Combining Genetics and Cell Biology to Crack the Code of Plant Cell Calcium Signaling

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(Published 2 October 2001)

Plant hormones, light receptors, pathogens, and abiotic signals trigger elevations in the cytosolic calcium concentration, which mediate physiological and developmental responses. Recent studies are reviewed here that reveal how specific genetic mutations impair or modify stimulus-induced calcium elevations in plant cells. These studies provide genetic evidence for the importance of calcium as a second messenger in plant signal transduction. A fundamental question arises: How can different stimuli use the same second messenger, calcium, to mediate different responses? Recent research and models are reviewed that suggest that several important mechanisms contribute to specificity in calcium signaling in plant cells. These mechanisms include (i) activation of different calcium channels in the plasma membrane and organellar membranes, (ii) stimulus-specific calcium oscillation parameters, (ii) cell type-specific responses, and (iv) intracellular localization of calcium gradients and calcium elevations in plant cells.

Plants as Models to Study Calcium Signaling

In the quest to understand how the concentration of free cytosolic calcium ($[Ca^{2+}]_{cyt}$) influences signal transduction at the single-cell level, higher plant cells provide interesting, genetically tractable systems, because most plant cells use $[Ca^{2+}]_{cyt}$ as a second messenger to transduce responses to diverse environmental and developmental stimuli (*1*, *2*). But how can this universal signaling element control the specificity of cellular responses to a multitude of diverse stimuli? This review focuses on how the combination of new molecular, genetic, cell biological, and imaging techniques is beginning to identify genes that affect Ca^{2+}-based signaling systems and unravel the fundamental questions of Ca^{2+} signaling and response specificity in higher plant cells.

Ca^{2+}-Signatures Provide a Model for Specificity Encoding

In plant cells, "Ca^{2+}-signatures," composed of differences in the spatiotemporal characteristics of Ca^{2+} signals, have been proposed to act as a central mechanism for encoding the specificity in cellular responses (*3*, *4*). However, the ability to measure $[Ca^{2+}]_{cyt}$ with the bioluminescent protein aequorin (*5*, *6*) or the reliance on technically difficult Ca^{2+}-dye microinjection techniques necessitated by the plant cell wall (*7*) has limited the investigation of Ca^{2+} signaling in plants at the single-cell level. Until 2 years ago, no plant mutants had been identified that modified stimulus-induced increases in $[Ca^{2+}]_{cyt}$. This picture is rapidly changing.

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Elements that may contribute to Ca^{2+}-signatures have been identified in nonplant cells, including differences in the spatial localization of Ca^{2+} signals arising from mobilization of different subcellular Ca^{2+} stores (*8*-*11*), differences in the kinetics of a Ca^{2+} signal or oscillation (*12*-*14*), or differences in the activation or sensitivity of downstream decoding elements, such as calcium/calmodulin kinase II (CaMKII) (*15*) or protein kinase C (*16*). However, the molecular machinery underlying Ca^{2+}-signature generation and cellular interpretation of the Ca^{2+}-signature has remained largely elusive, because most of the standard animal cell types used for these analyses are not easily genetically modified. Advances in using plant cells and mutants to study Ca^{2+} signaling are now providing genetically pliable cellular systems.

New Approaches for Determining Ca^{2+} Signaling Specificity in Plants

It has long been hypothesized that Ca^{2+} signaling in plant cells is more complex than a simple "on-off" switch, because differing strengths of a single stimulus—such as cold shock (*17*), mechanical stress (*18*), osmotic shock (*19*), oxidative stress (*20*, *21*), extracellular Ca^{2+} (*7*), strontium (*22*), or the hormone abscisic acid (ABA) (*23*)—could produce Ca^{2+} signals or oscillations of differing magnitudes or kinetics or could use different Ca^{2+} pools (*24*, *25*). In addition, different stimuli can elicit independent, mutually exclusive Ca^{2+} signals, presumably through activation of different signaling cascades or mobilization of different Ca^{2+} pools (*26*). Disrupting different phases of a Ca^{2+} signal pharmacologically can also affect the induction of physiological responses (*27*).

