Interaction between \textit{Arabidopsis} Ca\textsuperscript{2+}/H\textsuperscript{+} Exchangers CAX1 and CAX3\textsuperscript{**}

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Jian Zhao\textsuperscript{1}, Toshiro Shigaki\textsuperscript{1}, Hui Mei\textsuperscript{3}, Ying-qing Guo\textsuperscript{3}, Ning-Hui Cheng\textsuperscript{3}, and Kendal D. Hirschi\textsuperscript{1,3,5,1}

From the \textsuperscript{1}United States Department of Agriculture/Agricultural Research Service, Children’s Nutrition Research Center, Baylor College of Medicine, Houston, Texas 77030-23600 and the \textsuperscript{3}Vegetable and Fruit Improvement Center, Texas A&M University, College Station, Texas 77845

In plants, high capacity tonoplast cation/H\textsuperscript{+} antiport is mediated in part by a family of CAX (cation exchanger) transporters. Functional association between CAX1 and CAX3 has previously been inferred; however, the nature of this interaction has not been established. Here we analyze the formation of “hetero-CAX” complexes and their transport properties. Co-expressing both CAX1 and CAX3 mediated lithiated and salt tolerance in yeast, and these phenotypes could not be recapitulated by expression of deregulated versions of either transporter. Coincident expression of \textit{Arabidopsis} CAX1 and CAX3 occurs during particular stress responses, flowering, and seedling growth. Analysis of \textit{cax1}, \textit{cax3}, and \textit{cax1}/\textit{cax3} seedlings demonstrated similar stress sensitivities. When plants expressed high levels of both CAXs, alterations in transport properties were evident that could not be recapitulated by high level expression of either transporter individually. In \textit{ planta} coimmunoprecipitation suggested that a protein-protein interaction occurred between CAX1 and CAX3. \textit{In vivo} interaction between the CAX proteins was shown using a split ubiquitin yeast two-hybrid system and gel shift assays. These findings demonstrate cation exchange plasticity through hetero-CAX interactions.

Calcium (Ca\textsuperscript{2+}) is a cofactor for many enzymes, a vital signaling molecule and a structural component in providing the plant cell its strength (1, 2). Regulated Ca\textsuperscript{2+} fluctuations are presumably involved in every aspect of plant growth and adaptations. Ca\textsuperscript{2+} transporters on various membranes play an important role in orchestrating these diverse biological processes. Although a burgeoning number of Ca\textsuperscript{2+} transporters have been identified, it is often difficult to associate functions with particular transporters.

Ca\textsuperscript{2+} can accumulate to millimolar levels in the vacuole, whereas the concentrations are maintained in the micromolar range in the cytosol (3). This concentration gradient is established across the tonoplast in part by high capacity Ca\textsuperscript{2+}/H\textsuperscript{+} exchange and via Ca\textsuperscript{2+} pumping directly energized by ATP hydrolysis (4, 5). Plant Ca\textsuperscript{2+}/H\textsuperscript{+} exchangers were cloned by the ability of N-terminal truncated versions of the proteins to function in \textit{Saccharomyces cerevisiae} mutants defective in vacuolar Ca\textsuperscript{2+} transport (6–8). The term CAX (cation exchanger) is now used to identify CAX1 and CAX2 as well as four other CAX transporters in the \textit{Arabidopsis} genome (9). N-terminal truncations of these transporters are termed sCAXs. Interestingly, CAX3 (and sCAX3), which is most similar to CAX1 (77% identical at the amino acid level), is at best a weak vacuolar Ca\textsuperscript{2+} transporter when expressed in yeast cells (10, 11). Understanding of the biological roles these CAX transporters play in cell growth and in response to environmental stresses is only beginning to emerge.

Interplay between CAX1 and CAX3 has been suggested through genetic studies and yeast expression assays (11). In \textit{ planta}, deletions in CAX1 cause compensatory changes in gene expression among a battery of transporters, including heightened expression of CAX3 (12). Although \textit{cax1} and \textit{cax3} knock-out lines individually display subtle phenotypes, stunting and leaf chlorosis are readily apparent when both CAX1 and CAX3 are perturbed (11). In yeast suppression assays, when autoinhibited versions of CAX1 and CAX3 are co-expressed in yeast mutant cells, there is measurable Ca\textsuperscript{2+}/H\textsuperscript{+} exchange activity that is not present when these regulated transporters are expressed individually. The challenge is to delineate the nature and specificity of this association and determine how the action of endomembrane Ca\textsuperscript{2+}/H\textsuperscript{+} antiporters is integrated into various biological processes.

Frequently, transporters oligomerize to form pores. In some cases, the nature of the oligomer affects their function (13, 14). Recent work with ammonium transporters from \textit{Arabidopsis} suggests that allosteric interactions between isoforms may be essential for activity (15). In some cases, coupling between transporters may be a mechanism for increasing the dynamic range of transporter regulation and function.

Here we detail the interaction between the \textit{Arabidopsis} Ca\textsuperscript{2+}/H\textsuperscript{+} exchangers CAX1 and CAX3. We demonstrate an interaction between CAX1 and CAX3 in \textit{ planta} and define this interaction using both plant and yeast assays. We conclude that CAX1 and CAX3 can be combined to form functional transporters with distinct transport properties.

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\textsuperscript{1}To whom correspondence should be addressed: Baylor College of Medicine, USDA/ARS Children’s Nutrition Research Center, 1100 Bates St., Suite 9018, Campus Mail Stop: BCM-320, Houston, TX 77030-2600. Tel.: 713-798-7011; Fax: 713-798-7171; E-mail: kendalh@bcm.edu.
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EXPERIMENTAL PROCEDURES

Plant Materials and Transformation

The cax1-1, cax3-1, cax3-2, and cax1/cax3 (cax1/3) lines are in the Arabidopsis ecotype Col-0 (11, 12). The triple hemagglutinin (HA)2 epitope-tagged CAX3 was subcloned into a binary vector pH1121 by replacing the β-glucuronidase gene (11) and transformed into Agrobacterium tumefaciens GV3101 using the floral dip method (16).

