

Subcellular Localization of CAX Proteins in Plants

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Molecular Soil Biology, 2015, Vol.6, No.3 doi: 10.5376/msb.2015.06.0003

Received: 20 May, 2015

Accepted: 31 May, 2015

Published: 01 Jun., 2015

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Preferred citation for this article:

Chen and Zhang, 2015, Subcellular Localization of CAX Proteins in Plants, Molecular Soil Biology Vol.6, No.3 1-5 (doi: 10.5376/msb.2015.06.0003)

Abstract Inorganic cations play decisive roles in many cellular and physiological processes and are essential components of plant nutrition. The uptake and distribution of cations must be strictly controlled. Membrane antiporters are important elements in mediating the intracellular sequestration of these cations. These antiporters are energized by the proton gradient across the membrane and allow the rapid transport of cations into the organelles. CAXs (for CAtion eXchanger) are members of a multigene family and appear to predominately reside on vacuoles, while some reside on PM or other organelles.

Keywords CAX; Antiporter; Fluorescence; Localization; Organelle

Introduction

Transporters play one of the most fundamental roles in life, namely, selective import or removal of molecules across biological membranes. In Arabidopsis approximately 3% of the genome appears to code for transporters (The Arabidopsis Genome Initiative 2000). A vast majority of these transporters are secondary transporters, which are energized by the proton gradient and membrane potential. The plant cation/H⁺ exchangers (CAXs) are part of the ensemble of transporters that coordinate the redistribution of various cations including calcium (Ca²⁺) in exchange for the protons generated by H⁺-pumps.

To date, there are over 200 full-length CAX cDNAs or predicted open reading frames (ORFs) in the GenBank database (Manohar et al., 2010). CAXs are one of the five closely related families of transporters that together form a large phylogenetic clade, termed the Ca²⁺/cation antiporter (CaCA) superfamily (Shigaki and Hirschi, 2006). These CAXs have been classified into three major categories based on phylogenetic analysis: Types I (CAXs similar to *A. thaliana* CAX1), II (CAXs similar to *S. cerevisiae* VNX1) and III (CAXs similar to *E. coli* ChaA) (Shigaki et al., 2006).

The past several years has focused on the physiological roles, biochemistry and molecular biology of CAXs. CAXs are now recognised to be

involved in a number of important aspects of plant growth and development (Conn et al., 2011) and may be excellent tools for nutritional enhancement of crops and mitigating pollutants in soils. We will summarise in this review the knowledge of subcellular localization of CAX proteins in plants.

1 Subcellular localization of CAX proteins in *Arabidopsis thaliana*

To further establish the subcellular localization of CAX1 in plants, microsomal membranes from wild-type and transgenic lines harboring the hemagglutinin (HA)-tagged truncated CAX1 fusion protein (HA-sCAX1) were fractionated. Centrifugation through a continuous Suc gradient was first used to compare the distribution of the epitope-tagged transporter in Arabidopsis (Figure 1) and the native full-length CAX1 (Figure 1) with that of markers for the vacuole, plasma membrane, and endoplasmic reticulum. As shown in Figure 1, when membrane fractions were assayed for CAX1 and HA-sCAX1 accumulation, proteins of 50 kD increased at 30% Suc (most abundant in fractions corresponding to 28% and 37% Suc, respectively). Both endogenous CAX1 and HA-sCAX1 accumulated in fractions enriched in vacuolar membranes, which overlap with that of a resident protein (V-PPase) from this membrane.

