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Calcium Homeostasis: Role of CAXs Transporters in Plant Signaling

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ABSTRACT

Calcium is an essential macronutrient as well as an ubiquitous second messenger, playing a pivotal role in plant growth and development. The neutral cytosolic pH, acidic apoplastic and vacuolar pH is maintained by synergistic action of different channels/transporters in plant cells. In the cytosol, a submicro-molar range of calcium is maintained for efficient biochemical and physiological functioning including calcium-mediated signal transduction. A concerted interplay of channels/transporters, mediating influx and efflux of ions across membranes, tightly regulates the concentration of calcium in the cytosol by sequestering extra calcium into vacuole. For calcium homeostasis, the pre-requisite is to balance and maintain high calcium level in cytoplasm during signaling events and subsequently counterbalanced after the removal of the signal. Hence, the major mechanism in plant cell for calcium homeostasis is redistribution of calcium and other cation in exchange for the H^+ generated by various H^+ pumps and antiporters. Calcium/cation antiporter (CaCA) superfamily consist of five families, one of them is CAX multigene family (H^+/*ca*tion e*x*changers). In last two decades, several studies have been reported involving discovery of biochemical, physiological and molecular characterization of CAX family members extensively. CAX proteins are mainly constituted in vacuolar membrane and responsible for maintaining low cytosolic Ca^{2+} and/or other cations against their concentration gradient in cells. CAX family play an important role in calcium signaling, ion compartmentalization, sequestering of essential and heavy metal ions in vacuole. CAXs could be agriculturally important to increase the calcium content in edible part of plant and sequester heavy metals from polluted soil. In this review, we are primarily elaborating the functional aspect of CAX protein family in calcium homeostasis and stress mediated signaling in plants.

Keywords: abiotic stress, antiporters, calcium, exchanger, signal transduction, transporters
Abbreviations: Ca^{2+}, calcium; **CaCA**, Ca^{2+}/cation antiporter; **CaD**, Ca^{2+} domain; **CaM**, calmodulin; **CAX**, Ca^{2+}/H^+ exchanger; **CBL**, calcineurin B-like protein; **CDPK**, Ca^{2+}-dependent protein kinase; **CIPK**, CBL-interacting protein kinase; **CrCAX1**, *Chlamydomonas reinhardtii CAX1*; **CXIP**, CAX interacting protein; **NRR**, N-terminal regulatory region; **sCAX**, short CAX; **SOS2**, salt overly sensitive 2; **TMD**, transmembrane domain; **VCAX1**, mung bean Ca^{2+}/H^+ antiporter

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INTRODUCTION

Calcium (Ca^{2+}) is a pivotal cation and major nutrient element, necessary for plant growth and development. Ca^{2+} maintains organic and inorganic ionic balance in cytoplasm and vacuole by various counter cation import and export mechanism (Marschner 1995). In soil, primarily Ca^{2+} enters in root through Ca^{2+}-permeable channels; located on plasma membrane, and transported to shoot via xylem either by apoplastic transport or plasmodesmata mediated symplastic transport (White 2000, 2003). The cytosolic Ca^{2+} concentration is the determining factor for Ca^{2+} uptake by roots from the soil. In the resting stage cells, cytosolic sub-micromolar Ca^{2+} concentration is required to regulate various metabolic and signaling pathways (Klusener *et al.* 1995; Blatt 2000; Sze *et al.* 2000; Harper 2001; Ritchie *et al.* 2002; Wu *et al.* 2002). In general, the cytosolic Ca^{2+} level (<0.1 µm) is maintained by synergistic action of various transporters in the plant cells. When plants are exposed to external stimuli such as biotic, abiotic, nutrient deficiency, or developmental cues, cytosolic calcium $[Ca^{2+}]_{cyt}$ level is increased to several hundred folds in comparison to resting stage of cells. It is thought that a unique 'calcium signature' or 'calcium wave' is generated to accomplish a specific physiological response elicited by a specific condition (Sander 2002). This specific 'calcium signature' may trigger a large number of genes, involved in different signaling pathways. In the cell, there is a plethora of networks and signaling components, which decode the calcium signature, generated in response to a particular stimulus. One

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of such mechanisms is the prevalence of calcium binding proteins, which act as calcium sensors such as calcineurin B-like proteins (CBLs), calmodulins (CaM), Ca^{2+}-dependent protein kinases (CDPKs) (Luan *et al.* 2002; Hrabak *et al*. 2003; Pandey 2008; Das and Pandey 2010; Pandey *et al.* 2010). These calcium binding proteins bind to calcium and transduce the signal further downstream by interacting or affecting a large number of effectors or responders to regulate calcium dependent signaling pathways.

In response to specific stimulus, $[Ca^{2+}]_{cyt}$ levels increase more than 10 µM near endo-membrane. This $[Ca^{2+}]_{cyt}$ burst is due to release of Ca^{2+} from intracellular organelles and efflux through Ca^{2+} channels from plasma membrane (Gilroy and Jones 1992; McAinsh and Hetherington 1998; White 2000; Sanders 2002). The synergistic action of Ca^{2+} transporters, present on plasma membrane (PM), endoplasmic reticulum (ER) and tonoplast, is important not only to maintain the resting level of $[Ca^{2+}]_{cyt}$ but also to transiently increase the Ca^{2+} level to initiate specific signaling events. For proper cell functioning, this $[Ca^{2+}]_{cyt}$ balance is maintained by both high and low affinity Ca^{2+} transporters (Ueoka-Nakanishi *et al.* 1999).

During $[Ca^{2+}]_{cyt}$ burst in the cell, CAXs (Ca^{2+}/H^+ exchangers) play an important role to maintain optimal Ca^{2+} level by sequestering it into vacuoles because CAXs are low affinity (Km = 10-15 µM), high capacities Ca^{2+} antiporters. This antiport activity brings cytosolic Ca^{2+} concentration below 10 µM, which is optimal concentration for functioning of Ca^{2+}-ATPases. The Ca^{2+}-ATPases, high affinity (Km = 1-10 µM), low capacity Ca^{2+} transporter, fine-tune $[Ca^{2+}]_{cyt}$ to maintain sub-micromolar concentration, approximately ≤ 0.1 µm in the cells (Evan and Williams 1998; Miseta *et al.* 1999; Ueoka-Nakanishi *et al.* 1999; Hirschi 2001). Therefore, Ca^{2+}-ATPases and CAXs maintain optimum $[Ca^{2+}]_{cyt}$ either by apoplastic export or by sequestering excess Ca^{2+} to the lumen of vacuole against electrochemical gradient in the activated cells (Hirschi 2000; Sze *et al.* 2000; Hirschi *et al.* 2001). The other function of H^+-pumps and antiporters is to maintain pH gradient across different organellar compartments. In plant cells, different organellar compartment has different pH, for example, vacuole maintains acidic pH (pH 3-5) while cytosolic pH is approximately neutral in plant cell (pH >7.0), which is maintained by various H^+-pumps and exchangers. The Ca^{2+}/H^+ antiporters exchange two H^+ per Ca^{2+} in yeast while three H^+ per Ca^{2+} into plant vacuole against proton gradient to maintain low level of calcium concentration in the cytoplasm (Blackford *et al.* 1990; Dunn *et al.* 1994; Pittman 2011).

This review focuses on *Arabidopsis* and other plant Ca^{2+}/H^+ antiporters or cation/H^+ exchangers (CAXs) by discussing the diversity, phylogenetic classification, structure, heterologous expression, functional complementation, biochemical characterization in yeast and physiological functions in plants.

