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 antiporers. Calcium/cation antiporter (CaCA) superfamily consist of five families, one of them is CAX multigene family (H+/cation exchangers). 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 Ca2+ 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: Ca2+, calcium; CaCA, Ca2+/cation antiporter; CaD, Ca2+ domain; CaM, calmodulin; CAX, Ca2+/H+ exchanger; CBL, calcineurin B-like protein; CDPK, Ca2+-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; VCA1, mung bean Ca2+/H+ antiporter

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INTRODUCTION

Calcium ([Ca2+]i) is a pivotal cation and major nutrient element, necessary for plant growth and development. Ca2+ maintains organic and inorganic ionic balance in cytoplasm and vacuole by various counter cation import and export mechanisms (Marschner 1995). In soil, primarily Ca2+ enters in root through Ca2+-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 Ca2+ concentration is the determining factor for Ca2+ uptake by roots from the soil. In the resting stage cells, cytosolic submicromolar Ca2+ 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 Ca2+ 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 [Ca2+]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 calciurein B-like proteins (CBLs), calmodulins (CaM), Ca²⁺-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 various calcium signaling pathways.

In response to specific stimulus, [Ca²⁺]cyt levels increase more than 10 μM near endo-membrane. This [Ca²⁺]cyt burst is due to release of Ca²⁺ from intracellular organelles and efflux through Ca²⁺ channels from plasma membrane (Gilroy and Jones 1992; McCain and Hetherington 1998; White 2000; Sanders 2002). The synergistic action of Ca²⁺ transporters, present on plasma membrane (PM), endoplasmic reticulum (ER) and tonoplast, is important not only to maintain the resting level of [Ca²⁺]cyt but also to transiently increase the Ca²⁺ level to initiate specific signaling events. For proper cell functioning, this [Ca²⁺]cyt balance is maintained by both high and low affinity Ca²⁺ transporters (Ueoka-Nakanishi et al. 1999).

During [Ca²⁺]cyt burst in the cell, CAXs (Ca²⁺/H⁺ exchangers) play an important role to maintain optimum [Ca²⁺]cyt level by sequestering excess Ca²⁺ to the lumen of vacuole against electrochemical gradient to maintain low level of calcium concentration in the cytoplasm (Blackford et al. 1990; Dunn et al. 1994; Pittman et al. 2000; Shigaki et al. 2006). In the yeast complementation library screening, five bonafide CAXs as OsCAX1a-c, OsCAX2 and OsCAX3 are present in Arabidopsis genome. On the basis of transport function, Shigaki et al. (2006) classified CAXs into two phylogenetic groups, CAX1-6 and CAX7-11. They proposed that only CAX1-6 transporters are bonafide CAXs because they have H⁺/Ca²⁺ exchange activity. Whereas, CAX7-11 are classified into distinct phylogenetic groups and are more closely related to K⁺-dependent Na⁺/Ca²⁺-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 transport functional properties (Shigaki et al. 2006). Type IA CAXs (CAX1, CAX3 and CAX4) specifically transport Ca²⁺ while type IB CAXs (CAX2, CAX5 and CAX6) shows broader substrate specificity for divalent cation such as Ca²⁺, Mg²⁺ and Cd²⁺ (Hirschi et al. 2000; Shigaki 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.

DIVERSITY AND PHYLOGENETIC CLASSIFICATION OF CAXs

Ca²⁺/Ca²⁺-ion transporter (CaCA) superfamily consist of five related families (YRBG, NCX, NCKX, CCX and CAX). The YRBG, putative Na⁺/Ca²⁺ exchangers, present in bacteria and Archea, while NCX, Na⁺/Ca²⁺ exchanger is present in eukaryotes. The members of NCKX, K⁺ dependent Na⁺/Ca²⁺-exchangers are encoded by other eukaryotic genome but absent in land plants. The CCX, cation/Ca²⁺-exchangers, are encoded by eukaryotic genome and are also present in land plants (Emery et al. 2012). Besides five representative family of CaCA superfamily, 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-Ca²⁺-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 Hirschi 2000; Emery et al. 2012). In last decade, many representative gene from different 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 archaea bacteria, 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) (Shigaki and Hirschi 2006; Manohar et al. 2011). Numerous type I CAXs from plants and microorganisms have been functionally characterised, whereas single yeast type I 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 Ca²⁺ when other transporters are missing in yeast (Manohar et al. 2010). In higher plants, type II CAX is not present. The type III Ca²⁺/H⁺ antiporter, ChaA from E. coli also catalyzes direct Na⁺/H⁺ and K⁺/H⁺ antiport and provide resistance to high concentration of Na⁺ and K⁺ in medium (Ivey et al. 1993; Radechenko 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⁺/Ca²⁺ exchange activity. Whereas, CAX7-11 are classified into distinct phylogenetic groups and are more closely related to K⁺-dependent Na⁺/Ca²⁺-exchangers. Rice genome encodes five bonafide CAXs as OsCAX1a-c, OsCAX2 and OsCAX3.