For seed germination test, seed lots for Col-0, cax1-1, cax3-1, cax3-2, and cax1/cax3 seeds were treated as described previously (17) and sown on one-half strength MS medium (18) agar plates (1.5% sucrose, 0.8% agar, pH 5.6) with or without CaCl2 stored daily, and at least three independent experiments (about 60 seeds in each experiment) were performed. Seeds with protruded radicles were regarded as germinated. Germination rate was expressed as a percentage of total sown seeds.

Histochemical Assay of CAX::GUS Gene Expression

Histochemical assays for CAX::GUS activity were performed according to the protocol described previously (11). These CAX::GUS seeds were germinated on one-half strength MS medium (18) and monitored daily. Samples were photographed using a Nikon (Tokyo, Japan) E600W microscope.

Yeast Strains and Split Ubiquitin Vectors

The Ca2+-sensitive Saccharomyces cerevisiae strain K667 (MATa cnb1::LEU1 pmcl1::TRP1 vxi1Δ ade2-1 can1-100 his3-11,15 leu2-3,112 trpl-1 ural-3) was used for most yeast assays (19). The yeast NHX1 mutant WXY (Matu leu2-13 112 ural-3 trpl-1 his3-11, 15 ade2-1 can1-100 nhx1::TRP1) was used for additional sodium sensitivity analysis (20). The Nub and Cub vectors, KAT1-Cub and Nub-KAT1 constructs, and yeast strains THY.AP4 (MATa leu2-3,112 ural-3-52 trpl-1-289 lexa::HIS3 lexa::ADE2 lexa::lacZ) and THY.AP5 (MATa ura3 leu2-3,112 trpl-1-289 his3-Δ1 ade2::loxP) used for the mating-based split ubiquitin system were obtained from Dr. Wolf B. Frommer at Stanford University (13). CAX1, CAX3, sCAX (where the first 36 amino acids of CAX1 and CAX3 have been removed), and sCAX1H338N have been described previously (21, 22).

Construction of CAX Split Ubiquitin Plasmids

Cloning of CAX1 and CAX3, was conducted using in vivo recombinational cloning (23). Standard PCR conditions were used to amplify and clone CAX open reading frames into mBSUS vectors. The primers contained a B1 or B2 linker, and CAX1B1 (5′-ACAAAGTTTGTACAAAAAGCGAGCTTCCAAACCACATGGCGGAAATCGTGACAGAG-3′) and CAX1B2 (5′-TCCGCCACCAACACACTTTGGTACAAGAAAGCTGGTAAAGATGAGAAAATCTCTCC-3′) were used for full-length CAX1 amplification. Similarly, a pair of primers, CAX3B1 (5′-ACAAGTTTGTACAAAAAGCGAGCTTCCAAACCACATGGCGGAAATCGTGACAGAG-3′) and CAX3B2 (5′-TCCGCCACCAACACACTTTGGTACAAGAAAGCTGGTAAAGATGAGAAAATCTCTCC-3′), were used for full-length CAX3 amplification. For the construction of NubX and NubWT fusions, the split ubiquitin vectors NubX and NubWT were cleaved with EcoRI/Smal and mixed with the purified PCR products for CAX1, CAX3, N-CAX1, and C-CAX3 to transform THY.AP5 cells, and transformants were selected on SC medium lacking tryptophan and uracil. For Cub fusions, the vector metYcgate was cleaved with PstI/HindIII and mixed with the same PCR products to transform yeast strain THY.AP4, and transformants were selected on SC medium lacking leucine. After the initial interaction assay, plasmids were extracted from the yeast strains and amplified in E. coli. The interactions were then verified by repeating the assays using the purified plasmids, whose inserts were sequenced for confirmation.

Plant Microsomal Protein Extraction and Co-Immunoprecipitation

Microsomal membranes were extracted from Col-0, cax1-1, 35S::HA-CAX3-transgenic Arabidopsis plant leaves (3 weeks old). Tissues were ground to a fine powder in liquid nitrogen and resuspended in ice-cold extraction buffer: 50 mM MOPS-KOH, pH 7.6, 0.5 mM sorbitol, 5 mM EDTA, 5 mM EGTA, 1.5% polyvinylpyrrolidone 40,000, 0.5% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM DTT. The homogenate was filtered through four layers of cheesecloth and then centrifuged at 12,000 × g for 10 min. The supernatant was diluted in 10% glycerol, 5 mM Tris-MES, pH 7.6, 1 mM PMSF, and 1 mM DTT and then centrifuged at 120,000 × g for 45 min. The microsomal pellet was resuspended in membrane solubilization buffer containing 10 mM Tris-MES, pH 7.6, 1% Triton X-100, 75 mM KCl, 2 mM DTT, 1 mM PMSF, and 1 mg/liter leupeptin. After microsomal proteins were completely solubilized, samples were centrifuged at 4 °C to remove debris, and the supernatants were used for co-immunoprecipitation. Microsomal proteins were incubated with 20 μl of monoclonal mouse IgG against HA (Covance, Berkeley, CA) or 50 μl of CAX1 peptide antibody raised in rabbit against the N terminus of CAX1 (12), or 50 μl of V-ATPase B subunit antibody (24), 20 μl of vacuolar TPC1 (two-pore channel 1) antibody (25), TIP1.2 (tonoplast intrinsic protein aquaporin antibody) (26), or AVP1 (Arabidopsis vacuolar H+/pyrophosphatase antibody) (27) raised against rabbit IgG in CIP buffer (50 mM Tris-HCl, pH 7.5, 1 mM PMSF, 100 mM NaCl) with protein inhibitor mixture (Sigma). Samples were placed on ice for 2 h, and then 50 μl of protein A-agarose beads (pre-equivalent with CIP buffer without protease inhibitors) for 1 h at 4 °C. After four washings with CIP buffer and phosphate-buffered saline (PBS) by centrifugation at 4 °C, 500 × g for 2 min, bound proteins in beads were eluted by boiling the beads in SDS sample-loading buffer, and proteins were resolved by 12% SDS-PAGE followed by transfer to polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked with 5% milk in PBS-Tween 20 (PBST, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) at room temperature and incubated with primary antibodies, diluted in
2% milk in PBST (anti-HA at 1:2000, anti-V-ATPase at 1:1000, anti-CAX1 at 1:500, anti-TPC1 at 1:1000, anti-AVP1 at 1:3000, anti-TIP1;2 at 1:2000). Following washing, membranes were incubated with hors eradish peroxidase-conjugated anti-mouse or -rabbit secondary antibodies (Amersham Biosciences) for 1 h and detected by chemiluminescence using an ECL kit (Amersham Biosciences). The AVP1, TIP1;2, and TPC1 antibodies have been described previously (25–27).

