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REVIEW

Calcium Signals: The Lead Currency of Plant Information Processing

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Ca^{2+} signals are core transducers and regulators in many adaptation and developmental processes of plants. Ca^{2+} signals are represented by stimulus-specific signatures that result from the concerted action of channels, pumps, and carriers that shape temporally and spatially defined Ca^{2+} elevations. Cellular Ca^{2+} signals are decoded and transmitted by a toolkit of Ca^{2+} binding proteins that relay this information into downstream responses. Major transduction routes of Ca^{2+} signaling involve Ca^{2+}-regulated kinases mediating phosphorylation events that orchestrate downstream responses or comprise regulation of gene expression via Ca^{2+}-regulated transcription factors and Ca^{2+}-responsive promoter elements. Here, we review some of the remarkable progress that has been made in recent years, especially in identifying critical components functioning in Ca^{2+} signal transduction, both at the single-cell and multicellular level. Despite impressive progress in our understanding of the processing of Ca^{2+} signals during the past years, the elucidation of the exact mechanistic principles that underlie the specific recognition and conversion of the cellular Ca^{2+} currency into defined changes in protein–protein interaction, protein phosphorylation, and gene expression and thereby establish the specificity in stimulus response coupling remain to be explored.

INTRODUCTION

Calcium (Ca^{2+}) likely represents the most versatile ion in eukaryotic organisms. It is involved in nearly all aspects of plant development and participates in many regulatory processes. Because of its flexibility in exhibiting different coordination numbers and complex geometries, Ca^{2+} can easily form complexes with proteins, membranes, and organic acids. On the one hand, this feature renders Ca^{2+} a toxic cellular compound at higher concentrations because it would readily form insoluble complexes with phosphate (as present in ATP), but on the other hand, the required tight spatial and temporal control of cellular Ca^{2+} concentration may have paved the way for the evolutionary emergence of Ca^{2+} signaling.

Considerable interest and research on this ion has been sparked by the apparent antagonism between the obvious cellular abundance of Ca^{2+} in certain organelles and cell structures and its required rareness in the cytoplasm. Since the first report in the green algae *Chara* that changes of cytosolic Ca^{2+} indicate a function of Ca^{2+} as a second messenger in plants (Williamson and Ashley, 1982), transient elevations in cytosolic Ca^{2+} concentration have been documented to be involved in a multitude of physiological processes, including responses to abiotic stresses, hormones, and pathogens. During the last two decades of the 20th century, advances in Ca^{2+} monitoring techniques have allowed detailed analyses of cellular Ca^{2+} dynamics. Several groups reported that defined changes of cytosolic Ca^{2+} concentration are triggered by cellular second messengers, such as NAADP, IP3, IP6, Sphingosine-1-Phospate, and cADPR (Drøbak and Ferguson, 1985; Schumaker and Sze, 1987; Blatt et al., 1990; Gilroy et al., 1990; Allen and Sanders, 1995; Navazio et al., 2000; Lemtiri-Chlieh et al., 2003), and it became evident that the identity and intensity of a specific stimulus impulse results in stimulus-specific and dynamic alterations of cytosolic Ca^{2+} concentration (Allen et al., 1995; McAinsh et al., 1995). This heterogeneity of increases in cytosolic-free Ca^{2+} ion concentration in terms of duration, amplitude, frequency, and spatial distribution lead A.M. Hetherington and coworkers to formulate the concept of "Ca^{2+} signatures" (Webb et al., 1996). Herein, signal information would be encoded by a specific Ca^{2+} signature that is defined by precise control of spatial, temporal, and concentration parameters of alterations in cytosolic Ca^{2+} concentration. The spectrum of stimuli that evoke such Ca^{2+} elevations and their stimulus-specific characteristics has been cataloged and critically discussed in a number of informative reviews (Rudd and Franklin-Tong, 1999; Sanders et al., 1999; Knight and Knight, 2001; Sanders et al., 2002; Scrase-Field and Knight, 2003). Subsequent research suggested that while the shape and spatio-temporal distribution of Ca^{2+} elevations could be of critical importance for stimulus response coupling (Allen et al., 2001), an additional level of regulation and specificity is achieved by Ca^{2+} binding proteins that function as signal sensor proteins (Batistič and Kudla, 2004). These proteins decode and relay the information encoded by Ca^{2+} signatures into specific protein–protein interactions, defined phosphorylation cascades, or transcriptional responses (Luan et al., 2002; Sanders et al., 2002; Finkler et al., 2007a). Consequently, the dynamic interplay between Ca^{2+} signatures and Ca^{2+} sensing proteins contributes to generating stimulus specificity of Ca^{2+}

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signaling. Since the principles and cellular tool kits of Ca^{2+} signaling were last reviewed in this journal (Luan et al., 2002; Sanders et al., 2002), remarkable progress has been achieved especially in elucidating the mechanisms that contribute to decoding of Ca^{2+} signals, and complete Ca^{2+}-triggered regulatory modules have been identified. In this review, we will focus on the description of scientific insights and the discussion of emerging concepts that have been arising over the past few years.

FUNCTIONS OF Ca^{2+} SIGNALING

Ca^{2+} is involved in various responses to abiotic and biotic stimuli, including light, high and low temperature, touch, salt and drought, osmotic stress, plant hormones, fungal elicitors, and nodulation factors (Sanders et al., 1999). These stimuli induce a distinct spatio-temporal pattern of changes in cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$). Single-cell systems, such as guard cells, growing pollen tubes, or root hairs, represent excellent models to investigate primary and autonomous Ca^{2+} responses. However, the final response of the plant to external stimuli is manifested by regulation of complex growth processes in distinct tissues and organs. Concurrently to the diversity of stimulus-specific Ca^{2+} signatures at the single-cell level, differentiation gives rise to another layer of cell type–specific Ca^{2+} responses in tissues or organs. This additional level of complexity may contribute to more diversity in local or systemic responses. Therefore, research on plant Ca^{2+}signaling has taken advantage of single-cell model systems but in parallel moves forward to elucidate Ca^{2+} dynamics in the tissue context and in the whole organism. Consequently, here, we review and discuss how Ca^{2+} signatures contribute to signaling processes at the single-cell level and the multicellular level.

Ca^{2+} Signaling at the Single Cell Level

Stomatal Closure and Opening

In *Arabidopsis thaliana* guard cells, stimuli for stomatal closure, including abscisic acid (ABA), hydrogen peroxide, cold, elevation of external Ca^{2+}, and atmospheric CO_2, induce cytosolic Ca^{2+} oscillations (Allen et al., 2001; Young et al., 2006). Artificially imposed Ca^{2+} oscillations have revealed that cytosolic Ca^{2+} regulates stomatal closure by two mechanisms: short-term Ca^{2+}-reactive closure and long-term Ca^{2+}-programmed closure (Figure 1A) (Allen et al., 2001; Sanders et al., 2002). The short-term Ca^{2+}-reactive closure is a rapid reaction to exceeding a certain level of $[Ca^{2+}]_{cyt}$ elevation and is irrespective of the elevation pattern (Ca^{2+} functions likely in a threshold manner). Meanwhile, long-term Ca^{2+}-programmed closure (i.e., prevention of stomatal reopening) is controlled by Ca^{2+} oscillations within a defined range of amplitude, frequency, duration, and

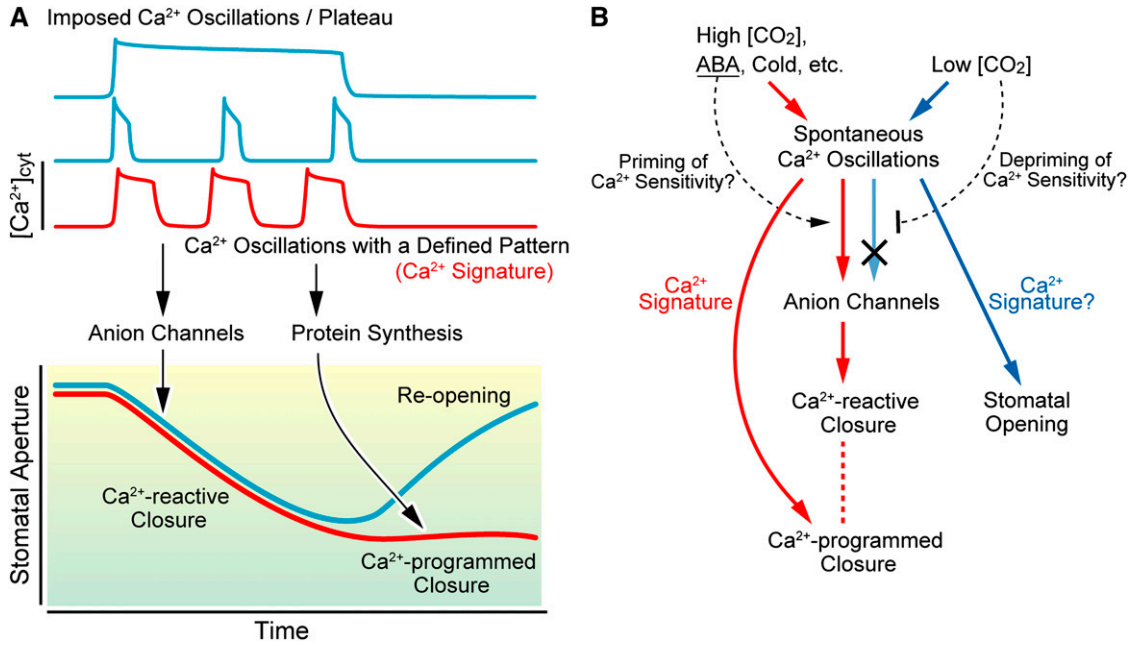

Figure 1. Ca^{2+} Signaling in Guard Cell Regulation.

(A) Schematic representation of artificially imposed Ca^{2+} oscillations/plateau and the corresponding temporal changes in stomatal aperture. The long-term Ca^{2+}-programmed closure is caused by Ca^{2+} oscillations with a defined pattern (Ca^{2+} signature), whereas the short-term Ca^{2+}-reactive closure is induced by $[Ca^{2+}]_{cyt}$ elevation, regardless of the pattern.

(B) Simplified model for Ca^{2+} controlled stomatal closure and opening. Stomatal closing stimuli, such as ABA, high CO_2, and cold, induce spontaneous Ca^{2+} oscillations, resulting in Ca^{2+}-reactive closure and subsequent Ca^{2+}-programmed closure. On the other hand, low CO_2-induced Ca^{2+} oscillations result not in Ca^{2+}-reactive closure but stomatal opening, implying that the stimulus may desensitize the signaling pathways of the Ca^{2+}-reactive closure (Ca^{2+} sensitivity priming hypothesis). It is unknown whether low CO_2-induced Ca^{2+} oscillation itself encrypts specific information of stomatal opening.

overall transient number (Ca^{2+} functions as a signature). Recent studies have revealed several elements involved in these mechanisms. The *Arabidopsis* Ca^{2+}-dependent protein kinase (CDPK) double mutant *cpk3 cpk6* exhibits impaired activation of S-type anion channels in response to cytosolic Ca^{2+}. Guard cells in the double mutant are defective in short-term closure but not in long-term closure (Mori et al., 2006). Mutation of the SLOW ANION CHANNEL-ASSOCIATED1 guard cell anion efflux channel abrogates Ca^{2+}-reactive stomatal closure and impairs stomatal responses to CO_2, ABA, ozone, light/dark transitions, humidity change, calcium ions, hydrogen peroxide, and nitric oxide (Negi et al., 2008; Vahisalu et al., 2008). By contrast, overexpression of At GLR3.1, a glutamate (Glu) receptor homolog, causes a defect in long-term closure but not in short-term closure (Cho et al., 2009). These findings indicate that the short-term and long-term responses are functionally separable. Interestingly, the translational inhibitor cycloheximide partially inhibits long-term closure but not short-term closure, suggesting no requirement of de novo protein synthesis in the short-term response (Cho et al., 2009). Taken together, these findings imply that certain threshold levels of Ca^{2+} elevations activate the preexisting cellular machinery, including Ca^{2+} sensor proteins and ion channels, to regulate rapid stomatal closure. Moreover, defined patterns of Ca^{2+} oscillations likely activate preexisting proteins and also induce the expression of required genes, resulting in inhibition of stomatal reopening (Figure 1A).

