Diverse Stomatal Signaling and the Signal Integration Mechanism

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Abstract

Guard cells perceive a variety of chemicals produced metabolically in response to abiotic and biotic stresses, integrate the signals into reactive oxygen species and calcium signatures, and convert these signatures into stomatal movements by regulating turgor pressure. Guard cell behaviors in response to such complex signals are critical for plant growth and sustenance in stressful, ever-changing environments. The key open question is how guard cells achieve the signal integration to optimize stomatal aperture. Abscisic acid is responsible for stomatal closure in plants in response to drought, and its signal transduction has been well studied. Other plant hormones and low-molecular-weight compounds function as inducers of stomatal closure and mediators of signaling in guard cells. In this review, we summarize recent advances in research on the diverse stomatal signaling pathways, with specific emphasis on signal integration and signal interaction in guard cell movement.

Contents

INTRODUCTION

Stomatal pores, which are formed by pairs of guard cells, function as gateways for controlling gas exchange and transpirational water loss. Stomatal opening promotes plant growth by enhancing carbon dioxide uptake and transpirational water loss, which are essential for photosynthesis and the uptake of nutrients from soil to the plant body, respectively. However, stomatal opening also causes undesirable and excess water loss from plants under drought stress conditions and allows microorganisms to invade plants through stomatal pores. Therefore, plants have evolved sophisticated and refined mechanisms to regulate stomatal movements, especially stomatal closure (**Figure 1**).

The opening and closing of pores are regulated by changes in guard cell volume (148). Stomatal opening is mediated by influx of water and solutes such as K^+ into guard cells, and stomatal closure is regulated by efflux of water and solutes from those cells (105). To optimize plant growth under multiple stress conditions in nature, guard cells have developed sophisticated signal integration mechanisms that enable appropriate control of stomatal apertures. The plant hormone abscisic acid (ABA) induces stomatal closure, a signal mechanism that has been well studied. The recent discovery of PYRABACTIN RESISTANCE/PYR1-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR (PYR/PYL/RCAR) has accelerated our understanding of ABA signaling (102, 143), and several reviews on guard cell ABA signaling have been published in recent years (62, 87). Here, we focus on stress signal transduction of other plant hormones and small chemical compounds in guard cells and discuss the mechanisms of signal integration.

Pyrabactin: a synthetic agonist of abscisic acid

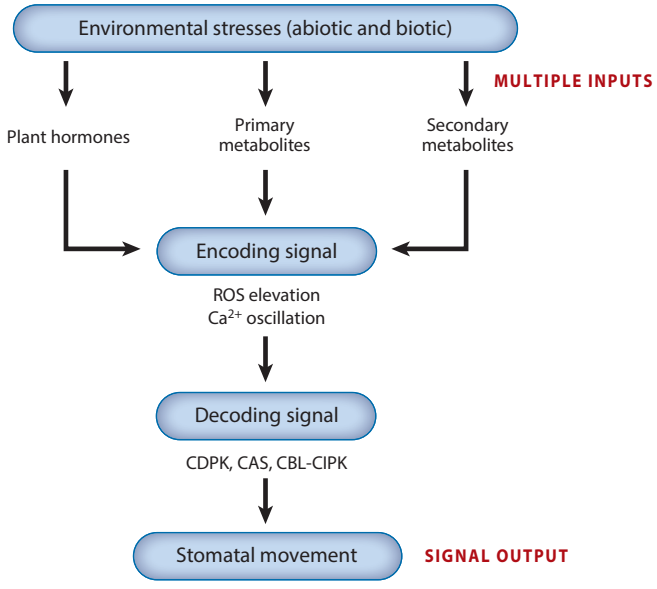

Figure 1

Signal encoding and decoding in guard cell signal integration. Abbreviations: CAS, CALCIUM-SENSING RECEPTOR; CBL, CALCINEURIN B–LIKE PROTEIN; CDPK, calcium-dependent protein kinase; CIPK, CBL-INTERACTING PROTEIN KINASE; ROS, reactive oxygen species.

