J Plant Growth Regul (2005) 24:296–307
DOI: 10.1007/s00344-005-0095-x

J P G R
Journal of Plant Growth Regulation

Early ABA Signaling Events in Guard Cells

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Abstract

The plant hormone abscisic acid (ABA) regulates a wide variety of plant physiological and developmental processes, particularly responses to environmental stress, such as drought. In response to water deficiency, plants redistribute foliar ABA and/or upregulate ABA synthesis in roots, leading to roughly a 30-fold increase in ABA concentration in the apoplast of stomatal guard cells. The elevated ABA triggers a chain of events in guard cells, causing stomatal closure and thus preventing water loss. Although the molecular nature of ABA receptor(s) remains unknown, considerable progress in the identification and characterization of its downstream signaling elements has been made by using combined physiological, biochemical, biophysical, molecular, and genetic approaches. The measurable events associated with ABA-induced stomatal closure in guard cells include, sequentially, the production of reactive oxygen species (ROS), increases in cytosolic free Ca^{2+} levels ($[Ca^{2+}]_i$), activation of anion channels, membrane potential depolarization, cytosolic alkalinization, inhibition of K^+ influx channels, and promotion of K^+ efflux channels. This review provides an overview of the cellular and molecular mechanisms underlying these ABA-evoked signaling events, with particular emphasis on how ABA triggers an ''electronic circuitry'' involving these ionic components.

Key words: Abscisic acid; Cytosolic Ca^{2+}; Drought stress; Guard cell; Ion channel; Signal transduction; Stomatal closure

Introduction

The phytohormone abscisic acid (ABA) regulates long-term developmental processes, such as seed dormancy, as well as rapid responses to environmental changes, such as drought and salt stress. Stomatal closure is detectable within about 5 min after ABA application, which represents the fastest ABA-induced response known and one that presumably takes place without any changes in gene expression.

It has long been of interest to plant biologists to elucidate how ABA induces stomatal closure. Stomata are formed by pairs of guard cells in the leaf epidermis, and their opening and closing are driven by guard cell turgor. High solute concentrations in both the cytosol and vacuoles increase guard cell volume and turgor, leading to stomatal opening; whereas loss of solutes from guard cells reduces the cell volume and turgor, resulting in guard cell shrinkage and stomatal closure.

Received: 26 July 2005; accepted: 10 August 2005; Online publication: 12 December 2005
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The solutes involved in stomatal movements include sucrose, K^+, and anions, such as Cl^-, malate, and possibly NO_3^- (MacRobbie 1998; Talbott and Zeiger 1998; Blatt 2000; Guo and others 2003a, 2003b). It is clear that K^+ and its counterions in guard cells play a key role in osmoregulation, as more than 85% of cellular K^+ is lost during stomatal closure. Sucrose has also been implicated in this process, particularly in the afternoon, in contrast to the primary role of K^+ in the morning (Talbott and Zeiger 1998). The ABA-induced loss of K^+ and its counterions in guard cells have long been established (MacRobbie 1998). The questions now being asked relate to how ABA acts to induce the loss of these solutes in guard cells.

Recently, many ABA signaling intermediates have been identified in guard cells using combined biophysical, biochemical, molecular, and genetic approaches. Although ABA receptor(s) have not been identified molecularly and the subcellular localization of ABA perception remains controversial, it has been established that the early events in guard cell ABA signal transduction following receptor activation involve ion channel activation, membrane potential depolarization, cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) elevation, and cytosolic pH alkalinization (Blatt 2000; Schroeder and others 2001a, 2001b; Hetherington and Woodward 2003; Fan and others 2004).

The progress in understanding stomatal biology in general and guard cell ABA signaling in particular has been reviewed frequently over the last two decades (Zeiger 1983; MacRobbie 1992; Assmann 1993; Leung and Giraudat 1998; McAinsh and Hetherington 1998; Blatt 2000; Schroeder and others 2001a, 2001b; Hetherington and Woodward 2003; Outlaw 2003; Fan and others 2004; Tallman 2004). During the past 5 years, however, our understanding of guard cell ABA signaling has extended from simple signaling pathways to a sophisticated signaling network that contains a wide spectrum of components (Schroeder and others 2001a, 2001b; Fan and others 2004). These components include genes identified from genetic mutants, small signaling molecules identified using pharmacological approaches, and previously known ionic components, such as ion channels, $[Ca^{2+}]_i$, membrane potential, and pH. This review describes these ionic components and their regulation, aiming to reinforce identification of an ABA-triggered signaling network involved in ionic components. In addition, some recent advances in ABA signal transduction in guard cells are highlighted.