However, it is important to note that nonoscillatory increases in cytosolic Ca^{2+} are also important features of stomatal responses that result in stomatal closure and that the actual pattern of oscillations evoked by a specific stimulus exhibits some variation and is not "tight" (Hetherington and Brownlee, 2004). Consequently, the Ca^{2+} decoding machinery that translates these Ca^{2+} elevations and oscillations into defined downstream responses must be able to decode these variable oscillations (Hetherington and Brownlee, 2004).

Surprisingly, recent studies have shown that Ca^{2+} SENSING-RECEPTOR (CAS), a thylakoid membrane-localized protein, is crucial for the Ca^{2+} response, suggesting involvement of chloroplasts in $[Ca^{2+}]_{cyt}$-mediated stomatal closure (Nomura et al., 2008; Weinl et al., 2008). The elucidation of the functional interplay between Ca^{2+} releases from different intra- and extracellular sources certainly represents an important goal to further our understanding of guard cell signaling.

Ca^{2+} signaling likely also contributes to the regulation of stomatal opening (Irving et al., 1992; Schroeder et al., 2001). For example, low atmospheric CO_2, which mediates stomatal opening, induces high-frequency Ca^{2+} oscillation. Treatment with the cytosolic Ca^{2+} chelator BAPTA-AM abolishes the oscillation and attenuates the stomatal opening in response to low CO_2 (Young et al., 2006). It would be interesting to investigate whether the rapid oscillation pattern encrypts specific information. As described above, artificially imposed Ca^{2+} oscillations induce the short-term Ca^{2+}-reactive closure regardless of their pattern. However, the rapid Ca^{2+} oscillation induced by low CO_2 does not cause such reaction (Young et al., 2006). A model was derived in which low CO_2 stimuli may negatively modulate Ca^{2+} sensitivities of signaling components, which regulate the short-term closure, such as CDPKs. On the other hand, during the stomatal closure response, preexposure to ABA enhances the response of S-type anion channels to $[Ca^{2+}]_{cyt}$ elevation, implying that ABA may positively modulate sensitivities of Ca^{2+} sensor proteins (Siegel et al., 2009). A Ca^{2+} sensitivity priming model has been proposed as a hypothesis to explain these observations in the context of mechanisms that contribute to the generation of specificity in Ca^{2+} signaling (Figure 1B) (Young et al., 2006).

The Establishment of Symbiosis in Root Hairs

In legume root hair cells, exposure to rhizobial-derived nodulation (Nod) factors induces biphasic changes $[Ca^{2+}]_{cyt}$ that comprise an initial Ca^{2+} influx and a subsequent (10 to 20 min later) long-term Ca^{2+} oscillation (also designated as Ca^{2+} spiking) in the perinucleus (Shaw and Long, 2003). The *Medicago truncatula* mutants *does not make infection1* (*dmi1*) and *dmi2* are defective in the Ca^{2+} spiking but retain the initial Ca^{2+} influx. Furthermore, low concentration (10^{-11} to 10^{-12} M) of Nod factor induces Ca^{2+} spiking but not Ca^{2+} influx, suggesting that they are separable responses (Shaw and Long, 2003). Induction of *Early Nodulation 11* (*ENOD11*) is impaired in the Ca^{2+} spiking-defective mutants *dmi1* and *dmi2*, as well as in *dmi3*, which is defective in a gene encoding Ca^{2+} calmodulin-dependent kinase (CCaMK). Remarkably, specific removal of the autoinhibitory domain of this kinase leads to autoactivation of the downstream nodulation signaling pathway with the resultant induction of nodules and nodulation gene expression in the absence of bacterial elicitation (Gleason et al., 2006). This demonstrates not only the essential function of this CCaMK in the regulation of nodule development but also indicates that the activation of this kinase through the oscillatory Ca^{2+} signal is necessary and sufficient to elicit this process (Gleason et al., 2006). Although the mechanism of generation of Ca^{2+} spikes is not well understood, some blockers for Ca^{2+} channels and Ca^{2+} pumps have been shown to inhibit both Ca^{2+} spiking (Engstrom et al., 2002) and *ENOD11* expression (Charron et al., 2004). This evidence suggests that Ca^{2+} spiking is essential for regulation of nodulation, raising the question of how the spiking could encode specific information. Miwa et al. (2006) proposed a correlation between the number of Ca^{2+} spikes and *ENOD11* expression levels. In this work, *ENOD11* inductions were observed only when the Ca^{2+} spiking lasted for at least 60 min. The average period between each spike was ~100 s; therefore, the authors estimated that a minimum of ~36 spikes is required for *ENOD11* induction. Jasmonic acid treatment lengthens the period between each Ca^{2+} spike but does not affect the required number of spikes for *ENOD11* expression, further supporting the notion that the number of Ca^{2+} spikes functions to encode information (Miwa et al., 2006).

Ca^{2+} also plays important roles in the symbiosis of legumes with arbuscular micorrhizal fungi. Incubation with micorrhizae induces Ca^{2+} spiking in legume root hair cells. Ca^{2+} spiking is abolished in *M. truncatula dmi1* and *dmi2*, suggesting that nodulation and mycorrhizal infection involve common signaling components. Despite the overlap, micorrhizal-induced Ca^{2+} spiking exhibits a shorter period and smaller amplitudes compared with that in the Nod factor response (Kosuta et al., 2008).

Remarkably, bacterial elicitors of plant pathogens also induce a Ca^{2+} release in the cytosol and nucleus. Here, the nitric oxide signal following elicitor application is important for the Ca^{2+} release in the cytosol, but it does not trigger a nuclear Ca^{2+} response (Lamotte et al., 2004). The nucleus also exhibits specific calcium signals in response to different elicitors. Harpin and flagellin resulted in a different Ca^{2+} release than observed in response to carbohydrate elicitors such as oligogalacturonides. Remarkably, the cytosolic Ca^{2+} response to these elicitors was comparable (Lecourieux et al., 2005). These observations suggest that the nucleus does indeed harbor an independent Ca^{2+} machinery that may involve P-ATPases and nucleotide gated channels located at the inner membrane of the nucleus to regulate the nuclear Ca^{2+} reservoir (Mazars et al., 2009).

Tip Growth in Pollen Tubes and Root Hair Cells

Pollen tubes are one of the most extensively studied tip-growing model systems. In pollen tubes, $[Ca^{2+}]_{cyt}$ is highly concentrated at the apex by an extracellular influx (Sanders et al., 1999; Hepler et al., 2001). In the Ca^{2+} gradient, $[Ca^{2+}]_{cyt}$ oscillates with a lag phase behind the corresponding growth rate oscillation, suggesting that stretch-activated Ca^{2+} channels may be involved in the maintenance of the Ca^{2+} gradient (Dutta and Robinson, 2004). In accordance with an essential function of stretch-activated channels, pharmacological inhibition of channel activity disrupts the Ca^{2+} influx at the apex and terminates pollen tube elongation (Picton and Steer, 1985). Moreover, *Petunia inflata* and *M. truncatula* mutant lines that are defective in CDPKs exhibit loss of polarity (Ivashuta et al., 2005; Yoon et al., 2006). Therefore, the formation of a Ca^{2+} gradient as well as the decoding of Ca^{2+} signals by Ca^{2+} sensing proteins appear to play important roles in generating spatial determinants for pollen tube growth. Moreover, several studies have suggested a close interaction of intracellular Ca^{2+} and the cytoskeleton in that the growth regulatory effect of this ion is exerted by Ca^{2+}-dependent regulation of the structure and activity of F-actin (Snowman et al., 2002; Cardenas et al., 2008).

Recent studies have also revealed the importance of Ca^{2+} signaling in the root hair single-cell model system. Similar to growing pollen, root hair cells exhibit a tip-focused Ca^{2+} gradient with Ca^{2+} oscillation lagging behind growth oscillation (Monshausen et al., 2008). Moreover, the Ca^{2+} oscillation is followed by oscillation of apoplastic reactive oxygen species (ROS) production (Monshausen et al., 2007, 2008). Intriguingly, the RHD2 NADPH oxidase (also known as RBOH C) is localized to the plasma membrane of the tip-growing site and is required for appropriate growth of root hair cells (Takeda et al., 2008). RHD2 contains an EF hand-like Ca^{2+} binding domain and phosphorylation sites that appear to be targets of calcium-regulated protein kinases (Takeda et al., 2008). Therefore, the Ca^{2+} oscillation may regulate RHD2 activity. Inhibition of the Ca^{2+} oscillation by La^{3+} causes bursting growth, while increasing the Ca^{2+} gradient by Ca^{2+} ionophore arrests growth of root hairs (Monshausen et al., 2008). Consequently, it has been proposed that Ca^{2+} and ROS compose a positive feedback loop to sustain tip growth of root hair cells (Takeda et al., 2008).

Ca^{2+} Signaling at the Multicellular Level

Abiotic Stress Responses

In the endodermis and the pericycle of *Arabidopsis* roots, salt stress causes a biphasic Ca^{2+} response consisting of an initial transient elevation and a subsequent oscillation that differs in phase and/or period in individual cells (Kiegle et al., 2000). By contrast, only a monophasic $[Ca^{2+}]_{cyt}$ elevation is induced in the epidermis and the cortex (Kiegle et al., 2000). Interestingly, the initial Ca^{2+} response in the pericycle occurs a few seconds later than that in the other cell types. Moreover, the magnitude in the pericycle is significantly lower. This is likely because the endodermis limits the flow of water and ions to the xylem. Accordingly, whole-plant $[Ca^{2+}]_{cyt}$ measurements have suggested a direct correlation between the strength of NaCl stress and the magnitude of $[Ca^{2+}]_{cyt}$ elevation (Tracy et al., 2008). The function of the subsequent Ca^{2+} oscillation in the endodermis and the pericycle remains to be elucidated.

In contrast with salt stress, cold stress causes only monophasic $[Ca^{2+}]_{cyt}$ elevation in the four types of *Arabidopsis* root cells without significant temporal difference, suggesting that all cells sense temperature changes simultaneously (Kiegle et al., 2000). The magnitude of cold-induced Ca^{2+} responses is dependent on the cooling rate ($\Delta T/\Delta t$) (Plieth et al., 1999). However, the pericycle exhibits a more pronounced Ca^{2+} response to cold stress (Kiegle et al., 2000), suggesting that different cell types harbor different Ca^{2+} homeostasis and signaling components. Interestingly, when cold-induced Ca^{2+} responses were analyzed by specific expression of the reporter protein Aequorin in guard cells, Dodd et al. (2006) observed circadian modulations of the cold-induced Ca^{2+} elevation that were significantly more pronounced during the mid-photoperiod than at the beginning or the end of the day. Therefore, it would be interesting to determine if circadian modulation of Ca^{2+} responses also occurs in root tissues and during responses to other environmental cues.