DIVERSITY AND PHYLOGENETIC CLASSIFICATION OF CAXs

Ca^{2+}/cation antiporter (CaCA) superfamily consist of five related families (YRBG, NCX, NCKX, CCX and CAX). The YRBG, putative Na^+/Ca^{2+} exchangers, present in bacteria and Archea, while NCX, Na^+/Ca^{2+} exchanger is present in eukaryotes. The members of NCKX, K^+ dependent Na^+/Ca^{2+} exchangers are encoded by other eukaryotic genome but absent in land plants. The CCX, cation/Ca^{2+} exchangers, are encoded by eukaryotic genome and are also present in land plants (Emery *et al*. 2012). Besides five representative family of CaCA superfamiliy, recently Emery *et al*. (2012) identified a number of proteins by BLAST search having distinct but CAX like topology and sequence percentage homology. The amino acids alignment of these CAX like proteins with CAXs showed higher sequence similarity with C-ter half than N-ter half of CAXs. These CAX-like proteins, also called EF-CAXs, having one or more EF-hand type-Ca^{2+}-binding domains in central loop. These types of genes are presents in *Arabidopsis*, poplar, grape, bryophytes, pteridophytes and in many algal species but absent in bacteria, fungus and animal genomes (Emery *et al*. 2012). The functions of these novel CAX-like proteins are not yet known (Emery *et al*. 2012).

The CAX family is one of the five families of CaCA superfamily (Cai and Lytton 2004; Emery *et al*. 2012). In the last decade, many representative genomes from all kingdoms were sequenced and reveal the presence or absence of CAXs. CAXs are presents in various taxa including bacteria, fungi, most of animals and plants (Shigaki *et al*. 2006). In recent past, homologues of CAX were also reported in lower vertebrates such as *Xenopus*, zebrafish, pufferfish and amphibians while CAXs were found to be absent in genomes of *Archaebacteria*, *Caenorhabditis elegans*, *Drosophila* and higher vertebrates like mouse and human (Manohar *et al.* 2011). On the basis of phylogenetic analysis, CAXs are further divided into three types. Type I (similar to *Arabidopsis* CAXs), type II (similar to *Saccharomyces cerevisiae* VNX1) and type III (similar to *Escherichia coli* ChaA) (Shikagi and Hirschi 2006; Manohar *et al.* 2011). Numerous type I CAXs from plants and microorganisms have been functionally characterised, whereas single yeast type II CAX, VNX1p, has been characterised (Cagnac *et al.* 2007, 2010; Manohar *et al.* 2010). The VNX1p has ability to transport Na^+ and K^+ into the vacuole (Cagnac *et al.* 2007). Yeast VNX1p can also transport Ca^{2+} when other transporters are missing in yeast (Manohar *et al.* 2010). In higher plants, type II CAX is not present. The type III Ca^{2+}/H^+ antiporter, ChaA from *E. coli* also catalyzes direct Na^+/H^+ and K^+/H^+ antiport and provide resistance to high concentration of Ca^{2+}, Na^+ and K^+ in medium (Ivey *et al*. 1993; Radchenko *et al*. 2006; Cagnac *et al*. 2010).

CAX genes exist as multigene family in plants. Maser *et al.* (2001) identified 11 *CAX* genes in *Arabidopsis* genome. On the basis of transport function, Shigaki *et al.* (2011) proposed new classification to further divide the 11 *Arabidopsis* CAXs into two phylogenetic groups, CAX1-6 and CAX7-11. They proposed that only CAX1-6 transporters are bonafide CAXs because they have H^+/Ca^{2+} exchange activity. Whereas, CAX7-11 are classified into distinct phylogenetic groups and are more closely related to K^+-dependent Na^+/Ca^{2+}-exchangers. Rice genome encodes five bonafide CAXs as OsCAX1a-c, OsCAX2 and OsCAX3.

Arabidopsis type I CAXs are further divided into two distinct phylogenetic groups, type IA and type IB, based on distinct functional properties (Shigaki *et al.* 2006). Type IA CAXs (CAX1, CAX3 and CAX4) specifically transport Ca^{2+} while type IB CAXs (CAX2, CAX5 and CAX6) shows broader substrate specificity for divalent cation such as Ca^{2+}, Mn^{2+} and Cd^{2+} (Hirschi *et al.* 2000; Shikagi *et al.* 2003; Pittman *et al.* 2004; Cheng *et al.* 2005; Edmond *et al.* 2009). The biochemical characterization and physiological functions of *Arabidopsis* and other plant CAXs are discussed in following sections.

STRUCTURE OF CAXs

A typical plant CAXs have 11 transmembrane domain, N-terminal regulatory or autoinhibitory domain, Ca^{2+} domain (CaD), acidic motifs and cation selectivity filter, required for cation selection and transport (**Fig. 1**) (Shikagi and Hirschi 2006). In the yeast complementation library screening, Hirschi *et al.* (1996) have identified two expressed sequence tags (ESTs), which were able to suppress the yeast Ca^{2+} uptake deficient mutant K665, named as CAX1 and CAX2. The deletion of endogenous vacuolar PMC1 and VCX1 makes K665 yeast strain defective in vacuolar Ca^{2+} transport, rendering it unable to grow on high- Ca^{2+} containing media (Cunningham and Fink 1996). Fortunately EST of CAX1 encoding 447 amino acids was truncated protein lacking N-terminus autoinhibitory domain. This truncated protein, lacking N-terminus 36 amino acids, rescued

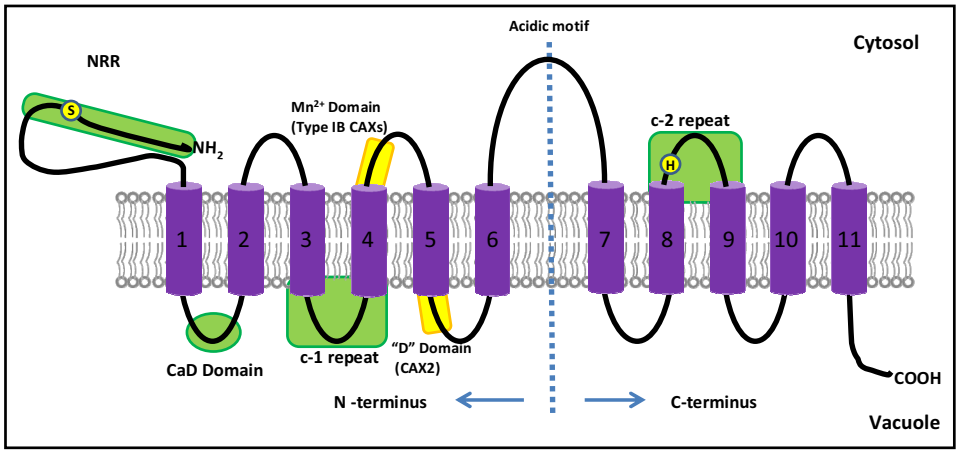

Fig. 1 Diagrammatic membrane topology of typical CAX. Typical CAX has 11 putative transmembrane helices (numbered 1-11 from N-terminus) with cytosolic N-terminal autoinhibitory domain and vacuolar C-terminal tail. This N-terminal regulatory domain interacts with different CXIPs and SOS2, which relieve autoinhibitory constrain and regulates CAXs. The green shaded regions represents the domains presents in typical CAXs. A negatively charged acidic motif divides CAXs into approximately two equal half as N and C-terminus halves.

the growth defect of K665 yeast strain while full length CAX1 ORF was unable to suppress the Ca^{2+} sensitivity of K667 (Hirschi *et al*. 1996; Pittman and Hirschi 2001). Thus, it confirmed that *Arabidopsis* CAXs have cytosolic, N-terminal autoinhibitory or regulatory domain. The VCX1 of *S. cerevisiae* does not appear to have N-terminal regulatory domain while most of the plant CAXs have N-terminal regulatory domain. N-terminal regulatory domain seems to be present in most of the *Arabidopsis* CAXs. *Arabidopsis* CAX1 (1-36), CAX2 (1-42) and CAX3 (1-36) showed a distinctly similar N-terminal regulatory region in CAXs sequence alignment (Pittman and Hirschi 2001). The N-terminal regulatory domain of VCAX1 (mung bean Ca^{2+}/H^{+} antiporter) does not repress Ca^{2+} transport activity in yeast mutant, which suggested possibly a different mechanism of regulation for Ca^{2+}/H^{+} transport in *Arabidopsis* (Ueoka-Nakanishi *et al.* 1999).