Stomatal Guard Cells as a Model System for ABA Signaling

The opening and closing of stomatal pores represent a crucial physiological process in higher plants. For instance, plants lose over 90% of their water by transpiration through stomatal pores. In response to water deficiency (drought stress), which is detrimental to plant growth and development and limits agricultural production worldwide, the concentration of ABA in the guard cell apoplast is increased up to 30-fold (Zhang and Outlaw 2001; Outlaw 2003). The elevated ABA triggers a signaling cascade in guard cells, which results in stomatal closure, eventually decreasing transpirational water loss (Zeevaart and Creelman 1988; Assmann 1993; Leung and Giraudat 1998; Schroeder and others 2001a, 2001b).

In addition to regulation of plant water status, stomatal movements play an important role in controlling CO_2 uptake. Furthermore, mineral nutrient uptake, such as Ca^{2+}, is largely dependent on the transpiration stream. To maximize photosynthesis and mineral uptake but minimize water loss, plants must precisely regulate stomatal apertures in response to both internal and external conditions. In fact, stomata open and close in response to many environmental signals, including light, temperature, and humidity. Therefore, stomatal guard cells are a good system for dissecting signal transduction cascades in plants.

Several technical advantages make stomatal guard cells among the best model systems for signal transduction studies. For example, stomata can be separated from other tissues/cells by peeling the epidermis or by blending whole leaves (MacRobbie 1981; Kruse and others 1989). The response of stomata to various stimuli can be replicated under *in situ* conditions, possibly because guard cells are highly differentiated, functionally limited, and physically isolated due to the lack of plasmodesmatal connection between guard cells and surrounding cells (Wille and Lucas 1984). The separation of stomatal guard cells from their surrounding cells allows the application of cell biological approaches, such as electrophysiology and Ca^{2+} imaging. In fact, several early ABA-signaling events in guard cells were originally discovered using isolated guard cells and their protoplasts. Furthermore, simple microscopic measurements of stomatal apertures provide a reliable assay of the biological functioning of guard cells (MacRobbie 1981), and the whole process of ABA-induced stomatal closure, which occurs

within 30 min, can be monitored and analyzed in live cells (Staxen and others 1999; Allen and others 2000; Roelfsema and others 2004).

Monitoring of Early ABA Signaling Events

Despite the substantial information gained recently on ABA signaling in guard cells (Schroeder and others 2001a, 2001b; Fan and others 2004), it remains difficult to define precisely what the early ABA signaling events are because of the lack of clear criteria. Nevertheless, these events are normally described by taking three different considerations: timing, functional connection, and direct physical interaction. Considering first the timing of events, many efforts have been made to directly record the events occurring sequentially after application of ABA to guard cells. Based on the time frame of their detection, the early observed events are, in order, reactive oxygen species (ROS) production (~ 1 min) (Pei and others 2000; Zhang and others 2001), membrane depolarization (~ 2 min) (Roelfsema and others 2004; Levchenko and others 2005), anion channel activation (2–5 min) (Levchenko and others 2005), cytosolic pH alkalinization (2–8 min) (Irving and others 1992; Blatt and Armstrong 1993), and $[Ca^{2+}]_i$ increases and oscillations (~ 10 min) (Allen and others 1999a, 1999b; Staxen and others 1999) (Figure 1).

This approach is primarily limited by the small number of cellular processes that can be monitored directly. Most of the measurable processes involve ionic component-related events. In contrast, other signaling processes, such as G protein activation and protein phosphorylation (Assmann 2002), which also have a key role in ABA signaling, cannot be readily monitored in such a temporal fashion. However, recent advances in biophysical and biochemical techniques have made it possible to monitor some of these events under very close to physiological conditions (Roelfsema and Hedrich 2002). For example, ABA activation of anion channels was found in guard cell protoplasts using patch clamp techniques (Pei and others 1997), intact guard cells in isolated epidermal strips (Grabov and others 1997), and guard cells in intact leaves using impalements with multi-barreled electrodes (Levchenko and others 2005). Despite some differences in the data generated using these techniques, the results should help to clarify the debate about these techniques (Roelfsema and Hedrich 2002).