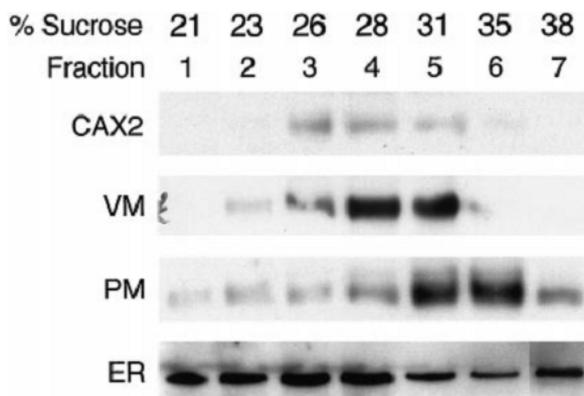


Figure 1 Subcellular Localization of CAX1 in Arabidopsis. Immunoblot analysis of CAX1 in Arabidopsis. Equal amounts of protein (10 g) isolated from HA-sCAX1-expressing transgenic Arabidopsis, wild-type Arabidopsis, and plant membrane markers: the plant endoplasmic reticulum luminal protein (BiP), mung bean vacuolar pyrophosphatase (V-PPase). Wild-type Arabidopsis membranes were prepared and fractionated in the absence or presence of 5 mmol/L MgCl₂, and then assayed for changes in CAX1 and BiP expression. Numbers above the gels indicate the Suc concentration of each fraction

CAX2 contains 11 putative transmembrane domains and has a predicted molecular mass of 39 kD (Hirschi et al., 1996). In yeast this protein appears to function at the tonoplast membrane (Hirschi et al., 1996). To identify the cellular localization of CAX2 in plants, researchers produced polyclonal antibodies against a peptide from the deduced amino acid sequence of the central nonmembranal loop. As shown in Figure 2, western-blot analysis of Arabidopsis membranes fractionated on Suc gradients show that CAX2 cofractionates with the vacuolar membrane marker tonoplast intrinsic protein, and not with plasma membrane or endoplasmic reticulum markers. Thus, CAX2 is predominately localized in the vacuolar membrane.

The localization of HA: CAX4 on the vacuolar membrane of plants was shown by the heterologous expression of the CAX4 fusion protein in a suspension of tobacco cells. The fusion protein under the control of the cauliflower mosaic virus 35S promoter was expressed in tobacco BY-2 cells. In the case of BY-2 cells, many vacuoles are generally observed in a single cell (Kost et al., 1998). As shown in Figure 3, A and B, confocal images of red fluorescent signals stained by Texas Red conjugated antibody revealed that HA: CAX4 is localized in the vacuolar membrane.

To confirm vacuolar membrane localization of the

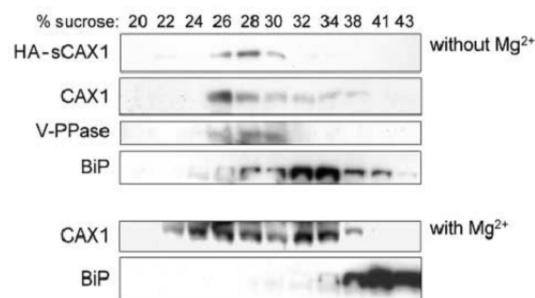


Figure 2 Intracellular localization of CAX2 in wild-type Arabidopsis plants. Arabidopsis membranes were extracted and fractionated in a Suc gradient. The fractions (fraction 1=21%; fraction 7=38% [v/v] Suc) were subjected to western-blot analyses using the following antibodies: CAX2, affinity-purified antibodies against a peptide from CAX2 deduced amino acid sequence; VM, antibodies against a vacuolar membrane marker VM23, a homolog of tonoplast intrinsic protein from radish (*Raphanus sativus*), which is a species closely related to Arabidopsis (Maeshima, 1992); PM, antibodies against the Arabidopsis plasma membrane marker protein RD-28 (Yamaguchi et al., 1992); ER, antibodies against the endoplasmic reticulum yeast BiP protein that specifically recognize plant endoplasmic reticulum BiP (Shimoni et al., 1995)

To confirm vacuolar membrane localization of the fusion protein, the crude membrane fraction of tobacco cells was subjected to Suc gradient centrifugation and immunoblotting with the anti-HA antibody. The distribution of the fusion protein (fractions from 26% to 33%) was paralleled by that of a vacuolar marker (mung bean vacuolar pyrophosphatase) and not that of an endoplasmic reticulum marker (plant endoplasmic reticulum luminal protein) and a plant plasma membrane marker (radish plasma membrane aquaporin; Figure 3C).

