The Language of Calcium Signaling

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Abstract

Ca^{2+} signals are a core regulator of plant cell physiology and cellular responses to the environment. The channels, pumps, and carriers that underlie Ca^{2+} homeostasis provide the mechanistic basis for generation of Ca^{2+} signals by regulating movement of Ca^{2+} ions between subcellular compartments and between the cell and its extracellular environment. The information encoded within the Ca^{2+} transients is decoded and transmitted by a toolkit of Ca^{2+}-binding proteins that regulate transcription via Ca^{2+}-responsive promoter elements and that regulate protein phosphorylation. Ca^{2+}-signaling networks have architectural structures comparable to scale-free networks and bow tie networks in computing, and these similarities help explain such properties of Ca^{2+}-signaling networks as robustness, evolvability, and the ability to process multiple signals simultaneously.

Contents

INTRODUCTION

Ca^{2+} is an astonishingly versatile signaling ion that is poised at the core of a sophisticated network of signaling pathways. These pathways integrate information from biotic and abiotic sources and have a resultant impact on gene expression and cell physiology. In plants, calcium signals take the form of transient increases in cytosolic free Ca^{2+} (specifically, the activity of cytosolic free Ca^{2+}, $[Ca^{2+}]_{cyt}$) that arise from the flux of Ca^{2+} into the cytosol, from the external medium and from subcellular compartments in which the concentration of Ca^{2+} is high compared with the cytosol. Stimulus-induced $[Ca^{2+}]_{cyt}$ increases in plant cells often occur as repetitive oscillations or spiking of $[Ca^{2+}]_{cyt}$ where the frequency (period), amplitude and shape (e.g. sinusoidal, square-wave) of the Ca^{2+} increase are determined by the nature of the stimulus. It is thought that stimulus-specific temporal changes in $[Ca^{2+}]_{cyt}$ enable the ion to encode stimulus-specific information within this so-called calcium signature, and thus define the nature and magnitude of the response (4, 108). An additional level of regulation and specificity is achieved by Ca^{2+}-binding proteins that function as Ca^{2+} signal sensors, which sense Ca^{2+} alterations by Ca^{2+} binding to domains such as EF hands (10, 98, 141). These Ca^{2+}-binding proteins decode and relay the information encoded within calcium signatures. Diverse proteins encompassing cellular transporters, enzymatic and signaling proteins including protein kinases, and transcription factors are targets of calcium-binding proteins (44, 136). The interplay between Ca^{2+} signatures and Ca^{2+} sensing thereby contributes to the stimulus specificity of Ca^{2+} signaling. Here, we identify emerging functions for Ca^{2+} signaling in plants, examine the

$[Ca^{2+}]_{cyt}$: activity of cytosolic-free Ca^{2+}

membrane transport mechanisms that control Ca^{2+} movement within the cell thereby driving Ca^{2+} signaling, and explore how Ca^{2+}-binding proteins recognize specific Ca^{2+} increases in order to translate these into specific cellular responses. Finally, we consider the new types of knowledge concerning cell signaling that have emerged from studies of the integrated functioning of entire Ca^{2+} signaling networks.

Evolution of Ca^{2+} Signaling

Maintenance of $[Ca^{2+}]_{cyt}$ at submicromolar activities would have been an early constraint during the evolution of cells. Intermediary metabolism based on energy contained within phosphoanhydride esters (e.g. phosphate-phosphate bonds within ATP) requires that free Ca^{2+} be sustained at a low level otherwise precipitation of calcium salts ensues as a result of the low solubility product of Ca^{2+} with P_i (139). This constraint drove the evolution of transport systems that export Ca^{2+} from the cytosol. In the earliest unicellular organisms, calcium homeostasis would have required export across the plasma membrane. In all eukaryotes, sequestration systems have also evolved at endomembranes. Importantly, the evolution of transport systems that maintain extremely low cytosolic free Ca^{2+} created a cellular environment for the evolution of a signaling mechanism that elevates cytosolic free Ca^{2+} very rapidly by capitalizing upon the enormous electrochemical potential difference for Ca^{2+} across membrane systems that separate "stores" of Ca^{2+} from the cytosol (139).

CELLULAR FUNCTIONS OF Ca^{2+} SIGNALS

Ca^{2+} signals regulate a large number of abiotic stress responses (108), as well as stomatal aperture (121), self-incompatibility during fertilization (49), interactions with pathogenic and symbiotic microorganisms (103, 124), and the development of tip-growing structures such as pollen tubes and root hairs (63). Ca^{2+} signals also participate in light and circadian signaling (39, 72, 147, 149). We highlight recent advances in the functions of Ca^{2+} signals that raise important questions for future research.

Organ- and Cell-Type Specificity of Abiotic Stress Signaling

Ca^{2+} elevations with stimulus-specific properties are evoked by extracellular sodium, osmotic stress, oxidative stress, low temperature, ozone, and mechanical cues (108). Dose-dependent relationships between the concentration of external NaCl and NaCl-induced $[Ca^{2+}]_{cyt}$ spike magnitude in *Arabidopsis* roots (155) recently demonstrated that information concerning abiotic stimulus strength is encoded within this $[Ca^{2+}]_{cyt}$ signal. Stress-induced Ca^{2+} signals have intriguing cell type–specific properties. $[Ca^{2+}]_{cyt}$ oscillations with different properties occur in the pericycle and endodermis after challenge by NaCl or osmotic stress with mannitol. Mannitol causes repetitive $[Ca^{2+}]_{cyt}$ increases in the endodermis with a peak in $[Ca^{2+}]_{cyt}$ every 20–30 s, compared with a sustained $[Ca^{2+}]_{cyt}$ increase in the pericycle (76), but the outcome of the different calcium signatures in these cell types in terms of cellular acclimation to abiotic stress remains unknown. An alternative explanation for the different cell-specific Ca^{2+} signatures of the endodermis and pericycle is that these cell types experience differing levels of exposure to the stress as a result of their location within the root structure, and so the different Ca^{2+} signatures in the cell types are caused by differing stimulus magnitudes. Whether cell type–specific Ca^{2+} signals cause cell type–specific stress responses remains an important and open question.

Regulation of Stomatal Aperture

Ca^{2+} signals are core regulators of stomatal aperture (121). Information encoded in $[Ca^{2+}]_{cyt}$ oscillations alone can program stomatal aperture because artificially imposed oscillations in guard cell $[Ca^{2+}]_{cyt}$ close stomata, and the $[Ca^{2+}]_{cyt}$ oscillation frequency and amplitude determine steady-state aperture (4). The greatest degree of steady-state closure is caused by artificial $[Ca^{2+}]_{cyt}$ increases

CAMTA: calmodulin-binding transcription factor (strictly, calmodulin-binding transcription activator)

that have a period of 10 minutes and when each increase lasts for approximately 5 minutes (4), which is comparable with ABA-induced $[Ca^{2+}]_{cyt}$ oscillations in guard cells that close stomata and have periods of 6 to 8 minutes. An emerging hypothesis is that preexposure of guard cells to ABA and CO_2 increases the sensitivity of Ca^{2+} sensors to subsequent Ca^{2+} increases (71, 148). For example, guard cell preexposure to ABA increases both the magnitude of S-type anion efflux currents and the down-regulation of K^+_{in} currents in response to Ca^{2+} (148). The authors (71) therefore propose that specific stimuli prime specific Ca^{2+} sensors so that the sensor is more readily activated by an increase in $[Ca^{2+}]_{cyt}$. The mechanistic basis for the priming of Ca^{2+} sensors by ABA and CO_2 is unclear but could involve direct interactions between Ca^{2+} sensor(s) and an ABA/CO_2-responsive protein or protein kinase, or alternatively the convergence of Ca^{2+}-dependent and Ca^{2+}-independent components of ABA signaling downstream from Ca^{2+} increases. Stomatal closure is attenuated but not prevented by suppression of ABA-induced $[Ca^{2+}]_{cyt}$ oscillations (148). This does not necessarily indicate that there is a Ca^{2+}-independent pathway to stomatal closure, because if Ca^{2+} sensors are primed, resting $[Ca^{2+}]_{cyt}$ might be sufficient to activate guard cell ion efflux. This is consistent with reports of ABA activation of anion channels in the absence of $[Ca^{2+}]_{cyt}$ increases (91).