Dodd et al. (2006) also performed mathematical modeling analysis of calcium signatures to investigate the relationship between single-cell calcium analyses and whole population (or whole plant) calcium monitoring. This study revealed that the population Ca^{2+} signature may be diagnostic for underlying single-cell Ca^{2+} oscillations, but it is unlikely to be representative of single-cell Ca^{2+} signatures. This is a very important finding that needs to be considered when interpreting work of Ca^{2+} dynamics that has used Aequorin as a (whole plant) reporter protein. For example, the second sustained Ca^{2+} elevation of biphasic Ca^{2+} responses that has been observed in such studies may in fact represent an amalgamation of asynchronous Ca^{2+} oscillations that occur in individual cells of the plant after the initial synchronous Ca^{2+} transient (Dodd et al., 2006).

Recently, it has been demonstrated that heat shock induces prolonged $[Ca^{2+}]_{cyt}$ elevation ($\sim$20 min duration) via putative heat-sensitive plasma membrane Ca^{2+}-permeable channels in the moss *Physcomitrella patens* (Saidi et al., 2009). It appears that a larger increase in temperature induces a more intense $[Ca^{2+}]_{cyt}$ elevation and results in a stronger response (e.g., expression of heat shock proteins) compared with a less pronounced temperature elevation shift. In *Arabidopsis*, Ca^{2+} and

calmodulin proteins have been shown to be involved in the heat shock response (Zhang et al., 2009). It is likely that flowering plants also sense temperature increments with heat-sensitive channels. It would be interesting to elucidate whether heat shock induces cell type–specific $[Ca^{2+}]_{cyt}$ elevations in *Arabidopsis* and to compare the Ca^{2+}-related mechanisms of heat stress responses with that of the cold response.

Response to Mechanical Stimuli

There is ample evidence that mechanical stimuli, such as touch or wind, induce $[Ca^{2+}]_{cyt}$ elevations in plant cells (Braam, 2005). Recently, Monshausen et al. (2009) reported that different types of mechanical stimuli, such as touch and bending, induce distinct patterns of Ca^{2+} responses in the *Arabidopsis* root. Whereas touch stimuli induce monophasic $[Ca^{2+}]_{cyt}$ elevations in the cells at the touch site, bending elicits biphasic transient elevations in the cells on the convex (stretching) side. These Ca^{2+} responses are essential for apoplastic alkalinization as well as RBOH C-dependent apoplastic ROS production that may contribute to resistance of plants to mechanical stresses (Monshausen et al., 2009). Bending of the root recruits pericycle cells on the convex side to become founder cells of a new lateral root (LR) primordium. Interestingly, blocking the bending-induced $[Ca^{2+}]_{cyt}$ elevation inhibits the recruitment of new LRs (Richter et al., 2009), indicating an essential role of Ca^{2+} signals in this process. However, it remains to be elucidated how the biphasic pattern of Ca^{2+} elevation encrypts specific information for bending-induced LR production.

GENERATION OF Ca^{2+} SIGNALS

A Ca^{2+} signal is defined by the balanced activation of Ca^{2+} channels at different cellular membranes, which is followed by the subsequent inactivation of channels and activation of efflux transporters to terminate Ca^{2+} influx and to rebalance the cellular Ca^{2+} homeostasis. Both processes are strictly regulated and define the physiological outcome of Ca^{2+} signaling.

Influx of Ca^{2+}

Distinct ion channel types capable of mediating Ca^{2+} fluxes coexist in different cell types and tissues. According to their activation mechanism, these channels can be classified as voltage-dependent, voltage-independent/ligand-dependent, and stretch-activated Ca^{2+} channels (Cosgrove and Hedrich, 1991; White et al., 2002; White and Broadley, 2003; Dutta and Robinson, 2004; Nakagawa et al., 2007). Depending on their specific activation properties, Ca^{2+} channels can shape the parameters of Ca^{2+} influx and the resulting Ca^{2+} signature (White et al., 2002; Demidchik and Maathuis, 2007). This enables the plant to translate a wide range of different signals into distinct Ca^{2+} signatures (Miedema et al., 2001, 2008). Moreover, variability in the specific abundance of the different channel types likely reflects the special needs of a cell type or tissue (Demidchik et al., 2002). A schematic summary of the channels and transporters that are discussed in this review is illustrated in Figure 2.

Voltage-Dependent Channels at the Plasma Membrane

Voltage-dependent Ca^{2+}-permeable channels have been classified as depolarization-activated Ca^{2+}-permeable channels (DACCs) and hyperpolarization-activated Ca^{2+}-permeable channels (HACCs) (White et al., 2002). Although the properties of DACCs and HACCs are well studied electrophysiologically (Grabov and Blatt, 1998; Thion et al., 1998; Thuleau et al., 1998; Hamilton et al., 2000; Pei et al., 2000; Klusener et al., 2002), the molecular identity of these channels is still unknown (White et al., 2002). It is assumed that DACCs contribute to the short transient influx of Ca^{2+} in response to various stimuli, including chilling and microbe interaction (Thion et al., 1998). HACCs contribute to a sustained Ca^{2+} influx in response to ABA (Hamilton et al., 2000; Pei et al., 2000), blue light (Harada and Shimazaki, 2009), and Ca^{2+} nutrition (Miedema et al., 2001, 2008).

Plant annexins are ubiquitous, soluble proteins capable of Ca^{2+}-dependent and Ca^{2+}-independent binding to endomembranes and the plasma membrane (Demidchik and Maathuis, 2007; Mortimer et al., 2008). Surprisingly, a recent study indicated that cytosolic annexins create a Ca^{2+} influx pathway directly, particularly during stress responses involving acidosis in *Zea mays* (Laohavisit et al., 2009). This suggests that annexins modulate or even represent a part of voltage-gated Ca^{2+}-permeable channels. However, further characterization of plant annexins and especially the molecular identification of voltage-gated Ca^{2+} channels are required to enable more insights of the contribution of annexins and voltage-gated channels to Ca^{2+} signaling (Cheong et al., 2007).

Ligand-Gated Channels at the Plasma Membrane

Cyclic nucleotide-gated channels (CNGCs) are ligand-gated channels that are important for cellular homeostasis of cations and can mediate fluxes of Ca^{2+} ions (Hua et al., 2003a; Ali et al., 2006). Twenty CNGC genes have been identified in *Arabidopsis* (White et al., 2002). CNGCs are activated by binding of the cyclic nucleotides cAMP and cGMP and harbor a binding site for calmodulin that partially overlaps with the binding domain for cyclic nucleotides (cNMPs). Consequently, binding of Ca^{2+}/calmodulin results in inactivation of CNGCs due to blocking of the cNMP binding domain (Hua et al., 2003b; Ali et al., 2006). Thereby, Ca^{2+} itself, by binding to calmodulins/calmodulin-like proteins (CMLs), can modulate the influx of Ca^{2+} mediated by CNGCs. Several CNGCs, including CNGC4, CNGC11, and CNGC12, have been implicated in response reactions to pathogens (Balague et al., 2003; Yoshioka et al., 2006; Urquhart et al., 2007). CNGC2 represents an extensively characterized channel that was originally identified by cloning of the gene responsible for the *defense no death1* (*dnd1*) mutant phenotype. Mutant plants fail to induce the Ca^{2+}-mediated hypersensitive response to an avirulent strain of the pathogen *Pseudomonas syringae* and exhibit enhanced resistance to pathogens (Yu et al., 1998; Clough et al., 2000). Specifically, *cngc2* mutants are impaired in nitric oxide production that is required for the occurrence of a hypersensitive response (Ali et al., 2007) and that was previously reported to depend specifically on Ca^{2+} influx from extracellular stores (Lamotte et al., 2004).

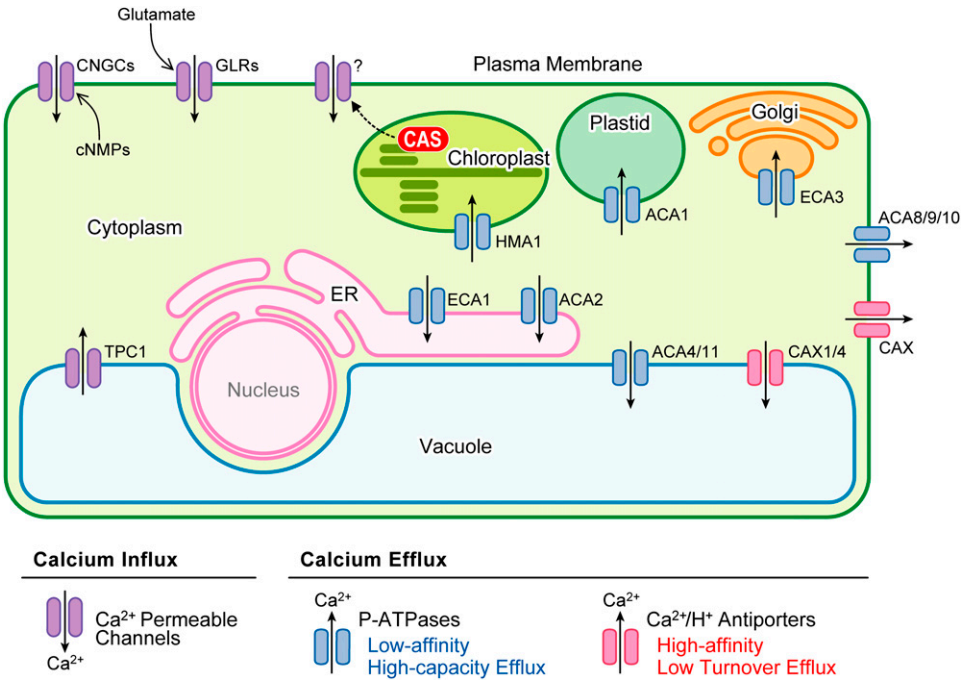

Figure 2. Overview of Ca^{2+} Transport Systems in an *Arabidopsis* Cell.

Shown are Ca^{2+} influx/efflux pathways that have been identified at the molecular level. See text for further details. CNGC, cyclic nucleotide channel; GLR, glutamate receptor; TPC1, two pore channel 1; CAS, Ca^{2+}-sensing receptor; ACA, autoinhibited calcium ATPase; ECA, ER type calcium ATPase; HMA1, heavy metal ATPase1; CAX, cation exchanger.

The function of CNGCs appears not to be restricted to plant pathogen interactions. CNGC3 and CNGC10 are implicated in regulating ion homeostasis especially for establishing the proper Na^+/K^+ ratio during salt stress adaptation (Gobert et al., 2006; Guo et al., 2008). CNGC18 is asymmetrically localized to the tip region of growing pollen tubes and has been shown to be indispensable for proper tip growth of pollen, suggesting that this channel is important to establish the tip-focused gradient of Ca^{2+} (Frietsch et al., 2007). Consequently, CNGC18 provides a mechanism for direct transduction of a cNMP signal into an ion flux that can produce a localized signal capable of regulating the pollen tip growth machinery. Considering the mechanistic similarities in the signal transduction processes governing tip growth in pollen tubes and root hairs, it would be most interesting to address the function of CNGCs in root hairs. Moreover, the investigation of Ca^{2+} dynamics in *cngc* mutant lines that would express the Ca^{2+} reporter protein cameleon would be most helpful for further elucidating the contribution of CNGC toward the generation of cellular Ca^{2+} signals.

