose. 1 unit of Zymolyase/A$_{600}$ per unit of cells was added and incubated at 30°C for 1-2 hrs to generate spheroplasts. The spheroplasts were washed twice with 30 ml ice cold spheroplast buffer. The spheroplasts are then suspended in 5 ml ice cold buffer A (10 mM MES-Tris pH 6.9, 0.1 mM MgCl$_2$, 12% ficoll PM 400, Sigma Aldrich St. Louis, MO, USA) and homogenized. The lysate was then centrifuged at 3,000 rpm for 10 minutes and the supernatant was collected. For vacuole fractionation, 5 ml of ice cold buffer A was layered over the supernatant and centrifuged at 60,000 g for 30 min. A thin white floating wafer of vacuoles was collected and resuspended in 5 ml of fresh buffer A and overlayed with 5 ml of buffer B (10 mM MES-Tris pH 6.9, 0.5 mM MgCl$_2$, 8% Ficoll 400). It was then centrifuged at 60,000 g for 30 min and the floating white wafer was collected and resuspended in 5 ml of ice cold buffer C (10 mM MES-Tris pH 6.9, 5 mM MgCl$_2$, 25 mM KCl). Afterwards it was spun at 60,000 g for 30 min, and the pellet was finally suspended in buffer C supplemented with 10% glycerol and stored at -80°C until use. Protein concentrations were determined using the Bio-Rad protein assay
(Bio-Rad, Hercules, CA). Time-dependent $^{45}\text{Ca}^{2+}/\text{H}^+$ transport into endomembrane vesicles was later measured using the filtration method as described previously (Pittman and Hirschi, 2001). The $K_m$ measurement was performed by using Graphpad Prism 5, version 5.4, La Jolla, CA, USA.

**RT-PCR and Western Blotting Analyses.** RT-PCR and Western blotting analysis were performed as previously described (Pittman & Hirschi 2001; Pittman et al. 2005). RT-PCR was done using a primer set designed to amplify $\Delta 90$-CAX3. In Western blot analysis, monoclonal antibody against HA (Berkeley Antibody, Richmond, CA) was used at the 1:1000 dilution.

**ICP-AES Analysis of Yeast $\text{Ca}^{2+}$ Content.** Yeast cultures were processed according to a previous report (Eide et al. 2005; Manohar et al. 2010). Briefly, Yeast cultures were inoculated in 5 ml YPD and 1/100 volume of 100X 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 Scientific, Pittsburgh, PA). Cells were washed three times with 1 ml of 1 µM ethylenediaminetetraacetic acid disodium salt solution (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 48 h ICP-AES analysis (Lahnar et al. 2003).

**Plant Materials, Transformation, and Growth Conditions.** The CAX3, sCAX3, and $\Delta 63$-CAX3 cDNAs were subcloned into the 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 (Hirschi 1999).

**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 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 (Fig. 2.1). Considering the sequence conservation among all CAXs, we speculate that the secondary structure of CAX1 is similar to CAX3 (Shigaki & Hirschi 2006). However, this assumption does not preclude 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 removing 57 amino acids, some CAX3 truncations (N-terminal 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 (Fig. 2.2). 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-CAX3), demonstrated suppression of the Ca\(^{2+}\) sensitivity of the yeast cells (Fig. 2.2). The assay of yeast growth on high Ca\(^{2+}\) medium was similar to the results when the cells were grown in liquid
media containing high Ca$^{2+}$ concentrations (data not shown). However, these assays do not allow us to make precise comparisons of Ca$^{2+}$/H$^+$ antiport activity.

**Fig. 2.1. 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/](http://www.cbs.dtu.dk/services/TMHMM/)) is indicated by red vertical bars. Blue line and magenta line indicate inside (cytosol) and outside (vacuolar lumen) location, respectively. The positions of truncation for Δ36 and Δ90 are also indicated. Figure by Toshiro Shigaki.
Fig. 2.2. Suppression of Ca\(^{2+}\) sensitivity of the Yeast Mutant by CAX3 Deletion Constructs. Saturated liquid cultures of pmcl vcx1 enbl 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 48 hour incubation at 30°C. Figure by Toshiro Shigaki.
To monitor expression levels in yeast, both CAX3 RNA and protein levels were measured. RT-PCR analysis established differences in CAX expression levels in yeast cells expressing CAX3, sCAX3, and Δ63-CAX3, Δ64-CAX3 and Δ90-CAX3 (Fig. 2.3A). Variation in CAX3 RNA abundance among these cells was also confirmed by northern blot using a CAX3 specific probe (Data not shown). 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 (Pittman & Hirschi 2001). The protein levels were different among the strains expressing the different constructs (Fig. 2.3B) but did not correlate with activity levels as high level expression was seen in yeast cells expressing CAX3 (inactive) and Δ90-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 inductively coupled plasma atomic emission spectrometry (ICP-AES) (Eide at al. 2005; Manohar et al. 2010). ICP data may be correlated with the data from yeast tolerance assays and the uptake of \(^{45}\text{Ca}^{2+}\) into vacuolar-enriched microsomes (Mei et al. 2007). When yeast mutant cells were grown in media supplemented with Ca\(^{2+}\), the expression of CAX3 variants caused Ca\(^{2+}\) content to increase over four-fold when compared to yeast cells expressing the vector, CAX3, or sCAX3 (Fig. 2.4). Of note, Δ63-CAX3 accumulates more Ca\(^{2+}\) than Δ90-CAX3 in 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).

