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REVIEW ARTICLE

CAX control: multiple roles of vacuolar cation/H^+ exchangers in metal tolerance, mineral nutrition and environmental signalling

J. K. Pittman[1] & K. D. Hirschi[2]

1 Department of Earth and Environmental Sciences, School of Natural Sciences, The University of Manchester, Manchester, UK
2 Children's Nutrition Research, Baylor College of Medicine, Houston, TX, USA

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Correspondence
K. D. Hirschi, Pediatrics-Nutrition, Children's Nutrition Research, Baylor College of Medicine, 1100 Bates Street, Houston, TX 77030, USA.
E-mail: kendalh@bcm.edu
J. K. Pittman, Department of Earth and Environmental Sciences, School of Natural Sciences, The University of Manchester, Michael Smith Building, Oxford Road, Manchester M13 9PT, UK.
E-mail: jon.pittman@manchester.ac.uk

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ABSTRACT

Plant vacuolar transporters, particularly CAX (Cation/H^+ Exchangers) responsible for Ca^{2+}/H^+ exchange on the vacuole tonoplast, play a central role in governing cellular pH, ion balance, nutrient storage, metal accumulation, and stress responses. Furthermore, CAX variants have been employed to enhance the calcium content of crops, contributing to biofortification efforts. Recent research has uncovered the broader significance of these transporters in plant signal transduction and element partitioning. The use of genetically encoded Ca^{2+} sensors has begun to highlight the crucial role of CAX isoforms in generating cytosolic Ca^{2+} signals, underscoring their function as pivotal hubs in diverse environmental and developmental signalling networks. Interestingly, it has been observed that the loss of CAX function can be advantageous in specific stress conditions, both for biotic and abiotic stressors. Determining the optimal timing and approach for modulating the expression of CAX is a critical concern. In the future, strategically manipulating the temporal loss of CAX function in agriculturally important crops holds promise to bolster plant immunity, enhance cold tolerance, and fortify resilience against one of agriculture's most significant challenges, namely flooding.

INTRODUCTION

Plants rely on regulated movement of ions across tissues and within cells to ensure ion homeostasis, including for nutritional needs and biochemical functions. Ca^{2+} is one such ion that is readily accumulated in plants as an essential mineral nutrient, is required for various biochemical and structural needs, and plays a critical role in signalling in response to environmental stimuli and developmental processes (White & Broadley 2003; McAinsh & Pittman 2009; Tian *et al.* 2020). The vacuole is a major store for Ca^{2+} within cells to prevent cytosolic toxicity caused by excess free Ca^{2+} but also to allow re-release as required during a Ca^{2+} signalling event (Peiter 2011). Various transporters mediate Ca^{2+} uptake into and release from the vacuole, with a key player being the proton-coupled cation/H^+ exchanger (CAX), which alongside the P-type Ca^{2+}-ATPases, are responsible for Ca^{2+} sequestration into the vacuole (Pittman 2011). However, CAXs are versatile proteins with many isoforms able to transport various cations including Ca^{2+}, Mn^{2+}, Zn^{2+}, and Cd^{2+} (Pittman & Hirschi 2016a). CAXs are not the only proteins that provide cation/H^+ exchange activity for metal sequestration into the vacuole; for example, Zn^{2+}, Mn^{2+}, and Fe^{2+} transport by VIT (vacuolar iron transporter) and MTP (metal transport protein) isoforms are also proposed to be proton-coupled (Arrivault *et al.* 2006; Connorton *et al.* 2017; Eroglu *et al.* 2017), but only CAXs appear to mediate vacuolar Ca^{2+}/H^+ exchange activity. Moreover, although some plant CAXs have occasionally been suggested to reside at membranes other than the tonoplast (Luo *et al.* 2005; Zou *et al.* 2021), the evidence for non-vacuolar CAX localization is very limited.

The CAX transporters are members of the Ca^{2+}/Cation Antiporter (CaCA) superfamily of ion-coupled transporters (Emery *et al.* 2012). Following the identification of the first plant CAX gene from *Arabidopsis* (*A. thaliana*) (Hirschi *et al.* 1996), individual gene cloning and genome-wide sequence analyses have demonstrated that CAX genes are ubiquitous across the plant kingdom, also in non-vascular plants, algae, and cyanobacteria (Emery *et al.* 2012; Pittman & Hirschi 2016b; Mao *et al.* 2021; Zheng *et al.* 2021). In addition to these photosynthetic organisms, CAXs are prevalent within fungi, protists, bacteria, and some animals, but not mammals (Shigaki *et al.* 2006; Pittman & Hirschi 2016a). Higher plant genomes typically possess CAX gene families of around five to ten genes, such as five CAX genes in *Arabidopsis* and six in rice (*Oryza sativa*) (Emery *et al.* 2012; Pittman & Hirschi 2016b; Zheng *et al.* 2021). A

wealth of biochemical and genetic research over recent decades has established the functional characteristics of individual CAX genes, especially in *Arabidopsis*. Typically, there is variation in substrate specificity across CAX gene families within a plant. As such, isoforms like AtCAX1 and AtCAX3 are mainly Ca^{2+} specific and critical to cellular calcium content (Cheng *et al.* 2005; Conn *et al.* 2011), while AtCAX2 and AtCAX5 have a broader cation substrate specificity and so may be involved in multiple aspects of mineral nutrient homeostasis (Pittman *et al.* 2004; Edmond *et al.* 2009). For more detailed background information on plant CAXs, the reader is directed to previous reviews (Manohar *et al.* 2011a; Bickerton & Pittman 2015; Pittman & Hirschi 2016a; Demidchik *et al.* 2018).

