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Guard Cell Signal Transduction Network: Advances in Understanding Abscisic Acid, CO_2, and Ca^{2+} Signaling

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Abstract

Stomatal pores are formed by pairs of specialized epidermal guard cells and serve as major gateways for both CO_2 influx into plants from the atmosphere and transpirational water loss of plants. Because they regulate stomatal pore apertures via integration of both endogenous hormonal stimuli and environmental signals, guard cells have been highly developed as a model system to dissect the dynamics and mechanisms of plant-cell signaling. The stress hormone ABA and elevated levels of CO_2 activate complex signaling pathways in guard cells that are mediated by kinases/phosphatases, secondary messengers, and ion channel regulation. Recent research in guard cells has led to a new hypothesis for how plants achieve specificity in intracellular calcium signaling: CO_2 and ABA enhance (prime) the calcium sensitivity of downstream calcium-signaling mechanisms. Recent progress in identification of early stomatal signaling components are reviewed here, including ABA receptors and CO_2-binding response proteins, as well as systems approaches that advance our understanding of guard cell-signaling mechanisms.

Keywords

stomata; ion channel; ABA; calcium; drought; carbon dioxide

INTRODUCTION

Plants need to assimilate CO_2 for photosynthesis while simultaneously preventing excessive loss of water. Because the plant cuticle is impermeable to both water and CO_2, transpirational water loss and CO_2 influx in plants are tightly regulated by the opening and closing of stomatal pores in aerial tissues. The stomatal pore is formed by two specialized guard cells, which in some plant species are surrounded by subsidiary cells (12). The transport of ions and water through channel proteins across the plasma and vacuolar membranes changes turgor and guard cell volume, thereby regulating stomatal aperture (138,148,161).

Guard cells continuously sense information from the leaf environment, including abiotic and biotic stimuli, as well as long-distance signals from roots. Guard cells integrate all of these signals and convert them into appropriate turgor pressure changes. Several important

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environmental factors induce stomatal opening in C3 and C4 plants, including blue and red light. Stomates also open in response to high humidity and low CO_2 in order to maintain CO_2 intake. Stomatal closure, on the other hand, is promoted by darkness in C3 and C4 plants. In order to preserve water, CAM-plants do not close their stomates in response to darkness. Instead, they accumulate CO_2 during the nighttime by converting it into organic molecules such as malate. Elevated CO_2 leads to stomatal closure because less opening is required for efficient CO_2 influx. Stomata are also closed in response to drought, as well as elevated ozone, thus protecting the inside of leaves from ozone-induced oxidative damage to plants (62,72,170). Drought causes production of the plant hormone abscisic acid (ABA), which promotes stomatal closure and thereby reduces transpirational water loss. Other plant hormones, including auxin, cytokinin, ethylene, brassinosteroids, jasmonates, and salicylic acid (in response to pathogenic bacteria), can have effects on stomatal function; these have recently been reviewed in detail elsewhere (1,115).

Elevated CO_2 concentrations (C_i) in intercellular spaces of leaves cause stomatal closure. The effect of CO_2 on stomatal movements has been known for over 90 years, but mutations that strongly impair CO_2-induced stomatal closure have only recently been described (55,66,96,128,190,214). The effect of ABA on stomatal movements was reported in the late 1960s. Both stimuli have gained a more focused interest over recent years as the continued rise in atmospheric CO_2 levels and ensuing climate change can cause drought stress in plants, as well as limit freshwater availability in many regions. Elevated atmospheric CO_2 concentrations may provide plants with increased water-use efficiency due to reduced stomatal conductance (66,69). However, a consequence of reduced stomatal conductance is higher leaf temperatures, which have been predicted to contribute to heat stress in plants, reducing crop yield (11,16,69). The predicted effects of global climate change on stomatal function call for an in-depth understanding of both drought- and CO_2-regulated stomatal signaling networks.

Several recent reviews have provided excellent accounts of advances made in understanding stomatal development (12,143), light-induced stomatal opening (170), and the roles of ion channels in stomatal regulation (72,138,161,173). In this review we focus on the molecular guard cell signaling mechanisms that have been uncovered in recent years on ion channel regulation, signaling, and perception of the stomatal closure signals ABA and CO_2. We include discussion of newly emerging models in CO_2 signal transduction, ABA reception, specificity in Ca^{2+}-signaling, and novel mechanisms in ABA signal transduction.

ION CHANNELS IN GUARD CELLS

An Overview of Guard Cell Ion Channels and Their Functions

When guard cells perceive increased ABA levels, their turgor and volume are reduced by efflux of anions and potassium ions and by gluconeogenic conversion of malate into starch, causing stomatal closure (110) (Figure 1). ABA triggers cytosolic $[Ca^{2+}]_{cyt}$ increases and enhances $[Ca^{2+}]_{cyt}$ sensitivity (172), which activates two different types of anion channels, slow-activating sustained (S-type) and rapid-transient (R-type) anion channels (56,107,162,165). Whereas S-type anion channels generate slow and sustained anion efflux, R-type anion channels are activated transiently within 50 ms, suggesting that two different types of anion channels provide distinctive mechanisms for anion effluxe (165). Activation of anion channels at the plasma membrane of guard cells has been regarded as a critical step in stomatal closure (46,140,160). Anion efflux via anion channels causes membrane depolarization, which subsequently drives K^+ efflux from guard cells through outward-rectifying K^+_{out} channels (65,164,166,184). Among the solutes released from guard cells, more than 90% originate from vacuoles (110). $[Ca^{2+}]_{cyt}$-activated vacuolar K^+ (VK) channels function in vacuolar K^+ release (44,197) (Figure 1).

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Stomatal opening requires the activation of H^+-ATPases in the plasma membrane of guard cells (10,171) (see Figure 1). Membrane hyperpolarization caused by H^+-ATPases induces K^+ uptake through inward-rectifying K^+_{in} channels (91,94,164,166). Influx of K^+, Cl^-, NO_3^-, and production of malate from osmotically inactive starch increases turgor and volume in the guard cell and induces stomatal opening. In guard cells, K^+ is accumulated in vacuoles by H^+/K^+ antiporter activities, and anions can be transported into vacuoles through both low-affinity anion channels and a H^+/anion exchange mechanism (29,48,87,142). ABA inhibits stomatal opening through downregulation of K^+_{in} channels and H^+-ATPases (80,162) (see the section Calcium Sensitivity Priming Hypothesis, below).

Updates on Ion Channels and Regulation during Stomatal Closure

In this section we review recent findings of mechanisms that mediate guard cell ion channel activity and regulation. Early patch clamp, cell signaling, and genetic studies suggested that S-type anion channels play a key role in stimulus-induced stomatal closure (46,77,140,162,165) (see Figure 1). A gene encoding the anion-conducting subunit of S-type anion channels has recently been identified. *SLAC1* (*SLOW ANION CHANNEL-ASSOCIATED 1*) was genetically isolated from independent mutant screens for ozone-sensitive mutants and CO_2-insensitive stomatal closure mutants (128,190). The *SLAC1/SLAH* (*SLAC1 HOMOLOGUE*) gene family encodes proteins with 10 predicted transmembrane domains, with similarity to bacterial and fungal dicarboxylate/malate transporters (128,190). *slac1* mutants exhibit reduced stomatal closure responses to ABA, CO_2, Ca^{2+}, and ozone treatments. In addition, Ca^{2+}-and ABA-activation of S-type anion channels are impaired in *slac1* guard cells, providing genetic evidence that *SLAC1* encodes a major anion-transporting component of S-type anion channels in guard cells (190). Heterologous expression of *Arabidopsis* SLAC1 in *Xenopus* oocyte illustrates that SLAC1 functions as an anion channel with selective permeability to Cl^- and NO_3^- (42,97). Furthermore, retention of R-type anion channel activities in *slac1* (190) provides genetic support for the model that two types of anion channels are present in guard cells (165).

The guard cell-expressed transmembrane ABC (ATP binding cassette) protein AtMRP5 (*MULTIDRUG RESISTANCE PROTEIN 5*) has also been shown to function in ABA-induced stomatal closure (38,82). In contrast to *slac1*, impairment of ABA regulation of Ca^{2+}-permeable cation (I_{Ca}) currents, as well as defects in ABA- and cytosolic Ca^{2+}-activation of S-type anion channels in *atmrp5,* suggests that AtMRP5 may function as a regulator of several guard cell signal transduction mechanisms rather than directly as an ion channel (178). It is intriguing to note that disruption of the *AtMRP5* homologous gene *AtMRP4* (*MULTIDRUG RESISTANCE PROTEIN 4*) produced an impairment in stomatal opening (81).