A highly polymorphic nine amino acid region between transmembrane (TM) 1 and TM2, named "Ca^{2+} domain" (CaD) is the determining region for Ca^{2+} transport and also cation transport specificity in plant CAXs (**Fig. 1**) (Shigaki *et al.* 2001; Shigaki and Hirschi 2006; Manohar *et al.* 2011). None of the nine amino acid of CaD domain of CAX1 (residue 87-95), CAX2 (47-55) and CAX3 (87-95) are identical and while flanking region of this domain are similar in corresponding aligned region. The Ca^{2+} domain is responsible of Ca^{2+} selectivity, supported by modification of CaD domain of CAX2 in yeast. To a large extent, the variable CaD domains of CAX1 and CAX3 are responsible for difference in vacuolar Ca^{2+} transport in yeast. The fusion of CaD domain of CAX1 to CAX2, results in an increase in vacuolar Ca^{2+} transport with no noticeable change in transport of other ions. So, the CAX antiporters may contain several Ca^{2+} domains that collectively coordinate ion transport. N-terminal regulatory domain and CaD domain do not interact and act independently to regulate vacuolar Ca^{2+} transport (Shigaki *et al.* 2001).

Acidic motif is also present in *E. coli* exchanger ChaA, calcium binding protein calreticulin and calsequesterin (Ivey *et al*. 1993). A negatively charged, approximately 20 amino acids residues spanning between TMD six and seven is known as acidic motif. Acidic motif divides CAXs polypeptide into nearly two half (**Fig. 1**). All CAXs have weak but recognizable homology to each other with respect to acidic motif. The AtCAX5 has a longer loop region between TMD 6-7 and has multiple acidic motifs (Edmond *et al*. 2009).

The higher plant CAXs show two highly conserved sequence repeat, known as cation selectivity filter, required for cation selectivity and transport (Shigaki *et al.* 2006). This region shows sequence similarity and conservation with cation binding region of mammalian Na^{+}/Ca^{2+} exchanger (Philipson and Nicoll 2000). Thus, there is possibility to explore the transport of other cations by CAXs. In the yeast complementation assay of mutant K667, it has been observed that the sCAX2 and sCAX5 could suppress the Mn^{2+} sensitivity, while CAX1, CAX3 and CAX4 were not able to complement (Edmond *et al.* 2009). The Mn^{2+} specificity of CAX2 is determined by specific domain, known as 'C-domain' or 'Manganese domain' responsible for vacuolar metal/H^{+} transport. The alteration in 'C-domain' (177-186 amino acids) of CAX2 abolished Mn^{2+} transport capability. The 'C-domain' lies within the TMD4 (**Fig. 1**). The residues Cys-Ala-Phe of TMD4 seems to be part of a pore, which provides Mn^{2+} specificity (Shigaki *et al.* 2003). The Cys-Ala-Phe residue are also present in CAX5, CAX6 and ZmCAX2 (Zea mays CAX2) suggest that they might play an important role in Mn^{2+} transport and functional redundancy of Mn^{2+} transport (Shigaki *et al.* 2003; Edmond *et al*. 2009).

A hydrophilic 'D-domain' (11 amino acids) between TMD 5 and TMD 6 was identified by domain swapping of CAX1 and CAX2. CAX2 having D-domain of CAX1 (CAX2-D) had a CAX1-like pH profile while CAX2-like cation transport activity (Ca^{2+} and Mn^{2+} transport) was unchanged (Shigaki *et al.* 2003; Pittman *et al.* 2005). The CAX2-H222K (substitution of histidine 222 residue with lysine in the 'D-domain' of CAX2) retained Ca^{2+} and Mn^{2+} antiport activity but pH sensing was affected significantly. Thus, 'D-domain' has a potential to regulate CAX2 antiport activity in pH dependent manner (**Fig. 1**) (Pittman *et al.* 2005). Although CAXs showed higher percentage sequence homology, similar canonical structures but uniquely single CAX have properties to transport various cations and their specificity depends on amino acid diversity among different CAXs (Shigaki *et al.* 2005; Shigaki and Hirschi 2006).

HETEROLOGOUS EXPRESSION OF ARABIDOPSIS CAXs IN YEAST

Yeast (*Saccharomyces* sp.) is eukaryotic single cell fungi, having small genome and complex cell and organellar organisation like higher eukaryotic cells. In last decades, various projects were extensively involved in sequencing of yeast genome, genome wide deletion mutagenesis of yeast ORFs and generation of yeast ORF tandem affinity purification lines for interactome study have been successfully accomplished. These yeast based knowledge resources and yeast collection databases made yeast as a highly suitable experimental model system. The yeast strain lacking endogenous genes can be used to discover new orthologues and also can be used for their functional characterization. To

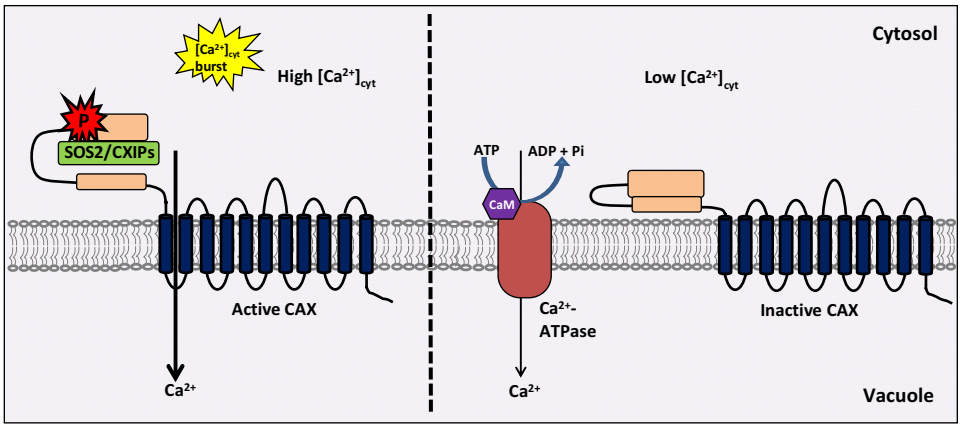

Fig. 2 Diagrammatic representation of typical active and inactive CAX. In response to specific stimulus, cytosolic ca^{2+} level increase several hundreds folds. At this particular condition, CAXs, low affinity calcium transporter, get activated by interacting with different CXIPs/SOS2 to relieve autoinhibitory constrains. CAXs sequester Ca^{2+} into vacuole and lowers cytosolic Ca^{2+} below 10 µM, which is optimal for vacuolar Ca^{2+}-ATPase. The Ca^{2+}-ATPases, high affinity transporters, interact with CaM and fine-tuned Ca^{2+} concentration in cytosol. At lower Ca^{2+} concentration CAX become inactive.