Fig. 2.3. RT-PCR and Western Blot Showing Relative Levels of CAX3, sCAX3, Δ63-CAX3, Δ64-CAX3 and Δ90-CAX3. (A) RT-PCR was performed on total RNA extracted from the yeast strains expressing CAX3, sCAX3, Δ63-CAX3, Δ64-CAX3, Δ90-CAX3 using specific primers against Δ90-CAX3. Actin primers were used as a control. (B) Western blot analysis was performed on vacuolar enriched protein extracted from C-terminal triple-HA-tagged full-length CAX3, sCAX3, Δ63-CAX3, Δ64-CAX3 or Δ90-CAX3. Twenty micrograms of microsomal samples were separated by SDS-PAGE, blotted, and subjected to Western blot analysis using a monoclonal antibody reactive to hemagglutinin. The exposure time used for Δ90-CAX3 expression is five fold faster than the other CAX variants.
The resemblance of CAX3 to CAX1 and the finding that suppression of the \textit{S. cerevisiae} mutant by expression of CAX3 variants implies that CAX3 is a bona-fide cation exchanger. In the yeast based \ce{Ca^{2+}} suppression assays, \(\Delta 90\text{-CAX3}\) mediated phenotypes appear weaker than those mediated by \(\text{sCAX1}\) expression (Fig. 2.5A). To test this directly, endomembrane-enriched vesicles were purified from \(\text{sCAX1}\) and \(\Delta 90\text{-CAX3}\) transformed K667 yeast cells and their capacity for pH-dependent \ce{Ca^{2+}} uptake was examined. Addition of MgCl$_2$/ATP and establishment of a steady-state pH by the V-ATPase associated with the vacuolar membrane followed by the addition of \(^{45}\ce{Ca^{2+}}\) resulted in uptake into endomembrane vesicles from \(\Delta 90\text{-CAX3}\) expressing K667.
vesicles. The Δ90-CAX3 expressing K667 had approximately 50% of the uptake of sCAX1 expressing vesicles, which further suggest that this CAX3 variant has less Ca\(^{2+}/H^+\) activity than sCAX1 (Fig. 2.5B). In Δ90-CAX3 expressing K667 vesicles, the inclusion of gramicidine (an uncoupler of the proton gradient) in the uptake medium decreased \(^{45}\)Ca\(^{2+}\) uptake to a level similar to that seen in the absence of MgCl\(_2\)/ATP as described previously (Hirschi et al. 1996). 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, bafilomycin as previously described (Pittman & Hirschi 2001). These results, together with the release of \(^{45}\)Ca\(^{2+}\) observed with the addition of the Ca\(^{2+}\) ionophore A23187 to Δ90-CAX3 vesicles, versus the small increase observed in \(^{45}\)Ca\(^{2+}\) uptake with vector control vesicles, demonstrate that Δ90-CAX3-generated uptake is concentrative (Fig. 2.5B). To further analyze the transport of Δ90-CAX3, Michaelis-Menten kinetic analysis was performed. A pH dependent \(^{45}\)Ca\(^{2+}\) transport in yeast endomembranes expressing Δ90-CAX3 over the range of 0 to 100 µM of Ca\(^{2+}\) demonstrated a Km value for the transporter of 14.01 ±2.8 µM (Fig. 2.5C).

To analyze the substrate specificity of Δ90-CAX3, competition experiments with yeast strains expressing Δ90-CAX3 were performed. This approach allowed us to compare and contrast sCAX1 and Δ90-CAX3 transport, pH-dependent 10 µM \(^{45}\)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 the \(^{45}\)Ca\(^{2+}\) uptake determined in the presence of two concentrations (10X and 100X) of various
nonradioactive metals (Fig. 2.5D). Inhibition of $^{45}\text{Ca}^{2+}$ uptake by nonradioactive Ca$^{2+}$ was used as an internal control, and as expected, $^{45}\text{Ca}^{2+}$ uptake was strongly inhibited by excess Ca$^{2+}$; however, the 10X concentration did not completely inhibit $^{45}\text{Ca}^{2+}$ uptake, highlighting the low Ca$^{2+}$ affinity of the CAX transporters. Like, sCAX1, ∆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, ∆90-CAX3 transport showed little inhibition by Mn$^{2+}$. Furthermore, ∆90-CAX3 did not show significant inhibition to any monovalent ion tested (Li$^+$, Na$^+$ and K$^+$).