This review article discusses new research insights from the last 5 to 6 years that have expanded our knowledge of CAX function. This includes our growing awareness of the critical involvement of CAX transporters as key hubs in various cell signalling pathways, as well as uncovering new mechanisms of CAX regulation. Moreover, studies have identified more roles of CAX transport activity in environmental stress response, and two example case studies (responses to low-oxygen and cadmium) are described in detail. Finally, we summarize some of the recent research that has explored the use of CAXs as gene targets for agronomic improvement, particularly for nutrient biofortification.

CAX TRANSPORTERS AS SIGNALLING HUBS FOR ABIOTIC AND BIOTIC STRESS RESPONSE

The CAX proteins from various plant species have been implicated in an extensive array of abiotic stress events; in most cases related to their proposed roles as modulators of Ca^{2+} signals. These include responses to cold (Yang *et al.* 2023), salinity (Navarro-León *et al.* 2021), alkalinity (Navarro-Leon *et al.* 2023), and flooding (Bakshi *et al.* 2023), as well as their roles in tolerance to non-essential metals, including cadmium (Modareszadeh *et al.* 2021) and barium (Mei *et al.* 2024). The involvement of a CAX isoform in stress pathways has typically been determined through observations of altered gene expression following a stress event, or by CAX gene overexpression or silencing experiments that give the plant altered sensitivity or tolerance to the stress. In some situations, stress tolerance due to CAX activity does not seem to involve Ca^{2+} signalling, such as cadmium, manganese or barium tolerance due to direct CAX-mediated Cd^{2+}, Mn^{2+} or Ba^{2+} transport into the vacuole (Koren'kov *et al.* 2007; Zhang *et al.* 2016; Zou *et al.* 2021; Mei *et al.* 2024). However, in many other instances, CAXs have been identified as positive or negative regulators of stress tolerance caused by altered Ca^{2+} transport leading to modification of a cytosolic Ca^{2+} 'signature'. The mechanisms by which Ca^{2+} efflux transporters such as CAXs may shape cytosolic Ca^{2+} signals are reviewed elsewhere (McAinsh & Pittman 2009; Tian *et al.* 2020). However, while many previous studies have indicated cytosolic Ca^{2+} changes due to CAX activity indirectly, it is only very recently that evidence has started to become available to support the model of CAX requirement for stress-stimulated Ca^{2+} signals (Bakshi *et al.* 2023; Wang *et al.* 2024).

By making use of green fluorescent protein-based Ca^{2+} sensors, it has been demonstrated that loss of *AtCAX1* alters anoxia-generated cytosolic Ca^{2+} signals, both in terms of the timing and spatial distribution of the Ca^{2+} signal (Yang *et al.* 2022), and these signals are altered further when other CAX genes are removed concurrently (Mathew *et al.* 2024). In contrast, Ca^{2+} signals generated in response to hypoxia were altered when *AtCAX2* was knocked out, giving rise to an elevated and prolonged Ca^{2+} signal (Bakshi *et al.* 2023). High concentrations of external Ca^{2+} cause a transient rise in cytosolic Ca^{2+} that quickly returns to resting levels, but *Arabidopsis cax1* single or *cax1*/*cax3* double knockout mutants prevent this reset (Wang *et al.* 2024). The exact relevance of cytosolic Ca^{2+} changes caused by CAX inhibition or activation requires further research to determine whether these are truly signals and, if so, identify the downstream components that are responsible for transducing the signal. However, CAX activity can also alter other components that may act as a cellular signal, including levels of apoplastic Ca^{2+} (Conn *et al.* 2011), apoplastic pH (Cho *et al.* 2012), or cytosolic reactive oxygen species (ROS) (Yang *et al.* 2022).

The CAX proteins are not just components of abiotic stress adaptation but are also implicated in biotic stress responses. Many studies have shown the importance of Ca^{2+} signalling for plant immunity, and various Ca^{2+} transport components including Ca^{2+}-permeable channel isoforms and autoinhibited Ca^{2+}-ATPase (ACA) isoforms are required for cytosolic Ca^{2+} signal generation in response to pathogen infection or elicitors (Tian *et al.* 2020). For example, the vacuolar Ca^{2+} pumps AtACA4 and AtACA11 play a critical role in the generation of Ca^{2+} signals induced by the bacterial elicitor flg22, which triggers an immune response (Hilleary *et al.* 2020). The flg22 elicitor also induces mRNA expression of *AtCAX3* but not *AtCAX1* (Hocking *et al.* 2017). In contrast, resistance to infection by the pathogens *Pseudomonas syringae* and *Botrytis cinerea* was increased when *AtCAX1* was disrupted, but not *AtCAX3* (Zhang *et al.* 2020). A CAX3 isoform from cotton (*Gossypium hirsutum*) has also been implicated in pathogen resistance, such that a microbial compound that induces resistance to cotton *Verticillium* wilt (by the pathogen *Verticillium dahliae*) does so by inhibiting *GhCAX3* expression, causing cytosolic Ca^{2+} elevation and induction of a hypersensitive response (Zhou *et al.* 2022). Furthermore, this resistance phenotype can be confirmed by genetically silencing *GhCAX3*. These outcomes are consistent with earlier work showing increased resistance to stem rust in barley (*Hordeum vulgare*) when *HvCAX1* is mutated causing an increased hypersensitive response (Zhang *et al.* 2009). More recently it has been demonstrated that both AtCAX1 and AtCAX3 act as Ca^{2+} signalling hubs during pattern-triggered immunity (PTI) pathways, downstream of well-studied molecular components of the PTI pathway, including the plasma membrane receptors FLS2 and BAK1, and the kinases BIK1 and PBL1 (Wang *et al.* 2024). Taken together these studies indicate that plant vacuolar Ca^{2+} exchangers are also involved in pathogen defence, but in an isoform-specific manner, and in most cases due to negative regulation.