For detection of the CAX1-CAX3 complex in plants, the detergent-solubilized plant microsomal fractions were prepared as described above from 3-week-old 35S::HA-CAX3 transgenic seedlings with or without 15 mM LiCl for 8 h. As previously detailed, anti-CAX1 antibody was used to immuno-precipitate the CAX1-CAX3 protein complex. Extensively washed precipitates with CIP and PBST buffer were dissolved in protein loading buffer without DTT and resolved in native gel for PAGE. Similar to the yeast gel shift assay, after transfer to PVDF membrane, anti-HA and anti-CAX1 antibodies were used to detect protein complex formation.

**Yeast Growth Conditions**

**Suppression Assay—Saccharomyces cerevisiae** strain K667 or WX1 was transformed with yeast expression constructs as described previously (21). Transformed cells were tested for their capability to suppress the K667 Ca\(^{2+}\)-sensitive phenotype (6) or WX1 NaCl-sensitive phenotype (20). Growth assays using K667 cells were conducted using YPD medium supplemented with or without CaCl\(_2\) (50, 100, 150, or 200 mM), LiCl (30, 50, 60, or 100 mM), or NaCl (400, 500, or 600 mM). Lithium and CaCl\(_2\) plates were made in YPD agar medium. The LiCl stock solution was filter-sterilized and added to the autoclaved YPD agar medium to final concentrations of 30, 50, 70, or 100 mM. K667 cells expressing various constructs were grown in SC selection medium overnight at 30 °C. After a series of 5-fold dilutions with water, 5-μl cell suspensions were spotted on the indicated media, and the cells were grown for 3 days. A growth assay with the WX1 strain was conducted on AP medium supplemented with or without 15 mM LiCl for 8 h. As previously described (31). Data from five repeats were calculated with a formula, (ion\(_{\text{cax}}\) − ion\(_{\text{vector}}\))/ion\(_{\text{vector}}\) × 100, and expressed as means ± S.E. (n = 5).

**Gel Mobility Shift Assay**

Microsomal fractions from yeast cells expressing CAX variants were prepared using the glass bead method (32). Briefly, glass bead buffer (25 mM Hepes-KOH, pH 7.5, 10% sucrose, 3 mM EGTA, 2 mM DTT, 1 mM PMSF, 10 mM benzamidine, and 5 μg/ml leupeptin) was added to the cells with an equal volume of glass beads (Sigma), and the mixture was vortexed for 3 × 1 min at maximum speed with intervals of 10 min on ice. Finally, Triton X-100 was added to the lysate to a final concentration of 0.5%. The lysate was centrifuged at 5,000 × g for 5 min, and the supernatant was saved. Equal amounts of protein (about 15 μg) from samples were resolved on native 12% PAGE with SDS-PAGE running buffer, followed by transferring membranes and immunoblotting as described above. Proteins were detected with HA, c-Myc, and GFP antibodies (Covance, Berkeley, CA).

**Structure and Topology Prediction for CAX1 and CAX3**

Transmembrane domains and topology of CAX1 and CAX3 were predicted using the ARAEMENON data base (available on the World Wide Web) and the transmembrane hidden Markov model (TMHMM version 2.0) program.

**Split Ubiquitin Assay**

The split ubiquitin system used in this assay was a mating-based split ubiquitin system developed previously (23, 33). Approximately 40 clones of each THY.AP5 and THY.AP4 transformation with various Nub and Cub constructs were mixed and incubated in appropriate selective SC with and without G418. Cultures of the lag phase were used for mating, as described previously (13). After mating THY.AP4 and THY.AP5 strains on YPD for 8 h of incubation at 28 °C, diploid cells were selected by replica plating on SC without tryptophan, leucine, and uracil for 2–3 days. Diploid cells were used to test on SC medium supplemented with 150 μM methionine for 3 days. After sequencing confirmation, these Nub and Cub constructs containing CAX1 and CAX3 constructs were used for retransformation, and the interaction assay was repeated using the confirmed Nub and Cub constructs.

For the filter assay, diploid cells were grown for 3 days on sterilized filter sets placed on SC medium supplemented with His, adenine, and 150 μM methionine. β-Galactosidase activity assays were conducted according to the standard X-gal filter assay protocol described previously (23). Color changes in the positive interactions could be detected within 3 h.

**Preparation of Membrane Vesicles and Calcium Transport Measurements**

Plants were grown hydroponically and membrane vesicles for yeast and plant Ca\(^{2+}\) uptake were performed as described previously (12, 21, 34). Three-week-old plants (mainly roots) were collected after 16 h of treatment with or without 100 mM CaCl\(_2\) (34). For the measurement of pH-dependent Ca\(^{2+}\) uptake, vacuole-enriched membrane vesicles were incubated in buffer containing 0.3 mM sorbitol, 5 mM Tris-MES (pH 7.6), 25 mM KCl, 0.1 mM sodium azide, and 0.2 mM sodium orthovana-
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date. The vesicles were added to 1 mM MgSO₄ and 1 mM ATP to reach steady state pH gradient for 5 min at 25 °C before the addition of ⁴⁵Ca²⁺ (6 mCi/ml; American Radiolabeled Chemicals, St. Louis, MO). The final concentration of Ca²⁺ in the reaction mixture was 10 μM and 1 mM. At the indicated times, aliquots of the reaction mix were removed and filtered through premoistened 0.45-μm filters (Millipore, Bedford, MA), followed by washing with 1 ml of ice-cold wash buffer (0.3 M sorbitol, 5 mM Tris-MES, pH 7.6, 25 mM KCl, and 0.1 mM CaCl₂), the filters were air-dried, and radioactivity was determined by liquid scintillation counting. For metal competition experiments, ΔpH-dependent 10 μM ⁴⁵Ca²⁺ uptake was measured at a 10 min time point in the presence of 100 μM or 1 mM nonradioactive metals.