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STRUCTURE OF CAXs

A typical plant CAXs have 11 transmembrane domain, N-terminal regulatory or autoinhibitory domain, Ca²⁺ domain (CaD), acidic motifs and cation selectivity filter, required for cation selection and transport (Fig. 1) (Shigaki 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 Ca²⁺ uptake deficient mutant K665, named as CAX1 and CAX2. The deletion of endogenous vacuolar PMC1 and VCX1 makes K665 yeast strain defective in vacuolar Ca²⁺ transport, rendering it unable to grow on high- Ca²⁺ 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
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 VCA1 (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 is 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 CaD 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 calsequestrin (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 divies 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 (Phillipson 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 vacuum 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 organelar 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
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 (g168VCX1 and g168PMC1; 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. 2005).
The expression of CAX3 does not suppress CaCl₂ hypersensitivity in yeast mutant K667 and does not produce change in Ca²⁺ 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 (A2-37 amino acid) could grow poorly in Ca²⁺ 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 antipporter 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 Ca²⁺-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 Ca²⁺-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 cDNA that allow full-length CAX1-expressing K667 yeast strain to grow in medium containing high level of Ca²⁺. Independently, these six genes or full-length CAX do not suppress yeast Ca²⁺ hypersensitivity to Ca²⁺ but when co-expressed, were able to suppress Ca²⁺ 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. 2008; Rahlf et al. 2001), which might interact with N-terminal of CAX1 and modify vacuolar Ca²⁺ 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 Ca²⁺ transport (Cheng and Hirschi 2003). Thus CXIP1 might activate CAX1 mediated Ca²⁺ transport through a direct interaction, which disrupts autoinhibitory configuration of CAX1 (Cheng and Hirschi 2003). The Ca²⁺ transport activity mediated by CXIP1-activated CAX1 was much lesser than that of deregulated transport by sCAX1. The higher Ca²⁺ transport mediated by sCAX1 might not correspond to in planta vacuolar Ca²⁺ 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 Ca²⁺-ATPase, transports significantly less Ca²⁺ than ACA2 lacking CaM binding domain activated CAX1. The sCAX1-activated CAX1 showed much less antipporter activity than the activity measured in Arabidopsis tissue. It might be 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 Ca²⁺ transport but does not activate CAX1 mediated Ca²⁺ transport in yeast mutant K667. CXIP1 might also weakly interact with CAX4 and activate Ca²⁺ transport to suppress yeast Ca²⁺ 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 Ca²⁺ hyperactivity of yeast mutant K667. The CXIP4 could not activate full-length CAX2, CAX3 and CAX4, which suggested that CAX1 vacuolar Ca²⁺ transport is specifically regulated by CXIP4 (Cheng et al. 2004).