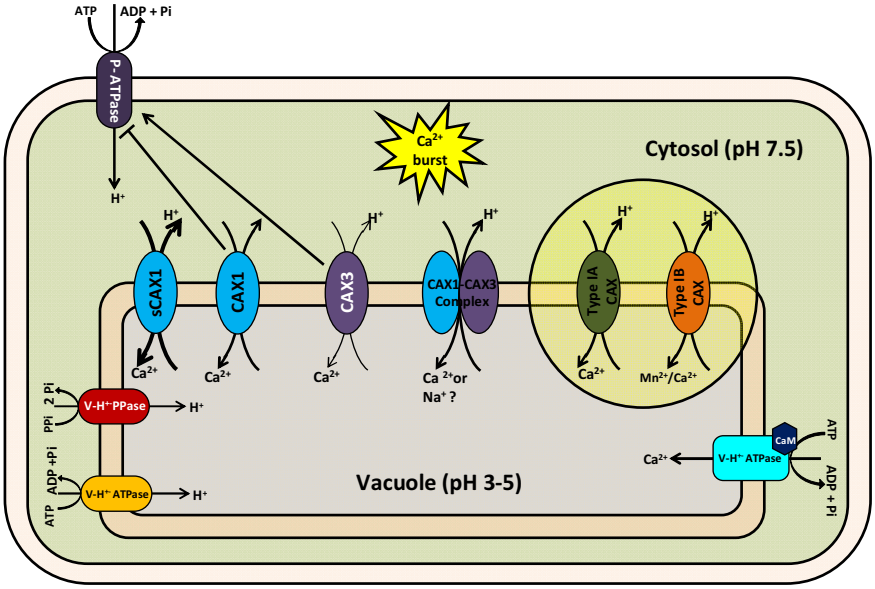

Fig. 3 Diagrammatic representation of *Arabidopsis* CAXs and CAXs-mediated Ca^{2+} sequestration in the plant vacuole. Vacuolar localized CAXs import Ca^{2+} and other cation into vacuolar lumen against proton motive force generated by vacuolar H^+-ATPase and H^+-PPase. Type IA CAXs specifically transport Ca^{2+} while type IB CAX sequester metal ions along with Ca^{2+} (shown in yellow circle). Deregulated CAX (sCAX) has higher Ca^{2+} transport activity than CAXs (represented by thickness of arrow). The CAXs have also indirect role in regulation of proton flux generated by plasma membrane H^+-ATPase. CAX3 positively regulate P-H^+-ATPase while negatively by CAX1. CAX1 and CAX3 either work independently or may interact and make heteromer to regulate calcium transport into vacuole. These heteromers have distinct transport properties.

understand and characterized the plant Ca^{2+} transport functions, yeast mutant lacking homologous transporter were used as a tool (Hirschi *et al.* 1996; Harper *et al.* 1998; Geisler *et al.* 2000; Ueoka-Nakanishi *et al.* 2000; Pittman and Hirschi 2001; Pittman *et al.* 2002; Kamiya *et al.* 2005; Qudeimat *et al.* 2008; Edmond *et al.* 2009; Pittman *et al.* 2009). The vacuolar calcium uptake deficient yeast mutant (ΔVCX1 and ΔPMC1; Cunningham and Fink 1994) were unable to sequester excess Ca^{2+} in the yeast vacuole and this mutant was explored as a tool to study vacuolar Ca^{2+}/H^+ antiport function.

Plant CAXs (CAX1 and CAX2) were first time identified in *Arabidopsis* by yeast vacuolar Ca^{2+} uptake deficient mutant (K665, ΔVCX1 and ΔPMC1; Cunningham and Fink 1994) by yeast mutant complementation screening (Hirschi *et al.* 1996). These genes have capability to suppress calcium hypersensitivity of yeast mutants, defective in vacuolar Ca^{2+} transport. Later on, it was found that CAX1 ORF was partial and it lacks 36 amino acid residues at N-terminus, and was named as short CAX1 (sCAX1). The sCAX1 was constitutively active (**Fig. 3**). The full length ORF of CAX1 has weaker vacuolar Ca^{2+} transport activity than sCAX1 and was not able to complement the yeast mutant (Cheng *et al.* 2005). This N-terminal domain of CAX1 (Δ1-36 amino acid) is a N-terminal regulatory region (NRR) or N-terminal autoinhibitory domain (Pittman and Hirschi 2001). The N-terminal (Δ10 Amino acids) region also has ability to suppress yeast hypersensitivity to $CaCl_2$ (Pittman and Hirschi 2001). The ACA2 (auto-inhibitory calcium ATPase) also has N-terminal autoinhibitory domain. Exogenous calmodulin bind to N-terminal autoinhibitory domain and activate ACA2 (Harper *et al.* 1998) while CAX1 does not have a calmodulin binding site in its NRR. Putative CDPK-binding sites are present in the NRR region of CAX1 (Pittman and Hirschi 2001; Cheng *et al.* 2004). It is quite possible that CAX1 might be regulated by CDPKs, or other regulatory molecules. The N-terminus of CAX physically interacts with the adjacent 52-62 amino acid region and makes CAX inactive (Pittman *et al.* 2002; Shigaki and Hirschi 2006). The NRR region of CAX1 also interacts with CXIP1 (CAX interacting protein 1) and CBL-interacting protein kinase, SOS2 (salt overly sensitive 2) and might regulate the vacuolar Ca^{2+} transport in plants (**Fig. 2**) (Cheng and Hirschi 2003; Cheng *et al.* 2004; Manohar *et*

al. 2011). The C-terminal region of CAX1 is responsible to determine substrate specificity and to transport Cd^{2+} in the yeast. The C-terminal of sCAX1 having point mutation at H338A residue decrease Ca^{2+} uptake but increase Cd^{2+} uptake (Shigaki *et al.* 2005). For heterologous expression studies, various forms of cDNA have been investigated to determine whether 3′ or 5′ UTR have role in regulation of transcription and translation. The 3′ UTR of CAX1 negatively regulate transcription and translation of CAX1 (Shigaki *et al.* 2010; Manohar *et al.* 2011). Although CAX3 showed very high sequence similarity to CAX1, it was very difficult to establish similar type of functional complementation assay for CAX3 as CAX1 in yeast (Shigaki and Hirschi 2006). Unlike full-length CAX2, sCAX2, CAX5, and sCAX5 ex-pressing yeast mutant K667 were able to confer growth on high Ca^{2+} and Mn^{2+}-containing medium and suppress Ca^{2+} and Mn^{2+} hypersensitivity (Hirschi *et al.* 1996; Edmond *et al.* 2009).

The expression of CAX3 does not suppress $CaCl_2$ hypersensitivity in yeast mutant K667 and does not produce change in Ca^{2+} transport (Shigaki *et al.* 2001). Replacement of the CAX3 with calcium domain (CaD) of CAX1 confer calcium transport but to lesser extent than CAX1 in yeast mutant (Shigaki *et al.* 2001). The full-length CAX4 could not complement and suppress hypersensitive phenotype of yeast mutant K667 while sCAX4 (Δ2-37 amino acid) could grow poorly in Ca^{2+} containing media (Cheng *et al.* 2002). Some of the antiporters were shown to be regulated by pH in earlier reports (Schumaker and Sze 1985; Blumwald and Poole 1986; Padan *et al.* 2001; Wiebe *et al.* 2001). Interestingly, CAX1 and CAX2 of *Arabidopsis* were also reported to be showing pH dependence antiport activity when expressed in yeast vacuolar membrane fractions (Pittman *et al.* 2005).

