Heterodimerization of Arabidopsis calcium/proton exchangers contributes to regulation of guard cell dynamics and plant defense responses

Bradleigh Hocking1,2*, Simon J. Conn1,*, Murli Manohar3,*, Bo Xu1,2*, Asmini Athman1,2, Matthew A. Stancombe4, Alex R. Webb4, Kendal D. Hirschi3,# and Matthew Gilliham1,2,#

1 Waite Research Institute and School of Agriculture, Food and Wine, University of Adelaide, Glen Osmond, SA, Australia
2 ARC Centre of Excellence in Plant Energy Biology, University of Adelaide, Glen Osmond, SA, Australia
3 US Department of Agriculture/Agricultural Research Service, Children’s Nutrition Research Center, Baylor College of Medicine, Houston, TX, USA
4 Department of Plant Sciences, University of Cambridge, Cambridge, UK

* These authors contributed equally to this work
† Present address: Centre for Cancer Biology, University of South Australia, Adelaide, SA, Australia
‡ Present address: Boyce Thompson Institute, Cornell University, Ithaca, NY, USA
# Correspondence: matthew.gilliham@adelaide.edu.au; kendalh@bcm.edu

Received 21 December 2016; Editorial decision 30 May 2017; Accepted 2 June 2017

Editor: Ian Dodd, Lancaster University

Abstract

Arabidopsis thaliana cation exchangers (CAX1 and CAX3) are closely related tonoplast-localized calcium/proton (Ca2+/H+) antiporters that contribute to cellular Ca2+ homeostasis. CAX1 and CAX3 were previously shown to interact in yeast; however, the function of this complex in plants has remained elusive. Here, we demonstrate that expression of CAX1 and CAX3 occurs in guard cells. Additionally, CAX1 and CAX3 are co-expressed in mesophyll tissue in response to wounding or flg22 treatment, due to the induction of CAX3 expression. Having shown that the transporters can be co-expressed in the same cells, we demonstrate that CAX1 and CAX3 can form homomeric and heteromeric complexes in plants. Consistent with the formation of a functional CAX1-CAX3 complex, CAX1 and CAX3 integrated into the yeast genome suppressed a Ca2+-hypersensitive phenotype of mutants defective in vacuolar Ca2+ transport, and demonstrated enzyme kinetics different from those of either CAX protein expressed by itself. We demonstrate that the interactions between CAX proteins contribute to the functioning of stomata, because stomata were more closed in cax1-1, cax3-1, and cax1-1/cax3-1 loss-of-function mutants due to an inability to buffer Ca2+ effectively. We hypothesize that the formation of CAX1-CAX3 complexes may occur in the mesophyll to affect intracellular Ca2+ signaling during defense responses.

Key words: Calcium, guard cells, homeostasis, mesophyll, protein interaction, signaling, transport.

Introduction

The tight control of calcium concentration ([Ca2+]i) within the apoplast (cell wall) and symplast (cytosol, vacuole, and other endomembrane compartments) are critical for plant nutrition, structure, development, signaling, and physiology (Pittman and Hirschi, 2003; White and Broadley, 2003; Hetherington and Brownlee, 2004; Pittman et al., 2005; Dodd et al., 2010; Conn et al., 2011b; Gilliham et al., 2011). Transporters that reside in the plant vacuolar membrane (the tonoplast) play a
major role in the regulation of \([\text{Ca}^{2+}]\) within the apoplastic and symplastic compartments (Pottosin and Schönhnlecht, 2007; Conn et al., 2011b). The \(\text{Ca}^{2+}/\text{H}^+\) antiporters CAX1 and CAX3 were previously identified as tonoplast-localized transporters that are important in controlling tissue \(\text{Ca}^{2+}\) homeostasis (Hirschi et al., 1996; Shigaki et al., 2001; Cheng et al., 2005; Conn et al., 2011b; Manohar et al., 2011a, 2011b; Pittman and Hirschi, 2016). These proteins share 87% sequence similarity and 79% sequence identity, and function as low-affinity, high-capacity \(\text{Ca}^{2+}\) transporters that use the protonotive force generated by the vacuolar \(\text{H}^+\)-ATPase and PPI-dependent \(\text{H}^+\) pumps to sequester \(\text{Ca}^{2+}\) from the cytosol into the vacuole (Hirschi et al., 1996).

Both CAX1 and CAX3 proteins have been ascribed a functional role based on \textit{in planta} expression analysis, ectopic expression, and mutant analysis in plants, and by heterologous expression in yeast (Shigaki and Hirschi, 2000; Manohar et al., 2011b). Until now, \(\text{CAX}1\) and \(\text{CAX}3\) expression has been shown to overlap in reproductive tissues at the organ level, but to localize differentially within the vegetative organs. \(\text{CAX}3\) expression has been shown to localize primarily to root tips, whereas \(\text{CAX}1\) expression is predominantly localized to leaf tissues. CAX1 regulates elemental accumulation across specific leaf cell types and subcellular compartments (Hirschi et al., 1996; Catalá et al., 2003; Conn et al., 2011b), whereas root growth in \(\text{cax}1-1\) plants is lower than that of wild-type or \(\text{cax}1-1\) plants under saline conditions, a phenotype that has been attributed to the greater inhibitory effects of \(\text{Na}^+\) (and \(\text{Li}^+\)) on \(\text{CAX}3\) \(\text{Ca}^{2+}\) transport compared with \(\text{CAX}1\) (Cheng et al., 2003; Manohar et al., 2011b).

\(\text{CAX}\) expression in leaves appears to be variable and subject to unresolved regulatory mechanisms (Conn and Gilliam, 2010; Gilliam et al., 2011). For example, in \(\text{cax}1-1\) plants, expression of \(\text{CAX}3\) and \(\text{CAX}4\) increase along with a vacuolar \(\text{Ca}^{2+}\)-ATPase \(\text{ACA}4\) (Cheng et al., 2003). It has been hypothesized that enhanced \(\text{CAX}3\) expression complements for the loss of \(\text{CAX}1\), as \(\text{cax}1-1\) plants do not show the major physiological perturbations of \(\text{cax}1-1\text{cax}3-1\) plants; however, it has not been shown whether \(\text{CAX}3\) directly replaces \(\text{CAX}1\) in the mesophyll (Cheng et al., 2003; Conn et al., 2011b). Such a complex system of cross-talk among genes is proposed to account for the subtleties in phenotypes that are often associated with loss-of-function genetic studies for transport proteins (Connorton et al., 2012). In order to elucidate these compensatory changes, in the present study we designed experiments to analyze the potential interactions between \(\text{CAX}\) transporters.

Despite the existence of phenotypic differences between \(\text{cax}\) knockout plants, there are also notable similarities. For instance, germination in both \(\text{cax}1-1\) and \(\text{cax}3-1\) knockouts is abscisic acid (ABA) and sugar sensitive, and ethylene inhibits seedling growth of both mutants, which suggests some shared functionality of these proteins (Zhao et al., 2008). Previous functional assays of \(\text{CAX}1\) and \(\text{CAX}3\) proteins in yeast, although insightful, have limitations because they used engineered \(\text{CAX}\) variants that lack N-terminal autoregulatory domains (Pittman and Hirschi, 2016). The functional significance of \(\text{CAX}1\) and \(\text{CAX}3\) interactions suggested by genetic and yeast assays is yet to be fully understood (Zhao et al., 2009b). Such interactions may occur in the guard cell, as expression of both \(\text{CAX}1\) and \(\text{CAX}3\) has been detected in guard cell protoplasts (Leonhardt et al., 2004; Cho et al., 2012). Moreover, genetic analysis has implied that a putative \(\text{CAX}1\)-\(\text{CAX}3\) complex may influence guard cell closure and apoplastic p\(\text{H}\) (Cho et al., 2012).