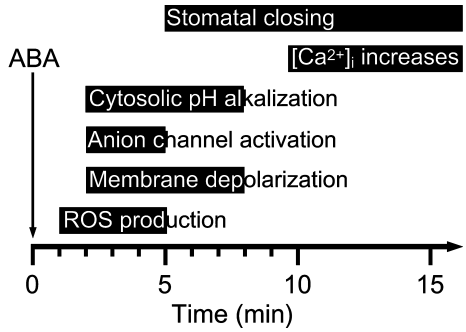

Figure 1. The sequence of measurable early ABA-signaling events in guard cells. Black boxes indicate the beginning and duration of an event. ROS, reactive oxygen species.

Each of these techniques has both advantages and disadvantages. To accurately measure ion channel currents and selectivities, patch clamping protoplasts probably provides the most appropriate approach. Nonetheless, because protoplast preparation itself causes various stresses to plant cells, and because protoplasts have lost some important physiological properties, such as turgor and the presence of a cell wall and its associated environs, some of the observations using protoplasts might not be exactly representative of the physiological processes occurring *in vivo*. On the other hand, monitoring ion channel activities and the membrane potential in intact guard cells embedded in intact leaves does preserve most of the physiological environments, although experimentally induced ion leakage may affect both intracellular and extracellular conditions. The currents recorded under these conditions could represent a mixture of various types of channels (possibly including both cation and anion channels). Furthermore, it is almost impossible to systematically manipulate both intracellular and extracellular conditions to assess ion channel properties as well as their regulation mechanisms. Therefore, it is important when addressing a specific question that the most appropriate technique be selected and used.

Sequential and Functional Connection of ABA Signaling Components

Our understanding of guard cell ABA signaling has unquestionably benefited from identification of the early sequential events induced by ABA. However, it is important to recognize that timing cannot be used as the sole standard to determine which events

occur upstream or downstream of others. For instance, Levchenko and others (2005) have shown that ABA-induced anion channel activation occurs at 2 to 5 min in guard cells of intact plants, whereas ABA-triggered $[Ca^{2+}]_i$ changes have been known to take place at about 10 min (Allen and others 1999a. 1999b; Staxen and others 1999) (Figure 1). They thus reasoned that anion channel activation is independent of the $[Ca^{2+}]_i$ changes; that is, $[Ca^{2+}]$ changes are a downstream event. Under physiological conditions, such as drought stress, it is likely that ABA accumulates gradually in the guard cell apoplast, and this process may take well over 10 min. Hence ABA-triggered $[Ca^{2+}]_i$ increases could also possibly occur prior to the activation of anion channels (Schroeder and Hagrwara 1989; Hedrich and others 1990). Technical advances using intact plants would make it possible to assess whether anion channel activation still occurs first under the osmotic stress in the roots, which stimulates ABA accumulation physiologically.

The second criterion used to define the sequence of ABA signaling components that include early events is functional connection. All ABA-signaling components identified so far can be placed in one or more positions within a sophisticated signaling network (Schroeder and others 2001a, 2001b; Fan and others 2004). The network backbone contains the major ionic components described above, but with more of an emphasis on the role of $[Ca^{2+}]_i$ in the perception of ABA (Figure 2).

It is well established that $[Ca^{2+}]_i$ is a ubiquitous second messenger in perceiving various extracellular signals in animals and plants (Berridge and others 2000; Sanders and others 2002). Abscisic acid triggers increases and oscillations in $[Ca^{2+}]_i$ in guard cells that result from both Ca^{2+} influx from extracellular spaces and Ca^{2+} release from internal stores (McAinsh and Hetherington 1998; Schroeder and others 2001a, 2001b) (Figure 2).

The elevated $[Ca^{2+}]_i$ activates anion channels, leading to anion efflux out of the cells, and membrane potential depolarization. Subsequently, the depolarized membrane potential enhances K^+ efflux via K^+ outward rectifying channels (K^+_{out}), but inhibits K^+ influx via K^+ inward rectifying channels (K^+_{in}) (Blatt 2000). The sustained K^+ and anion efflux leads to the loss of solutes, guard cells thus shrink, and eventually stomata close (Figure 2). A wide variety of ABA signaling intermediates and components have been identified to regulate these ionic components (Schroeder and others 2001a, 2001b; Fan and others 2004), which increase the complexity from a linear signaling pathway to an interactive signaling network.