2 Subcellular localization of CAX protein in *Vigna radiata*

The localization of VCAX1p on the vacuolar membrane was confirmed by the heterologous expression of VCAX1p in a suspension of tobacco cells. In this experiment, the VCAX1 sequence was fused at the C-terminus of sGFP and introduced into tobacco BY-2 cells via *Ag. tumefaciens* to express the fusion protein under the control of the CAMV 35S promoter. In the case of BY-2 cells, many vacuoles were generally observed in a single cell. Confocal imaging of GFP fluorescence emitted from transformed cells revealed that sGFP-VCAX1p is

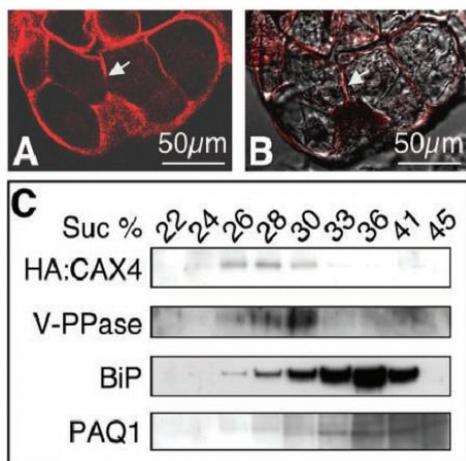


Figure 3 Expression of HA: CAX4 in tobacco BY-2 cells. A and B, Immunostaining of HA: CAX4 in BY-2 cell. A, HA: CAX4 was detected on vacuolar membrane by HA antibody and Texas Red conjugated secondary antibody (indicated by arrow). B, A superimposed image of red channel and transmitted light demonstrated only vacuolar membranes were stained (indicated by arrow). C, Immunoblotting of HA: CAX4 in tobacco BY-2 cells. Monoclonal antibodies against HA and a plant endoplasmic reticulum luminal protein (BiP), an indicator of endoplasmic reticulum, were used at dilutions of 1:1,000 and 1:1,500, respectively. Polyclonal antibodies against mung bean (*Vigna radiata*) vacuolar pyrophosphatase (V-PPase) and radish plasma membrane aquaporin (PAQ1) were used at 1:1,000 dilutions. The numbers indicate the concentration of the Suc fraction

localized in the vacuolar membrane and small vesicles (Figure 4). No green fluorescence was detected on the plasma membrane, nuclear envelopes or free cytosol.

3 Subcellular localization of CAX protein in *Glycine max*

A green fluorescent protein (GFP)-tagged version of GmCAX1 was made into a plant expression vector and then transformed and expressed in *Arabidopsis*. The GFP fluorescence in the guard cells of mature leaf was the most intensive. Optical sectioning through the center of the cell verified that the fluorescent signal was localized to the periphery of stomata in guard cells (Figure 5D~F), whereas the GFP alone was localized in the entire guard cells (Figure 5A~C). The result suggests that the GmCAX1-GFP fusion protein may be localized on the plasma membrane. To further investigate the GmCAX1 localization, the GmCAX1-GFP fusion gene was also introduced into the onion epidermal cells by particle bombardment. Before plasmolysis, the green fluorescence was mainly localized in the peripheral region of the cell (Figure 5I, J). After plasmolysis, the green fluorescence in the



Figure 4 Localization of sGFP-VCAX1p fusion protein expressed in tobacco suspension cells. A fusion protein of sGFP and VCAX1p was expressed in tobacco BY-2 cells, and then sGFP green fluorescence of the cells was imaged under a laser scanning confocal microscope. Scale bar: 20 μ m

GmCAX1-GFP fusion protein appeared to be localized on the plasma membrane (Figure 5K, L). The strong fluorescence aggregates in Figure 5K possibly indicate the shrinkage of the membranes at the two poles of the cell and around the nucleus. The control GFP protein was mainly present in the cytosol portion (Figure 5G, H).