ARABIDOPSIS CAXs: REGULATION THROUGH INTERACTIONS

The higher plants CAXs are regulated by cytosolic N-terminal regulatory domain by post translational regulation similar to plant Ca^{2+}-ATPases (Harper *et al.* 1998; Pittman *et al.* 2001, 2002). The full length ORF of CAX1 was unable to suppress the yeast calcium sensitivity. This autoinhibition of N-terminal domain inhibition was released by binding of various proteins like CAX interacting proteins (CXIP) (Pittman *et al.* 2002; Cheng *et al.* 2003, 2004a, 2004b). CAX1 showed interaction with many proteins (CXIPs) and seems to be not regulated by calmodulin like Ca^{2+}-ATPases. The coexpressed full-length CAX1 and CXIPs, have ability to activate full-length CAX1 in yeast mutant K667 (**Fig. 2**) (Cheng and Hirschi 2003; Cheng *et al*. 2004a, 2004b).

By yeast mutant complementation analysis, Cheng and Hirschi (2003) isolated six different *Arabidopsis* cDNAs that allow full-length CAX1-expressing K667 yeast strain to grow in medium containing high level of Ca^{2+}. Independently, these six genes or full-length CAX do not suppress yeast mutant hypersensitivity to Ca^{2+} but when co-expressed, were able to suppress Ca^{2+} hypersensitive phenotype of yeast mutant K667. These six cDNAs encoded different proteins termed as CAX-interacting proteins (CXIPs). CXIP1 protein (19.2 kDa) showed sequence homology to the C-terminus of PICOT (protein kinase C-interacting cousin of thioredoxin) domain containing proteins (Witte *et al.* 2000; Rahlf *et al.* 2001), which might interact with N-terminal of CAX1 and modify vacuolar Ca^{2+} transport in yeast K667. The N-terminal 1-36 amino acids physically interact with amino acids 56-62 at the N terminus of CAX1 to facilitate autoinhibition of CAX1 (Pittman *et al.* 2002) and CXIP1 bind to region 56-62 to regulate CAX1 vacuolar Ca^{2+} transport (Cheng and Hirschi 2003). Thus CXIP1 might activate CAX1 mediated Ca^{2+} transport through a direct interaction, which disrupts autoinhibitory configuration of CAX1 (Cheng and Hirschi 2003). The Ca^{2+} transport activity mediated by CXIP1-activated CAX1 was much lesser than that of deregulated transport by sCAX1. The higher Ca^{2+} transport mediated by sCAX1 might not corresponds to *in planta* vacuolar Ca^{2+} transport, suggesting the physiological regulation might be achieved by fine-tuned action of multiple components involved to regulate the transport function by removing the inhibitory modules. Similarly, ACA2, CaM regulated Ca^{2+}-ATPase, transports significantly less Ca^{2+} than ACA2 lacking CaM binding autoinhibitory domain (Hwang *et al.* 2000). The CXIP1-activated CAX1 showed much less antiport activity than the activity measured in *Arabidopsis* tissue. It might be quite possible that this complex protein may require additional proteins to fully activate CAX1 (Cheng and Hirschi 2003). The CXIP2, a 32 kDa PICOT-HD domain containing protein, weakly activated CAX4 for Ca^{2+} transport but does not activate CAX1 mediated Ca^{2+} transport in yeast mutant K667. CXIP1 might also weakly interact with CAX4 and activate Ca^{2+} transport to suppress yeast Ca^{2+} hypersensitivity of K667. It is quite possible that CXIP1 and CXIP2 might be involved in the regulation of CAX4. The CXIP3, a 15 kDa, FKBP15-2, a member of the FKBP-type immunophilin family, involved in protein folding (Luan *et al.* 1996) were able to activate CAX1 in yeast mutant K667 (**Fig. 2**) (Cheng and Hirschi 2003). The full-length CAX1 was activated by another protein CXIP4, a novel plant-specific 37.8 kDa protein of unknown function in yeast while CXIP4 alone or full-length CAX1 could not suppress Ca^{2+} hypersensitivity of yeast mutant K667. The CXIP4 could not activate full-length CAX2, CAX3 and CAX4, which suggested that CAX1 vacuolar Ca^{2+} transport is specifically regulated by CXIP4 (Cheng *et al.* 2004).

In response to hyperosmotic or salt stress, cytosolic Ca^{2+} level are elevated in yeast. The elevated Ca^{2+} activate calcineurin, a phosphatase, to regulate many transporters including the vacuolar Ca^{2+}/H^{+} transporter VCX1 for ion homeostasis and salt tolerance (Mendoza *et al.* 1994; Matsumoto *et al.* 2002; Cunningham and Fink 1996). The yeast calcineurin A (CNA) orthologues are not identified yet in plants. The SOS2/CIPK24 (ser/thr SnRK kinase) shows specific interaction with N-terminus (1-73 amino acids) of CAX1 in yeast two-hybrid assays. SOS2 has the ability to suppress the Ca^{2+} hypersensitivity of yeast K667 strain when coexpressed with CAX1. To mimic constitutive phosphorylation, a point mutation (S25D) in the N-terminus constitutively activates CAX1 (Pittman *et al.* 2002). Yeast mutant K667 co-expressing SOS2 and S25A-CAX1 was unable to grow on high Ca^{2+} containing media (Cheng *et al.* 2004). When SOS2 was co-expressed with CAX2, CAX3 and CAX4, it could not suppress calcium hypersensitivity of yeast mutant K667, which suggested that SOS2 specifically interact with CAX1 to regulate vacuolar Ca^{2+} transport. The SOS3/CBL4, a calcium sensor, interact with SOS2 and target this complex to plasma membrane to activate SOS1, a Na^{+}/H^{+} antiporter, in response to salt stress in *Arabidopsis* (Halfter *et al*. 2000; Qiu *et al*. 2002; Quintero *et al.* 2002). It has been suggested that SOS2 might act in SOS3-independent manner to directly regulate the CAX1 transporter or SOS2 might be interacting with some other CBLs, which target this complex to vacuolar membrane to activate the CAX1 *in planta* (Cheng *et al*. 2004). Therefore, SOS2 might be an important central regulator, which might be mediating both Na^{+} and Ca^{2+} homeostasis in plants under normal and stress condition (**Fig. 2**) (Cheng *et al*. 2004).

It has been observed that in many cases, several functional transporter proteins have evolved by gene duplication events, made up of more similar modules and retain their function after reconstitution of its half protein (Zhao *et al*. 2009b). As an example, CAXs protein family members can also be divided into two weakly homologous halves at “acidic motif” (Shigaki *et al.* 2006; Manohar *et al.* 2011). Functionally, the sCAX1 was able to suppress the Ca^{2+} hypersensitivity of yeast strain K667 meanwhile sCAX3 could not achieve this function (Hirschi *et al.* 1996; Shigaki and Hirschi 2000). The N- and C-terminus split protein of CAX1 and CAX3 respectively showed functional reconsti-