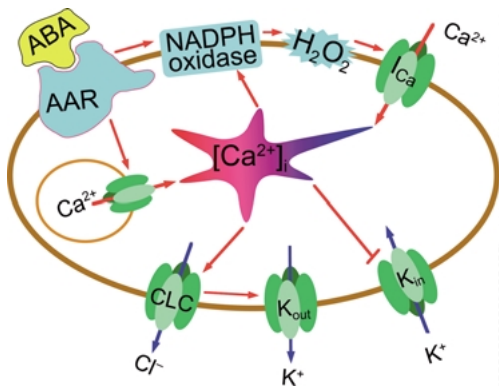

Figure 2. A model for the early ABA-signaling events in guard cells. Abscisic acid is perceived by an unknown receptor (AAR), which triggers $[Ca^{2+}]_i$ increases via H_2O_2-activated Ca^{2+} influx channels (I_{Ca}) or Ca^{2+} release from internal stores. The elevated $[Ca^{2+}]_i$ induces further H_2O_2 production via stimulating NADPH oxidases, leading to a positive feedback of $[Ca^{2+}]_i$ increases. Both S-type and F-type anion channels (CLC) are activated by elevated $[Ca^{2+}]_i$, resulting in anion efflux and depolarization of the membrane potential. The depolarized membrane potential promotes K^+ efflux via K^+ outward channels (K_{out}) and inhibits K^+ influx from K^+ inward channels (K_{in}). Meanwhile, K^+ influx via K_{in} is inhibited by elevated $[Ca^{2+}]_i$. The efflux of cations and anions reduces the solute concentration in guard cells, and thus leads to water efflux and stomatal closing.

Because many of the functional connections among signaling components are likely to operate some distance away from each other, open questions remain as to how these components interact. For instance, forward genetic approaches have revealed ABA-insensitive and -hypersensitive mutants. Some of these mutants show stomatal phenotypes, including *abi1*, *abi2*, *gca2*, *ost1*, *era1*, and *abh1*. ABI1 and ABI2 encode protein phosphatase 2C (Leung and others 1994; Meyer and others 1994). ERA1 encodes a farnesyltransferase (Cutler and others 1996). It has been show that these enzymes are required for ABA-induced activation of anion channels (Pei and others 1997; Pei and others 1998a, 1998b) as well as Ca^{2+} mobilization (Allen and others 1999a, 1999b). OST1 is a protein kinase of SnRK2 type which acts upstream of ROS production to regulate ABA-induced stomatal closure (Mustilli and others 2002; Yoshida and others 2002). Other types of protein phosphatases and kinases are involved in ion channel regulation and downstream events (Li and others 2000; Kwak and others 2002). Similarly, the mRNA cap binding protein ABH1 is required for ABA regulation of anion and K^+ channels in guard cells (Hugouvieux

and others 2001; Hugouvieux and others 2002). Apparently some of these proteins, if not all of them, regulate ion channels indirectly. Therefore, identification of proteins interacting with individual components will extend these genetic ''islands,'' and possibly bridge them to make a convincing signaling network with directly interacting members.

Molecular Identities of Ion Channels

Finally, the third criterion used to define the early ABA signaling is concerned with direct biochemical and physical interactions among these ABA signaling components. In this respect, the guard cell ABA signaling network remains poorly understood. Despite a wealth of plant genomic information, several key components still need to be identified molecularly.

Many genes encoding plasma membrane K^+_{in} and K^+_{out} channels have been identified (Very and Sentenac 2002). These genes encode sustained K^+_{in} and K^+_{out} channels, although it is not known which gene encodes the transiently activated K^+_{out} channels (Pei and others 1998a, 1998b). It is expected that some of them may work together to mediate K^+ flux during stomatal movements. In contrast to K^+ channels, it is unclear which genes encode guard cell anion channels, although some candidates have been isolated/identified (Barbier-Brygoo and others 2000).

The molecular identity for Ca^{2+}-permeable channels remains largely unknown in plants. Recently, a putative Ca^{2+}-permeable channel, AtTPC1, was identified in *Arabidopsis thaliana* (Furuichi and others 2001) as an ortholog of TPC1 from rat (Ishibashi and others 2000). The TPC1 family members were identified from various plant species. The rice homolog, OsTPC1, possesses Ca^{2+} uptake activity both in yeast cells and *in planta* (Kurusu and others 2004). OsTPC1 as well as the tobacco orthologs have been suggested to be involved in elicitor-induced Ca^{2+} mobilization and play crucial roles in defense signal transduction pathways and regulation of programmed cell death (Kadota and others 2004; Kurusu and others 2005).