4 Subcellular localization of CAX protein in *Oryza sativa*

Subcellular localization prediction (<http://psort.nibb.ac.jp>) showed that OsCAX3 is located on the plasma membrane of plant. The pBI221-GFP and pBI221-OsCAX3-GFP fusion vector were transformed into *Arabidopsis mesophyll* protoplast. Results showed, in the GFP control cells, fluorescence was distributed in cytoplasm and nucleus; while in the OsCAX3-GFP cells, fluorescence was distributed in plasma membrane (Figure 6). This shows that OsCAX3 is a plasma membrane $\text{Ca}^{2+}/\text{H}^{+}$ antiporter.

5 Subcellular localization of CAX protein in *Puccinellia tenuiflora*

We cloned a Cation/ H^{+} antiporter gene (*PutCAX2*) from *Puccinellia tenuiflora*. The recombinant plasmids of pBI121-GFP and pBI121-GFP-PutCAX2 were transformed into *Arabidopsis mesophyll* protoplast. Upper shows the expression of the GFP protein in *Arabidopsis* protoplasts, green fluorescence was distributed in cytoplasm; while in the GFP-PutCAX2 cells (below), green fluorescence was distributed on *Golgi apparatus*, which merged with the red fluorescence of Golgi-red dye (BODIPY® TR C5-ceramide complexed with BSA) (Figure 7). It

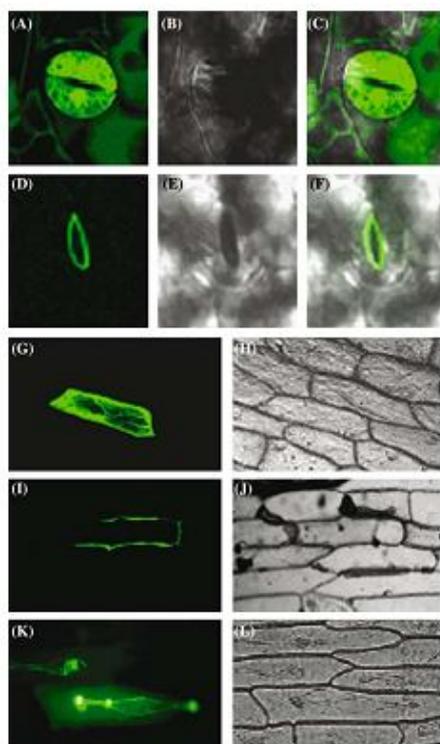


Figure 5 Subcellular localization of the GmCAX1-GFP fusion protein in plant cells. A~C, Localization of the control GFP protein in guard cells of transgenic Arabidopsis plants overexpressing the GFP gene. D~F, Localization of the GmCAX1-GFP fusion protein in guard cells of the transgenic plants overexpressing the GmCAX1-GFP fusion gene. G and H, Localization of the control GFP protein in an onion epidermal cell. I and J, Localization of the GmCAX1-GFP in the peripheral region of an onion epidermal cell before plasmolysis. K and L, Localization of the GmCAX1-GFP in the plasma membrane of an onion epidermal cell after plasmolysis. The strong fluorescence aggregates indicate the shrinkage of the membrane at the two poles and around the nucleus. The photographs were taken in the dark field for green fluorescence (parts A, D, G, I and K), in the bright light for the morphology of the cells (parts B, E, H, J and L), and in combination (parts C, F)

indicates that *PutCAX2* locates on the Golgi of *Arabidopsis protoplas*.

6 Conclusion

In this review, we showed the subcellular localization of different CAX proteins in different plants. The localization varies a lot from species to species. Both AtCAX1 and AtCAX2 are located on vacuolar membrane of Arabidopsis. Also, AtCAX4 and VCAX1p are located on vacuolar membrane of transgenic tobacco. But the difference is that GmCAX1 mainly resides on the plasma membrane of transgenic Arabidopsis and onion epidermal cells.

