Review Article
Modulating Plant Calcium for Better Nutrition and Stress Tolerance

Dominique (Niki) Robertson

Department of Plant Biology, North Carolina State University, P.O. Box 7612, Raleigh, NC 27695, USA

Correspondence should be addressed to Dominique (Niki) Robertson; niki@ncsu.edu

Received 10 January 2013; Accepted 2 February 2013

Copyright © 2013 Dominique (Niki) Robertson. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

External Ca\(^{2+}\) supplementation helps plants to recover from stress. This paper considers genetic methods for increasing Ca\(^{2+}\) to augment stress tolerance in plants and to increase their nutritional value. The transport of Ca\(^{2+}\) must be carefully controlled to minimize fluctuations in the cytosol while providing both structural support to new cell walls and membranes, and intracellular stores of Ca\(^{2+}\) for signaling. It is not clear how this is accomplished in meristems, which are remote from active transpiration—the driving force for Ca\(^{2+}\) movement into shoots. Meristems have high levels of calreticulin (CRT), which bind a 50-fold excess of Ca\(^{2+}\) and may facilitate Ca\(^{2+}\) transport between cells across plasmodesmatal ER. Transgenes based on the high-capacity Ca\(^{2+}\)-binding C-domain of CRT1 have increased the total plant Ca\(^{2+}\) by 15%–25% and also increased the abiotic stress tolerance. These results are compared to the overexpression of sCAX1, which not only increased total Ca\(^{2+}\) up to 3-fold but also caused Ca\(^{2+}\) deficiency symptoms. Coexpression of sCAX1 and CRT1 resolved the symptoms and led to high levels of Ca\(^{2+}\) without Ca\(^{2+}\) supplementation. These results imply an important role for ER Ca\(^{2+}\) in stress tolerance and signaling and demonstrate the feasibility of using Ca\(^{2+}\)-modulating proteins to enhance both agronomic and nutritional properties.

1. Introduction

Plants sense and respond to environmental stimuli using networks of sensors, second messengers, kinases, and transcription factors to regulate gene expression and adapt to the new conditions. Ca\(^{2+}\) is perhaps the best-known second messenger but is also required for proper cell wall structure and membrane integrity [1]. Although Ca\(^{2+}\) is present at relatively high concentrations (0.1–80 mM) in cell walls and organelles, cytoplasmic levels of Ca\(^{2+}\) are maintained at ~100 nM [2–4]. Signal transduction in plants requires the ability to mobilize and sequester Ca\(^{2+}\) from both internal and external Ca\(^{2+}\) stores. Because both deficiency and high concentrations of Ca\(^{2+}\) cause localized cell death, the transport of Ca\(^{2+}\) throughout the plant must be tightly regulated [5, 6].

Plants grown under Ca\(^{2+}\) deficient conditions are more susceptible to plant pathogens and show reduced growth of apical meristems, chlorotic leaves, and cell wall breakdown leading to softening of tissues [2]. But adding Ca\(^{2+}\) does more than just alleviate these symptoms, it bolsters plant growth by increasing root length and helps them to withstand or recover from stress [7–14]. Supplemental Ca\(^{2+}\) is also used to improve fruit characteristics and can function to delay ethylene-induced senescence [15]. This information is not new, a report in Science published over 40 years ago described the effect of 1 mM Ca\(^{2+}\) in preventing severe NaCl toxicity in beans [16].

The precise effects of extracellular Ca\(^{2+}\) on a plant system is likely to be complex, because Ca\(^{2+}\) has multiple roles, and because different plants show different responses to supplemental Ca\(^{2+}\). For example, in most plants extracellular Ca\(^{2+}\) reduces Na\(^{+}\) accumulation, which alleviates salt stress. But in some plants (such as maize), Na\(^{+}\) levels remain constant, but a beneficial effect on plant growth is still apparent [17]. Supplemental Ca\(^{2+}\) in a few plants, such as rice, has no apparent effect on salt tolerance [18] (see [19]). Supplementation with K\(^{+}\) has either no effect or is detrimental [20].

In a recent report, a solution of Ca\(^{2+}\) was found to be beneficial when sprayed directly onto the leaves of
drought-stressed tea plants [21]. How does simply spraying Ca\(^{2+}\) onto leaves benefit plants? Why have not plants figured out how to increase their own stores, since Ca\(^{2+}\) is readily available in most environments? Alternatively, is this a part of what makes some plants “weedy”? Is there a barrier to the effective long-distance transport of Ca\(^{2+}\)? Can we engineer a “work-around” or alternative mechanism for Ca\(^{2+}\) transport, to help them recover from stress or even to prevent damage in the first place?

To begin to understand how extracellular Ca\(^{2+}\) benefits plants, it is necessary to understand more about the function and mobility of Ca\(^{2+}\) at both the cellular and the whole plant level. Once we understand the different roles of Ca\(^{2+}\), how Ca\(^{2+}\) is sequestered and transported within the plant, released for cellular signaling—and then rapidly sequestered away from detrimental interactions—then we can begin to think about revising or tailoring some of its pathways. This paper will provide a brief, whole-plant overview of Ca\(^{2+}\)-regulated pathways and functions with the goal of identifying potential strategies for engineering additional Ca\(^{2+}\) ions into soluble plant reserves, so that they are readily available for signaling and growth. It is hoped that this approach can be part of a strategy to design more nutritional crop plants that are also more resilient to stress.

2. Ca\(^{2+}\) Stores and Signaling

It is commonly believed that Ca\(^{2+}\), one of the most abundant minerals in the earth, evolved as a signaling molecule because of the dual needs of the cell for soluble phosphate and Ca\(^{2+}\) and the propensity for the two to precipitate out as an insoluble salt [22]. Phosphate also plays a critical role in signal transduction, but its role as an energy intermediate requires a presence in the cytoplasm [23]. In plants, metabolic pathways that use ATP are found largely in the cytoplasm and are kept separate from Ca\(^{2+}\) stores, which are found primarily in the apoplastic, vacuole, and endoplasmic reticulum (ER) and to a lesser extent in mitochondria, chloroplasts, and the nucleus [4]. In animal cells and early in the plant lineage, the ER was the major source of Ca\(^{2+}\), and its release was controlled by another second messenger, inositol (1,4,5) triphosphate (IP\(_3\)), through activation of ER-localized IP\(_3\) receptors [24, 25]. Similar IP\(_3\) receptors have not been found in plants; however, the phosphoinositide pathway is conserved in plants [26–29]. Members of the phosphoinositide signaling pathway show transcriptional regulation by environmental and developmental stimuli in Arabidopsis [30], and Ca\(^{2+}\) release by IP\(_3\) is conserved [31, 32].

In addition to the apoplast, the ER and vacuoles are the major and metabolically relevant sources of cellular Ca\(^{2+}\) [33–35]. Cytosolic Ca\(^{2+}\) levels fluctuate and are controlled by a system of membrane-localized Ca\(^{2+}\) pumps and Ca\(^{2+}\) channels located in the plasmalemma, vacuole, and ER [4, 5, 36]. The electrochemical potential for Ca\(^{2+}\) to enter the cytoplasm, across the plasma membrane, was calculated by Spalding and Harper to be about $-52 \text{kJ/mol}$ [22]. Therefore, Ca\(^{2+}\) can enter cells passively through ion channels but requires energy to be pumped out of the cytoplasm. Although energetically unfavorable, removal of Ca\(^{2+}\) is rapid and efficient, resulting in 1000-fold and higher [Ca\(^{2+}\)] differences between the cytosol and surrounding organelles and apoplast [37].

Unlike animal systems, mutations in Ca\(^{2+}\) transport proteins often do not produce dramatic phenotypes [22], suggesting that plants are more tolerant of cytosolic Ca\(^{2+}\) or that they have overlapping and redundant systems. This has made it difficult to correlate electrophysiological experiments with genetics to identify exactly which Ca\(^{2+}\) channels function in signaling (or storage) and when. Ca\(^{2+}\) was shown to be released from the ER and possibly other membranes by cADP-ribose, an NAD\(^{+}\) metabolite, similar to what happens in animal cells, over a decade ago [34], however, it now seems clear that cyclic nucleotide-gated channels (CNGC) are found in the plasmalemma [48]. One of the few proteins that do have a phenotype, the phenotype of cngc2, is similar to cax1/cax3 (see Section 3) suggesting that it plays a major role in allowing nonsignaling Ca\(^{2+}\) entry into leaf cells [49]. There are 20 CNGC genes in Arabidopsis and an additional 20 genes that encode glutamate receptor-like channels (GLR), another type of Ca\(^{2+}\) channel found in the plasmalemma [48]. A third type of channel, the two-pore Ca\(^{2+}\) channel (TPC1), was first identified as a plasmalemma protein but is now known to be localized to the tonoplast membrane.

There are two major groups of proteins that function in Ca\(^{2+}\) removal from the cytoplasm [50]. Autoinhibitory Ca\(^{2+}\) ATPase (ACA) uses the energy of ATP to pump Ca\(^{2+}\) out of the cytoplasm and into organelles such as the vacuole and ER. The second group of proteins function as antiporters and are called Cation eXchange proteins (CAX), found on the tonoplast membrane. CAX exchanges two protons for one Ca\(^{2+}\), using the energy of the proton gradient to dampen cytoplasmic Ca\(^{2+}\) signals [51].

2.1. Calcium Signatures. Cytosolic increases in Ca\(^{2+}\) in response to high concentrations of salt were noted at least 25 years ago in plants [52], but the specificity of Ca\(^{2+}\) signaling is still not well understood. There are two nonexclusive models for how Ca\(^{2+}\) functions as a second messenger. The Ca\(^{2+}\) signature model posits that information is encoded in the shape, duration, and frequency of Ca\(^{2+}\) transients and the diversity of cellular Ca\(^{2+}\) stores, all of which may facilitate the formation of microdomains that support and respond to localized Ca\(^{2+}\) changes [4, 53]. These localized changes are specific to the inducing stimulus and result in specific changes to Ca\(^{2+}\)-modulated proteins and their targets [5, 39, 54–56]. A second model suggests that Ca\(^{2+}\) transients function as a simple binary switch, either on or off, and it is the Ca\(^{2+}\) sensor (a Ca\(^{2+}\)-modulated protein) that links different stimuli to the adaptive response [22].

The best-studied examples of Ca\(^{2+}\)-mediated signal transduction include guard cell opening, nodulation, and tip growth of polarized structures such as pollen tubes [57–66]. Specific Ca\(^{2+}\) signatures have also been reported, for example, in response to different chemicals in the root (aluminum,
glutamic acid, and ATP [67]) and, at the whole plant level, in response to ozone [68]. Examples of other stimuli that cause transient increases in cytosolic Ca\textsuperscript{2+} concentrations include touch, cold shock, heat shock, oxidative stress, anoxia, hypoxic shock, salinity, wounding, gravity, and pathogen infection [37, 56, 69–81]. Developmental signals including fertilization, senescence, abscission, and ripening also involve Ca\textsuperscript{2+}-regulated proteins [82–88].

There is evidence for tissue-specific differences in Ca\textsuperscript{2+} flux in response to the same stimulus, for example, salt stress. Salt tolerance is a complex trait involving responses to cellular osmotic and ionic stresses and their consequent secondary stresses (e.g., oxidative stress) [89, 90]. Roots show a biphasic transient increase in cytosolic Ca\textsuperscript{2+} following exposure to acute salt stress [73]. In contrast to cold shock, which is restricted to areas near the root meristem, salt shock increases cytosolic Ca\textsuperscript{2+} along the entire root [91]. To distinguish tissue-specific differences in Ca\textsuperscript{2+} flux, different transgenic plants transformed with a gene encoding aquorin (a reporter gene for Ca\textsuperscript{2+}) targeted to the cytoplasm of the epidermis, endodermis, or pericycle of Arabidopsis roots were used [73]. Prolonged oscillations in aquorin luminescence in the endodermis and pericycle occurred that were distinct from the epidermis [73]. This demonstrated that the same stimulus was transduced differently depending on the cell type, which could be due in part to the evolution of multiple family members in genes that transport Ca\textsuperscript{2+} (Section 2).

2.2. Calcium Sensors. Understanding the transduction of Ca\textsuperscript{2+} signatures has increased in the past decade due to rapid progress in deciphering the cellular network of Ca\textsuperscript{2+}-responsive proteins. There are several families of Ca\textsuperscript{2+}-binding proteins in plants [92–95]. Proteins such as calmodulin, calcineurin B-like proteins (CBLs), and Ca\textsuperscript{2+}-dependent protein kinases (CDPKs) “sense” Ca\textsuperscript{2+}, having one or more EF-hand domains that bind Ca\textsuperscript{2+} with high affinity. The Arabidopsis genome encodes 250 EF-hand-containing proteins [96], although it should be noted that the presence of an EF-hand domain does not necessarily mean that a protein is activated by Ca\textsuperscript{2+} [97]. Calmodulins can interact with transcription factors, directly transducing Ca\textsuperscript{2+} signals into changes in gene expression [98–103]. There is also evidence of Ca\textsuperscript{2+} signals within the nucleus, where CDPKs can phosphorylate and activate transcription factors [104, 105], and in the chloroplast [4, 106]. It is becoming clear that the cellular location of all parts of the signal transduction pathway plays an important role in proper signal transduction [105]. Sensors “relay” information from Ca\textsuperscript{2+} signatures (or the binary switch) into downstream events that include phosphorylation, changes in gene expression and protein–protein interactions [107]. The variety of Ca\textsuperscript{2+} binding proteins in plants suggests that intracellular Ca\textsuperscript{2+} levels, transport, release, and uptake are interdependent and tightly regulated [92].

2.3. CIPK/CBL Network. Batistic and Kudla [23] argue that a new system of Ca\textsuperscript{2+}-regulated proteins has evolved to replace the IP\textsubscript{3} receptor network as plants adapted to life on land. In Arabidopsis this system comprises 10 calcineurin B-like proteins (CBLs), which function as Ca\textsuperscript{2+} sensors, and 26 CBL-interacting protein kinases (CIPKs) [23]. Elegant experiments combining microscopy and biochemistry have been used to decipher the logistics of this pathway [108]. In addition to Ca\textsuperscript{2+} sensing, variations in both the cellular distribution and the interaction partners of members in this pathway contribute to an elaborate system capable of interpreting information from a variety of different stimuli [109]. To date, CBL/CIPK complexes have been shown to participate in the transduction of signals caused by the abiotic stress response, abscisic acid, potassium and nitrate uptake mechanisms, anaerobic response, cold, salt, sugar, cytokinin, and light [44, 47, 74, 110–122].

Kudla’s group has demonstrated that CIPK6/CBL4 interactions can lead to relocation of the K\textsuperscript{+} channel, AKT2, from the ER membrane to the plasmalemma [113]. Two lipid modifications of CBL4, myristoylation and palmitoylation, are required for it to associate with the ER to begin the relocation. CIPK6 serves as a scaffold in this process as phosphorylation of CBL4 is not required [113]. Lipid modifications are also required for CBL1 association with the plasmalemma, where it interacts with CIPK23 to activate a second K\textsuperscript{+} channel, AKT1 [124]. This interaction results in K\textsuperscript{+} uptake under low K\textsuperscript{+} conditions [124] while the CIPK6/CBL4 interaction is needed for normal growth [113].

There is indirect evidence for the role of the CBL/CIPK network in biotic stress as members of this family respond to salicylic acid [125]. CIPK6L was induced by Ca\textsuperscript{2+} in apples, and exogenous Ca\textsuperscript{2+} also induced both CIPK and CBL from pea [45, 125] and a CIPK from rice [122].

The overexpression of different CIPK/CBL proteins involved in abiotic stress has been shown to confer increased drought tolerance (Table 1). In addition to nutrient deprivation and abiotic stress, some CIPK/CBL members target particular developmental pathways during abiotic stress including root growth, pollination, and germination [47, 112, 126]. The impact of ectopic CIPK6 expression on root growth was shown to be mediated through auxin [44, 126]. Although CIPK6 expression was shown to confer tolerance to salt, the positive impact of its overexpression in Arabidopsis and tobacco on root growth suggests that those plants may also do well under water-limiting conditions. This is discussed in more details in Section 6.

2.4. Ca\textsuperscript{2+} Binding Proteins and Modulation of Ca\textsuperscript{2+} Stores. Suberization of the cell walls in the endodermis might prevent apoplastic Ca\textsuperscript{2+} from participating in cytosolic signaling events, because the deposition of the wax onto the cell walls would inhibit Ca\textsuperscript{2+} mobility. White and Knight used this insight to demonstrate that different stimuli do result in the cell accessing different stores of Ca\textsuperscript{2+} [91]. Transgenic plants that expressed apoaequorin only in the endodermis were used, and the root tips, which had different levels of suberization, were examined for luminescence in the presence of luciferin, which is directly proportional to the concentration of Ca\textsuperscript{2+}. While salt stress resulted in the production of
a continuous luminescent Ca\textsuperscript{2+} signal along the endodermis, cooling the roots produced a signal that was confined to a terminal 4-mm region of the root tip, where suberization was incomplete or lacking [91]. This was an elegant demonstration that signal propagation from salt and cooling require access to different Ca\textsuperscript{2+} stores. Moore et al. concluded that cytoplasmic signaling in response to salt stress utilized intracellular stores of Ca\textsuperscript{2+}, although it is still not clear what part of the cell contained the store [91].