Advances in cell biological techniques are providing new avenues for deciphering Ca^{2+} signatures in plant cells. Enhancer trap expression lines have been used to target aequorin to specific cell types in the *Arabidopsis* root (*28*). Significant differences in the amplitude and duration of elevations in $[Ca^{2+}]_{cyt}$ were apparent for cortex, steele, epidermal, or pericycle cells in response to drought, saline, or cold stresses, implying differences in perception and response to stresses in these different cell types. Similarly, in tobacco, aequorin has been targeted specifically into guard cells (the two cells that border and regulate the stomatal pore in plant leaves), by using guard-cell-specific promoters (*29*). The source of Ca^{2+} that was mobilized and the amplitude and duration of the increase in $[Ca^{2+}]_{cyt}$ in these guard cell populations differed, depending on whether stomatal closure was elicited by ABA, cold shock, or mechanical stress, again pointing toward specificity in the generation and interpretation of the Ca^{2+}-signature. However, because these new systems measure Ca^{2+} signals only from a population of cells, complex Ca^{2+} dynamics, such as oscillations in $[Ca^{2+}]_{cyt}$, can be masked by cells responding out of phase.

Improved imaging techniques and the advent of pH-insensitive green fluorescent protein (GFP)-based "cameleon" $[Ca^{2+}]_{cyt}$

indicators (*30*, *31*) have allowed the question of differential Ca^{2+} signaling to be addressed noninvasively at the single-cell level in plants. Oscillations in $[Ca^{2+}]_{cyt}$ in *Arabidopsis* guard cells expressing yellow cameleon 2.1 differed in their amplitude, frequency, transient number, and transient duration in response to the four stomatal closing stimuli ABA, extracellular Ca^{2+}, cold, and oxidative stress (*32*), suggesting that different stimuli can induce different Ca^{2+}-signatures in a single plant cell type, possibly by activation of separate signaling pathways. This is further supported by the observation that only particular Ca^{2+} signals were disrupted in guard cells of the V-ATPase mutant *det3* (see below), suggesting that particular stimuli are "hard wired" (*33*) to use a specific set of Ca^{2+} pools, channels, and transporters that constitute separate Ca^{2+} signaling pathways in guard cells and that the effects of mutations may be limited to individual signaling pathways (*32*).

A Novel Class of Hyperpolarization-Activated Ca^{2+} Channels

Calcium elevations and oscillation patterns are generated by activation of diverse types of Ca^{2+} channels in organelle membranes and in the plasma membrane (*2*). A specific class of hyperpolarization-activated Ca^{2+} channels is widely distributed in plants and may be part of a Ca^{2+} oscillator and contribute to Ca^{2+} gradients formed during polar growth. In contrast to the better-known eukaryotic Ca^{2+} channels, which are activated by depolarization, these plant Ca^{2+} currents (I_{Ca}) show enhanced opening when the plasma membrane is polarized to potentials negative of about −80 mV. Because most plant cells have resting potentials negative of −100 mV, I_{Ca} channels must be highly regulated to prevent Ca^{2+} influx at resting potentials and allow Ca^{2+} influx only when stimulated.

Several stimuli can increase $[Ca^{2+}]_{cyt}$ and activate I_{Ca}. Fungal elicitors stimulate hyperpolarization-activated Ca^{2+} channels in tomato cells in suspension culture (*34*). In guard cells, hyperpolarizations induced by lowering the extracellular KCl concentration (*35*, *36*) or by impalement microelectrodes (*36*) can elevate $[Ca^{2+}]_{cyt}$. In *Arabidopsis* guard cells, ABA and reactive oxygen species (ROS) activate I_{Ca}, and ABA causes ROS production (*21*). In *Vicia* guard cells, ABA stimulates hyperpolarization-activated Ca^{2+} channels (*37*) and enhances ROS production (*38*). Whether Ca^{2+} signaling in *Vicia* is affected by ROS remains to be determined. In *Vicia*, elevation of $[Ca^{2+}]_{cyt}$ decreases currents carried by I_{Ca} channels, providing a negative-feedback mechanism that could contribute to the generation of oscillations in $[Ca^{2+}]_{cyt}$ (*37*). Hyperpolarization-activated channels similar to I_{Ca} were also found in *Arabidopsis* root cortex cells from the elongation zone and the epidermis of growing root tips, but not in root pericycle cells and in mature epidermal cells (*39*). Interestingly, channels with properties similar to those of I_{Ca} were found in the apex of *Arabidopsis* root hair cells, indicating a possible role in maintaining Ca^{2+} gradients that control polar growth (*40*). Together, all of these recent studies lead to the hypothesis that hyperpolarization-activated Ca^{2+} channels are widely distributed in plants and may play important roles in diverse signal transduction, growth, and developmental responses.

