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Review Article

Modulating Plant Calcium for Better Nutrition and Stress Tolerance

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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 apoplast, 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 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. Cytoplasmic 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^{2+} concentrations include touch, cold shock, heat shock, oxidative stress, anoxia, hypoosmotic shock, salinity, wounding, gravity, and pathogen infection [37, 56, 69–81]. Developmental signals including fertilization, senescence, abscission, and ripening also involve Ca^{2+}-regulated proteins [82–88].

There is evidence for tissue-specific differences in Ca^{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^{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^{2+} along the entire root [91]. To distinguish tissue-specific differences in Ca^{2+} flux, different transgenic plants transformed with a gene encoding aequorin (a reporter gene for Ca^{2+}) targeted to the cytoplasm of the epidermis, endodermis, or pericycle of Arabidopsis roots were used [73]. Prolonged oscillations in aequorin 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^{2+} (Section 2).

2.2. Calcium Sensors. Understanding the transduction of Ca^{2+} signatures has increased in the past decade due to rapid progress in deciphering the cellular network of Ca^{2+}-responsive proteins. There are several families of Ca^{2+}-binding proteins in plants [92–95]. Proteins such as calmodulin, calcineurin B-like proteins (CBL), and Ca^{2+}-dependent protein kinases (CDPK) "sense" Ca^{2+}, having one or more EF-hand domains that bind Ca^{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^{2+} [97]. Calmodulins can interact with transcription factors, directly transducing Ca^{2+} signals into changes in gene expression [98–103]. There is also evidence of Ca^{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^{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^{2+} binding proteins in plants suggests that intracellular Ca^{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^{2+}-regulated proteins has evolved to replace the IP_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^{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^{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^+ 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 relocalization. CIPK6 serves as a scaffold in this process as phosphorylation 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^+ channel, AKT1 [124]. This interaction results in K^+ uptake under low K^+ 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^{2+} in apples, and exogenous Ca^{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^{2+} Binding Proteins and Modulation of Ca^{2+} Stores. Suberization of the cell walls in the endodermis might prevent apoplastic Ca^{2+} from participating in cytosolic signaling events, because the deposition of the wax onto the cell walls would inhibit Ca^{2+} mobility. White and Knight used this insight to demonstrate that different stimuli do result in the cell accessing different stores of Ca^{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^{2+}. While salt stress resulted in the production of

Table 1: Ectopic expression of CIPK/CBL members; the effect on abiotic stress.

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]

a continuous luminescent Ca^{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^{2+} stores. Moore et al. concluded that cytoplasmic signaling in response to salt stress utilized intracellular stores of Ca^{2+}, although it is still not clear what part of the cell contained the store [91].

2.4.1. The Vacuole as a Ca^{2+} Store. Although a considerable amount of Ca^{2+} is present in the apoplast, the vacuole is the main storage organelle for Ca^{2+} within the plant cell. However, there is little direct evidence for the vacuole as a source of Ca^{2+} for signaling [4, 127, 128], although the identification of Ca^{2+} channels in the tonoplast membrane is not complete either. Furthermore, most of the Ca^{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^{2+}, which is critical for preventing association with phosphate and for shaping putative Ca^{2+} signatures. Using mathematical modeling, Bose et al. suggest that the activity of the known major Ca^{2+} efflux proteins (two members, each of the ACA and *CAX* gene families) is sufficient to describe a wide variety of Ca^{2+} signatures, including all of the current experimental results, without having to take into consideration how Ca^{2+} enters the cytosol [50]. Figure 1 shows a diagram of the major Ca^{2+} efflux proteins in a leaf cell.

Two vacuolar Ca^{2+} ATPases, ACA4 and ACA11, have been shown experimentally to be important for removing excess cytoplasmic Ca^{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^{2+} by itself was not toxic [129]. It could be that PCD has the lowest threshold for sensing an activating cytoplasmic Ca^{2+} signal. While many stimuli could activate the release of Ca^{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^{2+} on a PCD-related Ca^{2+} sensor, or if ACA4 and ACA11 are specifically involved in PCD.