Transgenic Tobacco Plants Expressing CAX3 Variants Displayed 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 (∆63) and compared this to CAX3 and sCAX3. If active, we envisioned phenotypes similar to sCAX1 expression including stress sensitivities and Ca$^{2+}$ deficiency like symptoms (Hirschi 1999). We generated at least eight independent lines of tobacco expressing the empty vector, CAX3, sCAX3, and ∆63-CAX3 and DNA integration and gene expression were confirmed (Fig. 2.6A B). Ninety-two percent (34 out of 37 plants) of the plants expressing empty vector, CAX3, or sCAX3 grew normally. However, all plants (n=8) expressing ∆63-CAX3 displayed stunted growth as seedlings (Fig. 2.7) and none of these lines survived to produce flowers and seeds.
Fig. 2.5. Phenotypes of Yeast Cells Expressing CAX3 Transporters. (A) Suppression of Ca\(^{2+}\) sensitivity in yeast mutant cells that are defective in 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 media. This picture was taken after 3 days of incubation at 30° C. The sCAX1 indicates a 36 amino acid truncation from the N-terminal half of the CAX1. Similarly, the Δ90- indicates amino acid truncations from the N-terminal half of CAX3. (B) Time course of \(^{45}\)Ca\(^{2+}\) uptake into vacuolar vesicles prepared from the yeast strain K667 expressing sCAX1 or Δ90-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. This data is 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 Δ90-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 concentrations. The data is representative of three independent experiments. (D) Inhibition of Ca\(^{2+}\) uptake by Δ90-CAX3 into yeast vacuolar enriched vesicles in the presence of other metals. Uncoupler sensitive (ΔpH-dependent) uptake of 10 µM \(^{45}\)Ca\(^{2+}\) was measured in the absence (control with 100% activity shown with broken line) or presence of 10X or 100X 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 form two independent membrane preparations, and the bars indicates S.E.
Fig. 2.6. Genomic DNA Integration and Expression of Different Truncations of CAX3 in Tobacco. (A) Genomic DNA integration was confirmed by PCR analysis. PCR was performed on total DNA extracted from the CAX3, sCAX3 and $\Delta 63$-CAX3 using CAX3 specific primers. (B) Transcript levels were detected by RT-PCR analysis. RT-PCR was performed on total RNA extracted from the tobacco plants expressing CAX3, sCAX3 and $\Delta 63$-CAX3 using CAX3 specific primers. Integration and expression of three representative lines from different truncation has been shown.
Discussion

Regulatory elements must often be removed before protein activities can be measured (Manohar et al. 2011). In this report, we have made a series of N-terminal CAX3 deletions beyond the N-terminal ~40 amino acid NRR. Yeast strains expressing CAX3 variants lacking at least the first 57 amino acids displayed some apparent Ca$^{2+}$ transport (Fig. 2.2). Of note, deletions beyond the first 57 amino acids appeared to have

![Fig. 2.7. Tobacco Seedlings (Cv. K14) Ectopically Expressing (A) CAX3, (B) sCAX3, or (C) Δ63-CAX3 Under the Control of CaMV 35S Promoter. These plants are representative of at least eight independent transgenic lines. Figure by Sunghun Park and Joy Marshall.](image)
increased CAX activity based on these yeast growth assays. An interesting result from this study was that the TM1 of CAX3 was not required for Ca\(^{2+}\) transport (Fig.2.1) TM1 of CAXs have no apparent homology with other related transporters, such as NCX (Na\(^+\)/Ca\(^{2+}\) antiporters) and NCKX (K\(^+\)-dependent Na\(^+\)/Ca\(^{2+}\) antiporters) (Cai & Lytton 2004). TM1 is not part of the symmetrical arrangement of these transporters as symmetry is found between TM2-TM6 and the TM7-TM11 region (Fig 2.1). Recently, it was hypothesized that antiporters evolved through a duplication of half-sized progenitors which act as a dimer arranged in an antiparallel topology (Rapp et al. 2007). 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 & colleagues (2002) reported a CAX2 variant that lacks the first TM can still transport both Ca\(^{2+}\) and Mn\(^{2+}\) (Schaaf et al. 2002). In the future it will be interesting to test the transport of other CAXs when TM1 is 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 as small as 10 amino acids in the N-terminus activate transport (Pittman & Hirschi, 2001), 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 (Cheng & Hirschi 2006; Cheng et al. 2004a; Cheng et al. 2004b). The difference in the sequences and the lengths of NRR may account for these differences in regulation. While CAX1 is predominately expressed in leaves, CAX3 is expressed mostly in roots (Cheng et al. 2005). Our present
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 (Leonhardt et al. 2004; Cheng et al. 2005). 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 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 CAX1) (Shigaki et al. 2001). 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, $^{45}$Ca$^{2+}$ transport mediated by ∆90-CAX3 was inhibited by 100X concentration of Cd$^{2+}$, Zn$^{2+}$, Fe$^{2+}$ and Co$^{2+}$ (Fig. 2.5D). In fact, some studies in transgenic plants demonstrate CAXs can transport Cd$^{2+}$ (Korenkov et al. 2007a, b). The inability of monovalent cations to inhibit Ca$^{2+}$ transport suggests that ∆90- CAX3 does not transport these metals. Previous work *in planta* suggests CAX3 may transport Na$^+$ or Li$^+$ but these results in 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 because of its indirect effects on P-ATPase or V-ATPase activity (Zhao et al. 2008).

The role of transporters across the tonoplast in maintaining Ca$^{2+}$ homeostasis depends on their kinetic properties. CAX1 is a high capacity and low affinity transporter with a Km
value between 10-15 µM (Shigaki & Hirschi, 2006). Our data here suggest that Δ90-CAX3 has a Km value of approximately 14.01 µM. As mentioned previously, a lingering question from our studies is how well does this variant of CAX3 represent activated CAX3 transport in planta?