Here, we provide evidence that heteromeric \(\text{CAX}\) complexes have physiological roles in plants. A split luciferase assay demonstrated that \(\text{CAX}1\) and \(\text{CAX}3\) form homo- and heterodimers in plant tissue, and, using yeast-based functional assays, we have shown for the first time that full-length \(\text{CAX}1\)-\(\text{CAX}3\) has distinct transport characteristics compared with homomeric truncated-deregulated \(\text{CAX}\) proteins. The functional role for a \(\text{CAX}1\)-\(\text{CAX}3\) complex in the plant was probed with stomatal assays, and both \(\text{CAX}1\) and \(\text{CAX}3\) appear to be required for correct functioning of the stomata. Our results highlight that expression of \(\text{CAX}3\) and, to a lesser extent, \(\text{CAX}1\) is induced in the mesophyll during defense responses, and that both proteins are required in the guard cell for the control of gas exchange. The interactions between \(\text{CAX}1\) and \(\text{CAX}3\) identified here suggest the possibility of regulatory plasticity in tonoplast \(\text{Ca}^{2+}\) transport during signaling events.

**Materials and methods**

**Plant materials and growth**

All chemicals were obtained from Sigma-Aldrich unless stated otherwise. Plant materials were Arabidopsis thaliana wild-type Columbia-0 (Col-0) and Col-0 background T-DNA insertional loss-of-function mutants \(\text{cax}1-1\), \(\text{cax}3-1\), and \(\text{cax}11\text{cax}3-1\) (\(\text{cax}1\) \(\text{cax}3\)) (Cheng et al., 2005). For soil growth, seeds were sown on zero-nutrient-containing coco-peat-based soil and supplied weekly with a defined basal nutrient solution (BNS: 2 mM \(\text{NH}_4\text{NO}_3\), 3 mM \(\text{KNO}_3\), 0.1 mM \(\text{CaCl}_2\), 2 mM \(\text{KCl}\), 2 mM \(\text{Ca(NO}_3\text{)}_2\), 2 mM \(\text{MgSO}_4\), 0.6 mM \(\text{KH}_2\text{PO}_4\), 1.5 mM \(\text{NaCl}\), 50 \(\mu\text{M}\) \(\text{NaFe(III)EDTA}\), 50 \(\mu\text{M}\) \(\text{H}_2\text{BO}_3\), 5 \(\mu\text{M}\) \(\text{MnCl}_2\), 10 \(\mu\text{M}\) \(\text{ZnSO}_4\), 0.5 \(\mu\text{M}\) \(\text{CuSO}_4\), 0.1 \(\mu\text{M}\) \(\text{Na}_2\text{MoO}_4\), adjusted to pH 5.6 by the addition of KOH) as previously described by Conn et al. (2013). Hydroponic growth also followed the method described by Conn et al. (2013), with the following exceptions. After root emergence from modified microcentrifuge tubes containing low-nitrate germination medium at \(\approx2\) weeks, these tubes were transferred to aerated hydroponics tanks containing either BNS (2 mM \(\text{Ca}^{2+}\)) or 300 \(\mu\text{M}\) sufficient but low calcium solution (SLCS) for another 3 weeks before a further \(\text{Ca}^{2+}\) treatment in BNS, modified BNS using 11 mM high \(\text{Ca}^{2+}\) Solution (HCS), or SLCS; see Supplementary Table S1 at JXB online for full solution composition. All plants were grown in a short-day growth room (9.5 h light/15.5 h dark, 110 \(\mu\text{mol m}^{-2} \text{s}^{-1}\), 19 °C). Calcium concentration measurements were performed as previously described by Cheng et al. (2002).

**RNA extraction**

Total RNA was extracted from shoot tissue or mesophyll protoplasts of 5–6-week-old Col-0 plants treated as indicated in the respective figure legends, using TRIzol reagent (Invitrogen) and the DNase-treated by Turbo DNA-free kit (Ambion). Reverse transcription was used to synthesize cDNA from 2 \(\mu\text{g}\) RNA from each sample using SuperScript® III Reverse Transcriptase (Invitrogen) with Oligo(dT)\(_{20}\) as previously described by Conn et al. (2011b).
Gene cloning and plasmid construction

PCR was used to amplify DNA fragments from Arabidopsis cDNA to clone the CAX1 and CAX3 coding sequences without start or stop codons (primers listed in Supplementary Table S2). Then, the DNA fragments were cloned into the pCR8/GW/TOPO TA cloning vector and transformed into TOP10 chemically competent Escherichia coli (Invitrogen). The genes of interest in pCR8/GW/TOPO vectors were recombined into serial pDuEx-Bait/Prey expression vectors for a split luciferase interaction assay (Nakagawa et al., 2007), and a subsequent Cre-Lox recombinase reaction was performed to produce dual gene expression vectors for simultaneous expression of NLuc-CAX1 and CAX3-CLuc (or CLucN-CAX3-CLuc) (Creator™ DNA Cloning Kit, Clontech). CAX1 and CAX3 promoters (2 kb region upstream of the gene start codon ATG) as described by Cheng et al. (2005) were amplified from A. thaliana (Col-0) genomic DNA. Primers incorporated EcoRI/HindIII restriction sites (Supplementary Table S2) with the ampiclon sub-cloned into pNO::Luc vectors using T4 DNA Ligase (New England Biolabs). The CAX1 and CAX3 artificial miRNA (amiRNA) was designed to achieve CAX-specific transcript reduction. CAX1 and CAX3 amiRNA sequences were designed using Web MicroRNA Designer v2 (Schwab et al., 2006) and cloned into the pCR8/GW/TOPO vector. Subsequently, CAX1 and CAX3 amiRNA was recombined into a 2× CaMV 35S overexpression vector (pTOOL2) and used for miRNA expression (Plett et al., 2010).

Semi-quantitative PCR

Semi-quantitative PCR (semi-qPCR) for cell-specific CAX1 and CAX3 expression analysis was performed on cDNA separately synthesized from RNA of 5–6-week-old Arabidopsis mesophyll and epidermal cells. RNA preparation was performed as described previously using single cell sampling (SiCSA) (Conn et al., 2011b). Transcripts amplified were Actin2 (At3g18780; normalization control for both epidermis and mesophyll), CAX1 (At1g22690), and CAX3 (At3g18780; a marker for guard cells) with primers listed in Supplementary Table S2. Amplification for this analysis was performed using Phire Hot Start Taq DNA Polymerase (Finnzymes) with the following cycling conditions: first round: 98 °C for 1 min, then 25 cycles of 98 °C for 10 s, 50 °C for 10 s, and 72 °C for 30 s. Primers were then removed using Nucleospin Extract II (Macherey-Nagel), and 1 µl of the eluate was used as the template for the second round: 98 °C for 1 min, then 25 cycles of 98 °C for 10 s, 55 °C for 10 s, and 72 °C for 10 s.

Semi-qPCR for CAX1 and CAX3 expression analysis was performed on cDNA samples reverse-transcribed from mesophyll protoplast RNA isolated from 5–6-week-old Arabidopsis leaves, following the protoplast isolation method described below. Transcripts amplified were Actin2, CAX1, CAX3, and GCI (At1g22690; a marker gene for guard cells) with primers listed in Supplementary Table S2. Amplification of Actin2, CAX1, CAX3, and GCI transcripts was performed using Phire Hot Start DNA Polymerase (Finnzymes) with the following cycling conditions: 98 °C for 1 min; 35 cycles of 98 °C for 5 s, 56 °C for 5 s, and 72 °C for 15 s; and then 72 °C for 1 min.