AtTPC1 has recently been reported as a candidate for vacuolar Ca^{2+}-activated K^+ channels (SV) (Peiter and others 2005). AtTPC1 is expressed in guard cells. Although a *tpc1* knockout mutant affects ABA-induced repression of germination, it does not affect ABA-induced stomatal closure. Nonetheless, extracellular Ca^{2+}-induced stomatal closure is affected in the mutant. It will be interesting to characterize the properties of TPC1 in a heterologous expression system, and compare whether TPC1 has the properties of the well-documented SV channel (Pottosin and others 1997; Pei and others 1999; Miedema and others 2003; Scholz-Starke and others 2004-; Ivashikina and Hedrich 2005) or other Ca^{2+} channel(s), such as the one activated by ABA (Hamilton and others 2000; Pei and others 2000).

ABA Perception

Among the many ABA signaling components that need to be identified molecularly, ABA receptors are likely to remain at the top of the list for some time in the future. Receptors for some plant hormones have been identified as plasma membrane protein kinases. Signal transduction of ethylene and cytokinin are mediated by two-component His/Asp phosphorelay systems and their receptors are histidine kinases (Grefen and Harter 2004). Receptors for brassinosteroids and phytosulfokine are leucine-rich repeat serine/threonine receptor kinases (Li and Chory 1999; Matsubayashi and others 2002; Shiu and others 2004; Torii 2004; Kinoshita and others 2005).

The mechanism of ABA perception, however, has been controversial and remains largely unknown. Some evidence has suggested that ABA perception occurs intracellularly. For example, application of ABA to the cytosol of guard cell protoplasts inhibited inward K^+ currents, preventing stomatal opening (Schwartz and others 1994). Stomatal closure follows intracellular release of microinjected caged ABA after UV photolysis (Allan and others 1994). Recently experiments using multi-barreled microelectrodes introduced ABA into guard cells of intact plants suggested the importance of ABA binding to a cytosolic receptor (Levchenko and others 2005).

In contrast, although extracellular application of ABA was effective in inhibiting light-induced stomatal opening, microinjected ABA had less of an effect, suggesting that ABA perception exists mainly extracellularly (Anderson and others 1994). Extracellular perception of ABA was also suggested from experiments in which biologically active but membrane-impermeable ABA-protein conjugates induced ion channel activation (Jeannette and others 1999; Ghelis and others 2000) and gene expression (Schultz and Quatrano 1997; Jeannette and others 1999). Although several reports on ABA-binding proteins have been published (Hornberg and Weiler 1984; Zhang and others 2002; Razem and others 2004), the physiological functions of these proteins are unknown and no ABA receptor(s) has ever been described.

Novel technologies are required for the characterization and molecular identification of ABA receptor(s). Biotinylated ABA (bioABA) has been recently synthesized to characterize the ABA-perception sites in the guard cells (Yamazaki and others 2003). Treatment with bioABA induced stomatal closure and shrinkage of guard cell protoplasts in *Vicia faba*. The ABA-perception sites were visualized in patches on the surface of the guard cell protoplasts by confocal laser scanning microscopy, using bioABA and avidin labeled with a fluorophore. Binding of bioABA was inhibited by ABA in a dose-dependent manner, as was pretreatment of guard cell protoplasts with a protease. Experiments using ABA analogs and antagonists suggested that ABA is perceived on the plasma membrane of guard cells. Similar ABA perception sites were found on the cell surface of barley aleurone protoplasts using bioABA (Kitahata and others 2005).

The ABA perception sites show a patchy pattern of localization (Yamazaki and others 2003). Lipid signal mediators such as sphingolipids have been suggested to play critical roles in ABA signaling (Coursol and others 2003). Such lipids are also involved in formation of microdomains such as lipid rafts on the plasma membrane (Mongrand and others 2004). Abscisic acid receptors may interact with some specific membrane lipids and are localized to specific domains on the plasma membrane.

Heterotrimeric G proteins composed of α, β, and γ subunits link ligand perception by G protein–coupled receptors (GPCRs) with downstream effectors, providing a ubiquitous signaling mechanism in eukaryotes. The *Arabidopsis thaliana* genome encodes single prototypical Gα (GPA1) and Gβ (AGB1) subunits, and two probable Gγ subunits (AGG1 and AGG2) (Perfus-Barbeoch and others 2004). The heterotrimeric G protein plays an important role in ABA signaling in *Arabidopsis* (Wang and others 2001). Unlike mammals, the number of GPCRs is limited in the plant genome. One *Arabidopsis* gene, GCR1, encodes a protein having significant sequence similarity to nonplant GPCRs and a predicted 7-transmembrane domain structure (Pandey and Assmann 2004). GCR1 has been reported to interact with GPA1 and regulates ABA signaling. The *gcr1* null mutants exhibit hypersensitivity to ABA in root growth, gene regulation, and stomatal response. *gcr1* guard cells are also hypersensitive to the lipid metabolite, sphingosine-1-phosphate (S1P), which is a transducer of the ABA signal upstream of GPA1. Because *gpa1* mutants exhibit insensitivity in aspects of guard cell ABA and S1P responses, whereas *gcr1* mutants exhibit hypersensitivity, GCR1 may act as a negative regulator of GPA1-mediated ABA responses in guard cells.