Plant-Pathogen Interactions

Ca^{2+} signals are an early response by cells to the presence of pathogenic and symbiotic microorganisms. Surprisingly, defense responses are both suppressed and activated by Ca^{2+} signals, which suggests that Ca^{2+}-responsive but antagonistic signaling mechanisms are present. Molecules with microbe-associated molecular patterns (MAMPs) mobilize Ca^{2+} from both extracellular (apoplast) and intracellular (vacuole/endoplasmic reticulum) stores of Ca^{2+} and cause alterations in nuclear $[Ca^{2+}]$. The increases in nuclear Ca^{2+} that are induced by specific MAMPs are prolonged compared with those induced in the cytosol (e.g. harpin causes 5 min and 150 min increases in cytosolic and nuclear $[Ca^{2+}]$, respectively) and the Ca^{2+} increases measured from cell cultures are sustained rather than oscillatory (86). MAMP-induced $[Ca^{2+}]$ increases lead to the combined Ca^{2+}-dependent activation of mitogen-, salicylic acid (SA)–, and wound-activated protein kinases (86, 103). In contrast to the general principle that stimulus-induced Ca^{2+} increases can encode stimulus-specific information, it is proposed that MAMP-specific patterns of $[Ca^{2+}]_{cyt}$ increase are unusual because they do not encode MAMP-specific information. This is primarily because prolonged $[Ca^{2+}]_{cyt}$ increases induce similar defense responses irrespective of the elicitor (103 and references within). In contrast to MAMP-induced Ca^{2+} induction of defense responses, Ca^{2+} signals also suppress SA-mediated acquisition of systemic acquired resistance (41). The positive regulator of basal resistance and SA levels, *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*), is repressed following Ca^{2+}/calmodulin binding to the Ca^{2+}/calmodulin-binding transcription activator (CAMTA) CAMTA3 (41). In combination these studies suggest that the integration of Ca^{2+} signaling with defense responses is extraordinarily complex and could incoroporate several independent signaling pathways.

Nodulation and Other Symbioses

Nitrogen-fixing bacteria in proximity to legume roots secrete nodulation (Nod) factors. Nod factors cause perinuclear $[Ca^{2+}]_{cyt}$ spiking in root epidermal cells, which initiates cellular internalization of rhizobia and root nodule development (123). The core Nod factor receptor comprises the NOD FACTOR RECEPTOR1 (NFR1)/NFR5 heterodimer and is required for Nod factor–induced $[Ca^{2+}]_{cyt}$ spiking and membrane depolarization (123). Interactions occur between ABA and Nod factor signaling upstream of Ca^{2+} spiking (38), so it would be interesting to discover whether inositol 1,4,5-trisphosphate

($InsP_3$), which mobilizes Ca^{2+} and participates in ABA signaling, is involved in this interaction. Nod factor–induced Ca^{2+} spikes result in Ca^{2+}/calmodulin binding to the Ca^{2+}/calmodulin–dependent protein kinase (CCaMK) DOESN'T MAKE INFECTIONS3 (DMI3) in *Medicago truncatula* (123). This activates DMI3 which promotes early nodulation (*ENOD*) genes. *ENOD* genes are activated by induction of (*a*) the GRAS-family domain transcription factors NSP1 and NSP2 that together form a DNA-binding complex that binds the AATTT promoter motif, and (*b*) the ERF-subfamily transcription factor ERN (65, 123).

$[Ca^{2+}]_{cyt}$ transients also occur in plant cells during the formation of symbiotic relationships between plants and other micro-organisms. $[Ca^{2+}]_{cyt}$ transients occur during development of symbioses with other arbuscular-mycorrhizal (AM) fungi (82, 120) and after exposure to cell wall extracts (CWEs) from the growth-promoting fungus *Piriformospora indica* (158). These signals interact with, but are not identical to, defense signaling because both *P. indica* CWEs and MAMPs increase expression of defense-related *MAP KINASE6* (*MPK6*), but CWEs do not cause downstream defense responses and instead increase the transcript abundance of *CYCLIC NUCLEOTIDE GATED CHANNEL10* (*CNGC10*), *CNGC13*, *CALMODULIN-LIKE PROTEIN42* (*CML42*), and *CML38* (158). This implies that Ca^{2+} signaling is a common feature of plant-microbe interactions.

The Circadian Clock and Phototransduction

Ca^{2+} signals contribute to red light (RL), blue light (BL), and UV-B signaling. Ca^{2+} signaling during phytochrome-mediated RL phototransduction has been reviewed elsewhere (147, 149) so here we focus on the involvement of Ca^{2+} in BL signaling. BL and RL cause brief (60 s) $[Ca^{2+}]_{cyt}$ transients without apparent oscillations (13, 149) and the BL spectrum causes $[Ca^{2+}]_{cyt}$ transient maxima at the 440 nm and 470 nm wavelengths. BL causes Ca^{2+} influx through plasma membrane (lanthanum-sensitive) voltage-gated Ca^{2+} channels but does not cause Ca^{2+} release from the ER (thapsigargin-insensitive) (13). This Ca^{2+} increase is mediated by BL-activation of phototropin blue light photoreceptors (PHOTs) rather than cryptochrome blue light photoreceptors (CRYs) (13, 58, 59). BL Ca^{2+} signals are also required for BL inhibition of seedling growth (46). Several proteins might participate in sensing BL-induced Ca^{2+} increases. Ca^{2+}-binding proteins that have been linked to BL include SHORT UNDER BLUE LIGHT1 (SUB1), an EF-hand–containing protein involved in control of HY5-mediated seedling de-etiolation by phytochrome and cryptochrome (55). However, since SUB1 is involved in CRY and PHY signaling, but CRYs do not appear to mediate BL-induced Ca^{2+} increases (13), the involvement of SUB1 with light induced Ca^{2+} signals might relate primarily to the decoding of phytochrome-mediated Ca^{2+} alterations. PLASTID MOVEMENT IMPAIRED1 (PMI1) has regions with homology to binding domains for interaction with C-domain Ca^{2+}-binding proteins and is required for BL-induced chloroplast rearrangement (34). In contrast to higher plants, in the bryophyte *Physcomitrella patens* BL-induced $[Ca^{2+}]_{cyt}$ increases are mediated by both CRYs and PHOTs (156). This raises the intriguing possibility that in higher plants CRYs might contribute to BL-induced Ca^{2+} signals, but this is not always detected with the aequorin-based technology used for Ca^{2+} measurements. Future comparative studies involving the measurement of BL-induced Ca^{2+} increases in single cells could expand our understanding of whether CRYs are associated with BL-induced Ca^{2+} signals in higher plants or whether this mechanism was lost during the evolution of higher plants.

Circadian oscillations of $[Ca^{2+}]_{cyt}$ occur in continuous light and are controlled by the molecular circadian oscillator (72, 168). Under constant light there is a sinusoidal variation in $[Ca^{2+}]_{cyt}$ over 24 h that is estimated

to reach peak concentrations of 300–700 nM between the middle and end of the subjective day (72, 96). Similar $[Ca^{2+}]_{cyt}$ variations occur under light–dark cycles (96) but do not persist under continuous darkness, requiring light input via PHYTOCHROME B (PHYB), CRYPTOCHROME1 (CRY1), and CRY2 (168). Circadian rhythms of Ca^{2+} are thought to be positioned downstream of the calcium-mobilizing molecule cyclic ADP ribose (cADPR) (39). In light–dark cycles, there is a rhythm of ADP ribosyl cyclase activity in *Euglena gracilis* with peak activity during the light period (106); in continuous light, circadian cADPR fluctuations occur in *Arabidopsis* (39). In *Arabidopsis*, perturbation of circadian Ca^{2+} oscillations or cADPR signaling alter circadian clock function, which suggests that Ca^{2+}/cADPR forms a feedback loop within the clock because the circadian clock is required for circadian $[Ca^{2+}]_{cyt}$ oscillations to occur (39, 168). As with many Ca^{2+} signals in plants, the downstream Ca^{2+}-binding proteins that decode circadian $[Ca^{2+}]_{cyt}$ oscillations are unidentified. It is not known whether circadian $[Ca^{2+}]_{cyt}$ dynamics are distributed uniformly within the cytosol or are alternatively an aggregation of frequency- or amplitude-modulated $[Ca^{2+}]_{cyt}$ spikes. The extensive cross talk between circadian timing and stress signaling networks (39) indicates that it will be important in the future to discover whether circadian $[Ca^{2+}]_{cyt}$ oscillations participate in abiotic stress responses, particularly given the contribution of cADPR-mediated Ca^{2+} release to abscisic acid signaling.

Control of Polar Tip Growth by Tip-Focused Ca^{2+} Gradients

Tip-focused Ca^{2+} gradients are important determinants of polarity in tip-growing cells such as root hairs, pollen tubes, fungal hyphae, and algal rhizoids (63). Here, we consider research developments in Ca^{2+} signaling during root hair extension because there have been several recent developments in our knowledge of Ca^{2+} signaling in this cell type. The NADPH oxidase ROOT HAIR DEFECTIVE2 (RHD2, also AtRBOHC) is localized to the plasma membrane of the growing tip of root hairs (152). RHD2 produces reactive oxygen species (ROS) that stimulate hyperpolarization-activated Ca^{2+} channels of unknown molecular identity, leading to formation of a tip-focused Ca^{2+} gradient (47). This is thought to target the cytoskeleton and secretory apparatus to the growing tip. Polarity is proposed to be self-sustained though positive feedback, in which ROS-induced Ca^{2+} influx maintains elevated Ca^{2+} at the root hair tip, which results in synergistic activation of RHD2 by both Ca^{2+} binding to two EF-hands on RHD2 and Ca^{2+}-dependent phosphorylation of two serine residues on RHD2 (152). Consistent with this hypothesis, there are oscillations in the elevated tip-focused $[Ca^{2+}]_{cyt}$ gradient and in root hair extension, separated by a lag of approximately 5 s (112). The oscillations are proposed to arise from a burst of root hair extension causing a pulse of Ca^{2+} influx that subsequently induces ROS production, further Ca^{2+} influx and the next pulse of growth (112). This model is dependent upon the localization of RHD2 and relevant channels to the tip so that Ca^{2+} influx occurs only in this region, but the mechanisms controlling this distribution are unclear and this represents an important area for future research. Annexins, which can generate Ca^{2+}-permeable conductances (85), are concentrated in tip-growing structures (114) and are an attractive potential regulator of root hair polarity. The Rho GTPase GDP dissociation inhibitor (RhoGDI) SUPERCENTIPEDE1 (SCN1) regulates tip-focused ROS production (18), and the Rop GTPase ROP2 might do so as well (73).