2.4.1. The Vacuole as a Ca\textsuperscript{2+} Store. Although a considerable amount of Ca\textsuperscript{2+} is present in the apoplast, the vacuole is the main storage organelle for Ca\textsuperscript{2+} within the plant cell. However, there is little direct evidence for the vacuole as a source of Ca\textsuperscript{2+} for signaling [4, 127, 128], although the identification of Ca\textsuperscript{2+} channels in the tonoplast membrane is not complete either. Furthermore, most of the Ca\textsuperscript{2+} in the vacuole is complexed with chelators such as malate, isocitrate, and citrate and is, therefore, not readily available for signaling [4].

There is evidence for an important role for the vacuole in depleting cytosolic Ca\textsuperscript{2+}, which is critical for preventing association with phosphate and for shaping putative Ca\textsuperscript{2+} signatures. Using mathematical modeling, Bose et al. suggest that the activity of the known major Ca\textsuperscript{2+} efflux proteins (two members, each of the ACA and CAX gene families) is sufficient to describe a wide variety of Ca\textsuperscript{2+} signatures, including all of the current experimental results, without having to take into consideration how Ca\textsuperscript{2+} enters the cytosol [50]. Figure 1 shows a diagram of the major Ca\textsuperscript{2+} efflux proteins in a leaf cell.

Two vacuolar Ca\textsuperscript{2+} ATPases, ACA4 and ACA11, have been shown experimentally to be important for removing excess cytosolic Ca\textsuperscript{2+} [129]. When genes for both of these pumps were mutated, groups of cells in the mesophyll began undergoing programmed cell death (PCD). This phenotype requires salicylic acid, suggesting that the increased cytoplasmic Ca\textsuperscript{2+} by itself was not toxic [129]. It could be that PCD has the lowest threshold for sensing an activating cytoplasmic Ca\textsuperscript{2+} signal. While many stimuli could activate the release of Ca\textsuperscript{2+} into the cytoplasm (light, gravity, etc.), without appropriate dampening by ACAs the signal could spread to other parts of the cell to trigger unintended responses. It will be interesting to know if the propensity for cell death is an indirect effect of altered cytosolic Ca\textsuperscript{2+} on a PCD-related Ca\textsuperscript{2+} sensor, or if ACA4 and ACA11 are specifically involved in PCD.

2.4.2. The ER as a Ca\textsuperscript{2+} Store. The ER also contains high levels of Ca\textsuperscript{2+} and is an attractive candidate for storing signaling Ca\textsuperscript{2+} [130]. Calreticulin (CRT) is an ER luminal chaperone that has two Ca\textsuperscript{2+} binding domains. The P-domain contains a high affinity, EF hand-like structure that binds 1-2 moles of Ca\textsuperscript{2+} per mole protein [131]. The C-domain is the least conserved among organisms but contains a disproportionately high number of acidic amino acid residues that function to bind large amounts of Ca\textsuperscript{2+} with weak affinity. The C-domain has been estimated to bind 30–50 moles of Ca\textsuperscript{2+} per mole of protein [131]. Because of its low affinity, C-domain binding requires a relatively high concentration of Ca\textsuperscript{2+}, such as in the ER. Although estimates are scarce, the concentration in the ER of pollen tubes has been estimated to be ∼100–500 μM; about 1000-fold higher than in the cytoplasm [130]. The ER of animal cells contains ~1 mM Ca\textsuperscript{2+} but the concentration is nonuniform [132]. This is also likely to be true in plants due to the conservation of ER pumps and Ca\textsuperscript{2+} binding proteins such as CRT and Calnexin (CXN) [133]. CXN is a membrane-bound ER protein that functions with CRT and

| Gene   | Source of gene | Target organism | Impact                                           | Reference |
|--------|----------------|-----------------|--------------------------------------------------|-----------|
| AtCBL1 | Arabidopsis    | Arabidopsis     | Reduces transpiration, increases abiotic stress tolerance | [38]      |
| AtCBL1 | Arabidopsis    | Arabidopsis     | Increased salt and drought tolerance, reduced freezing tolerance | [39]      |
| AtCBL2 | Arabidopsis    | Arabidopsis     | Enhanced susceptibility to low K⁺                  | [40]      |
| AtCBL3 | Arabidopsis    | Arabidopsis     | Enhanced susceptibility to low K⁺                  | [40]      |
| ZmCBL4 | Zea mays       | Arabidopsis     | Increased salt tolerance                          | [41]      |
| AtCBL5 | Arabidopsis    | Arabidopsis     | Increased drought tolerance                       | [42]      |
| OsCBL8 | Rice           | Rice            | Increased salt tolerance                          | [43]      |
| CaCIPK6| Chickpea       | Tobacco         | Increased salt tolerance, enhanced root development | [44]      |
| MdCIPK6L| Apple         | Apple, Arabidopsis, tomato | Enhanced tolerance to salt, osmotic, drought and chilling stress; no effect on root growth | [45]      |
| OsCIPK03| Rice          | Rice            | Enhanced tolerance to cold by increased proline and soluble sugars | [46]      |
| AtCIPK9| Arabidopsis    | Arabidopsis     | Enhanced susceptibility to low K⁺                  | [40]      |
| OsCIPK12| Rice          | Rice            | Enhanced tolerance to drought by increased proline and soluble sugars | [46]      |
| OsCIPK15| Rice          | Rice            | Enhanced tolerance to salt                        | [46]      |
| OsCIPK23| Rice          | Rice            | Increased drought tolerance                       | [47]      |
Figure 1: Major Ca\textsuperscript{2+} efflux systems in a leaf cell, and structure of the ER spanning two cells. CAX1 is the major cation exchanger in leaf cells, but CAX3 can compensate if CAX1 activity is compromised. Not shown is a vacuolar proton ATPase that uses ATP to pump protons into the vacuole. The energy from the proton gradient is used to pump Ca\textsuperscript{2+} into the vacuole. There are also two Ca\textsuperscript{2+} pumps on the tonoplast membrane, ACA4 and ACA11. The ER and plasma membrane also have Ca\textsuperscript{2+} pumps (lower right). Ca\textsuperscript{2+} pumps are also found on the nuclear envelope and chloroplast (not shown). The reticulate nature of the ER is modeled next to the plasma membrane but reticulation (and the ER) is found throughout the cell. Cortical ER is found near the cell wall and is less dynamic than ER in the interior. A desmotubule spans a single plasmodesma between the upper cell and a partial cell on the bottom, but of course there are multiple plasmodesmatal connections between most cells (except guard cells and between the epidermis and mesophyll). Both the cytosol and the ER lumen are continuous across the plasmodesmata.

BiP (another chaperone) in glycosylation and quality control of ER proteins [133, 134].

Most plants have two forms of CRT [135, 136]. In Arabidopsis, CRT1\textsubscript{a} and CRT1\textsubscript{b} (also called CRT2) have the highest homology and form the first group, while CRT3, which is specifically needed for viral cell-to-cell movement [137], is in the second group. All three CRTs function as chaperones and play an important role in protein folding and glycosylation [136, 138–141]. The C-domain of CRT3 is reduced in size compared to CRT1\textsubscript{a} and CRT1\textsubscript{b}, but was specifically required for proper folding of the brassinosteroid receptor, BRII [142]. All three CRTs have been implicated in innate immunity for proper folding of different receptor proteins [143–148], and CRT1 appears to participate in signaling [149]. CRT has also been associated with increased tolerance to abiotic stress [147, 150].

CRT is highly expressed in meristematic and reproductive tissues. It shows lower expression associated with vascular tissue. CRT1 and CRT2 are largely coexpressed, except that CRT2 is high in senescing leaves, perhaps as a mechanism for retrieving Ca\textsuperscript{2+}. CRT2 also shows guard cell-specific expression.

The ER also contains at least one Ca\textsuperscript{2+} ATPase, ACA2, that is activated by calmodulin and inhibited by a CDPK [151, 152]. Inhibition of an ER-type Ca\textsuperscript{2+} ATPase (ECA1) in pollen tubes decreased ER Ca\textsuperscript{2+} and inhibited pollen tube growth suggesting that the ER serves as a Ca\textsuperscript{2+} store for signaling [130]. In addition, mutants with 4-fold lower ECA1 activity showed poor growth on medium with low Ca\textsuperscript{2+} (0.2 mM versus 1.5 mM, normal) [153]. It is not clear why ECA1 is needed to pump Ca\textsuperscript{2+} into the ER under low Ca\textsuperscript{2+} conditions.

In animal cells, Ca\textsuperscript{2+} is constantly leaking out of the ER and constantly being pumped back in by SERCA, membrane pumps that are similar to ECA [132]. But the major mechanism for ensuring adequate ER levels of Ca\textsuperscript{2+} is a specialized plasma membrane pump that responds only to low ER Ca\textsuperscript{2+}. In a mechanism called store-operated Ca\textsuperscript{2+} entry [154], the pump (Orai) forms a structure adjacent to an ER protein (STIM1) that contains an EF hand to sense ER Ca\textsuperscript{2+} levels. Together, they allow ER Ca\textsuperscript{2+} levels to be refilled [132]. It is not known if a similar mechanism could function in plants.

2.5. Ca\textsuperscript{2+} Transduction and Regulation of Gene Expression. How many genes and proteins are associated with Ca\textsuperscript{2+} regulation? In addition to the ~250 EF-hand containing proteins, ~700 are thought to be involved with Ca\textsuperscript{2+} signaling for Arabidopsis, according to proteomic data [155]. These proteins generate Ca\textsuperscript{2+} signatures and transduce the signal into changes in protein phosphorylation, protein localization, protein-protein interactions, and changes in gene expression. It is the latter that is most difficult to identify due to
the difficulty in testing Ca$^{2+}$ without other secondary effects that result when stimuli such as NaCl are used that also cause chemical and ionic perturbations of the system. Knight’s group addressed this by using an applied voltage to alter membrane permeability in combination with transgenic aequorin to monitor changes in cytoplasmic Ca$^{2+}$ levels [156]. Conditions for a transient increase in cytoplasmic Ca$^{2+}$ from less than 100 nM to almost 600 nM were established, and microarrays were used to profile genetic changes. A combination of transient and oscillating Ca$^{2+}$ fluxes produced the greatest number of genes (269) with increased expression levels, while a single long increase in Ca$^{2+}$ to 200 nM produced only 10 genes with increased expression.

Analysis of the promoter regions of the Ca$^{2+}$-upregulated genes revealed a surprising bias for genes that respond to abiotic stress. Three out of the four Ca$^{2+}$-regulated promoter motifs were previously identified as being important for abiotic stress responses and included the ABA-response element and the drought-responsive element [156]. This bias could be due to the nature of the Ca$^{2+}$ flux, which may have resembled signatures produced from an apoplastic source of Ca$^{2+}$, or could be a feature of Ca$^{2+}$ regulation.

In addition to the cytoplasm, transient Ca$^{2+}$ fluctuations have also been reported in the nucleus, chloroplast, mitochondrion, and peroxisome [4]. Ca$^{2+}$ oscillations in the cytosol and chloroplast have been linked to circadian rhythms [32, 157]. It is not known whether these fluctuations also lead to changes in gene expression.

We used a genetic method to specifically increase Ca$^{2+}$ in the ER by taking advantage of the high capacity, low affinity Ca$^{2+}$ binding activity of the C-domain from CRT. A green fluorescent protein-calcium binding peptide (GFP-CBP) fusion protein consisting of the C-domain from Zea mays CRT1 was fused to the C-terminal region of GFP [158]. The GFP-CBP construct included a signal protein for ER-targeting and the C-terminal region of CRT1, which contains an HDEL sequence for ER retention. Total Ca$^{2+}$ in seedling shoots was increased by ~25%, when GFP-CBP was expressed in Arabidopsis using a constitutive promoter. Microarray analysis of seedlings expressing GFP-CBP compared to seedlings expressing GFP showed that 31 genes were upregulated by >3.5-fold. As expected, none of these genes included the cytosolic Ca$^{2+}$-regulated genes identified by Whalley et al. Only one of the genes was involved in Ca$^{2+}$ regulation—CIPK6 [158]. Whalley et al. also identified a single CIPK, CIPK9 [156]. The other genes we found were enriched for microsome-associated proteins and glycinin-rich proteins, which are often targeted to the cell wall [158]. One of the proteins encoded a subunit of the anaphase-promoting complex [158]. This expression pattern could indicate a regulatory role for ER Ca$^{2+}$ levels in mitosis. We will come back to this in Section 5.2.

Of course steady-state modulation of Ca$^{2+}$ levels in an organelle is quite different from generating a cytosolic Ca$^{2+}$ signal. According to the eFP browser [159], CIPK6 is induced by salt, drought, and abscisic acid and is expressed at a low level in guard cells, leaves, flowers, and developing fruit and seed. Although some of the genes coexpressed with CIPK6 in the GFP-CBP plants showed similar expression profiles to CIPK6, there is nothing to suggest a connection with ER Ca$^{2+}$.

2.6. Summary of Cellular Ca$^{2+}$ Dynamics. Cells contain stores of Ca$^{2+}$ in the apoplast and in various compartments within the cell. Cytoplasmic Ca$^{2+}$ is kept low to prevent interference with phosphate-containing pathways. Signal transduction uses discrete Ca$^{2+}$ fluxes to connect stimuli with adaptive responses. Different stores of Ca$^{2+}$ are used in the generation of these fluxes and the location, magnitude, and duration of the fluxes appear to contain information for the appropriate response. Vacular pumps and antiporters participate in removing Ca$^{2+}$ from the cytoplasm before deleterious interactions occur. It has been difficult to determine which intracellular stores participate in different kinds of signaling, but the ER is an attractive candidate because of its distribution throughout the cell, and the ability of CRT to bind large quantities of Ca$^{2+}$ with low affinity.

We still need more information on the plant’s ability to generate stimulus-specific Ca$^{2+}$ signatures. What is the source of the Ca$^{2+}$ used for different signals? What dampens the signature? How is information about the signal (magnitude, oscillations, and duration) transduced into specific responses? With respect to the original question—what, exactly, could the presence of supplemental Ca$^{2+}$ contribute to increase stress tolerance? Are certain Ca$^{2+}$ stores normally limited, or does spraying Ca$^{2+}$ onto a plant trigger oscillations as Ca$^{2+}$ is assimilated? Understanding Ca$^{2+}$-regulated networks is plagued by the ubiquity of the molecule, and dissecting pathways in different cells and tissues is still tedious and difficult. However, the combination of biochemistry, Ca$^{2+}$ reporter genes, and genetics is providing tremendous information that is building a solid foundation for understanding Ca$^{2+}$ regulation.

The next section begins to discuss tissue-specific differences in Ca$^{2+}$ levels to better understand how exogenous Ca$^{2+}$ is assimilated.

3. Calcium Distribution within the Leaf

Eating roots and leaves is the best way for vegans (people who do not eat meat, fish, or dairy products) to increase Ca$^{2+}$ intake [160]. This makes sense because Ca$^{2+}$ is transported from roots to shoots through transpiration, and leaves carry out the bulk of transpiration. But not all cells within a leaf have equivalent Ca$^{2+}$ levels. In grasses, Ca$^{2+}$ is found mainly in the upper epidermis [161]. In dicots, Ca$^{2+}$ levels are low in both upper and lower epidermis, but are higher in mesophyll, a distribution that facilitates Ca$^{2+}$ control over stomatal aperture [161, 162].

A landmark study looked at the distribution of Ca$^{2+}$ in different cell types of the leaf and found that mesophyll cells have ~6-fold more Ca$^{2+}$ than epidermal cells, due largely to the differential expression of CAXI in those cells [162]. CAXI is located on the tonoplast membrane and couples proton export with Ca$^{2+}$ transport into the vacuole.
cax1/3 double mutants not only had reduced growth, reduced photosynthesis, and thicker cell walls, but also had higher apoplastic levels of Ca$^{2+}$ [162]. This resulted in reduced stomatal apertures, which led to reduced growth due to a lack of carbon assimilation compared to nonmutant lines [162]. Although the cell walls were thicker, they were also more brittle and contained more pectin. Supplementation with low Ca$^{2+}$ media reduced free apoplastic Ca$^{2+}$ levels and suppressed the phenotype, while returning the plants to normal Ca$^{2+}$ caused the phenotype to return. Free Ca$^{2+}$ (sorbitol-exchangeable) was ~3-fold higher in the apoplast of cax1/3 double mutants compared to the nonmutant line. In fact, CAX1, CAX3, CAX4, and ACA4 (encoding a Ca$^{2+}$ ATPase) and ACA11 are coregulated to make sure total Ca$^{2+}$ levels are constant [162].