ABA activates Ca^{2+}-permeable I_{Ca}-channels in the plasma membrane of guard cells (49,141). PP2Cs, NADPH oxidases, glutathione peroxidase, and Ca^{2+}-dependent protein kinases function in ABA-activation of these Ca^{2+}-permeable channels (85,90,117,120,124). These I_{Ca} Ca^{2+} channels have been proposed to function as a failsafe mechanism against stomatal opening, since these channels are activated at hyperpolarized membrane potentials (49,72,141). Thus, enhanced Ca^{2+} influx by activated Ca^{2+}-permeable I_{Ca}-channels may ensure a conditional regulation of stomatal movements.

During stomatal closure, $[Ca^{2+}]_{cyt}$-activated vacuolar K^+ (VK) channels contribute to K^+ release from vacuoles (44,197) (see Figure 1). It was previously shown that heterologous expression of TPK1 (TWO PORE K^+ CHANNEL 1) in yeast produced vacuolar K^+ currents with similar characteristics to VK channels (13). Recent genetic evidence shows that TPK1 mediates guard cell VK channel currents (44). ABA-induced stomatal closure is slowed in

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the *tpk1* mutant (44). However, a residual ABA response in stomatal closure in *tpk1* suggests that additional vacuolar K^+ release pathways exist in plants.

Recent Updates on Ion Channels and Regulation during Stomatal Opening

Stomatal opening is initiated by hyperpolarization of the guard cell plasma membrane, which is caused by H^+-ATPase-dependent proton efflux (10,171) (see Figure 1). Membrane hyperpolarization activates inward-rectifying K^+_{in} channels and induces solute influx followed by water uptake into guard cells (see Figure 1). Two dominant alleles of *Arabidopsis AHA1/OST2* (*ARABIDOPSIS H*$^+$ *ATPASE 1/OPEN STOMATA 2)* were identified and provide genetic evidence supporting the role of H^+-ATPases in stomatal movements. The dominant *ost2–1* and *ost2–2* mutants produce constitutively activated H^+-ATPases, persistent stomatal opening, and thus ABA insensitivity (116). The defect found in stomatal closure in the dominant *ost2* correlates with ABA-inhibition of H^+-ATPases (171).

Subunits of a heterotrimeric G protein complex were shown to be required for ABA-inhibition of K^+_{in} channels in guard cells (32,196). Mutations in *AtGPA1* (*G PROTEIN ALPHA SUBUNIT 1*) (196) and *AGB1* (*GTP BINDING PROTEIN BETA 1*) reduce ABA-inhibition of K^+_{in} currents (32), which correlates with impairment in ABA-inhibition of stomatal opening. The reader is also referred to other detailed reviews on previous findings of guard cell ion channels (72,138,161,170).

ABA-Regulation of Ion Channel Activities by Protein Trafficking

During stomatal movements, changes in guard cell volume affect the surface area of guard cells by up to 40% (63). Previously, it was found that an increase in the plasma membrane surface area of guard cells is proportional to an addition of active inward- and outward-rectifying K^+ channels to the plasma membrane of guard cells (64). However, until recently, it was not clear whether this membrane trafficking contributes specifically to ABA regulation of ion channels.

Microscopic observation using a photoactivatable GFP fusion to the K^+_{in} channel α-subunit KAT1 (POTASSIUM CHANNEL IN *ARABIDOPSIS THALIANA* 1) showed that ABA causes endocytotic internalization of KAT1 (181). KAT1 movement from the plasma membrane to the endosome contributes to a reduction in K^+_{in} channel activity and limited stomatal opening (181). Endosomal KAT1 proteins are subsequently redelivered to the plasma membrane to complete the vesicle traf-ficking cycle, and this process is dependent on the SNARE protein SYP121 (SYNTAXIN OF PLANTS 121) (180). Taken together, protein trafficking of membrane ion channels provides a parallel mechanism to downregulate K^+_{in} channels during ABA signaling in guard cells (181).

CO_2 SIGNALING IN GUARD CELLS

Elevated concentrations of CO_2 decrease stomatal conductance via rapid physiological responses, as well as via sustained developmental mechanisms. In the short term, CO_2 provokes stomatal closure. In addition, long-term exposure to elevated CO_2 decreases stomatal density in leaves, thus further reducing stomatal conductance (47,92). However, the mechanism by which CO_2 controls stomatal movements and stomatal development have remained largely unknown and the first plant mutants that robustly affect CO_2 control of stomatal movements have only recently been identified (55,66,96,128,190,214).

Site of CO_2 Signaling

Elevated CO_2 (C_i) concentrations that occur in leaves at night due to respiration and the continuing rise in atmospheric [CO_2] mediate reduction in stomatal apertures on a global

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scale. CO_2, as a lipophilic, nonpolar molecule, appears to diffuse across the cell membrane. Recent research on the chloroplast membrane, however, has demonstrated that aquaporins function in transmembrane CO_2 transport for photosynthesis (33,186,187).

The physiology of CO_2 control of stomatal apertures has been discussed in previous reviews (9,72,192). In brief, elevated [CO_2] activates anion channels and K^+_{out} efflux channels in *Vicia faba* guard cells (14,147), and consistent with this, CO_2 triggers chloride release from guard cells and depolarization in intact leaves (51,152). Ca^{2+} is required for CO_2-induced stomatal closure (167,200,214) and high CO_2 causes no detectable change in cytosolic pH in *V. faba* (14). However, the question of whether the CO_2 signal is sensed directly by guard cells (96,153) or by leaf mesophyll cells (57,121) has been a matter of active debate and could be advanced through genetic investigation. The idea that both cell types contribute to this stomatal CO_2 response cannot currently be excluded.

Analyses of stomatal movements in epidermal strips, which were removed from the mesophyll cell environment, showed that elevated CO_2 can mediate closure of stomatal pores, indicating a direct functional role for guard cells in mediating the CO_2 response (96,203,214). In other research, however, stomatal movements in isolated and mesophyll-grafted epidermes indicated that mesophyll tissue mediates the stomatal CO_2 response (121). Identification of specific CO_2 signaling components and mechanisms by genetic approaches is required for further insights into the cell type specificity of CO_2 signaling mechanisms.

Arabidopsis Mutants in the CO_2 Signaling Network

Recently, mutant screening and functional characterizations in *Arabidopsis* have led to identification of plant mutants and genes that mediate CO_2 control of stomatal movements. The ABA-insensitive mutant *gca2* (*growth controlled by abscisic acid 2*) (61) is strongly impaired in CO_2-induced stomatal closure in response to elevated CO_2 (800 ppm) both in leaf epidermes and in intact leaves of plants (214). In addition, [CO_2] shifts did not elicit significant changes in the $[Ca^{2+}]_{cyt}$ transient rate in *gca2* mutant guard cells, indicating an impairment in CO_2-induced depolarization of the membrane potential (214). Together with previous research, showing that *gca2* mutant plants are impaired in ABA-induced stomatal closure (4,61), GCA2 likely functions downstream of the convergence point of CO_2 and ABA signaling transduction networks (Figure 2).

Ozone, like CO_2, enters the leaf mainly through stomata. Ozone-sensitive and CO_2-insenstive *Arabidopsis* mutant alleles in the *SLAC1* gene were recently identified. *slac1* mutant plants are strongly impaired in the high CO_2-induced stomatal closure response, illustrating that the SLAC1 protein is a positive mediator of the CO_2-induced stomatal closure signaling pathway (128,190). *slac1* mutant alleles are more susceptible to ozone due to impairment in ozone- and ROS (reactive oxygen species)-induced stomatal closure. As a result, more ozone can enter leaves and cause cell death (190). As discussed in Ion Channels in Guard Cells, above, ABA-induced stomatal closure and, specifically, S-type anion channel activation are impaired in *slac1* alleles, but R-type anion channel activity and ABA-activated Ca^{2+} channel activity are retained in these mutants (190). These findings provide genetic evidence for the model that S-type anion channels function as a central control mechanism for ABA- and CO_2-induced stomatal closure (162) (Figures 1 and 2).

The HT1 (HIGH LEAF TEMPERATURE 1) protein kinase is the first identified molecular component that functions as a major negative regulator in the high CO_2-induced stomatal closure pathway (55). Stomatal responses to CO_2 changes in leaf epidermes and in intact leaf gas-exchange analyses show that the recessive *ht1–2* mutation causes a constitutive high-[CO_2] stomatal closure (55). Although HT1 protein kinase activity is greatly reduced in *ht1–1* and *ht1–2* mutants, they retain responsiveness to ABA and blue light, indicating that

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HT1 may function upstream of the convergence of the CO_2- and ABA-induced stomatal closure pathways (55) (Figure 2).