Semiquantitative Reverse Transcription and Gene Expression Analysis

For testing CAX1 and CAX3 expression, Col-0 seeds were analyzed at different times during germination. For LiCl induction, germinating seeds on day 4 were treated with 15 mM LiCl, and seedling RNA was extracted 8, 16, and 24 h post-treatment. For CAX1 expression in cax1-1, Col-0, and 35S::HA-CAX3 transgenic plants, roots of 3-week-old plants grown hydroponically in B5 medium were harvested for RNA extraction. Total RNA was extracted with the RNeasy plant mini kit (Qiagen, Valencia, CA). First strand cDNA was synthesized with Superscript II RNase H-reverse transcriptase kit with (Qiagen, Valencia, CA). First strand cDNA was synthesized with Superscript II RNase H-reverse transcriptase kit with oligo(dT)₁₂₋₁₈ primer (Invitrogen) according to the manufacturer’s instructions. PCR was performed with the following program: 94 °C for 2 min to denature DNA and then from 94 °C to 55 °C for 30 s, 94 °C for 10 min. A 10 min time point in the presence of 100 μM or 1 mM nonradioactive metals.

RESULTS

Phenotypes When Co-expressing CAX1 and CAX3 in Yeast—

We demonstrated previously that co-expression of CAX1 and CAX3 can suppress yeast vacuolar Ca²⁺ transport defects, whereas expression of either transporter individually failed to do so (11). In fact, in yeast assays, we have never been able to measure Ca²⁺/H⁺ transport mediated by CAX1 or CAX3. Expression of the deregulated version of CAX1, sCAX1, confers high levels of Ca²⁺/H⁺ transport; however, we have not yet isolated a deregulated version of CAX3 that functions as a Ca²⁺/H⁺ transporter. In the present study, we were interested in the potential change in substrate specificity that might occur during the putative CAX1 and CAX3 association. As shown in Fig. 1, co-expression of CAX1 and CAX3 could produce Li⁺ tolerance, a phenotype that was not observed when deregulated versions of the transporters were expressed individually (Fig. 1A). Data suggest that both proteins localize to the yeast vacuole (supplemental Fig. 1), and we have previously shown equivalent expression levels of these transporters in yeast (10, 21). We also consistently obtained this phenotype regardless of the promoters and vectors used to express either CAX1 or CAX3 (data not shown; supplemental Table 1). Furthermore, we were unable to recapitulate these phenotypes when using other Ca²⁺/H⁺ transporter pairs (data not shown). For example, CAX1 or CAX3 co-expressed with the tonoplast-localized Arai-bidopsis CAX2 or CAX4 (8) did not confer any measurable changes in yeast ion tolerance (data not shown). These phenotypes imply that CAX1 and CAX3 may interact to form oligomers that confer distinct transport properties.

We also assessed the impact of co-expressing both CAX1 and CAX3 transporters on yeast metal accumulation (Fig. 1). This experiment allows us to determine if the metal accumulation in the cells was consistent with the tolerance phenotypes. We expressed sCAX1 + vector, CAX1 + vector, CAX3 + vector, or CAX1 + CAX3 and the vector control in the K667 (vec1 pmc1 cnb1) mutant yeast strain, which is deficient in vacuolar Ca²⁺ transporters (19) and grew the cells in liquid medium supplemented with LiCl to levels sufficient to facilitate detection in cell extracts by inductively coupled plasma mass spectroscopy. Increased Ca²⁺, Na⁺, and Li⁺ accumulation was consistently observed in CAX1 + CAX3-expressing yeast cells compared with the cells expressing these transporters individually (Fig. 1A). Although CAX3-expressing cells demonstrated some increased Li⁺ content, the yeast cells expressing CAX3 were not tolerant to LiCl-containing media (Fig. 1A).

We then measured Ca²⁺ uptake in the vector control, sCAX1-1, and CAX1 + CAX3-expressing cells (Fig. 1B). The ΔpH-dependent 10 μM ⁴⁵Ca²⁺ uptake was measured at several time points into yeast vacuole-enriched membrane vesicles isolated from strains expressing vector, sCAX1, and CAX1 + CAX3. We were able to measure uptake with vesicle from sCAX1-1 and CAX1 + CAX3-expressing cells (Fig. 1B). However, we were unable to measure Ca²⁺ uptake from CAX1-1 or CAX3-expressing cells, presumably because these transporters are autoinhibited (11). The ability to measure vacuolar Ca²⁺ transport in the co-expressing cells suggests that both transporters are still predominantly localized on the tonoplast.

To analyze the properties of the putative CAX1 + CAX3 transporter, competition experiments were performed (Fig. 1C). The ΔpH-dependent 10 μM ⁴⁵Ca²⁺ uptake was measured at a single 10 min time point into yeast membrane vesicles isolated from strains expressing vector, sCAX1, and CAX1 + CAX3. This approach allowed us to determine the effect of co-expressing the CAXs in terms of cation selectivity in comparison with sCAX1. Ca²⁺ uptake determined in the absence of excess nonradioactive metal (control) was compared with Ca²⁺ uptake determined in the presence of two concentrations (10 × and 100 ×) of various nonradioactive metals (Fig. 1C). Inhibition of Ca²⁺ uptake by nonradioactive Ca²⁺ was used as a control, and as expected, Ca²⁺ uptake was inhibited in both sCAX1 and CAX1 + CAX3 transporters by excess Ca²⁺. Nonradioactive Ca²⁺, particularly 10 × concentrations, did not completely inhibit Ca²⁺ uptake, further highlighting the low Ca²⁺ affinity
of the transporters. Ca\(^{2+}\) uptake by sCAX1-expressing cells was inhibited by Cd\(^{2+}\), whereas the CAX1 + CAX3-mediated Ca\(^{2+}\) transport was not significantly inhibited by Cd\(^{2+}\). In contrast, CAX1 + CAX3-expressing cells displayed more Ca\(^{2+}\) uptake inhibition by Li\(^+\) (Fig. 1C). Both sCAX1- and CAX1 + CAX3-expressing cells displayed similar levels of Ca\(^{2+}\) uptake inhibited by excess Na\(^+\) (Fig. 1C).