Why was high apoplastic Ca$^{2+}$ a problem? Guard cells use Ca$^{2+}$ to signal downstream components to close or open stomata. In the presence of excess Ca$^{2+}$, stomata remain closed even under conditions favorable for gas exchange and carbon fixation. The exact mechanism for how extracellular Ca$^{2+}$ interferes with guard cell signaling is not known. As mentioned in Section 2, the electrochemical gradient for Ca$^{2+}$ across the cell membrane strongly favors passive Ca$^{2+}$ entry—it is the removal of Ca$^{2+}$ from the cytoplasm that requires energy. Thus, the presence of high levels of free Ca$^{2+}$ on the other side of the plasmalemma may either make it difficult to remove Ca$^{2+}$ from the cytoplasm or make it too easy for Ca$^{2+}$ to enter it. Extracellular Ca$^{2+}$ has been shown to cause guard cells to close by generating H$_2$O$_2$ and NO, which generate an intracellular Ca$^{2+}$ spike, leading to stomatal closure [63].

Thus, keeping free Ca$^{2+}$ out of the apoplast enables proper guard cell function and allows normal plant growth. CAX1 keeps apoplastic Ca$^{2+}$ low by storing it in the vacuole [162]. Rather than viewing the apoplast as a separate entity that protected plant cells from extracellular threats, it now seems important to acknowledge that unbound extracellular Ca$^{2+}$ must be maintained in equilibrium across the apoplast/symplast boundary. At least in leaves, it is the vacuole, a membrane-bound organelle on the symplastic side of the divide, not the cell wall, that serves as the reservoir for excess accumulation of Ca$^{2+}$.

Where does Ca$^{2+}$ come from? In leaf cells, Ca$^{2+}$ is transported through the xylem by transpiration [2]. Ca$^{2+}$ is one of the most immobile ions in the plant, with Mg$^{2+}$ and Mn$^{2+}$ not far behind [2]. In the leaf, Ca$^{2+}$ is thought to diffuse through the apoplasm up to about 15 cells away from the xylem. Transpiration would seem to direct Ca$^{2+}$ to guard cells, which are mostly on the lower side of leaves, but the pattern of veins, anatomy of the leaf, and presence of air spaces all help to dissipate the pattern of water flow [163].

The pattern of Ca$^{2+}$ transport is thought to vary with the developmental stage of the leaf, the species, and environmental conditions [163]. In eudicots, Ca$^{2+}$ is trapped in the vacuoles of mesophyll cells by CAX1 [163], while in monocots higher relative levels of Ca$^{2+}$ are found in the epidermis [2, 123]. Root pressure can contribute to the transport of Ca$^{2+}$, especially when humidity is high and transpiration low [164]. Ca$^{2+}$ deficiency is first noticed as tip burn, and diseases such as blossom end rot in tomato are a visual demonstration of the limited mobility of Ca$^{2+}$. Since leaves develop acropetally, the apex is the last to differentiate. This suggests that dividing cells may be particularly vulnerable to Ca$^{2+}$ depletion. We will come back to this in Section 5.

### 4. Ca$^{2+}$ Is Transported from the Roots to the Shoot by Transpiration through the Xylem

There could be three points of control for transpiration—uptake in the root apoplast, entry into the xylem across the endodermis, and exit through guard cells. The apoplast shows very little electrical resistance and allows the free exchange of most ions. Ca$^{2+}$ is absorbed from the soil by the apoplast and by cation channels in the root epidermis [165]. The extent of symplastic transport of Ca$^{2+}$ between cells is not known, although a cadmium resistant channel was recently identified that facilitates radial movement of Ca$^{2+}$ in roots [166].

Two pathways for Ca$^{2+}$ transport to the shoot can be experimentally tested, a symplastic or cell-to-cell pathway and an apoplastic pathway. The symplastic pathway involves passage through at least one membrane. The Casparian strip of the endodermis, which contains suberin, restricts solute passage through the apoplast, and promotes passage through the symplastic pathway. Studies with radio-labeled Ca$^{2+}$ suggest that this pathway predominates in onion [6]. Identification of enhanced suberin (esb) mutants in Arabidopsis allowed the role of the endodermis to be directly tested [167]. Shoot Ca$^{2+}$ levels decreased ~50% compared to wild type. If there was no change, it could be concluded that transport was entirely apoplastic or entirely symplastic. So the reduction in Ca$^{2+}$ transport suggests that restriction by the Casparian strip of the endodermis is incomplete—some apoplastic flow is permitted through the Casparian strip in its wild type state. There was no change in Mg$^{2+}$ in the esb mutants, which is also transported through the phloem, but Zn$^{2+}$ and Mn$^{2+}$ also decreased [167]. Surprisingly, accumulation of the monovalent ions Na$^+$, S$^+$, and K$^+$ increased. Transpiration was also decreased and the plants were less susceptible to wilting.

The existence of the apoplastic pathway was demonstrated from experiments that showed that the ratios of Ca$^{2+}$, Br$^{-}$, and Sr$^{2+}$ do not change after they are applied to roots, although channels and pumps have a clear preference for Ca$^{2+}$ [168]. In many plants, the amount of Ca$^{2+}$ transported depends on the rate of transpiration, which is consistent with solvent drag, not symplastic processes [168]. In some plants under certain conditions, Ca$^{2+}$ transport may be almost entirely apoplastic with channels at the destination cell controlling cellular Ca$^{2+}$ entry, followed by rapid assimilation into different organelles by pumps and antiporters. Ca$^{2+}$ transport through the endodermal cytosol in the symplastic pathway is thought to be achieved using Ca$^{2+}$ channels and pumps, but must be carefully regulated to avoid interfering...
with signaling pathways. According to White, apoplastic transport may be necessary to meet the demand for adequate Ca\(^{2+}\) in the shoot [168]. Breaks in the endodermis, for example where lateral roots emerge, allow Ca\(^{2+}\) transport without an intervening sympodial step.

Transpiration is considered to be the driving force for Ca\(^{2+}\) transport into shoots and leaves, and Ca\(^{2+}\) travels with the bulk water flow [2, 6, 163, 169, 170]. The pattern of Ca\(^{2+}\) deficiency symptoms can be explained by a combination between demand for Ca\(^{2+}\) and variation in transpiration. Tip burn, which affects the leaf margin and the undeveloped distal region of the leaf, is thought to result from a lack of well-developed veins in the undifferentiated part of the leaf and high rates of cell wall deposition.

Recent experiments actually compared the shoot accumulation of several minerals in members of 7 different plant families grown together under different fertilizer regimes [123]. The correlation with phylogeny (versus fertilizer treatment or residual) was the strongest for Ca\(^{2+}\) (70%) and total Ca\(^{2+}\) varied over 5-fold (Table 2). In contrast, Mg (with a 32.8% correlation with phylogeny) showed little more than a 2-fold variation. Dicotyledonous plants are known to accumulate more Ca\(^{2+}\) than monocots, partly as a function of the structure of their cell walls, and there only was ~3-fold variation in Ca\(^{2+}\) in different dicot families (Table 2). To put this in perspective, there was a ~2-fold variation in Ca\(^{2+}\) among Arabidopsis ecotypes, which are all members of the same species [171]. The molecular basis for the difference in Ca\(^{2+}\) levels between different families is not known, but the data suggest that factors are at play that ultimately limit the amount of Ca\(^{2+}\) absorbed from the soil.

The endodermis clearly has a role in regulating water transport, and likely helps the plant to conserve water by preventing unrestricted transpiration. Gilliham et al. argue that Ca\(^{2+}\) transport and transpiration are linked—Ca\(^{2+}\) regulates both stomatal activity in leaves and aquaporin (water channel) density and function in roots [163]. Thus, Ca\(^{2+}\) could increase its own transport by affecting aquaporin function [14]. Global mechanisms such as this may also play a role in limiting the amount of Ca\(^{2+}\) that ultimately reaches the shoot. In support of this, the overexpression of an aquaporin in Arabidopsis increased Ca\(^{2+}\) levels by ~33% under normal conditions and almost doubled Ca\(^{2+}\) under 100 mM NaCl [172]. The regulation of hydraulic conductivity (aquaporin function) under stress is reviewed by Aroca et al. [173].

A second mechanism for Ca\(^{2+}\) regulation of Ca\(^{2+}\) leaf concentration has been proposed [174]. A plasma membrane-localized Calcium Sensing receptor, CAS, is upregulated in guard cells. High levels of apoplastic Ca\(^{2+}\) cause stomata to close, a process that requires CAS. When transpiration levels are high, Ca\(^{2+}\) has the potential to be too high. CAS mutants grown in soil had ~40% more Ca\(^{2+}\) than wild type plants [174]. Together with the aquaporin overexpression [172], this suggests that global regulation of Ca\(^{2+}\) levels occurs primarily through mechanisms found in the shoot, not through the endodermis in the root.

5. An ER Ca\(^{2+}\) Network for Meristems

Meristems are critically important for plant growth and reproduction. Meristems require high amounts of Ca\(^{2+}\) because of cell wall deposition and organelle biogenesis, but it is not clear how Ca\(^{2+}\) moves from areas with high rates of transpiration (leaves) into the protected region of the meristem (Figure 2). An alternative mechanism for Ca\(^{2+}\) transport is through the endoplasmic reticulum (ER). The ER is contiguous with the nuclear envelope and forms a symplastic continuum throughout the plant by spanning cell walls through plasmodesmata. Consistent with the idea of CRT as a Ca\(^{2+}\) transporter/regulator, high levels of CRT are found in plasmodesmata [175, 176] and in meristems [177]. This may be especially important in meristems, where the need for Ca\(^{2+}\) is high due to the formation of new cell walls, but the ability to transpire Ca\(^{2+}\) is limited by the lack of differentiated xylem. Transport through the ER would avoid the problem of cytoplasmic transit disrupting signaling pathways and could either augment apoplastic transport to ensure the protection of developing areas of the plant or bypass it, depending on where Ca\(^{2+}\) enters the ER.

If the ER functions in Ca\(^{2+}\) transport, why has not this been detected in leaves? A key aspect of the proposed Ca\(^{2+}\) network in meristems is the presence of CRT, whose gene shows high expression in meristematic tissues [177]. As described in Section 2.4.2, CRT has three conserved domains, one of which binds 30–50 Ca\(^{2+}\) ions with low affinity (the C-domain). CRT may function in intercellular Ca\(^{2+}\) distribution by acting as a buffer, partly neutralizing the charge. CRT is further proposed here to act as a sort of matrix to facilitate Ca\(^{2+}\) absorption and movement by the cell and to provide a gradient for additional Ca\(^{2+}\) to be transported cell-to-cell from mature tissues. But because CRT is not expressed at high levels in mature leaves, Ca\(^{2+}\) transport appears to follow a bulk flow pattern of distribution with the rate of transpiration dictating where it accumulates.

5.1. Desmotubules Allow Movement through the Plasmodesmata

Cytosplasmic Ca\(^{2+}\) transients have been demonstrated to result in rapid closure of plasmodesmata [178]. The biggest obstacle to Ca\(^{2+}\) transport through an ER network is the plasmodesmata. Plasmodesmata consist of a central desmotubule (see Figure 1), which is derived from the compaction of the two sides of the ER tubule that traverses the cell wall. A thin

| family             | Ca\(^{2+}\)  | Mg\(^{2+}\) |
|--------------------|-------------|------------|
| Plantaginaceae (48)* | 17.38 (3.59)* | 1.69 (0.13) |
| Polygonaceae (6)    | 5.90 (1.23) | 2.87 (0.41) |
| Poaceae (6)         | 3.33 (0.25) | 1.33 (0.07) |

*Number in parenthesis is n.
**Data are the average value of the mineral in mg/g dry weight, with SE in parenthesis.
cytoplasmic sleeve that lies between the desmotubule and the plasma membrane serves as the conduit for cytoplasmic proteins and solutes that show intercellular trafficking [175].

CRT has been localized to plasmodesmata [175, 176] and could serve as a Ca\(^{2+}\) donor to maintain an internal network of stored Ca\(^{2+}\). High concentrations of CRT on either side of the plasmodesmata may result in a Ca\(^{2+}\) gradient, which could facilitate the distribution of Ca\(^{2+}\) to adjacent cells. Any cytoplasmic Ca\(^{2+}\) transients would occur independently of luminal concentrations [178].

Despite the narrow aperture of the desmotubule, transit of fluorescent molecules across the desmotubule appeared to be rapid. Microinjection studies were used to study the spread of the small molecular weight fluorescent tracers carboxyfluorescein and FITC-conjugated trinitramic acid in epidermal cells of tobacco and Torenia [179]. About 10% of the injections resulted in a punctate pattern of label that corresponded to the pattern obtained with DiOC\(_6\), a fluorescent dye that labels ER. This was explained by insertion of the needle into the lumen of the ER. In each case, the fluorescent molecules rapidly spread into adjacent cells through the desmotubule of the plasmodesmata. Spread of the fluorescence was more rapid through the desmotubule than through the cytoplasmic sleeve of the plasmodesmata and occurred more readily (100% of the cases versus ~88% for injections into the cytoplasm) [179].

Fluorescent dextran corresponding to 10 kDa showed luminal transport in Torenia in 3 out of 3 injections. This demonstrates that sufficient space exists within the desmotubule for cell-to-cell Ca\(^{2+}\) transport. Although movement of ER-targeted GFP through the desmotubule was not demonstrated, Martens et al. discuss the possibility of the desmotubule functioning both as a conduit for cell-to-cell Ca\(^{2+}\) transport and as a mechanism for whole-plant signaling [180].

GFP fusions have also been used to study intercellular trafficking in leaf epidermal cells following microinjection. A CRT-GFP fusion protein in the ER lumen did not traffic into adjacent cells, but calnexin-GFP, an ER membrane-localized protein, did spread cell to cell [181]. CXN also binds Ca\(^{2+}\) and functions with CRT as a protein chaperone. It contains an N-terminal Ca\(^{2+}\)-binding domain on the luminal side and an acidic tail of ~90 amino acids. These characteristics could enable it to transport Ca\(^{2+}\) across the plasmodesmata.

Why would transport through the desmotubule be needed? Plasmodesmata are regulated by Ca\(^{2+}\). When a cold shock was used to increase cytoplasmic Ca\(^{2+}\) from 100 to 200 mM, there was a 4-fold increase in resistance, but the resistance returned to normal within 10 sec [182]. Thus, cytoplasmic Ca\(^{2+}\) transients would be expected to close plasmodesmata. By compartmentalizing Ca\(^{2+}\) away from the cytoplasm, it could equilibrate between cells at levels that would interfere with plasmodesmata function if it were on the cytosolic side of the plasmodesmata.

5.2. Ca\(^{2+}\) and Cell Division. Vascular tissue forms de novo and differentiates acropetally (phloem) and basipetally (xylem) in developing leaves after they have begun to expand and differentiate. The leaf midvein does not connect to the stem until after xylem and phloem have differentiated, and the leaves have begun to actively photosynthesize. The high rates of cell division in developing leaf primordia require significant amounts of Ca\(^{2+}\) to bind to cell wall pectin, stabilize the plasma membrane, and ensure completion of mitosis.

Ca\(^{2+}\) plays a major role in mitosis at anaphase, where it concentrates at the spindle poles at levels that cause microtubule depolymerization [183]. Interestingly, two proteins, one of them a CRT-like protein, have been identified in plants that could facilitate this process. Tonsoku (TSK) localized to the nucleoplasm while tonsoku-associated protein (TSA) has a signal peptide and was found in cytoplasmic vesicles derived from the ER [184]. During anaphase, the two proteins colocalized and appeared to interact. TSA has 10 repeats of an EFE motif consisting of acidic amino acids and was shown to bind Ca\(^{2+}\) in vitro. Although there was no homology with CRT, it may have a very similar function—to provide a matrix for storing Ca\(^{2+}\) until it is needed. Although a function has not been reported for these proteins, other than to bind Ca\(^{2+}\) and colocalize, it seems possible that they would be needed, along with kinesins [185], for depolymerization of microtubules during anaphase.

When plant cells enter prophase, the nuclear envelope (which is contiguous with the ER) disintegrates into vesicles. Following anaphase, a new cell wall is deposited, which requires vesicle secretion and membrane fusion. The ER is well positioned to provide Ca\(^{2+}\) during this process, which would be needed for stabilizing the developing cell wall by binding to pectate. The dynamic nature of cell-to-cell movement through desmotubules could ensure that the ER
has a ready supply of Ca\textsuperscript{2+} available for the new cell wall that could be delivered through the vesicles.

\textit{CRT} is known to be expressed at high levels in dividing cells, but the reason for this has not been obvious. Clearly, there is a higher need for glycosylated proteins as new cells are formed, but it appears to play more than a structural role, as overexpression of \textit{CRT} has been shown to increase regeneration [186]. One possibility is that cell division, and possibly regeneration, may have become linked to the expression of \textit{CRT}, such that if ER Ca\textsuperscript{2+} levels were not adequate to support new growth, the process of cell division would arrest. Our microarray results provide some tantalizing evidence in favor of this hypothesis. We found that GFP-CBP caused a 3.7-fold increase in the expression of At5g26635, which encodes one subunit of a putative anaphase-promoting complex. However, this is probably too late in the cell cycle to arrest development. A more likely explanation is that ER Ca\textsuperscript{2+} levels need to be high enough to facilitate the depolymerization of microtubules.