ABA stimulates the production of ROS in guard cells (*21*) and in maize embryos (*41*). Both studies together suggest that ROS may be an important second messenger in ABA signaling. The recessive ABA-insensitive mutant *gca2*, which disrupts all known ABA responses (*42*), impaired H_2O_2 activation of I_{Ca}, providing genetic evidence for a role of ROS and I_{Ca} in ABA signaling (*21*). Pathogen elicitors cause ROS production in plants (*43*). Ca^{2+} influx is triggered by elicitors both before and after ROS production (*5*, *34*, *44*, *45*), suggesting that more than one Ca^{2+} channel or activation mechanism may contribute to this response. The finding that elicitors can stimulate hyperpolarization-activated Ca^{2+} channels suggests that I_{Ca} contributes to signals initiated by pathogens (*34*). Interestingly, in the absence of elicitor in a subpopulation of cells from tomato and in the absence of H_2O_2 in a subpopulation of guard cells, spontaneous activation of hyperpolarization-activated Ca^{2+} channels was observed and could be inhibited by dithiothreitol (*21*, *46*). Elicitor-activated I_{Ca}, therefore, may contribute to the secondary Ca^{2+} response that follows ROS production.

It will be important to determine mechanisms in addition to elicitors, ABA, and ROS that activate I_{Ca} in different plant cell types. Although the molecular identity of these channels is still unknown, it is of interest that both glutamate and cyclic nucleotides can elevate $[Ca^{2+}]_{cyt}$ in plant cells (*47*, *48*). Genes for candidate glutamate receptor and cyclic nucleotide-gated ion channels that might mediate these $[Ca^{2+}]_{cyt}$ elevations are found in the *Arabidopsis* genome (*49*-*52*).

Building Genetic Maps of Cytosolic Ca^{2+} Signaling Pathways

The ability to position or "map" specific mutations in relation to the cytosolic Ca^{2+} signal in distinct signaling pathways is emerging as a powerful approach for dissecting the genetic basis of Ca^{2+} signaling in plant cells. Only recently, a number of mutations in plants have been positioned in relation to Ca^{2+} elevations in their respective signaling pathways. In host legumes, infection of root hairs by *Rhizobium* bacteria induces the formation of nodules that house the bacteria required for symbiotic nitrogen fixation. An early signaling event in the infection process is the induction of a series of $[Ca^{2+}]_{cyt}$ spikes by bacterial lipochitin oligosaccharide nodulation factors (nod factors). These $[Ca^{2+}]_{cyt}$ spikes are predominantly localized in the nuclear area and precede the morphological changes required for nodulation (*53*). In *Medicago truncatula*, mutants defective in the nodulation response have been mapped with respect to the Ca^{2+} signal (*54*). The mutants *dmi1*, *dmi2*, and *dmi3* all show the same phenotype—root hair swelling in response to *Rhizobium*—but are defective in subsequent root hair morphology changes, changes in gene expression, and nodulation. The *dmi1* and *dmi2* mutants abolished or repressed nod factor-induced $[Ca^{2+}]_{cyt}$ spikes in root hairs and thus are positioned upstream of the induction of $[Ca^{2+}]_{cyt}$ signals. This implies that root hair swelling is a Ca^{2+}-independent process that precedes $[Ca^{2+}]_{cyt}$ spiking. The *dmi3* mutant showed normal $[Ca^{2+}]_{cyt}$ spiking and therefore affects signaling downstream of, or parallel to, $[Ca^{2+}]_{cyt}$ and may be involved in sensing changes in $[Ca^{2+}]_{cyt}$, because very early morphological changes are disrupted in this mutant (*54*). Another complementation group of mutants, the *nsp* and *hcl* mutants, exhibit early root hair branching, morphological changes, and normal $[Ca^{2+}]_{cyt}$ spike induction, suggesting that they lie parallel to or further downstream of the Ca^{2+} signal, but still prevent the nodulation response. In a similar study in pea roots of seven nonnodulating mutants, three acted upstream, abolishing nod factor-induced $[Ca^{2+}]_{cyt}$ spiking, whereas the others were hypothesized to act at various positions

downstream of the Ca^{2+} signal (*55*).