To obtain a clearer understanding of the potential function of CAX1 + CAX3 in NaCl tolerance, we repeated these experiments in yeast strains lacking only the prevacuolar localized Na\(^+\)/H\(^+\) transporter NHX1p (35). In this strain, only yeast cells co-expressing CAX1 + CAX3 displayed sodium tolerance (Fig. 1D). This phenotype was obtained regardless of the promoters and vector sets used to co-express either CAX1 or CAX3 (data not shown; supplemental Table 1). When this experiment was performed using CAX1 or CAX3 co-expressed with the tonoplast-localized Arabidopsis CAX2 or CAX4, the cells did not display a sodium-tolerant phenotype (data not shown). Furthermore, using whole cell metal accumulation measurements, these same cells grown in high levels of NaCl accumulated slightly more Na\(^+\) and Li\(^+\) than controls or cells expressing either transporter individually (Fig. 1D).

**Functional Interactions between CAX1 and CAX3 in Planta**—Our previous findings suggested that CAX1 and CAX3 functionally associate in Arabidopsis cells (11). In mature plants, CAX1 is expressed predominantly in green tissues, whereas CAX3 is expressed in roots (11). However, there are several instances when the two transporters are co-expressed temporally and spatially. We have shown that CAX3 expression is generally low in shoot/leaf tissue but not completely absent. In fact, CAX3 is up-regulated by abscisic acid in guard cells where CAX1 is highly expressed (36). Additionally, CAX1 and CAX3 are co-expressed in floral tissue and many other tissues during empty vectors were expressed as controls. In the top panel, saturated liquid cultures of K667 containing various plasmids were diluted and then spotted onto selection medium or the yeast extract peptone dextrose medium supplemented with 50 mM LiCl. Photographs were taken after 2–3 days of growth at 30 °C. Data from the metal content are from five repeats and were calculated with a formula, (ion\(_{\text{cax}}\) - ion\(_{\text{vector}}\))/ion\(_{\text{vector}}\) × 100, and expressed as means ± S.E. (n = 5). B, Ca\(^{2+}\) uptake mediated by sCAX1 and CAX1 + CAX3. Time course of \(^{45}\)Ca\(^{2+}\) uptake into vacuolar vesicles prepared from the yeast strain K667 co-expressing sCAX1 with vector, CAX1 + CAX3, and vector controls. Solid circle, pH-dependent \(^{45}\)Ca\(^{2+}\) uptake; empty square, uptake in the presence of the protonophore gramicidin. The Ca\(^{2+}\) ionophore, A23187 (5 μM), was added at 12 min. The data represent means of three replications, and the bars indicate S.E. C, Ca\(^{2+}\) uptake by sCAX1 or CAX1 + CAX3 into yeast endomembrane vesicles in the presence of other metals. Uncoupler-sensitive (A2pH-dependent) uptake of 10 μM \(^{45}\)Ca\(^{2+}\), estimated as the difference between uptake with and without 5 μM gramicidin, was measured in the absence (control) or presence of 100 X NaCl or NaCl after 10 min. Ca\(^{2+}\) uptake values are shown following subtraction of the gramicidin background values and expressed as percentages of the control in the absence of any excess nonradiolabeled metals. The data represent the means of at least four repeats from three independent membrane preparations and are expressed as means ± S.E. (n = 3). B, the top panel shows that co-expression of CAX1 + CAX3 in mhx1 mutant (WX1) suppressed the NaCl-sensitive phenotype. In the lower panel, lithium and sodium content of WX1 cells expressing CAX1 + vector and CAX3 + vector and co-expressing CAX1 + CAX3 in YPD medium supplemented with 30 and 50 mM NaCl is shown. The plasmids were expressed as described above, but the yeast strains were grown in AP medium supplemented with NaCl. Photographs were taken after 3–4 days of growth at 30 °C. Data from five repeats were calculated with a formula, (ion\(_{\text{cax}}\) - ion\(_{\text{vector}}\))/ion\(_{\text{vector}}\) × 100, and expressed as means ± S.E. (n = 5).

**FIGURE 1. Co-expression of CAX1 and CAX3 in yeast.** A, the top panel shows suppression of LiCl sensitivity of the pmc1cnb1vcx1 yeast mutant (K667) by various CAX constructs. The bottom panel shows the lithium, sodium, and calcium content in K667 cells expressing sCAX1 + vector, CAX1 + vector, CAX3 + vector, and CAX1 + CAX3 with cells grown in YPD medium supplemented with 500 μM LiCl. CAX1, sCAX1, and CAX3 were expressed in various yeast vectors and transformed into K667 cells (supplemental Table 1). The
than CAX1 transport properties from lines expressing CAX1, CAX3, and CAX1-CAX3 complex, we sought to compare and contrast the contigent germination rates among control, cax1, cax3, and cax1/cax3 lines on lithium- and calcium-containing media (Fig. 3A). As shown in Fig. 2A, analysis of CAX promoter::GUS (β-glucuronidase) reporters and RT-PCR demonstrated that both transporters were expressed in young seedlings during germination. This coincident expression during germination could signify that the CAX1-CAX3 complex has an important role in development. We thus investigated germination rates among control, cax1, cax3, and cax1/cax3 lines on lithium- and calcium-containing media (Fig. 3A). In all stress conditions, germination rates from wild-type Col-0 were significantly different from cax1 or cax3 single or double mutant lines (p < 0.05). On 50 mM CaCl2-containing media, germination rates for cax1 or cax3 single mutants were significantly different from cax1/cax3 double mutants (p < 0.05), as determined by Student’s t test (Fig. 3A). In contrast, on lithium-containing media, the cax1/cax3 double mutant had similar germination rates compared with both cax1 and cax3 lines. Using RT-PCR, we demonstrated that during calcium treatments, CAX1 expression significantly increased, whereas CAX3 was slightly induced (10, 37) (Fig. 3B), and exposure to lithium increased both CAX1 and CAX3 expression levels (Fig. 3C).