tution of CAX in the yeast mutant K667. The N-sCAX1 and C-CAX3 could be localized to similar endomembrane and fold properly to reconstitute functional CAX in yeast and plant cells similar to other proteins (Ottolia *et al.* 2001; Reinders *et al.* 2002; Zhao *et al.* 2009b). One of the major applications of this methodology would be to design a novel transporter, having unique substrate affinities and specificities, which could fulfil the nutritional requirement of the plants. When yeast split assays were done, it was found that N-sCAX1 along with C-terminus of CAX3 provides higher Na^+ and lower Ca^{2+} tolerance than N-sCAX1 plus C-CAX1, which suggested that N-terminal half of CAXs mediate Ca^{2+} transport activity, whereas the C-terminal halves of CAXs determine Na^+ or Li^+ tolerance (Zhao *et al.* 2009b). The different sCAXs have different transport properties. It has been suggested that the heteromeric chimera of CAX1-CAX2 might impart different biochemical properties, ion transport specificity and transport activities (Pittman *et al.* 2004; Shigaki and Hirschi 2006). In future, heterochimeric or point mutated heteromeric transporter can be designed for specific transport activity (Zhao *et al.* 2009b; Manohar *et al.* 2011). Although, CAX1 is tightly regulated by autoinhibition in plant cells and activated by many interacting proteins *in planta* but CAXs might also form heteromer of CAX1 and CAX3 by direct protein-protein interaction to alter Ca^{2+} transport activity (Cheng and Hirschi 2003; Cheng *et al.* 2004a, 2004b; Zhao *et al.* 2009a). In most of the cases, CAX1 and CAX3 showed differential expression in vegetative tissues but both CAX1 and CAX3 showed overlapping expression in germinating seedlings, leaf guard cells, reproductive organs, and during senescence. During osmotic stress, wounding and UV light treatment, CAX3 expression level reaches almost like CAX1 in aerial part of plant (Leonhardt *et al.* 2004; Cheng *et al.* 2005; Zhao *et al.* 2009a). One of the interesting finding attributed to CAX1 and CAX3 hetero-CAX complex is that it could increase Li^+ accumulation and salt tolerance, which was not shown either by sCAX1 or sCAX3 in yeast (Zhao *et al.* 2009a). The physical interaction of CAX1 with CAX3 was also confirmed by CAX1-CAX3 split ubiquitin assay and functional complementation by coexpression in yeast. The co-immunoprecipitation confirms CAX1 and CAX3 interaction *in planta*. The functional association between CAX1 and CAX3 was also confirmed by genetics studies in plants (Cheng *et al.* 2005; Zhao *et al.* 2008, 2009a). The CAX1, CAX3, and their heterocomplexes in vacuoles might be present simultaneously in different ratio, which might be dependent upon growth and physiological conditions (**Fig. 3**) (Zhao *et al.* 2009a).

PHYSIOLOGICAL FUNCTIONS OF CAXs IN ARABIDOPSIS

The CAXs gene family is well characterised in *Arabidopsis* and rice. The CAXs gene family consist of genes encoding proton coupled Ca^{2+} antiporter. These vacuole localized transporters have ability to sequester Ca^{2+}, Mn^{2+} in vacuole to maintain Ca^{2+} and cation homeostasis in the cell. Plant Ca^{2+}/H^+ exchangers were first time isolated from *Arabidopsis* by yeast mutant complementation screening and these exchangers had pH dependent Ca^{2+} transport activity in yeast vacuole (Hirschi *et al.* 1996). The *CAX1* gene expression was highly upregulated by exogenous Ca^{2+} application while weakly upregulated by Ni^{2+}, salt, low temperature and osmotic stress treatment in plants (Hirschi 1999; Catalá *et al.* 2003). Calcium deficiency causes short brownish root, necrosis of young leaves followed by apical meristem necrosis and increased cation sensitivity to other cations (Scaife and Turner 1984). The deregulated *CAX1*-expressing tobacco plants (sCAX1, devoid of autoinhibitory domain) showed tip burning, Ca^{2+} deficiencies symptoms and sensitivity towards K^+ and Mg^{2+} (Hirschi 1999). It is quite possible that excess K^+ and Mg^+ ions might compete with Ca^{2+} uptake because exogenous Ca^{2+} suppresses the cation sensitivity of s*CAX1* expressing plants (Hirschi 1999). The s*CAX1*-expressing tobacco plants also showed high Ca^{2+}/H^+ antiport activity and increased total Ca^{2+} accumulation in vacuole while *cax1* mutant plants were tolerant to serpentine soils, having low Ca^{2+} and high Mg^{2+} and other metals. Therefore CAX activity modulation in plants can provide tolerance to toxic cations (Hirschi 1999; Bradshaw 2005; Visscher *et al.* 2010). Such kind of antiport activity was also seen when yeast vacuolar Ca^{2+}/H^+ antiporter, *VCX1*, was expressed in *Arabidopsis* and tobacco. The *VCX1*- expressing *Arabidopsis* plants exhibited Ca^{2+} transport activity, sensitivity towards Na^+ and other ions, which can be suppressed by exogenous Ca^{2+} application (Hirschi *et al.* 2001).

The autoinhibitory function of N-terminal regulatory domain in CAX1 was already established in yeast (Pittman and Hirschi 2001). The tobacco plant expressing deregulated CAX1 showed increased Ca^{2+}/H^+ antiport activity, thus, more calcium accumulation in plant cell (Mei *et al.* 2007). Hence, sCAX1 could activate and retained transporter activity *in planta* but it is noteworthy to raise a question how exactly the autoinhibition imparted by NRR domain will be relieved in *Arabidopsis*? (Mei *et al.* 2007).

The *cax1* mutants did not show salt sensitivity while constitutive overexpression of sCAX1 in *cax1* mutants showed mild salt sensitivity and growth defect (Cheng *et al.* 2004). Moreover, the *cax1* mutant showed freezing tolerance after cold acclimation (Catalá *et al.* 2003). The *cax1* mutants were sensitive to exogenous Ca^{2+} while they were more tolerant to Ca^{2+} deficient conditions compared to wild type. This suggested that *cax1* mutants were not able to grow in high Ca^{2+} because they were unable to sequester excess Ca^{2+} into vacuole from cytosol. This cytosolic Ca^{2+} burst might affect several signaling pathway and disturb calcium homeostasis in the cells (Hirschi 1999; Catalá *et al.* 2003; Cheng *et al.* 2003). In *cax1* mutants, a significant decrease in Ca^{2+}/H^+ antiport activity and V-type H^+-translocating ATPase activity was observed in contrary to a prominent increase in tonoplastic Ca^{2+}-ATPase activity (Cheng *et al.* 2003). The *cax1* mutants also showed upregulation of other Ca^{2+}/H^+ antiporters like *CAX3* and *CAX4*, which might help in adaptation and plant development in absence of CAX1 to compensate the CAX1 antiport function. Thus, the CAX1-expressing plants displayed altered calcium homeostasis, and increased stress and ions sensitivities, which suggested that it might be performing vital role in plant growth and stress adaptation (Hirschi 1999).

The *CAX2* gene expression did not increase in response to exogenous Ca^{2+} application (Hirschi *et al.* 2000). CAX2 had dual characteristics to transport Ca^{2+}, Mn^{2+} by Ca^{2+}/H^+ and Mn^{2+}/H^+ antiport activity in yeast vacuole respectively (Pittman *et al.* 2004). The *CAX2*-expressing tobacco plants accumulate more Ca^{2+} in plant vacuole as compared to *CAX1*-expressing plants. In contrary to *CAX1*-expressing plants, the *CAX2*-expressing plants did not showed Ca^{2+} deficiency symptoms but provide Mn^{2+} tolerance. The vacuolar membrane fraction of *cax2* mutant plant showed lower Mn^{2+} transport activity than wild type while vacuolar membrane fractions of *CAX2*-expressed tobacco plant showed higher accumulation of Ca^{2+}, Cd^{2+} and Mn^{2+} than wild type, which possibly suggested that CAX2 might be functioning as heavy metal transporter (Hirschi *et al.* 2000; Pittman *et al.* 2004).

The specific function of CAX3 has been difficult to determine (Cheng *et al.* 2003, 2005; Conn *et al.* 2011). The CAX3 expression does not suppress Ca^{2+} hypersensitivity in yeast mutant and CAX3 overexpressing tobacco plants did not show altered sensitivity towards exogenous Mg^{2+} ions (Shigaki *et al.* 2001, 2002). Phenotypically, *cax1* and *cax3* mutants were sensitive to both sugar and ABA during germination and showed an increased tolerance to ethylene during early seedling development (Cheng *et al.* 2005; Zhao *et al.* 2008). The *cax3* mutant plants showed salt, lithium and low pH sensitivity, which could be due to a reduced vacuolar Ca^{2+}/H^+ transport during salt stress (Zhao *et al.* 2008). It was already established that nine amino acids long 'calcium domain' of CAXs had important role in calcium

transport (Shigaki *et al*. 2001). The yeast and tobacco plant overexpressing *CAX3-9* (*35S::CAX3* having nine amino acid long 'calcium domain' of *CAX1*) showed increased Ca^{2+} transport and lesser ions sensitivity than *CAX1*-expressing tobacco plants (Hirschi 1999; Shigaki *et al.* 2002). Hence, the role of CaD domain of CAXs in Ca^{2+} transport activity has been determined both in yeast and plants (Shigaki *et al.* 2002).