While Δ63-CAX3 expression in yeast phenocopies aspects of sCAX1 expression, the phenotypes in planta suggest differences among the activated transporters. Genomic DNA integration and expression in tobacco plants was confirmed by PCR and RT-PCR, respectively (Fig. 2.6). Ectopic expression of Δ63-CAX3 in planta caused severe tip burning and stunting like sCAX1-expressing lines; however, these Δ63-CAX3 expressing lines displayed more dramatic alterations in growth and failed to make viable seeds (Fig. 2.7). These phenotypes may cause altered compartmentalization of several different nutrients (Conn et al. 2011). In order 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 (Fig. 2.2). For example, Δ57-CAX3 expressing cells displayed some growth on Ca^{2+} containing media and expression of this variant in planta may cause less severe growth defects. This milder activated variant of CAX3 may aid in 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 (Fig. 2.5). Analysis of loss-of-function mutants demonstrate 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 (Conn et al. 2011). 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 (Conn et al. 2011).

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 (Shigaki & Hirschi 2006). 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 (Morris et al. 2008). However, excessive sequestration of Ca\(^{2+}\) may produce undesirable phenotypes for agronomic applications (Park et al. 2005) and attenuated CAX1 activity may be important for tolerance to serpentine soil (soils with a low Ca\(^{2+}\):Mg\(^{2+}\) ratio) (Bradshaw 2005). Therefore, modulated transport is necessary under specific environmental conditions. Here we have demonstrated that N-terminal truncation of CAX3 generate 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 (Fig. 2.3).

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. By removing 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 an Arabidopsis Zn\(^{2+}\)/H\(^{+}\) exchanger AtMTP1 stimulates transport (Kawachi et al. 2008). Alternatively, if one is looking to
temper activity, a transporter may be modulated by inserting an appropriate loop between two TMs.

**Conclusions**

In conclusion, 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.
III. EXPRESSION AND PURIFICATION OF CAX PROTEINS FOR X-RAY CRYSTALLOGRAPHIC ANALYSIS

Introduction

Calcium transporters localized across the various membranes play an important role in maintaining both spatial and temporal calcium concentrations (Manohar et al. 2011). Cation/Ca\(^{2+}\) exchangers (CaCAs) are an essential component of Ca\(^{2+}\) signaling pathways and function to transport cytosolic Ca\(^{2+}\) across membranes against its electrochemical gradient utilizing the gradient of H\(^+\), Na\(^+\), or K\(^+\) generated by primary transporters (Cai & Lytton 2004). Cation/Ca\(^{2+}\) exchangers are found in archaea, bacteria, fungus, plants and animals. All CaCA proteins share a similar topological model: 10 to 11 transmembrane (TM) domains are predicted to form a functional Ca\(^{2+}\) transporter. All CaCA proteins have two conserved clusters of hydrophobic domains, designated as the \(\alpha-1\) in TM helices 2-3 and \(\alpha-2\) in TM helices 7-8 (Shigaki & Hirschi 2006). Based on functional and bioinformatics studies, CaCAs are classified into five sub families: YRBGs (found in prokaryotes), cation/H\(^+\) exchangers (CAXs), Na\(^+\)/Ca\(^{2+}\) exchangers (NCXs), K\(^+\) dependent Na\(^+\)/Ca\(^{2+}\) exchangers (NCKXs) and cation/Ca\(^{2+}\) exchanger (CCXs) (Cai & Lytton 2004). The size of the deduced transporters varies from approximately 300-1000 amino acids.

CAXs are a group of proteins that export cations out of the cytosol to regulate cytosolic ion homeostasis. Recent genome sequencing projects suggest the presence of CAXs in a range of taxa; however, CAXs are absent from the genomes of mammals and
insects (http://www.ncbi.nlm.nih.gov/sites/genome). The reason for the absence and presence of CAXs in various taxa offers an interesting target for pharmacological studies. For example, specific drugs designed against pathogenic bacteria or fungal CAXs would not affect a mammalian host. Furthermore, a putative mitochondrial $\text{Ca}^{2+}/\text{H}^+$ transporter (PfCHA) from the human malaria parasite *Plasmodium falciparum*, has also been characterized (Rotmann et al. 2010). Currently in the absence of an efficient vaccine, the medical treatment of malaria depends on the use of drugs; however, the information obtained from three dimensional structures of CAXs will certainly provide insight into future structure based drug design against malaria parasite.

Altering residues in a protein helps infer the role of specific amino acids or motifs; however, the structure of a protein is required to unravel deeper insights into the relationship between structure and its function. *Arabidopsis* has six CAXs (CAX1-CAX6) and their functional analyses have identified specific domains involved in cation specificity (Shigaki & Hirschi 2006). Chimeric analysis of *Arabidopsis* CAX transporters based on switching desirable amino acid domains between CAX1 and CAX3 has identified a nine amino acid region, termed the $\text{Ca}^{2+}$ domain (CaD), essential for $\text{Ca}^{2+}$ transport in AtCAX1. Similar approaches by switching domains between CAX1 and CAX2 have identified a three amino acid region from AtCAX2, which is found to be critical for $\text{Mn}^{2+}$ transport (Shigaki et al. 2005). Aside from these domains, other studies have also delineated additional regions required for substrate specificity (Shigaki & Hirschi 2006). Several single amino acid residues, such as His$^{338}$ in AtCAX1 and His$^{330}$ in OsCAX1a, are critical in $\text{Ca}^{2+}$ transport. Furthermore, when His$^{338}$ is replaced to Asn,
the variant transporter has increased affinity for Zn$^{2+}$ and Cd$^{2+}$ (Shigaki et al. 2005). The wide substrate range and the ability to alter substrate specificity of the CAX proteins demonstrate its potential in nutrient mining and phytoremediation (Manohar et al. 2011). Numerous studies have also implicated altered CAX activity to be involved in plant responses to biotic and abiotic stresses (Park et al. 2005; Ho & White, 2005; Kamiya et al. 2005, 2006; Zhang et al. 2009; Freitas et al. 2010; Conn et al. 2011). We envision that the knowledge obtained from the three dimensional structure of CAXs can be used to manipulate nutrient partitioning to positively impact production in agriculture.