CASE STUDY: CAX MUTANTS AND THEIR INFLUENCE ON LOW-OXYGEN/FLOODING TOLERANCE

Flooding poses a significant risk to worldwide crop production, and plants employ various adaptive mechanisms to mitigate this stress (Bailey-Serres *et al.* 2012; Voesenek *et al.* 2016). One of these cellular pathways involves the modification of Ca^{2+}

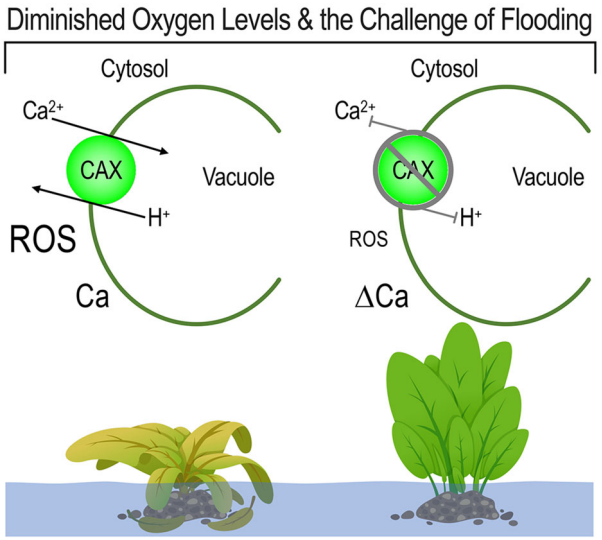

Fig. 1. Loss of CAX function leads to enhanced tolerance to low oxygen and water stress. The absence of CAX isoforms can enhance plant ability to withstand both waterlogging and submergence. CAXs use the H^+ gradient to transfer Ca^{2+} into the vacuole. Mutants lacking CAXs exhibit changes in cytosolic Ca^{2+} signalling and reduced accumulation of ROS during the recuperation phase following exposure to low oxygen conditions.

signalling (Wang *et al.* 2016; Bakshi *et al.* 2023). Several studies highlight how CAX activity may be engineered to regulate flooding and submergence tolerance (Yang *et al.* 2022; Bakshi *et al.* 2023). By analysing available transcriptomic data from *Arabidopsis* plants, the *AtCAX2* gene was one of 28 Ca^{2+} related genes that appeared to be a promising candidate for study: the transcript was rapidly upregulated in datasets following low oxygen stress or flooding (Bakshi *et al.* 2023). Through mutant analysis, disruption of *AtCAX2* had the most robust phenotype among the 28 genes, while *cax2* null mutants displayed improved plant survival during soil waterlogging, altered expression of hypoxic response genes, and altered Ca^{2+} signals (Bakshi *et al.* 2023). These findings suggest that AtCAX2 plays a role in coordinating rapid plant responses to flooding and/or low oxygen responses. However, the rapid transcriptional expression of a transporter whose loss of function appears to help the plant during flooding is puzzling. This suggests that these assays (or the transcriptional profiling data) may not capture the intricacies of the flooding process. It is conceivable that AtCAX2 oversees a subsection of the various cellular processes linked to longer-term flooding responses that were not examined in this research (Pedersen *et al.* 2017).

While *AtCAX2* transcript abundance is induced during flooding and low oxygen conditions, the more highly expressed *AtCAX1* is not. However, mutants in *AtCAX1* have related phenotypes: tolerance to submergence and anoxic conditions (Yang *et al.* 2022; Mathew *et al.* 2023). Phenotypic evaluations, RNA-sequencing, and proteomics demonstrate that the anoxia-induced alterations in *cax1* mutant *Arabidopsis* lines resemble changes observed in anoxia-tolerant crops: shifts in metabolic processes, decreased generation of ROS following anoxia, and adjustments in hormone signalling (Yang *et al.* 2022). When comparing wild-type and *cax1* plants expressing transgenic Ca^{2+} indicators, differences in cytosolic Ca^{2+} signals in *cax1* plants were evident during re-oxygenation (Fig. 1).

Given that both AtCAX1 and AtCAX2 appear to have a role in low-oxygen related stress responses, the next step was to impair multiple CAXs, also including AtCAX3 and AtCAX4 (Mathew *et al.* 2024). These combinatorial *Arabidopsis* CAX mutants had enhanced anoxia tolerance compared to the single knockout *cax1* plant. By progressively reducing CAXs to generate a quadruple knockout mutant, plants showed increased mRNA expression and protein changes related to ROS and stress signalling pathways (Mathew *et al.* 2024). Analysis showed that the concentrations of elements in leaves correlated with the number of CAX genes removed and that lower Ca^{2+} levels enhance anoxia tolerance, as wild-type plants grown under low Ca^{2+} conditions show increased anoxia tolerance (Mathew *et al.* 2024).