In *Arabidopsis*-expressing aequorin, blue light induces a transient increase in $[Ca^{2+}]_{cyt}$. Of the mutants *cry1*, *cry2*, and *nph1*, each disrupted in one of three blue-light photoreceptors, only the phototropism-defective mutant *nph1* showed a severe disruption of blue light-induced elevation of $[Ca^{2+}]_{cyt}$, which suggests that NPH1 acts upstream of increases in $[Ca^{2+}]_{cyt}$ in controlling phototropism (*56*). Conversely, other mutations in *Arabidopsis* lie downstream of, or parallel to, changes in $[Ca^{2+}]_{cyt}$. For example, the *stf6* mutation renders *Arabidopsis* hypersensitive to cold shock, but does not affect the cold-induced increases in $[Ca^{2+}]_{cyt}$, as measured by aequorin in whole seedlings (*57*).

Arabidopsis mutants that affect stomatal closure in response to the plant hormone ABA have also been positioned relative to changes in $[Ca^{2+}]_{cyt}$. The dominant protein phosphatase 2C mutants *abi1-1* and *abi2-1* render stomata insensitive to ABA and repress ABA-induced increases in $[Ca^{2+}]_{cyt}$ (*58*). To directly test whether the *abi* mutants affect other components downstream of Ca^{2+}, experimental elevation of $[Ca^{2+}]_{cyt}$ led to Ca^{2+}-activated anion channel activity and stomatal closure, showing that the *abi* mutations impair ABA-induced increases in $[Ca^{2+}]_{cyt}$, but not processes downstream of the Ca^{2+} signal. Thus, *abi1-1* and *abi2-1* can be placed downstream of ABA and upstream of the increase in $[Ca^{2+}]_{cyt}$. ABA stimulates production of the Ca^{2+}-mobilizing second messenger inositol trisphosphate (IP_3) in seeds (*59*), and ABA activation of plasma membrane Ca^{2+} influx is required for ABA induction of *rab18* gene expression in *Arabidopsis* suspension culture cells (*60*). These studies suggest that ABA-induced increases in $[Ca^{2+}]_{cyt}$ may play general roles in ABA signaling in various tissues. This is consistent with general ABA insensitivity and the suppression of the increase in $[Ca^{2+}]_{cyt}$ in *abi1-1* and *abi2-1* (*58*).

Mutations that Impinge on Cytosolic Ca^{2+} Signals

Rather than acting upstream or downstream of changes in $[Ca^{2+}]_{cyt}$, other *Arabidopsis* mutants have been identified that affect the Ca^{2+} signal or Ca^{2+} homeostasis more directly and can be thought of as impinging upon the Ca^{2+} signaling machinery. For example, a tip-focused gradient in $[Ca^{2+}]_{cyt}$ is required for root hair growth, but this gradient is disrupted in the *rhd-2* mutant, and sustained root hair growth is abolished (*61*). Similarly, a tip-focused gradient in $[Ca^{2+}]_{cyt}$ in pollen tubes is required for normal growth and oscillates close to the phase of oscillations in growth (with a 6-s delay), whereas the Ca^{2+} influx that replenishes the Ca^{2+} stores is completely phase-shifted relative to growth oscillations (*62-64*). Oscillations in $[Ca^{2+}]_{cyt}$ and pollen tube growth can be disrupted by microinjection of antibodies against (or overexpression of) the small guanosine triphosphate (GTP)-binding protein Rop1At (*65*), indicating the requirement for Rop1 in tip growth. Additionally, gamete fusion in maize leads to Ca^{2+} influx and initiates a Ca^{2+} wave in the egg cell that appears to be required for successful fertilization (*66*). Together, these studies show that cytosolic Ca^{2+} gradients, oscillations, and waves are important in controlling polarized growth and fertilization in plant cells.

The role of oscillations in $[Ca^{2+}]_{cyt}$ in stomatal closure was investigated in *Arabidopsis* guard cells expressing the GFP-based, molecularly encoded Ca^{2+} indicator yellow cameleon 2.1. Oscillations in $[Ca^{2+}]_{cyt}$ were stimulated in response to ABA, oxidative stress, cold, or increases in external Ca^{2+} (*32*). However, in guard cells of the V-ATPase mutant *det3*, external Ca^{2+} and oxidative stress elicited prolonged Ca^{2+} increases (plateaus), which did not oscillate, and long-term stomatal closure was abolished. However, oscillations in $[Ca^{2+}]_{cyt}$ and stomatal closure were normal in response to ABA and cold in *det3* guard cells. The findings that ROS stimulate partial stomatal closure (*20*, *21*) and that H_2O_2 and ABA generate different Ca^{2+} responses in *det3* (*32*) support the model that branched Ca^{2+} influx and release pathways are controlled in parallel by ABA (*21*).