To gain additional insights into the function of the putative CAX1-CAX3 complex, we sought to compare and contrast the transport properties from lines expressing CAX1, CAX3, and CAX1 + CAX3. Given that CAX3 expression levels are lower than CAX1, we ectopically expressed a HA-tagged CAX3 fusion driven by the CaMV 35S promoter in Col-0. After we verified expression of HA-CAX3 in the F3 generation (supplemental Fig. 1) and the induction of CAX1 by CaCl2 (Fig. 3B), we prepared vacuole-enriched membrane vesicles from 35S::HA-CAX3 lines, vector controls, and cax1-1 lines with and without CaCl2 treatment. Vector controls without CaCl2 express low levels of both CAX3 and CAX1, and we have previously demonstrated that we were able to measure any Ca2+/H+ antiport in vacuole-enriched membrane vesicles from these lines (12, 37). Control lines treated with CaCl2 predominantly express CAX1, and we have shown that we can measure Ca2+/H+...
exchange in these vesicles (Fig. 4A) (12, 37). With cax1-1 lines, we could assess the influence of CaCl2 treatment without CAX1 present. In the 35S::HA-CAX3 lines, we could obtain vesicles predominantly expressing HA-CAX3 (without CaCl2 treatment) and vesicles expressing CAX1 + HA-CAX3 (treatment with CaCl2). Without CaCl2 treatment, Col-0, cax3-1, and cax1-1 lines displayed similar low levels of Ca2+/H+ transport activity, and the 35S::HA-CAX3 lines showed very modest activity (Fig. 4A) (data not shown). CaCl2 treatment significantly increased vacuolar Ca2+/H+ transport activity from both Col-0 and 35S::HA-CAX3 lines (Fig. 4A). We postulate that by comparing the activity in these conditions, we can compare and contrast the activity of CAX1 with that of the putative CAX1-CAX3 complex.

To analyze the transport properties in these vacuole-enriched membranes, we performed competition studies similar to those done previously in yeast (Fig. 1C). As stated earlier, in the 35S::HA-CAX3 lines without CaCl2, we were unable to measure substantial Ca2+/H+ transport (Fig. 4A); thus, the competition measurements were not done under these conditions. Only during CaCl2 treatment, when we propose that CAX1 is active, were we able to measure uptake and perform competition measurements. In tonoplast-enriched vesicles from CaCl2-treated Col-0 lines (CAX1 activity high), Cd2+ appeared to inhibit Ca2+ transport; however, the inhibition was not as robust in CaCl2-treated 35S::HA-CAX3 lines (putative CAX1-CAX3 complex formation; Fig. 4C). In addition, lines expressing both CAX1 and CAX3 appeared to be more strongly inhibited by Li+ and Na+ than CAX1-expressing lines (CaCl2-treated Col-0). At 100× concentrations of Li+, the HA-CAX3 CaCl2-treated lines (where we postulate CAX3 + CAX1 interactions) had only 30% of the Ca2+/H+ transport activity, compared with the CAX1-expressing lines (CaCl2-treated Col-0), which were 55% as active under these conditions (Fig. 4, B and C).

Co-immunoprecipitation of CAX1 and CAX3 in Plants—To analyze the interaction between the transporters in plants, we performed co-immunoprecipitation using proteins from microsomal preparations of transgenic plant leaves and HA- and CAX1-specific antibodies (12) (Fig. 5). These microsomal fractions contain vacuoles, prevacuolar compartments, and other light vesicles; however, these membrane preps should allow preliminary observations regarding co-localization of CAXs. As controls, the V-ATPase antibody and other tonoplast antibodies were used to precipitate these protein samples (supplemental Fig. 2). All immunoprecipitates were resolved by SDS-PAGE and detected by immunoblotting with either HA, CAX1, or V-ATPase antibodies. As shown in Fig. 5, detection with CAX1 antibody demonstrated that both CAX1 and HA antibodies clearly precipitated CAX1 proteins of the appropriate size (~50 kDa) from controls and HA-CAX3-expressing plants but failed to precipitate the same protein from cax1-1 lines, suggesting high efficiency and specificity of immunoprecipitation and binding between CAX1 and CAX3. Detection with the HA antibody showed HA-CAX3 proteins immunoprecipitated with both CAX1 and HA antibodies from HA-CAX3 transgenic plants but not from controls and cax1-1 lines, further suggesting that CAX1 and CAX3 physically interact (Fig. 5B). As expected, the V-ATPase antibody failed to precipitate either CAX1 or HA-CAX3, although it did precipitate V-ATPase proteins from all plants (Fig. 5C). Similar negative interactions with the CAX transporters were found using antibodies against the Arabidopsis H+-pyrophosphatase (APV1), TPC1 (two-pore channel 1), and TIP (tonoplast intrinsic protein) (supplemental Fig. 2). Thus, a nonspecific interaction due to incomplete solubilization between CAX1 and CAX3 is unlikely, since neither HA-CAX3 nor CAX1 interacted with several other tonoplast proteins.

To determine if LiCl treatment altered CAX1-CAX3 interactions, we carried out gel shift assays with 35S::HA-CAX3 plant protein extracts with and without LiCl treatment. As shown in Fig. 5D, after treatment with 15 mM LiCl for 8 h, 35S::HA-CAX3 plants accumulated much more putative CAX1-CAX3 complex resolved by a native gel shift assay than control lines. However, even in control lines, the complex could be detected in SDS-polyacrylamide gels after immunoblotting (Fig. 5D).