During the last few decades, structure determination of soluble proteins has become routine; however, understanding the structure of membrane proteins remains a challenging task. (Baker 2010; Bill et al. 2011). There are four methods for determining three dimensional structures: X-ray crystallography, atomic force microscopy, nuclear magnetic resonance, and electron diffraction (Leviatan et al. 2010). X-ray crystallography is currently the most widely used technique to solve the structure of membrane proteins. The major bottlenecks in determining structure of membrane proteins by x-ray crystallography are getting sufficient amounts of stable purified protein and obtaining its well ordered crystals (Roosild et al. 2005; Bill et al. 2011). As a result, membrane structural biology is lagging some 20-30 years behind the study of soluble proteins (Baker 2010) and the structure of only a small number of membrane proteins have been determined. The natural abundance of most of the membrane proteins is usually not sufficient for structural studies; therefore, sufficient amount of membrane proteins must be obtained by heterologous expression. The expression of membrane
proteins in hosts such as *E. coli*, leads to challenges at all stages, including its expression, membrane incorporation, solubilization, purification and crystallization. Therefore, every step must be addressed by protein specific optimization of the process. Our work here is designed to address the large scale protein purification and production of crystals to provide structural models of CAX proteins.

**Materials and Methods**

**Cloning and Expression.** The open reading frames for full length and N-terminal truncated CAX proteins were PCR amplified and cloned into pET28a (Novagen, Billerica, MA), a bacterial expression vector, to add a hexa-His tag at the N-terminus, and sequences were verified (Table 1). The different CAX constructs were expressed in BL21 (DE3) or BL21 (DE3) harboring pTif6 chaperone (Takara Bio Inc. Mountain view, CA) *E. coli* bacterial strains. Freshly transformed bacterial cells were grown in Luria-Bertani medium at 37°C until OD<sub>600nm</sub>=0.6 was reached. The temperature was then lowered to 25°C and protein expression was induced by addition of 0.5 mM IPTG (Isopropyl-β-D-thio-galactoside), followed by overnight incubation. Bacterial cells were lysed by three sonication cycles 20 seconds each with 1 second pulse. Protein expression was analyzed by immunoblotting using a monoclonal anti-His tag antibody (Fisher Scientific, Pittsburgh, PA).

**Purification of CAX Proteins.** Protein purification was performed from *E. coli* strains BL21 (DE3) or BL21 (DE3) harboring pTif6 (Takara Bio Inc. Mountain view, CA) in auto-induction medium (Studier et al. 2005). Freshly transformed cells were inoculated to 12 liter of auto-induction medium (Studier 2005) and grown to 37°C for 3
hr followed by overnight growth at 25°C for protein production. Bacterial cell pellets were harvested by centrifugation and suspended in 200 ml of lysis buffer (50 mM Potassium Phosphate pH 7.8, 500 mM NaCl, 20 mM imidazole and 10% glycerol). Cells were disrupted using a high pressure homogenizer (Avestin Inc. Canada). Unbroken cells and debris are removed. The supernatant was centrifuged at 40,000 rpm for 60 min at 4°C to collect a membrane enriched pellet, which was then resuspended in lysis buffer (10 ml buffer/g of pellet). Membrane protein was solubilized by adding 1% Foscholine-12 (n-Dodecylphosphocholine) or LDAO (n-Dodecyl-N,N-Dimethylamine-N-Oxide) (Anatrace, Maumee, OH). Unsolubilized membranes were removed by centrifugation at 40,000 rpm for 25 min at 4°C. The solubilized fraction was incubated with nickel-nitrilotriacetic acid (Ni-NTA) (GE healthcare, Piscataway, NJ) or TALON resin (Clonetech, Mountain view, CA) for 2 hr at 4°C with constant agitation. The resin was then washed with 50 volumes of lysis buffer containing 0.05% of detergent. Proteins were eluted from resin using the lysis buffer containing 0.05% detergent and 400 mM imidazole. The protein was concentrated by Centricon (Millipore, Billerica, MA) and further purified by size exclusion chromatography (SEC) using a 24 ml analytical Superdex 75 10/300GL column (GE Healthcare, Piscataway, NJ).