Similar to CNGCs, glutamate receptors (GLRs) are nonselective cation channels that have attracted considerable attention as important regulators of Ca^{2+} influx. In *Arabidopsis*, 20 genes encode for GLRs that are differentially activated by Glu and Gly, as well as by other amino acids, and mediate an increase of cytosolic Ca^{2+} (Chiu et al., 2002; Qi et al., 2006; Stephens et al., 2008). It is assumed that GLRs are important for plant Ca^{2+} nutrition (Kim et al., 2001; Demidchik and Maathuis, 2007) but also in mediating Ca^{2+} responses upon cold stress (Meyerhoff et al., 2005) or excess aluminum (Sivaguru et al., 2003). At GLR1.1 regulates the expression of enzymes involved in carbon and nitrogen metabolism and regulates ABA biosynthesis (Kang and Turano, 2003; Kang et al., 2004). At GLR1.1 antisense lines contain elevated ABA levels and are hypersensitive to ABA or glucose (Kang and Turano, 2003; Kang et al., 2004). Overexpression of another GLR, At GLR3.1, impaired long-term stomatal closure but did not affect the short-term stomatal closure response or the kinetics of Ca^{2+} oscillations that were imposed by extracellular Ca^{2+} (Cho et al., 2009). In rice (*Oryza sativa*), disruption of the Os *GLR3.1* gene results in reduced growth of the primary root and of adventious roots especially in the early seedling stage due to reduced mitotic activity of the root apical meristem (Li et al., 2006a). GLRs also have been implicated in root development in *Arabidopsis* since Glu can affect root architecture (Walch-Liu et al., 2006). Additionally, GLRs might have a role in Ca^{2+}-dependent generation of electrical potentials in the root (Masi et al., 2009). Moreover, plants that have been treated with antagonists of GLRs display impaired light-induced signal transduction, harbor enlarged hypocotyls, and accumulate less chlorophyll when grown in light, suggesting that GLRs may also function in photomorphogenesis (Lam et al., 1998; Brenner et al., 2000).

These findings suggest that extracellular Glu not only provides a signal for Ca^{2+} nutrition but in addition regulates different developmental aspects, electrical transmission, supply of nutrients, and responses to abiotic stress. Therefore, it would be of considerable interest to investigate the functional interrelation of

plant CNGCs and GLRs and to investigate whether they act in concert to mediate Ca^{2+} signaling.

Voltage-Dependent Channels at Other Cellular Membranes

The coexistence of voltage-dependent channels and ligand-gated channels appears not to be restricted to the plasma membrane since compelling evidence points to a function of both channel types also in the vacuolar membrane. An important voltage-dependent channel activity was identified as a slow vacuolar type (SV) channel under depolarized conditions (Johannes et al., 1992; Allen and Sanders, 1994). This SV channel originally was described as voltage-dependent channel that is regulated by cytoplasmic Ca^{2+} concentrations (Hedrich and Neher, 1987), and subsequent work reported the Ca^{2+} release capability of this channel (Allen and Sanders, 1994). In addition, this channel seems to be regulated by Ca^{2+} in two opposing ways. While elevation of cytoplasmic Ca^{2+} can activate the channel, an increase in vacuolar luminal Ca^{2+} inactivates the channel (Pottosin and Schonknecht, 2007). This electrophysiologically characterized channel activity was recently shown to be conferred by the protein Two-pore channel1 (TPC1) (Peiter et al., 2005). Although the SV channel is the most abundant vacuolar channel and *TPC1* represents a single gene in the *Arabidopsis* genome, loss of SV channel function in *Arabidopsis* does not result in a lethal phenotype or severe growth defects. However, loss of *TPC1* function has a significant effect on germination inhibition by ABA, and while there is no effect on guard cell ABA signaling, there is a highly significant effect on stomatal responses to external Ca^{2+} (Peiter et al., 2005; Ranf et al., 2008). Although the SV channels appear to make only a minor contribution to a global Ca^{2+} release into the cytosol by Ca^{2+} influx from the vacuole, it was proposed that TPC1 channels could form clusters within the tonoplast to form local Ca^{2+} microdomains (Perez et al., 2008). In contrast with the situation in *Arabidopsis*, knockout mutation of *TPC1* in rice results in reduced plant growth on soil (Kadota et al., 2004). Moreover, overexpression of Os *TPC1* resulted in prolonged activation of the mitogen-activated protein kinase (MAPK) pathway, indicating that in rice (Kurusu et al., 2005) and also in tobacco (*Nicotiana tabacum*; Kadota et al., 2004), channel function could have a role in pathogen response. Similarly, knockout of *TPC1* in *Arabidopsis* results in weaker induction of defense genes after pathogen infection but does not significantly affect pathogen resistance (Bonaventure et al., 2007b). By contrast, a hyperactive gain-of-function allele of *TPC1*, designated as *fou2*, leads to enhanced defense gene expression and resistance to pathogens (Bonaventure et al., 2007a, 2007b). Although the exact physiological role of TPC1 in *Arabidopsis* is not fully understood, the fact that TPC1 mediates the flux of K^+ and other cations through the vacuolar membrane (Bihler et al., 2005; Ivashikina and Hedrich, 2005) may point to an important role of TPC1 in regulating cytosolic K^+ homeostasis in a Ca^{2+}-dependent manner (Pottosin and Schonknecht, 2007). In agreement with such a function, SV channel activity is down-regulated at low vacuolar potassium concentrations (Pottosin et al., 2005), and the *fou2* transcriptome resembles the transcript profile of plants after potassium starvation (Bonaventure et al., 2007b). Moreover, *fou2* mutant plants contain less K^+ and more Ca^{2+} within the vacuole (Beyhl et al., 2009). Several recent investigations reported that NAADP binds to and confers the activation of animal TPC channels that results in Ca^{2+} release from lysosomal and endosomal compartments (Brailoiu et al., 2009; Calcraft et al., 2009). Since these animal TPC channels are structurally closely related to plant TPC1 but lack the EF hand motif in their central domain (Ishibashi et al., 2000), it will be most interesting to investigate if the respective plant channels are also responsive to this second messenger.

In addition to TPC1, several other less well characterized voltage-dependent channels appear to exist at the vacuolar membrane (Allen and Sanders, 1994), which were characterized as a fast vacuolar channel (Hedrich and Neher, 1987) and as a Ca^{2+}-insensitive vacuolar channel (Ranf et al., 2008).

Ligand-Gated Channels at the Vacuolar and Endoplasmic Reticulum Membranes

Several electrophysiological studies supported the existence of ligand-gated channels at the tonoplast and suggested regulation of these channels by IP3/IP6 and cADPR (Schumaker and Sze, 1987; Allen et al., 1995; Muir and Sanders, 1996; Martinec et al., 2000; Navazio et al., 2000; Lemtiri-Chlieh et al., 2003). However, despite the existence of multiple full-genome sequences for several higher plant species, no genes encoding for an ADP ribosyl cyclase that would be required for cADPR production have been identified. In addition, ryanodine receptors that are the targets of cADPR in animal cells appear to be missing in higher plants. In this regard, experimental determination and direct proof of cADPR abundance by gas chromatography–mass spectrometry techniques are urgently needed.

Similarly, no sequences with significant similarity to animal IP3 receptors have been identified in genomes of higher plants. Interestingly, while higher plants lack classical endoplasmic reticulum (ER)-localized IP3 receptors, which are well known and characterized in animals (Berridge, 2009), several algae species, such as *Volvox* and *Chlamydomonas*, appear to harbor these receptor channels, suggesting that this channel type was present in the ancient plant progenitor of the evolutionary plant lineage and may have been lost during the further evolution of the plant lineage (Wheeler and Brownlee, 2008). This raises the obvious question of how IP3 can function in plant Ca^{2+} signaling in the absence of canonical IP3 receptors. The absence of IP3 receptors in higher plants in combination with the extremely low levels of phosphatidyl-inositol-4,5-bisphosphate in plant tissues, that in animal cells is converted by PLC into IP3, has raised concerns about the simple assignability of mechanistic principles of IP3-induced Ca^{2+} release from animal systems to the situation in plant cells (Munnik and Testerink, 2009). Therefore, future research in this field of plant Ca^{2+} signaling may unravel unexpected mechanisms that interconnect Ca^{2+} and IP3.

In addition to the previously mentioned ligand gated channels, a unique ligand-gated channel that appears to reside in the ER membrane is activated by NAADP (Navazio et al., 2000). However, since the molecular identity of all these channels is still unknown, it remains to be established if these ligands directly activate channels or, alternatively, function via receptors that indirectly modulate the activity of channels.

Efflux of Ca^{2+}

Much of the research on Ca^{2+} signaling has focused on advancing our understanding of the generation of Ca^{2+} elevations by Ca^{2+}-releasing channels. However, to represent a distinct signal, the regulation of Ca^{2+} efflux that not only terminates but also shapes the Ca^{2+} signature is as important as the Ca^{2+}-releasing events. During the past years, we have witnessed significant insights into the regulation of Ca^{2+} extrusion. However, in the future, it will be most important to reveal the interconnected regulation of Ca^{2+} release and extrusion that is required to generate defined signals.

Extrusion of Ca^{2+} from the cytosol is achieved by P-type Ca^{2+}-ATPases and by the Ca^{2+}/proton antiporter systems. While antiporters mediate a high-affinity low turnover efflux, ATPases mediate a low-affinity high-capacity efflux. Therefore, it is assumed that antiporters reduce the Ca^{2+} concentration back to a few micromolar after signal mediated influx, while Ca^{2+}-ATPases are important to maintain the low resting concentration of Ca^{2+} (Hirschi, 1999).

The coordinative regulation of the cellular extrusion systems still is not fully understood. Transport activity is clearly activated after influx of Ca^{2+}, as in response to different hormones (Erdei et al., 1979; Bush et al., 1993; Zocchi and Rabotti, 1993), salt stress (Gao et al., 2004), or mechanical stimulation (Bourgeade et al., 1991). Enhanced Ca^{2+} extrusion was reported to occur in senescent potato (*Solanum tuberosum*; Fakhrai and Hall, 1984). Moreover, enhanced transcription of ATPases was observed in response to ABA (Cerana et al., 2006), sugar (Mito et al., 1996), or by sodium (Wimmers et al., 1992).

Hormones can also differentially activate transporter systems of different cellular membranes. Ca^{2+} released by GA seems to be transported mainly via ER transporters out of the cytoplasm, while ABA activates transport activity at the ER as well as at the tonoplast (Bush and Sze, 1986; Bush et al., 1989a, 1993). Interestingly, overall, the Ca^{2+} extrusion system seems to be less effective in protoplasts or cells from epidermal strips than in intact leaves (Levchenko et al., 2008). This important observation should be considered in the design of future experiments addressing the regulation of Ca^{2+} extrusion.

Ca^{2+}-Proton Antiporter

In the *Arabidopsis* genome, six genes encode for putative Ca^{2+}-proton antiporters, also named cation exchangers (CAX) (Maser et al., 2001; Shigaki et al., 2006), which regulate the homeostasis of Ca^{2+} and other divalent cations, such as cadmium (Catala et al., 2003; Cheng et al., 2003; Koren'kov et al., 2007; Zhao et al., 2008). Five additional antiporters that were previously designated as CAX7-11 resemble potassium (Na^{2+})-dependent Na^{2+}/Ca^{2+} antiporters and were therefore renamed cation Ca^{2+} exchanger proteins (Shigaki et al., 2006). Additionally, four putative antiporters encoded in the *Arabidopsis* genome contain EF hand Ca^{2+} binding motifs, implicating that these transporters are directly regulated by Ca^{2+} (Shigaki et al., 2006). It is unknown to which extent the latter two types of antiporters are important for Ca^{2+} extrusion.