Nevertheless, the relationship between ER Ca\textsuperscript{2+}, \textit{CRT} expression, and mitotic activity would be interesting to study—to determine why “meristem burn” is not a problem, for example. It would also be interesting to examine \textit{CRT} expression in tomato, since it suffers from the occurrence of blossom end rot, discussed in Section 6. Blossom end rot is a Ca\textsuperscript{2+}-related disorder that results in tissue softening and necrosis at the distal end of the tomato fruit, which contains the highest proportion of dividing cells. Tomato is not the only fruit to undergo extensive cell division during fruit development (papayas, watermelons, and jack fruit are also quite large); what makes it more susceptible?

\subsection*{5.3. Summary.} In summary, a gradient of Ca\textsuperscript{2+} ions in the ER is proposed to help plant guard its vulnerable meristem from fluctuations in the transpiration of Ca\textsuperscript{2+}. \textit{CRT} networks in the ER could provide a conduit for Ca\textsuperscript{2+} transport to ensure that adequate levels of Ca\textsuperscript{2+} reach the meristem to support growth. \textit{CRT} could serve as a buffer to help neutralize charge and to draw Ca\textsuperscript{2+} towards the meristem. Cell division may be coupled to \textit{CRT} expression to ensure that adequate levels of Ca\textsuperscript{2+} are present when the cell divides. Transport through the ER would avoid competition with the vacuole and protect the cytoplasm while ensuring that enough Ca\textsuperscript{2+} is transported to meet the demands of the cell wall and organelles.

\section{Genetic Manipulation of Ca\textsuperscript{2+} Stores}

Many postmenopausal women take supplemental Ca\textsuperscript{2+} to help prevent osteoporosis, a crippling disease related to aging. With the demographics of most developed countries showing a rise in the aging population, the impact of nutrient deficiencies on human health is likely to increase. Many people do not like to take Ca\textsuperscript{2+} in the form of a pill because of its large size, which is needed due to the relatively poor absorption of chemical Ca\textsuperscript{2+}. The best way to obtain more nutrients is to consume more fruits and vegetables, especially roots and leaves for Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, and K\textsuperscript{+} [160, 187]. Unfortunately, almost 10\% of the adult population of the USA and UK are deficient for those three elements [160], due, in part, to consumption of cereal grains rather than vegetables (although breakfast cereals are often sprayed with supplemental Ca\textsuperscript{2+}). Although other countries who rely on rice as a major staple face a similar problem, they are much more likely to combine it with vegetables, if they have the money. Thus, Ca\textsuperscript{2+} deficiencies are more of a problem in developed countries, which are also more likely to have an aging population. Since fortifying plants with supplemental Ca\textsuperscript{2+} increases their tolerance to stress, it would be prudent to consider the genetic alteration of Ca\textsuperscript{2+} stores with transgenes that benefit consumers as well as farmers.

Ca\textsuperscript{2+} levels show a high degree of heritability but vary from species to species. Ca\textsuperscript{2+} distribution was shown to vary 2-fold among Arabidopsis ecotypes and was correlated with Mg\textsuperscript{2+} in all tissues except seeds, [171]. In general, there is more Ca\textsuperscript{2+} in shoots than in roots, and the distribution within leaves is nonuniform. In grasses, Ca\textsuperscript{2+} is found only in the upper epidermis. In dicots, Ca\textsuperscript{2+} levels are low in both upper and lower epidermis, but are higher in mesophyll, a distribution that facilitates Ca\textsuperscript{2+} control over stomatal aperture. Much of the variation in Ca\textsuperscript{2+} levels could be traced to the expression of \textit{CAX1} [162]. So far, Ca\textsuperscript{2+} levels have been altered by mutation or overexpression in the vacuole, ER, and apoplast using two proteins—\textit{CAX1} and a derivative of \textit{CRT}. This section will describe these results and examine their collateral impact on abiotic stress responses and make some recommendations for future experiments.

\subsection*{6.1. Transgenic Expression of CAX Family Members.} The protein family with the best potential for increasing bioavailable Ca\textsuperscript{2+} in plants is \textit{CAX}, located on the tonoplast membrane [188]. As previously mentioned, \textit{CAX} expression levels are the primary determinant for Ca\textsuperscript{2+} levels in Arabidopsis [162, 189]. In leaves, \textit{CAX} functions to clear free Ca\textsuperscript{2+} from the apoplast, so that guard cell signaling, which requires extracellular Ca\textsuperscript{2+}, can be regulated properly (Section 3). In \textit{sCAX1}, the N-terminal autoinhibitory loop has been removed so that it can transport increased amounts of Ca\textsuperscript{2+} into the vacuole [190].

Ectopic \textit{sCAX1} expression increased Ca\textsuperscript{2+} in potato tubers by 2-3 folds with no change in morphology or yield when supplemented with 2 mM CaCl\textsubscript{2} during the first 3 months [191]. However, \textit{CAX} transports other cations in addition to Ca\textsuperscript{2+}, which are not as beneficial from a nutritional standpoint. Hirsch’s group, therefore, modified the \textit{CAX2} gene, which shows a greater specificity for Ca\textsuperscript{2+}, to eliminate its Mn\textsuperscript{2+} transport function and then showed 55-60\% increase in Ca\textsuperscript{2+} in transgenic potatoes [192].

Tomato was transformed with \textit{CAX4}, which is more specific for Ca\textsuperscript{2+} than \textit{CAX1} [193]. This resulted in a 40\% increase in total Ca\textsuperscript{2+} and was not associated with Ca\textsuperscript{2+} deficiency symptoms even in the absence of CaCl\textsubscript{2} supplementation. \textit{CAX4} increased fruit firmness (and, therefore, postharvest life), but did not impact ethylene production or sugar content [193]. In addition, root growth was enhanced [193]. Later experiments in Arabidopsis demonstrated that \textit{CAX4}
expression, which is uniquely confined to roots, is needed for normal root growth and that cax4 mutants had reduced DR5:GUS expression [194]. DR5 is a synthetic promoter that responds to auxin. The authors postulate that cytosolic Ca\(^{2+}\) levels may have increased, due to altered CAX4-mediated efflux into the vacuole. This may have affected polar transport of auxin, which is regulated by CDPKs [194]. The impact of CAX genes on root growth is important and deserves further study. Although CAX1 and CAX4 are thought to act primarily by depleting cytosolic Ca\(^{2+}\), roots of CAX transformants were less sensitive to inhibition by applied auxin than the wild type [195], while roots of CAX4 transformants were more sensitive [194]. It would be very interesting to know how these alterations in root phenotype affect tolerance to abiotic stress.

There are no deleterious effects of sCAX1 expression under normal conditions if supplemental Ca\(^{2+}\) is added. Otherwise, Ca\(^{2+}\) deficiency symptoms result, which include increased sensitivity to salt and cold stress. The yeast 2-hybrid experiments demonstrated that CAX1 interacts with SOS2, a CIPK that usually requires SOS3 (a CBL) for activity, through its N-terminal domain [196]. This may help to deplete the cytosol of excess Ca\(^{2+}\), following salt stress, which is known to produce a transient increase in Ca\(^{2+}\). Overexpression of sCAX1 increased the plant's sensitivity to salt, perhaps by being too efficient in the removal of excess Ca\(^{2+}\), leading to store depletion [196]. The impact of drought and osmotic stress on sCAX1 overexpression has not been studied, but would be expected to show similar responses (enhanced sensitivity). In contrast to sCAX1 transformants, mutants of CAX3 show increased salt sensitivity [197]. Both decreased Ca\(^{2+}\) transport into the vacuole during salt stress and decreased H\(^+\) ATPase activities at the plasma membrane were associated with the cax3 mutation.

Overexpression of CAX1 also resulted in increased sensitivity to salt while mutations in this gene produce salt and drought tolerant plants [195]. Interestingly, exogenous Ca\(^{2+}\) can reverse salt sensitivity in CAX transgenic plants and can also reverse the salt tolerance of cax1 mutants [195]. CAX1 may be involved in sequestering Ca\(^{2+}\) to the vacuole following release into the cytoplasm. If Ca\(^{2+}\) signals cannot be dampened by transport into the vacuole, cytosolic levels may remain high, activating salt tolerance pathways. Conversely, if Ca\(^{2+}\) is sequestered into the vacuole at a faster rate than normal (as in the CAX1 over-expressors), cytosolic levels may never reach the threshold required to activate pathways for salt tolerance. cax1 mutants showed developmental abnormalities including reduced root growth and delayed flowering [195].

Ectopic expression of sCAX1 in tobacco was also associated with increased sensitivity to cold shock [188]. This correlated with the positive impact of mutations in cax1 on cold tolerance [51]. The negative impact of CAX1 on cold tolerance was shown to be due to decreased upregulation (relative to wild type) of DREBI and a subset of cold-responsive genes induced by DREBI [51]. These results are interesting because they are the first to demonstrate altered gene expression by CAX1, although the signal transduction pathway has yet to be demonstrated. Although DREBI was upregulated by cold in the cax1 mutants, there were no changes in gene expression associated with exposure to dehydration or salt [51].

There are also beneficial effects of ectopic CAX expression. Both CAX1 and CAX4 expressions have been associated with enhanced tolerance to heavy metals [194, 198–201]. The potential impacts on other traits are difficult to assess. CAX1 and CAX3 have been shown to regulate phosphate homeostasis by repressing phosphate starvation-associated genes [202]. A cax1/3 double mutant resulted in increased shoot phosphorous accumulation [202]. Grafting experiments suggested that CAX1 and CAX3 could be involved in the generation of a shoot to root signal that represses phosphate transport [202], but the impact of sCAX1 over-expressing plants on phosphate transport has not been determined.

Unfortunately, sCAX1 expression has not contributed towards mitigation of Ca\(^{2+}\) deficiency diseases. Massive cell death is associated with Ca\(^{2+}\) deficiency resulting, for example, in fruit that is not suitable for consumption. Tomato fruit development is especially susceptible to cell death (blossom end rot) caused by Ca\(^{2+}\) deficiency [203] a situation aggravated by increased salinity [204]. Blossom end rot in tomato is known to be related to Ca\(^{2+}\) deficiency [205]. Instead of helping to prevent blossom end rot, sCAX1 expression resulted in 100% of the tomato fruits developing the disorder [206]. This may have been due to reduced free Ca\(^{2+}\) in the apoplast, where it likely helps to stabilize membrane structure, among other things. Ca\(^{2+}\) deficiency near the plasma membrane causes destabilization, which could precipitate the disorder [206]. Although sCAX1 expression may make Ca\(^{2+}\) more bioavailable to humans, it does not appear to have the same effect in plants.

In contrast to Arabidopsis, over expression of soybean CAX1 homolog in Arabidopsis increased salt tolerance [207]. GmCAX1 has a N-terminal autoinhibitory loop, also found in AtCAX1, but shows only 65% homology to it and 68% homology to CAX2 [207]. In contrast to Arabidopsis, GmCAX1 was not induced by cold suggesting that the regulation and function of different CAX homologs may show considerable variation across species [207]. It is not clear what this means for predicting the impact of overexpression of sCAX1 in other species. As acknowledged [197], it may be difficult to predict the effects of overexpression of a major transporter on the phenotype of any plant.

6.2. CRT and CBP. CRT is a multifunctional protein that is highly conserved in eukaryotic cells [208–210]. It has at least three functional domains: a globular N-domain, a proline rich, high affinity (K_d = 1.6 \mu M), low capacity (B_{max} = 1 mol/mol of protein) Ca\(^{2+}\)-binding domain (the P-domain), and a highly acidic, low affinity (K_d = 0.3–2 mM), high capacity (B_{max} = 20–50 mol/mol of protein) Ca\(^{2+}\)-binding domain (the C-domain) [211]. In animals, CRT has been suggested to be involved in Ca\(^{2+}\) signaling [212, 213], chaperone activity [211], cell adhesion [214], gene expression [215], apoptosis [216], and in controlling store-operated fluxes through the plasma membrane [217–219]. Overexpression of CRT in both plants [220] and animals [221] increases total ER Ca\(^{2+}\) stores.
We found that ectopic expression of the maize CRT1 or a Ca\(^{2+}\)-Binding Peptide (CBP) consisting of only the CRT C-domain can not only increase Ca\(^{2+}\) stores, but also enhance the survival of Arabidopsis plants grown in low Ca\(^{2+}\) medium [222, 223], suggesting that the extra Ca\(^{2+}\) could be used by the plant in times of stress. The hypothesis guiding this research is that the CBP sequesters Ca\(^{2+}\) in the ER in a manner similar to CRT. However, Ca\(^{2+}\) may bind the CBP protein in the ER, but then travel as a complex through the secretory system to the vacuole, cytoplasm, or even the nucleus [224]. It is highly unlikely that Ca\(^{2+}\) will be bound by ER-CBP in the cytoplasm, because of its low affinity. It is, therefore, reasonable to use the ER-CBP as a tool for altering intracellular stores of Ca\(^{2+}\).

Our previous work demonstrated that intracellular Ca\(^{2+}\) levels could be manipulated in Arabidopsis by heat shock induction of an ER-targeted GFP-CBP peptide constructed by translationally fusing the green fluorescent protein gene to a sequence corresponding to 126 amino acids derived from the maize calreticulin C-domain [223]. ER-CBP plants induced on Ca\(^{2+}\)-containing medium survived longer than similarly heat-shocked ER-GFP control plants when transferred to Ca\(^{2+}\)-depleted medium [223]. This work suggested that the ER capacity for Ca\(^{2+}\) could be directly related to a physiological response, early senescence in the absence of Ca\(^{2+}\). Importantly, ER Ca\(^{2+}\) could be modulated without the addition of external Ca\(^{2+}\) and deleterious effects due to Ca\(^{2+}\) depletion were not apparent. To further examine physiological differences in these plants and to avoid the complications of heat shock induction, we transformed Arabidopsis with the same GFP-CBP construct (or CBP without GFP, for indole-1 experiments) but under the control of the constitutive 35S cauliflower mosaic virus promoter.

Why not over-express CRT to increase Ca\(^{2+}\)? Overexpression of ZmCRT1 in tobacco cells increased Ca\(^{2+}\) by 2-fold, and transformation of Arabidopsis with ZmCRT1 reduced the rate of senescence following transfer to low Ca\(^{2+}\) media [222]. There are two potential problems with over-expressing full-length CRT, silencing of the endogenous gene, and deleterious effects under some conditions. Overexpression of CRT2 resulted in the production of dwarfed plants, caused by high levels of salicylic acid [145]. Although overexpression of Chinese cabbage CRT1 enhanced shoot and root regeneration in tobacco, the subsequent growth of tobacco plants was retarded [225]. CRTI overexpression was also shown to be deleterious in rice [186].

My group initially used a soybean heat shock promoter to drive the expression of a maize CRT1 C-domain, which we called CBP for Ca\(^{2+}\) binding peptide, fused to GFP to stabilize it. This turned out to be unnecessary although it was very useful for detecting gene silencing. Nevertheless, we were able to increase Ca\(^{2+}\) in heat-shocked plants by ~15%. Now we know that total Ca\(^{2+}\) levels can be increased by ~25% using constitutively expressed ER-localized CBP [158]. Arabidopsis plants transformed with 35S : CBP showed better salt and drought tolerance and had longer roots, even in the absence of stress [158]. There were no detectable differences in GFP-CBP plants compared to GFP or control plants under normal conditions except that seed production was slightly higher and seedling root growth was increased [158].

Preliminary experiments using both cytoplasmic aequorin-expressing plants and indole-1 ratio imaging suggested that there were no significant differences in [Ca\(^{2+}\)]\(_{cyt}\) concentrations between 35S : CBP-expressing Arabidopsis and wild type or 35S : GFP control plants [226]. However, after 4-5 days growth in Ca\(^{2+}\)-deficient media, the peak [Ca\(^{2+}\)]\(_{cyt}\) in control plants was significantly lower than in CBP-expressing plants in response to a 150–300 mM NaCl challenge [226]. This suggested that expression of CBP allowed plants to respond to stimuli over a longer period of time due to the excess ER-localized reserves of Ca\(^{2+}\). This was a very interesting result that could provide a mechanism for how CBP benefits plants with respect to stress tolerance.

Microarray results of 35S : GFP-CBP compared to 35S : GFP plants showed that genes for endomembrane and cell wall-associated proteins were upregulated [158]. One Ca\(^{2+}\)-regulated gene was strongly upregulated (greater than 3.5-fold), CIPK6. As described in Section 2.3, CIPK6 is a protein kinase that interacts with a Ca\(^{2+}\) sensor protein, CBL. Mutants in ArcIPK6 are sensitive to salt [44], and overexpression of a constitutively active mutant of ArcIPK6 in tobacco confers salt tolerance and also increases root length [44, 126], which are both found in CBP-expressing plants. We, therefore, asked if the enhanced salt tolerance was due to co-expression of CIPK6. When CBP was crossed with a cipk6 knockout mutant (50% reduction in mRNA) and then challenged with NaCl, it showed the same response as wild type plants. This was somewhat disappointing, as we believed that CBP would enhance stress tolerance by providing a Ca\(^{2+}\) reserve. Of course the induction of CIPK6 may have been caused by the presence of additional ER Ca\(^{2+}\); but the eradication of the response by a single mutation was surprising. It remains possible that there is an extra advantage of CBP expression in drought tolerance or under different conditions. The cipk6 mutant has been complemented with a CIPK6 transgene (D. Chattopadhyay, pers. Comm.).