2.4.2. The ER as a Ca^{2+} Store. The ER also contains high levels of Ca^{2+} and is an attractive candidate for storing signaling Ca^{2+} [130]. Calreticulin (*CRT*) is an ER luminal chaperone that has two Ca^{2+} binding domains. The P-domain contains a high affinity, EF hand-like structure that binds 1-2 moles of Ca^{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^{2+} with weak affinity. The C-domain has been estimated to bind 30–50 moles of Ca^{2+} per mole of protein [131]. Because of its low affinity, C-domain binding requires a relatively high concentration of Ca^{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^{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^{2+} binding proteins such as CRT and Calnexin (CXN) [133]. CXN is a membrane-bound ER protein that functions with CRT and

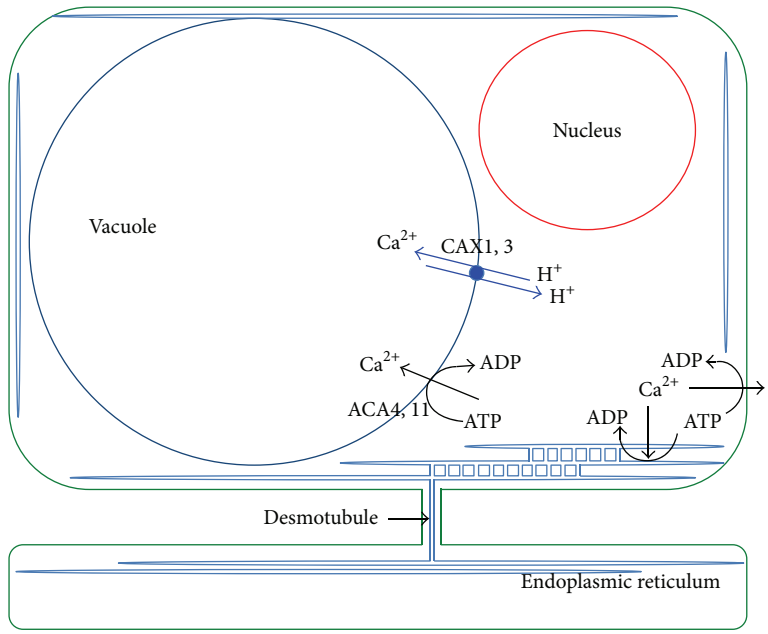

FIGURE 1: Major Ca^{2+} efflux systems in a leaf cell, and structure of the ER spanning two cells. CAX1 is the major cation exchanger in leaf cells, but CAX3 can compensate if CAX1 activity is compromised. Not shown is a vacuolar proton ATPase that uses ATP to pump protons into the vacuole. The energy from the proton gradient is used to pump Ca^{2+} into the vacuole. There are also two Ca^{2+} pumps on the tonoplast membrane, ACA4 and ACA11. The ER and plasma membrane also have Ca^{2+} pumps (lower right). Ca^{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, *CRT1a* and *CRT1b* (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 *CRT1a* and *CRT1b*, but was specifically required for proper folding of the brassinosteroid receptor, BRI1 [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^{2+}. *CRT2* also shows guard cell -specific expression.

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

In animal cells, Ca^{2+} is constantly leaking out of the ER and constantly being pumped back in by SERCAs, membrane pumps that are similar to ECA [132]. But the major mechanism for ensuring adequate ER levels of Ca^{2+} is a specialized plasma membrane pump that responds only to low ER Ca^{2+}. In a mechanism called store-operated Ca^{2+} entry [154], the pump (Orai1) forms a structure adjacent to an ER protein (STIM1) that contains an EF hand to sense ER Ca^{2+} levels. Together, they allow ER Ca^{2+} levels to be refilled [132]. It is not known if a similar mechanism could function in plants.