In situ PCR

Leaves of 5–6-week-old Arabidopsis plants grown in short-day conditions were infiltrated with either ultrapure H2O or 2 µM Flg22 in ultrapure H2O and left for 12 h. Then, leaves were detached, cut into 5 mm strips, and fixed in ice-cold formalin-acetic-alcohol solution [63% (v/v) ethanol, 5% (v/v) acetic acid, 2% (v/v) formalin] and washed in 1× PBS before being embedded in 5% agarose. Embossed leaf tissue was cross-sectioned using a VT 1200 S Vibrating Microtome (Leica) into 70 μm sections and transferred into a PCR tube. Then, the in situ PCR protocol of Athman et al. (2014) was followed using gene-specific qPCR primers as listed in Supplementary Table S2, with the following cycling conditions: 98 °C for 1 min; 35 cycles of 98 °C for 5 s, 56 °C for 5 s, and 72 °C for 15 s; and then 72 °C for 1 min.

Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) was performed on 0.2 µl cDNA using an iCycler Thermal cycler equipped with an iQ multi-color optical assembly module (Bio-Rad) and using KAPA SYBR® FAST qPCR Kits (KAPA Biosystem), according to the following program: 95 °C for 3 min; 40 cycles of 95 °C for 20 s, 55 °C for 20 s, and 72 °C for 20 s; with melt curve analysis from 52 °C to 92 °C in 0.5 °C increments. Primers for RT-qPCR analysis are listed in Supplementary Table S2. RT-qPCR result analysis followed the method described by Schmittgen and Livak (2008) using 2-ΔΔCt to calculate gene expression level normalized to Actin2 (At3g18780) as an internal control.

Protoplast isolation

Isolation and transformation of protoplasts was carried out according to a previously described method (Yoo et al., 2007). Briefly, mesophyll protoplasts were isolated from leaf strips of 5–6-week-old A. thaliana by a 3-hour digestion in an enzyme solution containing 1.5% cellulase R10 and 0.4% macerozyme R10 (Yakult Pharmaceutical). Protoplasts were transformed via the polyethylene glycol 1450-mediated introduction of plasmid DNA in buffer solution. Modifications to this method included the use of one cell incubation medium, W2 [4 mM 2-(N-morpholino)ethanesulfonic acid (MES), 0.4 M mannitol, 10 mM CaCl2, and 5 mM MgCl2, adjusted to pH 5.7 with KOH], as a replacement for both W1 and W3 solutions. Protoplasts were incubated at room temperature for 0 or 24 h before harvesting for RNA extraction as indicated in the legend of Fig. 1.

Split luciferase protein–protein interaction and native promoter luciferase reporter assay

Direct protein–protein interactions between CAX1 and CAX3 were probed using a split luciferase complementation assay (Fujikawa and Kato, 2007). Mesophyll protoplasts were transformed with an equal amount of expression pDuEx-Bait and/or pDuEx-Prey plasmids and incubated for at least 16 hours. Native promoter reporter assays were performed using an equal amount of pN0::Luc expression plasmid fused to either the 35S, CAX1, or CAX3 promoter transformed into mesophyll protoplasts; pN0::Luc containing the promoter-free LUC gene was used as a negative control. Luciferase activity in the protein–protein interaction and promoter activity assay was detected using the ViviRen Live Cell Substrate (Promega) in a PolarStar Optima plate-reading spectrophotometer with luminescence detection capabilities (BMG Labtech). Following overnight incubation of transfected protoplasts, the ViviRen substrate was dissolved in DMSO and added to 500 µl protoplasts (2 × 10⁶ cells ml⁻¹) to 60 µM, mixed briefly, and aliquots of 100 µl (equivalent to 4 × 10⁶ cells) were dispensed into white 96-well plates, in triplicate. Luminescence was measured immediately. Peak luminescence was observed at 300 s after substrate addition (gain=4095), and data from this time point were used for further analysis.

Ca²⁺ tolerance and uptake assay in Saccharomyces cerevisiae

Saccharomyces cerevisiae strain K667 (wcl:hisG canb::LEU2 pmel::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trpl-1 ura3-1) (Cunningham and Fink, 1996) was transformed with sCAX1 or CAX1 and CAX3 using (SC-Ura) transformation (Sherman et al., 1986). The Ca²⁺ growth tolerance assay of S. cerevisiae was performed as previously described by Manohar et al. (2011b). Briefly, the assay was carried out via growing yeast expressing genes of interest at 30 °C for 3 days on solid YPD medium and supplemented with the appropriate amount of CaCl₂. A vacuum-enriched membrane fraction was prepared from yeast, following the method described by Manohar et al. (2011b). Yeast cells were collected by centrifugation at 4000×g for 5 min until the density reached an OD₆₀₀ of ~1.5.
The collected cell pellet was washed in spheroplast buffer (100 mM potassium phosphate buffer, 1.2 M sorbitol, pH 7.0) and resuspended in the same buffer plus 10 mM dithiothreitol (DTT) and 1% dextrose. Membrane vesicles of yeast cells were isolated using 1.5 units of zymolyase and incubated at 30 °C for up to 2 h. Time-dependent 45Ca2+/H+ transport into these endomembrane vesicles was measured as described previously by Pittman et al. (2005).

Western blotting analysis
Western blotting analysis was performed as previously described by Manohar et al. (2011b). A monoclonal antibody to human influenza hemagglutinin (HA) (Berkeley Antibody Co., Richmond, CA, USA) was used at a 1:1000 dilution.

Gas exchange and photosynthesis measurements
Gas exchange and photosynthesis rate of whole rosettes were measured in 5- to 8-week-old Arabidopsis plants with treatments as indicated in the corresponding figure legends, using a LI-6400 infrared gas exchange analyzer (LiCOR) equipped with a Arabidopsis whole-plant chamber. Individual plants were exposed to light intensity of ~350 μmol m−2 s−1 at least 30 minutes prior to the start of measurement. The rosette was allowed to acclimatize inside the Arabidopsis whole-plant chamber for at least 5 minutes before gas exchange data were recorded, with reference CO2 concentration set at 500 μmol mol−1, flow rate at 500 μmol s−1, light intensity at 350 μmol m−2 s−1, and relative humidity at 56%. Leaf area of the whole plant was calculated using MATLAB on the basis of the image of whole plants, as described by Conn et al. (2013).

Guard cell aperture measurement
Stomatal aperture was measured in wild-type Col-0, cax1-1, cax3-1, and cax1/cax3 lines, as described by Conn et al. (2011b). Briefly, epidermal peels were prepared from 3–4-week-old seedlings, grown on 0.5× Murashige and Skoog medium, and incubated in buffer containing 5 mM KCl, 10 mM MES-KOH (pH 6.2) with or without 1 mM CaCl2 supplement.

Agrobacterium-mediated transformation
Agrobacterium-mediated Arabidopsis seedling transformation followed the Fast Agrobacterium-mediated Seedling Transformation (FAST) method described by Li et al. (2009). Briefly, Arabidopsis seedlings were grown on 0.25× Murashige and Skoog medium for 5–6 weeks before being transferred into fresh 0.25× Murashige and Skoog liquid medium in a Petri dish containing an additional 100 μM acetosyringone and 0.005% (v/v) Silwet L-77, and co-cultivation with Agrobacterium tumefaciens cells at OD600=0.5 for 2 days. Assays were then performed on these seedlings.

Data and statistical analysis
Statistical tests are described in the figure legends. All graphing and statistical analysis were performed in GraphPad Prism v6 and 7.
Results

CAX3 expression can be induced in mesophyll cells by cax1 knockout, wounding, or pathogen stress.

To directly test whether it is possible for CAX3 to replace and compensate for the loss of CAX1 expression in cax1-1 plants, we examined the expression profile of CAX1 and CAX3 in mesophyll cells of A. thaliana ecotype Col-0 and cax1-1 using laser capture microdissection (LMD) qRT-PCR (Fig. 1A). We confirmed that CAX1 was expressed at high levels in the mesophyll cells of wild-type (Col-0) plants, whereas CAX3 was not detected. In contrast, CAX3 expression was significantly induced in cax1-1 mesophyll, when CAX1 was absent, as predicted by Conn et al. (2011b). This suggests that it is possible for CAX3 to be expressed in the mesophyll under some conditions. However, the lack of CAX3 expression detected in Col-0 mesophyll cells (Fig. 1A), contrasts with previous observations made from protoplasts, where both CAX1 and CAX3 have been detected (Leonhardt et al., 2004; Cho et al., 2012). We further explored this disparity.