A plasma membrane ABC malate uptake transporter, AtABCB14 (ABC TRANSPORTER B FAMILY MEMBER 14), in guard cells was identified as another negative regulator of CO_2-induced stomatal closure (96). CO_2-induced stomatal closure in detached leaves was slightly accelerated in *atabcb14* mutants and decreased in *AtABCB14* overexpressing plants (96), suggesting that malate uptake into guard cells by AtABCB14 plays a role in the CO_2-induced regulation of stomatal closure. Since extracellular malate enhances anion channel activity (57) and CO_2- and ABA-induced stomatal closure (58,160), knockout of the guard cell malate uptake transporter AtABCB14 may increase extracellular malate, thus slightly accelerating CO_2-induced stomatal closure (96) (Figure 2). In addition, knockout of guard cell plasma membrane–localized malate import in *atabcb14* plants may reduce the osmotic increase in intracellular malate levels, thus reducing stomatal apertures (96). Dominant negative repression of the inward-rectifying K^+_{in} channel subunit KAT2 (POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 2) caused CO_2-insensitive stomatal conductance regulation (93). The question of why dominant K^+_{in} channel downregulation impairs CO_2 responsiveness requires further investigation.

Mechanisms of CO_2 Signaling in Guard Cells

Several *Arabidopsis* mutants have been identified that mediate CO_2-regulated stomatal signaling in guard cells, but the mechanism by which the physiological stimulus of CO_2 is transduced to regulate stomatal apertures is only beginning to be understood.

Solubilized, CO_2 is converted to carbonic acid, bicarbonate and protons. Thus the sensing mechanism could either rely on measuring CO_2, protons and bicarbonate or monitor the interconversion of a protein via CO_2 binding. Experiments using either the pH-sensitive dye BCECF or fluorescence microphotometry found no evidence for a change in cytosolic pH after elevation of [CO_2] up to 1000 ppm (14). These data suggested that CO_2 response is not mediated through changes of cytosolic pH.

A mechanism mediating CO_2-induced stomatal closure had previously been proposed, in which malate released from mesophyll cells in response to elevated CO_2 mediates the stomatal CO_2 response, by extracellular malate-induced activation of guard cell anion channels (57–59), resulting in anion loss and subsequent stomatal closure. However, since guard cells release malate into the cell wall during stomatal closure (77,128,191) and malate enhances R-type anion channel activity in guard cells (58,146), an alternative model can be considered, in which malate released from guard cells provides positive feedback by further stimulating anion channels (96) (Figure 2). Consistent with the latter positive feedback model, extracellular malate also enhances ABA-induced stomatal closure in *V. faba* (160), and isolated guard cell protoplasts respond to CO_2 (9,203).

CO_2 binding proteins that function at the apex of CO_2-regulated stomatal movements have remained unknown and their identification is needed to understand the mechanism mediating this response that affects plant gas exchange in response to the global CO_2 increase. Research in other species has suggested that CO_2 signaling is mediated by receptor-ligand mechanisms. For example, in *Drosophila*, CO_2 was reported to be sensed as an olfactory stimulus by a novel G protein-coupled receptor, although direct CO_2 binding/interaction remains to be analyzed (75). Research in mice proposed that carbonic anhydrases function as olfactory CO_2-binding proteins to trigger an avoidance behavior response to elevated CO_2 (67). In plants CO_2-binding/interacting proteins that mediate CO_2-induced stomatal closure remain unknown and genetic redundancy may have prevented their identification. A recent study revealed that *Arabidopsis* mutant plants—disrupted in two

carbonic anhydrases, βCA1 (BETA CARBONIC ANHYDRASE 1) and βCA4 (BETA CARBONIC ANHY-DRASE 4)—are strongly impaired in stomatal CO_2 responses (66). Guard cell-specific expression of either carbonic anhydrase restores the CO_2 responsiveness, indicating that carbonic anhydrases can mediate the CO_2 response directly in guard cells. Interestingly, *ca1ca4ht1–2* triple mutant plants exhibit the same constitutive high-[CO_2] response as *ht1–2*, demonstrating that *HT1* is epistatic to βCA1 and βCA4 (Figure 2). CAs are also involved in detection of CO_2 in animal taste receptors (18). Interestingly, expression of an unrelated mammalian α-carbonic anhydrase specifically in guard cells restored stomatal CO_2 signaling and high intracellular bicarbonate and CO_2 concentrations activated S-type anion channels, providing strong evidence that CA-mediated CO_2 catalysis is the mechanism for transmission of the CO_2 signal (66).

GUARD CELL ABA SIGNAL TRANSDUCTION

ABA Receptors and Early Signaling Components

Several candidate ABA receptors have been reported, including the Mg-chelatase H subunit (169) and GCR2 (G-PROTEIN COUPLED RECEPTOR 2) (108). Whether they represent authentic ABA receptors however, remains controversial (see 39,122,150,205 for discussion). Two other recently identified candidate ABA receptors are the GPCR (G-PROTEIN COUPLED RECEPTOR)-TYPE G PROTEINS GTG1 and GTG2 (137) and the Betv1/START domain family proteins PYR/PYL/RCAR (PYRABACTIN RESISTANCE/ PYR1 LIKE/REGULATORY COMPONENT OF ABA RECEPTOR) (109,139).

GTG1 and GTG2, with (respectively) 45% and 68% protein sequence similarity to the mammalian membrane protein GPHR (Golgi pH regulator) bind ABA (137). In plants GTG1 and GTG2 are targeted to the plasma membrane and interact with the Gα subunit GPA1. Previous research has shown that the *gpa*1 mutant causes ABA insensitivity in guard cells (196) and ABA hypersensitivity in seeds (19,136). *gtg1gtg2* double mutants are ABA hyposensitive in seed germination, root growth, gene expression, and stomatal movement. ABA binds to a fraction (~1%) of GTG1 and GTG2 recombinant proteins. Since *gtg1gtg2* plants are only partially insensitive to ABA, either genetic redundancy of GTG genes or the presence of additional independent ABA receptors is likely.

The PYR/PYL/RCAR family of proteins was recently identified as ABA binding and signaling proteins by two independent groups using different methods (109,139). The *pyr1* (*pyrabactin resistance 1*) mutant was isolated from a genetic screen for mutants resistant to the ABA agonist pyrabactin (139). PYR1 encodes a Bet v 1 family protein, which is known as a major birch pollen allergen. To identify molecular targets of PYR1 in a ligand-dependent manner, yeast two-hybrid screening in the presence of pyrabactin was performed. These experiments led to isolation of the type 2C protein phosphatase (PP2C), HAB1 (HOMOLOGY TO ABI1), as an interaction partner of PYR1. HAB1 functions as a negative regulator of ABA signaling, including ABA-induced stomatal closure (99,151,155). ABA-induced interaction of PYR1 with HAB1 and ABI1 (ABA-INSENSITIVE 1) was also confirmed in tobacco (139) and in *Arabidopsis* (132). Furthermore, yeast-two hybrid analyses showed that the PYR1 family members, PYL1 to PYL4, interact with HAB1 only in the presence of ABA (139). However, PYL5 to PYL12 could interact with HAB1 in yeast regardless of ABA presence (139,157). The single *pyr1* mutant has no ABA response phenotype. Notably, however, the *pyr1/pyl1/pyl2/pyl4* quadruple mutant exhibits strong ABA-insensitive phenotypes in seed germination, root growth, gene expression (139) and stomatal opening and closing responses (132), indicating a functional redundancy within the PYR/PYL/RCAR family.

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Independently, the RCAR1/PYL9 (REGULATORY COMPONENT OF ABA RECEPTOR 1/PYR1 LIKE 9) gene was identified as an interactor of the PP2C ABI2 (ABA INSENSITIVE 2) in a yeast two-hybrid screen (109). ABA binds to the RCAR1/PYL9-ABI2 complex in vitro (109). ABA causes inhibition of PP2C activity when either recombinant RCAR1 or PYR1 protein is added to the reaction (109,139). These findings provide in vitro evidence that perception of ABA signaling by the PYR/PYL/RCAR proteins shuts down negative regulation of ABA signaling by PP2Cs.