1. PLANT HORMONES THAT REGULATE STOMATAL MOVEMENTS

1.1. Jasmonates

Jasmonates (JAs) regulate stomatal aperture. Exogenous application of jasmonic acid and its methyl ester [methyl jasmonate (MeJA)] induces stomatal closure in various plant species (128). Genetic studies of *Arabidopsis thaliana* mutants have suggested that JAs share a common signaling pathway with ABA in guard cells. Similar to ABA, MeJA requires the NAD(P)H oxidases RESIRATORY BURST OXIDASE HOMOLOG D (AtRBOHD) and AtRBOHF to induce reactive oxygen species (ROS) production in guard cells and stomatal closure (165). Patch-clamp studies revealed that MeJA activates outward-rectifying K^+ channels (K^+_{out} channels) and inactivates inward-rectifying K^+ channels (K^+_{in} channels) in guard cells (40, 151). It also activates slow-type (S-type) anion channels in guard cells (127, 130), thereby depolarizing the plasma membranes of the guard cells, which in turn evokes K^+ extrusion through the depolarization-activated K^+_{out} channels (170).

Clade A protein phosphatase 2Cs (PP2Cs) such as ABA-INSENSITIVE 1 (ABI1) and ABI2 are negative regulators of ABA signaling and interact with PYR/PYL/RCAR ABA receptor proteins in the presence of ABA (102, 143). The stomata of two ABA-insensitive dominant-negative mutants of *ABI1* and *ABI2*, *abi1-1* and *abi2-1*, are insensitive to MeJA (130). Thus, JAs might affect regulation of the ABA receptor complexes in guard cells (128). However, in spite of the robust signal crosstalk between ABA and JAs in guard cells, no significant reduction of drought tolerance was detected in the *Arabidopsis coronatine-insensitive 1* (*coi1*) mutant (S. Munemasa & Y. Murata, unpublished data). Although the *coi1* mutation blocked guard cell MeJA signaling, it did not influence ABA signaling (130). In addition, pretreatment of *Arabidopsis* plants with MeJA did not improve drought tolerance

NAD(P)H oxidase: a transmembrane enzyme that generates superoxide anions in extracellular space by consuming the reduction energy of NADH or NADPH

Patch clamp: an electrophysiological technique that is able to record very small ion currents, including the current of a single ion channel

(S. Munemasa & Y. Murata, unpublished data). These facts suggest that JA-induced stomatal closure does not play a crucial role in drought tolerance, at least in *Arabidopsis*, and that JAs do not simply activate guard cell ABA signaling, although they share several signaling elements. The results also imply that guard cells display a diversified response to environmental stimuli even though some signaling elements are common, which may be important for understanding the multiresponsivity of plants to environmental stimuli. By contrast, exogenous application of JAs and coronatine, a JA-mimicking phytotoxin, induces stomatal opening and inhibits ABA-induced stomatal closure instead of inducing stomatal closure (112, 113). Plant pathogens such as *Pseudomonas syringae* hijack the JA-mediated stomatal opening signal pathway for invasion through the opened pores. However, the physiological role and significance of endogenous JA-mediated stomatal opening are not clear.

Superoxide anion: a one-electron-reduced oxygen molecule

***NahG*:** a gene encoding salicylate hydroxylase, which converts salicylic acid to catechol

Both the JA precursor 12-oxo-phytodienoic acid (12-OPDA) and MeJA induce stomatal closure (121, 155), but pretreatment with MeJA inhibits the 12-OPDA-induced stomatal closure (121). These results suggest that 12-OPDA triggers a signaling distinct from JA signaling in guard cells, which might be due to its electrophilic properties (6, 41).

1.2. Salicylic Acid

Salicylic acid (SA) is a phenolic compound that plays key roles in pathogen defense, thermogenesis, and flowering. An attack by any of a broad spectrum of pathogens induces an elevation of SA levels that triggers systemic acquired resistance (SAR), which reprograms the transcriptome and immune responses (173). Endogenous SA levels can be increased 10–100-fold in local leaves and up to 10-fold in systemic leaves in response to pathogens (115). Stomata are vulnerable pores in the epidermal tissues of plants that are unprotected against microbes; an increased level of endogenous SA induces stomatal closure, preventing the invasion of bacterial and fungal pathogens through the aperture.

Stomatal closure induced by SA is accompanied by the production of extracellular superoxide anions. Superoxide anion production around guard cells is inhibited by the peroxidase inhibitor salicylhydroxamic acid (SHAM) but not by the NAD(P)H oxidase inhibitor diphenylene iodonium (DPI) (85, 125). This suggests that superoxide anion production is associated with cell wall–bound peroxidase activity. However, SHAM is not a specific inhibitor of cell wall–bound peroxidases. Therefore, the mechanism of SA-induced superoxide production will need to be further investigated by molecular genetic studies. In turn, SA induces intracellular ROS accumulation and inactivation of plasma membrane K^+_{in} channels (85). A distinct difference between SA-induced ROS generation and ABA- and MeJA-induced ROS generation is that NAD(P)H oxidase is necessary for the latter but not for the former.