The antiporters CAX1 to CAX4 are localized to the vacuole (Hirschi et al., 2000; Cheng et al., 2002, 2003, 2005), but antiporter activity was also reported to reside at the plasma membrane (Kasai and Muto, 1990; Luo et al., 2005). CAX proteins harbor an N-terminal regulatory/autoinhibitory domain, which binds to an adjacent region within the N terminus (Pittman et al., 2002; Mei et al., 2007). The exact mechanistic regulation by the N terminus is not well understood, and differential N-terminal–dependent regulation of CAX protein activity may occur. Autoinhibition can be relieved by formation of heteromers of, for example, CAX1 and CAX3 (Zhao et al., 2009), and different regulatory proteins could additionally interact with CAX proteins to regulate transport activity (Cheng and Hirschi, 2003; Cheng et al., 2004a, 2004b). The importance of specific transport activity regulation by the N-terminal region was demonstrated by overexpressing an N-terminal truncated, deregulated version of the vacuolar Ca^{2+}/proton antiporter CAX1 from *Arabidopsis* in tobacco. Although tobacco plants contained more total Ca^{2+}, these plants showed Ca^{2+} deficiency symptoms and displayed hypersensitivity to magnesium, sodium, and cold shock (Hirschi, 1999). It was assumed that overexpression of At CAX1 leads to overloading of Ca^{2+} into the vacuole and to a severe reduction of cytosolic Ca^{2+} concentration, which caused the observed deficiency symptoms.

The formation of functional heteromers does not exclude the possibility that single CAX proteins are important for responses to specific signals. While both *cax1* and *cax3* mutant plants are hypersensitive to ABA during germination (Zhao et al., 2008), *cax3* mutants exhibited increased sensitivity toward NaCl or LiCl and low pH levels, while *cax1* mutants displayed normal wild-type responses (Catala et al., 2003; Zhao et al., 2008). This difference can be partially explained by the observation that CAX3 is prominently expressed in roots, whereas CAX1 is predominantly expressed in leaves (Zhao et al., 2008).

P-ATPases

Classical Ca^{2+} P-ATPases belong to the second subclass (II) of phosphorylated (P)-type ATPases. They are classified as P_{IIA}- or ER-type Ca^{2+} ATPases (ECAs; which include four members in *Arabidopsis*) and P_{IIB} ATPases (10 family members in *Arabidopsis*, all of which contain an autoinhibtory N-terminal region; Sze et al., 2000). Therefore, the latter ATPases also have been named autoinhibited Ca^{2+} ATPases (ACAs) (Sze et al., 2000). The autoinhibitory domain in P_{IIB}-type proteins can be relieved by binding of calmodulin to the regulatory domain, which results in activation of the pump (Harper et al., 1998). On the other hand, the activity of the P_{IIB}-type Ca^{2+} ATPase ACA2 can be inhibited by phosphorylation within the N-terminal regulatory domain by other Ca^{2+}-regulated proteins, such as CDPKs (Hwang et al., 2000).

P_{IIA}-type ATPases are localized at the ER (ECA1) (Liang et al., 1997), the Golgi (ECA3) (Mills et al., 2008), and endosomes (also ECA 3) (Li et al., 2008), implicating that the latter organelles can function as mobile Ca^{2+} stores that could contribute to the spatial specificity of Ca^{2+} signaling (Menteyne et al., 2006).

P_{IIB} types are localized at the ER (ACA2) (Harper et al., 1998), vacuole (ACA4 and ACA11) (Geisler et al., 2000; Lee et al., 2007b), plasma membrane (ACA8, ACA9, and ACA10) (Bonza et al., 2000; Schiott et al., 2004; George et al., 2008), and at the plastid envelope (ACA1) (Huang et al., 1993). The importance of a

P_{IIa}-type Ca^{2+}-ATPase activity regulating the cytoplasmic Ca^{2+} dynamics was recently impressively exemplified by the analysis of a Ca^{2+}-ATPase loss-of-function mutant in the moss *P. patens* (Qudeimat et al., 2008). Whereas wild-type plants exhibit a transient cytosolic Ca^{2+} signature after applying sodium stress, loss-of-function mutant lines displayed a sustained elevation of Ca^{2+} (Qudeimat et al., 2008). Interestingly, the sustained level of Ca^{2+} leads to a reduced upregulation of salt stress–induced genes and renders mutant plants specifically less tolerant to sodium stress (Qudeimat et al., 2008). These findings not only establish the importance of regulated Ca^{2+} extrusion for appropriate formation of Ca^{2+} signatures, they also implicate a direct interaction between the shape of a Ca^{2+} signature and proper stress responsiveness. In *Arabidopsis*, analysis of loss-of-function mutants of ACA9 and ACA10 indicated that the pumps specifically function in pollen tube growth and in inflorescence development of plants, respectively (Schiott et al., 2004; George et al., 2008)

Besides ECAs and ACAs, P_I-type ATPases, which are known as heavy metal transporters, were recently implicated in Ca^{2+} transport. At HMA1, a heavy metal transporter involved in detoxification processes for heavy metals, is a P_I-ATPase that localizes to the chloroplast envelope. At HMA1 transports Ca^{2+} and heavy metals, such as copper, with high affinity and, similar to Ca^{2+} pumps from animals, is specifically inhibited by thapsigargin (Seigneurin-Berny et al., 2006; Moreno et al., 2008).

All of these observations support the notion that finely regulated Ca^{2+} extrusion is as important as the influx of Ca^{2+}, as the deregulated hyperextrusion of Ca^{2+} as well as downregulated extrusion result in deregulated Ca^{2+} dynamics and homeostasis. Certainly, a minimal concentration of the toxic cation Ca^{2+} is required to sustain correct signaling and metabolic functions, while decelerated extrusion of Ca^{2+} disturbs the formation of defined Ca^{2+} signatures and impairs the capabilities of the plants to correctly respond to a signal.

Calcium Signal Modulation by Organelles

Plastids can accumulate high levels of Ca^{2+} (Nobel et al., 1966) in the milimolar range and thereby can contribute to the homeostasis of cellular Ca^{2+} and other ions (Portis and Heldt, 1976). Ca^{2+} within plastids, especially the correct distribution between stroma and thylakoid lumen, is important for the regulation of plastidial enzymes (Brand and Becker, 1984; Kreimer et al., 1988). The level of Ca^{2+} within plastids is regulated and can raise upon illumination (Muto et al., 1982; Kreimer et al., 1988) or during transition to dark (Sai and Johnson, 2002).

A protein identified as the Ca^{2+} binding protein CAS may influence the loading capacity of chloroplasts or may be important for sensing the loading status of Ca^{2+} within the chloroplasts, thereby modulating cytoplasmic Ca^{2+} signaling (Figure 2) (Han et al., 2003; Nomura et al., 2008; Weinl et al., 2008). CAS was first reported as an extracellular Ca^{2+}-sensing receptor, exhibiting a high capacity to bind Ca^{2+} (10 to 12 Ca^{2+} ions per molecule) (Han et al., 2003). However, the protein contains an N-terminal chloroplast targeting signal peptide, and subsequent reports identified CAS as a chloroplast-localized protein regulating cytoplasmic Ca^{2+} levels (Nomura et al., 2008; Vainonen et al., 2008; Weinl et al., 2008). Within the chloroplast, CAS is targeted to the thylakoid membrane (Vainonen et al., 2008; Weinl et al., 2008). CAS can be phosphorylated in a light-dependent manner depending on the activity of the light-regulated kinase STN8 (Vainonen et al., 2005, 2008). Although the activity of the photosystem is unaltered, *cas* knockout plants show retarded growth. Especially when grown under low Ca^{2+} conditions, plants show delayed bolting and impaired induction of flowering (Han et al., 2003). Mutant plants also show a strong deficit in regulating cytoplasmic Ca^{2+} levels (Vainonen et al., 2008) and are not able to induce stomatal closure provoked by extracellular Ca^{2+} (Han et al., 2003; Nomura et al., 2008; Weinl et al., 2008). However, *cas* knockout plants can respond to externally imposed Ca^{2+} oscillations and then display normal stomatal closure compared with the wild type, indicating a defect in the generation of Ca^{2+} transients that are required for stomatal closure, rather than the response machinery per se (Weinl et al., 2008). This indicates that the chloroplast-targeted Ca^{2+} sensor protein somehow connects cytoplasmic and chloroplast Ca^{2+} levels. This function resembles that of the Ca^{2+} buffer protein CRT within the ER lumen (Persson et al., 2001; Jia et al., 2009). Similarly, loss of CAS may lead to a reduced buffer capacity of the chloroplasts, suggesting that less Ca^{2+} can be allocated from the chloroplasts to the transient cytoplasmic increase of Ca^{2+}. Therefore, it will be of interest to analyze the concentration and dynamics of Ca^{2+} in chloroplasts of *cas* mutant lines.

DECODING AND RELAY OF Ca^{2+} SIGNALS

Ca^{2+} signals evoked by a specific stimulus are presented as defined Ca^{2+} signatures on the cellular level (as for example in guard cells) as well as in distinct cell types and tissue regions (for example, in roots after mechanical stimulation). It is apparent that the ability of a given cell or tissue to translate these Ca^{2+} signals into defined molecular and biochemical responses primarily depends on the presence, concentration, cellular localization, and Ca^{2+} binding affinity of signaling components that can sense such Ca^{2+} signatures and convey specific output reactions for further information processing (McAinsh and Hetherington, 1998). In plants, a diverse and extensive set of Ca^{2+} binding proteins that function as cellular Ca^{2+} sensors represent these first information translation points (Luan et al., 2002; Batistič and Kudla, 2004; McCormack et al., 2005; Kim et al., 2009).

Plant Ca^{2+} sensor proteins have been classified conceptually into sensor relays and sensor responders (Sanders et al., 2002). Sensor responder proteins, for example, CDPKs, combine within one protein a sensing function (Ca^{2+} binding and Ca^{2+}-induced conformational changes) with a response activity (e.g., protein kinase activity). By contrast, sensor relay proteins, like calmodulin, also effectively bind Ca^{2+} ions and usually undergo Ca^{2+}-induced conformational changes but lack other effector domains. To transmit the Ca^{2+} signal, sensor relay proteins interact with target proteins and regulate their activity (Luan et al., 2002; Sanders et al., 2002).

Initially, this concept of functional classification for Ca^{2+} sensing proteins was applied to the conversion of Ca^{2+} signals into phosphorylation responses (Sanders et al., 2002). However,

considering new insights into the function of Ca^{2+}-sensing proteins, we propose extending this concept to the conversion of Ca^{2+} signals into transcriptional responses. Whereas Ca^{2+} binding to calmodulin 7 (CAM7) appears to result in direct promoter interaction and regulation, other calmodulins are likely to mediate gene regulation by interacting with calmodulin binding transcription factors (CAMTAs) that function as transcriptional coregulators (see below). Consequently, CAM7 would represent a bona fide sensor responder directly contributing to activating gene expression, while other calmodulins function as sensor relays modulating gene expression via their interaction with proteins that function as transcription factors (Kushwaha et al., 2008).

In addition to calmodulins, CMLs, which are represented by >50 diverse calcium sensor proteins in *Arabidopsis*, appear to fulfill important functions in plant development and responses to environmental cues (McCormack et al., 2005). Loss of CML42 function leads to aberrant trichomes with increased branching, while mutation of CML24 causes alterations in flowering time (Delk et al., 2005; Tsai et al., 2007; Dobney et al., 2009). Moreover, CML24 functions in responses to ABA and ionic stress, and mutation of CML9 alters plant responses to ABA and abiotic stresses (Delk et al., 2005; Magnan et al., 2008). Exploring the function of the other members of this diverse protein family and especially identifying targets of these proteins therefore represents a promising area of plant signaling research.