Previous findings showed that the dominant *PP2C* mutants, *abi1–1* and *abi2–1*, impair several of the earliest known ABA signaling responses, including Ca^{2+} signaling, reactive oxygen species production, and OST1/SnRK2.6/SnRK2E (OPEN STOMATA 1) kinase and S-type anion channel activation (5,46,124,125,140) (Figure 4). Based on these earlier studies, an ABI1 complex purification approach was pursued and independently led to identification of the PYR/PYL/RCAR proteins using proteomic analysis (132). This study showed that the major and most robust in vivo ABI1 copurified proteins in *Arabidopsis* were nine members of the PYR/PYL/RCAR protein family and that ABA rapidly stimulates PYR1-ABI1 interaction within 5 min in *Arabidopsis* (132).

Consistent with the findings, present results suggest that a major early step in ABA signal transduction is the inactivation of the cluster A subgroup members of the *Arabidopsis* PP2C family (Figure 3) (109,139,157). ABA perception by PYR/PYL/RCAR proteins induces protein complex formation between PYR/PYL/RCAR proteins and the PP2Cs, and that subsequently inactivates the negative regulatory function of PP2Cs (Figure 3). This early signaling model is also genetically supported because the *hab1-1abi1–2abi2–2* and *hab1–1abi1–2pp2ca-1* triple mutants cause partially constitutive ABA responses in the absence of exogenous ABA (154). Furthermore, it was shown that downregulation of the *PP2CA* mRNA level in *abh1* (*aba hypersensitive 1*) loss-of-function alleles contributed to the ABA hypersensitivity of *abh1* (88). Mutations in the mRNA cap binding protein ABH1 cause ABA hypersensitivity (68).

Recently, a series of crystallographic studies on PYR/PYL/RCAR proteins have determined structural bases of ABA perception to PYR1 (131,158), PYL1 (114,119,211), and PYL2 (114,211). PYR1 and PYL2 exist as homodimers in crystals, in solution and in planta (131,158,211). Resolution of the unbound (ABA-free) structure of these receptors reveals that the ABA covering lid structures of the PYR1 homodimer exhibit direct intersubunit PYR1-PYR1 interactions (131,158). ABA binding to the internal cavity of PYR1, PYL1, and PYL2 induces closing of lid structures through conformational changes (114,119,131,158,211). Closing of the ABA binding cavity exposes a hydrophobic surface on the ABA receptors that associates with the active site of PP2Cs (114,119,211). Interaction of PP2Cs with the hydrophobic surface of ABA-bound receptors inhibits PP2C phosphatase activity (114,119,211). Furthermore, the structure of the unnatural (−)-ABA stereo-isomer bound to the ABA receptors was resolved, providing a structural basis for classical observations that this ABA stereo-isomer can trigger physiological responses (131). Together these findings provide structural mechanisms of early ABA signaling events (Figure 3).

The ABA-activated protein kinase OST1 and the *V. faba* homolog, AAPK (abscisic acid-activated protein kinase), function as positive regulators of ABA-induced stomatal closure (101,125,212). Interestingly, ABI1 interacts with OST1 in vitro and negatively regulates ABA-activated OST1 kinase activity (125,213). Recent research has shown that the ABI1 protein phosphatase co-immunoprecipitates with the SnRK2.2 and SnRK2.3 protein kinases in *Arabidopsis* (132) and that the ABI1/ABI2/HAB1 PP2Cs interact with the OST1 and SnRK2.3 protein kinases (188,194), confirming in vivo interactions between ABI1 and

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SnRK2s. With in vitro studies showing that ABI1 or HAB1 inactivates the OST1 kinase by dephosphorylation of the activation loop (188,194), these findings further support the early ABA signaling model (Figure 3). Genetic studies using *snrk2.2snrk2.3ost1* triple mutants further support major roles of SnRK2 protein kinases, exhibiting strong ABA insensitive phenotypes (34,126). Moreover, ABA-induced activation of SnRK2.2 and SnRK2.3 was reduced in the *pyr1pyl1pyl2pyl4* quadruple mutant, providing a link from ABA receptors to the activation of SnRK2 kinases (139). These findings together with the finding that OST1 also interacts with and activates the SLAC1 anion channel (42,97) and the AtRBOHF (RESPIRATORY BURST OXIDASE HOMOLOGUE F) NADPH oxidase (174) provide strong evidence that the SnRK2 protein kinases can interact with and regulate multiple target proteins (Figure 4), including transcription factors (35,37) (discussed further below).

Important topics for future research are the identification of the network of protein targets of both the cluster A PP2Cs and the SnRK2 protein kinases.

Calcium in Guard Cell Signaling

A number of second messengers regulate ABA signaling (62,72,161), including reactive oxygen species (ROS), nitric oxide (NO), phosphatidic acid (PA), phosphatidyl-inositol-3-phosphate (PIP3), inositol-3-phosphate (IP3), inositol-6-phosphate (IP6), and sphingolipids. Plant homologs for some of the predicted components for plant Ca^{2+} signaling in diverse plant cell types have not yet been found in land plant genomes, including IP3-receptors, ADP-ribosyl cyclases, and the cADPR-regulated ryanodine receptor channels, in contrast to algal genomes; further research is needed to determine the underlying land plant–specific signaling mechanisms (202). Recent reviews provide detailed discussions of the various small-molecule second messengers and their roles in guard cell signaling responses and are recommended for further reading (60,72,138,161,170).

ABA elevates ROS levels via mechanisms that include the NADPH oxidases AtRBOHD and AtRBOHF (90) (Figure 4). The OST1 protein kinase was shown to directly interact with and phosporylate the AtRBOHF NADPH oxidase (174), which is consistent with findings that these NADPH oxidases function in early ABA-mediated ROS signaling (90). Notably, through feedback, ROS directly regulates early ABA signaling. ROS downregulate the phosphatase activity of the ABI1 and ABI2 PP2Cs in vitro (113) (Figure 4). MPK9 (MAP KINASE 9) and MPK12 were identified as downstream factors that integrate ABA-ROS signaling, leading to anion channel activation (73). Guard cell expressed MPK9 and -12 are activated by ABA and H_2O_2 treatments and *mpk9/12* double mutants are ABA and H_2O_2 insensitive in stomatal movements (73). In addition, ROS activate Ca^{2+} channels in the plasma membrane of guard cells (90,141) and promote NO and PIP3 signaling in response to ABA (15,40,41,129,195). NO and PIP3 act by modulation of $[Ca^{2+}]_{cyt}$ levels in the cell. The roles of nitric oxide and reactive oxygen species in ABA signaling and components of this signaling pathway have been recently reviewed (129).

In this review we focus on the role of $[Ca^{2+}]_{cyt}$ in guard cell signaling. $[Ca^{2+}]_{cyt}$ acts in a rapid Ca^{2+}-reactive-stomatal closure response as well as in a long-lasting Ca^{2+}-programmed inhibition of reopening of stomatal pores (4,72).

Calcium-Dependent and Calcium-Independent Signaling

It has been known for some time that ABA induces $[Ca^{2+}]_{cyt}$ elevations in guard cells of C*ommelina communis* prior to stomatal closure (112). Later experiments, however, showed that ABA induces $[Ca^{2+}]_{cyt}$ elevations only in part of the cells [37% in *V. faba* (163), 40–80% in *C. communis* (43), and 70% in *Paphiopedilum tonsum* (70)]. The absence of a tight coupling between ABA-induced stomatal closure and ABA-induced Ca^{2+} increases

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therefore indicated a Ca^{2+}-independent mechanism existing in the ABA signaling network (3) (Figure 4).

A recent study has now quantified the relative importance of $[Ca^{2+}]_{cyt}$-elevation-dependent and -independent signaling in ABA-induced stomatal closure in *Arabidopsis* (172). After inhibition of spontaneous and ABA-induced $[Ca^{2+}]_{cyt}$ elevations, ABA-induced stomatal closure was greatly attenuated and showed only ~30% of the response, compared to control conditions with $[Ca^{2+}]_{cyt}$ elevations (172). The remaining 30% change in stomatal aperture still required physiological, resting intracellular Ca^{2+}concentrations of 100–150 nM (172), consistent with results from *V. faba* (100,199). This research points to the possible relevance of a Ca^{2+}-elevation-independent but resting Ca^{2+} requirement for ABA signaling (172). Thus, whether the proposed Ca^{2+}-independent pathway actually requires ABA enhancement (priming) of the sensitivity to resting Ca^{2+} levels is an important question for future research. Mutants with reduced ABA sensitivity (*ost1, abi2–1*) had an even further reduced ABA sensitivity after blocking $[Ca^{2+}]_{cyt}$ elevations (172). These results are consistent with the findings that these signaling components act upstream of calcium-dependent and calcium-independent signaling (172) (Figure 4).