SA-induced stomatal closure is not associated with $[Ca^{2+}]_{cyt}$ elevation in guard cells but is blocked by a Ca^{2+} chelator, ethylene glycol tetraacetic acid (EGTA), and by a Ca^{2+} antagonist, $LaCl_3$ (85). La^{3+} is known to compete with Ca^{2+} for Ca^{2+}-binding sites and to block Ca^{2+}-permeable channels. These results suggest that extracellular Ca^{2+} binding rather than $[Ca^{2+}]_{cyt}$ elevation is crucial for SA-induced stomatal closure. Hence, the ROS-Ca^{2+} signaling system in the control of SA-induced stomatal closure appears to be different from that in the control of stomatal closure in response to ABA (93, 144) and MeJA (130, 165).

Contact between an *Arabidopsis* leaf and a pathogenic bacterium induces stomatal closure. This closure is impaired in SA-deficient transgenic *NahG* plants and in the SA biosynthetic mutant *enhanced disease susceptibility 16-2* (*eds16-2*) (113), suggesting that SA is essential for stomatal defense against bacterial infection; that is, SA-mediated stomatal closure is an innate immune response elicited by contact with pathogenic bacteria. In addition, it has been proposed that the

combined action of SA and ABA signaling pathways mediates stomatal closure in response to bacterial pathogens (113, 122). *P. syringae* pv. *tomato* strain DC3000 induced stomatal closure, which was impaired in the ABA-deficient mutant *aba3* (113), but SA-induced stomatal closure was not impaired in another ABA-deficient mutant, *aba2* (71). The mechanism of signal crosstalk between ABA and SA during the innate immune response remains to be clarified.

Drought stress is known to increase SA accumulation levels in plants. The stomatal apertures of the *Arabidopsis* mutants *sap and miz 1* (*siz1*), *accelerated cell death 6* (*acd6*), and *constitutive pathogen response 5* (*cpr5*), which accumulate higher levels of SA, are narrower than those of wild-type plants, and consequently the accumulation of endogenous SA improves drought tolerance (119, 141). Moreover, the narrow-stomata phenotype of the mutants is compromised by application of the peroxidase inhibitors SHAM and azide but not by application of the NAD(P)H oxidase inhibitor DPI (119, 141). These results suggest that ROS generation mediated by SHAM-sensitive peroxidases is involved in SA-related drought responses. In contrast to these results, however, Catala et al. (21) reported that the *siz1* mutant has a drought-sensitive phenotype. In addition, the SA-accumulating *cpr22* mutant showed reduced stomatal sensitivity to ABA and enhanced dehydration, and the *NahG* transgene suppressed the *cpr22* phenotype (126). *SIZ1* encodes a small ubiquitin-like modifier (SUMO) E3 ligase (120) and sumoylates various proteins, including the MYB transcription factor PHOSPHATE STARVATION RESPONSE 1 (PHR1) (120), the basic leucine zipper (bZIP) transcription factor ABI5 (118), and the nitrate reductases NIA1 and NIA2 (142). Thus, SIZ1 regulation of SA signaling may not be direct, but rather mediated by an indirect complex mechanism.

1.3. Ethylene

The gaseous plant hormone ethylene functions in plants under certain biotic stress responses. Rapid drought stress elicits production of ethylene in wheat (132) but not in common bean, cotton, or miniature rose (123). Ethylene production is induced by moderate drought stress in maize with a compromised ability to produce ABA, although it is not induced by severe drought stress (174). Flooding induces stomatal closure, which is accompanied by ethylene production in nonaquatic plants (30, 72). These results suggest that ethylene regulation of stomatal movements under stress conditions is highly species dependent (1).

Ethylene has been shown to induce stomatal closure (36). In *Arabidopsis*, ethylene-induced stomatal closure is dependent on ROS production mediated by the NAD(P)H oxidase AtRBOHF (36). Ethylene-induced stomatal closure is impaired in the ethylene-insensitive mutants *etr1-1* and *ethylene-insensitive 2-1* (*ein2-1*). Interestingly, ethylene-induced ROS production in guard cells is impaired in *etr1-1* but not in *ein2-1*, suggesting that activation of AtRBOHF occurs upstream of EIN2 in guard cell ethylene signaling (36). By contrast, however, ethylene has been shown to counteract ABA-induced stomatal closure (167, 168). Watkins et al. (178) showed recently that ethylene increases the flavonol content in guard cells, suggesting that the accumulated flavonols act as antioxidants, reducing guard cell ROS levels and suppressing stomatal closure. Therefore, although how plants decide whether to open or close stomata in response to ethylene is unknown, it is clear that ROS homeostasis is a main target of ethylene signaling in guard cells. Desikan et al. (36) also suggested that an unknown cell-to-cell communication between guard cells and mesophyll cells is required by ethylene-induced stomatal closure but not by ethylene inhibition of ABA-induced stomatal closure. Chen et al. (22) showed that ethylene limits stomatal response to ABA in aging leaves. These results suggest that ethylene regulation of stomatal movements is highly dependent on surrounding environmental conditions.