Receptor-like protein kinase1 (RPK1), a Leu-rich repeat (LRR) receptor kinase in the plasma membrane, is upregulated by ABA in *Arabidopsis* (Hong and others 1997). Repression of *RPK1* expression decreased sensitivity to ABA during germination, growth, and stomatal closure (Osakabe and others 2005). Microarray analyses showed that many ABA-inducible genes are downregulated in these plants. Furthermore, overexpression of the RPK1 LRR domain alone or fused with the BRI1 kinase domain resulted in phenotypes indicating ABA sensitivity. These findings suggest that RPK1 is involved in the main ABA signaling pathway and in early ABA perception in *Arabidopsis*.

Many different kinds of molecules are perceived by plants as being an auxin, a cytokinin, or a gibberellic acid. In contrast, the number of molecules possessing ABA activity is very limited, and abscisic acid seems so far to be the only naturally occurring molecule among them. However, the structural specificity of these ABA analogs seems to be different among tissues, organs, and phenomena triggered, suggesting that several kinds of receptors are present in plants, and that their distribution is specifically regulated among various cells and tissues. Another article in this issue by Zaharia and coworkers (2005) notes that naturally occurring 7', 8' and 9' hydroxy ABAs have biological activity; it also discusses the activity of a number of ABA analogs. The hormone activity of ABA analogs and of naturally occurring hydroxylated ABAs is discussed in detail in that report (Zaharia and others 2005).

Cytosolic Ca^{2+} as a Second Messenger

Ca^{2+} is required for guard cell ABA signaling. Direct measurement of $[Ca^{2+}]_i$ in living cells using Ca^{2+}-sensitive dyes (Tsien 1980; Tsien and others 1982) provided not only a technical breakthrough but also validated a revolutionary concept: cytosolic Ca^{2+} is an important and ubiquitous second messenger. Long before the techniques could be applied to plant cells, experiments using pharmacological approaches showed that Ca^{2+} might be involved in ABA-induced stomatal closure (for a review see MacRobbie 1992). Elimination of external Ca^{2+} by Ca^{2+} chelators or addition of Ca^{2+}-channel blockers abolished ABA inhibition of stomatal opening, suggesting that Ca^{2+} influx across the plasma membrane is involved in guard cell ABA signaling (De Silva and others 1985). A later study showed

that EGTA and Ca^{2+}-channel blockers could only partially inhibit ABA-induced stomatal closure, which argued that release of Ca^{2+} from internal stores might also be important (McAinsh and others 1991). In contrast, ABA-induced shrinkage of guard cell protoplasts can occur in the absence of external Ca^{2+} (Fitzsimons and Weyers 1987), indicating a Ca^{2+}-independent ABA signaling pathway in guard cells (Allan and others 1994). Despite these controversial observations, it seems that elimination of external Ca^{2+} inhibits, at least to some extent, ABA-induced stomatal closure.

Abscisic acid induces a transient increase as well as oscillations in $[Ca^{2+}]_i$. A measurable increase in $[Ca^{2+}]$i induced by ABA was first observed by micro-injection of Ca^{2+}-sensitive fluorescent dyes into guard cells (McAinsh and others 1990; Gilroy and others 1991). This finding was later confirmed using transgenic plants expressing Ca^{2+} indicators of aequorin and cameleon (Allen and others 1999a, 1999b; Wood and others 2000). Furthermore, it has been shown that ABA triggers repetitive increases (oscillations) in $[Ca^{2+}]_i$ (Allen and others 1999a, 1999b; Staxen and others 1999), suggesting that the dynamics of $[Ca^{2+}]_i$ may be critical for the transduction of the ABA signal.