Calcium Signal Transducers: CDPKs and CIPK/CBLs

Although the role of Ca^{2+} as a second messenger in ABA signaling is well established, we are only beginning to understand the molecular components underlying this network. A large number of abiotic and biotic stress factors, plant hormones, and light utilize localized intracellular $[Ca^{2+}]_{cyt}$ transients to elicit specific responses in plants (60), pointing to the central cell biological question being investigated in both plant and animal systems, namely, how specificity in Ca^{2+} signaling is achieved.

Plants possess several families of Ca^{2+} sensors to link upstream $[Ca^{2+}]_{cyt}$ elevations to downstream signaling events. CALCIUM-DEPENDENT PROTEIN KINASES (CDPKs, or in *Arabidopsis,* CPKs) act as sensor responders by combining Ca^{2+}-binding and kinase activity in the same polypeptide (20,53). The *Arabidopsis* genome encodes 34 CDPK isoforms. In reverse genetic approaches, 4 CDPKs have been identified with functions in guard cell and ABA signaling (120,221). Mutations in the guard cell–expressed CDPKs *CPK3* and *CPK6* led to partial impairment in ABA and Ca^{2+} activation of S-type anion channels and, interestingly, ABA activation of plasma membrane Ca^{2+} channels (120) (Figure 4). In addition, the calcium-reactive stomatal closure response of *cpk3cpk6* double mutants was impaired by ~64–81%, whereas the long-lasting calcium-programmed response was not clearly affected (120). CPK4 and CPK11 have also been identified as positive transducers of Ca^{2+}-dependent ABA signaling (221). Strong ABA insensitivity in stomatal closure and increased drought sensitivity were reported in the *cpk4* and *cpk11* single and double mutants, with opposite phenotypes observed in *CPK4* and *CPK11* overexpression lines (Figure 4). The nuclear and cytosolic localizations of CPK4 and CPK11 (221) suggest possible dual nuclear/cytoplasmic roles for these CDPKs. CPK4 and CPK11 phosphorylate two members of the ABA-RESPONSIVE ELEMENT BINDING FACTORS (ABFs), namely, ABF4 and ABF1 in vitro (221). Besides CPK4 and CPK11, several *Arabidopsis* CDPKs, including CPK10, CPK30, and CPK32, have been shown to interact with ABF4 in vitro (25). Furthermore, CPK32 has been shown to phosphorylate ABF4 in vitro and to interact with ABF1, ABF2, and ABF3 (25). This may indicate either a general mechanism or a lack of specificity among CDPKs. In vivo confirmation of phosphorylation events is presently needed.

CALCINEURIN-B LIKE PROTEINS (CBLs) are sensor relay proteins that, upon Ca^{2+} binding, interact with and modulate the activity of CBL-INTERACTING PROTEIN KINASES (CIPKs). Ten *CBLs* and 25 *CIPKs* are expressed in the *Arabidopsis* genome, and

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interactions between individual members of the CBL family with various CIPKs allow cross talk between abiotic stress and phytohormone signaling pathways at the molecular level (28). Two CBLs have been identified thus far as playing a role during ABA signaling in guard cells, CBL1 and CBL9. CBL1 was identified as a relay for multiple stress responses (2,21) and acts as a positive regulator of drought signaling (2,21). CBL1-overexpressing plants exhibit enhanced drought tolerance and constitutive expression of stress genes. However, loss of *cbl1* function did not affect ABA responsiveness (2,21). *cbl9* mutant plants are hypersensitive to ABA in seed germination, seedling growth, and gene expression (134). CBL9 has been shown to interact with CIPK3 and might in this way regulate ABA responses at the level of seed germination (78,135). Although neither CBL single mutant is ABA hypersensitive in guard cells, the *cbl1cbl9* double mutant was reported to be more drought tolerant in wilting assays, and the stomatal closure response in the double mutant was hypersensitive to ABA (22). As an interaction partner of CBL1 and CBL9, CIPK23 was identified as a negative regulator of ABA signaling in guard cells. The *cipk23* mutant is ABA hypersensitive during stomatal opening and closing responses and has reduced transpirational water loss in leaves (22). Based on the *cbl1cbl9* double mutant phenotype, CBL1 and CBL9 might synergistically activate CIPK23 during Ca^{2+}-dependent signaling in guard cells (22). CBL1 and CBL9 bind to CIPK23 and target it to the plasma membrane (22). It is proposed that CIPK23 negatively regulates ABA signaling in guard cells by activating an inward potassium channel (22). A candidate for this mechanism is AKT1, an inward-conducting potassium channel that is activated by CIPK23 (102,207).

The presence of functional redundancy of Ca^{2+}-binding proteins (22,120,221) supports the observation of an overall robustness of the guard cell signaling network. In the current model CDPKs act as confirmed positive regulators (120,221) (Figure 4) and CBLs/CIPKs as negative regulators (22,78,135) of Ca^{2+}-dependent ABA signaling.

Calcium-Sensitivity Priming Hypothesis

Ca^{2+}-imaging experiments have shown that spontaneous repetitive $[Ca^{2+}]_{cyt}$ transients occur in guard cells under nonstimulated conditions (6,45,83,176,210,214) (Figure 4). These spontaneous repetitive $[Ca^{2+}]_{cyt}$ transients have been observed in guard cells in intact plants (210). This raises the question, How can CO_2- and ABA-induced stomatal closure be Ca^{2+}-dependent (30,98,167,200,214) if guard cells have repetitive spontaneous $[Ca^{2+}]_{cyt}$ transients? One new hypothesis (214) is that the physiological stomatal closure signals, elevated CO_2 and ABA, enhance (prime) the Ca^{2+} sensitivity of guard cells (214), as has recently been demonstrated for ABA signaling (172) (Figure 4). While measured in a low-extracellular-Ca^{2+} bath, guard cell S-type anion channels show little response to an increase in $[Ca^{2+}]_{cyt}$ to 2 μM (7,172). However, preincubation of guard cells in the same solution, containing ABA, strongly increased the ability of 2μM $[Ca^{2+}]_{cyt}$ to activate anion channel currents (172). These findings provide evidence that ABA enhances/primes the ability of guard cells to respond to increased $[Ca^{2+}]_{cyt}$ levels and to activate anion channels (172) (Figure 4).

The Ca^{2+}-sensitivity priming effect of ABA is not restricted to S-type anion channel activation but also regulates inward potassium channels (172). The priming effect uncovered in stomatal CO_2 and ABA responses (172,214) may not be restricted to guard cell signaling. During rice seed swelling a Ca^{2+}-dependent protein kinase activity could be enhanced by addition of Ca^{2+} to the kinase reaction and could be further enhanced by treatment with phosphatidylserine (86). In the same experiment kinase activity from seedlings pretreated with 5 μM ABA was dependent on only Ca^{2+} and could not be induced further by phosphatidylserine (86). Ca^{2+} sensitivity priming of specific Ca^{2+} sensors may provide an important mechanism for specificity in Ca^{2+} signaling in plants and animals. In *cpk3cpk6* mutants ABA could not prime S-type anion channel activation (120), further suggesting that

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priming could occur in the Ca^{2+}-dependent signaling pathway or in a closely associated parallel signaling pathway (Figure 4).

Interestingly, experimentally imposed $[Ca^{2+}]_{cyt}$ transients, regardless of the $[Ca^{2+}]_{cyt}$ transient pattern, have been shown to trigger a Ca^{2+}-reactive stomatal closure response in *Arabidopsis thaliana* and *V. faba* (4,105,209). These findings, and enhanced anion channel activation after extracellular preexposure to high Ca^{2+} (7), indicate that high extracellular $[Ca^{2+}]_{ext}$ itself can also prime guard cells for permissive (primed) intracellular Ca^{2+} signaling. The chloroplastic protein CAS (CALCIUM SENSING RECEPTOR) was recently reported to regulate $[Ca^{2+}]_{cyt}$ elevations in response to elevated extracellular $[Ca^{2+}]_{ext}$ (198,201). Oscillations of $[Ca^{2+}]_{cyt}$ were absent in response to elevated $[Ca^{2+}]_{ext}$ in *cas* mutant plants, thereby abolishing stomatal closure (50,201).