Future work should address the molecular mechanisms by which CAX transport influences plant responses to low oxygen and flooding. Why does impairment of this low affinity, high-capacity Ca^{2+} transport system at the tonoplast improve this stress phenotype more than alterations in tonoplast high affinity, low-capacity transporters (the Ca^{2+}-ATPases ACA4 and ACA11)? A priority should also be discerning the potential for genetic engineering to enhance flood and submergence tolerance in crop plants. Additionally, further studies should address the relationship between Ca^{2+} levels and anoxia tolerance and how altering the concentrations of other elements may impact stress responses in plants. As more data accumulate, bioinformatics tools and computational modelling will be able to predict and analyse the effects of various CAX mutations under different environmental scenarios and determine if the concepts identified during low oxygen tolerance can be applied to improving stress tolerance in crops facing other environmental stresses, such as drought and salinity.

FURTHER INSIGHTS INTO MECHANISMS OF CAX REGULATION

As described above, individual CAX genes have been implicated in numerous signalling pathways, which leads to the question of how specificity is determined to ensure that a particular CAX isoform functions to modify a Ca^{2+} signal only when needed. Furthermore, continuous activation of Ca^{2+}/H^+ exchange activity is deleterious to the plant (Hirschi 1999; Mei *et al.* 2007), therefore the ability of the cell to regulate CAX function is crucial. While CAX gene abundance can be controlled transcriptionally, transport activity is regulated post-translationally. CAX proteins such as AtCAX1 and AtCAX3 possess an N-terminal autoinhibitory domain that regulates CAX activity, such that when this domain is removed or its structural conformation changed, the transporter is active (Pittman & Hirschi 2001; Manohar *et al.* 2011b). Similarly, CAX proteins from other plant species seem to share this mode of regulation (Martins & Gerós 2020; Han *et al.* 2022). One model proposes that CAX interacting proteins (CXIP) bind to the N-terminal CAX domain in response to a specific stimulus to release autoinhibition and activate cation transport (Fig. 2). In support of this model, putative CXIPs were identified in *Arabidopsis* (Cheng & Hirschi 2003; Cheng *et al.* 2004a,b), and more recently, orthologs of one of these proteins (CXIP4) were identified in other plants, and have been validated to be *bona fide* CAX binding proteins (Chen *et al.* 2019; Martins & Gerós 2020). CXIP4 is a nuclear-localized protein that can