Yeast Split Ubiquitin Assay of CAX1 and CAX3 Interaction—To test whether CAX1 and CAX3 can physically interact in yeast, an optimized split ubiquitin system was used (13). This system allows detection of interactions between membrane proteins in vivo.
Protein fusions of ubiquitin were constructed (Fig. 6, A and B), and the interaction of CAX1 and CAX3 was monitored by the release of the artificial transcription factor PLV, activating lexA-driven reporter genes in the nucleus. As a positive control, we demonstrated the interaction between AtKAT1 subunits (13, 23) (Fig. 6). As negative controls, CAX1 and CAX3 did not interact with the membrane protein AtKAT1. Using this system, interactions were observed between CAX1 and CAX3 (Fig. 6). The interaction of CAX1 (Cub or Nub) and CAX3 (Cub or Nub) was also detected by measuring β-galactosidase activity of LacZ (Fig. 6D). These results provided independent evidence that CAXs were capable of assembling in a heterodimeric protein complex.
**DISCUSSION**

CAX1 is a tonoplast Ca$^{2+}$/H$^+$ exchanger (12, 37, 38) that appears to be autoinhibited in planta, requiring trans-acting factors for optimal activity (8, 30, 34). Here we identify a potentially new mechanism for CAX regulation through the formation of “hetero-CAX” complexes, which may alter CAX transport properties. We show that CAX1 and CAX3 can be present in the same cells of plants and that they can interact directly, and we provide some arguments why this interaction could be physiologically important.

**Tissue Expression of CAX1 and CAX3**—Although CAX1 and CAX3 differentially accumulate in vegetative tissue, there are several conditions where their expression patterns overlap. For example, CAX1 and CAX3 both accumulate in reproductive organs and germinating seeds and during senescence (11). Furthermore, CAX3 expression can be enhanced more than 4-fold in the leaf guard cells, where CAX1 is highly expressed (36). The data in guard cells are the most definite example of coincident expression of CAX1 and CAX3 in the same cells. Other microarray data suggest that during osmotic stress, UV light treatment, and wounding, CAX3 expression in the aerial portions of the plants can reach levels equivalent to CAX1 (see the Arabidopsis Membrane Protein Library on the World Wide Web). Here, we have used CAX1:GUS and CAX3:GUS lines as well as RT-PCR analysis to demonstrate overlap in expression patterns during germination (Fig. 2). In general, CAX1 expression is higher than CAX3 and does not display the dynamic fluctuations in gene expression displayed by CAX3. We speculate that CAX3 may sometimes act as a co-factor with CAX1 to modulate transport function in response to various hormonal or environmental stresses. Guard cells may be the ideal model to further explore this dynamic Ca$^{2+}$/H$^+$ transport, given that basal levels of CAX1 are high in this tissue, and during abscisic acid treatment, both CAX1 and CAX3 are highly expressed.

**CAX1 and CAX3 Forms a Transporter with Distinct Functions**—Our results indicate that the CAX1-CAX3 complex in the plant may possess distinct transport functions. Here we show that co-expression of CAX1 with CAX3 produced LiCl tolerance in yeast, a unique phenotype not associated with expression of any of the deregulated CAXs (Fig. 1). It is interesting to note that sCAX1 expression in yeast conferred increased sensitivity to LiCl and NaCl levels as opposed to the salt tolerance for the CAX1 + CAX3-expressing cells (Fig. 1). The ability to confer LiCl tolerance in yeast without producing appreciable NaCl tolerance has been documented (39). Presumably, LiCl accumulation at the yeast vacuole (mediated by NHX1) confers only a degree of LiCl tolerance (39), and such accumulation/tolerance can be improved by expression of the CAX1-CAX3 complex. We have used transport assays to further demonstrate the differences among CAX1, CAX3, and CAX1 + CAX3 (Fig. 1). Only yeast cells expressing CAX1 + CAX3 demonstrated Ca$^{2+}$ uptake rates that could be inhibited by Li$^+$, further suggesting that this hetero-CAX complex has different transport properties.

The inability to measure CAX3-mediated Ca$^{2+}$/H$^+$ exchange suggests that the Ca$^{2+}$/H$^+$ measurements presented here are not the product of the additive effect of the individual transporters. To date, we have been unable to measure the CAX3-mediated Ca$^{2+}$/H$^+$ exchange in yeast, and CAX3 does not suppress yeast mutants defective in vacuolar Ca$^{2+}$ transport. In Arabidopsis, cax3-1 lines treated with CaCl$_2$ had Ca$^{2+}$/H$^+$ exchange activity similar to that of controls (11). Furthermore, 35S::HA-CAX3 lines did not exhibit high levels of Ca$^{2+}$/H$^+$ exchange. It should be noted that our in planta transport assays lack precision, given that other transporters are certainly active in these membrane preparations. Furthermore, our previous work has shown that mutants in CAX transporters cause altered activity of other transporters. Despite these limitations, together with our data regarding the physical interactions (see below), we interpret the changes in Ca$^{2+}$/H$^+$ transport in planta as being a consequence of the interaction between the transporters.

In our yeast assays, co-expression of both CAX1 and CAX3 (CAX1 + CAX3) demonstrated more salt accumulation than either transporter expressed individually (Fig. 1, A and D). We have thus used whole yeast metal accumulation as a rapid inference of these CAX complex function(s). For example, where we measured vacuolar Ca$^{2+}$ uptake inhibited by Li$^+$, we were also able to measure increased accumulation of Li$^+$ (Fig. 1A). Taken together, these observations further suggest that we are measuring phenotypes caused by an interaction of the transporters rather than an additive effect of the two individual transporters.

Additionally, we demonstrated that CAX1 + CAX3 cells could suppress the NaCl sensitivity of nlx1 yeast strains (Fig. 1C), a phenotype that could not be recapitulated by expressing sCAX1, CAX1, or CAX3, as well as several other combinations of CAX transporters (data not shown).