In *det3* guard cells, experimentally imposing external Ca^{2+}-induced oscillations with a "Ca^{2+} clamp" technique that uses repetitive exchanges in the extracellular KCl concentration to elicit hyperpolarization-activated $[Ca^{2+}]_{cyt}$ transients in guard cells rescued stomatal closure. Moreover, imposing a plateau in $[Ca^{2+}]_{cyt}$ in wild-type cells abolished long-term stomatal closing, suggesting that oscillations in $[Ca^{2+}]_{cyt}$ are required for persistent closure (*32*). However, if the total increase in $[Ca^{2+}]_{cyt}$ during a plateau was too high, it might abolish stomatal closure independently of whether $[Ca^{2+}]_{cyt}$ oscillates or not (*67*). To address this possibility, plateaus in $[Ca^{2+}]_{cyt}$ or oscillations in $[Ca^{2+}]_{cyt}$ with the same total increase in $[Ca^{2+}]_{cyt}$ were imposed in guard cells (*68*). No long-term stomatal closure was induced by the plateau in $[Ca^{2+}]_{cyt}$. Furthermore, by abolishing cytosolic Ca^{2+} transients by removing extracellular Ca^{2+}, the extracellular KCl exchanges associated with the Ca^{2+} clamp produced only a minimal change in stomatal aperture (*68*, http://www.nature.com/nature/journal/v411/n6841/suppinfo/4111053a0.html), further illustrating that cytosolic Ca^{2+} oscillations mediate stomatal closure. Together, these data provide genetic and experimental evidence that oscillations in the $[Ca^{2+}]_{cyt}$ are required for stomatal closure.

But do cells really perceive Ca^{2+} oscillation parameters, such as frequency, or is an oscillating Ca^{2+} signal alone enough to elicit the response of stomatal closure? Experiments with the Ca^{2+} clamp technique in wild-type guard cells revealed dual mechanisms of Ca^{2+}-dependent stomatal closure (*68*). A short-term "Ca^{2+} reactive" stomatal closure occurred rapidly when $[Ca^{2+}]_{cyt}$ transients were imposed, irrespective of the oscillation kinetics. Subsequently, however, stomata remained closed only if the oscillations in $[Ca^{2+}]_{cyt}$ had parameters within a defined window of frequency , transient number, duration, and amplitude. These data show that the degree of long-term stomatal closure is programmed by the Ca^{2+} oscillation parameters, suggesting that oscillation parameters are indeed perceived by guard cells (*68*).

A Ca^{2+}-independent pathway has been proposed for ABA-induced stomatal closing on the basis of negative results showing lack of resolved ABA-induced Ca^{2+} increases in guard cells (*69*). However, injection of the Ca^{2+} chelator BAPTA abolished ABA-induced stomatal closing and gene expression in *Arabidopsis* guard cells (*70*). To unequivocally determine whether ABA can close stomata in the absence of $[Ca^{2+}]_{cyt}$ increases, analysis of mutants that are defective in ABA-induced Ca^{2+} signals but continue to show ABA-induced stomatal closure would demonstrate the presence of and allow direct analysis of a Ca^{2+}-independent pathway.

Genetic Alteration of the Guard Cell Ca^{2+} Oscillator

In guard cells of the ABA-insensitive *gca2* mutant (*42*), the kinetics of the oscillations in $[Ca^{2+}]_{cyt}$ elicited by ABA and extracellular Ca^{2+} were altered, having increased frequencies and reduced transient durations compared with the wild type (*68*). Long-term stomatal closure was abolished in response to these stimuli in