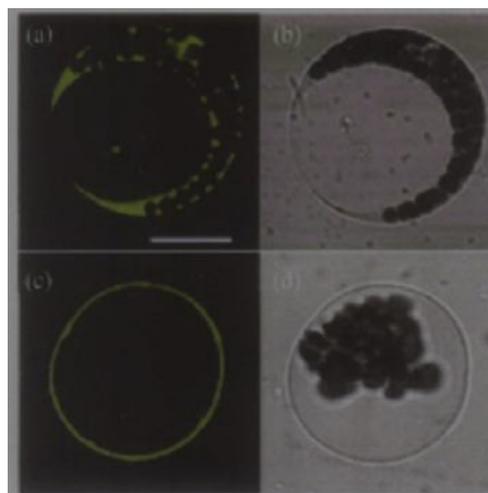


Figure 6 Transient expression and subcellular localization analysis of OsCAX3-GFP fusion protein in mesophyll cell protoplast of Arabidopsis (a) Confocal microscopy images of green fluorescence in protoplasts expressing GFP alone. (c) Optical sectioning microscopy image of green fluorescence in protoplasts expressing OsCAX3-GFP. (b), (d) the same cells of (a) and (c) in brightfield. Bar in (a) = 20 μ m. (b), (d) Cells were photographed in brightfield shown in (a) and (b)

When expressed in mesophyll cell protoplast of Arabidopsis, OsCAX3 also resides on the plasma membrane. The interesting thing is that *PutCAX2* from *Puccinellia tenuiflora* surprisingly locates on the Golgi of transgenic Arabidopsis. We still yet don't know what makes all this difference, and what the key point is to clear the mystery behind. In the near future, it is important to combine the localization and the expression of the CAX genes in various growth and developmental mutants to explore the mechanism of CAX.

Acknowledgments

This study was supported by a grant from the National Natural Science Foundation of China (NSFC, No. 31270311) awarded to Xinxin Zhang.

Reference

- Cheng N.H., Pittman J.K., Bronwyn J.B., Shigaki T., and Hirschi K.D., 2003, The Arabidopsis *cax1* Mutant Exhibits Impaired Ion Homeostasis, Development, and Hormonal Responses and Reveals Interplay among Vacuolar Transporters, *Plant Cell*, 15: 347-364
<http://dx.doi.org/10.1105/tpc.007385>
- Cheng N.H., Pittman J.K., Shigaki T., and Hirschi K.D., 2002, Characterization of CAX4, an Arabidopsis H⁺/Cation Antiporter, *Plant Physiology*, 128: 1245-1250
<http://dx.doi.org/10.1104/pp.010857>

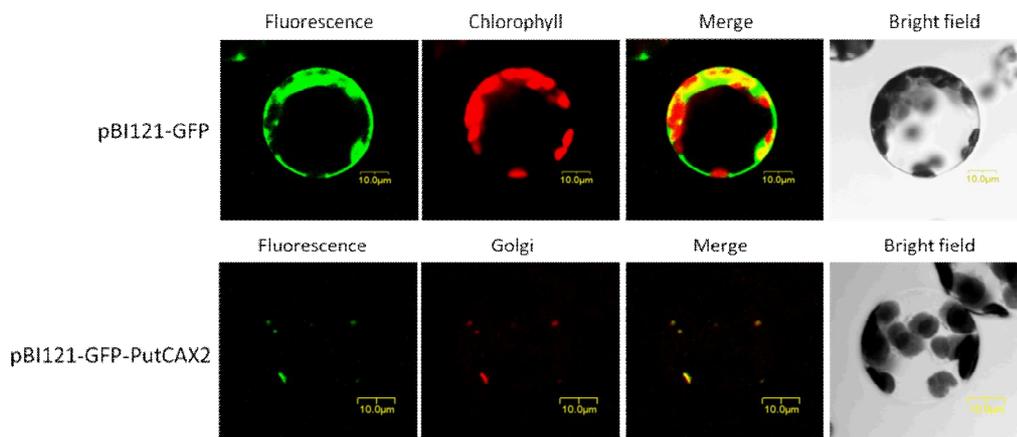


Figure 7 Subcellular localization of the GFP fusion PutCAX2 protein in Arabidopsis protoplasts. Live cells were stained using BODIPY® TR C5-ceramide complexed with BSA, and the localization was observed using confocal microscopy. Bars=10 µm. (Upper) Expression of the GFP protein in Arabidopsis protoplasts. (Below) Expression of the GFP-PutCAX2 protein in Arabidopsis protoplasts