The Self-Incompatibility Response

Ca^{2+} signals are involved in reproductive self-incompatibility in poppy (*Papaver rhoeas*). To prevent inbreeding, self-produced pollen that shares the same *S*-allele as the stigma is recognized by the growing pollen tube when it develops on the stigma. This causes

the self-incompatibility (SI) response that stops pollen tube growth and prevents self-fertilization. In poppy $[Ca^{2+}]_{cyt}$ alterations act as an early signal in prevention of self-fertilization and activate several inhibitory mechanisms. Within seconds of pollen tube challenge with self- (i.e., incompatible) pistil S-proteins (PrsS), $[Ca^{2+}]_{cyt}$ in the pollen tube shank increases from ~200 nM to 1.5 μM for several minutes and the oscillatory tip-focused $[Ca^{2+}]_{cyt}$ gradient dissipates (48). Artificial $[Ca^{2+}]_{cyt}$ elevation within the pollen tube mimics the SI response (49). The pollen SI determinant is the plasma membrane protein PrpS and this interacts with the pistil self-determinant PrsS to trigger the SI response when pollen PrpS interacts with pistil self-PrsS (164). The molecular identity of the Ca^{2+} channels that cause Ca^{2+} influx during the SI response remains to be discovered. The SI response also involves Ca^{2+}/calmodulin-dependent inhibitory hyperphosphorylation of the soluble inorganic pyrophosphatases Pr-p26.1a and Pr-p26.2b. This is proposed to cause P_i to increase during the SI response and so inhibit pollen tube metabolism (33). The SI Ca^{2+} signal also promotes caspase (cysteine-aspartic protease)-like activity, which is a component of mechanisms causing programmed cell death (153).

TRANSPORT SYSTEMS THAT ENCODE Ca^{2+} SIGNALS

Calcium transport systems have to maintain low $[Ca^{2+}]_{cyt}$ against a significant electrochemical potential difference for Ca^{2+} (i.e., not only a concentration difference but also an electrical potential) and thus are energized (141). In plants energization is accomplished either through Ca^{2+} pumps powered by ATP hydrolysis or through Ca^{2+}-H^+ antiporters powered by a proton-motive force (141). A conventional view of these energized systems in plant cells is that they provide the homeostatic background against which Ca^{2+}-release channels operate transiently to elevate free $[Ca^{2+}]_{cyt}$ (139). Opening of such channels should therefore provide at least the initial spike for the elevation of $[Ca^{2+}]_{cyt}$. In general, this paradigm remains intact. However, as discussed in the next section, research involving mutants in genes encoding energized transport systems has yielded intriguing results demonstrating that energized transport of Ca^{2+} from the cytosol might provide much more than a housekeeping background to Ca^{2+} signaling.

Energized Systems: Ca^{2+}-Pumping ATPases

ATP-dependent export of Ca^{2+} from the cytosol is accomplished by P-type ATPases of the P_2 class. P_2 Ca^{2+}-pumping ATPases comprise two distinct clades. In *Arabidopsis* these are ER (endoplasmic reticulum)-type Ca^{2+}-ATPases (ECAs) of the P_{2A} group, and AUTOINHIBITED Ca^{2+}-ATPases (ACAs) of the P_{2B} group. Two major features distinguish ECAs from ACAs (17). First, an N-terminal cytosolic domain present only in ACAs binds calmodulin that is bound to Ca^{2+}, and this interaction activates Ca^{2+} pumping (8). Second, there are differences in membrane-located residues that are thought to be involved in Ca^{2+} binding.

This latter difference might account for the fact that insertional mutants in at least two of the four *Arabidopsis ECA* genes have Mn^{2+}- as well as Ca^{2+}-related phenotypes. Thus, while plants lacking the ER-localized ECA1 pump (166) and those lacking the Golgi- or endosome/post-Golgi-localized ECA3 (93, 111) exhibit growth-sensitive phenotypes to low Ca^{2+} concentrations, all mutants also exhibit phenotypes with respect to Mn^{2+}. Therefore, this group of Ca^{2+}-ATPases seems to be involved principally in delivery of cations to intracellular compartments where there is a requirement for secretion or as a cofactor for enzymatic activity, rather than in cytosolic Ca^{2+} homeostasis and signaling per se.

By contrast, ACA pumps, which comprise a 10-member gene family in *Arabidopsis*, are emerging as potential key players in plant Ca^{2+} signaling. Hints at this role come from expression profiling. For example, *ACA12* and

ACA13 transcripts are dramatically upregulated by pathogen stress (17). Furthermore, transcript abundance of the closely-related ACA8 and ACA10 is differentially regulated by cold (142), whereas transcripts of ACA8 and ACA9 are both acutely upregulated by ABA (21).

ACAs 8, 9, and 10 are plasma membrane localized (16, 142, 143), and distinct roles in signaling and development have become apparent. T-DNA insertional mutants in the *ACA9* gene exhibit partial male sterility, consistent with expression primarily in pollen (143). This finding need not of itself indicate a key signaling role for ACA9, despite the central function of Ca^{2+} in dictating pollen tube growth and guidance (63): It is possible that a general disruption of cytosolic Ca^{2+} homeostasis in *aca9* mutants impacts the phenotypes of reduced pollen tube growth and discharge of sperm cells into ovules.

However, an intriguing phenotype of *aca10* mutants suggests that ACA pumps play critical developmental roles. A compact inflorescence (cif) phenotype in *Arabidopsis* is specific to *aca10* mutants but not to *aca8* or *aca9* mutants (53). Nevertheless, overexpression of ACA8 in an *aca10* background will complement the cif phenotype, suggesting that differences in expression among members of this subgroup of P_{2B} ATPases can impact development.

ACA pumps are also subject to complex posttranslational regulation, including activation by calmodulin, regulation by acidic phospholipids (15), and in the case of the ER-localized ACA2, phosphorylation (68). These multiple modes of control, differential intracellular locations (e.g., ACA4 and ACA11 localize to vacuoles; 52, 88), and differing tissue-specific expression of ACAs are challenging in terms of understanding the extent to which ACAs have specific roles in Ca^{2+} signaling. In *Physcomitrella* an ACA-type deletion of Ca^{2+}-ATPase gene results in enhanced sensitivity to NaCl and this is associated with aberrant regulation of $[Ca^{2+}]_{cyt}$ (134). To date, there have been no such studies in higher plants, making this a fruitful area for further investigation.

Energized Systems: Calcium–Proton Exchangers

With respect to P_2 ATPases, calcium-proton exchangers are low-affinity cytosolic export systems, coupled to the thermodynamically downhill exchange flux of H^+. It is likely that there are six bona fide calcium–proton exchangers, known as CATION EXCHANGER1 to -6 (CAX) (146), encoded in the *Arabidopsis* genome. CAX transporters are members of the Major Facilitator Superfamily and are predicted to have 11 transmembrane domains (TMDs). Where membrane location has been characterized, in the cases of CAX1 to CAX4, the localization is vacuolar (146).

In *Arabidopsis*, CAX1 and CAX3 appear to be prominent in Ca^{2+} homeostasis. *cax1* mutants exhibit developmental reductions in lateral root length and number, a dramatic reduction in the length of the primary inflorescence (24), and increased capacity for cold acclimation associated with enhanced expression of *CBF/DREB1* (20). Although CAX1 is more strongly expressed in shoot tissue and CAX3 more so in roots, *cax1/cax3* double mutants have significantly more severe phenotypes than either of the single mutants, including leaf tip necrosis and ionic content (25). *cax3* mutants are also more susceptible to salt stress (171).

CAX transporters, like ACA pumps, are subject to posttranslational regulation in plants through an autoinhibitory N-terminus (110) and possibly also through regulatory protein–protein interactions (23, 26). In combination with *cax* mutant phenotypes, this posttranslational regulation suggests an acute role for CAXs in Ca^{2+} homeostasis. A critical current question revolves around the nature of the mutant phenotypes of these energized cytosolic export systems: Are they merely disrupted in Ca^{2+} homeostasis, as upregulation of alternative Ca^{2+} transporters can imply in some mutants (24, 25), or is there a more fundamental role in Ca^{2+} signaling?

Whether energized Ca^{2+} transport systems are more than background players in

the Ca^{2+} signaling toolbox, and, like PCAI in *Physcomitrella*, play an active role in shaping Ca^{2+} signals, will only be established once measurements of $[Ca^{2+}]_{cyt}$ have been performed in mutants lacking these transport systems.