CBP-expressing plants also downregulate CIPK23, which is also involved in salt tolerance, by 2-fold [158]. We believe this is why the CBPxcipk6 plants showed a similar response to NaCl as the controls, despite the presence of ~50% CIPK6 in the knockout mutant.

How does CIPK6 enhance salt tolerance? Recent experiments from Kudla’s group have shown that CIPK6 interacts with AKT2, a K\(^{+}\) channel [113]. Interaction occurs on the ER membrane, although both proteins are translated in the cytosol. CIPK6 interacts specifically with CBL4, which was originally identified as an SOS (salt overly sensitive) mutant [227–229]. When CBL4 is modified by both myristoylation and palmitoylation, the AKT2/CIPK6/CBL4 complex moves from the ER membrane to the plasma membrane, where AKT2 participates as a K\(^{+}\) channel. Mutations in CIPK6, AKT2, and CBL4 confer similar phenotypes when grown under short days, reduced leaf number and size and delayed flowering [113]. K\(^{+}\) is needed for phloem transport, and the reduced size of the mutant plants is restored under long
day conditions [113]. This phenotype is consistent with a reduction in phloem transport, but does not provide an explanation for the altered response by cipk6 to NaCl.

The role of AKT1, which is modulated by CIPK23/CBL1/CBL9 in a similar manner as AKT2, was recently called into question. Mutants defective in akt1 or cipk23 showed better drought tolerance than wild type plants, suggesting that CIPK23/CBL1/CBL9 regulation of AKT1 may actually decrease abiotic stress tolerance [230]. However, overexpression of CBL1 and CIPK23 has been shown to increase tolerance to abiotic stress [39, 47]. Clearly, more experiments are needed to understand the relationship between K⁺ and abiotic stress.

In addition to Arabidopsis, CBP has been transformed into potato and rice ([231], S. Y. Lee, R. Qu, and D. Robertson, in preparation). The goal for CBP expression in potato was to prevent internal heat necrosis (INH), a disorder affecting the quality of potato tubers [232]. There is strong but indirect evidence for an involvement of Ca²⁺ in this disorder. The application of antitranspirants to potato leaves reduced total Ca²⁺ levels and increased Ca²⁺ in tubers. This led to a decreased incidence of the disorder. However, when 3 independent transgenic potato lines (cv. Atlantic) expressing a 35S:CBP gene were grown under greenhouse conditions, the incidence of INH correlated positively with expression of 35S:CBP, which also increased potato tuber yield and total Ca²⁺ in leaves [231]. It was not possible to measure Ca²⁺ in tubers. There were also increased levels of Mg²⁺ and Mn²⁺ in the CBP-expressing plants, and reduced levels of K⁺ [231]. Although the increased yield was statistically significant, the experiment would need to be repeated. It is not known if it was the increased yield that was responsible for greater incidence of INH, but it is unlikely that it could be separated from the expression of CBP.

It would be interesting to know if CBP expression in other plants (besides Arabidopsis) causes an increase in CIPK6 orthologs, and these experiments are currently in progress for rice (Lee, Qu, and Robertson, unpublished). Does the induction of CIPK6 depend on a flux or an increase of ER Ca²⁺? Could this result from ACA and ECA activity in removing Ca²⁺ from the cytosol? Confocal microscopy of the GFP-CBP fusion protein showed ER and, to a lesser extent, nuclear activity [158]. Although CBP would not be expected to bind Ca²⁺ in the cytosol, it could bind Ca²⁺ in the nucleus. Acidic domains can act as transcriptional coactivators [233], providing a possible mechanism for CBP action. These results illustrate the difficulty of using genetic methods to modulate specific stores of Ca²⁺. Although targeting of CBP to the nucleus could be used as a control, the molecular weight of CBP is estimated to be ∼5 kDa so it should enter the nucleus without a targeting sequence.

6.3. Coexpression of sCAX1 and CRT1. 100% of tomato plants expressing sCAX1 developed blossom end rot, a Ca²⁺ deficiency related disorder that leads to necrosis in the distal, developing end of the fruit [206]. These plants were grown in a greenhouse under conditions where none of the nontransgenic control plants developed the syndrome. The expression of sCAX1 was shown to reduce apoplastic Ca²⁺ levels, which increased membrane leakiness [234]. Coexpression of CRT resulted in a significant decrease in Ca²⁺ deficiency symptoms in both tomato and tobacco without the addition of supplemental Ca²⁺ [235]. This is very interesting and, if it can be repeated in other species, may suggest several things about the ER and vacuole with respect to signaling. Questions that this observation raises include the following:

1. How is the Ca²⁺ level in the shoot increased, without an increase in transpiration? (This is relevant to all sCAX-expressing plants.)
2. Does the ER form a symplastic Ca²⁺ network distinct from the apoplast and vacuole?
3. Is CRT needed to keep a bioavailable pool of Ca²⁺ inside the ER for signaling? If so, then extra CRT may have successfully competed with CAX1 for the limited pool of free Ca²⁺ in the apoplast in the dual transgenic plants.
4. Can the vacuole serve as the source of Ca²⁺ for some stimuli?
5. What would CRT overexpression in a cax1/3 mutant do? Could it help to bind excess apoplastic Ca²⁺?

6.4. Other Transgenes for Manipulating Ca²⁺ Stores in Plants. Several Ca²⁺-related proteins have the potential to serve as a mechanism for altering Ca²⁺ stores in plants. Theoretically, any part of the cell except the cytoplasm could sustain increased levels of Ca²⁺ without deleterious consequences, although this needs to be experimentally verified. As a group, plants vary in Ca²⁺ content and show differential sensitivity to Ca²⁺ as a nutrient [236]. Since we know there is variation in Ca²⁺ levels between plants, even between ecotypes of Arabidopsis (and that variation correlates with CAXI expression [189]), we should be able to genetically manipulate it.

One of the benefits of large-scale scientific experiments (“omics”) is the availability of data for gene expression and ion concentrations for a variety of closely related plants. Arabidopsis ecotypes have been collected from around the world, and there are hundreds of accessions, each of which shows less genetic variation than would be found between two species, but together there is a large pool of variation that can be correlated with a variety of different phenotypes. The leaf ionome of 31 of these accessions has now been completed, and Conn and his colleagues have outlined methods for using this data to identify candidate genes controlling elemental accumulation [237]. This promises to be a very productive avenue of research, especially if some of the candidates can be correlated with positive agronomic properties.

In addition to proteins found in Arabidopsis, there are other Ca²⁺ binding proteins that have been identified in various species. Examples include a celery vacuole-associated dehydrin-like protein [238] and a radish vacuolar Ca²⁺ binding protein [239] that is induced by lack of Ca²⁺. Neither of these proteins has mutants nor has been overexpressed, so it is not clear how much Ca²⁺ can be increased by using them.
Recently, TPC1, the slow vacuolar channel found in all plants, has been shown to contain a novel Ca\(^{2+}\) binding site that senses Ca\(^{2+}\) and alters its activity. Mutants have been created that are insensitive to feedback inhibition by luminal Ca\(^{2+}\), which leads to an increase in the store of vacuolar Ca\(^{2+}\) [240].

Simply adding Ca\(^{2+}\) to fertilizers can increase leaf Ca\(^{2+}\) levels by up to 3-fold [241], and there is an argument that transgene manipulation may be unnecessary as breeding for increased Ca\(^{2+}\) levels should be sufficient to meet nutritional requirements for Ca\(^{2+}\). There are two arguments against this notion: adding Ca\(^{2+}\) to the right compartment has the potential to boost the resiliency of plants to stress and providing Ca\(^{2+}\) loosely complexed to protein might result in enhanced nutritional absorption. Since overexpression of CRT can be detrimental to plant growth [145, 225], transgenic approaches that separate out the C-domain are the most straightforward approach to boosting ER Ca\(^{2+}\).

### 6.5. Biofortification Studies

The potential role for CAX in biofortification has been demonstrated in carrots expressing sCAX1 [242]. Human consumption of the genetically engineered carrots resulted in a 41% increase in Ca\(^{2+}\) absorption compared to controls, demonstrating the bioavailability of vacuumal Ca\(^{2+}\) in this system [242]. Lettuce was also transformed with sCAX1 and contained 25%-32% more Ca\(^{2+}\) than controls [243]. The response of a human panel to the engineered lettuce was positive for its sensory characteristics [243]. As long as sCAX1-expressing plants have good agronomic properties and can be grown in the presence of excess Ca\(^{2+}\) or cotransformed with CRT (or, better, CBP), this is a very promising method for biofortification.

The absorption of Ca\(^{2+}\) from vegetables can be complicated by the presence of “antinutrients” such as oxalic acid, which forms insoluble Ca\(^{2+}\) oxalate crystals. As long as the diet is varied, it should not have a significant impact. Antinutrients are more important when choosing a plant for transgenic modification. These requirements are fulfilled in carrots, a good choice for one of the first plants to be transformed for increased Ca\(^{2+}\) absorption [242].

It has never been tested in clinical trials, but the delivery of Ca\(^{2+}\) ions complexed with protein, such as found in the ER in the form of the C-domain of CRT (CBP), could increase the absorption rate of Ca\(^{2+}\). Although the use of sCAX1 to increase vacuolar Ca\(^{2+}\) has achieved remarkable increases in Ca\(^{2+}\) absorption on a per gram basis [242], the overall efficiency of Ca\(^{2+}\) absorption was 10% less than for controls. The reason for this is not clear, unless the level of antinutrients increased (which would be important to know). Comparing the efficiency of absorption between CBP transgenic and sCAX1 transgenic carrots could help to determine if Ca\(^{2+}\) absorption efficiency decreases as its concentration increases, or whether the cellular context of the extra Ca\(^{2+}\) plays a role in absorption. In the long run, it will be important to be able to use Ca\(^{2+}\) as efficiently as possible. Since CBP and the combination of CBP and sCAX1 lead to higher total Ca\(^{2+}\) levels without external supplementation, the added nutritional benefit may not require supplemental Ca\(^{2+}\) to be added.

Because the CRT C-domain is not highly conserved, it should be possible to choose sequences that retain a high number of acidic amino acids, which are known to bind Ca\(^{2+}\), without causing silencing of the endogenous CRT genes. The potential for CBP expression alone to increase Ca\(^{2+}\) absorption from food should be tested, because Ca\(^{2+}\) loosely bound to a protein may be even more bioavailable than Ca\(^{2+}\) salts in the vacuole. CBP has not been associated with Ca\(^{2+}\) deficiency symptoms under normal or stress conditions in the laboratory. It would be interesting to compare Ca\(^{2+}\) uptake from sCAX-expressing plants to those expressing CBP, along with a combination of the two transgenes. The long-term goal for sustainable agriculture should be to maximize the efficiency of Ca\(^{2+}\) supplementation in the human diet, so that the effective use of Ca\(^{2+}\) as a fertilizer can be maximized.

### 6.6. Summary

Transgenic expression of sCAX1 or CAX4 may be the best way to increase vegetative sources of Ca\(^{2+}\) but this can require supplementation with CaCl\(_2\). When coexpressed with CRT1, the need for Ca\(^{2+}\) supplementation appears to be reduced, but more studies are needed to determine the effect of two Ca\(^{2+}\) binding transgenes on agronomic properties, because CRT1 overexpression by itself can have deleterious effects on plant growth under certain conditions.

Transgenic expression of CBP also increases total Ca\(^{2+}\) but not by as much as CAX1. This may be a better transgene to co-express with CAX1 than CRT1 because it retains Ca\(^{2+}\) binding but lacks most of the functions of CRT1. CBP expression by itself increases root growth under nonstress conditions and reduces the effects of drought and salt stress, perhaps in part by increasing root growth but we think also by providing a more extensive store of bioavailable Ca\(^{2+}\).

### 7. Conclusion

As described in the beginning, many studies show that Ca\(^{2+}\) applied externally can benefit plants by increasing stress tolerance. Even postharvest fruit characteristics are improved following a CaCl\(_2\) soak. It is still not known where in the plant this supplemental Ca\(^{2+}\) is absorbed and distributed, or how it is used to benefit the plant. How much is actually necessary for the enhanced growth and stress responses? Is it the change in Ca\(^{2+}\) concentration or the absolute amount of Ca\(^{2+}\) available to the plant that is relevant?

One explanation for the beneficial response is that Ca\(^{2+}\) induces genes involved in abiotic stress tolerance, such as members of the CIPK/CBL family, some of which are known to be induced by exogenous Ca\(^{2+}\) [45, 125]. But rather than overexpressing Ca\(^{2+}\)-regulated genes, it may be more beneficial to increase the Ca\(^{2+}\) stores that are used to cause their induction. Finding ways to genetically increase Ca\(^{2+}\) levels in plants may allow us to capture the Ca\(^{2+}\)-stimulated enhancement under normal conditions or with minimal Ca\(^{2+}\) supplementation. Additional research on targeting Ca\(^{2+}\)
binding proteins to various organelles may, therefore, be useful.

More robust signaling pathways and stress responses would seem to be a good thing in the face of global climate change. By increasing just the second messenger, one could conceivably preserve the ability of the plant to adapt to different stresses. By increasing the degree of stress response, but not the specific pathway, plants may be better able to deploy valuable reserves into tolerating a wide variety of different stresses. This would make the ubiquity of Ca\(^{2+}\) an asset rather than an impediment to research. The more we understand about Ca\(^{2+}\)-regulated pathways, the more we can optimize the response to adverse conditions. One thing is clear, more exploratory research on the ectopic expression of Ca\(^{2+}\) binding or exchange proteins could be very promising for plants, agronomists, and consumers.

Acknowledgments

The author would like to thank Dr. Sang Yoon Lee for his dedication and initiative in working on the CBP project and for many interesting discussions. Drs. Pei-Lan Tsou and Sarah Wyatt started this work, and it was Dr. Wendy Boss’s idea to use the CRT C-domain as a transgene for manipulating ER Ca\(^{2+}\). I would also like to thank Dr. George Allen for his critical comments and encouragement and Dr. Steven Nagar for Figure 2. The CBP project was originally funded by NASA.

References

[1] F. J. Maathuis, “Physiological functions of mineral macronutrients,” Current Opinion in Plant Biology, vol. 12, no. 3, pp. 250–258, 2009.

[2] P. J. White and M. R. Broadley, "Calcium in plants," Annals of Botany, vol. 92, no. 4, pp. 487–511, 2003.

[3] K. D. Hirschi, “The calcium conundrum. Both versatile nutrient and specific signal,” Plant Physiology, vol. 136, no. 1, pp. 2348–2424, 2002.

[4] S. Stael, B. Wurzinger, A. Mair, N. Mehler, U. C. Vothknecht, and M. Teige, "Plant organellar calcium signalling: an emerging field," Journal of Experimental Botany, vol. 63, pp. 1525–1542, 2012.

[5] C. K. Y. Ng and M. R. Macintosh, "Encoding specificity in plant calcium signalling: hot-spotting the ups and downs and waves," Annals of Botany, vol. 92, no. 4, pp. 477–485, 2003.

[6] E. Cholewa and C. A. Peterson, “Evidence for synplastic involvement in the radial movement of calcium in onion roots,” Plant Physiology, vol. 134, no. 4, pp. 1793–1802, 2004.

[7] H. Upadhayaya, B. K. Dutta, L. Sahoo, and S. K. Panda, "Comparative Effect of Ca, K, Mn and B on Post-Drought Stress Recovery in Tea (Camellia sinensis (L.) O. Kuntze)," American Journal of Plant Sciences, vol. 3, no. 4, pp. 443–460, 2012.

[8] T. Jiang, X. Zhan, Y. Xu, L. Zhou, and L. Zong, "Roles of calcium in stress-tolerance of plants and its ecological significance," Chinese Journal of Applied Ecology, vol. 16, no. 5, pp. 971–976, 2005.

[9] C. A. Jaleel, P. Manivannan, B. Sankar et al., "Water deficit stress mitigation by calcium chloride in Catharanthus roseus: effects on oxidative stress, proline metabolism and indole alkaloid accumulation," Colloids and Surfaces B, vol. 60, no. 1, pp. 110–116, 2007.

[10] H. Nayyar and S. K. Kaushal, “Alleviation of negative effects of water stress in two contrasting wheat genotypes by calcium and asbiscic acid,” Biologia Plantarum, vol. 45, no. 1, pp. 65–70, 2002.

[11] S. M. Juice, T. J. Fahey, T. G. Siccama et al., “Response of sugar maple to calcium addition to northern hardwood forest,” Ecology, vol. 87, no. 5, pp. 1267–1280, 2006.

[12] C. Sulochana and N. Sanithramma, “Effect of Calcium in Amelioration of PEG, (600) Induced Water Stress in Ground Nut (Arachis hypogaea L.) Cultivars during Seedling Growth,” Journal of Plant Biology, vol. 38, 2001.