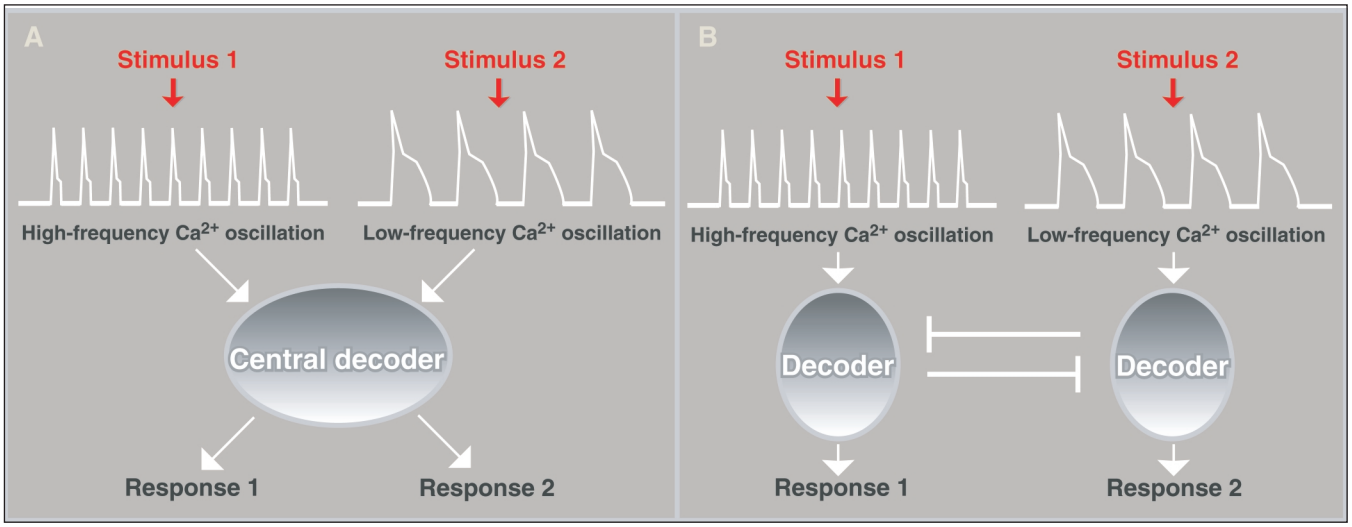

Fig. 1. Models for molecular mechanisms that read oscillations in $[Ca^{2+}]_{cyt}$ in plant cells. (**A**) Separate stimuli elicit specific cytosolic Ca^{2+} signatures by generating oscillations in $[Ca^{2+}]_{cyt}$ with different kinetics. These oscillations are "sensed" by a common decoder that activates different downstream pathways, depending on the oscillation kinetics. (**B**) Distinct oscillations in $[Ca^{2+}]_{cyt}$ induced by separate stimuli activate different decoders by virtue of their different kinetics. These separate decoders in turn activate independent response pathways. Blunt arrows indicate negative cross-regulation of separate decoders.

this mutant. Long-term stomatal closure could be recovered in *gca2* when oscillations in $[Ca^{2+}]_{cyt}$ with parameters that cause closure in the wild type were experimentally imposed in *gca2* guard cells by the Ca^{2+} clamp technique (*68*). Additionally, when oscillations in $[Ca^{2+}]_{cyt}$ that mimicked the rapid frequency and transient duration of oscillations in *gca2* were imposed on wild-type guard cells, no long-term stomatal closure occurred. These data show that the failure of *gca2* stomata to close in response to ABA or extracellular Ca^{2+} is predominantly due to the mutation disrupting the function of the guard cell Ca^{2+} oscillator.

The ABA-insensitive *gca2* mutant is one of only two known eukaryotic mutants for which desynchronized Ca^{2+} oscillations have been shown to affect the subsequent physiological response (*68*, *71*). Therefore, further analysis of *gca2* can provide a genetic model system for analyzing how oscillations are synchronized that may yield results applicable to many eukaryotic cells. As discussed earlier, *gca2* impairs ROS activation of I_{Ca} (*21*). The delay between H_2O_2 application and activation of I_{Ca} (*21*) and the presence of residual I_{Ca}-like currents in some *gca2* guard cells (*72*) suggest that GCA2 encodes an intermediate signal transducer rather than the I_{Ca} channel itself. Together, these data suggest that I_{Ca} activation contributes to the ABA-induced Ca^{2+} oscillator in guard cells. Because negative-feedback loops are essential for generating oscillations in $[Ca^{2+}]_{cyt}$, it is possible that mutants that desynchronize oscillations in $[Ca^{2+}]_{cyt}$ code for Ca^{2+}-sensitive regulatory feedback proteins. The combination of *Arabidopsis* molecular genetics and cell biological analyses in guard cells provides powerful tools for addressing these classical questions in cellular Ca^{2+} signaling (*73*).