In *Arabidopsis*, the calcium concentration was found to be higher in the mesophyll cells than epidermal cells. The cell specific Ca^{2+} concentration difference is due to higher accumulation of Ca^{2+} in vacuole of mesophyll cell. In the cell-specific microarray of epidermal verses mesophyll cells, CAX1 was found to be most abundantly and differentially expressing Ca^{2+}/H^{+} exchanger in mesophyll cells (Conn *et al.* 2011). In the double mutant of *cax1cax3*, a dramatic reduced accumulation of Ca^{2+} was found in the mesophyll cells while a higher accumulation of Ca^{2+} was seen in the apoplast. This altered concentration of Ca^{2+} in mesophyll and apoplast leads to reduced stomatal aperture, gas exchange, leaf growth rate and cell wall extensibility, which could be due to altered expression of cell wall modifying enzymes (Conn *et al.* 2011). Therefore, it has been concluded that CAXs are playing important role in plant growth and development by maintaining Ca^{2+} concentration in vacuole of mesophyll cells and apoplast (Conn *et al.* 2011).

One of the important functions of antiporters such as Na^{+}/H^{+} exchanger and CAX is to provide salt tolerance and ions homeostasis. The salt tolerance and ion homeostasis is directly or indirectly mediated by calcium signaling. Saline condition leads to increase in cytosolic Ca^{2+} level from resting level of Ca^{2+} in cells and specific Ca^{2+} signature is maintained by efflux of Ca^{2+} by Ca^{2+}/H^{+} antiport activity in vacuole. Under the salt stress, the CAX3 gene was up-regulated and might be involved in salt tolerance indirectly (Cheng *et al.* 2005; Barkla *et al.* 2008; Zhao *et al.* 2008; Edmond *et al.* 2009). It has been observed that in contrast to *cax1* mutants, *cax3* mutants have reduced Ca^{2+} transport activity in salt stress, which suggested that CAX3 might have predominant Ca^{2+}/H^{+} transport activity in salt stress, also supported by transcripts upregulation of CAX3 in salt (Shigaki and Hirschi 2000). The knockout mutant of *cax1* and *cax3* showed sensitivity to abiotic stresses, which indicated that these transporters might be important regulator of calcium mediated stress tolerance in plants (Cheng *et al.* 2003, 2005; Zhao *et al.* 2008).

In an interesting study, an altered H^{+}-ATPase activity at both the plasma membrane and the tonoplastic membrane in *cax* mutants was observed which suggested a tight interplay between the Ca^{2+}/H^{+} exchangers and proton pumps (Cheng *et al.* 2003, 2005; Zhao *et al.* 2008). For example, *cax1* mutants showed decreased V-type ATPase activity and an increased P-type ATPase activity while *cax3* mutants showed decrease in both P-ATPase and V-ATPase activity (Cheng *et al.* 2003, 2005; Zhao *et al.* 2008). The *cax3* plant showed sensitivity towards acidic pH, which could also be correlated with reduced P-ATPase and V-ATPase activity (Cheng *et al.* 2005; Zhao *et al.* 2008). CAX1 and CAX3 appeared to be important regulators of tonoplastic and plasma membrane H^{+} pumps, which regulate H^{+} coupled transporters to maintain pH in the cellular compartment (Cheng *et al.* 2005; Zhao *et al.* 2008). Therefore, an indirect regulation of proton flux across membrane is also attributed to CAX functions along with Ca^{2+} sequestration in the vacuole (**Fig. 3**).

As reported by Mei *et al.* (2009), one of the type IA CAXs, CAX4 was uniquely expressed in roots apex and lateral primordia in contrast to other CAXs. It has similar biochemical properties as other CAXs and *cax4* mutant displayed altered root growth in response to Cd^{2+} and Mn^{2+} suggested its possible role as a cation/H^{+} antiporter that has an important function in root growth under heavy metal stress conditions (**Fig. 3**) (Mei *et al.* 2009).

As reported previously that the vacuolar membrane fractions of *cax2* mutant showed reduced but not complete absence of Mn^{2+}/H^{+} transport activity, which suggested that there could be other CAX2 homologues that might be regulating the Mn^{2+}/H^{+} antiport activity in vacuole (Pittman *et al.* 2004; Edmond *et al.* 2009). Similarly, in *Arabidopsis*, two other genes (*AtCAX5* and *AtCAX6*) were identified where they cluster with type IB member CAX2. The high amino acid sequence similarity of AtCAX5 and AtCAX6 with CAX2 (>87%) suggested a possible similar function in plants. Moreover, *AtCAX5* showed ubiquitous expression in all tissues similar to CAX2. It was reported that CAX2 can transport divalent cations Ca^{2+}, Cd^{2+} and Mn^{2+} but its expression was not induced by these metal ions (Hirschi *et al.* 2000). *AtCAX5* gene expression was increased under Ca^{2+} deficient condition (Edmond *et al.* 2009). In contrast to CAX2, the expression of *AtCAX5* was induced significantly by exogenous Mn^{2+} and slightly by Cd^{2+} and Ca^{2+} treatment (Hirschi *et al.* 2000; Edmond *et al.* 2009). In contrast to full length AtCAX5, sAtCAX5 was able to suppress both the Ca^{2+}- and Mn^{2+}-hypersensitivity of K667. When analysed for Ca^{2+} transport activity, sAtCAX5 had lesser Ca^{2+} and Mn^{2+} transport activity into vacuole than sCAX2 expressing yeast cells (**Fig. 3**) (Edmond *et al.* 2009).

PHYSIOLOGICAL AND BIOCHEMICAL FUNCTION OF OTHER PLANT CAXs

The orthologues of *Arabidopsis* CAXs were also isolated and characterized in other plants species such as *Chlamydomonas reinhardtii* (CrCAX1), mung bean (VCAX1), *Capsella bursapastoris* (CbCAX51), rice (Ueoka-Nakanishi *et al.* 1999; Kamiya *et al.* 2005; Lin *et al.* 2008; Pittman *et al.* 2009). The Unicellular alga *Chlamydomonas reinhardtii* CAX, CrCAX1, is a Ca^{2+}/H^{+} and Na^{+}/H^{+} exchanger (Pittman *et al.* 2009). The CrCAX1 is localised to vacuole and shows more sequence homology to fungal *CAX*, *VCX1* than *Arabidopsis CAX1*. The CrCAX1 has N-terminal tail like higher plant CAXs (Pittman *et al.* 2009). CrCAX1 has Ca^{2+}/H^{+} and Na^{+}/H^{+} exchange activity along with Co^{2+} and Cd^{2+} transport ability. It can suppress Ca^{2+} sensitivity of yeast mutant like higher plant CAXs (Pittman *et al.* 2009). Due to Na^{+}/H^{+} exchange activity, CrCAX1 could provide Na^{+} tolerance both in yeast and *Arabidopsis thaliana*. As reported by Pittman *et al.* (2009), the Na^{+}/H^{+} exchange activity was not regulated by the N-terminus of the CrCAX1 protein. Thus, CrCAX1 can transport both monovalent (Na^{+}) and divalent cations (Ca^{2+}) into vacuole to maintain cationic balance in the cell.