Immediately following the isolation of mesophyll protoplasts, CAX3 expression was barely detected, but the expression was significantly increased 24 h after protoplast isolation. Interestingly, CAX1 was highly abundant at both stages, with reduced expression after 24 h (Fig. 1B). A guard-cell-specific marker (GC1) (Yang et al., 2008) was close to the detection limits at both time points (Fig. 1B). These results indicate that there was no or minimal guard cell contamination in our mesophyll preparation, and that CAX3, but not CAX1, showed inducible expression within mesophyll cells during the protoplasting procedure.

To assess whether these changes were due to either the stability of the mRNA or an increase in CAX promoter activity, we generated native CAX promoter::luciferase reporter constructs for transient expression in mesophyll protoplasts. To make this construct, we cloned the CAX1 and CAX3 promoter fragments, identified in Cheng et al. (2005) as reporting native expression patterns. We then inserted a promoter upstream of a luciferase protein derived from Renilla reniformis (sea pansy), and transfected the constructs into Arabidopsis mesophyll protoplasts. At 24 h after transfection, equivalent to the time point used in Fig. 1B, we could detect the expression of luciferase in the protoplasts driven by either the CAX1 or the CAX3 promoter (Fig. 1C), or via three sets of a truncated promoter for each CAX (F1, F2, and F3) (see Supplementary Fig. S1). Interestingly, the CAX3 promoter drove 3- to 15-fold stronger luciferase activity compared with the respective CAX1 promoter (Fig. 1C). This suggests that gene transcription of CAX3 increases during protoplasting.

We then investigated whether we could induce CAX3 expression in the mesophyll of Col-0 plants under any conditions. Using SiCSA and semi-qRT-PCR, under our standard conditions (i.e. growth in BNS), CAX1 transcript was detected in both adaxial epidermal and palisade mesophyll cells of Col-0 leaves, whereas CAX3 was detected only from cax1-1 plant mesophyll (Fig. 1D). CAX1 transcript was abundant in RNA extracted from whole leaves of Col-0 plants, consistent with its expression in epidermis and mesophyll, while the presence of CAX3 transcript was below the level of our assay’s detection limits under our standard growth conditions (see Supplementary Fig. S2). In an attempt to overload the leaf with apoplastic Ca2+, to mimic the situation in cax1/cax3 plants (Conn et al., 2011b), we increased the concentration of Ca2+ in the root growth solution from 2 to 11 mM Ca2+ (HCS). Although both transcripts were induced by HCS (Fig. 1D; Supplementary Fig. S2), we did not observe a change in the cell-type localization of CAX1 and CAX3 expression under high Ca2+ conditions (Fig. 1D). Furthermore, we compared the leaf vacuolar and apoplastic Ca2+ content of Col-0, cax1-1, and cax3-1, and no significant differences were observed between the genotypes (Supplementary Fig. S3).

To determine whether the changes in CAX transcript abundance translated into increases in CAX protein levels, we used immunochemistry to measure the abundance of translational pCAX(1 or 3):CAX::HA fusions. In Col-0, the relative amount of CAX1 and CAX3 protein reflected the differences in transcript abundance, with CAX1 ~24-fold as abundant as CAX3 (Fig. 2). In cax1-1 plants transformed with these constructs, CAX1 protein abundance was comparable to wild-type, whereas CAX3 protein abundance was increased ~17-fold compared with wild-type (Fig. 2). These results strongly indicate that the changes in transcript abundance for CAX1 and CAX3 in whole leaves and in protoplasts reflect changes in protein abundance. This finding encouraged us to conduct a broader analysis to determine whether there were other stimuli that alter CAX expression.

A survey of existing Arabidopsis expression data (eFP BAR; http://bbc.botany.utoronto.ca) identified several biotic and abiotic stresses that might regulate CAX1 and CAX3 expression. CAX3 abundance was increased in leaves/shoots by osmotic stress, salt stress, and infection with Pseudomonas syringae or Botrytis cinerea (Table 1). To further examine the effect of P. syringae on CAX expression, we infiltrated 5-week-old Col-0 leaves with 1 μM flg22 (as a proxy for Pseudomonas infection) or water (as a control) 12 h before performing qPCR. Consistent with the result in the eFP BAR database, CAX3 expression was increased in flg22-infiltrated leaves, whereas CAX1 expression was unchanged (Fig. 3A). In addition, using in situ PCR, CAX3 was detected in the mesophyll of flg22-infiltrated leaves but not in the leaves of water-infiltrated controls (Fig. 3B). Protoplasting induces wound responses (Ecker and Davis, 1987) and flg22 is a pathogen mimetic; both induce CAX3 expression, which implies that CAX3 has a role in plant defense responses. Using the BAR Expression Angler, we identified 236 genes strongly co-regulated with CAX3 (r2>0.75) in Arabidopsis leaves in response to P. syringae, B. cinerea, and their corresponding elicitors, whereas there were only 16 genes co-regulated in response to abiotic stresses (Austin et al., 2016). Furthermore, microarray analysis of cax1/cax3 plants shows alteration in expression of many pathogen-related genes (see Supplementary Table S3). These findings suggest that co-expression of CAX1 and CAX3 within the mesophyll occurs during defense responses
Levels of CAX1 and CAX3 protein expression in Col-0 and cax1-1 plants as assessed using the FAST technique (Li et al., 2009). The following native promoter/gene/reporter fusions were constructed: pCAX1::AtCAX1::YFP::2xHA (pSIM1; GenBank accession number: HM750245.1) and pCAX3::AtCAX3::YFP::2xHA (pSIM3; GenBank accession number: HM750246.1). pSIM1 and pSIM3 proteins were predicted to have molecular weights of 81.9 and 81.6 kDa, respectively (ExpASy Compute pi/Mw tool). Arabidopsis Col-0 seedlings were co-cultivated with Agrobacterium carrying (1) pSIM3, (2) pSIM1; the cax1-1 line was transformed with (3) pSIM3 and (4) pSIM1; and (5) d35S::AtCAX3 served as a negative control. (A) Western blot of total protein extracted from the shoots of eight transformed seedlings, transferred to nitrocellulose membrane, and probed with anti-HA, HRP-conjugated primary antisera (Cell Signalling Technology, CS2999), demonstrates that CAX3 compensates at the protein level for the loss of CAX1 in the A. thaliana cax1-1 T-DNA insertion line. (B) Coomassie-stained 10% SDS-PAGE gel as a loading control for the Western blot. (C) Quantification of band intensities across three biological replicates using QuantityOne software (Bio-Rad Laboratories), normalized to pSIM3-transfected Col-0 (lane 1). and as such there is potential for a CAX1-CAX3 complex to have a physiological role.

CAX3 in guard cells affects stomatal responses

To determine whether the putative CAX1-CAX3 interaction may have functional roles in plants, we surveyed the cellular expression of CAX1 and CAX3 in leaves. CAX1 expression was detected in mesophyll cells, vascular bundles, adaxial epidermal cells, and abaxial epidermal peels (Fig. 4). The only tissue assayed in which CAX3 expression was detected was abaxial epidermal peels; these peels will contain viable stomatal guard cells, indicating that CAX1 and CAX3 are co-expressed in guard cells.