Reconstitution of CAXs in Proteoliposome. Liposomes are prepared from soybean lipid, azolectin (Sigma, St. Louis, MO). 5 mg of soybean lipid was washed with 1 ml cold diethyl ether and dried under argon gas and for additional 30 min under vacuum. The dried thin layer of lipid was suspended in 178 µl of a reconstitution buffer (200 mM NH₄Cl, 50 mM potassium oxalate, 50 mM Tris-HCl, pH 8.0, 1mM
dithiothreitol and 10% glycerol) and sonicated to clarity. The liposome was then solubilized by the addition of 0.5% octylglucoside (OG) (Anatrace, Maumee, OH). The external divalent cations from the purified protein was removed by treating with 10 mM EDTA (ethylenediaminetetraacetic acid) followed by gel filtration and reconstituted into clear lipid by mixing protein and lipid in a ratio of 1:500 (up to 1:20). Protein-phospholipid mix was rapidly injected into 20 ml of reconstitution buffer (without DTT) at 22°C. The solution was mixed again by drawing liquid and rapidly injecting by Pasteur pipet and incubated for 20 min at 23°C. After incubation, proteoliposomes are collected by centrifugation for 1 hr at 4°C at 22,500 rpm and the pellet was suspended in storage buffer (Tris-HCl pH 8.0, 200 mM NH₄Cl) and used to perform Ca²⁺ transport assays. The proteoliposomes are diluted to 200 fold in transport assay buffer (200 mM BTP-HCl pH 7.5, 50 mM Tris-HCl pH 8.0, 5 µM quinacrine (6-chloro-9-[(4-(diethylamino)-1-methylbutyl] amino)-2-methoxyacridine). Diluted proteoliposomes are allowed to reach steady state with respect to the pH gradient before addition of putative substrates (cations). The cation/H⁺ exchange activity was measured by metal dependent recovery of quinacrine fluorescence as described previously (Shigaki et al. 2005). Fluorescence was monitored in a thermostated cell at 25°C using a fluorescence spectrometer with excitation and emission wavelengths of 430 and 500 nm respectively, both with a slit width of 5 mm. As described previously (Shigaki et al. 2005), the rate of fluorescence recovery was directly proportional to proton flux. Thus, initial rate of fluorescence recovery represents the initial rate of proton-dependent metal transport. Additionally, radioactive Ca²⁺ was used to measure transport by addition of ⁴⁵Ca²⁺ at 50
µM final concentrations. At various times after addition of $^{45}\text{Ca}^{2+}$, 100 µl of reaction mixture were filtered on 25 mm nitrocellulose filter (0.45 µM pore size, Millipore, Billerica, MA) and washed with 5 ml of the wash buffer (50 mM potassium phosphate pH 8.0, 200 mM NaCl, 1 mM DTT). The filters were dried, and the radioactivity was measured by liquid scintillation spectrometry.

**Preparation of Inverted Bacterial Vesicles and Transport Assays.** Inverted membrane vesicles were prepared by a method described previously with slight modification (Rosen & McClees 1974). In brief, bacterial cultures were harvested in mid-log phase by centrifugation. The bacterial cells were first washed with buffer A (10 mM Tris-HCl, pH 7.3, 140 mM KCl, 0.5 mM DTT and 250 mM sucrose) and then resuspended to five volumes per gram of cell pellet. The cells were lysed by single passage through a high pressure homogenizer (Avestin Inc. Canada) at 3000-4000 kpsi. The debris and unbroken cells were removed by centrifugation at 15,000 rpm for 15 min. The supernatants were collected and subjected to high speed centrifugation at 55,000 rpm for 90 min. The membrane pellet was collected and suspended in buffer A to a final concentration of 2-3 mg/ml of protein. The vesicles were flash-frozen in liquid nitrogen and stored at -80°C. For transport assays, membrane vesicles were thawed and diluted 10-fold in assay buffer containing 10 mM Tris-HCl, pH 8.0, 140 mM KCl, 250 mM sucrose and 5 mM potassium phosphate. After 15 min incubation at 23°C, NADH (reduced nicotinamide adenine dinucleotide) was added to 5 mM final concentration, followed by immediate addition of $^{45}\text{Ca}^{2+}$ to 50 µM final concentrations. At various times after addition of $^{45}\text{Ca}^{2+}$, 100 µl of reaction mixture were filtered on 25 mm
nitrocellulose filter (0.45 µM pore size; Millipore, Billerica, MA) and washed with 5 ml of the assay buffer. The filters were dried, and the radioactivity was measured by liquid scintillation spectrometry.

**Isothermal Titration Calorimetry (ITC).** The external divalent metals from the purified protein were removed by treating with 10 mM EDTA and by subsequent gel filtration. Protein activity was determined by heat released or absorbed by VP-ITC (Microcal, Piscataway, NJ) after injecting 5 or 10µl of 500µM-1mM metals with 25 or 50 repetitions.

**CAX Protein Crystallization Screening.** The initial screen was performed in 96 well plates using the sitting-drop vapor diffusion method at 18°C (Wu et al. 2010). CAX protein was subjected to gel-filtration using buffer containing 5 mM Tris-HCl pH 7.8, 200 mM NaCl, 10% glycerol and 0.05% Fos-choline-12, and the peak protein fraction was collected and concentrated by Centricon (Millipore, Billerica, MA) to 8 mg/ml protein. CAX crystal formation was screened manually under compound microscope.