2.5. Ca^{2+} Transduction and Regulation of Gene Expression. How many genes and proteins are associated with Ca^{2+} regulation? In addition to the ~250 EF-hand containing proteins, ~700 are thought to be involved with Ca^{2+} signaling for Arabidopsis, according to proteomic data [155]. These proteins generate Ca^{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 glycine-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. Vacuolar 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 *CAX1* in those cells [162]. CAX1 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_2O_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 apoplast 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^{2+}, 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

Table 2: Concentration of Ca^{2+} and Mg^{2+} in shoots of different angiosperm families (data taken from [123]).

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.

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 symplastic 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. Cytoplasmic 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

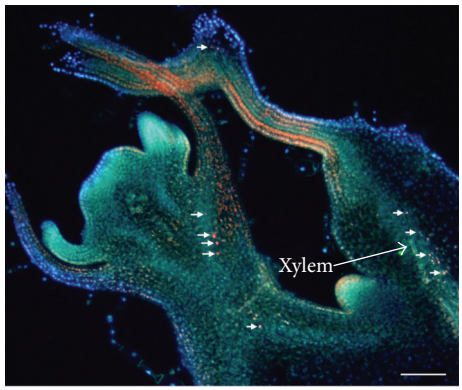

Figure 2: How can transpiration, which requires differentiated xylem, deliver sufficient Ca^{2+} to sustain meristematic growth? Longitudinal section of *Nicotiana benthamiana* stained with DAPI (blue) to detect DNA and hybridized to fluorescent oligos complementary to tomato golden mosaic virus DNA (pink, short arrows). Viral DNA is transported through phloem and makes a nice marker for developing vascular tissue. Image was visualized with a triple-fluorescence cube for DAPI, FITC, and Texas Red. Orange and green colors are the result of autofluorescence. Most of the cells in this section are undifferentiated. Long arrow points to a tracheary element that has differentiated, but most of the leaf is still developing (section through this leaf is oblique). Bar = 50 microns.

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 triglutamic 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 dextrans 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^{2+} available for the new cell wall that could be delivered through the vesicles.

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 *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 *CRT*, such that if ER Ca^{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^{2+} levels need to be high enough to facilitate the depolymerization of microtubules.

Nevertheless, the relationship between ER Ca^{2+}, *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 *CRT* expression in tomato, since it suffers from the occurrence of blossom end rot, discussed in Section 6. Blossom end rot is a Ca^{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?

5.3. Summary. In summary, a gradient of Ca^{2+} ions in the ER is proposed to help the plant guard its vulnerable meristem from fluctuations in the transpiration of Ca^{2+}. *CRT* networks in the ER could provide a conduit for Ca^{2+} transport to ensure that adequate levels of Ca^{2+} reach the meristem to support growth. *CRT* could serve as a buffer to help neutralize charge and to draw Ca^{2+} towards the meristem. Cell division may be coupled to *CRT* expression to ensure that adequate levels of Ca^{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^{2+} is transported to meet the demands of the cell wall and organelles.

6. Genetic Manipulation of Ca^{2+} Stores

Many postmenopausal women take supplemental Ca^{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^{2+} in the form of a pill because of its large size, which is needed due to the relatively poor absorption of chemical Ca^{2+}. The best way to obtain more nutrients is to consume more fruits and vegetables, especially roots and leaves for Ca^{2+}, Mg^{2+}, and K^{+} [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^{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^{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^{2+} increases their tolerance to stress, it would be prudent to consider the genetic alteration of Ca^{2+} stores with transgenes that benefit consumers as well as farmers.

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

6.1. Transgenic Expression of CAX Family Members. The protein family with the best potential for increasing bioavailable Ca^{2+} in plants is *CAX*, located on the tonoplast membrane [188]. As previously mentioned, *CAX1* expression levels are the primary determinant for Ca^{2+} levels in Arabidopsis [162, 189]. In leaves, *CAX1* functions to clear free Ca^{2+} from the apoplast, so that guard cell signaling, which requires extracellular Ca^{2+}, can be regulated properly (Section 3). In *sCAX1*, the N-terminal autoinhibitory loop has been removed so that it can transport increased amounts of Ca^{2+} into the vacuole [190].