Many biological processes, such as metabolic starch degradation by α-amylase (Bush et al., 1989b); biosynthetic processes, such as brassinosteroid synthesis (Du and Poovaiah, 2005); or even the mechanical occlusion of sieve tube elements (Furch et al., 2009) are likely important targets of direct Ca^{2+}-dependent modulation. However, with respect to the signaling function of Ca^{2+} ions, Ca^{2+}-dependent phosphorylation events and Ca^{2+}-dependent gene regulation represent the major cellular currencies for converting defined Ca^{2+} signatures into specific downstream reactions. Recent advances in these two facets of Ca^{2+} signaling will be discussed in detail here.

Translating Ca^{2+} Signatures into Protein Phosphorylation

Signaling requires messengers whose concentration varies in time and space. Ca^{2+} ions and phosphate ions have come to dominate cellular signaling. Ca^{2+} binding triggers changes in protein shape and charge. Similarly, phosphorylation imparts a negative charge, modulating protein conformations and protein interactions (Hunter, 1995; Soderling, 1999; Clapham, 2007). Ca^{2+}-dependent kinases and protein kinases regulated by interaction with Ca^{2+} binding proteins functionally combine these two major cellular currencies of signal transduction and allow for the perception and transmission of Ca^{2+} signatures directly into phosphorylation events that orchestrate downstream signaling responses (Harmon et al., 2000; Batistič and Kudla, 2004). Plants possess an extensive repertoire of calcium-regulated protein kinases, which are represented by the three families of CCaMKs, CDPKs, and CBL-interacting protein kinases (CIPKs) (Sanders et al., 2002; Batistič and Kudla, 2004; Gleason et al., 2006). While CDPKs and CCaMKs (the later appear not to exist in the *Arabidopsis* genome) represent typical sensor responders, the CIPKs are targets of Calcineurin B-like (CBL) sensor relay proteins. These kinases and Ca^{2+} binding proteins form an intricate cellular network for decoding Ca^{2+} signals in diverse cellular processes (Batistič and Kudla, 2009; Weinl and Kudla, 2009).

The CDPK Signaling System

The *Arabidopsis* genome encodes 34 CDPKs and eight additional CDPK-related kinases (Hrabak et al., 2003). The biochemistry and regulation of CDPKs have been comprehensively reviewed (Harper et al., 2004; Ludwig et al., 2004; Harper and Harmon, 2005). Binding of Ca^{2+} to the C-terminal EF hand–containing regulatory domain leads to conformational changes relieving the active site of the kinase domain from masking by an autoinhibitory domain, resulting in activation of the respective CDPK. This process is enhanced by autophosphorylation of the CDPKs that contribute to full activation of the kinases (Ludwig et al., 2004).

The first conclusive evidence that linked the activation of CDPKs with the induction of signaling responses in vivo was performed on the Cf9-avr9 disease response of tomato (*Solanum lycopersicum*) and was obtained by suppression of Nt CDPK2 by viral-induced gene silencing in *Nicotiana benthamiana* (Romeis et al., 2001). CDPK-silenced plants displayed a reduced and delayed hypersensitive response after race-specific Avr9 elicitation in a gene-for-gene interaction and lacked an accompanying wilting phenotype. Subsequent further analysis of Nt CDPK2 function by T. Romeis and coworkers additionally indicated that elevated CDPK signaling compromises stress-induced MAPK activation and that this inhibition requires ethylene synthesis and perception (Ludwig et al., 2005). These important findings suggest that CDPK and MAPK pathways do not function independently and that a concerted regulation of both pathways controls response specificity to biotic and abiotic stress. Clearly, such aspects of interconnection and interplay between different plant signaling systems will need to be investigated in more depth in the future.

The application of reverse genetics techniques has recently extended our knowledge of the physiological function of several members of the CDPK protein family (see Figure 3 for an illustrated summary). *Arabidopsis* CPK3 and CPK6 function synergistically in the regulation of stomatal closure in response to ABA and external Ca^{2+} elevation (Mori et al., 2006). Moreover, in independent alleles of single and double mutants of *cpk3* and *cpk6*, ABA and Ca^{2+} activation of slow-type anion channels and ABA activation of plasma membrane Ca^{2+}-permeable channels were impaired in guard cells (Mori et al., 2006). In addition to CPK3 and CPK6, *Arabidopsis* CPK4 and CPK11 are critical for proper ABA responsiveness of guard cells and have been shown to phosphorylate the ABA-responsive transcription factors ABF1 and ABF4 in vitro (Zhu et al., 2007). Research in tobacco revealed that CDPK1 regulates the transcription factor RSG in response to gibberellins (Ishida et al., 2008). Phosphorylation of Ser-114 in RSG by CDPK1 promotes interaction with 14-3-3 proteins that appears to regulate the intracellular localization of this transcription factor. Work in potato suggests that two CDPKs (St CDPK4 and St CDPK5) phosphorylate NADPH oxidases and thereby positively regulate the production of ROS (Kobayashi et al., 2007). Accordingly, ectopic expression of a constitutively active

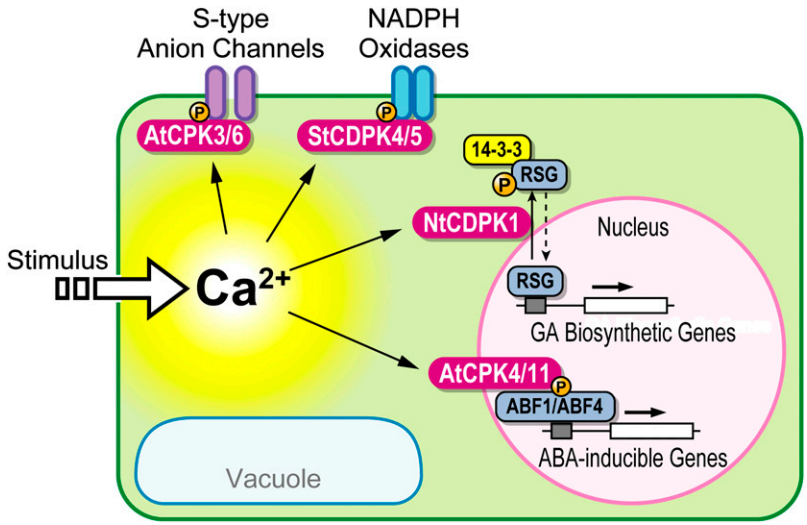

Figure 3. The CDPK Signaling System for Translating Ca^{2+} Signatures into Protein Phosphorylation.

Shown are characterized components of the CDPK system. See text for further details. RSG, repression of shoot growth; ABF, ABA response element-binding factor.

mutant of St CDPK5 provoked ROS production in *N. benthamiana* leaves, and this CDPK-mediated ROS production was disrupted by knockdown of St CDPK5. Together, these findings point to a critical role of CDPK-mediated Ca^{2+} signaling in a diverse set of biological processes. The identification of additional CDPK target proteins and CDPK-regulated process should in the near future allow us to address more precisely how specific CDPKs mechanistically contribute to a specific decoding of distinct Ca^{2+} signatures.

The CBL/CIPK Signaling System

CBL proteins and their interacting protein kinases (CIPKs) were originally identified in *Arabidopsis* (Kudla et al., 1999; Shi et al., 1999). Subsequent comprehensive bioinformatics analyses have identified a complement of 10 CBLs and 26 CIPKs in *Arabidopsis* and 10 CBLs and 30 CIPKs in the genome of rice, respectively (Kolukisaoglu et al., 2004; Weinl and Kudla, 2009). In contrast with the CDPK sensor responders, the families of CBL proteins and their interacting CIPKs separate Ca^{2+} binding function (sensor relay function) and kinase activity (response activity) into two flexible combinable modules. In addition, preferential complex formation of individual CBLs with defined subsets of CIPKs appears to be one of the mechanisms generating the temporal and spatial specificity of Ca^{2+} signals in plant cells (Albrecht et al., 2001). Together, these features allow for the formation of a complex and dynamic Ca^{2+}-decoding signaling network. Since its initial discovery, this signaling system has been subject to intensive research. Advances in our understanding of the physiological function and structural features of these proteins and functional principles of this Ca^{2+}-decoding system have been discussed in several reviews (Luan et al., 2002; Batistič and Kudla, 2004, 2009; Luan, 2009; Luan et al., 2009; Weinl and Kudla, 2009).

CBL proteins exhibit significant similarity to the regulatory B subunit of calcineurin and Neuronal Ca^{2+} Sensor proteins from animals and yeast (Kudla et al., 1999). All CBL proteins share a rather conserved core region consisting of four EF hand Ca^{2+} binding sites that are arranged in completely invariant spacing within the protein (Kolukisaoglu et al., 2004). All CIPK-type kinases are composed of a conserved N-terminal kinase domain and a C-terminal regulatory domain, which are separated by a variable junction domain. Within the rather divergent regulatory domain, the conserved NAF domain has been identified as required and sufficient for mediating CBL interaction (Albrecht et al., 2001). It is assumed that binding of CBL proteins to the NAF domain of CIPKs releases the C-terminal (autoinhibitory) domain from the kinase domain, thereby transforming the kinase into an active state (Guo et al., 2001; Gong et al., 2002). Additional phosphorylation of the activation loop within the kinase domain by an unidentified kinase further contributes to the activation of CIPKs (Gong et al., 2002). An interesting new facet of CIPK–CBL interaction is provided by recent reports of phosphorylation of CBL proteins by their interacting CIPKs that appears to enhance the CBL–CIPK interaction (Mahajan et al., 2006; Lin et al., 2009). Lin et al. (2009) reported that the kinase CIPK24/SOS2 specifically phosphorylates CBL10 (which these authors renamed SCaBP8), but not any other investigated CBL protein, at position Ser-237, a finding that is rather surprising considering that this amino acid position and the surrounding sequence motif is conserved in eight out of the 10 CBL proteins in *Arabidopsis* (Lin et al., 2009). In addition, a protein-phosphatase interaction (PPI) domain mediating CIPK interaction with phosphatases of the PP2C group has been identified within the C terminus of these kinases (Ohta et al., 2003). Unfortunately, the functional implications of this interaction are currently unknown. It appears conceivable that CIPKs may phosphorylate PP2Cs or that

PP2Cs dephosphorylate CIPKs in vivo, as it has been shown for sucrose nonfermenting-related kinases (SnRKs) of the SnRK2 family that are dephosphorylated by a subgroup of PP2Cs (Umezawa et al., 2009). Alternatively, CIPK/PP2C complexes could serve as signaling kinase/phosphatase modules allowing for rapid alternating phosphorylation/dephosphorylation of target proteins. However, crystallization studies of CBL4 (SOS3) in complex with the regulatory domain of CIPK24 (SOS2) suggest that either CBLs or PP2Cs may interact in a mutually exclusive manner with the regulatory domain of CIPKs, thereby preventing the formation of trimeric complexes (Sanchez-Barrena et al., 2007). Consequently, PP2C interaction with the PPI domain of CIPKs would lead to replacement of the CBL protein, which binds to the NAF and partly to the PPI domain, and would allow for competitive formation of either CBL/CIPK or CIPK/PP2C complexes. Considering the recent identification of PP2C phosphatases as direct targets of the PYR/RCAR ABA receptors (Ma et al., 2009; Park et al., 2009), it is tempting to speculate that PP2C-bound CIPKs contribute to early signaling steps after ABA perception, an assumption that would be in agreement with the observation of ABA response phenotypes in many analyzed CIPK mutants (see below).