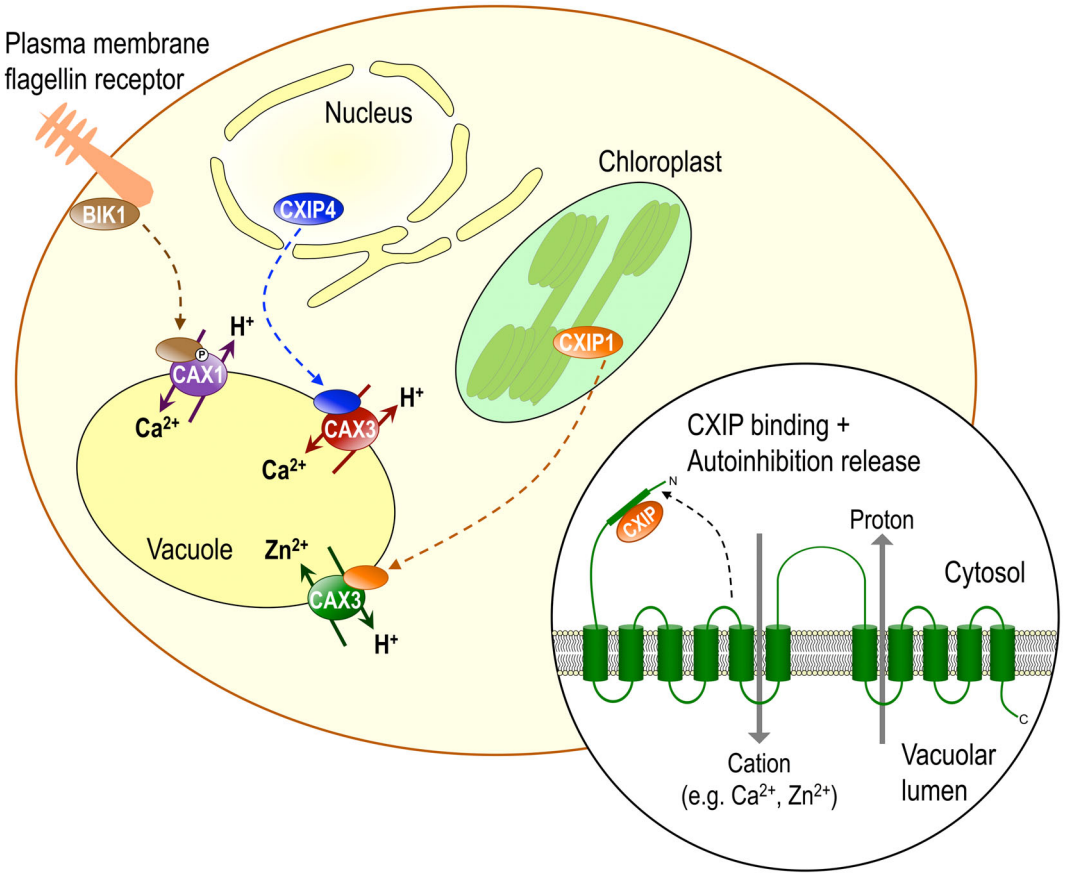

Fig. 2. Model of CAX interacting protein (CXIP) activation of CAX transporters. AtCAX1 (and AtCAX3) from *Arabidopsis* is regulated by CAX-interacting kinases, including AtBIK1, a cytoplasmic kinase that associates and is activated by plasma membrane pathogen immunity receptors, such as the flagellin receptor. AtBIK1 activates Ca^{2+}/H^+ exchange activity at the vacuole by binding and phosphorylating the N-terminal autoinhibitory domain of the CAX. VvCAX3 from grape is regulated by VvCXIP4, which is exported to the cytosol from the nucleus to activate Ca^{2+}/H^+ exchange activity at the vacuole. MdCAX3 from apple is regulated by MdCXIP1, which is normally in the chloroplast and is presumably exported to the cytosol to activate Zn^{2+}/H^+ exchange activity at the vacuole. CXIP1 is proposed to bind the N-terminal autoinhibitory domain of CAX, release autoinhibition, and then activate transport activity.

move to the tonoplast in response to an elevated Ca^{2+} stimulus, where it interacts and activates Ca^{2+}/H^+ exchange activity, such as by VvCAX3 from grape (*Vitis vinifera*) (Martins & Gerós 2020) (Fig. 2).

Some CXIPs activate CAX-mediated Ca^{2+} transport through phosphorylation. In response to elevated cytosolic Ca^{2+} the tonoplast-localized CBL2 and CBL3 Ca^{2+} sensors recruit and activate the protein kinases CIPK3, CIPK9, or CIPK26, which then phosphorylate AtCAX1 and AtCAX3 to activate vacuolar Ca^{2+} sequestration and prevent Ca^{2+} cytotoxicity (Wang *et al.* 2024). However, other kinases including the pathogen-triggered BIK1 and PBL1, which are activated by plasma membrane immune receptors, also interact with and phosphorylate AtCAX1 and AtCAX3 (Fig. 2). This is proposed to allow the CAX proteins, in concert with plasma membrane Ca^{2+} influx channels, to generate specific Ca^{2+} signals that ultimately elicit an immune response. Interestingly, both sets of kinases act through the same targets on the AtCAX1 and AtCAX3 N-terminal autoinhibitory domains, a conserved cluster of four serine residues that are proposed to cause a conformational change to the N-terminal tail following phosphorylation to allow Ca^{2+} transport activation (Wang *et al.* 2024).

The CXIP-mediated CAX regulation is not limited to modulation of Ca^{2+} transport activity. A recent study uncovered a mechanism of CXIP regulation of zinc partitioning and sequestration during iron starvation in a domesticated apple (*Malus domestica*) variety. MdCAX3 was shown to enhance Zn^{2+} accumulation in root cell vacuoles following activation by MdCXIP1 via direct protein interaction (Hao *et al.* 2022) (Fig. 2). CXIP1 (also known as GRXS14) is a glutaredoxin that functions in iron homeostatic redox control (Cheng *et al.* 2006; Wu *et al.* 2017). The increased Zn^{2+} removal from the cytosol due to activated MdCAX3 was proposed to prevent the zinc-dependent inhibition of the plasma membrane Fe^{2+} uptake transporter IRT1, which helps to alleviate iron deficiency. Moreover, *MdCAX3* mRNA was shown to be mobilized from leaves to roots during iron starvation to enhance its expression in root tissues, indicating an intriguing mode of post-transcriptional regulation of this CAX. Finally, the authors of this study propose that CAX3/CXIP1-mediated iron and zinc homeostasis may be a common occurrence in plants, while providing evidence that *Arabidopsis cax3* knockout mutant plants also show zinc sensitivity and reduced iron uptake capacity (Hao *et al.* 2022). This same apple CAX (also

known as *MdCAX3L-2*) is also regulated by the MdbHLH4 transcription factor in response to cold stress (Yang *et al.* 2023). Gene overexpression and silencing experiments demonstrated that this CAX3 protein negatively regulates cold tolerance, likely through modification of cold-induced Ca^{2+} signals, since this CAX can transport Ca^{2+}, in addition to its ability to transport Zn^{2+}. This is consistent with the negative regulation role of AtCAX1 in cold response (Catalá *et al.* 2003).