In response to hyperosmotic or salt stress, cytosolic Ca²⁺ level are elevated in yeast. The elevated Ca²⁺ activate calcineurin, a phosphatase, to regulate many transporters including the vacuolar Ca²⁺/H⁺ transporter VCY1 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 Ca²⁺ 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 Ca²⁺ 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 suggests that SOS2 constitutively interact with CAX1 to regulate vacuolar Ca²⁺ 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 (Haller 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 of CAX1 and might be involved in regulation of Ca²⁺ homeostasis in response to salt stress. Therefore, SOS2 might be an important central regulator, which might be mediating both Na⁺ and Ca²⁺ 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 other CBLs, and 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 CAX1 was able to suppress the Ca²⁺ 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. Parallel split assays were done in vitro and it was found that N-sCAX1 along with C-terminus of CAX3 provides higher Na+ and lower Ca2+ tolerance than N-sCAX1 plus C-CAX1, which suggested that N-terminal half of CAXs mediate Ca2+ 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 autoinhibiton 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 Ca2+ 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 plants. When yeast split assays were done, it was found that CAX1 and CAX3 showed differential expression in the cases, CAX1 and CAX3 showed differential expression. The physical interaction of CAX1 with CAX3 was also confirmed by genetics studies in plants (Zhao et al. 2009). The specific function of CAX1 has been difficult to determine (Zhao et al. 2009a). The physical interaction of CAX1 with CAX3 was also confirmed by sCAX1-CAX3 split ubiquitin assay and functional complementation by coexpression in yeast. The coimmunoprecipitation 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 Ca2+ antiporter. These vacuole localized transporters have ability to sequester Ca2+, Mn2+ in vacuole to maintain Ca2+ and cation homeostasis in the cell. Plant Ca2+/H+ exchangers were first time isolated from Arabidopsis by yeast mutant complementation screening and these exchangers had pH dependent Ca2+ transport activity in yeast vacuole (Hirschi et al. 1996). The CAX1 gene expression was highly upregulated by exogenous Ca2+ application while weakly upregulated by Ni2+, salt, low temperature and osmotic stress treatment in plants (Hirschi 1999; Catalá et al. 2001, 2002). CAX3 gene expression was highly upregulated by exogenous Ca2+, while weakly upregulated by Ni2+, salt, low temperature and osmotic stress treatment in plants (Hirschi 1999). The CAX3 expressing tobacco plants also showed high Ca2+/H+ antiport activity and increased total Ca2+ accumulation in vacuole while cax1 mutant plants were tolerant to serpentine soils, having low Ca2+ and high Mg2+ 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 Ca2+/H+ antiporters CAX1, CAX3 and the vacuole expressed Arabidopsis plants exhibited Ca2+ transport activity, sensitivity towards Na+ and other ions, which can be suppressed by exogenous Ca2+ 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 Ca2+/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. 2003). Moreover, the cax1 mutant showed freezing tolerance after cold acclimation (Catalá et al. 2003). The cax1 mutants were sensitive to exogenous Ca2+ while they were more tolerant to Ca2+ deficient conditions compared to wild type. This suggested that cax1 mutants were not able to grow in high Ca2+ because they were unable to sequester excess Ca2+ into vacuole from cytosol. This cytosolic Ca2+ 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 Ca2+/H+ antiport activity and V-type H+-translocating ATPase activity was observed in contrary to a prominent increase in tonoplastic Ca2+-ATPase activity (Cheng et al. 2003). The cax1 mutants also showed upregulation of other Ca2+/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 Ca2+ application (Hirschi et al. 2000). CAX2 had dual characteristics to transport Ca2+, Mn2+ by Ca2+/H+ and Mn2+/H+ antiport activity in yeast vacuole respectively (Cheng et al. 2004). The CAX2-expressing tobacco plants also accumulate more Ca2+ in plant vacuole as compared to CAX1-expressing plants. In contrary to CAX1-expressing plants, the CAX2-expressing plants did not showed Ca2+ deficiency symptoms but provide Mn2+ tolerance. The vacuolar membrane fraction of cax2 mutant plant showed lower Mn2+ transport activity than wild type while vacuolar membrane fractions of CAX2-expressing tobacco plant showed that CAX2-expressing tobacco plant accumulate Mn2+ in the cell (Cheng et al. 2004). This, 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 Ca2+ hypersensitivity in yeast mutant and CAX3 overexpressing tobacco plants did not show altered Ca2+ sensitivity towards 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 Ca2+/H+ transport during salt stress (Zhao et al. 2008). It was already established that among amino acids long ‘calcium domain’ of CAXs had important role in calcium physiology.
transport (Shigaki et al. 2001). The yeast and tobacco plant overexpressing CAX1-9 (35S::CAX3 having nine amino acid long ‘calcium domain’ of CAX1) showed increased Ca2+ 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 Ca2+ 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 Ca2+ concentration difference is due to higher accumulation of Ca2+ in vacuole of mesophyll cell. In the cell-specific microarray of epidermal versus mesophyll cells, CAX1 was found to be most abundantly and differentially expressing Ca2+/H+ exchanger in mesophyll cells (Conn et al. 2011). In the double mutant of cax1cax3, a dramatic reduced accumulation of Ca2+ was found in the mesophyll cells while a higher accumulation of Ca2+ was seen in the apoplast. This altered concentration of Ca2+ in mesophyll and apoplasm 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 Ca2+ concentration in vacuole of mesophyll cells and apoplasm (Conn et al. 2011).