Evolution and Functional Diversification of the CBL/CIPK Signaling System

The increasing number of available full-genome sequences has facilitated the study of the evolution of the CBL/CIPK signaling system. Single CBL and CIPK genes have been identified in green alga species, such as *Ostreococcus tauri* and *Chlorella* sp, whereas the moss *P. patens* contains four CBLs and seven CIPKs, and the genome of the fern *Selaginella moellendorfii* possesses a complement of five CBL and five CIPK genes (Batistič and Kudla, 2009; Weinl and Kudla, 2009; Batistič et al., 2010). These observations suggest that the complexity of the CBL/CIPK system evolved concurrently with the increasing morphological and developmental sophistication of land plants that enabled the colonization of ecologically diverse and environmentally fluctuating habitats. Surprisingly, CBLs and CIPKs also were recently identified in protozoan eukaryotic species, such as*Trichomonas vaginalis* and *Naegleria gruberi*, raising the question about the function of this Ca^{2+} decoding components in nonplant species (Batistič and Kudla, 2009). Remarkably, the occurrence and function of plant-specific Ca^{2+} signaling components in human pathogens appears not to be restricted to CBLs and CIPKs, as CDPKs have been identified in *Plasmodium falciparum* and *Plasmodium berghei*, where Pb CDPK4 fulfills a critical function during the life cycle of this human pathogen (Billker et al., 2004; Harper and Harmon, 2005). Considering the absence of related proteins in their human host, further advancements of our understanding of the regulation of plant Ca^{2+} sensor proteins may facilitate the identification of therapeutic inhibitors specifically affecting the plant-like proteins of these protozoan species.

Local Ca^{2+} signals at specific microdomains are assumed to be the basis for differential Ca^{2+} signaling (Berridge, 2006). Consequently, Ca^{2+}-decoding systems would be expected to reflect this spatial specification of Ca^{2+} signaling. The CBL/CIPK network appears to meet this demand. Localization studies of all 10 *Arabidopsis* CBL proteins revealed the importance of their variable N-terminal extensions for specific subcellular targeting (Batistič et al., 2010). Four CBL proteins are localized to the plasma membrane, while another four CBLs are localized to vacuolar membrane and two CBLs are detected in the cytoplasm and nucleus (Batistič et al., 2010). These distinct subcellular localizations suggest that CBL Ca^{2+} sensors might function as fast relays of 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. Dual lipid modification by myristoylation and *S*-acylation are required for CBL1 function and for localization of this Ca^{2+} sensor at the plasma membrane. This localization is achieved by a two-step targeting process in which initial myristoylation results in localization at the ER, and subsequent *S*-acylation is crucial for ER–to–plasma membrane trafficking (Batistič et al., 2008). As CBL4, CBL5, and CBL9 have been shown to undergo myristoylation and share the adjacent palmitoylation motif, a similar targeting mechanism can be predicted for these proteins (Batistič et al., 2008).

In contrast with CBL proteins, most CIPKs when expressed as green fluorescent protein fusions exhibit a cytoplasmic and nucleoplasmic localization (D'Angelo et al., 2006; Batistič et al., 2010). However, CBL–CIPK interaction analyses using bimolecular fluorescence complementation revealed that the subcellular localization of CIPKs incorporated into distinct CBL/CIPK complexes is determined by the identity of their CBL moiety (D'Angelo et al., 2006; Cheong et al., 2007; Batistič et al., 2008, 2010; Waadt et al., 2008). For example, CIPK1 is targeted to the plasma membrane by CBL1 or CBL9 (Cheong et al., 2007; Waadt et al., 2008). However, upon interaction with CBL2, the resulting CBL2/CIPK1 complexes are localized exclusively to the tonoplast (Batistič et al., 2008). Similarly, CIPK14/CBL2 complexes have been detected at the tonoplast, while the same kinase is targeted to the plasma membrane upon interaction with CBL8 (Batistič et al., 2010). Remarkably, the cellular targeting of plasma membrane– or tonoplast-localized CBL/CIPK complexes appears not to involve the conventional protein trafficking pathway because inhibition experiments using a dominant-negative form of the SAR1 protein (that interferes with COPII vesicle formation) or brefeldin A (that impedes COPI vesicle formation) did not affect the cellular targeting of singular CBL proteins or CBL/CIPK complexes (Batistič et al., 2008, 2010).

Physiological Functions of CBLs and CIPKs

Forward genetic screens aiming to identify critical components of plant salt tolerance have provided insights into the physiological function of CBLs and CIPKs. The CBL Ca^{2+} sensor SOS3 (At CBL4) and the CIPK-type kinase SOS2 (At CIPK24) appear to be part of a Ca^{2+}-regulated signaling pathway that specifically mediates salt stress adaptation by regulating the Na^+/H^+ antiporter SOS1 (Liu and Zhu, 1998; Halfter et al., 2000; Qiu et al., 2002). Recent studies revealed that mutation of CBL10 also renders plants salt sensitive and that CBL10 is also able to interact with the kinase CIPK24 (Kim et al., 2007; Quan et al., 2007). In vivo analyses revealed a tonoplast localization of the

CBL10/CIPK24 complex, thereby supporting a functional model wherein alternative complex formation of CIPK24 kinases with either CBL4 or CBL10 creates a dual functioning kinase. While CBL4/CIPK24 complexes mediate Na^+ extrusion via the regulation of the H^+/Na^+ antiporter SOS1 at the plasma membrane, formation of CBL10/CIPK24 likely results in Na^+ sequestration into the vacuole by regulating unknown targets (Kim et al., 2007; Weinl and Kudla, 2009). In addition, recent studies have suggested that CBL/CIPK24 complexes at the tonoplast membrane activate and regulate the vacuolar Ca^{2+}/H^+ antiporter CAX1 independently from CBL4 (Cheng et al., 2004b) and have identified subunits of the V-ATPase complex as interacting proteins of CIPK24 (Batelli et al., 2007).

Reverse genetics analyses have greatly advanced our understanding of CBLs and CIPKs and have uncovered crucial functions of these proteins for plant mineral nutrition and for responses to abiotic stresses and hormones, such as ABA (see Figure 4 for an illustrated summary). Characterization of *cbl1* loss-of-function mutant lines revealed a function of CBL1 as a central integrator of responses to abiotic stresses, including drought, cold, and salt (Albrecht et al., 2003; Cheong et al., 2003). 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 (Pandey et al., 2004). Alternative complex formation of the kinase CIPK1 with either CBL1 or CBL9 mediates ABA-dependent and ABA-independent signaling responses (D'Angelo et al., 2006). In addition, CBL9 appears to form a complex with CIPK3 for modulating ABA responses (Pandey et al., 2008).

A breakthrough study in 2006 by Wei-Hua Wu and colleagues uncovered a role of the CBL/CIPK system in regulating K^+ homeostasis and provided the first molecular insights into how plant ion channels might be regulated by phosphorylation (Xu et al., 2006). CIPK23 is targeted to the plasma membrane and activated by the two highly related Ca^{2+} sensors CBL1 and CBL9 (Xu et al., 2006; Cheong et al., 2007). These complexes then regulate the activity of the shaker-like potassium channel AKT1 (Li et al., 2006b; Xu et al., 2006). CIPK23 interacts exclusively with AKT1 but not with other K^+ transporters from *Arabidopsis* (Hedrich and Kudla, 2006; Lee et al., 2007a; Geiger et al., 2009). Interaction analysis in yeast in combination with electrophysiological studies indentified the 2C-type protein phosphatase AIP1 as a negative regulator of AKT1 that counteracts activation by CIPK23 (Lee et al., 2007a). Considering that AIP1 can interact with both CIPK23 and AKT1, it will be most interesting to distinguish if this activation/deactivation switch is brought about by phosphorylation/dephosphorylation or, alternatively, results from competitive binding of either the phosphatase or the kinase to the potassium channel. Besides the regulation of K^+ uptake in roots, the Ca^{2+}-decoding CBL1/CBL9/CIPK23 module appears to be involved in stomata regulation under dehydrating conditions (Cheong et al., 2007).

An exciting novel twist in our understanding of CIPK23 function results from the recent report that this kinase also phosphorylates the nitrate transporter CHL1 (also called NRT1.1). Taking advantage of a novel CHL1 mutant allele (*chl1-9*), Yi-Fang Tsay and colleagues provided compelling evidence that this mutation impairs the nitrate uptake function of CHL1 without affecting the signaling response to nitrate as analyzed by the transcriptional response of the NRT2.1 gene (Ho et al., 2009). Importantly, biochemical and reverse genetics analyses identified CIPK23 as a critical regulator mediating the switch between low- and high-affinity nitrate transport modes by phosphorylating residue Thr-101 of CHL1. Specifically, at low external nitrate concentrations,

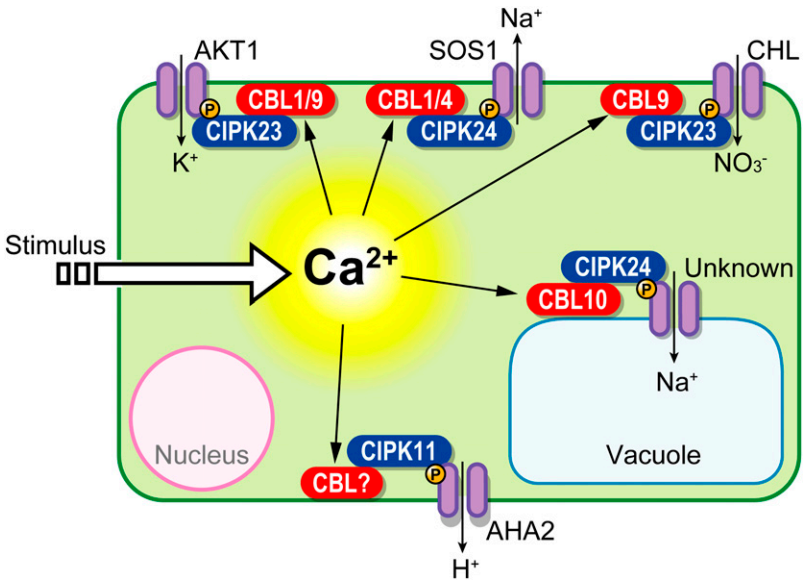

Figure 4. The CBL/CIPK Signaling System for Translating Ca^{2+} Signatures into Protein Phosphorylation.

Shown are characterized complexes consisting of CBL proteins and their interacting CIPKs. AKT1, *Arabidopsis* K^+ transporter 1; SOS1, salt overly sensitive 1; CHL, a nitrate transporter; AHA2, *Arabidopsis* H^+ ATPase 2.

CIPK23-mediated phosphorylation results in low-level nitrate signaling (Ho et al., 2009). Moreover, Ho et al. reported that CIPK23 is independently involved in potassium and nitrate responses, indicating a lack of crosstalk between both ions. Considering the critical involvement of the same CBL1 (and CBL9) Ca^{2+} sensor proteins and CIPK23-dependent phosphorylation in both processes, this puzzling observation underscores the urgent need for further investigation of the primary ion-sensing mechanism(s) that confer this remarkable specificity in plant signaling.

The plasma membrane H^+-ATPase AHA2 has been identified as an additional target of CBL/CIPK signaling complexes (Fuglsang et al., 2007). Phosphorylation of a Ser residue within the C-terminal domain of AHA2 by CIPK11 (designated as PKS5 by these authors) prevents binding of 14-3-3 proteins to this domain and leads to downregulation of AHA2 proton transport activity. Moreover, yeast two-hybrid interaction studies suggested a potential function of CBL2 in the CIPK11-dependent regulation of AHA2 (Fuglsang et al., 2007). However, since CBL2 has so far only been detected at the tonoplast in plants cells (Batistič et al., 2008, 2010), unambiguously determining the identity of the CBL protein mediating Ca^{2+}-dependent regulation of AHA2 in plants may require additional in planta or reverse genetics analyses.