1.4. Other Plant Hormones

Other plant hormones, such as brassinolide, auxin, gibberellin, strigolactone, and cytokinin, are not directly implicated in stomatal movements but may be indirectly involved. However, information about stomatal movements related to these plant hormones is limited, and we refer readers to other reviews on this subject (1, 29, 146).

2. ELICITORS

In this section, we summarize the regulation of stomatal apertures by small chemical compounds, including elicitors and secondary metabolites, and discuss the physiological significance of this regulation.

2.1. Flg22, Elf18, Elf26, Bacterial Lipopolysaccharide, and Chitin

It has been assumed that microscopic surface openings, such as stomata, serve as passive ports of bacterial entry during infection in plants. Like other plant cells, guard cells respond to microbes by detecting molecules derived from microbes, which are termed microbe-associated molecular patterns (MAMPs). Each MAMP elicitor is sensed by a specific plasma membrane–localized receptor termed a pattern recognition receptor (PRR). Recent studies have clearly shown that guard cells in various plant species respond to MAMPs, resulting in stomatal closure to restrict the invasion of microbes (88, 90, 95, 113). The well-known MAMPs that induce stomatal closure include flg22 (a conserved 22-amino-acid peptide near the N terminus of bacterial flagellin) (113), elf18 (an 18-amino-acid peptide near the N terminus of bacterial elongation factor Tu) (185), elf26 (a 26-amino-acid peptide near the N terminus of bacterial elongation factor Tu) (34), bacterial lipopolysaccharide (113), chitin (a fungal cell wall component) (98), chitosan (a partially deacetylated chitin) (95), and yeast elicitor (an ethanol precipitate of yeast extract) (88).

The *Arabidopsis* PRRs responsible for recognition of flg22, elf18 and elf26, and chitin are FLAGELLIN-SENSITIVE 2 (FLS2) (25, 51), ELONGATION FACTOR TU RECEPTOR (EFR) (193), and CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) (65, 121), respectively, and these three PRRs are expressed in *Arabidopsis* guard cells (97, 149). In addition, these three PRRs cannot perceive lipopolysaccharide components, lipid A, or core polysaccharides (161). For details on the mechanism of MAMP perception by PRRs, we refer readers to other recent reviews (12, 104, 182, 192).

Flagellin is the constituent protein of the bacterial flagellum. The highly conserved N-terminal epitope of flagellin, flg22 (42), is perceived by guard cells, leading to stomatal closure. Stomatal closure in response to flg22 is abolished in the loss-of-function mutant *fls2* (113) (**Figure 2**), suggesting that the PRR of flg22, FLS2, plays a critical role in flg22-induced stomatal closure. Production of hydrogen peroxide (H_2O_2) catalyzed by the NAD(P)H oxidase AtRBOHD is also essential for flg22-induced stomatal closure (34, 79, 96).

The NAD(P)H oxidase AtRBOHD associates with FLS2 and *BOTRYTIS*-INDUCED KINASE 1 (BIK1) and is phosphorylated by BIK1 at several amino acid residues in a Ca^{2+}-independent manner (80, 96). The phosphorylation of AtRBOHD by BIK1 is required for flg22-induced H_2O_2 production and stomatal closure (43, 80, 96). In addition, Dubiella et al. (37) and Gao et al. (45) reported that calcium-dependent protein kinases (CDPKs) phosphorylate and activate AtRBOHD in response to flg22 treatment. Both CDPK-dependent and BIK1-dependent regulation of AtRBOHD are likely required for a full ROS burst during flg22 response, but the interdependent mechanism is unknown. Production of H_2O_2 induced by flg22 is attributed not only to plasma membrane NAD(P)H oxidases but also to type III peroxidases (31, 138), suggesting