Ectopic *sCAX1* expression increased Ca^{2+} in potato tubers by 2-3 folds with no change in morphology or yield when supplemented with 2 mM $CaCl_2$ during the first 3 months [191]. However, CAX1 transports other cations in addition to Ca^{2+}, which are not as beneficial from a nutritional standpoint. Hirschi's group, therefore, modified the *CAX2* gene, which shows a greater specificity for Ca^{2+}, to eliminate its Mn^{2+} transport function and then showed ~50%–60% increase in Ca^{2+} in transgenic potatoes [192].

Tomato was transformed with *CAX4*, which is more specific for Ca^{2+} than *CAX1* [193]. This resulted in a 40% increase in total Ca^{2+} and was not associated with Ca^{2+} deficiency symptoms even in the absence of $CaCl_2$ supplementation. *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 *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 *CAX1* 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 *CAX1* 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 *DREB1* and a subset of cold-responsive genes induced by DREB1 [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 *DREB1* 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 an N-terminal autoinhibitory loop, also found in *AtCAX1*, but shows only 65% homology to it and 68% homology to *CAX*2 [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 indo-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]. *CRT1* 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 *AtCIPK6* are sensitive to salt [44], and overexpression of a constitutively active mutant of *AtCIPK6* 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* knockdown 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 knockdown 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 Ca^{2+} in this disorder. The application of antitranspirants to potato leaves reduced total Ca^{2+} levels and increased Ca^{2+} 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 Ca^{2+} in leaves [231]. It was not possible to measure Ca^{2+} in tubers. There were also increased levels of Mg^{2+} and Mn^{2+} 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 Ca^{2+}? Could this result from ACA and ECA activity in removing Ca^{2+} 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 Ca^{2+} in the cytosol, it could bind Ca^{2+} 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 Ca^{2+}. 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 Ca^{2+} 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 Ca^{2+} levels, which increased membrane leakiness [234]. Coexpression of *CRT* resulted in a significant decrease in Ca^{2+} deficiency symptoms in both tomato and tobacco without the addition of supplemental Ca^{2+} [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 Ca^{2+} 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 Ca^{2+} network distinct from the apoplast and vacuole?

(3) Is *CRT* needed to keep a bioavailable pool of Ca^{2+} inside the ER for signaling? If so, then extra *CRT* may have successfully competed with *CAX1* for the limited pool of free Ca^{2+} in the apoplast in the dual transgenic plants.

(4) Can the vacuole serve as the source of Ca^{2+} for some stimuli?

(5) What would *CRT* overexpression in a *cax1/3* mutant do? Could it help to bind excess apoplastic Ca^{2+}?

6.4. Other Transgenes for Manipulating Ca^{2+} Stores in Plants. Several Ca^{2+}-related proteins have the potential to serve as a mechanism for altering Ca^{2+} stores in plants. Theoretically, any part of the cell except the cytoplasm could sustain increased levels of Ca^{2+} without deleterious consequences, although this needs to be experimentally verified. As a group, plants vary in Ca^{2+} content and show differential sensitivity to Ca^{2+} as a nutrient [236]. Since we know there is variation in Ca^{2+} levels between plants, even between ecotypes of Arabidopsis (and that variation correlates with *CAX1* 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 Ca^{2+} binding proteins that have been identified in various species. Examples include a celery vacuole-associated dehydrin-like protein [238] and a radish vacuolar Ca^{2+} binding protein [239] that is induced by lack of Ca^{2+}. Neither of these proteins has mutants nor has been overexpressed, so it is not clear how much Ca^{2+} 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 vacuolar 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.

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