The rice genome encodes five CAXs, named as OsCAXs, having 35-73% sequence homology among them. The OsCAXs have typical CAXs like structure (Hirschi *et al.* 1996; Ueoka-Nakanishi *et al.* 1999; Kamiya and Maeshima 2004; Kamiya *et al.* 2005). The OsCAXs are phylogenetically clustered into two groups as type IA and type IB. OsCAX1a, OsCAX1b and OsCAX1c belongs to type IA class along with *Arabidopsis* CAX1, CAX3 and CAX4 while OsCAX2 and OsCAX3 are grouped into type IB class along with CAX2, CAX5 and CAX6 (Kamiya *et al.* 2005). The OsCAX1a has two domains, named c-1 and c-2, which are conserved throughout plant CAXs. The c-1 lies between TMD 3-4 while c-2 domain lies between TMD 8-9. These two domains are involved in gating mechanism or to form filter of cation pore (Kamiya and Maeshima 2004). The deregulated variants of OsCAXs (OsCAX1aΔ27, OsCAX1bΔ36 and OsCAX1cΔ47, and OsCAX3) have ability to suppress Ca^{2+} sensitivity of yeast strain K665 to different extents. The OsCAX1a, OsCAX1c and OsCAX3 expressing yeast strain showed higher tolerance to Ca^{2+} while OsCAX1b and OsCAX2Δ26 showed lesser tolerance to Ca^{2+} (Kamiya *et al.* 2005), whereas OsCAX1a and OsCAX3-expressing yeast showed Mn^{2+} tolerance in yeast K665 (Kamiya and Maeshima 2004; Kamiya *et al.* 2005). The OsCAX1a transports Ca^{2+} into vacuoles and maintained normal Ca^{2+} level in cells when plants were exposed to excess Ca^{2+} (Kamiya *et al.* 2006). In contrary to *Arabidopsis*, rice appears to accumulate Ca^{2+} in epidermal cells

rather mesophyll cells (Conn and Gillham 2010; Conn *et al.* 2011).

The *Arabidopsis* orthologues of CAX2 from barley (HvCAX2) and tomato LeCAX2 were also studied to understand conservation of functional characteristics among CAX2-like transporters in higher plants (Edmond *et al.* 2009). The LeCAX2 and HvCAX2 belong to type IB class of CAXs and showed sequence similarity with CAX2 and OsCAX3. They have typical putative structure of type IB CAX as 11 predicted TMD, an acidic motif, and Mn^{2+} transport determinant residues (Cys-Ala-Phe) of TMD4. They also contain c-1 and c-2, substrate-selectivity filters, which is conserved among CAX2-like genes (Kamiya *et al.* 2005; Edmond *et al.* 2009). In contrast to typical CAXs, HvCAX2 have shorter N-terminal regulatory domain. The N-terminal of HvCAX2 showed similar secondary protein structure as N-terminus of yeast VCX1 and OsCAX3 (Edmond *et al.* 2009).

The HvCAX2 expressing K667 yeast strains were able to grow on high-Ca^{2+} and Mn^{2+} containing medium but lesser growth was observed when compared to s*CAX2*. The tomato full-length LeCAX2 and NRR truncated LeCAX2 (sLeCAX2) have ability to suppress the Ca^{2+} hypersensitivity of yeast mutant K667 (Edmond *et al.* 2009).

Interestingly, the full-length LeCAX2 has ability to suppress Ca^{2+} hypersensitivity while Mn^{2+} transport is constrained by N-terminal regulatory region in yeast (Edmond *et al.* 2009). Most of the CAXs protein share similar coil-helix-coil region while LeCAX2 have clearly distinct secondary structure near N-terminal region. The mutagenesis analysis of this region could reveal the distinct regulatory mechanism of Ca^{2+} transport by LeCAX2 (Edmond *et al.* 2009). The expression of HvCAX2 transcripts was found to be upregulated by exogenous Ca^{2+} and high salt but not by exogenous metal ion treatment (Edmond *et al.* 2009). The vacuolar membrane fraction of yeast expressing HvCAX2, sHvCAX2, LeCAX2, sLeCAX2 and CAX2 showed distinct proton mediated Ca^{2+} and Mn^{2+} transport activity. The Ca^{2+}/H^+ transport activity of LeCAX2 and sLeCAX2 was similar to sCAX2 while a reduced Ca^{2+}/H^+ transport activity was observed for HvCAX2 expressing yeast mutant. This might be due to slightly higher K_m of HvCAX2 for Ca^{2+}. 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 and Pittman 2010). Overall, type IB CAXs proteins either from dicot or monocots (CAX2, CAX5, LeCAX2 or HvCAX2) have similar characteristic of Ca^{2+} and Mn^{2+} transport activity and this trait is conserved throughout this group (Edmond *et al.* 2009). Despite this overall similarity in functions, type IB group members had variation in the transport kinetics of Ca^{2+} and Mn^{2+} and thus their regulatory mechanism could be distinct among the different plant species (Edmond *et al.* 2009).

Remarkably, a functional diversity is observed among plants CAXs despite of their high structural similarity, which suggest that functional redundancy, might have acquired by these transporters in different plant species based on different growth and developmental requirement. Overall, the CAXs transporters have been extensively studied in *Arabidopsis* but their detailed physiological roles in other plant species requires further investigation in near future.

CONCLUSIONS AND FUTURE PERSPECTIVES

Ion homeostasis is maintained by cumulative action of different transporters/channels/pump present in membranes. The transporters present in vacuolar membranes plays important role in regulation of cytosolic pH and in accumulation of toxic ions and xenobiotics. The cytosolic Ca^{2+} is maintained by various transporters but Ca^{2+}-binding proteins also takes part in fine tuning of cytosolic Ca^{2+} concentration and regulate many signaling pathways in the cell. The CAXs protein family are diverse and characterized in most of the plant kingdoms. The algal CAXs, CrCAX1 shows distinct structural and biochemical properties than higher plant CAXs. The six *Arabidopsis* CAXs are well characterized for their regulation, substrate specificity, and physiological function while all rice CAXs are isolated and biochemically characterized in yeast but physiological function of most of the OsCAXs is yet to be discovered. CAXs are unique in their substrate specificity, transport activity and their regulation. The N-terminal autoinhibitory or regulatory domain is important for CAXs transport activity. Upon specific condition, CAXs have properties to form heteromer with other CAXs *in planta* and also to interact with other regulatory proteins to regulate the transport activity. The antiport activity of CAX protein might be regulated by interaction with regulatory proteins such as kinases or phosphatases, which upon phosphorylation-dephosphorylation leads to conformational changes to relieve the autoinhibitory constrains. One of the major bottleneck is to understand the mechanism of regulation of CAX antiporters under different developmental and physiological conditions by different regulatory pathways. Identification of new regulatory components such as kinases, phosphatases and other signaling proteins, which could regulate the activity of CAX *in planta* require detailed investigation.

The intrinsic property of CAXs protein is to sequester high Ca^{2+} content in plant tissues (especially in vacuoles). Genetically, multiple CAXs have been implicated to be involved in quite a number of physiological and developmental processes such as freezing tolerance after cold acclimation, increased sensitivity of germination to ABA, delayed flowering, regulate Ca^{2+} accumulation in apoplast, tolerance to serpentine soil (Catala *et al*. 2003; Cheng *et al*. 2003, 2005; Visscher *et al*. 2010; Conn *et al*. 2011). In the near future, genetically designed variants of CAX proteins can be used as potential candidate for enhancing the abiotic stress tolerance, increased level of calcium content in edible part of plant to eradicate calcium malnutrition, and for phytoremediation of heavy metals from polluted soil.

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