**Physiological Relevance of CAX Interactions**—Here we have addressed the interplay among CAXs within plant cells using both phenotype analysis and transport studies. We demonstrated that cax1, cax3, and cax1/3 lines have similar germination defects when grown under high lithium conditions (Fig. 3). In addition, cax1, cax3, and cax1/3 lines display similar responses to sugar stress, ethylene, and abscisic acid during seed germination (17). These genetic data are consistent with a model where both transporters are required together for these responses. In agreement with these observations, transgenic lines that simultaneously express high levels of HA-CAX3 + CAX1 had altered substrate specificity, as measured by cytochrome selectivity comparisons (Fig. 4). The Ca$^{2+}$/H$^+$ transport measured from tonoplast-enriched vesicles from the HA-CAX3 + CAX1-expressing lines demonstrated increased inhibition in the presence of excess nonradioactive Na$^+$ and/or Li$^+$ compared with lines expressing high levels of each transporter individually.

The intriguing question is how CAX1 and CAX3 interact and coordinate transport. To understand the function of any specific transporter, much less the function of transporter complexes, is difficult. The Arabidopsis genome contains over 150 cation transporters, many with redundant functions (40). We speculate that the CAX1-CAX3 complex formation is regulated temporally by alterations in transporter abundance (Fig. 2) (11). In this study, both CAX1 and CAX3 displayed similar expression levels during seed germination. This may signify that CAX1-CAX3 complexes have an important role during
germination. As a consequence, the cax1, cax3, and cax1/3 lines have similar germination phenotypes (Fig. 3) (17).

CAX1 and CAX3 also have discrete roles in plant growth and development. CAX1 and CAX3 can also form functional homo-oligomers. Previous studies with a mung bean CAX in yeast demonstrated homo-CAX formation (7). In planta, we speculate that in maturing roots, a homo-CAX3 complex is active. Only CAX3 is highly expressed in roots, and it appears to be responsible for 10-day-old plant responses to NaCl and low pH stress (17). Homo-CAX1 complexes may also be important for ion tolerance; however, 10-day-old cax1 seedlings display no difference in growth compared with wild-type grown on lithium-containing medium (12). Interestingly, ectopic expression of the soybean cation/H+ antiporter GmCAX1 in Arabidopsis does provide lithium tolerance (41), and 10-day-old cax3 lines display some alterations in lithium tolerance (17). These findings suggest that homo-Arabidopsis CAX transporters as well as the CAX1-CAX3 complex may have roles in particular stress tolerances.

**CAX1 and CAX3 Interact**—The CAX1-CAX3 interaction is demonstrated by the following findings. 1) In yeast assays, the co-expression of CAX1 and CAX3 conferred unique growth and transport properties not exhibited by the expression of either transporter individually (Fig. 1) (11). 2) Split ubiquitin assays demonstrated physical interaction among different CAXs (Fig. 6). 3) In vivo interaction in plant cells was shown through coimmunoprecipitation of CAX1 with HA-CAX3 (Fig. 5). 4) Genetic analysis between the loss-of-function alleles of cax1 and cax3 is consistent with the gene products operating together during stress responses (Fig. 3A) (17). 5) Transport studies in vacuole-enriched membrane vesicles isolated from Arabidopsis lines expressing HA-CAX3 and CAX1 demonstrate transport properties distinct from membranes expressing high levels of either CAX1 or CAX3 (Fig. 4).

Here we have focused our plant CAX1-CAX3 analysis on lithium phenotypes. Our data suggest that the putative CAX1-CAX3 complex may be required for tolerance to particular stress conditions. Both CAX1 and CAX3 are highly expressed when seedlings are exposed to high levels of LiCl (Fig. 3B). Furthermore, the complex formation appears to be enhanced during LiCl treatment (Fig. 5D). These complexes may be subject to degradation, as indicated by our inability to resolve distinct bands during the isolation procedure after prolonged LiCl treatments (Fig. 5D; data not shown). Both yeast and plant data support the idea that CAX1-CAX3 complexes can transport Li+ more efficiently than either transporter individually (Figs. 1 and 4). We postulate that the interaction among the CAXs alters substrate affinity though a conformational change. However, future work is required to directly measure Li+ transport kinetics.

The physical interaction between CAX1 and CAX3 in planta has been demonstrated using co-IP experiments using plant protein extracts derived from microsomes. We have utilized a number of tonoplast antibodies as negative controls (V-ATPase, AVP1, TPC1, and TIP) to document that this interaction is direct and specific. Although it remains a formal possibility that CAX1 and CAX3 could co-exist in the same membrane protein complex/domain, our co-IP data, in tandem with the membrane transport changes in CAX1 + HA-CAX3-expressing plants, strongly favor a model where CAX1 directly interacts with CAX3. Future experiments in planta with split-GFP or a fluorescence resonance energy transfer system using isolated vacuoles can be used to look at the dynamics of this interaction in more detail.

**CAX Regulation via Oligomerization**—Oligomerization may represent a regulatory mechanism for many transporters. Both plant and animal sugar transporters form regulatory complexes (42, 43). In lipid transport, the small CER5 ABC transporter may form a homo or heterodimer to facilitate fatty acid transport to the cuticle (44, 45). Oligomerization of S. cerevisiae Nha1p is essential for its Na+/H+ antiporter activity (46). Recently, a cytosolic trans-activation domain has been identified as being essential for ammonium uptake by AMT transporters (15, 47). A molecular model of AtAMT1;2 provides a mechanism where the C terminus of one monomer directly contacts the neighboring subunit. These alterations in the C-terminal domain may provide conformation coupling between monomers to allow tight regulation of transport and sensing (15, 48).

Like oligomerization of other transporters, CAX1 and CAX3 interactions could allow dynamic flexibility for membrane transport. In plant tonoplasts, CAX1, CAX3, and CAX1-CAX3 complexes may simultaneously exist, and during growth and adaptation the ratio of these complexes may be regulated. Perturbing both CAX1 and CAX3 causes significant morphological phenotypes (11); however, not all endomembrane Ca2+/H+ activity is abolished. These observations suggest that other CAX transporters may also be involved in this regulatory interplay.

**Conclusions**—We have demonstrated that hetero-CAX interactions can have distinct functions when expressed in yeast and plant cells. These complexes may provide plants with dynamic transport flexibility and help in adaptation to environmental stresses.

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