**Results**

**Screening of CAX Proteins for Expression.** We have concentrated our efforts on overexpressing multiple eukaryotic CAX transporters because expression of almost identical proteins can vary significantly. Additionally, it is often necessary to screen a variety of hosts for expression (Wagner et al. 2006). Therefore, thirteen CAX proteins with 11 transmembrane spanning domains were initially chosen for cloning into the *E. coli*, *Saccharomyces cerevisiae* and *Pichia pastoris* expression vectors. Because full
length CAX proteins are autoinhibited, active CAXs were also cloned into the appropriate vectors (Manohar et al. 2011).

Table 1. Bacterial Expression of CAX Proteins. His-tag was fused in frame with CAX protein at N-terminus. Expression was scored by estimating the western-blot using anti-His antibody HRP conjugate. No expression detected by western-blot was labeled with “N”.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Organism</th>
<th>Fused with hexa-His tag at N-terminus (in pET28a)</th>
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<td></td>
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<td>T33A-AtCAX1</td>
<td><em>Arabidopsis thaliana</em></td>
<td>Y/Y</td>
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<td>S25D-AtCAX1</td>
<td><em>Arabidopsis thaliana</em></td>
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Following the cloning of multiple CAX proteins into the different expression vectors, small scale expression experiments were performed with *E. coli* because it is the
most widely used host for membrane protein expression and purification. Expression levels of CAX proteins are analyzed by Western blotting (Fig. 3.1).

Out of thirteen CAXs analyzed, at least five of them, AtCAX1, CbCAX51, VCAX1, LeCAX1 and OsCAX1a, yielded detectable expression. This suggests that screenings multiple proteins are necessary to identify a protein and expression system suitable for functional and structural studies.

**Fig. 3.1.** Detergent Screening, Solubilization and Purification of CAX Protein Using Fos-Choline-12. (A). Detergent solubilization screening for CAX protein. P, Pellet; S, Supernatant. (B). Detergent solubilization and purification of CAX protein using Fos-choline-12: CL, Cell lysate; P, Pellet; S, Supernatant; FT, Flow through; E, Elution.
Optimization of CAX Protein Detergent Solubilization. Native membrane environments provide stability to the membrane proteins through its hydrophobic interactions. Since, the lipid bilayer is required for proper folding and functioning of the membrane proteins, solubilization in aqueous media often leads to rapid denaturation. However, membrane proteins can be extracted from host cell membranes by addition of
detergents, which cover the hydrophobic transmembrane spanning domains, allowing its solubilization (Carpenter et al. 2008). For each CAX protein that was successfully expressed in bacteria, we tested whether the proteins could be solubilized by the commercially available detergents. Seven relatively mild detergents were tested for efficiency of CAX protein extraction. Most of the detergents we tested have previously been used successfully in membrane protein solubilization and crystallography. Western blot analysis of solubilized and unsolubilized fractions indicated that Fos-Choline-12 successfully extracted most of the CAX protein from the *E. coli* membrane (Fig. 3.1). Note that LDAO also extracted CAX proteins from the bacterial cell membrane; however, proteins are stable only for a short time. To obtain large amounts of protein, the initial purification and solubilization steps were done mostly by using Fos-Choline-12. Purified CAX proteins maintained their stability and solubility throughout size exclusion gel filtration, which enabled separation and purification from the heterogeneous mixture (Fig. 3.2). We were able to obtain 1-2 mg of purified, stable CAX protein per liter of *E. coli* bacterial culture. Protein purity was also assessed by SDS-PAGE using Coomassie Brilliant Blue stain. (Fig. 3.2).

**Activity Measurement.** *E. coli* is the most widely used host for overexpressing eukaryotic membrane proteins; however, the absence of eukaryotic protein processing components may hamper proper folding and insertion into the membrane. Absence of protein translocation machinery, the nature and composition of membrane lipids, and post-translational modifications may be crucial for certain proteins. Additionally,
crystallization of misfolded and or non-functional membrane proteins may be difficult. Considering these factors, establishing the activity of the CAX proteins is desirable.