In addition to CAX modulation by small protein interaction, CAX regulation occurs through homo- and hetero-dimerization. AtCAX1 and AtCAX3 have functional overlap, as indicated by the severity of the *cax1*/*cax3* double mutant (Cheng *et al.* 2005; Conn *et al.* 2011), while when co-expressed either in yeast (*Saccharomyces cerevisiae*) or ectopically in plant cells they can directly interact and release autoinhibition (Zhao *et al.* 2009a; Zhao *et al.* 2009b). The physiological relevance of CAX hetero-dimerization was examined in guard cells and surrounding mesophyll cells where these proteins co-express, allowing them to interact and control stomatal opening, possibly via Ca^{2+} signalling (Cho *et al.* 2012; Hocking *et al.* 2017). In particular, it was proposed that the AtCAX1–AtCAX3 complex may modulate apoplastic Ca^{2+} signalling in order to regulate stomatal opening. Moreover, the interaction between the CAX proteins in mesophyll cells was shown to be dependent on bacterial elicitors, further highlighting the involvement of AtCAX1 and AtCAX3 in biotic stress response (Hocking *et al.* 2017). CAX hetero-dimerization was also observed for other CAX isoforms, such as in apple (Mao *et al.* 2021). An open question is whether there are chaperone proteins that control the formation of CAX hetero- or homo-dimerization, and whether these proteins overlap with any of the CXIPs that interact with CAX monomers. It is also unclear whether there are microdomains of CAX interaction across the tonoplast, and how transient or stable such interactions are.

CASE STUDY: CAX TRANSPORTERS IN CADMIUM SEQUESTRATION AND TOLERANCE

Previous studies have shown that *Arabidopsis* CAXs are crucial in sequestering cadmium into vacuoles, essential for limiting transport of this undesirable pollutant from plants into human diets (Salt & Wagner 1993; Koren'kov *et al.* 2007; Koren'kov *et al.* 2009). Recent work suggests that CAXs in rice and poplar (*Populus trichocarpa*) may also affect cadmium tolerance and transport (Zou *et al.* 2021; He *et al.* 2022). Although most CAXs appear able to transport Cd^{2+}, some CAX transporters exhibit a higher affinity for Cd^{2+}, and their expression can enhance the sequestration of Cd^{2+}, reducing its potential harm to both the plant and the environment (Koren'kov *et al.* 2007). In addition to cadmium tolerance derived from wild-type CAX sequences, modified variants made either by site-directed mutagenesis or the TILLING (Targeting Induced Local Lesions In Genomes) technique can display enhanced cadmium tolerance and phytoremediation potential (Wu *et al.* 2011; Navarro-León *et al.* 2020). For example, BrCAX1a TILLING mutants of *Brassica rapa* subsp. *trilocularis* (known as yellow sarson), have increased cadmium uptake capacity and enhanced tolerance, including through higher ROS detoxification activity, potentially via up-regulated Ca^{2+} transport since the mutations are located within the N-terminal autoinhibitory domain (Navarro-León *et al.* 2020).

Plants known for their cadmium hyperaccumulation also exhibit changes in the expression of CAX genes (Baliardini *et al.* 2015; Baliardini *et al.* 2016; Zhang *et al.* 2016). Furthermore, when a CAX transporter from *Sedum alfredii*, a plant that hyperaccumulates zinc and cadmium, was expressed in tobacco (*Nicotiana benthamiana*), it caused a rise in accumulation of cadmium (Zhang *et al.* 2016). High expression levels of a specific CAX isoform (*AhCAX1*) are an important cadmium hyperaccumulation factor in *Arabidopsis halleri*. Furthermore, ectopic expression of *AhCAX1* in roots of the non-hyperaccumulating *A. thaliana* increased the plant's ability to accumulate cadmium (Ahmadi *et al.* 2018). The increase in ionic Cd^{2+} in plants, like other stresses, generates ROS, resulting in changes in levels of cytosolic Ca^{2+} (Choi *et al.* 2017). A model has been proposed where AhCAX1 prevents positive feedback of Cd^{2+}-elicited ROS production (Ahmadi *et al.* 2018). This model is supported by work in *Arabidopsis*, where enhanced expression of AtCAX3 also enhances cadmium tolerance by decreasing Cd^{2+}-induced ROS production (Modareszadeh *et al.* 2021).

Further research is needed to investigate the mechanism underlying these changes related to ROS. A simple starting point would be to address whether mutants in yeast lacking its vacuolar CAX, ScVCX1 (Cunningham & Fink 1996; Miseta *et al.* 1999), have ROS-related phenotypes during cadmium stress. It could then be examined whether yeast cadmium stress responses change when plant CAX genes are expressed in the *vcx1* mutants. Another question is whether CAX-mediated ROS control in plants during cadmium exposure is a result of increased Cd^{2+} sequestration, changes in cytosolic Ca^{2+} levels, or alterations in cytoplasmic pH. One way to explore this would be to introduce previously generated CAX protein variants with modified Cd^{2+} or Ca^{2+} transport ability, or modified pH optima (Pittman *et al.* 2005; Shigaki *et al.* 2005) into the appropriate plant CAX mutant background. By doing so, we could investigate how these alterations affect the ROS changes induced by cadmium exposure.

CAX MUTANTS INFLUENCE ELEMENT DYNAMICS

The dynamics of the ionome, encompassing the comprehensive array of inorganic ions and elements within an organism, have become a burgeoning area of interest in plant physiology (Baxter 2015; Whitt *et al.* 2020). While *Arabidopsis cax1* and *cax3* single knockout lines exhibit relatively subtle shifts in their ion profiles, the combined *cax1*/*cax3* double knockout lines reveal striking deviations (Cheng *et al.* 2005). Specifically, within the shoot tissue, notable increases in concentrations of phosphate, manganese, and zinc are coupled with a simultaneous reduction in calcium and magnesium (Cheng *et al.* 2005). These changes are notably interconnected with the pivotal roles played by AtCAX1 and AtCAX3 in mediating a signal originating in the shoot, intricately regulating the root PO_4^{3-} transporter system (Liu *et al.* 2011). There is also an interesting observation that some bacterial CAX proteins are able to mediate the coupled transport of Ca^{2+} and PO_4^{3-} in exchange for H^+ under certain conditions (Niu *et al.* 2023). However, there is currently no evidence that plant CAXs can perform PO_4^{3-} transport.