Calcium Entry to the Cytosol

Passive return flow of Ca^{2+} down the electrochemical potential generated by ATPases and CAXs is thought to be the primary driver for Ca^{2+} signaling (141). This flux occurs through ion channels. For example, plasma membrane Ca^{2+}-permeable channels, which are in normal conditions relatively inactive, are activated by ABA or by ROS in guard cells (117, 127), two stimuli that effect stomatal closure. ROS also activate Ca^{2+}-permeable channels at the plasma membrane of root hairs during Ca^{2+}-regulated cell expansion (47). These ROS-related activation events might involve extracellular ATP signaling (36). However, depending on the stimulus, Ca^{2+} signals can be generated across membranes other than the plasma membrane: Cold shock induces mobilization of vacuolar Ca^{2+} (79), while Nod factors generate $[Ca^{2+}]_{cyt}$ signals that emanate from the perinuclear region ER of legume root hairs (123).

Electrophysiological studies, particularly during the 1990s, established that a variety of different Ca^{2+}-permeable channel types exist in plants (reviewed in 35, 141). The plasma membrane possesses Ca^{2+}-permeable channels with a range of voltage-dependencies: some are activated by membrane hyperpolarization or by depolarization, and some are voltage-insensitive. In accord with the notion that Ca^{2+} can be mobilized across endomembranes, voltage-dependent channels also reside in the vacuolar membrane and ER. Additionally, electrophysiological approaches with intact vacuoles and radiometric approaches using membrane vesicles have established the presence of ligand-gated Ca^{2+}-permeable pathways at endomembranes. Second messengers such as $InsP_3$ and cADPR release Ca^{2+} from the vacuolar lumen (e.g., 5). Both ligands also liberate Ca^{2+} from the ER (116, 119), along with nicotinic acid adenine dinucleotide phosphate (NAADP; 118).

$InsP_3$ participates in pollen tube orientation, salt and hyperosmotic stress signaling, ABA signaling and gravitropism, while cADPR mediates the activation of some defense genes and functions in ABA and circadian signaling (39, 141). These findings confirm the physiological relevance of the two ligands and, in combination with results demonstrating that $InsP_3$ and cADPR mobilize Ca^{2+}, suggest the presence of receptors that also function as Ca^{2+} channels, as is the case in mammalian cells. However, homologues of mammalian $InsP_3$ receptor and ryanodine receptor (the receptor for cADPR) are not encoded by higher plant genomes, and the molecular identities of the $InsP_3$- and cADPR-activated Ca^{2+}-release pathways in higher plants remain unknown. The absence of this molecular handle has impeded progress in understanding the roles of $InsP_3$ and cADPR in plant Ca^{2+} signaling.

Until relatively recently, the molecular identities of plasma membrane Ca^{2+}-permeable channels and those not controlled by ligands at endomembranes were unknown. Forward and reverse genetic approaches have now yielded specific information on the molecular identities and physiological roles of some plant Ca^{2+}-permeable channels (161).

Cyclic Nucleotide–Gated Channels

Arabidopsis possesses a large gene family of 20 members that encode cyclic nucleotide-gated channels (CNGCs) (105). These channels have six TMDs and a pore domain, and probably assemble tetramerically to form the pore. Some plant CNGCs are Ca^{2+}-permeable when expressed heterologously (90, 157), although some are also permeable to monovalent ions (9, 89). Besides a cyclic nucleotide-binding domain, CNGCs also bind calmodulin (144). All CNGCs studied to date localize to the plasma membrane (7, 54, 157).

Mutants in *Arabidopsis* CNGC2, CNGC4, CNGC11, and CNGC12 have aberrant regulation of pathogen defense responses (9, 29, 170),

and *cngc2* mutants additionally lack a cAMP-gated Ca^{2+} current at the plasma membrane of guard cells (3). The manner in which CNGCs are activated during defense signaling has yet to be determined. A more diverse role for members of the CNGC family has been indicated in *cngc18* mutants, which are defective in pollen tube growth (50).

Glutamate-Receptor-Like Channels

Plants contain homologues of animal ionotropic glutamate receptors that function as nonselective cation channels at postsynaptic membranes. The *GLUTAMATE RECEPTOR* (*GLR*) gene family in *Arabidopsis* comprises 20 members, each of which encodes a protein with three predicted TMDs. The entire channel is probably formed as a tetramer or pentamer (32).

Glutamate, as well as five other amino acids and even glutathione, applied to *Arabidopsis* seedlings generates an inward current and a cytosolic Ca^{2+} spike, and both responses are attenuated in *glr3.3* mutants (37, 132). Different members of the GLR family respond differently to activating ligands (150), but it is not clear which ligands are physiologically active. Nevertheless, exogenous glutamate and glycine have roles in processes as diverse as hypocotyl elongation (43) and the regulation of C and N metabolism (74).

For both GLR and CNGC ion channels, the potential for members of large gene families to form heteromultimeric complexes might explain the diversity of plant plasma membrane Ca^{2+}-permeable channels that have been reported in electrophysiological studies. However, a note of caution is also necessary. Channels that depolarize a membrane when activated might contribute to a cytosolic Ca^{2+} response merely by activating some other voltage-sensitive pathway. One example for which this is almost certainly the case relates to perinuclear Ca^{2+} spiking during Nod-factor perception. CASTOR and POLLUX (in *Lotus*) and DMI1 (in *Medicago*) are nucleus-localized channels, and mutants lack Nod-factor-induced Ca^{2+} spiking. However, all three channels, when studied heterologously, have characteristics that suggest they are regulators of Ca^{2+}-permeable channels but do not themselves form the physiological pathway for Ca^{2+} release (22, 129). Indeed, perinuclear Ca^{2+} spiking in *Medicago* can be elicited in *dmi1* mutants by mastoparan, a G-protein agonist (151).

Two-Pore Channels

The *Arabidopsis* genome contains a single member of the *TWO-PORE CHANNEL* (*TPC*) family, *TPC1*. The protein is predicted to have 12 TMDs and incorporate two pore domains (hence the name), and is likely to form a homodimer. A cytosolic loop between TMDs 6 and 7 includes two putative calcium-binding EF-hands and a 14-3-3 binding domain, and TMDs 4 and 10 have positively charged residues that suggest the channel should be voltage gated (128).

TPC1 localizes in *Arabidopsis* to the vacuolar membrane, and mutants lack activity of the so-called slow vacuolar (SV) channel (128) that dominates the vacuolar membrane conductance at high $[Ca^{2+}]_{cyt}$ (62). SV/TPC1 channels appear to be expressed ubiquitously (62). The TPC1 protein is unusually highly expressed for a channel protein, appearing in a number of vacuolar proteomics analyses (19, 165). Electrophysiological studies demonstrate that this Ca^{2+}-activated channel is Ca^{2+} permeable (6, 162), suggesting that the channel provides a pathway for Ca^{2+}-induced Ca^{+} release. This has been confirmed in a careful study involving patch clamp electrophysiology combined with noninvasive ion flux measurements using ion-selective microelectrodes (131).

Deletion mutants in *TPC1* are defective in two Ca^{2+} signaling pathways—Ca^{2+}-induced stomatal closure and ABA-delayed germination (128)—but not in a selective range of other types of Ca^{2+} signaling (135), demonstrating the stimulus specificity of Ca^{2+}-permeable channel activation. A constitutively active mutant of *TPC1* (*fou2*) exhibits jasmonate overproduction (14). One way that TPC1 channel

activation might occur at the vacuolar membrane is illustrated in **Figure 1** (140, 162).

Annexins as Novel Plant Ca^{2+}-Permeable Channels

Annexin proteins purified from maize can function as Ca^{2+}-permeable channels when incorporated into planar lipid bilayers and can also elicit elevations of $[Ca^{2+}]_{cyt}$ when added to protoplasts (85). The purified protein also has peroxidase activity, and it is suggested that annexins might form plasma membrane Ca^{2+}-permeable channels during stress responses.

PERCEPTION AND DECODING OF Ca^{2+} SIGNALS

The increases in cytosolic and nuclear free calcium that occur during signal transduction are detected by calcium sensor proteins. Proteins involved in the perception and decoding of Ca^{2+} signals are present in the cytosol and nucleus, and are both free and attached to membranes. The large number of calcium sensor proteins with different Ca^{2+}-binding characteristics, subcellular localizations and downstream signaling interactions is thought to provide a toolkit that enables the decoding of information within Ca^{2+} oscillations and spikes and the processing of this information into alterations in cell function.