[13] Y. E. Kolupaev, G. E. Akinina, and A. V. Mokrousov, "Induction of heat tolerance in wheat coleoptiles by calcium ions and its relation to oxidative stress," Russian Journal of Plant Physiology, vol. 52, no. 2, pp. 199–204, 2005.

[14] Y. Wu, X. Liu, W. Wang, S. Zhang, and B. Xu, “Calcium regulates the cell-to-cell water flow pathway in maize roots during variable water conditions,” Plant Physiology and Biochemistry, vol. 58, pp. 212–219, 2012.

[15] M. S. Aghdam, M. B. Hassanpouraghdam, G. Paliyath, and B. Farmani, “The language of calcium in postharvest life of fruits, vegetables and flowers,” Scientia Horticulturae, vol. 144, pp. 102–115, 2012.

[16] P. A. Lahaye and E. Epstein, “Salt tolerance by plants: enhancement with calcium,” Science, vol. 166, no. 3903, pp. 395–396, 1969.

[17] P. A. Essah, R. Davenport, and M. Tester, “Sodium influx and accumulation in Arabidopsis,” Plant Physiology, vol. 133, no. 1, pp. 307–318, 2003.

[18] C. M. Grieve and H. Fujiyama, “The response of two rice cultivars to external Na/Ca ratio,” Plant and Soil, vol. 103, no. 2, pp. 245–250, 1987.

[19] L. Shanyun, L. Yongchao, G. Zhenfei, L. Baosheng, and L. Mingqi, “Enhancement of drought resistance of rice seedlings by calcium,” Zhongguo Shuidao Kejixue, vol. 13, pp. 161–164, 1999.

[20] Z. Rengel, “The role of calcium in salt toxicity,” Plant, Cell and Environment, vol. 15, pp. 625–632, 1992.

[21] H. Upadhyaya, S. K. Panda, and B. K. Dutta, “CaCl\(_2\) improves post-drought recovery potential in Camellia sinensis (L) O. Kuntze,” Plant Cell Reports, vol. 30, no. 4, pp. 495–503, 2011.

[22] E. P. Spalding and J. F. Harper, “The ins and outs of cellular Ca\(^{2+}\) transport,” Current Opinion in Plant Biology, vol. 14, no. 6, pp. 715–720, 2011.

[23] O. Batistic and J. Kudla, “Analysis of calcium signaling pathways in plants,” Biochimica et Biophysica Acta, vol. 8, pp. 1283–1293, 1820.

[24] A. M. Cameron, J. P. Steiner, A. J. Roskams, S. M. Ali, G. V. Ronnett, and S. H. Snyder, “Calcineurin associated with the inositol 1,4,5-trisphosphate receptor- FKBP12 complex modulates Ca\(^{2+}\) flux,” Cell, vol. 83, no. 3, pp. 463–472, 1995.

[25] M. D. Sjaastad, R. S. Lewis, and W. J. Nelson, “Mechanisms of integrin-mediated calcium signaling in MDCK cells: regulation of adhesion by IP3- and store-independent calcium influx,” Molecular Biology of the Cell, vol. 7, no. 7, pp. 1025–1041, 1996.

[26] I. Y. Perera, I. Heilmann, and W. F. Boss, “Transient and sustained increases in inositol 1,4,5-trisphosphate precede the differential growth response in gravistimulated maize pulvini,” Proceedings of the National Academy of Sciences of the United States of America, vol. 96, no. 10, pp. 5838–5843, 1999.
[27] J. M. Stevenson, I. Y. Perera, I. Heilmann, S. Persson, and W. F. Boss, "Inositol signaling and plant growth," *Trends in Plant Science*, vol. 5, no. 6, pp. 252–258, 2000.

[28] R. Zhong, D. H. Burk, W. H. Morrison, and Z. H. Ye, “FRAG-ILE FIBER3, an Arabidopsis gene encoding a type II inositol polyphosphate 5-phosphatase, is required for secondary wall synthesis and actin organization in fiber cells,” *Plant Cell*, vol. 16, no. 12, pp. 3242–3259, 2004.

[29] F. M. Carland and T. Nelson, "Cotyledon Vascular Pattern-mediated inositol (1,4,5) triphosphate signal transduction is essential for closed venation patterns of Arabidopsis folar organs," *Plant Cell*, vol. 16, no. 5, pp. 1263–1275, 2004.

[30] W. H. Lin, R. Ye, H. Ma, Z. H. Xu, and H. W. Xue, "DNA chip-based expression profile analysis indicates involvement of the phosphatidylinositol signaling pathway in multiple plant responses to hormone and abiotic treatments," *Cell Research*, vol. 14, no. 1, pp. 34–45, 2004.

[31] I. Y. Perera, C. Y. Hung, C. D. Moore, J. Stevenson-Paulik, and W. F. Boss, "Transgenic Arabidopsis plants expressing the type I inositol 5-phosphatase exhibit increased drought tolerance and altered abscisic acid signaling," *Plant Cell*, vol. 20, no. 10, pp. 2876–2893, 2008.

[32] R. H. Tang, S. Han, H. Zheng et al., "Coupling diurnal cytosolic Ca" oscillations to the CAS-IP 3 pathway in Arabidopsis," *Science*, vol. 315, no. 5817, pp. 1423–1426, 2007.

[33] J. Groenendyk, J. Lynch, and M. Michalak, "Calreticulin, Ca"", and calcinurin—signaling from the endoplasmic reticulum, *Molecules and Cells*, vol. 17, no. 3, pp. 383–389, 2004.

[34] L. Navazio, P. Mariani, and D. Sanders, "Mobilization of Ca"" by cyclic ADP-ribose from the endoplasmic reticulum of cauliflower florets," *Plant Physiology*, vol. 125, no. 4, pp. 2129–2138, 2001.

[35] G. Mailhot, J. L. Petit, C. Demers, and M. Gascon-Barre, "Influence of the in vivo calcium status on cellular calcium homeostasis and the level of the calcium-binding protein calreticulin in rat hepatocytes," *Endocrinology*, vol. 141, pp. 891–900, 2000.

[36] T. Yang and B. W. Poovaiya, "Calcium/calmodulin-mediated signal network in plants," *Trends in Plant Science*, vol. 8, no. 10, pp. 505–512, 2003.

[37] D. Sanders, J. Pelloux, C. Brownlee, and J. F. Harper, "Calcium at the crossroads of signaling," *Plant Cell*, vol. 14, no. supplement 1, pp. S401–S417, 2002.

[38] V. Albrecht, S. Weinl, D. Blazevic et al., "The calcium sensor CBL1 integrates plant responses to abiotic stresses," *The Plant Journal*, vol. 36, no. 4, pp. 457–470, 2003.

[39] Y. H. Cheong, K. N. Kim, G. K. Pandey, R. Gupta, J. J. Grant, and S. Luan, "CBL1, a calcium sensor that differentially regulates salt, drought, and cold responses in Arabidopsis," *Plant Cell*, vol. 15, no. 8, pp. 1833–1845, 2003.

[40] L. L. Liu, H. M. Ren, L. Q. Chen, Y. Wang, and W. H. Wu, "A protein kinase CIPK9 interacts with calcium sensor CBL3 and regulates K' homeostasis under low-KK stress in Arabidopsis," *Plant Physiology*, vol. 161, pp. 266–277, 2013.

[41] M. Wang, D. Gu, T. Liu et al., "Overexpression of a putative maize calcineurin B-like protein in Arabidopsis confers salt tolerance," *Plant Molecular Biology*, vol. 65, no. 6, pp. 733–746, 2007.

[42] Y. H. Cheong, S. J. Sung, B. G. Kim et al., " Constitutive overexpression of the calcium sensor CBL5 confers osmotic or drought stress tolerance in Arabidopsis," *Molecules and Cells*, vol. 29, no. 2, pp. 159–160, 2010.

[43] Z. Gu, B. Ma, Y. Jiang, Z. Chen, X. Su, and H. Zhang, "Expression analysis of the calcineurin B-like gene family in rice (Oryza sativa L.) under environmental stresses," *Gene*, vol. 415, no. 1-2, pp. 1–12, 2008.

[44] V. Tripathi, B. Parasuraman, A. Laxmi, and D. Chattopadhyay, "CIPK6, a CBL-interacting protein kinase is required for development and salt tolerance in plants," *The Plant Journal*, vol. 58, no. 5, pp. 778–790, 2009.

[45] R. K. Wang, L. L. Li, Z. H. Cao et al., " Molecular cloning and functional characterization of a novel apple MdCIPK6L gene reveals its involvement in multiple abiotic stress tolerance in transgenic plants," *Plant Molecular Biology*, vol. 79, pp. 123–135, 2012.

[46] Y. Xiang, Y. Huang, and L. Xiong, "Characterization of stress-responsive CIPK genes in rice for stress tolerance improvement," *Plant Physiology*, vol. 144, no. 3, pp. 1416–1428, 2007.

[47] W. Yang, Z. Kong, E. Omo-Ikerodah, W. Xu, Q. Li, and Y. Xue, "Calcineurin B-like interacting protein kinase OsCIPK23 functions in pollination and drought stress responses in rice (Oryza sativa L.)," *Journal of Genetics and Genomics*, vol. 35, no. 9, pp. 531.S1–543.S2, 2008.

[48] K. R. Konrad, M. M. Wudick, and J. A. Feijo, "Calcium regulation of tip growth: new genes for old mechanisms," *Current Opinion in Plant Biology*, vol. 14, pp. 721–730, 2011.

[49] M. Gillilham, A. Athman, S. D. Tyerman, and S. J. Conn, "Cell-specific compartmentation of mineral nutrients is an essential mechanism for optimal plant productivity—another role for TPCII?" *Plant Signaling & Behavior*, vol. 6, pp. 1656–1661, 2011.

[50] J. Bose, I. I. Pottosin, S. S. Shabala, M. G. Palmgren, and S. Shabala, "Calcium efflux systems in stress signaling and adaptation in plants," *Frontiers in Plant Science*, vol. 2, p. 85, 2011.

[51] R. Catala, E. Santos, J. M. Alonso, J. R. Ecker, J. M. Martinez-Zapater, and J. Salinas, "Mutations in the Ca""/""H"" transporter CAXI increase CBF/DREB1 expression and the cold-acclimation response in Arabidopsis," *Plant Cell*, vol. 15, pp. 2940–2951, 2003.

[52] J. Lynch, V. S. Polito, and A. Lauchli, "Salinity stress increases cytoplasmic Ca activity in maize root protoplasts," *Plant Physiology*, vol. 90, pp. 1271–1274, 1989.

[53] A. J. Laude and A. W. M. Simpson, "Compartmentalized signalling: Ca"" compartments, microdomains and the many facets of Ca"" signalling," *FEBS Journal*, vol. 276, no. 7, pp. 1800–1816, 2009.

[54] A. Trewavas, "Le calcium, c'est la vie: calcium makes waves," *Plant Physiology*, vol. 120, no. 1, pp. 1–6, 1999.

[55] S. Papp, E. Dziak, M. Michalak, and M. Opas, "Is all of the endoplasmic reticulum created equal? The effects of the heterogeneous distribution of endoplasmic reticulum Ca""-handling proteins," *Journal of Cell Biology*, vol. 160, no. 4, pp. 475–479, 2003.

[56] J. M. Fasano, G. D. Massa, and S. Gilroy, "Ionic signaling in plant responses to gravity and touch," *Journal of Plant Growth Regulation*, vol. 21, no. 2, pp. 71–88, 2002.

[57] I. C. Mori, Y. Murata, Y. Yang et al., "CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and Ca""-permeable channels and stomatal closure," *PLoS Biology*, vol. 4, no. 10, p. e327, 2006.

[58] H. Marten, K. R. Konrad, P. Dietrich, M. R. G. Roelfsema, and R. Hedrich, "Ca""-dependent and -independent abscisic acid activation of plasma membrane anion channels in guard cells of Nicotiana tabacum," *Plant Physiology*, vol. 143, no. 1, pp. 28–37, 2007.
[59] S. J. Su, Y. F. Wang, A. Frelet et al., “The ATP binding cassette transporter AtMRP5 modulates anion and calcium channel activities in Arabidopsis guard cells,” Journal of Biological Chemistry, vol. 282, no. 3, pp. 1916–1924, 2007.

[60] L. Cárdenas, “New findings in the mechanisms regulating polar growth in root hair cells,” Plant Signaling and Behavior, vol. 4, no. 1, pp. 4–8, 2009.

[61] D. Cho, S. A. Kim, Y. Murata et al., “De-regulated expression of the plant glutamate receptor homolog AtGLR3.1 impairs long-term Ca²⁺-programmed stomatal closure,” The Plant Journal, vol. 58, no. 3, pp. 437–449, 2009.

[62] R. S. Siegel, S. Xue, Y. Murata et al., “Calcium elevation-dependent and attenuated resting calcium-dependent abscisic acid induction of stomatal closure and abscisic acid-induced enhancement of calcium sensitivities of S-type anion and inward-rectifying K⁺ channels in Arabidopsis guard cells,” The Plant Journal, vol. 59, no. 2, pp. 207–220, 2009.

[63] W. H. Wang, X. Q. Yi, A. D. Han et al., “Calcium-sensing receptor regulates stomatal closure through hydrogen peroxide and nitric oxide in response to extracellular calcium in Arabidopsis; J Exp Bot, vol. 63, pp. 177–190, 2011.

[64] W. H. Wang and H. L. Zheng, “Mechanisms for calcium sensing receptor-regulated stomatal closure in response to the extracellular calcium signal,” Plant Signaling & Behavior, vol. 7, pp. 289–291, 2012.

[65] W. Capoën, J. D. Herder, J. Sun et al., “Calcium spiking patterns and the role of the calcium/calmodulin-dependent kinase CcA1MK in lateral root base nodulation of sesbania rostrata,” Plant Cell, vol. 21, no. 5, pp. 1526–1540, 2009.

[66] P. K. Hepler, J. G. Kunkel, C. M. Rounds, and L. J. Winship, “Calcium entry into pollen tubes,” Trends in Plant Science, vol. 17, pp. 32–38, 2011.

[67] M. Rincón-Zachary, N. D. Teaster, J. Alan Sparks, A. H. Valster, C. M. Motes, and E. B. Blanco-Calderon, “Fluorescence resonance energy transfer-sensitized emission of yellow cameleon 3.60 reveals root zone-specific calcium signatures in Arabidopsis in response to aluminum and other trivalent cations,” Plant Physiology, vol. 152, no. 3, pp. 1442–1458, 2010.

[68] E. F. Short, K. A. North, M. R. Roberts, A. M. Hetherington, A. D. Shirras, and M. R. McInish, “A stress-specific calcium signature regulating an ozone-responsive gene expression network in Arabidopsis,” The Plant Journal, vol. 71, no. 6, pp. 948–961, 2012.

[69] K. Takahashi, M. Irobe, M. R. Knight, A. J. Trewavas, and S. Muto, “Hyposmotic shock induces increases in cytosolic Ca²⁺ in tobacco suspension-culture cells,” Plant Physiology, vol. 113, no. 2, pp. 587–594, 1997.

[70] J. C. Sedbrook, P. J. Kronenbusch, G. G. Borisy, A. J. Trewavas, and P. H. Masson, “Transgenic Aequorin reveals organ-specific cytosolic Ca²⁺ responses to anoxia in Arabidopsis thaliana seedling,” Plant Physiology, vol. 111, no. 1, pp. 243–257, 1996.

[71] M. C. Rentel and M. R. Knight, “Oxidative stress-induced calcium signaling in Arabidopsis,” Plant Physiology, vol. 135, no. 3, pp. 1471–1479, 2004.

[72] H. Song, R. Zhao, P. Fan, X. Wang, X. Chen, and Y. Li, “Overexpression of AtHsp90.2, AtHsp90.5 and AtHsp90.7 in Arabidopsis thaliana enhances plant sensitivity to salt and drought stresses,” Planta, vol. 229, no. 4, pp. 955–964, 2009.

[73] E. Kiegle, C. A. Moore, J. Haseloff, M. A. Tester, and M. R. Knight, “Cell-type-specific calcium responses to drought, salt and cold in the Arabidopsis root,” The Plant Journal, vol. 23, no. 2, pp. 267–278, 2000.
of the United States of America, vol. 101, no. 25, pp. 9502–9507, 2004.

[88] M. A. M. Aboul-Soud, A. M. Aboul-Enein, and G. J. Loake, "Nitric oxide triggers specific and dose-dependent cytosolic calcium transients in Arabidopsis," Plant Signaling & Behavior, vol. 4, no. 3, pp. 191–196, 2009.

[89] J. K. Zhu, "Salt and drought stress signal transduction in plants," Annual Review of Plant Biology, vol. 53, pp. 247–273, 2002.

[90] J. K. Zhu, "Regulation of ion homeostasis under salt stress," Current Opinion in Plant Biology, vol. 6, no. 5, pp. 441–445, 2003.

[91] A. A. Ludwig, T. Romeis, and J. D. G. Jones, "CDPK-mediated signalling pathways: specificity and cross-talk," Journal of Experimental Botany, vol. 55, no. 395, pp. 181–188, 2004.