One of the important functions of antiporptors 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 Ca2+ level from resting level of Ca2+ in cells and specific Ca2+ signature is maintained by efflux of Ca2+ by Ca2+/H+ antiporter activity in vacuole. Under the salt stress, the CAX3 gene was upregulated 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 Ca2+ transport activity in salt stress, which suggested that CAX3 might have predominant Ca2+/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 H+-/H+ exchanger and CAX activity (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. 2003, 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 Ca2+ sequestration in the vacuole (Fig. 3).

CAX1, CAX3 and CAX4 were found to be 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 Cd2+ and Mn2+ 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 Mn2+/H+ transport activity, which suggested that there could be other CAX2 homologues that might be regulating the Mn2+/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 both. Moreover, AtCAX5 showed ubiquitous expression in all tissues similar to CAX2. It was reported that CAX2 can transport divalent cations Ca2+, Cd2+ and Mn2+ but its expression was not induced by these metal ions (Hirschi et al. 2000). AtCAX5 gene expression was increased under Ca2+ deficient condition (Edmond et al. 2009). In contrast to CAX2, the expression of AtCAX5 was induced significantly by exogenous Mn2+ and slightly by Cd2+ and Ca2+ treatment (Hirschi et al. 2000; Edmond et al. 2009). In contrast to full length AtCAX5, sAtCAX5 was able to suppress both the Ca2+- and Mn2+-hypersensitivity of K667. When analysed for Ca2+ transport activity, sAICAX5 had lesser Ca2+ and Mn2+ 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 CrCAX1, CrCAX1, is a Ca2+/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 Ca2+/H+ and Na+/H+ exchange activity along with Co2+ and Cd2+ transport ability. It can suppress Ca2+ 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 (Ca2+) into vacuole to maintain cationic balance in the cell.

The rice genome encodes five CAXs, named as OsCAXs, having 55-73% 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 Ca2+/H+ exchange activity in vacuole (Hirschi et al. 1996; Kamiya et al. 2005), whereas OsCAX1a and OsCAX1c and CAX2 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; Edmond et al. 2009). In contrast to full length AtCAX5, sAtCAX5 was able to suppress both the Ca2+- and Mn2+-hypersensitivity of K667. When analysed for Ca2+ transport activity, sAICAX5 had lesser Ca2+ and Mn2+ transport activity into vacuole than sCAX2 expressing yeast cells (Fig. 3) (Edmond et al. 2009).
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. The putative features of type IB CAX as 11 predicted TMD, an acidic motif, and Mn2+ 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-Ca2+ and Mn2+ containing medium but lesser growth was observed when compared to sCAX2. The tomato full-length LeCAX2 and NRR truncated LeCAX2 (sLeCAX2) have ability to suppress the Ca2+ hypersensitivity of yeast mutant K667 (Edmond et al. 2009). In contrast, the full-length CAX2 have ability to suppress Ca2+ hypersensitivity while Mn2+ 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 Ca2+ transport by LeCAX2 (Edmond et al. 2009). The expression of HvCAX2 transcripts was found to be upregulated by exogenous Ca2+ 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 Ca2+ and Mn2+ transport activity. The Ca2+/H+ transport activity of LeCAX2 and sLeCAX2 was similar to sCAX2 while a reduced Ca2+/H+ transport activity was observed for HvCAX2 expressing yeast mutant. This might be due to slightly higher Km of HvCAX2 for Ca2+. Each of these CAXs can transport Ca2+ and Mn2+ 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 monocot (CAX2, CAX5, LeCAX2 or HvCAX2) have similar characteristic of Ca2+ and Mn2+ transport activity and this trait is conserved throughout this group (Edmond et al. 2009). Despite the overall similarity in functions, type IB CAX group members had variation in the transport kinetics of Ca2+ and Mn2+ 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 Ca2+ is maintained by various transporters but Ca2+-binding proteins also takes part in fine tuning of cytosolic Ca2+ 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 but 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-de-phosphorylation 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 CAXs in planta require detailed investigation.

The intrinsic property of CAXs protein is to sequester high Ca2+ 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 Ca2+ 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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