Calcium sensor proteins are classified as either sensor relays or sensor responders (141). Sensor responder proteins such as Ca^{2+}-dependent protein kinases (CDPKs) combine within a single protein (*a*) a sensing function, mediated by calcium-binding domains that often cause Ca^{2+}-induced conformational changes, and (*b*) a response activity (e.g., kinase activity). In contrast, sensor relay proteins such as calmodulin also contain multiple calcium-binding domains and usually undergo Ca^{2+}-induced conformational changes but lack other effector domains (**Figure 2**). To transmit the Ca^{2+} signal, sensor relay proteins

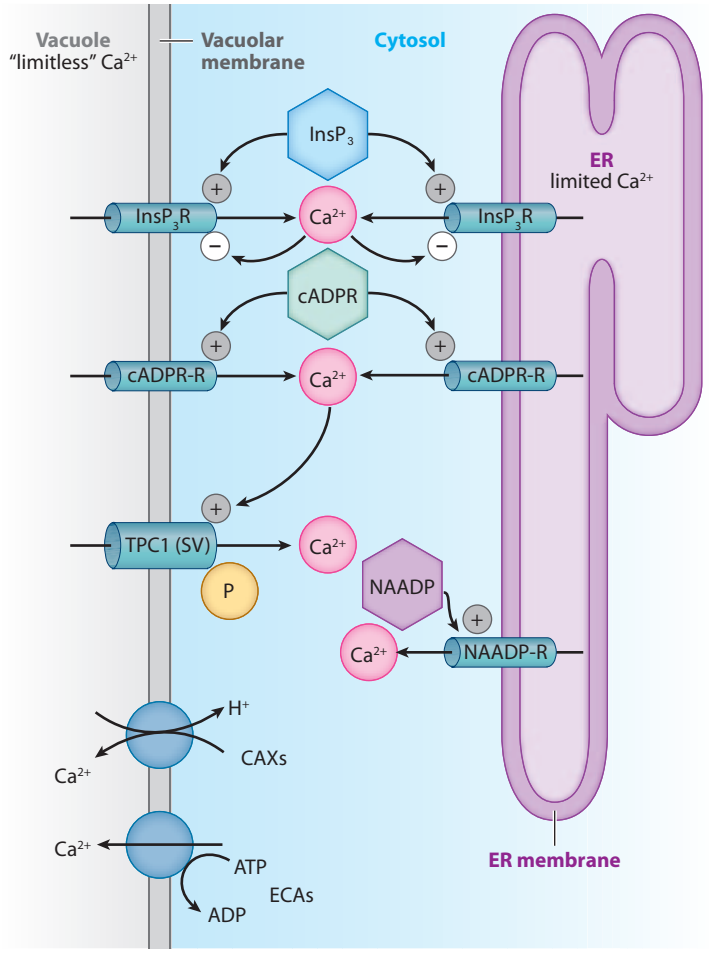

Figure 1

Ca^{2+}-induced Ca^{2+} release in plant cells involves the vacuole and ER. The vacuole and ER store different quantities of Ca^{2+} and their membrane systems are sensitive to different combinations of small molecules. Trigger Ca^{2+}, released through ligand-activated Ca^{2+} channels, might activate the TPC1/SV channel (*a*) directly through binding to the EF-hands, (*b*) indirectly through membrane depolarization resulting from cytosol-directed movement of Ca^{2+}, or (*c*) through eliciting Ca^{2+}-activated K^+ channels (not shown). This dual form of activation would trigger regenerative Ca^{2+} release, for which some kind of negative feedback—perhaps through kinase inhibition—would be required. Abbreviations: cADPR, cyclic adenosine diphosphate ribose; cADPR-R, receptor for cADPR (unknown in plants); CAX, Ca^{2+}/H^+ antiporter (*ca*tion *ex*changer); ECA, ER-type Ca^{2+} ATPase; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, receptor for inositol 1,4,5-trisphosphate (unknown in plants); NAADP, nicotinic acid adenine dinucleotide phosphate; NAADP-R, receptor for NAADP; TPC1, TWO-PORE CHANNEL1.

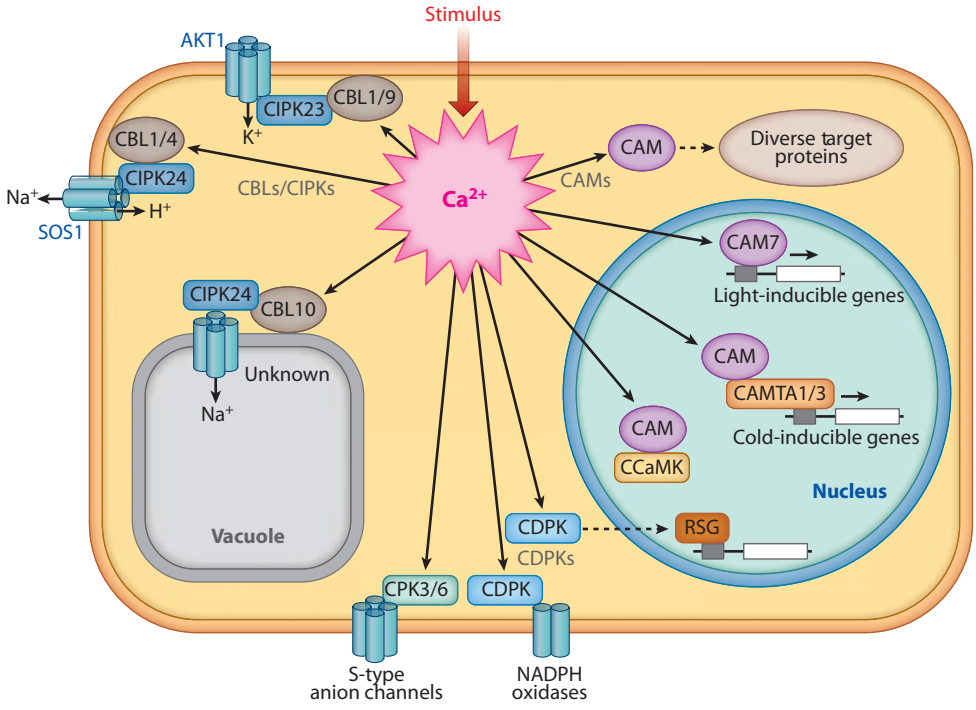

Figure 2

An array of mechanisms decode Ca^{2+} signals and elicit highly specific responses that depend on spatial and temporal $[Ca^{2+}]_{cyt}$ variations. Abbreviations: CAM, calmodulin; CAMTA, Ca^{2+}/calmodulin-binding transcription activator; CBL, calcineurin B-like protein; CCaMK, calcium/calmodulin-dependent kinase; CDPK, Ca^{2+}-dependent protein kinase; CIPK, CBL-interacting protein kinase; CPK, protein encoded by *CALCIUM DEPENDENT PROTEIN KINASE* gene family; RSG, promoter of *REPRESSION OF SHOOT GROWTH*; SOS1, the Na^+/H^+ antiporter SALT OVERLY SENSITIVE1.

must therefore interact with target proteins and regulate their activity (98). Although this concept of functional classification was initially applied to the conversion of Ca^{2+} signals into phosphorylation responses, its relevance for the regulation of transcriptional processes by Ca^{2+} signals is becoming apparent. While Ca^{2+} binding to CALMODULIN7 (CAM7) appears to result in direct promoter interaction and regulation, other calmodulins are likely to mediate gene regulation via interacting CAMTAs that function as transcriptional (co)regulators. Metabolic and biosynthetic processes like brassinosteroid synthesis are important targets of direct Ca^{2+}-dependent modulation (42), but Ca^{2+}-dependent phosphorylation and Ca^{2+}-dependent gene regulation represent the major cellular currencies for converting defined Ca^{2+} signatures into specific downstream reactions and are discussed in detail here.

CDPK: Ca^{2+}-dependent protein kinase

Connecting Ca^{2+} with Transcription

The mechanisms of Ca^{2+}-dependent transcriptional regulation involve numerous signal transducers including Ca^{2+}-binding proteins (69). However, despite the importance of defined Ca^{2+} signatures during reactions to, e.g., distinct abiotic stress conditions such as cold, drought, and salinity (108), the molecular identity of those genes subject to Ca^{2+}-dependent regulation and the molecular mechanisms mediating Ca^{2+}-responsive gene expression have remained little understood. In part, this is because of the difficulty in

distinguishing stress-dependent Ca^{2+} responses from stress-dependent but Ca^{2+}-independent reactions (45, 115). The induction of defined artificial Ca^{2+} transients by the use of the calmodulin antagonists WP7 and SKF-7171 allowed identification of 230 Ca^{2+}-responsive genes that were differentially expressed 1 h post stimulus (75). Considering that this study covered only 25% of known *Arabidopsis* genes, these results suggest that approximately 3.3% of *Arabidopsis* genes are subject to regulation by Ca^{2+}. Many of the Ca^{2+}-regulated genes identified by this study were known early stress-induced genes. Importantly, this investigation established that known ABRE (abscisic acid–responsive element)-related *cis*-promoter elements are sufficient to confer transcriptional regulation in response to cytosolic Ca^{2+} signatures. Since ABREs are present in the promoter of C-REPEAT/DRE BINDING FACTOR (CBF or DREB1) transcription factors that function as master regulators of abiotic stress responses (45), these findings point to a direct interconnection between Ca^{2+} regulation of transcription and abiotic stress responses but raise the question of how Ca^{2+} signals are transduced to the transcription machinery. The answer may be provided in part by the function of CAMTA proteins.