Decoding Cytosolic Ca^{2+} Oscillations

The effect of *gca2* and *det3* mutants on guard cell cytosolic Ca^{2+} signaling shows that the kinetics of slow oscillations in $[Ca^{2+}]_{cyt}$ in guard cells can carry the information necessary to control the degree of a graded physiological response (*3*, *4*). Low-frequency Ca^{2+} oscillations, with periods similar to those in guard cells, have also been measured in a variety of animal cells and have been demonstrated to control downstream processes and physiological responses [reviewed in (*74*)]. The identities of downstream components that "read" $[Ca^{2+}]_{cyt}$ oscillations in plant cells remain unknown. A variety of candidate proteins have been identified in guard cells and include Ca^{2+}-dependent protein kinases (*75*), Ca^{2+}-dependent ion channels on the vacuolar (*76*) or plasma membranes (*77*), Syntaxins (*78*), or transcription factors (*79*). Using molecular genetic approaches to modify the expression or activity of potential decoders will be invaluable for identifying the signaling elements that lie downstream of Ca^{2+}. Critically assessing the effect of oscillations that elicit the Ca^{2+}-programmed stomatal closure response (*68*) on guard cell transporter activities, such as K^+ channels, anion channels, and pumps, will indicate which of these downstream response elements are necessary for this long-term response.

Overall, two possible scenarios for oscillation decoding are either (i) a central decoder reads various oscillations and then differentially activates downstream response pathways to elicit separate physiological responses (Fig. 1A), or (ii) oscillations with different kinetics stimulate different downstream decoders, each of which in turn activates separate response pathways (Fig. 1B). Two decoders could act as positive and negative regulators in a single pathway, thus narrowing the window of oscillation parameters that mediate a response (*68*). If multiple decoders exist that mediate independent responses, it appears likely that they may additionally repress each other to prevent inappropriate cross-talk between pathways (Fig. 1B).

How divergent cytosolic Ca^{2+} signals are read by a single cell type to elicit different physiological responses is not well understood in animal or plant cells. Because single plant cells usually respond to multiple hormonal and exogenous signals, they may provide excellent model systems for future dissection of this classical question in cell biology. The use of molecular genetics to identify mutants that show normal cytosolic Ca^{2+} signals, but still have disrupted physiological responses, will be crucial for determining downstream decoders of the cytosolic

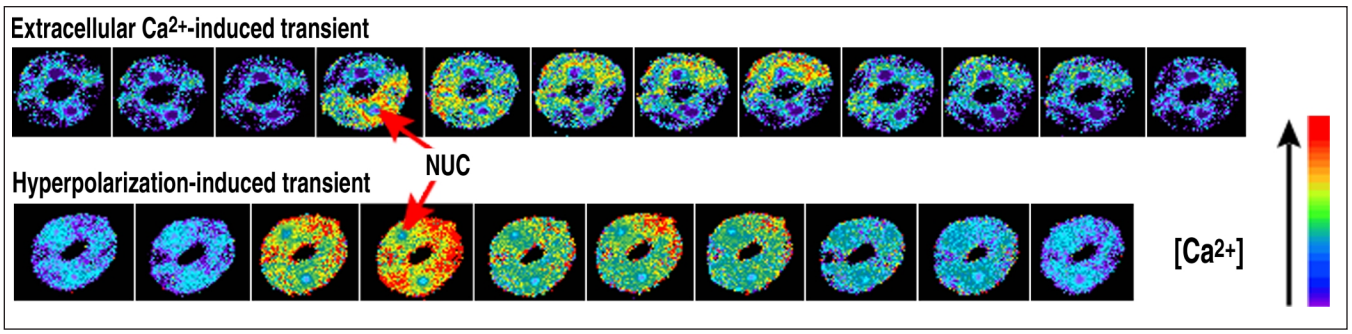

Fig. 2. Separate regulation of cytosolic and nuclear (NUC) Ca^{2+} signals in *Arabidopsis* guard cells. *Arabidopsis* guard cells expressing the GFP-based Ca^{2+} indicator YC2.1 accumulate YC2.1 in the cytosol and nucleoplasm (*31*). Guard cells were treated with 10 mM extracellular Ca^{2+} in a constant 5 mM KCl (pH 6.15) buffer (upper panels), or extracellular Ca^{2+} influx was promoted by hyperpolarizing the cell simultaneously with 10 mM Ca^{2+} addition by decreasing external KCl to 0.1 mM (lower panels). The ratiometric images indicate that nuclear Ca^{2+} remains lower than cytosolic Ca^{2+} during these elevations that lead to stomatal closure (*32*). Images were acquired at 12-s intervals.

Ca^{2+} signal in plant cells and for identifying Ca^{2+}-dependent enzymes that are differentially activated by variations in the cytosolic Ca^{2+} oscillation parameters.