Furthermore, the influence of CAXs on *Arabidopsis* seed ionome distribution patterns is evident, as element imaging

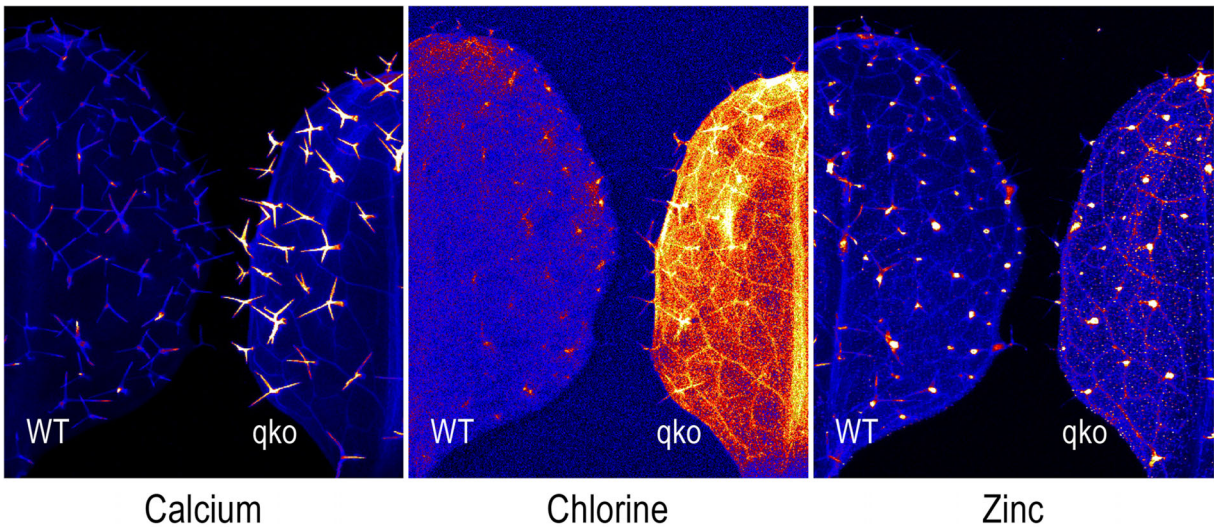

Fig. 3. Loss of CAX function alters partitioning of multiple elements within the leaf. The spatial distribution of calcium, chlorine, and zinc in wild-type (WT) and *cax1/cax2/cax3/cax4* quadruple knockout (qko) mutant *Arabidopsis* plants cultivated in standard growth media. Synchrotron X-ray fluorescence elemental imaging was used to capture images of half-leaf sections from 14-day-old plants. In the qko leaves there is a significant accumulation of calcium in trichomes, while chlorine is evenly dispersed throughout the leaves, and there is a distinctive punctate pattern of zinc distribution.

shows elevations in calcium content in both the seed coat and embryo of *cax1*, *cax3*, and *cax1/cax3* lines (Punshon *et al.* 2012). Employing these high-resolution element imaging techniques demonstrates that disruptions in CAX activity alter the partitioning of calcium within cells, resulting in shifts in organelle allocation and potentially affecting cytosolic Ca^{2+} levels, ultimately erasing tissue-level calcium gradients (Conn *et al.* 2011; Punshon *et al.* 2012). In leaf tissues, element imaging using a combination of synchrotron X-ray fluorescence microscopy (SXRF) and inductively-coupled plasma mass spectrometry (ICP-MS) demonstrates changes in element distribution patterns and a noticeable reduction in calcium levels in response to perturbations in CAX activity (Fig. 3) (Mathew *et al.* 2024). This imaging analysis offers valuable insights into the dynamic ionomic processes in plants and underscores the intricate regulatory roles played by CAXs in shaping element content and distribution patterns. Subsequent investigations will aim to determine whether these alterations are responsible for the variations in signalling observed in the CAX mutants.

In addition to the *Arabidopsis* CAX proteins, mutations to *B. rapa BrCAX1a* generated by TILLING altered the mineral nutrient profile of the plant (Navarro-León *et al.* 2018). In comparison to the parental line, TILLING mutants had increased content of calcium, magnesium and iron in the leaves. Likewise, nitrogen and sulfur content increased in one or more of these lines. In some cases, such as for iron and nitrogen, the increase in nutrient content caused by CAX1 mutation was irrespective of external calcium treatment. In contrast, the increase in content of calcium and magnesium was only observed in the TILLING lines when higher external calcium doses were applied. It was also observed that the content of minerals including copper and manganese was reduced in many of the *BrCAX1a* mutant lines (Navarro-León *et al.* 2018). However, the exact causes of these element variations in the TILLING lines and how they link to altered CAX1 activity require further study.