CAMTAs are a family of eukaryotic Ca^{2+}-dependent calmodulin-binding transcription factors with six members in *Arabidopsis* (44). These transcription factors share a conserved domain structure, including a C-terminal calmodulin-binding domain that mediates interactions with calmodulin and an N-terminal CG-1 domain that mediates binding to DNA *cis*-elements (CAMTA binding sites), the latter including ABREs and additional CGCG elements (44). CAMTA gene expression in *Arabidopsis* responds rapidly and transiently to various stresses (e.g., cold, salinity) and hormones (e.g., ABA, jasmonic acid) (169), suggesting their involvement in multiple signal transduction pathways that are critical for plant stress tolerance. First insights into the physiological function of plant CAMTA proteins were provided by a reverse genetic analysis of *Arabidopsis* CAMTA3 function that revealed a critical role for this protein in suppressing plant responses to pathogens such as *Pseudomonas syringae* and *Botrytis cinerea* (51). Importantly, a recent study (40) provided evidence for a direct link between Ca^{2+} signaling (via CAMTA1 and CAMTA3) and cold tolerance in plants by discovering that these CAMTA proteins bind to regulatory elements (CAMTA binding sites) in the promoter of the *DREB1c/CBF2* gene. Whilst cold induction of *CBF2* and other cold-induced genes is reduced in a single *camta3* mutant, *camta1/camta3* double mutants are impaired in their cold acclimation to freezing tolerance (40).

These findings establish a role for Ca^{2+}/calmodulin-regulated CAMTA transcription factors in controlling the CBF regulon of cold-regulated genes and promoting freezing tolerance. Moreover, they suggest a model in which CAMTAs may function directly in the transduction of cold-induced cytosolic Ca^{2+} signatures into the regulation of gene expression through interaction with one or more of the seven *Arabidopsis* Ca^{2+}/calmodulin sensors (109). Further investigations will need to address exactly how changes in $[Ca^{2+}]_{cyt}$ lead to changes in nuclear transcription.

Calmodulins are prototypical Ca^{2+} sensor relay proteins, and their genomics, structural properties, and functional principles have been reviewed comprehensively (98, 109). A surprising twist to our view about calmodulin function in plants was provided by a recent study of CALMODULIN7 (CAM7) from *Arabidopsis* (84). In *Arabidopsis* seven genes encode four CAM isoforms, of which CAM1/CAM4 differ by four amino acid substitutions from CAM7, whereas CAM2/3/5 and CAM 6 differ by one amino acid from CAM7 (109). Kushwaha et al. (84) established that CAM7, but not CAM2/3/5, is a transcriptional regulator that interacts directly with promoters of several light-inducible genes. *cam7* mutants did not have photomorphogenic growth alterations, most likely due to overlapping functions, but *cam7* mutants had reduced expression of light-inducible genes. Conversely, overexpression

CIPK: CBL-interacting protein kinase

of CAM7 caused an increase in expression of light-inducible genes and hyperphotomorphogenic growth. These findings suggest that the calcium sensor CAM7 translates cytosolic Ca^{2+} signatures into gene expression through DNA binding. However, future work needs to investigate whether this requires the interaction of CAM7 with additional (transcription factor) proteins and exactly how the interconnection between cytosolic Ca^{2+} signatures and nuclear gene regulation is achieved.

Connecting Ca^{2+} with Protein Phosphorylation

Phosphorylation cascades regulated by kinases and phosphatases are primary downstream interpreters of Ca^{2+} signals. Ca^{2+} transients are perceived and transmitted by Ca^{2+}-dependent kinases and phosphatases. These proteins can alter biochemical function directly and rapidly through reversible phosphorylation, and also cause alterations in gene expression by modulating transcription factor activity (139). Plants have a unique repertoire of Ca^{2+}-dependent protein kinases that comprise the families of CCaMKs (*C*alcium-*Calm*odulin-Dependent *K*inases), CDPKs (*C*alcium-*D*ependent *P*rotein *K*inases), and CIPKs (CBL-Interacting Protein Kinases) that form an intricate cellular network for decoding Ca^{2+} signals and regulating cellular processes, including ion homeostasis (see **Figure 2**) (10, 141). While CDPKs and CCaMKs are typical sensor responders, the CIPKs are targets of *C*alcineurin *B–l*ike (CBL) sensor relay proteins (note that CCaMKs are absent from the *Arabidopsis* genome).

The *Arabidopsis* genome encodes 34 CDPKs and 8 additional CDPK-related kinases (66). The biochemistry and regulation of CDPKs have been reviewed (60, 61, 100). Activation of CDPKs is assumed to occur after binding of Ca^{2+} to the C-terminal EF-hand-containing regulatory domain, causing conformational changes that relieve the active site of the kinase domain from masking by an autoinhibitory domain. This process is paralleled by autophosphorylation of the CDPKs that contributes to full activation of the kinases (100).

The first in vivo evidence for CDPK function was obtained by suppression of *NtCDPK2* by viral-induced gene silencing (VIGS) in *Nicotiana benthamiana* (137). CDPK-silenced plants had a reduced and delayed hypersensitive response after race-specific Avr9 elicitation in a gene-for-gene interaction, and lacked an accompanying wilting phenotype. Remarkably, further analysis of NtCDPK2 function suggests that elevated CDPK signaling inhibits stress-induced MAPK activation, and this inhibition requires ethylene synthesis and perception (101). This indicates that CDPK and MAPK pathways do not function independently and concerted regulation of both pathways controls response specificity to biotic and abiotic stress.

Reverse genetic analyses have substantially extended our knowledge on the physiological function of several CDPKs. *Arabidopsis* CALCIUM DEPENDENT PROTEIN KINASE3 (CPK3) and CPK6 function in ABA regulation of stomatal closure and modulate guard cell S-type anion channels (113). In addition, CPK4 and CPK11 are critical for ABA responsiveness of guard cells and they phosphorylate the ABA-responsive transcription factors ABSCISIC ACID RESPONSIVE ELEMENT-BINDING FACTOR1 (ABF1) and ABF4 in vitro (172). Experiments in tobacco revealed that CDPK1 regulates the transcription factor REPRESSION OF SHOOT GROWTH (RSG) in response to gibberellins (70), and work in potato suggests that several CDPKs regulate ROS production by NADPH oxidases (80). Together, these findings indicate critical roles for CDPK-mediated Ca^{2+} signaling in a very diverse array of processes. However, it will be most important to discover the mechanistic basis for how specific CDPKs contribute to the decoding of specific Ca^{2+} signatures (see **Figure 2**).

The CBL protein family and their interacting kinases (CIPKs) separate Ca^{2+}-binding functionality (sensor relay function) and kinase activity (response activity) into two flexible,

combinable modules. This allows for the formation of a complex and dynamic Ca^{2+}-decoding signaling network. Since the discovery of CBLs and CIPKs in *Arabidopsis* (83, 145), advances in our knowledge of the structural features, evolution, and functional principles of this Ca^{2+}-decoding system have been reviewed (10, 11, 97–99, 163). CBL proteins have significant similarity to the regulatory B subunit of calcineurin and neuronal calcium sensor (NCS) proteins from animals and yeast (83). CBLs contain four Ca^{2+}-binding EF-hand domains that are arranged with invariant spacing (81). CIPK-type kinases comprise a conserved N-terminal kinase domain with high similarity to yeast SNF1, and a C-terminal regulatory domain that is separated from the kinase domain by a variable junction domain. Within the rather divergent regulatory domain, the conserved NAF domain is required and sufficient for interaction with CBLs (1). Moreover, a protein-phosphatase interaction (PPI) domain that mediates CIPK interaction with PP2C phosphatases is present in the C-terminus of these kinases (122). It is assumed that CBL binding to the NAF domain of CIPKs releases the C-terminal (autoinhibitory) domain from the kinase domain, thereby transforming the kinase into an active state (56). Comprehensive bioinformatic analyses of both protein families have identified 10 *CBL*s and 26 *CIPK*s in the *Arabidopsis* genome, and 10 *CBL*s and 30 *CIPK*s in rice (1, 81, 163). Single *CBL* and *CIPK* genes are present in several species of green algae, while *Physcomitrella* contains four *CBL*s and seven *CIPK*s and the genome of the fern *Selaginella moellendorfii* has five *CBL*s and five *CIPK*s (11, 163). These findings suggest that the evolution of plants was accompanied by the evolution of complexity of the CBL and CIPK protein families.

Spatial specificity is an important aspect of cellular information processing. Localization studies of *Arabidopsis* CBL proteins revealed that four CBLs are present at the plasma membrane, four are localized to the vacuolar membrane, and two are present in the cytoplasm and nucleus (12, 28, 31, 77, 163). This suggests that CBL-CIPK complexes could function as fast responders to local Ca^{2+} release events from internal and external stores and that the spatial separation of distinct CBL-CIPK complexes contributes to spatial specificity in Ca^{2+} signaling. For the plasma membrane–localized CBL1, dual lipid modification by myristoylation and S-acylation are required for both its function and its localization to the plasma membrane. CBL1 localization is achieved by a two-step targeting process in which initial myristoylation results in localization to the endoplasmatic reticulum (ER) and subsequent S-acylation is crucial for ER-to-plasma membrane trafficking (12).

Most CIPK-GFP fusion proteins have cytosolic and nuclear localization (11, 31, 163). However, CBL-CIPK interaction analyses using bimolecular fluorescence complementation (BiFC) revealed that CIPKs are targeted to different compartments of the cell by their respective interacting CBL proteins (12, 28, 31, 160). For example, CIPK1 is targeted to the plasma membrane by CBL1 or CBL9 (28, 160) but upon interaction with CBL2 the resulting CBL2/CIPK1 complexes are exclusively vacuolar membrane-localized (12).