To investigate whether co-expression is required in planta for normal guard cell function, we examined the aperture of stomatal pores in Col-0, cax1-1, cax3-1, and cax1/cax3 plants. Apertures of cax1-1, cax3-1, and cax1/cax3 stomata from isolated epidermis were smaller than those of Col-0 (Fig. 5A). Moreover, none of the mutants had reduced apertures in the presence of supplemental extracellular [Ca\(^{2+}\)], unlike Col-0 (Fig. 5B). Coupled with the fact that EGTA treatment opens cax1/cax3 stomata (Conn et al., 2011b), this result indicates that stomata in these epidermal peels were already partially closed in a Ca\(^{2+}\)-dependent manner in both the single and double cax mutants. We previously demonstrated that while cax1/cax3 exhibits reduced leaf gas exchange as a result of higher apoplastic calcium, which causes reduced stomatal aperture (Conn et al., 2011b), apoplastic Ca\(^{2+}\) concentration was not different from wild type in cax1-1 or cax3-1 plants under steady-state conditions (see Supplementary Fig. 3). This is likely due to CAX1 protein, or CAX3 in the case of cax1-1 plants, effectively buffering apoplastic Ca\(^{2+}\) in the mesophyll of the single-knockout plants.

We therefore explored whether the Ca\(^{2+}\)-sensitive stomatal phenotype of cax1-1 and cax3-1 plants could be recreated in leaves of intact plants or whether it was an artifact of the epidermal peel system. In order to achieve this, we first optimized the growth of cax1/cax3 plants to ensure that the guard cell phenotype observed in intact cax1/cax3 plants was not a consequence of the dwarf stature or delayed development of these plants when they are grown in standard BNS (2 mM Ca\(^{2+}\)) for 1 week. While Col-0 plants did not appear to be adversely affected by this treatment and continued to develop normally, the growth of the cax1/cax3 rosette was greatly inhibited (Fig. 6A). In addition, the photosynthesis and transpiration rate of cax1/cax3 plants (per mm rosette surface

Fig. 2. Levels of CAX1 and CAX3 protein expression in Col-0 and cax1-1 plants as assessed using the FAST technique (Li et al., 2009). The following native promoter/gene/reporter fusions were constructed: pCAX1::AtCAX1::YFP::2xHA (pSIM1; GenBank accession number: HM750245.1) and pCAX3::AtCAX3::YFP::2xHA (pSIM3; GenBank accession number: HM750246.1). pSIM1 and pSIM3 proteins were predicted to have molecular weights of 81.9 and 81.6 kDa, respectively (ExpASy Compute pi/Mw tool). Arabidopsis Col-0 seedlings were co-cultivated with Agrobacterium carrying (1) pSIM3, (2) pSIM1; the cax1-1 line was transformed with (3) pSIM3 and (4) pSIM1; and (5) d35S::AtCAX3 served as a negative control. (A) Western blot of total protein extracted from the shoots of eight transformed seedlings, transferred to nitrocellulose membrane, and probed with anti-HA, HRP-conjugated primary antisera (Cell Signalling Technology, CS2999), demonstrates that CAX3 compensates at the protein level for the loss of CAX1 in the A. thaliana cax1-1 T-DNA insertion line. (B) Coomassie-stained 10% SDS-PAGE gel as a loading control for the Western blot. (C) Quantification of band intensities across three biological replicates using QuantityOne software (Bio-Rad Laboratories), normalized to pSIM3-transfected Col-0 (lane 1). and as such there is potential for a CAX1-CAX3 complex to have a physiological role.

CAX3 in guard cells affects stomatal responses

To determine whether the putative CAX1-CAX3 interaction may have functional roles in plants, we surveyed the cellular expression of CAX1 and CAX3 in leaves. CAX1 expression was detected in mesophyll cells, vascular bundles, adaxial epidermal cells, and abaxial epidermal peels (Fig. 4). The only tissue assayed in which CAX3 expression was detected was abaxial epidermal peels; these peels will contain viable stomatal guard cells, indicating that CAX1 and CAX3 are co-expressed in guard cells.

To investigate whether co-expression is required in planta for normal guard cell function, we examined the apertures of stomatal pores in Col-0, cax1-1, cax3-1, and cax1/cax3 plants. Apertures of cax1-1, cax3-1, and cax1/cax3 stomata from isolated epidermis were smaller than those of Col-0 (Fig. 5A). Moreover, none of the mutants had reduced apertures in the presence of supplemental extracellular [Ca\(^{2+}\)], unlike Col-0 (Fig. 5B). Coupled with the fact that EGTA treatment opens cax1/cax3 stomata (Conn et al., 2011b), this result indicates that stomata in these epidermal peels were already partially closed in a Ca\(^{2+}\)-dependent manner in both the single and double cax mutants. We previously demonstrated that while cax1/cax3 exhibits reduced leaf gas exchange as a result of higher apoplastic calcium, which causes reduced stomatal aperture (Conn et al., 2011b), apoplastic Ca\(^{2+}\) concentration was not different from wild type in cax1-1 or cax3-1 plants under steady-state conditions (see Supplementary Fig. 3). This is likely due to CAX1 protein, or CAX3 in the case of cax1-1 plants, effectively buffering apoplastic Ca\(^{2+}\) in the mesophyll of the single-knockout plants.

We therefore explored whether the Ca\(^{2+}\)-sensitive stomatal phenotype of cax1-1 and cax3-1 plants could be recreated in leaves of intact plants or whether it was an artifact of the epidermal peel system. In order to achieve this, we first optimized the growth of cax1/cax3 plants to ensure that the guard cell phenotype observed in intact cax1/cax3 plants was not a consequence of the dwarf stature or delayed development of these plants when they are grown in standard BNS (2 mM Ca\(^{2+}\)) for 1 week. While Col-0 plants did not appear to be adversely affected by this treatment and continued to develop normally, the growth of the cax1/cax3 rosette was greatly inhibited (Fig. 6A). In addition, the photosynthesis and transpiration rate of cax1/cax3 plants (per mm rosette surface...
area) was lower than that of Col-0 (Fig. 6B, C). Plants grown in SLCS for 6 weeks were used to compare the effects of root-fed Ca\(^{2+}\) on gas exchange as a proxy for stomatal aperture. Both photosynthesis and rosette conductance of the single cax1 knockout and wild-type lines were not significantly different in SLCS, but were decreased in the cax1/cax3 line treated with HCS for 18 hours (Supplementary Fig. 5); this difference was sustained 7 days after treatment (data not shown). We found a decrease in mean rosette conductance in cax1-1 and cax3-1 plants, although this was less significant than in cax1/cax3.

When the length of exposure to HCS was reduced from 18 to 2 hours, we found that the cax1-1, but not cax3-1, plants had significantly reduced photosynthetic and rosette conductance rates compared with Col-0 (Supplementary Fig. 6). This suggests that the transporters that buffer increases in apoplastic [Ca\(^{2+}\)] around mesophyll cells in cax1-1 plants, which include CAX3, are less effective than in wild-type plants containing CAX1, but only in the short term.

CAX1 and CAX3 interact as homo- and heterodimers in planta and facilitate Ca\(^{2+}\) transport when co-expressed in yeast

Despite previous reports identifying the capacity of CAX1 and CAX3 proteins to interact in yeast and in plants under the control of ubiquitous promoters (Zhao et al., 2009a, 2009b), the nature of their interactions remains largely unexplored. We sought to address this latter point by expressing full-length CAX1 and CAX3 using a split luciferase reporter construct in mesophyll protoplasts (Fig. 7). We found that both CAX1 and CAX3 could homodimerize and heterodimerize, and these interactions were abolished by co-transfecting with an artificial miRNA designed against one of the genes (see Supplementary Fig. 7). Furthermore, by switching the split luciferase between the N- and C-terminus, we found that the interaction was mediated by the N-termini of both proteins (head-to-head fashion) (Fig. 7; Supplementary Fig. 7).