Monitoring changes in $[Ca^{2+}]_i$ in plant cells in response to stimuli is often difficult (Rudd and Franklin-Tong 2001). Plant cell $[Ca^{2+}]_i$ can be detected using three types of Ca^{2+}-sensitive indicators, fluorescent dyes (McAinsh and others 1990), aequorin (Knight and others 1991), and cameleon (Allen and others 1999a, 1999b). Each of these indicators has advantages and disadvantages (Rudd and Franklin-Tong 2001). The Ca^{2+}-sensitive fluorescent dyes are the most sensitive and accurate Ca^{2+} indicators, and can be loaded into guard cells via acid loading (Allen and others 1999a, 1999b), acetoxymethylester form (Kuchitsu and others 2002), or microinjection (McAinsh and Hetherington 1998). However, technical difficulties and translocation of the fluorescent probes into some organelles such as vacuoles sometimes prevent measurement. Constitutively expressing aequorin in plants cells allows a noninvasive measurement of $[Ca^{2+}]_i$ at the tissue and whole plant levels; however, its low luminescent intensity largely prevents high temporal (s to min) and spatial (cellular to subcellular) resolutions. GFP-FRET-based cameleon provides moderate sensitivity and specificity to Ca^{2+} compared to fluorescent dyes and aequorin (Miyawaki and others 1999). The high fluorescent intensity of cameleon allows an analysis of the dynamics as well as variation of ABA-induced $[Ca^{2+}]_i$ changes in detail (Hugouvieux and others 2001; Kwak and others 2003).

Quantitative analyses have shown that increases in $[Ca^{2+}]_i$ occur in guard cells about 10 min after addition of 10 μM ABA (Allen and others 1999a, 1999b; Staxen and others 1999; Kwak and others 2003), suggesting that $[Ca^{2+}]_i$ changes may not be the earliest event in ABA signaling (Levchenko and others 2005). About 85% of all guard cells respond to the treatment of 5–10 μM ABA with one or more $[Ca^{2+}]_i$ transients, and among the responding guard cells, about 60% of them showed more than one transient, that is, oscillations, in $[Ca^{2+}]_i$ (Kwak and others 2003). The exact relationship between these ABA-induced $[Ca^{2+}]_i$ transients and stomatal closure has not yet been established. Yet, the relationship between $[Ca^{2+}]_i$ oscillations and stomatal apertures has been studied using $[Ca^{2+}]_i$ clamp techniques, which allow the systematic variation of the parameters associated with $[Ca^{2+}]_i$ oscillations in *Arabidopsis* guard cells (Allen and others 2001). Interestingly, this study showed that stomatal closure corresponding to $[Ca^{2+}]_i$ oscillations only occurred within a defined range of frequency, transient number, duration, and amplitude. In an ABA-insensitive mutant *gca2*, because ABA induced $[Ca^{2+}]_i$ transients with increased frequencies and reduced duration, it failed to trigger stomatal closure (Allen and others 2001).

Ca^{2+} signals are decoded by many kinds of Ca^{2+} binding proteins in the cytosol. In the plant genome, genes encoding EF-hand containing Ca^{2+} binding proteins are abundant (Reddy and Reddy 2004). Although calmodulin exists as a single molecular species in mammals, three distinct calmodulin proteins are differentially regulated and play specific physiological functions (Karita and others 2004). Calmodulin domain protein kinases (CDPKs) are activated by Ca^{2+} and regulate function of numerous target proteins including ion channels (Harper and others 2004). Some Ca^{2+} sensor–associated protein kinase (CIPK/SnRK3) family members, which are specifically regulated by Ca^{2+}-sensors (calcineurin B–like proteins; CBLs), have also been shown to be involved in ABA signaling (Luan and others 2002; Xiong and others 2002; Pandey and others 2004).

Extracellular Ca^{2+} Sensing Nonessential for ABA Signaling

It has long been established that external Ca^{2+} inhibits stomatal opening and promotes stomatal closing, and is required for ABA-induced stomatal closure (MacRobbie 1992). Recently, an extracellular Ca^{2+}-sensing receptor (CAS) has been cloned

from an *Arabidopsis* cDNA library using a functional screen in human embryonic kidney cells (Han and others 2003). Expression of *CAS* alone confers extracellular Ca^{2+}-induced $[Ca^{2+}]_i$ increases in these cells. CAS is localized to the plasma membrane and exhibits low-affinity/high-capacity Ca^{2+}-binding. CAS is expressed in guard cells and suppression of *CAS* in *CAS* antisense (*CASas*) lines results in defects in extracellular Ca^{2+}-induced $[Ca^{2+}]_i$ increases in guard cells and Ca^{2+}-induced stomatal closure (Han and others 2003). However, ABA-induced stomatal closure is not affected in these lines, suggesting that extracellular Ca^{2+} sensing is not involved in ABA signaling in guard cells.

pH Changes

The other global change detectable in response to ABA is a change in cytoplasmic pH, with increases of about 0.2–0.4 pH units (Irving and others 1992; Grabov and others 1997; Blatt 2000). Cytoplasmic pH has been suggested as a second messenger in various signal transduction pathways (Kurkdjian and Guern 1989).