Several distinctive mechanisms could provide a molecular basis for Ca^{2+}-, ABA-, and CO_2-induced Ca^{2+} sensitivity priming. During the measurements of K^{+}_{in} channel priming by ABA, guard cells were incubated in ABA-containing solution for about 45 minutes prior to patch clamping (172). This would leave sufficient time for transcription and translation of Ca^{2+}-binding proteins that participate in Ca^{2+}sensitivity priming either by binding Ca^{2+} directly or by facilitating Ca^{2+} sensing. Relocalization of proteins offers another possibility. As shown for CIPK23 (22), proteins can be relocalized in the cell via protein-protein interactions. Chemical modifications such as myristoylation can trigger protein relocalization. Parallel detection of Ca^{2+} elevation and an independent signal would provide a third mechanism for modulation of Ca^{2+} sensitivity (Figure 4). Only upon perception of both, Ca^{2+}-elevation and an independent signal, could downstream signaling occur. Such a signaling network would reduce spontaneous activation and an additional signaling component would allow for a tightly controlled layer of specificity in Ca^{2+} signaling.

The Ca^{2+} sensitivity priming hypothesis derived from guard cell signaling research (214) might explain specificity in other plant Ca^{2+} responses, given the over 200 Ca^{2+}-binding proteins found in the *Arabidopsis* genome alone, and may also explain how opposing signaling pathways like ABA-induced stomatal closure and blue-light- and low-$[CO_2]$-induced stomatal opening can both employ $[Ca^{2+}]_{cyt}$ elevations as a secondary messenger and nonetheless retain specificity (52,214).

Calcium-Programmed Stomatal Response

The long-term Ca^{2+}-programmed inhibition of stomatal reopening is distinct from the above discussed rapid Ca^{2+}-reactive response (24,120). In contrast to the Ca^{2+} reactive response, this slower programmed response does depend on the pattern of the preceding imposed $[Ca^{2+}]_{cyt}$ transients (4,105,209). Preceding Ca^{2+} transients of the appropriate pattern enhance inhibition of the reopening of stomatal pores, even after the Ca^{2+} transients have been terminated (4,24,105,209). The dampening of $[Ca^{2+}]_{cyt}$ transients during ABA-induced membrane potential depolarization and stomatal closure might reflect a $[Ca^{2+}]_{cyt}$ pattern that contributes to long-term Ca^{2+}-programmed inhibition of stomatal reopening (4,45,72,83,176,214). A first mutant that impairs this Ca^{2+}-programmed long-term Ca^{2+} inhibition of reopening of stomatal pores was recently identified via overexpression of the glutamate receptor-like channel AtGLR3.1 (GLUTAMATE RECEPTOR 3.1) (24). Data further suggest that both transcriptional and translational mechanisms are required for this long-term Ca^{2+}-programmed response of guard cells, further distinguishing it from the Ca^{2+}-reactive response (24).

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NEWLY IDENTIFIED COMPONENTS AND MECHANISMS IN ABA SIGNAL TRANSDUCTION

Regulation of ABA Metabolism

Considering the wide range of roles of ABA in abiotic stress and developmental responses (161,215), elucidating ABA metabolism is an important step in understanding the functions of ABA. Three important questions on regulation of ABA metabolism arise: How is ABA synthesized and degraded? Where is ABA synthesized? and How is ABA metabolism activated?

The enzymatic biosynthesis pathway of the sesquiterpenoid, abscisic acid, from C_{40} carotenoids has been well characterized biochemically and genetically (127,215). A rapid increase in the ABA concentration in response to abiotic stresses can be partly explained by transcriptional induction of ABA biosynthesis genes such as the rate-limiting step enzyme *NCED3* (*NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3*) (182). In addition to the transcriptional induction of ABA biosynthesis genes, stress-induced conversion of inactive ABA conjugates has been proposed to contribute to an increase in the net ABA concentration. ABA-glucose ester is an inactive ABA conjugate and is regarded as a candidate precursor for ABA signaling (74). It has been reported that hydrolysis of ABA-GE by β-glucosidase AtBG1 (BETA-1,3-GLUCANASE 1) is induced by dehydration-induced AtBG1 complex formation (95), indicating conjugation and decon-jugation of ABA. However, in order to explain the more than 30-fold induction in ABA concentrations by osmotic stresses (54), plants must have other regulatory mechanism(s) to adjust ABA levels in response to changing environments.

A key enzyme in ABA catabolism is ABA 8′-hydroxylase, which converts active ABAs into inactive 8′-hydroxy ABAs (127). Recent studies show that ABA catabolism is under the control of environmental conditions and can fine-tune ABA concentrations. The CYTOCHROME P450, FAMILY 707, SUBFAMILY A genes *CYP707A1* to *A4,* encoding ABA 8′-hydroxylases, are induced by rehydration of dehydrated plants (89). High humidity can also induce *CYP707A3* in vascular tissues and *CYP707A1* in guard cells (133). Generation of inactive ABA-GE from active ABA has been proposed to provide another mechanism for ABA inactivation, as indicated by overexpression of *UGT71B6* (*UDP-GLUCOSYL TRANS-FERASE 71B6*) (144). However, whether glucosyltransferases are regulated by stress signals remains to be determined.

The question has resurfaced as to which tissues ABA synthesis occurs in as a response to osmotic stresses (i.e., drought, salinity, and cold). Recent immunohistochemical localization studies of the ABA biosynthesis enzymes ABA2 (ABA DEFICIENT 2), NCED3, and AAO3 (ABSCISIC ALDEHYDE OXIDASE 3) indicated that shoot vascular tissues appear to be a major site of ABA biosynthesis in response to stress conditions in *Arabidopsis* (31). Consistent with these findings, luciferase reporter expression under the control of the ABA-responsive *AtHD6* (HISTONE DEACETYLASE 6) promoter was detected in the vasculature and in guard cells in response to drought, suggesting a role for tissue-autonomous ABA synthesis in addition to long-distance root-to-shoot movement of ABA.

Several mechanisms are considered as signaling cues to initiate ABA biosynthesis, including hydraulic signals and pH changes (26,74). However, information about the genes and the underlying mechanisms that detect primary stress signals and cause activation of ABA biosynthesis and ABA biosynthetic gene expression is just beginning to be revealed. In response to osmotic stress, the histidine kinase ATHK1 has been proposed to mediate induction of ABA biosynthesis genes and ABA accumulation because, compared to wild-type controls, sorbitol-treated *athk1* mutants contained lower ABA levels, whereas

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overexpression of *ATHK1* produced higher ABA levels (204). Overexpression of the RING-H2 gene *XERICO* (84), a putative E3 ligase, and the recessive mutant *saul1* (*senescence-associated E3 ubiquitin ligase 1*) (145) cause enhanced ABA accumulation. These findings indicate that an ubiquitin-based protein degradation pathway may be involved in upregulation of ABA biosynthesis. Moreover, reduced ABA levels in *sad1 (supersensitive to ABA and drought 1)* (206) indicate that regulation of RNA metabolism can affect ABA concentrations. As a whole, limited knowledge exists about the proteins that sense and translate osmotic stresses to ABA synthesis.

Transcription Factors Involved in ABA Signaling

ABA is known to strongly affect transcription of downstream target genes (99,168). The presence of ABA-responsive elements (ABREs) within the promoters of many ABA upregulated genes suggests that transcription factors binding to ABREs may represent major downstream targets of ABA signaling responses (99,168). A bZIP transcription factor, AREB1/ABF2 (ABSCISIC ACID RESPONSE ELEMENT-BINDING FACTOR 1), was identified as a binding protein to ABRE motifs and shown to be phosphorylated by ABA-activated SnRK2 kinases (37). Posttranslational modification of AREB1 might trigger induction of downstream ABA-responsive genes because enhanced general ABA responses were reported by over-expression of a constitutively active truncated form of AREB1 (36).

In addition to ABREs, MYBR (MYB-recognition site) and MYCR (MYC-recognition site) are *cis*-elements identified in the promoters of ABA-regulated genes (208). Two guard cell–expressed MYB transcription factors, MYB60 and MYB61, function in light-induced stomatal opening (27,106). *MYB60* expression is downregulated by ABA and upregulated by light, which correlates with the reduced stomatal opening of the *atmyb60* mutant (27). *MYB61* expression is upregulated in the dark, and overexpression of *MYB61* causes inhibition of light-induced stomatal opening (106).

Moreover, transcripts of *MYB44*, another guard cell–expressed transcription factor, accumulates in response to abiotic stresses (76). Transgenic plants overexpressing *MYB44* were hypersensitive to ABA in stomatal closure. Notably, stress induction of several cluster A *PP2C* mRNAs was severely compromised by *MYB44* overexpression, which correlates with the over-expression phenotype (Figures 3, 4).