### Table 1. In silico analysis of CAX1 and CAX3 expression in Col-0 under various treatments

| Treatment                              | Time after treatment (h) | Tissue                        | CAX1 (fold change) | CAX3 (fold change) |
|----------------------------------------|--------------------------|-------------------------------|--------------------|--------------------|
| Osmotic stress (300 mM mannitol)       | 24                       | Leaf                          | 0.45               | 14.33              |
| Salt (150 mM NaCl)                     | 24                       | Leaf                          | 0.71               | 4.57               |
| Wounding (needle stick)                | 24                       | Leaf                          | 2.06               | 5.98               |
| *Pseudomonas syringae* infiltration    | 1, 4                     | Leaf                          | 1.07               | 2.86               |
| 1 μM flg22 infiltration                | 2, 6, 24                 | Leaf                          | 0.73, 0.83 0.9     | 1.04, 0.56, 0.79   |
| Botrytis cinerea                       | 18, 48                   | Leaf                          | 0.62, 0.43         | 0.96, 0.77         |
| 50 μM ABA                              | 3                        | Leaf                          | 1.0, 1.27          | 7.74, 11.68        |
| 100 μM ABA                             | 4                        | Leaf                          | 1.22               | 5.18               |
| 10 μM ABA                              | 3                        | Guard cells from epidermal peels | 0.24              | 5.69               |
| 10 μM ABA                              | 3                        | Mesophyll protoplasts         | 0.57               | 1.14               |
| 10 μM ABA                              | 3                        | Guard cell protoplasts        | 1.04               | 2.59               |
| 10 μM ABA                              | 3                        | 7-day-old seedlings           | 0.82               | 5.38               |

This table includes expression data from a variety of experiments; as such, conditions are not standardized between experiments. Data are expressed as fold change compared with mock-treated tissue. ABA, abscisic acid. Adapted from eFP BAR; http://bbc.botany.utoronto.ca.
To gain insights into the functional relevance of the interaction between CAX1 and CAX3, we utilized yeast expression assays. Previously, it has been demonstrated that co-expression of CAX1 and CAX3 can suppress yeast vacuolar Ca\(^{2+}\) transport defects, whereas expression of either transporter individually fails to do so (Cheng et al., 2005; Manohar et al., 2011b). The negative regulatory domains within CAX1 and CAX3 prohibit the functional expression of either transporter when individually expressed in yeast cells (Cheng et al., 2005; Manohar et al., 2011b). To avoid potential artifacts arising from the plasmid-dependent CAX overexpression approaches used previously, we modified yeast to integrate both CAX transporters into the genome to ensure stable expression levels. Only strains harboring both constructs conferred Ca\(^{2+}\) tolerance to yeast mutants defective in vacuolar Ca\(^{2+}\) transport (Fig. 8). Immunoblot analysis demonstrated that both CAX1 and CAX3 proteins accumulated to comparable levels in yeast cells (see Supplementary Fig. 8).

We measured transport properties by measuring \(^{45}\text{Ca}^{2+}\) uptake activity in membrane vesicles isolated from yeast cells expressing integrated CAX1-CAX3 and the plasmid-based deregulated sCAX1, an artificially truncated form of CAX1 with the autoinhibitory domain removed. In this system, the pH gradient across yeast vacuolar membrane vesicles was generated by activation of the vacuolar H\(^+\)-ATPase. The vesicles of sCAX1- and CAX1-CAX3-expressing cells took up \(^{45}\text{Ca}^{2+}\) from the medium in a pH- and time-dependent manner for up to 12 min (Fig. 9A). The accumulated \(^{45}\text{Ca}^{2+}\) was released after the addition of the Ca\(^{2+}\) ionophore A23187. The addition of gramicidin, a protonophore that dissipates the pH gradient, eliminated membrane vesicle Ca\(^{2+}\) uptake activity. Membrane vesicles of yeast cells expressing an empty vector had negligible activity (data not shown). Interestingly, CAX1-CAX3-expressing yeast cells demonstrated transport activity that differed from that of the deregulated sCAX1-expressing cells (Fig. 9A). Moreover, Michaelis–Menten kinetic analysis of the data showed that CAX1-CAX3-expressing yeast cells demonstrated a \(K_m\) of 21.64 µM for Ca\(^{2+}\), while sCAX1-expressing cells demonstrated a \(K_m\) of 13.10 µM (Fig. 9B).

To analyze and compare the substrate specificity of the putative CAX1-CAX3 transporters, competition experiments were performed. This approach allowed us to determine the effect of co-expressing CAX proteins in terms of cation selectivity in comparison to the deregulated sCAX1. Initially, we measured Ca\(^{2+}\) uptake in sCAX1- and CAX1-CAX3-expressing cells. The pH-dependent 10 µM \(^{45}\text{Ca}^{2+}\) uptake into yeast microsomal vesicles isolated from strains

---

**Fig. 4.** Expression profiling of CAX1 and CAX3 in leaf tissues. qPCR analysis of cDNA isolated from laser capture microdissected leaf cell types and tissues (leaf 8 of 6-week-old plants) of Col-0 Arabidopsis plants. Data represent means±SD, \(n=3\) plants, performed in triple technical replicates. Gene transcript level was normalized to Actin2 (At3g18780). Asterisks indicate undetectable transcript level.

**Fig. 5.** Stomatal aperture and calcium responsiveness of Col-0, cax1-1, cax3-1, and cax1/cax3 isolated epidermal strips. (A) Stomatal pore aperture, expressed as width/length ratio, in Col-0, cax1-1, cax3-1, and cax1/cax3, \(n=175, 173, 173, 176\), respectively. (B) Change in stomatal pore apertures in adaxial epidermal peels of Col-0, cax1-1, cax3-1, and cax1/cax3 measured with an additional 1 mM extracellular Ca\(^{2+}\) (\(n=173, 183, 146, 166\)), plotted as the change in aperture between each genotype with or without the addition of 1 mM Ca\(^{2+}\). Asterisks indicate significant differences within a genotype with Ca\(^{2+}\) treatment: (A) two-way ANOVA with Tukey’s post-hoc test, \(*P<0.0054, **P<0.05\); (B) Sidak’s multiple comparisons test, \(**P<0.0001\).
Guard cell and pathogen-induced CAX expressing empty vector (data not shown), sCAX1, and CAX1-CAX3 (Fig. 9C) was measured at a single 10 min time point. Ca²⁺ uptake determined in the absence of excess non-radioactive metal (control) was compared with Ca²⁺ uptake determined in the presence of two concentrations (10× and 100× excess) of various non-radioactive metals (Zhao et al., 2008). Inhibition of Ca²⁺ uptake by non-radioactive Ca²⁺ was used as an internal control; as expected, excess Ca²⁺ inhibited
Ca\(^{2+}\) uptake in both sCAX1- and CAX1-CAX3-expressing yeast. Non-radioactive Ca\(^{2+}\), particularly the 10× concentration, did not completely inhibit Ca\(^{2+}\) uptake, further highlighting the low Ca\(^{2+}\) affinity of the transporters. Ca\(^{2+}\) uptake by sCAX1-expressing cells was strongly inhibited by a 10× concentration of Cd\(^{2+}\), whereas CAX1-CAX3-mediated Ca\(^{2+}\) transport was only moderately inhibited. Interestingly, microsomes from CAX1-CAX3-expressing cells, compared with sCAX1-expressing cells, displayed less Ca\(^{2+}\) uptake inhibition by Li\(^{+}\) and Na\(^{+}\). These data demonstrate that the CAX1-CAX3 complex has altered Ca\(^{2+}\) affinity and transport capacity compared with the deregulated sCAX1. These observations imply that the Ca\(^{2+}\) dynamics may be different in plant cells containing the CAX1-CAX3 complex compared with cells containing only CAX1 or CAX3.