- Conn S.J., Gillham M., Athman A., Schreiber A.W., Baumann U., Moller I., Cheng N.H., Stancombe M.A., Hirschi K.D., Webb A.R., Burton R., Kaiser B.N., Tyerman S.D., Leigh R.A., 2011, Cell specific vacuolar calcium compartmentation regulates apoplastic calcium concentration, gas exchange and plant productivity, *Plant Cell*, 23: 240-257
<http://dx.doi.org/10.1105/tpc.109.072769>
- Hanayo U.N., Tomohiro T., Maki S., Yoichi N., Kyle W.C., Masayoshi M., 2000, Functional expression of mung bean Ca^{2+}/H^{+} antiporter in yeast and its intracellular localization in the hypocotyl and tobacco cells, *Eur. J. Biochem.*, 267: 1-10
- Hirschi K.D., Zhen R.G., Cunningham K.W., Rea P.A., Fink G.R., 1996, CAX1 and H^{+}/Ca^{2+} antiporter for Arabidopsis, *Proc. Natl. Acad. Sci.*, 93: 8782-8786
<http://dx.doi.org/10.1073/pnas.93.16.8782>
- Kendal D.H., Victor D.K., Nathaniel L.W., George J. W., 2000, Expression of Arabidopsis CAX2 in Tobacco, Altered Metal Accumulation and Increased Manganese Tolerance, *Plant Physiology*, 124: 125-133
<http://dx.doi.org/10.1104/pp.124.1.125>
- Kost B.S., Spielhofer P., Chua N.H., 1998, A GFP-mouse talin fusion protein labels plant actin filaments in vivo and visualizes the actin cytoskeleton in growing pollen tubes, *Plant J*, 16: 393-401
<http://dx.doi.org/10.1046/j.1365-3113x.1998.00304.x>
- Maeshima M., 1992, Characterization of the major and integral protein of vacuolar membrane, *Plant Physiol*, 98: 1248-1254
<http://dx.doi.org/10.1104/pp.98.4.1248>
- Manohar M., Mei H., Franklin A.J., Sweet E.M., Shigaki T., Riley B.B., MacDiarmid C.W., Hirschi K.D., 2010, Zebrafish (*Danio rerio*) endomembrane antiporter similar to a yeast cation/ H^{+} transporter is required for neural crest development, *Biochemistry*, 49: 6557-6566
<http://dx.doi.org/10.1021/bi100362k>
- Manohar M., Shigaki T., Hirschi K.D., 2010, Plant cation/ H^{+} exchangers (CAXs): biological functions and genetic manipulations, *Plant Biology*, 13: 56-569
- Shigaki T., Hirschi K.D., 2006, Diverse functions and molecular properties emerging for CAX cation/ H^{+} exchangers in plants, *Plant Biology*, 8: 419-429
<http://dx.doi.org/10.1055/s-2006-923950>
- Shigaki T., Rees I., Nakhleh L., Hirschi K.D., 2006, Identification of three distinct phylogenetic groups of CAX cation/proton antiporters, *Journal of Molecular Evolution*, 63: 815-825
<http://dx.doi.org/10.1007/s00239-006-0048-4>
- Shimoni Y., Zhu X., Levanony H., Segal G., Galili G., 1995, Purification, characterization and intracellular localization of glycosylated protein disulfide isomerase from wheat grains, *Plant Physiol.*, 108: 327-335
<http://dx.doi.org/10.1104/pp.108.1.327>
- Yamaguchi S.K., Masahiro K., Satomi U., Kazuo S., 1992, Molecular cloning and characterization of 9 cDNAs for genes that are responsive for desiccation in *Arabidopsis thaliana*: sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein, *Plant Cell Physiol.*, 33: 217-224