The *cis*-element CCAAT box is found in 25–30% of all mammalian promoters and is recognized by nuclear factor-Y (183). It was recently reported that the *Arabidopsis* NFYA5 (NUCLEAR FACTOR Y, SUBUNIT A5) and the maize NF-YB2 function as positive regulators of drought-stress responses (104,130), suggesting a possible role of the CCAAT box element and its binding partner NF-Y in ABA/abiotic stress signaling. Besides transcriptional induction by ABA, *NFYA5* gene expression is further enhanced by posttranscriptional control of *NFYA5* mRNA stability. *NFYA5* transcripts contain a target site for the microRNA, *miR169*, which is downregulated by drought. Furthermore, overexpression of *miR169* and a T-DNA insertion mutation in *NFYA5* both caused drought sensitivity in *Arabidopsis* (104). The MYB101 and MYB33 transcription factors are also targets of a microRNA (*miR159)* and modulate ABA responses (149).

In addition to the positive regulation of ABA signaling by transcriptional activators, possible transcriptional repressors AtERF7 (ETHYLENE RESPONSE FACTOR 7) and NPX1 (NUCLEAR PROTEIN X 1) negatively modulate ABA signaling (79,175). Supporting AtERF7 and NPX1 as negative regulators of guard cell signaling, overexpression transgenic lines of *AtERF7* or *NPX1* exhibit a reduced ABA sensitivity in stomatal movements and an increased wilting phenotype in response to drought stress (79,175).

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Roles of 26S Proteasome-Dependent Protein Degradation in ABA Signaling

Specific target protein degradation by the 26S proteasome is a common regulatory mechanism in plant hormone and light signal transduction. Series of enzymes function in tagging target proteins with small ubiquitin modifiers for destruction. In particular, E3 ligases function to select specific target proteins by direct protein–protein interactions (193). Among different types of E3 ligases, E3 SCF (SKP1-CULLIN-F-BOX PROTEIN) complexes containing F-box proteins with LRR (leucine-rich-repeat) motifs have been identified as major hormone perception and response proteins in auxin, jasmonic acid (JA), gibberellin, and ethylene signal transduction (159,193). However, during ABA and abiotic stress signal transduction, RING or U-box type E3 ligases have instead been identified as regulatory components (177,217,218). For example, AIP2 (ABI3-INTERACTING PROTEIN 2) is a RING-type E3 ligase and regulates protein stability of ABI3 (ABA-INSENSITIVE 3) (217). In vitro ubiquitylation of ABI3 by AIP2 and ABA hypersensitive phenotypes of the *aip2* mutant suggest that AIP2-mediated ABI3 protein degradation downregulates ABA signal transduction (217). Similarly, the RING E3 ligase KEG (KEEP ON GOING) was shown to ubiquitylate ABI5 (ABA-INSENSITIVE 5) (177). Consistent with the direct interaction of KEG with ABI5, increased ABI5 protein levels were found in *keg* T-DNA insertion mutants (177).

Besides the specific ubiquitylation-dependent degradation of positive transcription factors such as ABI3 and ABI5, degradation of negative ABA signaling regulators has also been implicated in regulation of ABA signaling. Genetic mutants of the RING E3 ligase *SDIR1* (*SALT- AND DROUGHT-INDUCED RING FINGER 1*) produced reduced ABA responses in seed germination as well as in stomatal closure, suggesting SDIR1 targets negative regulators of ABA signaling (218).

In addition to ubiquitin-mediated protein stability, sumoylation of protein targets is also involved in ABA and abiotic stress signaling. SIZ1 is a component of a sumoylation-mediating E3 ligase (17,118). ABA hypersensitive seed germination, root growth, and gene expression phenotypes of *siz1* indicate that SIZ1 negatively regulates ABA signal transduction. In fact, sumoylation of ABI5 by SIZ1 produces inactive ABI5 and attenuates ABA responses during seed germination (118). In addition to ABI5, accumulation of sumoylated proteins by drought treatment (17) suggests more stress-response targets are regulated by SIZ1-mediated sumoylation. Given the importance of regulated protein degradation for ABA responses, research is needed to determine how these mechanisms may mediate ABA control of stomatal movements.

Epigenetic Regulation in ABA Signaling

Recent evidence indicates that epigenetic regulation is also involved in transcriptional control of plant stress responses (23). Chromatin modification and DNA methylation are the two most frequently observed epigenetic regulation mechanisms in eukaryotes that require coordinated actions of diverse sets of regulatory components. The ABA and cold stress–hypersensitive mutant *hos15* (*high expression of osmotically responsive genes 15*) encodes a WD40 motif containing protein with similarity to human TBL1 (TRANSDUCIN β–LIKE PROTEIN 1), which is known to repress gene expression by histone deacetylation (220). Consistent with this, HOS15 interacts with histone H4 directly, and *hos15* mutants contain more acetylated histone H4 than wild type. These data suggest that HOS15 negatively regulates ABA and abiotic signal transduction by deacetylation of histone H4 (220).

An *Arabidopsis* component of the SWI/SNF chromatin remodeling complex SWI3B was identified as an interacting protein of the PP2C HAB1 (156). The *swi3b* mutant exhibited reduced ABA sensitivity during seed germination and seedling growth by down-regulation

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of ABA-dependent gene expression. Chromatin-immunoprecipitation (ChiP) results using HAB1 as bait suggest that ABA-induced transcription is regulated by direct interactions between SWI3B and HAB1 in the presence of ABA. In addition to direct contributions of epigenetic regulation to ABA signal transduction, stress-induced epigenetic controls have been hypothesized to establish a "stress memory" in plants in preparation for upcoming stresses (23). More research is needed to determine the relative significance of this model.

Interaction with Jasmonic Acid Signaling

Novel roles of ABA signaling during pathogen infection and antagonistic control of ABA signaling in defense responses against biotic stresses have been found (185). JA is one of the major plant hormones that regulates plant biotic stress signal transduction. The JA-derivative, MeJA (methyl jasmonate), induces stomatal closure through a *COI1* (*CORONATINE INSENSITIVE 1*)- and *JAR1* (*JASMONATE RESISTANT 1*)-dependent signaling pathway (123,179). Other research indicates that MeJA treatment inhibits ABA-induced stomatal closure (115) rather than causes stomatal closure. More research is needed to clarify the proposed opposing MeJA responses.

MeJA-triggered activation of S-type anion channels and Ca^{2+}-permeable (I_{Ca}) channels is abolished in *abi2–1* (123), indicating that MeJA induces stomatal closure through ABA signaling. Guard cell–abundant TGG1 (THIOGLUCOSIDE GLUCOHYDROASE 1) also functions in regulation of ABA- and JA-triggered stomatal closure. *tgg1* was impaired in ABA-inhibition of inward K^+-channel activity and stomatal opening (219), and *ttg1/ttg2* double mutants were defective in ABA- and JA-induced stomatal closure responses (71), suggesting a role of glucosinolate metabolism in guard cell ABA signaling.

NEW APPROACHES AND PERSPECTIVES ON THE STUDY OF GUARD CELL SIGNALING

Cell biological/physiological and molecular genetic approaches have identified numerous components and regulatory mechanisms in guard cell signal transduction. Functional redundancies in major signaling components require alternative approaches that combine mechanistic characterizations of gene functions and parallel innovative systems approaches to advance our understanding of the guard cell signaling network.

Genomic scale analyses of guard cell gene expression (99,210) have led to the identification of guard cell signal transduction mechanisms, including redundant signaling mechanisms (71,90,120). In addition, tiling array-based analyses of whole plant samples, at the level of the whole genome, have identified comprehensive ABA-/stress-dependent transcriptomes (111,216). In particular, the identification of more than 7000 stress-inducible, noncoding elements (111) suggests important regulatory functions of noncoding transcripts in ABA and/or stress signal transduction. However, information regarding the roles of stress-inducible, noncoding transcripts in guard cells and other tissues is still elusive.

Complementary to guard cell transcriptome analyses, mass spectrometric profiling of guard cell–expressed proteins has identified 1734 proteins, including 336 newly detected proteins (219). Considering the critical roles of protein kinases such as SnRK2s, CDPKs, and HT1 in ABA and CO_2 signaling (55,101,125,160,213), phosphoproteome and ubiquitome profiling, in addition to total protein profiling, will provide new insights for downstream target identifications.

Although dissection of gene and protein expression modification networks can guide stress signaling pathway models, the real-time physiological status during stress responses is hard to predict. Metabolomic profiling of total plant cell extracts provides a tool for

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understanding physiological changes under abiotic stress conditions. Combined approaches of transcriptome/proteome analyses with metabolite profiling have identified dynamic metabolic changes during drought stress (8,189). With the accumulation of genetic, proteomic, and metabolomic profiling data sets, an integration of quantitative data by computational modeling (103) can guide the prediction of signaling interactions and novel regulatory mechanisms in ABA/stress signaling.