Effects of ABA on ion channels has been suggested to be mediated not only by $[Ca^{2+}]_i$ but also cytosolic pH as second messengers. Outward rectifying K^+ channels are activated by cytosolic alkalinization (Blatt and Armstrong 1993). The source of ABA-induced cytoplasmic alkalinization has not yet been established. Activation of decarboxylation mechanisms, such as malic enzyme, and activation of tonoplast H^+-pumps may be candidates.

Blue light–dependent H^+ extrusion by guard cells, which drives stomatal opening, is inhibited by ABA (Goh and others 1996). Cytosolic alkalinization may be associated with the inhibition of the plasma membrane H^+-ATPase (H^+-pump). Inhibition of blue light–dependent H^+ pumping by ABA was shown to be due to a decrease in the phosphorylation level of H^+-ATPase, and it was postulated that H_2O_2 might be involved in this response (Zhang and others 2004). Because H^+-ATPase is inhibited by cytosolic Ca^{2+} (Kinoshita and others 1995), ABA-induced cytosolic Ca^{2+} increases may also be responsible for regulation of the H^+-pump.

Nitric Oxide

Accumulated evidence has shown that nitric oxide (NO) is a versatile signaling molecule regulating basic physiological processes throughout the plant life cycle and playing a critical role in plant responses to abiotic and biotic stresses (Wendehenne and others 2001; Lamattina and others 2003; Crawford and Guo 2005). Nitric oxide is involved a wide spectrum of processes in plants, including seed germination, de-etiolation, root growth, leaf extension, flowering, fruit maturation, and senescence. Nitric oxide has also been found to induce stomatal closure (Mata and Lamattina 2001).

The endogenous NO level is elevated in response to ABA, suggesting that NO may act as a signaling intermediate for ABA (Neill and others 2002; Guo and others 2003b). A recent study has shown that NO elevates the resting $[Ca^{2+}]_i$ levels as well as voltage-evoked $[Ca^{2+}]_i$ increases in guard cells (Garcia-Mata and others 2003). Nitric oxide does not recapitulate ABA-evoked control of plasma membrane Ca^{2+} channels described previously (Hamilton and others 2000), but it may selectively regulate Ca^{2+}-permeable ion channels mediating Ca^{2+} release from intracellular stores. In addition, nitric oxide does not directly regulate K^+ channels and anion channels evoked by ABA (Garcia-Mata and others 2003), indicating that the action of NO may be mediated in large part by changes in $[Ca^{2+}]_i$. If NO is a major signaling intermediate for ABA signaling in guard cells, then saturation of the NO signaling pathway should drastically limit the sensitivity of stomatal movements to ABA. Thus, it should provide insight into the NO action in ABA signaling to analyze ABA-induced stomatal closure in an NO overproducing mutant, *nox1* (He and others 2004; Crawford and Guo 2005). As suggested by Garcia-Mata and others (2003), it is possible that a subset of the ABA signaling pathways does not overlap with the NO signaling pathway.

Conclusions and Perspectives

Significant recent advances in our understanding of ABA signal transduction in guard cells have come from the identification and characterization of genes and small signaling molecules involved, as well as from the efforts toward identifying the sequence of ABA-triggered ionic events under physiological environments. Despite these developments, several gaps in our understanding of ABA signaling still exist. First, virtually nothing is known about the molecular nature of ABA receptor(s) and the event that converts ABA into the earliest cellular second signal/messenger. Recent biochemical and genetic studies have identified several candidates for molecules involved in ABA perception. In addition, current progress in genomics, proteomics, biochemistry, and functional cloning may help to

isolate ABA receptors. Second, although several ion channels, such as these for Ca^{2+} influx and release have been studied electrophysiologically, the genes encoding these critical channels remain to be identified. Finally, the ABA signaling network is built in large part on functionally correlated components. Direct physical and biochemical interactions among these components have to be established to construct a precise signaling network in guard cells. Clearly, understanding of this physiologically important process will allow better identification of molecular genetic targets for engineering crops with drought resistance but without sacrificing photosynthesis and mineral uptake.

ACKNOWLEDGMENTS

We are grateful to Dr. James Siedow for reading the manuscript. The research in the authors' laboratories was supported by Duke University Startup Funds, NSF (MCB- 0451072), USDA (2005-02413) to Z-MP, and by CREST, JST and the Rice Genome Project (MP2134) to KK.

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