Finally, recent work performed in rice further extended the identified physiological functions of CIPKs (Lee et al., 2009). The results from this study indicate that protein kinase Os CIPK15 plays a key role in O_2 deficiency tolerance in rice and is required for growth of rice under flooded conditions. Moreover, CIPK15 regulates the plant global energy and stress sensor SnRK1A, thereby integrating responses to O_2 deficiency with sugar signaling and enabling rice growth under floodwater. The latter finding may point to a general role of the CBL/CIPK system in fine-tuning plant metabolism in response to adverse environmental conditions.

The results of all these studies suggest that the CBL/CIPK network represents a central and critical signaling system for decoding Ca^{2+} signals in response to a wide range of stimuli. So far, research on CBLs and CIPKs has been focused mainly on abiotic stress responses and ion uptake. However, it is safe to predict that future studies will most likely uncover a role of this Ca^{2+} decoding system in additional processes, such as plant pathogen interactions. It is now well established 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, furthering our understanding of this signaling system will require the combined application of state-of-the-art cell biological techniques that enable the monitoring of dynamic protein–protein interactions and protein targeting. Such approaches should also allow the elucidation of mechanistic factors that determine the decision making in this flexible interaction network and will be of primary importance to advance our understanding of Ca^{2+} decoding mechanisms.

Converting Ca^{2+} Signals into Transcriptional Responses

Transcriptional regulation is coupled with numerous signaling processes that can be conveyed by several second messengers and the function of Ca^{2+} binding proteins (Ikura et al., 2002). Despite the obvious importance of defined Ca^{2+} signatures in eliciting specific transcriptional response reactions in plants to stimuli, such as cold, drought, and salt stress, the molecular mechanisms mediating Ca^{2+}-responsive gene expression have long remained little understood (Scrase-Field and Knight, 2003). This is due in part to the difficulty in discriminating between stress-dependent Ca^{2+} responses per se and stress-dependent but Ca^{2+}-independent transcriptional reactions (Finkler et al., 2007b). Recently, the induction of defined artificial Ca^{2+} transients in response to calmodulin antagonists like WP7 and SKF-7171 has allowed the identification of 230 Ca^{2+}-responsive genes that were differentially expressed 1 h after stimulus in *Arabidopsis* (Kaplan et al., 2006). Since the microarrays used in this study covered only 25% of the known *Arabidopsis* genes, this finding suggests that ~3% of the protein-coding genes in the *Arabidopsis* genome are subject to regulation by Ca^{2+}. Remarkably, many of the genes identified in this study as being Ca^{2+} regulated were previously characterized as early stress-induced genes.

Within the promoter regions of Ca^{2+}-regulated genes, this study identified abscisic acid–responsive element (ABRE)-related *cis*-elements as being sufficient to confer transcriptional regulation in response to cytosolic Ca^{2+} signatures. This finding is significant because such ABREs have been identified in the promoter of CBF/DREB1 transcription factors that function as master regulators of abiotic stress responses (Finkler et al., 2007b). Therefore, these results point to a direct interconnection of Ca^{2+}-regulated gene transcription and abiotic stress responses. Such an immediate conversion of Ca^{2+} signatures into transcriptional regulation may be in part achieved by the function of calmodulin binding transcription factors (CAMTA proteins).

Ca^{2+}-Regulated Transcription Factors

CAMTAs are Ca^{2+}-dependent CaM binding transcription factors forming a family of six members in *Arabidopsis* (Finkler et al., 2007a). They share a conserved domain structure including an N-terminal CG-1 domain mediating binding to DNA *cis*-elements (CAMTA binding sites), including ABREs (Finkler et al., 2007a). The C-terminal CaM binding domain of CAMTAs mediates interactions with calmodulin. First insights into the physiological function of plant CAMTA proteins came from a reverse genetic analysis of *Arabidopsis* CAMTA3 function that revealed a critical role of this protein in suppressing plant responses to pathogens such as *P. syringae* and *Botrytis cinerea* (Galon et al., 2008). Most recent work on CAMTA3 (designated At SR1) uncovered that CAMTA3 directly interacts with the promoter of the *EDS1* gene, an established regulator of salicylic acid levels, and represses its expression (Figure 5) (Du et al., 2009). Moreover, Ca^{2+}/calmodulin binding to CAMTA3 was required for the suppression of plant defense, indicating a direct role of Ca^{2+}/calmodulin in regulating the function of CAMTA3 (Du et al., 2009). Important evidence for a direct link between Ca^{2+} signaling via CAMTA1 and CAMTA3 and the regulation of cold tolerance in plants was provided by the discovery that these CAMTA proteins bind to regulatory elements in the promoter region of the *DREB1c/CBF2* gene (Doherty et al., 2009). A single *camta3* mutant exhibited a

significant reduction in cold induction of a number of known cold-induced genes, including *CBF2*. Moreover, *camta1 camta3* double mutant plants were impaired in their cold acclimation to freezing tolerance (Doherty et al., 2009). These findings establish a crucial role of Ca^{2+}/calmodulin-regulated CAMTA transcription factors in controlling CBF-regulated cold-responsive gene expression in plants (Figure 5). Direct decoding of cold-induced Ca^{2+} signatures into the regulation of gene expression may occur through direct and Ca^{2+}-dependent interaction of CAMTAs with members of the *Arabidopsis* calmodulin Ca^{2+} sensors family (McCormack et al., 2005). The function of calmodulin in the regulation of gene expression by direct interaction with transcription factors appears not to be restricted to their interaction with CAMTAs because interactions of calmodulin with MYB and WRKY transcription factors has also been reported (Park et al., 2005; Yoo et al., 2005). In this regard, it will be important to investigate exactly how the stimulus-induced changes in Ca^{2+} concentration that occur in the cytoplasm are connected into transcriptional regulation that proceeds in the nucleus.

Transcriptional Regulation by Calmodulin

Calmodulins are conserved eukaryotic Ca^{2+} sensor relay proteins that upon Ca^{2+} binding undergo extensive conformational changes that result in protein interactions with their target proteins (Luan et al., 2002; McCormack et al., 2005). 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 CAM6 differ in one amino acid position from CAM7 (McCormack et al., 2005). Classical microinjection experiments into hypocotyl cells of the phytochrome deficient tomato *aurea* mutant suggested a function of Ca^{2+} and calmodulin in light signal transduction that controls regulation of light-responsive gene expression (Neuhaus et al., 1993; Bowler et al., 1994). A recent study of CAM7 from *Arabidopsis* revealed surprising insights into the regulation of light-induced gene expression by calmodulin (Kushwaha et al., 2008). The authors identified specifically CAM7 but not CAM2/3/5 as a transcriptional regulator that directly interacts with promoters of several light-inducible genes (Figure 5). In accordance with such a function of CAM7, *cam7* mutants exhibited a reduced expression of light-inducible genes. Conversely, overexpression of CAM7 resulted in an increase in the expression of light-inducible genes. The findings of this study suggest a direct translation of cytoplasmic Ca^{2+} signatures by the Ca^{2+} sensor responder protein CAM7 into regulation of gene expression by DNA binding. To further elucidate details of this regulatory circuit, it will be most interesting to investigate if this function requires the translocation of CAM7 from the cytoplasm to the nucleus and/or the interaction of CAM7 with additional (transcription factor) proteins.

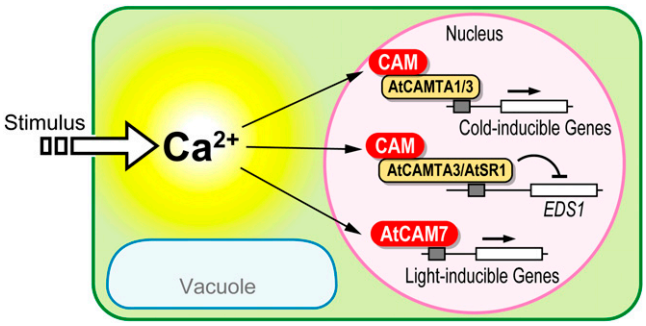

Figure 5. Converting Ca^{2+} Signals into Transcriptional Responses.

Recent studies have unveiled that Ca^{2+}/CAMs (calmodulins) regulate CAMTAs that interact with promoters of abiotic stress-responsive genes. Interestingly, *Arabidopsis* CAM7 is likely a direct converter of cytoplasmic Ca^{2+} signatures into regulation of gene expression. *EDS1*, enhanced disease susceptibility 1.

Conclusions and Future Perspectives

During the past years we have witnessed tremendous progress in our understanding of Ca^{2+} signaling processes in plants. When comparing research on Ca^{2+} signaling in animals and plants during the past decade, remarkably different developments become apparent. Whereas in animals the components and mechanisms that exercise primary Ca^{2+}-releasing events and shape Ca^{2+} signatures have seen the most significant advances, in plant research, most progress has been achieved in identifying and characterizing components that decode Ca^{2+} signals.

A central question around which Ca^{2+} signaling research in plants is centered is: How can one ion specifically control so many different processes and events? Current research is beginning to provide answers. Ca^{2+} signaling in plants involves many facets that can define and adjust responses in both time and space. The unequal distribution of this ion in the cell provides the basis for rapid Ca^{2+} fluxes and the resulting concentration changes. Many energized cation transporters and channels that were previously assumed to merely be regulators of Ca^{2+} homeostasis actually appear to play a role in Ca^{2+} signaling. However, a major bottleneck in our understanding of the generation of Ca^{2+} signals is the paucity of molecular information about true Ca^{2+} channels. It may require novel genetic screens or the application of chemical genetic screens (that have been successful, for example, in identifying plant ABA receptors) to successfully tackle this serious limitation of plant Ca^{2+} signaling research.

It is now widely accepted that complex families of Ca^{2+} binding proteins that function as calcium sensors provide the toolkit for deciphering various Ca^{2+} signatures. Calmodulins, CMLs, CDPKs, and CBL/CIPK complexes form intricate signaling networks for translating these signatures into downstream phosphorylation events and transcriptional responses. An important role of Ca^{2+}-regulated transcription factors like CAMTAs in these processes is emerging. It is very likely the network-like character of these signaling systems that warrants flexible but robust information processing. Accumulating evidence also points to two levels at which Ca^{2+} signaling is operative. Within specific cells, such as guard cell or root hairs, intracellular Ca^{2+} signaling processes occur at high spatial and temporal specificity. This layer of Ca^{2+} signaling appears to be superimposed on the tissue or organismic level where specific tissue layers or cell types exhibit defined and prominent changes in Ca^{2+} concentration.

It has become evident that parameters such as the specific Ca^{2+} binding affinity, the specific cellular concentration and

subcellular localization, and the specific interaction affinities of Ca^{2+} decoders are critical components that contribute to generating specificity in signal-to-response coupling. However, a major challenge that remains is to understand mechanistically how exactly calcium signatures or variable oscillations are sensed and transduced by calcium sensor proteins. Tackling this important task will require serious investments in structural analyses of Ca^{2+} sensors, NMR-based studies of their structural dynamics, extensive investigation of Ca^{2+} binding and Ca^{2+}-dissociation parameters of Ca^{2+} sensors, and novel cell biological approaches to visualize the dynamics of protein interactions and protein complex formation in planta. The integration of these data with advanced fluorescence resonance energy transfer-based monitoring of cellular Ca^{2+} dynamics and the mathematical modeling of such processes represent the route to be pursued in plant Ca^{2+} signaling research in the near future. Currently, despite all the exciting progress described in this review article, we are still not close to finally cracking the calcium code in plants.

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