CAXS CAN PROVIDE AGRONOMIC IMPROVEMENTS

The foundations of human nutrition predominantly arise from plant-based diets (Gibbs & Cappuccio 2022). A hopeful strategy to enhance the nutritional quality of plant-based foods involves manipulating plant transport systems (Schroeder *et al.* 2013). If effectively implemented, this technique has the potential to selectively extract targeted nutrients from the soil, filter out less desirable metals, and concentrate these nutrient-rich minerals in the edible parts of plants (Hirschi 2008).

Elevated levels of CAX expression can alter calcium and trace mineral content in various crops (Park *et al.* 2004; Kim *et al.* 2005; Park *et al.* 2005a; Park *et al.* 2005b; Kim *et al.* 2006; Park *et al.* 2009). Notably, potatoes (*Solanum tuberosum*), carrots (*Daucus carota*), lettuce (*Lactuca sativa*), and tomatoes (*Solanum lycopersicum*) all have exhibited higher calcium concentrations in their edible parts through enhanced CAX expression (Park *et al.* 2004, 2005a,b; Kim *et al.* 2006; Park *et al.* 2009). Employing stable isotope labeling methods with human feeding trials has proven that CAX-enhanced carrots possess more bioavailable calcium (Morris *et al.* 2008; Hawthorne *et al.* 2009). Furthermore, CAX-expressing lettuce varieties retain their taste and texture qualities, as demonstrated by a panel of taste testers (Park *et al.* 2009). Genomic analyses have further validated the utility of altering CAX expression in *B. rapa* for adjusting calcium composition and concentration (Graham *et al.* 2014; Navarro-León *et al.* 2018). In addition, detailed genomic analyses of rice CAX genes has identified candidate genes that link to specific agronomic traits related to seed development and rice grain yield, as well as specific abiotic stress responses, that are likely to be of value for future rice crop improvement (Lian *et al.* 2024).

Global concerns about dietary calcium intake levels are significant (Weaver 2000), and plant biotechnology offers a cost-effective solution to tackle this challenge (Garg *et al.* 2018). Nevertheless, a critical question emerges: can manipulating CAX transporters truly augment the nutritional value of food effectively to address this concern (Yang *et al.* 2012)? A noteworthy obstacle with this strategy is the potential for excess calcium accumulation in vacuoles, potentially leading to symptoms resembling calcium deficiency (Hirschi 1999; Gao *et al.* 2020). In the case of potato, increased CAX expression can lead to the formation of calcium oxalates, acting as 'anti-nutrients' that further hinder calcium bioavailability (Zorrilla *et al.* 2019). If the pursuit of heightened calcium content in crops compromises their yield and results in the creation of anti-nutrients, this approach becomes counter-productive. Moreover, there is a need for more feeding studies involving human subjects on plants displaying modified transporter expression. Considering the relatively modest improvements in calcium levels observed in various crops expressing CAX, doubts arise regarding the widespread adoption of this method (Yang *et al.* 2012). When addressing deficiencies in iron and zinc, where the necessary adjustments are less substantial, utilizing modified transporters and chelators in crops may emerge as a more viable path toward enhancing human nutrition (Connorton & Balk 2019).

In the past 10 years, there has been a transition from assessing the nutritional aspects of CAX expression in crops to a greater emphasis on understanding the effects of manipulating CAXs on crop productivity. In rice, impaired CAX expression damages the structure that bears the rice grains, which are crucial for reproduction (Gan *et al.* 2023). Modifying CAX levels in *B. rapa*, enhances salt tolerance, with potential improvements in alkalinity tolerance linked to specific mutations (Navarro-León *et al.* 2021; Navarro-León *et al.* 2023). In the case of Chinese cabbage (*B. rapa* subsp. *pekinensis*), adjusting CAX expression may lead to tip-burn issues (Cui *et al.* 2023). However, the relationship between CAX expression and tip-burning phenotypes in lettuce still needs to be more clearly defined (Beacham *et al.* 2023).

CONCLUSIONS AND FUTURE PERSPECTIVES

A wealth of plant science research over many decades has uncovered vacuolar Ca^{2+}/H^{+} exchange activity as a critical component of cellular function. This understanding of the importance of CAX-mediated processes continues as we uncover their roles, not just in the model plant *Arabidopsis*, but in agronomically important crop and horticultural plant species. It is becoming clearer that CAX proteins are not just involved in responses to abiotic stresses but also during plant immunity, alongside other components of the Ca^{2+} signalling toolkit. Moreover, the use of genetically encoded Ca^{2+} sensors is confirming that CAX proteins are indeed important for cytosolic Ca^{2+} signal generation, although the exact relevance of these signals still requires further research. It appears that CAX proteins can function as critical cellular hubs through which various environmental and developmental signal pathways can intersect, suggesting that CAXs are key players in the control of environmental resilience. This will become increasingly important in the development of climate resilient crops, either through breeding or genetic engineering, in order to future-proof our food security. It is also particularly noticeable that many tolerance phenotypes, such as tolerance to cold, flooding or resistance to pathogens, are enhanced when CAX genes are mutated, indicating negative regulation. As such, relatively simple gene mutation, rather than transgenic overexpression, can generate these phenotypes in crop lines, potentially avoiding complex regulatory approval.

AUTHOR CONTRIBUTIONS

JKP and KDH conceived and wrote the article.

CONFLICT OF INTEREST

There are no competing interests declared.

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