[92] N. A. Eckardt, "CAMTA proteins: a direct link between calcium elevation in roots and cold acclimation," The Plant Journal, vol. 50, no. 2, pp. 457–465, 2009.

[93] J. F. Harper, G. Breton, and A. Harmon, "Decoding Ca\(^{2+}\) signals through plant protein kinases," Annual Review of Plant Biology, vol. 55, pp. 263–288, 2004.

[94] A. A. Ludwig, T. Romeis, and J. D. G. Jones, "CDPK-mediated signalling pathways: specificity and cross-talk," Journal of Experimental Botany, vol. 55, no. 395, pp. 181–188, 2004.

[95] A. C. Harmon, "Calcium-regulated protein kinases of plants," Gravitational and Space Biology Bulletin, vol. 16, no. 2, pp. 83–90, 2003.

[96] E. M. Hrabak, C. W. M. Chan, M. Gribskov et al., "The Arabidopsis CDPK-SnRK superfamily of protein kinases," Plant Physiology, vol. 132, no. 2, pp. 666–680, 2003.

[97] L. Li, B. G. Kim, Y. H. Cheong, G. K. Pandey, and S. Luan, "A Ca\(^{2+}\) signaling pathway regulates a K\(^{+}\) channel for low-K response in Arabidopsis," Proceedings of the National Academy of Sciences of the United States of America, vol. 103, no. 33, pp. 12625–12630, 2006.

[98] J. Xu, H. D. Li, L. Q. Chen et al., "A Protein Kinase, Interacting with Two Calcineurin B-like Proteins, Regulates K\(^{+}\) Transporter AKT1 in Arabidopsis," Cell, vol. 125, no. 7, pp. 1347–1360, 2006.

[99] Y. Galon, O. Snir, and H. Fromm, "How calmodulin binding transcription activators (CAMTAs) mediate auxin responses," Plant Signaling and Behavior, vol. 5, no. 10, pp. 1311–1314, 2010.

[100] Y. Qiu, J. Xi, L. Du, J. C. Suttle, and B. W. Poovaliah, "Coupling calcium/calmodulin-mediated signaling and herbivore-induced plant response through calmodulin-binding transcription factor AtSR1/CAMTA3," Plant Molecular Biology, vol. 79, pp. 89–99, 2012.

[101] C. J. Doherty, H. A. Van Buskirk, S. J. Myers, and M. F. Thomashow, "Roles for Arabidopsis CAMTA transcription factors in cold-regulated gene expression and freezing tolerance," Plant Cell, vol. 21, no. 3, pp. 972–984, 2009.

[102] L. Du, G. S. Ali, K. A. Simons et al., "Ca\(^{2+}\)/calmodulin regulates salicylic-acid-mediated plant immunity," Nature, vol. 457, no. 7233, pp. 1154–1158, 2009.

[103] N. A. Eckardt, "CAMTA proteins: a direct link between calcium signals and cold acclimation?" Plant Cell, vol. 21, no. 3, p. 697, 2009.

[104] J. Vadassery, S. Ranf, C. Drzewiecki et al., "A cell wall extract from the endophytic fungus Piriformospora indica promotes growth of Arabidopsis seedlings and induces intracellular calcium elevation in roots," The Plant Journal, vol. 59, no. 2, pp. 193–206, 2009.

[105] J. Vadassery and R. Oelmüller, "Calcium signaling in pathogenic and beneficial plant microbe interactions: what can we learn from the interaction between Piriformospora indica and Arabidopsis thaliana," Plant Signal & Behavior, vol. 4, no. 11, pp. 1024–1027, 2009.

[106] S. Stuel, A. G. Rocha, T. Wimberger, D. Anrather, U. C. Vothknecht, and M. Teige, "Cross-talk between calcium signaling and protein phosphorylation at the thylakoid," Journal of Experimental Botany, vol. 63, pp. 1725–1733, 2011.

[107] K. Hashimoto and J. Kudla, "Calcium decoding mechanisms in plants," Biochimica et Biophysica Acta, vol. 193, no. 6, pp. 985–992, 2009.

[108] O. Batistic, M. Rehers, A. Akerman et al., "S-acylation-dependent association of the calcium sensor CBL2 with the vacuolar membrane is essential for proper abscisic acid responses," Cell Research, vol. 22, pp. 1155–1168, 2012.

[109] W. Z. Lan, S. C. Lee, Y. F. Che, Y. Q. Jiang, and S. Luan, "Mechanistic analysis of AKT1 regulation by the CBL-CIPK-PP2CA interactions," Molecular Plant, vol. 4, no. 3, pp. 527–536, 2011.

[110] H. L. Piao, Y. H. Xuan, S. H. Park et al., "OsCIPK3, a CBL-interacting protein kinase is involved in germination and seedling growth under abiotic stress conditions in rice plants," Molecules and Cells, vol. 30, no. 1, pp. 19–27, 2010.

[111] K. Held, F. Pascaud, C. Eckert et al., "Calcium-dependent modulation and plasma membrane targeting of the AKT2 potassium channel by the CBL4/CIPK6 calcium sensor/protein kinase complex," Cell Research, vol. 21, no. 7, pp. 1116–1130, 2011.

[112] L. Li, B. G. Kim, Y. H. Cheong, G. K. Pandey, and S. Luan, "A Ca\(^{2+}\) signaling pathway regulates a K\(^{+}\) channel for low-K response in Arabidopsis," Proceedings of the National Academy of Sciences of the United States of America, vol. 103, no. 33, pp. 12625–12630, 2006.

[113] J. Xu, H. D. Li, L. Q. Chen et al., "A Protein Kinase, Interacting with Two Calcineurin B-like Proteins, Regulates K\(^{+}\) Transporter AKT1 in Arabidopsis," Cell, vol. 125, no. 7, pp. 1347–1360, 2006.

[114] Y. H. Cheong, G. K. Pandey, J. J. Grant et al., "Two calcineurin B-like calcium sensors, interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in Arabidopsis," The Plant Journal, vol. 52, no. 2, pp. 223–239, 2007.

[115] B. G. Kim, R. Waadt, Y. H. Cheong et al., "The calcium sensor CBL10 mediates salt tolerance by regulating ion homeostasis in Arabidopsis," The Plant Journal, vol. 52, no. 3, pp. 473–484, 2007.

[116] S. C. Lee, W. Z. Lan, B. G. Kim et al., "A protein phosphorylation/dephosphorylation network regulates a plant potassium channel," Proceedings of the National Academy of Sciences of the United States of America, vol. 104, no. 40, pp. 15959–15964, 2007.

[117] G. K. Pandey, J. J. Grant, Y. H. Cheong, B. G. Kim, L. G. Li, and S. Luan, "Calcineurin-B-like protein CBL9 interacts with target kinase CIPK3 in the regulation of ABA response in seed germination," Molecular Plant, vol. 1, no. 2, pp. 238–248, 2008.

[118] H. C. Hu, Y. Y. Wang, and Y. F. Tsay, "AtCIPK8, a CBL-interacting protein kinase, regulates the low-affinity phase of the primary nitrate response," The Plant Journal, vol. 57, no. 2, pp. 264–278, 2009.
S. Luan, W. Lan, and S. Chul Lee, “Potassium nutrition, sodium toxicity, and calcium signaling: connections through the CBL-CIPK network,” *Current Opinion in Plant Biology*, vol. 12, no. 3, pp. 339–346, 2009.

K. N. Kim, J. S. Lee, H. Han, S. A. Choi, S. J. Go, and I. S. Yoon, “Isolation and characterization of a novel rice Ca2+-regulated protein kinase gene involved in responses to diverse signals including cold, light, cytokinins, sugars and salts,” *Plant Molecular Biology*, vol. 52, no. 6, pp. 1191–1202, 2003.

P. J. White, M. R. Broadley, J. A. Thompson et al., “Testing the distinctiveness of shoot ionomes of angiosperm families using the Rothamsted Park Grass Continuous Hay Experiment,” *New Phytologist*, vol. 196, pp. 101–109, 2012.

D. Geiger, D. Becker, D. Vosloh et al., “Heteromeric AtKCI-AKT1 channels in *Arabidopsis* roots facilitate growth under K+-limiting conditions,” *Journal of Biological Chemistry*, vol. 284, no. 32, pp. 21288–21295, 2009.

N. Tuteja and S. Mahajan, “Further characterization of calcineurin B-like protein and its interacting partner CBL-interacting protein kinase from Pismum sativum,” *Plant Signaling and Behavior*, vol. 2, no. 5, pp. 358–361, 2007.

V. Tripathi, N. Syed, A. Laxmi, and D. Chattopadhyay, “Role of CIPK6 in root growth and auxin transport,” *Plant Signaling and Behavior*, vol. 4, no. 7, pp. 663–665, 2009.

E. Peiter, “The plant vacuole: emitter and receiver of calcium signals,” *Cell Calcium*, vol. 50, no. 2, pp. 120–128, 2011.

R. Hedrich and I. Marten, “TPCI—SV channels gain shape,” *Molecular Plant*, vol. 4, no. 3, pp. 428–441, 2011.

Y. Boursiac, S. M. Lee, S. Romanowsky et al., “Disruption of the vacuolar calcium-ATPases in *Arabidopsis* results in the activation of a salicylic acid-dependent programmed cell death pathway,” *Plant Physiology*, vol. 154, no. 3, pp. 1158–1171, 2010.

M. Iwano, T. Entani, H. Shiba et al., “Fine-Tuning of the cytoplasmic calcium Ca2+ concentration is essential for pollen tube growth,” *Plant Physiology*, vol. 150, no. 3, pp. 1322–1334, 2009.

M. Michalak, J. Groenendyk, E. Szabo, L. I. Gold, and M. Opas, “Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum,” *Biochemical Journal*, vol. 417, no. 3, pp. 651–666, 2009.

D. E. Clapham, “Calcium Signaling;” *Cell*, vol. 131, no. 6, pp. 1047–1058, 2007.

L. E. V. Del Bem, “The evolutionary history of calreticulin and calnexin genes in green plants,” *Genetica*, vol. 139, no. 2, pp. 255–259, 2011.

J. P. Lièvremont, R. Rizzuto, L. Hendershot, and J. Meldolesi, “BiP, a major chaperone protein of the endoplasmic reticulum lumen, plays a direct and important role in the storage of the rapidly exchanging pool of Ca2+,” *Journal of Biological Chemistry*, vol. 272, no. 49, pp. 30873–30879, 1997.

S. Persson, M. Rosenquist, K. Svensson, R. Galvão, W. F. Boss, and M. Sommarin, “Phylogenetic analyses and expression studies reveal two distinct groups of calreticulin isoforms in higher plants,” *Plant Physiology*, vol. 133, no. 3, pp. 1385–1396, 2003.

A. Christensen, K. Svensson, L. Thelin et al., “Higher plant calreticulins have acquired specialized functions in *Arabidopsis*,” *PLoS ONE*, vol. 5, no. 6, p. e11342, 2010.

M. H. Chen, G. W. Tian, Y. Gafni, and V. Citovsky, “Effects of calreticulin on viral cell-to-cell movement,” *Plant Physiology*, vol. 138, no. 4, pp. 1866–1876, 2005.

Y. Saito, Y. Ihara, M. R. Leach, M. F. Cohen-Doyle, and D. B. Williams, “Calreticulin functions in vitro as a molecular chaperone for both glycosylated and non-glycosylated proteins,” *The EMBO Journal*, vol. 18, no. 23, pp. 6718–6729, 1999.

X. Y. Jia, L. H. He, R. L. Jing, and R. Z. Li, “Calreticulin: conserved protein and diverse functions in plants,” *Physiologia Plantarum*, vol. 136, no. 2, pp. 127–138, 2009.

I. L. Conte, N. Keith, C. Gutiérrez-González, A. J. Parodi, and J. J. Caramelo, “The interplay between calcium and the in vitro lectin and chaperone activities of calreticulin,” *Biochemistry*, vol. 46, no. 15, pp. 4671–4680, 2007.

A. Christensen, K. Svensson, S. Persson et al., “Functional characterization of *Arabidopsis* calreticulin1a: a key alleviator of endoplasmic reticulum stress,” *Plant and Cell Physiology*, vol. 49, no. 6, pp. 912–924, 2008.

H. Jin, Z. Hong, W. Su, and J. Li, “A plant-specific calreticulin is a key retention factor for a defective brassinosteroid receptor in the endoplasmic reticulum,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 32, pp. 13612–13617, 2009.

Y. Saijo, N. Tintor, X. Lu et al., “Receptor quality control in the endoplasmic reticulum for plant innate immunity,” *The EMBO Journal*, vol. 28, no. 21, pp. 3439–3449, 2009.

Y. Qiu, J. Xi, L. Du, and B. W. Poovaiah, “The function of calreticulin in plant immunity: new discoveries for an old protein,” *Plant Signaling & Behavior*, vol. 7, no. 8, pp. 907–910, 2012.

Y. Qiu, J. Xi, L. Du, S. Roje, and B. W. Poovaiah, “A dual regulatory role of *Arabidopsis* calreticulin-2 in plant innate immunity,” *The Plant Journal*, vol. 69, pp. 489–500, 2011.

J. Li, Z. H. Chu, M. Batoux et al., “Specific ER quality control components required for biogenesis of the plant innate immune receptor EFR,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 37, pp. 15973–15978, 2009.

Y. Q. An, R. M. Lin, F. T. Wang, J. Feng, Y. F. Xu, and S. C. Xu, “Molecular cloning of a new wheat calreticulin gene TaCRT1 and expression analysis in plant defense responses and abiotic stress resistance,” *Genetics and Molecular Research*, vol. 10, pp. 3576–3585, 2011.

J. L. Caplan, X. Zhu, P. Mamillapalli, R. Marathe, R. Anandakishmi, and S. P. Dinesh-Kumar, “Induced ER chaperones regulate a receptor-like kinase to mediate antiviral innate immune response in plants,” *Cell Host and Microbe*, vol. 6, no. 5, pp. 457–469, 2009.

H. G. Kang, C. S. Oh, M. Sato et al., “Endosome-associated CRT1 functions early in Resistance gene-mediated defense signaling in *Arabidopsis* and tobacco,” *Plant Cell*, vol. 22, no. 3, pp. 918–936, 2010.

X. Y. Jia, C. Y. Xu, R. L. Jing et al., “Molecular cloning and characterization of wheat calreticulin (CRT) gene involved in drought-stressed responses,” *Journal of Experimental Botany*, vol. 59, no. 4, pp. 739–751, 2008.

I. Hwang, J. F. Harper, F. Liang, and H. Sze, “Calmodulin activation of an endoplasmic reticulum-located calcium pump involves an interaction with the N-terminal autoinhibitory domain,” *Plant Physiology*, vol. 122, no. 1, pp. 157–167, 2000.

I. Hwang, H. Sze, and J. F. Harper, “A calcium-dependent protein kinase can inhibit a calmodulin-stimulated Ca2+ pump (ACA2) located in the endoplasmic reticulum of *Arabidopsis*,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 11, pp. 6224–6229, 2000.
[153] Z. Wu, F. Liang, B. Hong et al., “An endoplasmic reticulum-bound Ca\(^{2+}/\text{Mn}^{2+}\) pump, ECAI, supports plant growth and confers tolerance to Mn2+ stress,” Plant Physiology, vol. 130, no. 1, pp. 128–137, 2002.

[154] J. W. Putney, “Recent breakthroughs in the molecular mechanism of capacitative calcium entry (with thoughts on how we got here),” Cell Calcium, vol. 42, no. 2, pp. 103–110, 2007.

[155] V. S. Reddy and A. S. N. Reddy, “Proteomics of calcium-signaling components in plants,” Phytochemistry, vol. 65, no. 12, pp. 1745–1776, 2004.

[156] H. J. Whalley, A. W. Sargeant, J. F. Steele et al., “Transcriptomic analysis reveals calcium regulation of specific promoter motifs in Arabidopsis,” Plant Cell, vol. 23, pp. 4079–4095, 2011.

[157] C. H. Johnson, M. R. Knight, T. Kondo et al., “Circadian oscillations of cytosolic and chloroplastic free calcium in plants,” Science, vol. 269, no. 5232, pp. 1863–1865, 1995.

[158] P. L. Tsou, S. Y. Lee, N. S. Allen, H. Winter-Sederoiff, and D. Robertson, “An ER-targeted calcium-binding peptide confers salt and drought tolerance mediated by CIPK6 in Arabidopsis,” Planta, vol. 235, pp. 539–552, 2011.

[159] D. Winter, B. Vinegar, H. Nahal, R. Ammar, G. V. Wilson, and N. J. Provart, “An “Electronic Fluorescent Pictograph” browser for exploring and analyzing large-scale biological data sets,” PloS ONE, vol. 2, no. 1, p. e718, 2007.

[160] M. R. Broadley and P. J. White, “Eats roots and leaves. Can edible horticultural crops address dietary calcium, magnesium and potassium deficiencies?” Proceedings of the Nutrition Society, vol. 69, no. 4, pp. 601–612, 2010.

[161] S. Conn and M. Gilliam, “Comparative physiology of elemental distributions in plants,” Annals of Botany, vol. 105, no. 7, pp. 1081–1102, 2010.