**Discussion**

We found that both CAX1 and CAX3 are expressed in stomatal guard cells, unlike most other leaf tissues, under standard conditions. This corroborates previous studies that have detected CAX1 and CAX3 RNA in isolated guard cell protoplast preparations (Leonhardt et al., 2004; Yang et al., 2008). These studies also found CAX3 to be expressed in the mesophyll. However, when we extracted RNA from non-protoplasted leaf tissue, using LMD or SiCSA, we could not detect CAX3 transcript in mesophyll cells (Fig. 1A). Only when protoplast incubation was extended to 24 h could CAX3 expression be detected at moderate levels in the mesophyll (Fig. 1B). The cell wall damage that occurs during protoplasting has been suggested to mimic physiological perturbations that occur in the cell wall in response to pathogen infections (Ecker and Davis, 1987). Our finding that flg22, a bacterial elicitor peptide, stimulates CAX3 expression in the mesophyll cells supports the conclusion that CAX3 expression is induced by wounding and pathogens, but is otherwise normally absent from the mesophyll (Table 1; Fig. 3). As both CAX1 and CAX3 are present in mesophyll cells in response to bacterial elicitors, and a functional CAX1-CAX3 complex can form, it is likely that the transport properties of this complex could participate in the physiological response to pathogen attack.

It is interesting to note that cax1/cax3 plants, which have an altered capacity for Ca\(^{2+}\) secretion into mesophyll cells, had an increased apoplastic Ca\(^{2+}\) concentration, and an altered transcript profile enriched in pathogen-responsive genes (see Supplementary Table S3). For instance, PR1 and PR2, in addition to many cell-wall-related genes, were upregulated in the cax1/cax3 rosette; expression could be reduced to wild-type levels by transferring plants to a low Ca\(^{2+}\) condition (Fig. 6A; Supplementary Fig. S9) (Conn et al., 2011b). Altered Ca\(^{2+}\) compartmentation into the mesophyll and apoplast may also mimic some plant responses to pathogen infection. For example, heterologous expression of Arabidopsis sCAX1 in tomato results in upregulated expression of two pathogen-related proteins, PR P2 precursor and PR leaf protein 4-like, homologs of PRI and PR4 from A. thaliana (~8- and ~23-fold, respectively) (De Freitas et al., 2011). Similarly, four PR genes were induced in cax1/cax3 plants, including PRI (17-fold) and PR5 (11-fold) (Conn et al., 2011b). The increased membrane leakage and blossom end rot symptoms in tomato fruits were considered to be due to the impact of enhanced vacuolar Ca\(^{2+}\) storage on Ca\(^{2+}\)-signaling-related proteins and disturbed apoplastic [Ca\(^{2+}\)], as well as cell wall modification (De Freitas et al., 2011). The upregulation of PR genes in sCAX1-expressing tomato and Arabidopsis cax1/cax3 lines suggests that a modification in CAX-mediated Ca\(^{2+}\) transport in cells may also occur during pathogen responses of plants (Hocking et al., 2016). Variation in the intracellular Ca\(^{2+}\) concentration in a plant cell is known to be a critical step for early defense signaling pathways (Lecourieux et al., 2006).

The observation that CAX1 and CAX3 are expressed in guard cells (Fig. 4) suggests a role for the CAX1-CAX3 complex in stomatal function. A smaller mean stomatal aperture was found in epidermal strips of cax1-1, cax3-1, and cax1/cax3 plants relative to Col-0 (Fig. 5A). These findings are consistent with a previous study Cho et al. (2012), which found significantly reduced steady-state stomatal apertures in epidermal strips in cax1-1, cax3-1, and cax1/cax3 plants.
Fig. 9. Michaelis–Menten kinetic analysis and comparison of inhibition of Ca^{2+} uptake in the presence of other metals. (A) Time course of ^{45}Ca^{2+} uptake into vacuolar vesicles prepared from the yeast strain K667 expressing sCAX1 or CAX1/CAX3. Results are shown in the absence and presence of a protonophore (gramicidin; G). The Ca^{2+} ionophore A23187 (5 µM) was added at 12 min and uptake was measured at 22 min. Data are representative of three independent experiments. (B) Michaelis–Menten kinetic analysis of the initial rate of metal/H^{+} exchange. A preset steady-state pH gradient was generated in vacuolar-enriched vesicles from yeast cells expressing sCAX1 and CAX1-CAX3 by activation of the V-ATPase. Initial rates of H^{+}-dependent Ca^{2+} uptake were calculated over a range of Ca^{2+} concentrations from 0 to 50 µM. Data are representative of three independent experiments. (C) Inhibition of Ca^{2+} uptake by sCAX1 or CAX1-CAX3 into yeast vacuolar-enriched vesicles in the presence of other metal ions. Uncoupler-sensitive (∆pH-dependent) uptake of 10 µM ^{45}Ca^{2+} was measured in the absence (control with 100% activity, shown by the dotted line) or presence of 10× or 100× non-radioactive CaCl_{2}, LiCl, NaCl, NiSO_{4}, MnCl_{2}, CdCl_{2}, ZnCl_{2}, FeCl_{3}, or CoCl_{2} after 10 min. Data are averages of at least three replications from two independent membrane preparations, and are presented as means±SEM.
compared with wild-type. These mutants were also non-responsive to increased Ca²⁺ concentrations, indicating that each isoform is required in guard cells for a normal response to apoplastic Ca²⁺ (Fig. 5B). We propose that the CAX1-CAX3 complex may function in modulating apoplastic Ca²⁺ signaling and is required for maintenance of normal stomatal aperture in response to changes in external Ca²⁺ concentration; this might occur through the sensing of external Ca²⁺ signals through the CAS apoplastic sensor pathway or due to misregulation of cytosolic free Ca²⁺ affecting intracellular signaling in the guard cell (Wang et al., 2014).

Interestingly, however, we found no statistical difference in gas exchange rate in the single cax1-1 or cax3-1 mutants under most conditions (see Supplementary Figs S5 and S6). Previously, we have demonstrated that mesophyll CAX1 controls leaf apoplastic [Ca²⁺] and that CAX3 could compensate for loss of CAX1 in cax1-1 lines (Cheng et al., 2005; Conn et al., 2011b). Here, we demonstrate that CAX3 expression was induced in the mesophyll cells of cax1-1 plants, substituting for CAX1 (Fig. 1). Under standard Ca²⁺ conditions, ectopic expression of CAX3 in the mesophyll in cax1-1 plants prevents excessive Ca²⁺ accumulation in the apoplast and allows the maintenance of growth rate and gas exchange in cax1-1 plants (Supplementary Figs S3–S5) (Cheng et al., 2003). However, after a 2 h pulse of high Ca²⁺ to the roots of cax1-1 plants, the gas exchange rates were reduced compared with wild-type or cax3-1 plants (Supplementary Fig. S6); this indicates that CAX3 cannot fully complement cax1-1. This reduction in gas exchange was not observed in cax1-1 plants when high Ca²⁺ was supplied over 18 h or 7 days, suggesting that the plants can adapt to cope with this Ca²⁺ load over a longer time period.

In this study, we demonstrate that integrated expression of both CAX1 and CAX3 can catalyze vacuolar Ca²⁺ uptake and rescue the Ca²⁺-hypersensitive phenotype of yeast strains defective in Ca²⁺ transport (Fig. 8A). Further analysis on yeast vesicles showed that the heteromeric CAX1-CAX3 complex has similar Ca²⁺ transport properties to deregulated CAXs (Fig. 9; Supplementary Fig. S6) (Cheng et al., 2005; Manohar et al., 2011b). These yeast assays support the hypothesis that CAX3 may act as an activator of the negatively regulated CAX1 in specific plant tissues (Conn et al., 2011b; Cho et al., 2012). The transport affinity of the CAX1-CAX3 complex in yeast assays was different from that of the deregulated transporters and this finding suggests that coupling between CAX transporters could be a mechanism for increasing the range of transporter functions.