Initial insights into the physiological function of CBLs and CIPKs came from forward genetic screens. The CBL calcium sensor SOS3 (AtCBL4) and the CIPK-type kinase SOS2 (AtCIPK24) appear to be part of a Ca^{2+}-regulated signaling pathway that specifically mediates salt stress adaptation by regulating the Na^+/H^+ antiporter SOS1 (see **Figure 2**) (57, 94, 95). Recent studies revealed that the calcium sensor CBL10 also interacts with and activates the kinase CIPK24 (77, 133). CBL10/CIPK24 complexes are vacuolar membrane-localized, thereby supporting the functional concept that alternative complex formation of CIPK24 kinases with either CBL4 or CBL10 creates a dual-function kinase with separate functions at the plasma and vacuolar membranes (see **Figure 2**). While CBL4/CIPK24 complexes mediate Na^+ extrusion via the regulation of the H^+/Na^+ antiporter SOS1 at the plasma membrane, formation of CBL10/CIPK24 results in

vacuolar Na^+ sequestration by regulating unknown targets (see **Figure 2**).

Reverse genetics has greatly advanced our understanding of CBLs and CIPKs and revealed crucial functions of distinct CBL proteins and CIPKs for mineral nutrition, as well as responses to abiotic stresses and to ABA. Analysis of a CIPK3 loss-of-function allele established that this kinase regulates ABA responses during seed germination and regulates ABA-induced gene expression (78). Two independent reverse genetic analyses of CBL1 function revealed that CBL1 is a central integrator of responses to drought, cold, and salinity (2, 27). While the mutant studies of CBL1 revealed an ABA-independent function of this protein in several abiotic stress responses, loss of function of the closely related Ca^{2+} sensor CBL9 renders plants hypersensitive to ABA (125). Alternative complex formation between the kinase CIPK1 and either CBL1 or CBL9 mediates ABA-dependent and ABA-independent responses (31). CBL9 also appears to complex with CIPK3 for modulating ABA responses (126).

The CBL/CIPK system also regulates K^+ homeostasis. CIPK23 is targeted to the plasma membrane and is activated by the two highly related Ca^{2+} sensors CBL1 and CBL9 (28, 167), and the complexes regulate the activity of the shaker-like K^+ channel ARABIDOPSIS K^+ TRANSPORTER1 (AKT1). CIPK23 interacts exclusively with AKT1 and no other K^+ transporters from *Arabidopsis* (87). Besides the regulation of K^+ uptake in roots, the Ca^{2+}-decoding CBL1/CBL9/CIPK23 module is involved in stomatal regulation under dehydrating conditions (28).

The findings of all these studies indicate that the CBL-CIPK network is a central and critical system for decoding Ca^{2+} signals in response to a broad variety of stimuli. It is also becoming apparent that each CBL and each CIPK represents a multifunctional signaling component that can undergo alternative protein interactions, determining the flow of information processing through this signaling system. Therefore, elucidating the mechanistic factors that determine the "decision making" in this flexible interaction network will be of eminent importance to further our understanding of Ca^{2+}-decoding mechanisms.

Ca^{2+} SIGNALING SYSTEMS

Ca^{2+} signaling networks are complex and sophisticated. Prediction of network function is often nonintuitive due to the high degree of interconnectivity between network components. Here, we (*a*) consider systems-based investigations of network architecture, and (*b*) discuss the modeling of $[Ca^{2+}]$ alterations during the generation and decoding of Ca^{2+} signals.

Scale-Free Network Architecture

Some signaling networks are proposed to have properties similar to scale-free networks in computing. Scale-free networks have many interconnected nodes (i.e., signaling intermediates). A small number of the nodes are very highly connected and called hubs (64). Scale-free networks are robust to node removal, are sensitive to hub removal, and can process multiple signals simultaneously (64). In the guard cell, the extensive connectivity of Ca^{2+} with other network components and dependency of stomatal closure upon stimulus-induced Ca^{2+} alterations imply that Ca^{2+} is a hub (64). The paradigm of scale-free network architecture is not a formalism of network function but provides a tool for development of mathematical models, advances understanding of the evolutionary basis of the network, and allows identification of optimal manipulation targets for research or agricultural purposes.

Knowledge of interconnectivities between a large number of nodes (>1000) is necessary to conclude scale-free architecture (64). An *Arabidopsis* whole-genome network derived from transcriptome data has scale-free properties but is not completely scale-free (102). This network (102) reflects gene regulation and so did not specifically incorporate Ca^{2+}. Interestingly, the cold-regulated subnetwork includes known and uncharacterized proteins linked to Ca^{2+}

signaling (102). Transcripts associated with Ca^{2+} signaling also occupy separate and well-connected nodes within subnetworks associated with ER stress responses (*CALRETICULIN2*), biotic stress (*CAX3*), jasmonic acid/ethylene signaling (Ca^{2+}-binding *TSK-ASSOCIATING PROTEIN1*), and salicylic acid metabolism and pathogen responses (calmodulin-binding protein encoded by At1g73805) (102). When Ca^{2+} signaling is considered within gene networks, genes associated with Ca^{2+} signaling appear to occupy independent positions within several subnetworks. This might derive from the capacity of Ca^{2+} signals to encode specificity, which allows these signals to occupy multiple positions within the network. Scale-free network architecture is also a useful paradigm with which to consider smaller networks. For example, the CBL/CIPK Ca^{2+} sensor relay system (10) is proposed to have scale-free architecture because the majority of CBLs interact with a small number of CIPKs, while a limited number of hublike CBLs (e.g., CBL2) interact with many CIPKs (10). This provides the capacity to process multiple signals simultaneously though formation of alternative CBL/CIPK complexes, depending on localization and concentration of Ca^{2+} alterations and localization of CBL/CIPKs (31, 160).

Bow-Tie Network Architecture

Plant Ca^{2+} signaling networks have characteristics of bow-tie networks in neural network computing (30, 130) (**Figure 3**). These networks connect diverse inputs and outputs by processing information through a small number of core intermediates known as the hidden layer, using parameters that become defined during network training (**Figure 3*a***). The parameters that define relationships between inputs and the hidden layer, and between the hidden layer and outputs, cause classification, whereby a specific set of inputs leads to a specific set of outputs. In signaling, classification occurs when a specific Ca^{2+} signature is defined by the input parameter set, and also during subsequent Ca^{2+} signature decoding into a stimulus-specific output. For example, several abiotic stimuli cause $[Ca^{2+}]_{cyt}$ and ROS alterations, with different properties that induce stimulus-specific transcript sets (**Figure 3*b***). Whether Ca^{2+} signals in plant cells perform neural network–like classification has not been formally tested, although this appears to be the case for Ca^{2+} and cGMP signaling in some animal cells (130). This architecture is proposed to (*a*) control cell function efficiently because alterations in relatively few intermediates can evoke a variety of large-scale output alterations, (*b*) be evolvable because new

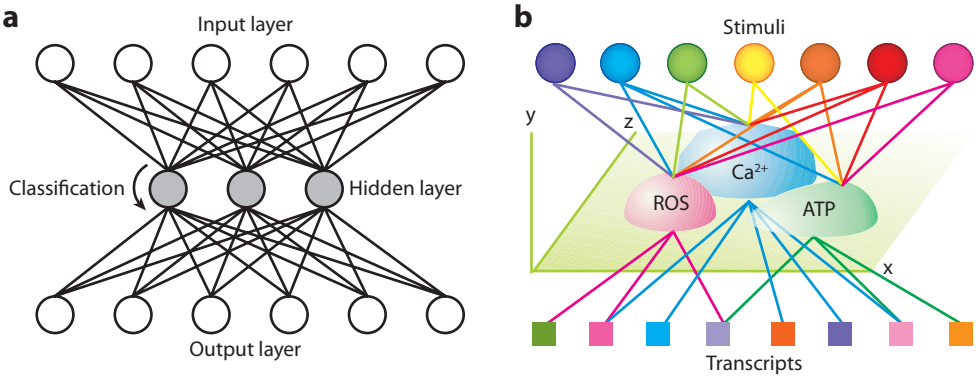

Figure 3

(*a*) Bow-tie feed forward neural network architecture in which diverse inputs regulate diverse outputs by processing (classification) within a core hidden layer. (*b*) Translation of this concept to plant Ca^{2+} signaling with classification involving spatiotemporal variations in Ca^{2+}, ROS, and ATP. Concept from (130). Abbreviations: ROS, reactive oxygen species; ATP, adenosine triphosphate.

inputs and outputs integrate readily with the toolbox of core molecules, and (*c*) have fragility to failure of the core mechanism and susceptibility to hijacking (30). This final property might explain the evolutionary recruitment of root hair Ca^{2+} signaling into initiation of root nodule formation.

Predictive Models of Network Function

The dynamic interactions within many signaling networks are well characterized, but quantitative descriptions of network function are infrequent (67). Here, we consider simulations of networks that include Ca^{2+} signaling.