[162] S. J. Conn, M. Gilliam, A. Athman et al., “Cell-specific vacuolar calcium storage mediated by CAXI regulates apoplastic calcium concentration, gas exchange, and plant productivity in Arabidopsis,” Plant Cell, vol. 23, no. 1, pp. 240–257, 2011.

[163] M. Gilliam, M. Dayod, B. J. Hocking et al., “Calcium delivery and storage in plant leaves: exploring the link with water flow,” Journal of Experimental Botany, vol. 62, no. 7, pp. 2233–2250, 2011.

[164] M. Kerton, H. J. Newbury, D. Hand, and J. Pritchard, “Accumulation of calcium in the centre of leaves of coriander (Coriandrum sativum L.) is due to an uncoupling of water and ion transport,” Journal of Experimental Botany, vol. 60, no. 1, pp. 227–235, 2009.

[165] V. Demidchik, H. C. Bowen, F. J. M. Maathuis et al., “Arabidopsis thaliana root non-selective cation channels mediate calcium uptake and are involved in growth,” The Plant Journal, vol. 32, no. 5, pp. 799–808, 2002.

[166] W. Y. Song, K. S. Choi, A. Alexis de, E. Martinoa, and Y. Lee, “Brassica juncea plant cadmium resistance 1 protein (BjPCR1) facilitates the radial transport of calcium in the root,” Proceedings of the National Academy of Sciences of the United States of America, vol. 108, pp. 19808–19813, 2011.

[167] I. Baxter, P. S. Hosmani, A. Rus et al., “Root suberin forms an extracellular barrier that affects water relations and mineral nutrition in Arabidopsis,” PloS Genetics, vol. 5, no. 5, Article ID e1000492, 2009.

[168] P. J. White, “The pathways of calcium movement to the xylem,” Journal of Experimental Botany, vol. 52, no. 358, pp. 891–899, 2001.
Z. Li and S. Komatsu, “Molecular cloning and characterization of calreticulin, a calcium-binding protein involved in the regener-
ation of rice cultured suspension cells,” European Journal of Biochemistry, vol. 267, no. 3, pp. 737–745, 2000.

A. J. Karley and P. J. White, “Moving cationic metals to edible tissues: potassium, magnesium, calcium,” Current Opinion in Plant Biology, vol. 12, no. 3, pp. 291–298, 2009.

K. D. Hirschi, “Expression of Arabidopsis CAXI in tobacco: altered calcium homeostasis and increased stress sensitivity,” Plant Cell, vol. 11, no. 2, pp. 2113–2122, 1999.

T. Punshon, K. Hirschi, J. Yang, A. Lanzirotti, B. Lai, and M. L. Guerinot, “The role of CAX1 and CAX3 in elemental distribution and abundance in Arabidopsis seed,” Plant Physiology, vol. 158, pp. 352–362, 2011.

J. K. Pittman and K. D. Hirschi, “Regulation of CAXI, an Arabidopsis Ca\(^{2+}\)/H\(^+\) antiporter. Identification of an N-terminal autoinhibitory domain,” Plant Physiologoy, vol. 127, no. 3, pp. 1020–1029, 2001.

S. Park, T. S. Kang, C. K. Kim et al., “Genetic manipulation for enhancing calcium content in potato tuber,” Journal of Agricultural and Food Chemistry, vol. 53, no. 14, pp. 5598–5603, 2005.

C. K. Kim, J. S. Han, H. S. Lee et al., “Expression of an Arabidopsis CAX2 variant in potato tubers increases calcium levels with no accumulation of manganese,” Plant Cell Reports, vol. 25, no. 11, pp. 1226–1232, 2006.

S. Park, N. H. Cheng, J. K. Pittman et al., “Increased calcium levels and prolonged shelf life in tomatoes expressing Arabidopsis H\(^+\)/Ca\(^{2+}\) transporters,” Plant Physiologoy, vol. 139, no. 3, pp. 1194–1206, 2005.

H. Mei, N. H. Cheng, J. Zhao et al., “Root development under metal stress in Arabidopsis thaliana requires the H\(^+\)/cation antiporter CAX4,” New Phytologistist, vol. 183, no. 1, pp. 95–105, 2009.

N. H. Cheng, J. K. Pittman, B. J. Barkla, T. Shigaki, and K. D. Hirschi, “The Arabidopsis cax1 mutant exhibits impaired ion homeostasis, development, and hormonal responses and reveals interplay among vacuolar transporters,” Plant Cell, vol. 15, no. 2, pp. 347–364, 2003.

N. H. Cheng, J. K. Pittman, J. K. Zhu, and K. D. Hirschi, “The protein kinase SOS2 activates the Arabidopsis H\(^+\)/Ca\(^{2+}\) antiporter CAX1 to integrate calcium transport and salt tolerance,” Journal of Biological Chemistry, vol. 279, no. 4, pp. 2922–2926, 2004.

J. Zhao, B. J. Barkla, J. Marshall, J. K. Pittman, and K. D. Hirschi, “The Arabidopsis CAX3 mutants display altered salt tolerance, pH sensitivity and reduced plasma membrane H\(^+\)-ATPase activity,” Planta, vol. 227, no. 3, pp. 659–669, 2008.

Q. Wu, T. Shigaki, K. A. Williams et al., “Expression of an Arabidopsis Ca\(^{2+}\)/H\(^+\) antiporter CAX1 variant in petunia enhances cadmium tolerance and accumulation,” Journal of Plant Physiology, vol. 168, no. 2, pp. 167–173, 2011.

T. Shigaki, H. Mei, J. Marshall, X. Li, M. Manohar, and K. D. Hirschi, “The expression of the open reading frame of Arabidopsis CAXI, but not its cDNA, confers metal tolerance in yeast,” Plant Biology, vol. 12, no. 6, pp. 935–939, 2010.

V. Korenkov, S. Park, N. H. Cheng et al., “Enhanced Ca\(^{2+}\)-selective root-tonoplast-transport in tobacco expressing Arabidopsis cation exchangers,” Planta, vol. 225, no. 2, pp. 403–411, 2007.

V. Korenkov, B. King, K. Hirschi, and G. J. Wagner, “Root-selective expression of AtCAX4 and AtCAX2 results in reduced lamina cadmium in field-grown Nicotiana tabacum L.,” Plant Biotechnology, vol. 7, no. 3, pp. 219–226, 2009.

T. Y. Liu, K. Aung, C. Y. Tseng, T. Y. Chang, Y. S. Chen, and T. J. Chiou, “Vascular Ca\(^{2+}\)/H\(^+\) transport activity is required for systemic phosphate homeostasis involving shoot-to-root signaling in Arabidopsis,” Plant Physiologoy, vol. 156, no. 3, pp. 1176–1189, 2011.

P. C. Dekock, D. Vaughan, a. Hall, and C. Ord, “Biochemical studies on blossom end rot [caused mainly by calcium deficiency] of tomatoes,” Plant Physiologoy, vol. 48, pp. 312–316, 1980.

H. E. Johnson, D. Broadhurst, R. Goodacre, and A. R. Smith, “Metabolic fingerprinting of salt-stressed tomatoes,” Phytochemistry, vol. 62, no. 6, pp. 919–928, 2003.

M. D. Taylor and S. J. Locascio, “Blossom-end rot: a calcium deficiency,” Journal of Plant Nutrition, vol. 27, no. 1, pp. 123–139, 2004.

S. T. de Freitas, M. Padda, Q. Wu, S. Park, and E. J. Mitcham, “Dynamic alternations in cellular and molecular components during blossom-end rot development in tomatoes expressing sCAX1, a constitutively active Ca\(^{2+}\)/H\(^+\) antiporter from Arabidopsis,” Plant Physiologoy, vol. 156, no. 2, pp. 844–855, 2011.

G. Z. Luo, H. W. Wang, J. Huang et al., “A putative plasma membrane cation/proton antiporter from soybean confers salt tolerance in Arabidopsis,” Plant Molecular Biology, vol. 59, no. 5, pp. 809–820, 2005.

K. H. Krause and M. Michalak, “Calreticulin,” Cell, vol. 88, no. 4, pp. 439–443, 1997.

P. D. Nash, M. Opas, and M. Michalak, “Calreticulin: not just another calcium-binding protein,” Molecular and Cellular Biochemistry, vol. 135, no. 1, pp. 71–78, 1994.

J. Meldolesi, K. H. Krause, and M. Michalak, “Calreticulin: how many functions in how many cellular compartments?” Cell Calcium, vol. 20, no. 1, pp. 83–86, 1996.

M. Michalak, P. Mariani, and M. Opas, “Calreticulin, a multifunctional Ca\(^{2+}\) binding chaperone of the endoplasmic reticulum,” Biochemistry and Cell Biology, vol. 76, no. 5, pp. 779–785, 1998.

M. S. Kwon, C. S. Park, K. R. Choi et al., “Calreticulin couples calcium release and calcium influx in integrin-mediated calcium signaling,” Molecular Biology of the Cell, vol. 11, no. 4, pp. 1433–1443, 2000.

P. B. Simpson, S. Mehota, D. Langley, C. A. Sheppard, and J. T. Russell, “Specialized distributions of mitochondria and endoplasmic reticulum proteins define Ca\(^{2+}\) wave amplification sites in cultured astrocytes,” Journal of Neuroscience Research, vol. 52, pp. 672–683, 1998.

M. Opas, M. Szewczenko-Pawlikowski, G. K. Jass, N. Mesiati, and M. Michalak, “Calreticulin modulates cell adhesiveness via regulation of vinculin expression,” Journal of Cell Biology, vol. 135, no. 6, pp. 1913–1923, 1996.

L. Perrone, G. Tell, and R. Di Lauro, “Calreticulin enhances the transcriptional activity of thyroid transcription factor-1 by binding to its homeodomain,” Journal of Biological Chemistry, vol. 274, no. 8, pp. 4640–4645, 1999.

H. Liu, R. C. Bowes, B. Van De Water, C. Sillence, J. F. Nagelkerke, and J. L. Stevens, “Endoplasmic reticulum chaperones GRP78 and calreticulin prevent oxidative stress, Ca\(^{2+}\) disturbances, and cell death in renal epithelial cells,” Journal of Biological Chemistry, vol. 272, no. 35, pp. 21751–21759, 1997.

L. Mery, N. Mesiati, M. Michalak, M. Opas, D. P. Lew, and K. H. Krause, “Overexpression of calreticulin increases intracellular...
Ca$^{2+}$ storage and decreases store-operated Ca$^{2+}$ influx, "Journal of Biological Chemistry," vol. 271, no. 16, pp. 9332–9339, 1996.

[218] H. L. Roderick, D. H. Llewellyn, A. K. Campbell, and J. M. Kendall, "Role of calreticulin regulating intracellular Ca$^{2+}$ storage and capacitative Ca$^{2+}$ entry in HeLa cells," Cell Calcium, vol. 24, no. 4, pp. 253–262, 1998.

[219] C. Fasolato, P. Pizzo, and T. Pozzan, "Delayed activation of the store-operated calcium current induced by calreticulin overexpression in RBL-1 cells," Molecular Biology of the Cell, vol. 9, no. 6, pp. 1513–1522, 1998.

[220] J. Denecke, L. E. Carisson, S. Vidal et al., "The tobacco homolog of mammal calreticulin is present in protein complexes in vivo," Plant Cell, vol. 7, pp. 391–406, 1995.

[221] C. Bastianutto, E. Clementi, F. Codazzi et al., "Overexpression of calreticulin increases the Ca$^{2+}$ capacity of rapidly exchanging Ca$^{2+}$ stores and reveals aspects of their luminal microenvironment and function," Journal of Cell Biology, vol. 130, no. 4, pp. 847–855, 1995.

[222] S. Persson, S. E. Wyatt, J. Love, W. F. Thompson, D. Robertson, and W. F. Boss, "The Ca$^{2+}$ status of the endoplasmic reticulum is altered by induction of calreticulin expression in transgenic plants," Plant Physiologogy, vol. 126, no. 3, pp. 1092–1104, 2001.

[223] S. E. Wyatt, P. L. Tsou, and D. Robertson, "Expression of the high capacity calcium-binding domain of calreticulin increases bioavailable calcium stores in plants," Transgenic Research, vol. 11, no. 1, pp. 1–10, 2002.

[224] F. Brandizzi, S. Hanton, L. L. Pinto DaSilva et al., "ER quality control can lead to retrograde transport from the ER lumen to the cytosol and the nucleoplasm in plants," The Plant Journal, vol. 34, no. 3, pp. 269–281, 2003.

[225] Z. L. Jin, K. H. Joon, A. Y. Kyung et al., "Over-expression of Chinese cabbage calreticulin 1, BrCRT1, enhances shoot and root regeneration, but retards plant growth in transgenic tobacco," Transgenic Research, vol. 14, no. 5, pp. 619–626, 2005.

[226] S. Y. Lee, The involvement of ER calcium in abiotic stress tolerance [Ph.D. thesis], 2010.

[227] U. Hafter, M. Ishitan, and J. K. Zhu, "The Arabidopsis SOS3 protein kinase physically interacts with and is activated by the calcium-binding protein SOS2," Proceedings of the National Academy of Sciences of the United States of America, vol. 97, no. 7, pp. 3735–3740, 2000.

[228] M. Ishitan, J. Liu, U. Hafter, C. S. Kim, W. Shi, and J. K. Zhu, "SOS3 function in plant salt tolerance requires N-myristoylation and calcium binding," Plant Cell, vol. 12, no. 9, pp. 1667–1677, 2000.

[229] D. Gong, Y. Guo, K. S. Schumaker, and J. K. Zhu, "The SOS3 family of calcium sensors and SOS2 family of protein kinases in Arabidopsis," Plant Physiological, vol. 134, no. 3, pp. 919–926, 2004.

[230] M. Nieves-Cordones, F. Caballero, V. Martinez, and F. Rubio, "Disruption of the Arabidopsis thaliana inward-rectifier K+ channel AKT1 improves plant responses to water stress," Plant and Cell Physiology, vol. 53, pp. 423–432, 2012.

[231] P. H. McCord, Genetic, genomic, and transgenic approaches to understand internal heat necrosis in potato [Ph.D. thesis], 2009.

[232] G. C. Yencho, P. H. McCorrd, K. G. Haynes, and S. B. R. Sterrett, "Internal heat necrosis of potato—a review," American Journal of Potato Research, vol. 85, no. 1, pp. 69–76, 2008.

[233] W. S. Blair, H. P. Bogerd, S. J. Madore, and B. R. Cullen, "Mutational analysis of the transcription activation domain of RelA: identification of a highly synergistic minimal acidic activation module," Molecular and Cellular Biology, vol. 14, no. 11, pp. 7226–7234, 1994.

[234] S. T. de Freitas, A. K. Handa, Q. Wu, S. Park, and E. J. Mitcham, "Role of pectin methylesterases in cellular calcium distribution and blossom-end rots development in tomato fruit," The Plant Journal, vol. 71, pp. 824–835, 2012.

[235] Q. Wu, T. Shigaki, J. S. Han, C. K. Kim, K. D. Hirschi, and S. Park, "Ectopic expression of a maize calreticulin mitigates calcium deficiency-like disorders in sCAX1-expressing tobacco and tomato," Plant Molecular Biology, vol. 80, pp. 609–619, 2012.

[236] R. Reid and J. Hayes, "Mechanisms and control of nutrient uptake in plants," International Review of Cytology, vol. 229, pp. 73–114, 2003.

[237] S. J. Conn, P. Berninger, M. R. Broadley, and M. Gillham, "Exploiting natural variation to uncover candidate genes that control element accumulation in Arabidopsis thaliana," New Phytologist, vol. 193, pp. 859–866, 2012.

[238] B. J. Heyen, M. K. Alsheikh, E. A. Smith, C. F. Torvik, D. F. Seals, and S. K. Randall, "The calcium-binding activity of a vacuole-associated, dehydrin-like protein is regulated by phosphorylation," Plant Physiology, vol. 130, no. 2, pp. 675–687, 2002.

[239] K. Yuasa and M. Maeshima, "Purification, properties, and molecular cloning of a novel Ca$^{2+}$-binding protein in radish vacuoles," Plant Physiology, vol. 124, no. 3, pp. 1069–1078, 2000.

[240] B. Dadacz-Narloch, D. Beyhl, C. Larisch et al., "A novel calcium binding site in the slow vacuolar cation channel TPCI senses luminal calcium levels," Plant Cell, vol. 23, pp. 2696–2707, 2011.

[241] J. J. Rios, S. O. Lochlainn, J. Devonshire et al., "Distribution of calcium (Ca) and magnesium (Mg) in the leaves of Brassica rapa under varying exogenous Ca and Mg supply," Annals of Botany, vol. 109, pp. 1081–1089, 2012.

[242] E. L. Connolly, "Raising the bar for biofortification: enhanced levels of bioavailable calcium in carrots," Trends in Biotechnology, vol. 26, no. 8, pp. 401–403, 2008.

[243] S. Park, M. P. Elless, J. Park et al., "Sensory analysis of calcium-biofortified lettuce," Plant Biotechnology Journal, vol. 7, no. 1, pp. 106–117, 2009.