Conclusions

Evidence from non-plant studies is beginning to provide confirmation that CAX proteins are able to modulate Ca²⁺ signals (Guttery et al., 2013; Melchionda et al., 2016). These studies have the advantage that the CAX proteins are encoded by single genes, and therefore genetic dissection of CAX Ca²⁺ signaling is not hampered by genetic redundancy. Our study highlights that the multigene CAX families found throughout the plant kingdom may allow the formation of complex functional heteromeric complexes. In yeast-based assays, CAX1-CAX3 displayed transport properties that could not be recreated by high-level expression of either native transporter individually. We also investigated the significance of these interactions for a variety of plant physiological responses. We found that the CAX1-CAX3 complex can occur in leaf mesophyll in response to pathogen attack. Additionally, CAX3 and the CAX1-CAX3 complex may be important in guard cells for maintenance of normal calcium responses and signaling pathways. Further work is required to determine the full extent of the signaling pathways in which CAX1-CAX3 may play a role.

Supplementary material

Supplementary data are available at JXB online.

Fig. S1. Profiling CAX1 and CAX3 promoter activity in mesophyll protoplasts.

Fig. S2. qPCR on whole-leaf RNA.

Fig. S3. Mesophyll vacuolar and leaf apoplastic Ca²⁺ concentrations.

Fig. S4. Physiological parameters affected by changes in [Ca²⁺]ₕυ.

Fig. S5. Gas exchange rates for Col-0, cax1-1, cax3-1, and cax1/cax3 with and without 18 h Ca²⁺ treatment.

Fig. S6. Gas exchange rates for Col-0, cax1-1, cax3-1, and cax1/cax3 after 2 h of Ca²⁺ treatment.

Fig. S7. Split luciferase protein–protein assay determining whether CAX1 and CAX3 interact.

Fig. S8. Western blots showing relative levels of CAX1 and CAX3.

Fig. S9. Expression of PRI and PR2 in 6-week-old Col-0 and cax1/cax3 shoots.

Table S1. Contents of media used for growth studies: BNS, HCS, and SLCS.

Table S2. PCR primers used in this study.

Table S3. Pathogen-related genes that are differentially expressed in cax1/cax3 plants compared with Col-0.

Acknowledgements

This study was supported by United States Department of Agriculture and National Science Foundation (award 1557890) funds to KDH, and Australian Research Council (ARC) CE1400008 and FT130100709 awarded to MG. We also acknowledge Roger Leigh, Steve Tyerman, and Brent Kaiser, and their support from the ARC (DP0774603) under which this project was initiated. We also acknowledge Maclin Dayod for assistance with gas exchange measurements, and Charlotte Jordans and Sam Henderson for assistance with qPCR.

References

Athman A, Tanz SK, Conn VM, Jordans C, Mayo GM, Ng WW, Burton RA, Conn SJ, Gilliham M. 2014. Protocol: a fast and simple in situ PCR method for localising gene expression in plant tissue. Plant Methods 10, 29.

Austin RS, Hiu S, Waese J, et al. 2016. New BAR tools for mining expression data and exploring cis-elements in Arabidopsis thaliana. The Plant Journal 88, 490–504.
guard cells and isolation of a recessive absicic acid hypersensitive protein phosphatase 2C mutant. The Plant Cell 16, 596–615.

Li JF, Park E, von Arnim AG, Nebenführ A. 2009. The FAST technique: a simplified Agrobacterium-based transformation method for transient gene expression analysis in seedlings of Arabidopsis and other plant species. Plant Methods 5, 6.

Manohar M, Shigaki T, Hirschi KD. 2011a. Plant cation/H+ exchangers (CAXs): biological functions and genetic manipulations. Plant Biology 13, 561–569.

Manohar M, Shigaki T, Mei H, Park S, Marshall J, Aguilar J, Hirschi KD. 2011b. Characterization of Arabidopsis Ca2+/H+ exchanger CAX3. Biochemistry 50, 6189–6195.

Melchionda M, Pittman JK, Mayor R, Patel S. 2016. Ca2+/H+ exchange by acidic organelles regulates cell migration in vivo. Journal of Cell Biology 212, 803–813.

Nakagawa T, Suzuki T, Murata S, et al. 2007. Improved Gateway binary vectors: high-performance vectors for creation of fusion constructs in transgenic analysis of plants. Bioscience, Biotechnology, and Biochemistry 71, 2095–2100.

Pittman J, Hirschi K. 2016. CAX-ing a wide net: cation/H+ transporters in metal remediation and abiotic stress signalling. Plant Biology 18, 741–749.

Pittman JK, Hirschi KD. 2003. Don’t shoot the (second) messenger: endomembrane transporters and binding proteins modulate cytosolic Ca2+ levels. Current Opinion in Plant Biology 6, 257–262.

Pittman JK, Shigaki T, Hirschi KD. 2005. Evidence of differential pH regulation of the Arabidopsis vacuolar Ca2+/H+ antiporters CAX1 and CAX2. FEBS Letters 579, 2648–2656.

Plett D, Safwat G, Gillilahm M, Skrmsmger Moller I, Roy S, Shirley N, Jacobs A, Johnson A, Tester M. 2010. Improved salinity tolerance of rice through cell type-specific expression of ATHK1:1. PLoS One 5, e12571.

Pottosin II, Schönhnecht G. 2007. Vacular calcium channels. Journal of Experimental Botany 58, 75–156.

Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative Ct method. Nature Protocols 3, 1101–1108.

Schwab R, Ossowski S, Riemer M, Warthmann N, Weigel D. 2006. Highly specific gene silencing by artificial microRNAs in Arabidopsis. The Plant Cell 18, 1121–1133.

Sherman F, Fink GR, Hicks JB. 1986. Laboratory course manual for methods in yeast genetics. Cold Spring Harbor: Cold Spring Harbor Laboratory.

Shigaki T, Cheng NH, Pittman JK, Hirschi K. 2001. Structural determeme of Ca2+/H+ transport in the Arabidopsis H+/Ca2+ antiporter CAX1. Journal of Biological Chemistry 276, 43152–43159.

Shigaki T, Hirschi K. 2000. Characterization of CAX-like genes in plants: implications for funcational diversity. Gene 257, 291–298.

Wang WH, Chen J, Liu TW, Chen J, Han AD, Simon M, Dong XJ, He JX, Zheng HL. 2014. Regulation of the calcium-sensing receptor in both stornatal movement and photosynthetic electron transport is crucial for water use efficiency and drought tolerance in Arabidopsis. Journal of Experimental Botany 65, 223–234.

White PJ, Broadley MR. 2003. Calcium in plants. Annals of Botany 92, 487–511.

Yang Y, Costa A, Leonardt N, Siegel RS, Schroeder JI. 2008. Isolation of a strong Arabidopsis guard cell promoter and its potential as a research tool. Plant Methods 4, 6.

Yoo SD, Cho YH, Sheen J. 2007. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nature Protocols 2, 1565–1572.

Zhao J, Barkia BJ, Marshall J, Pittman JK, Hirschi KD. 2008. The Arabidopsis CAX; Ca2+ transporters display altered salt tolerance, pH sensitivity and reduced plasma membrane H+-ATPase activity. Plants 227, 659–669.

Zhao J, Connorton JM, Guo Y, Li X, Shigaki T, Hirschi KD, Pittman JK. 2009a. Functional studies of split Arabidopsis Ca2+/H+ exchangers. Journal of Biological Chemistry 284, 34075–34083.

Zhao J, Shigaki T, Mei H, Guo YQ, Cheng NH, Hirschi KD. 2009b. Interaction between Arabidopsis Ca2+/H+ exchangers CAX1 and CAX3. Journal of Biological Chemistry 284, 4605–4615.