**Fig. 3.3. Activity Measurement of CAX Protein.** (A). Isothermal titration calorimetry in the presence of 500 μM Cd²⁺. The thermodynamic changes were measured in the absence or presence of protein. Ligands were injected either 25 times, 10 μl per injection or 50 times, 5 μl per injection. 20 nM (1 mg) proteins were used in each experiment. (B). Time course of ⁴⁵Ca²⁺ uptake into vacuolar vesicles prepared from the bacterial strain BL21(DE3) expressing CAXs. Results are shown in the absence and presence of the protonophore FCCP and vector control. (C). Proton dependent metal transport in reconstituted CAX proteoliposomes. The reconstituted proteoliposomes are allowed to reach steady state with respect to pH gradient for a few minutes before addition of CdCl₂ (left) and CaCl₂ (right). Cation/H⁺ exchange activity was measured by metal dependent recovery of quinacrine until saturation. The pH gradient was then disrupted with triton X-100.
CAX protein activity was measured by three independent methods. 1). To measure the thermodynamic nature of CAX-ligand binding, 25 or 50 injections of ligand (divalent cations) solution were added to the purified CAX protein solution in the ITC (isothermal titration calorimetry) cylinder. The area under each injection peak (top panel) was measured which is equal to the total heat released for that injection. This integrated heat is plotted against the molar ratio of ligand added to the protein in the cylinder to obtain a complete binding isotherm for the interaction (bottom panel). The amount of heat released after ligand titration indicates that the purified CAX protein maintained the capacity to bind divalent cations during purification (Fig. 3.3A). 2). Inverted vesicles were prepared from CAX expressing *E. coli* bacteria, and their capacity for pH-dependent Ca$^{2+}$ uptake was examined. Addition of NADH (Nicotinamide adenine dinucleotide; Sigma, St. Louis, MO) and potassium phosphate established a steady-state proton gradient across the membrane, which resulted in $^{45}$Ca$^{2+}$ uptake into the bacterial vesicles. In CAX expressing vesicles, the inclusion of FCCP (carbonylcyanide p-trifluoromethoxyphenylhydrazone, an uncoupler of the proton gradient) in the uptake medium decreased the rate of uptake of $^{45}$Ca$^{2+}$ to a level similar to that seen in the vector control (Fig. 3.3B). 3). Dilution of proteoliposomes in reconstitution buffer without NH$_4$Cl resulted in the instantaneous generation of pH gradient across the liposome. Ca$^{2+}$/H$^+$ antiport activity was initiated upon the addition of Ca$^{2+}$ and rate of transport was measured by quinacrine fluorescence recovery (Fig. 3.3C). Additionally, the transport activity was measured by the addition of $^{45}$Ca$^{2+}$ in the assay medium which resulted into the accumulation of $^{45}$Ca$^{2+}$ in the sealed proteoliposomes. The inclusion of triton X-100
in the uptake medium disrupts the proteoliposome and brings both quinacrine fluorescence and Ca\textsuperscript{2+} accumulation to the base level. These preliminary results, together with Ca\textsuperscript{2+} uptake by bacterial vesicles and substrate binding in ITC, demonstrate that CAX protein is functional in bacterial expression system and maintains correct folding and activity during the purification process. More extensive studies are required to verify the activity of purified CAX proteins.

Discussion

The determination of the high-resolution structure of eukaryotic membrane proteins is challenging because of the technical difficulties at each step, from expression to structural solution. For successful structural studies of a membrane protein, hundreds of milligrams of purified protein are necessary. Only a few membrane proteins are abundant in their native membranes at such high levels. Examples include such as mammalian and bacterial rhodopsin, aquaporins, respiratory complexes, ATPase, photosynthetic complexes, reaction centers and light harvesting complexes (Bill et al. 2011). For other membrane proteins, heterologous expression is necessary; however, membrane proteins usually lose their three dimensional structure in such approaches. There are a number of factors that influence the success of heterologous expression of eukaryotic membrane proteins (Carpenter et al. 2008). The choice of a host organism is appear to be the most crucial for membrane protein expression. Better understanding of the host organisms and modify them to suit requirement to express specific membrane protein is an emerging trend for achieving the high yield for structural studies (Bill et al. 2011).
Delineating the structure of CAX transporters will provide valuable insights into the transporter’s role in Ca\(^{2+}\) signaling, biotic and abiotic stresses. Herein, we have demonstrated that eukaryotic Ca\(^{2+}/H^+\) exchanger can be successfully expressed and purified from \textit{E. coli} expression system. Moreover, we have shown that the amount of purified protein obtained from \textit{E. coli} is enough for functional and structural determination. Our preliminary activity assay with inverted bacterial vesicles and purified protein suggests that CAX protein is correctly folded and incorporated in the cell membrane; however, multiple replications with independently purified proteins are required to support these preliminary activity data. In our initial crystallization trails, the purified protein precipitated lightly to heavily (40-60\%) and only 20-30\% crystallization conditions tested resulted in clear drops (no precipitation). Further optimization of the crystallization condition is necessary. These observations indicate that correctly folded, soluble and stable CAX protein can be produced through bacterial expression system.

**Conclusions**

CAX proteins overexpressed and purified from \textit{E. coli} can be used for transport activity and crystallization experiments. These promising results may allow great progress in structure-function studies for Ca\(^{2+}\) transporters.
IV. SUMMARY

Plant Ca\(^{2+}\) levels are regulated in part by high-capacity vacuolar cation/H\(^{+}\) antiporters (CAXs). CAXs are integral membrane proteins that transport Ca\(^{2+}\) or other cations by using proton gradients. Analysis of CAX transporters in plants revealed their roles in myriad physiological functions such as cell wall extensibility, stomatal aperture, growth rate and ionome content. *Arabidopsis* CAXs appear to contain an approximately 40- amino acid N-terminal regulatory region (NRR) that modulates transport through N-terminal autoinhibition. Our study demonstrated that AtCAX3, similar to previously characterized AtCAX1, has a unique, long N-terminal autoinhibitory domain. Truncation of the longer NRR revealed the functional and kinetic properties of CAX3. Although, the biological importance of CAXs is almost clear, the molecular mechanism by which CAXs mediate transport activities remains unclear. Delineating the structure of CAX transporters will provide valuable insight into the transporter’s role in Ca\(^{2+}\) signaling, biotic and abiotic stress. To provide structural framework of CAXs, we are attempting to obtain CAX crystals for X-ray crystallography. The most successful structure predictions, known as homology modeling, rely on the existence of a "template" structure with sequence similarity to the protein being modeled. A solved structure of CAXs will provide a valuable three-dimension template for modeling of other membrane transporters.


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