One model for ABA-induced stomatal closure has generated new questions concerning Ca^{2+} signaling in the guard cell (92). In this model interactions of signaling intermediates are described with Boolean logic, with the ultimate outcome that the pore is either open or closed (92). The most highly connected components are $[Ca^{2+}]_{cyt}$ (12 interconnections), pH_{cyt} (9 interconnections), and plasma membrane depolarization (9 interconnections). Within this network there are at least two semiindependent pathways connecting ABA perception with stomatal closure, involving changes in $[Ca^{2+}]_{cyt}$ and pH_{cyt}, respectively (92). One hypothesis formed from this model is that stomata are closed by ABA-induced $[Ca^{2+}]_{cyt}$ increases, but $[Ca^{2+}]_{cyt}$ increases are not a prerequisite for stomatal closure (92). Testing this hypothesis revealed that ABA causes slow and partial stomatal closure when $[Ca^{2+}]_{cyt}$ increases were experimentally prevented (148). The Boolean model includes OST1, ABI1, and phospholipase D as components of the closure pathway that are relatively Ca^{2+} independent (92). However, inhibition of ABA-induced closure in the ABA-insensitive mutants *ost1–2* and *abi1–1* is more pronounced when $[Ca^{2+}]_{cyt}$ increases are prevented, and a comparable response occurred in the ABA-hyposensitive *pldα1* mutant, suggesting that the Ca^{2+}-dependent ABA signaling pathway is dominant or that a Ca^{2+}-independent mechanism primes the signaling network to Ca^{2+} increases (148).

Several circadian clock transcripts are up- or downregulated by cADPR, suggesting that cADPR or Ca^{2+} affects circadian clock function (39, 138). To investigate this, parameters within an existing model for the circadian clock were constrained to force the simulated abundance of the clock transcripts *CCA1/LHY*, *GI* and *TOC1* to adopt the fold changes caused by induction of cADPR synthesis (39, 138). The simulations predicted that (*a*) temporary parameter constraints (induction of cADPR synthesis) would cause transient alterations in clock function, (*b*) continuous parameter constraint (constitutive cADPR synthesis) would alter the circadian period, depending on the model parameters constrained, and (*c*) for many parameter pairs, an inverted parameter constraint (inhibition of cADPR synthesis) would cause a longer simulated period (39). It was subsequently found that manipulation of cADPR synthesis can alter circadian clock function (39). Future modeling may uncover candidate mechanistic links between cADPR/Ca^{2+} and the circadian clock.

Properties of Ca^{2+} oscillations thought to confer specificity include the period, amplitude, and waveform; these are amenable to mathematical analysis. Arbuscular-mycorrhizal (AM) fungi cause irregular patterning of Ca^{2+} spiking compared with consistently repetitive Ca^{2+} oscillations induced by Nod factor (compare **Figure 4*a*** with **4*b***) (82). The authors

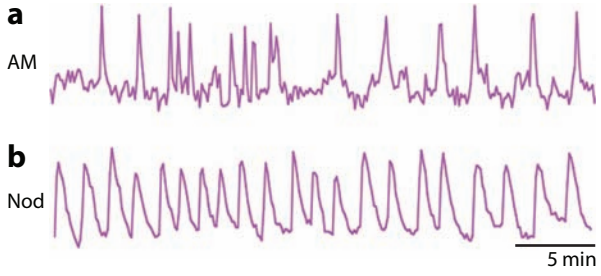

Figure 4

(*a*) Arbuscular-mycorrhizal (AM), and (*b*) Nod-factor-induced $[Ca^{2+}]_{cyt}$ spiking in root hairs; from (82). (Copyright 2008 National Academy of Sciences, U.S.A.)

investigated mathematically whether AM-induced Ca^{2+} spikes are unpredictable (stochastic) or deterministic but with chaotic properties, and concluded from multiple measures that both AM- and Nod-induced Ca^{2+} spikes have recurrent properties. The variable patterning of AM-induced Ca^{2+} spikes is therefore likely due to chaotic rather than stochastic properties of the system (82). This suggests that the transport processes causing the oscillations have chaotic regulatory properties that allow slight differences in the stimulus to evoke highly differentiated output responses (82), a conclusion that may help explain how many distinct Ca^{2+} signatures can be caused by a small set of channels.

Simulation of Ca^{2+} Homeostasis and Dynamics

Classical simulations of stimulus-induced Ca^{2+} oscillations in animal cells parameterize two or more Ca^{2+} stores, a Ca^{2+} release mechanism, cytosolic Ca^{2+}-scavenging Ca^{2+} ATPases, and membrane leak currents (154). These models simulate Ca^{2+} oscillations with properties similar to Ca^{2+} oscillations in animal cells. More sophisticated models incorporate features such as the ligand binding kinetics of Ca^{2+} release channels (154). Important differences between plant and animal cells require incorporation into simulations. cADPR and $InsP_3$ are thought to activate separate Ca^{2+} stores in animal cells, whereas common cADPR- and $InsP_3$-sensitive Ca^{2+} stores exist in plant cells because the vacuolar membrane is cADPR- and $InsP_3$-sensitive (5), whereas ER membranes are $InsP_3$-, cADPR-, and NAADP-sensitive (see **Figure 1**) (5, 116, 118, 119). The notion of an ER store that contains finite quantities of Ca^{2+} that functions in combination with a vacuolar store containing virtually limitless Ca^{2+} was incorporated within a simulation of ABA-induced $[Ca^{2+}]_{cyt}$ oscillations (159). The study concluded that $[Ca^{2+}]_{cyt}$ oscillations with comparable properties are evoked by ABA concentrations that span several orders of magnitude because the different kinetics of Ca^{2+} scavenging Ca^{2+} ATPases and Ca^{2+}/H^+ antiporters mean that at high [ABA], Ca^{2+}/H^+ antiporters remove Ca^{2+} from the cytosol and allow $[Ca^{2+}]_{cyt}$ oscillations; whereas, at low [ABA], $[Ca^{2+}]_{cyt}$ oscillations can occur with only Ca^{2+}-ATPase activity (see **Figure 1**) (159). The long period of $[Ca^{2+}]_{cyt}$ oscillations in plant cells compared with animal cells might arise from differences in the rate of activation by $InsP_3$ of $InsP_3$-sensitive Ca^{2+} channels (strictly, relief from Ca^{2+} inhibition of the channel by $InsP_3$), and different Ca^{2+} signatures may arise from variations in this parameter (159). The simulation assumed that Ca^{2+} release was entirely mediated by cADPR and $InsP_3$-gated channels, so future simulations incorporating regulatory kinetics of TPC1-mediated Ca^{2+} release (128) would be informative (see **Figure 1**). The contribution of extracellular Ca^{2+} influx to stimulus-induced Ca^{2+} signals, which is important in at least those guard cells that are relatively insensitive to ABA (104), could provide further information concerning the contribution of each Ca^{2+} store to Ca^{2+} oscillations.

Four-dimensional simulation of Ca^{2+} waves has been performed using as a template the 3D geometry of membrane systems in animal cells from tomographic electron microscopy (107). Simulations predicted that regions of close membrane proximity participate in formation of signaling microdomains. Application to plant cells seems appropriate given the distinctive arrangement of a large central vacuole surrounded by a relatively small-volume, ER-containing cytosol, which provides a very different topology for the propagation of Ca^{2+} signals compared with animal cells. We suggest that, in future, computational approaches will be essential for advancing our understanding of the complex intracellular language of Ca^{2+} signaling.

SUMMARY POINTS

1. Many energized transporters previously thought to function only in Ca^{2+} homeostasis may have signaling roles.
2. Specific Ca^{2+} signatures are decoded by a complex toolkit of Ca^{2+}-binding proteins that translate the information encoded within Ca^{2+} signals into phosphorylation events and defined transcriptional responses.
3. Ca^{2+}-signaling networks could have architectural and functional properties that are comparable to scale-free networks and bow-tie networks.

FUTURE ISSUES

1. Understand the transport and signaling functions of specific gene products that are likely to form multisubunit Ca^{2+}-permeable channels.
2. Identify genes encoding ligand-gated channels to establish the mechanistic basis for cADPR, $InsP_3$ and NAADP signaling in plant cells and the evolutionary basis for these mechanisms, particularly given the absence of animal cADPR and $InsP_3$ receptors from the plant genome.
3. Measure $[Ca^{2+}]_{cyt}$ in Ca^{2+} pump and Ca^{2+} exchanger mutants to position these proteins within the Ca^{2+} signaling network.
4. Understand the biological significance of the spatial distribution of Ca^{2+} signals within single plant cells during signaling.
5. Develop predictive models for Ca^{2+} signals that incorporate the kinetics and regulation of specific gene products.
6. Understand how circadian Ca^{2+} oscillations are decoded: (*a*) What is the identity of the Ca^{2+} sensor protein(s) that are involved in this pathway? (*b*) What are the downstream mechanisms that convert the signals arising from activation of these proteins into alterations in gene expression or enzyme activity?
7. Uncover the functional interconnections between the systems that decode Ca^{2+} signals and other cell signaling systems such as PP2C-type phosphatases, receptor-like kinases, and MAPK modules.

DISCLOSURE STATEMENT

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