Despite the major advances made in understanding guard cell signaling, there are still many questions remaining before a comprehensive understanding of stomatal regulation is at hand. What are the gene identities encoding other major regulators and ion channels in guard cells (e.g., R-type anion and I_{Ca} channels) (Figures 1 and 4)? How do stress signals increase ABA concentrations in guard cells? What are the precise structure and protein–protein interactions of guard cell signaling networks, and how are diverse signals such as ABA, CO_2, light, and ozone integrated at the mechanistic level? What is the mechanistic basis of Ca^{2+} sensitivity priming and Ca^{2+} specificity? In the future, further research into individual signaling mechanisms, combined with genomics and systems biology analyses of guard cell signaling, will advance our knowledge of ABA and CO_2 signal transduction. For example, protein–protein interaction screens of membrane proteins will generate a regulatory interaction map of plant cell signaling. While stomatal movement analyses have the potential of quantifying and detecting mechanisms that affect guard cell signaling, either directly or indirectly, analyses of the modulation of downstream signaling targets such as membrane potential, ion channels, protein kinases, and transcription are needed for gaining an understanding of the underlying signaling mechanisms. Furthermore, real-time measurements of parameters such as stomatal conductance in signaling mutants using intact leaves and plants point to mechanisms that have strong physiological effects (e.g., 55, 66, 214), and such intact plant response analyses will lead to physiologically significant and integrated information.

Continued expansion of the mechanistic understanding of the guard cell signal transduction network is also of relevance, considering global population growth and predicted environmental changes due to the continuing rise in atmospheric CO_2, increasing temperatures (11), and limited availability of fresh water. Thus, guard cell signaling research will both enrich our general understanding of basic mechanisms that mediate plant cell signaling and likely illuminate new approaches for engineering improved water-use efficiency and dessication avoidance in crop and biomass-producing plants.

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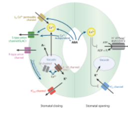

Figure 1.

Summary of guard cell signaling and ion channel regulation. This model focuses on guard cell ion channel functions and ABA-induced signal transduction across the plasma membrane and vacuolar membrane of guard cells. Signaling events during stomatal closing are shown in the left guard cell, and major regulation steps for ABA-inhibition of stomatal opening mechanisms are shown in the right guard cell. Abbreviations: ABA, abscisic acid; I_{Ca}, inward Ca^{2+} current; S-type, slow-type; SLAC1, SLOW ANION CHANNEL ASSOCIATED 1; R-type, rapid-type; SV, slow vacuolar; VK, vacuolar K^+ selective; TPK1, TWO PORE K^+ CHANNEL 1; AHA1, *ARABIDOPSIS* H^+ ATPASE 1; OST2, OPEN STOMATA 2.

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Figure 2.

A simplified model illustrating the functions of recently identified genes and mechanisms in guard cells mediating CO_2 control of stomatal movements. In this model, the HT1 protein kinase and ABCB14 proteins function as negative regulators (*red*), and CA1 and CA4, GCA2, and SLAC1 function as positive mediators (*green*) of high CO_2-induced stomatal closing. Convergence with abscisic acid (ABA) signaling is also indicated. Abbreviations: HT1, HIGH LEAF TEMPERATURE 1; GCA2, GROWTH CONTROLLED BY ABSCISIC ACID 2; CA, CARBONIC ANHYDRASE; SLAC1, SLOW ANION CHANNEL ASSOCIATED 1.

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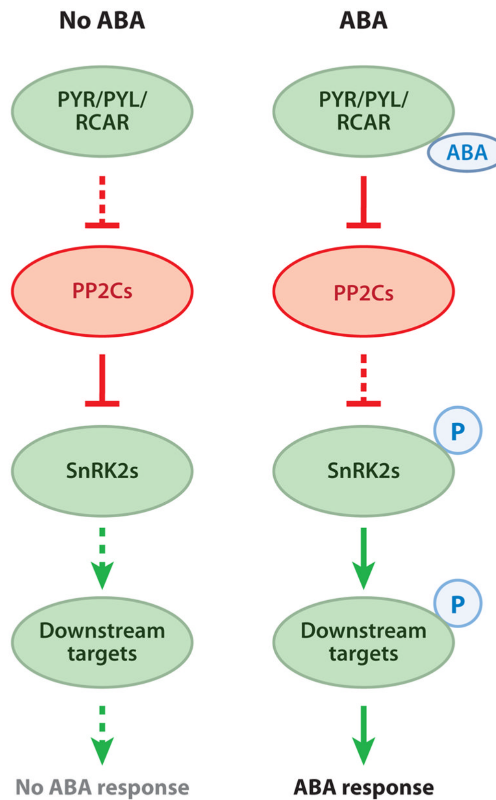

Figure 3.
A proposed simplified model for early ABA signaling events. In the absence of ABA, PP2Cs negatively regulate activation of SnRK2 kinases. Without activation of SnRK2s, downstream ABA signaling targets are inactive. In the presence of ABA, ABA binds to PYR/PYL/RCAR proteins. The ABA ligand-PYR/PYL/RCAR-PP2C complex then inhibits PP2Cs and that activates SnRK2 kinases. Active SnRK2 kinases phosphorylate downstream target proteins, including NADPH oxidases, the SLAC1 anion channel and the ABF family proteins, and generate ABA responses. Abbreviations: ABA, abscisic acid; PYR/PYL/RCAR, PYRABACTIN RESISTANCE/PYR1 LIKE/REGULATORY COMPONENT OF ABA RECEPTOR; PP2C, type 2C protein phosphatase; SnRK2, sucrose non-fermenting 1-related protein kinase 2; ABF, ABA-RESPONSE ELEMENT BINDING FACTOR.

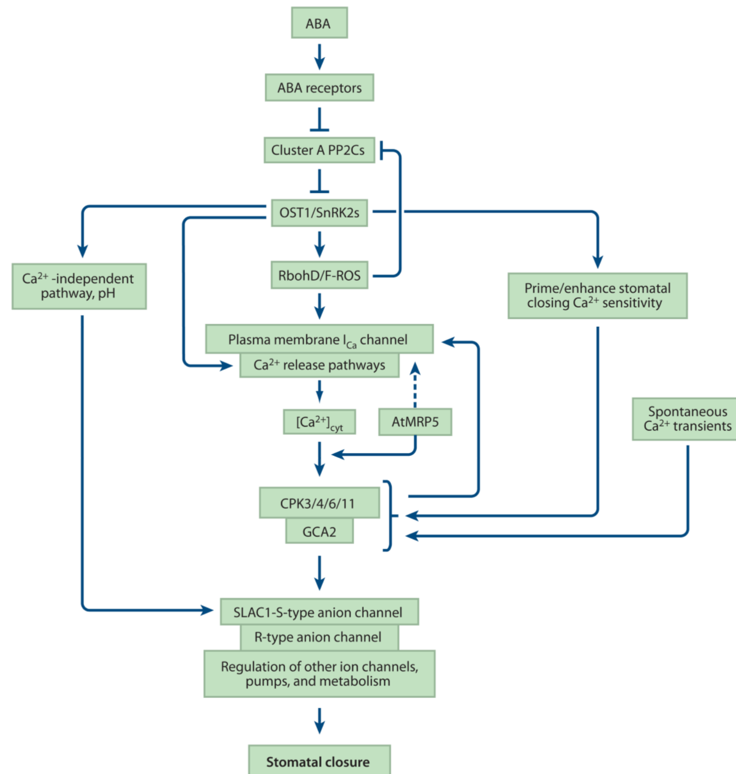

Figure 4.
ABA-induced Ca^{2+}-dependent (*middle* and *right*) and Ca^{2+}-independent (*left*) signal transduction mechanisms in guard cells (see text for the details). Abbreviations: ABA, abscisic acid; PP2C, type 2C protein phosphatase; OST1, OPEN STOMATA 1; RbohD/F, RESPIRATORY BURST OXIDASE HOMOLOGUE D/F; I_{Ca}, inward C^{a2+} current; AtMRP5, MULTIDRUG RESISTANCE PROTEIN 5; CPK, CALCIUM-DEPENDENT PROTEIN KINASE; GCA2, GROWTH CONTROLLED BY ABSCISIC ACID 2; S-type, slow-type; SLAC1, SLOW ANION CHANNEL ASSOCIATED 1; R-type, rapid-type.

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