Specificity Encoded by Intracellular Ca^{2+} Signal Localization

Although specificity in cytosolic $[Ca^{2+}]_{cyt}$ signaling can be determined by the kinetics of the oscillations in $[Ca^{2+}]_{cyt}$, the subcellular localization of Ca^{2+} signals, the intracellular Ca^{2+} store mobilized, or a combination of these can also affect the subsequent response (*8-10*, *80*). In plant cells, as in animal cells, multiple intracellular stores may exist for Ca^{2+} mobilization, and a number of Ca^{2+}-permeable channels have been identified in plant intracellular membranes, such as the vacuole (*76*) and endoplasmic reticulum (ER) (*81*, *82*). Intracellular second messengers—such as IP_3 (*83*), cyclic adenosine diphosphate ribose (cADPR) (*84*, *85*), sphingosine-1-phosphate (*86*), and nicotinic acid adenine dinucleotide phosphate (NAADP) (*87*), which predominantly affect endomembrane Ca^{2+} release—are known to function in plants. Examples of selective mobilization also have been described: ER Ca^{2+} can be mobilized during pollen tube growth (*62*) or mechanical stimulation (*81*), and vacuolar Ca^{2+} is released during cold shock (*24*).

Nuclear Ca^{2+} is highly regulated in animal cells (*88*), and the nuclear Ca^{2+} concentration ($[Ca^{2+}]_{nu}$) can be regulated separately from $[Ca^{2+}]_{cyt}$ (*89*, *90*). Ca^{2+} can be loaded into plant nuclei in an adenosine triphosphate (ATP)-dependent manner (*91*). Using aequorin targeted into the nucleus of tobacco protoplasts, it has been demonstrated that nuclear Ca^{2+} can be regulated separately in response to different stimuli, and the resulting changes in gene expression are specific for Ca^{2+} signals limited to the nucleus or cytosol (*92*). In addition, in these plants, $[Ca^{2+}]_{nu}$ and $[Ca^{2+}]_{cyt}$ are differentially sensitive to elevation by the pharmacological inhibitor of heterotrimeric GTP binding proteins, mastoparan and $[Ca^{2+}]_{nu}$ increases in the absence of an intact cytosolic Ca^{2+} regulatory system, indicating that the nucleus has an independent Ca^{2+} regulatory system (*93*). In correlation with these findings, in stomatal guard cells increases in $[Ca^{2+}]_{cyt}$ appear to be excluded or delayed and reduced in the nucleus in *Commelina* (*7*) and *Arabidopsis* (Fig. 2), suggesting that Ca^{2+} regulation in these plant nuclei also occurs separately from cytosolic Ca^{2+} signals.

That differential regulation of nuclear and cytosolic Ca^{2+} can effect the specificity of the response has recently been elegantly demonstrated in a plant cell by using two-celled fucus embryos where one cell is undergoing polarized tip growth (*19*). Mild osmotic shock resulted in increased $[Ca^{2+}]_{cyt}$ localized to the growing tip and in decreased cell volume through turgor regulation, but did not affect cell division rates. However, more severe osmotic shock led to increased $[Ca^{2+}]_{nu}$ and resulted in a cessation of cell division. These two responses could be mimicked by localized increases in $[Ca^{2+}]_{cyt}$ initiated by flash photolysis of caged IP_3. IP_3 release in the growing tip led to a localized increase in $[Ca^{2+}]_{cyt}$ that affected volume regulation only, whereas IP_3 release in the nuclear region led to a decrease in cell division rates. These data clearly show for the first time that the subcellular localization of Ca^{2+} signals can control the specificity of the response in plant cells.

Conclusions

It has long been a mystery how cells can specifically respond to a plethora of biotic and abiotic stimuli through the second messenger Ca^{2+}. Major inroads to the solution of this puzzle have been achieved in plants by combining the power of genetics and newly arising imaging and Ca^{2+} manipulation techniques. Individual plant cells are known to rely heavily on cross-talk and specificity in signaling pathways, suggesting that they will contribute further to unraveling outstanding questions in eukaryotic cell Ca^{2+} signaling. The highly developed molecular genetic approaches in *Arabidopsis* and other emerging plant model systems, together with the completed *Arabidopsis* genome sequence and the ease of generating and sharing mutant seeds, provides fertile ground for future studies.

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94. Research in the authors' laboratory was supported by grants from DoE (94ER201807), NIH (GM60396), and NSF (MCB 0077791).