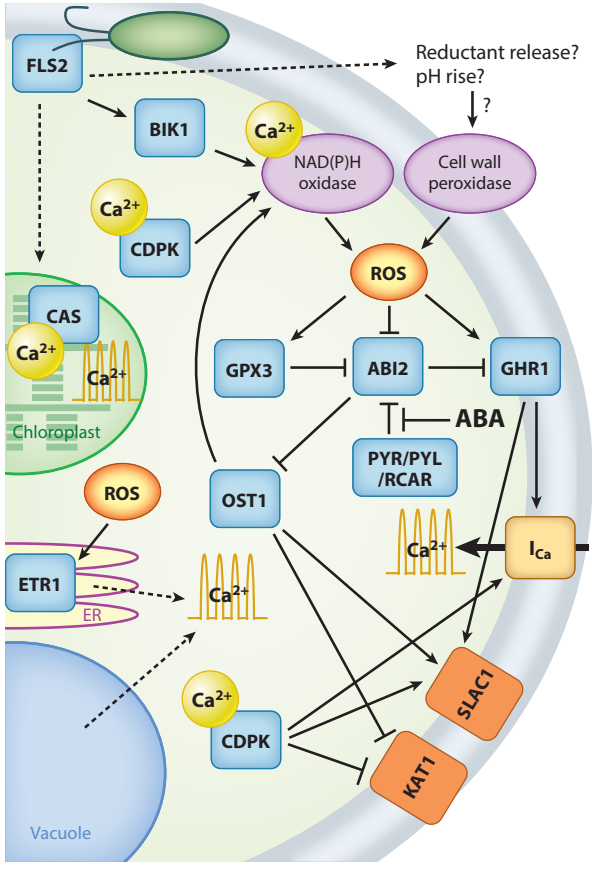

Figure 2

A proposed simplified model for signal integration mechanisms in guard cells. Representative abiotic and biotic stimuli, flagellin, and ABA are shown. Plasma membrane NAD(P)H oxidases and apoplastic peroxidases are major ROS sources that activate the downstream signaling components. Activation of plasma membrane calcium current (I_{Ca}) channels causes $[Ca^{2+}]_{cyt}$ elevation, which composes a part of the $[Ca^{2+}]_{cyt}$ oscillation with a defined pattern (Ca^{2+} signature). The ER and vacuole are also considered to be major intracellular Ca^{2+} stores. Stromal Ca^{2+} elevation mediated by CAS is observed during stomatal closure induced by microbe-associated molecular patterns. The activities of NAD(P)H oxidases and CDPKs are regulated by the direct binding of Ca^{2+}. At the thylakoid membrane, CAS binds to Ca^{2+}, but the functional role of this binding is unknown. ABI2, GPX3, and ETR1 are involved in ROS sensing. The dashed arrows show possible signaling pathways. Abbreviations: ABA, abscisic acid; ABI2, ABA-INSENSITIVE 2; BIK1, *BOTRYTIS*-INDUCED KINASE 1; CAS, CALCIUM-SENSING RECEPTOR; CDPK, calcium-dependent protein kinase; ER, endoplasmic reticulum; FLS2, FLAGELLIN-SENSITIVE 2; GHR1, GUARD CELL HYDROGEN PEROXIDE–RESISTANT 1; GPX3, GLUTATHIONE PEROXIDASE 3; KAT1, POTASSIUM CHANNEL IN *ARABIDOPSIS THALIANA* 1; OST1, OPEN STOMATA 1; PYR/PYL/RCAR, PYRABACTIN RESISTANCE/PYR1-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR; ROS, reactive oxygen species; SLAC1, SLOW ANION CHANNEL–ASSOCIATED 1.

that ROS production in the plasma membrane and in the cell wall function collaboratively in flg22-induced stomatal closure.

A positive regulator of ABA signaling, OPEN STOMATA 1 (OST1) protein kinase, is required for flg22-induced stomatal closure when flg22 concentrations are at or below 5 μM (113, 122); based on the results of an in-gel kinase assay, however, flg22 does not increase OST1 activity at a

concentration of 100 nM (122). These results suggest that flg22 does not induce ABA biosynthesis to facilitate stomatal closure and that a basal level of ABA serves to prime flg22-induced stomatal closure.

Like flg22, elf18 induces stomatal closure and H_2O_2 production in guard cells, both of which are impaired by the *AtrbohD* mutation (103), suggesting that AtRBOHD participates in elf18-induced stomatal closure. However, type III peroxidases may also mediate elf18-induced stomatal closure, because type III peroxidases contribute to H_2O_2 production in plants in response to flg22 (31, 138).

2.2. Chitosan and Yeast Elicitor

Chitosan is found in the walls of fungi. Exogenous application of chitosan also induces ROS accumulation in guard cells, leading to stomatal closure in *Arabidopsis*, *Commelina communis*, and *Pisum sativum* (88, 102, 163). Chitosan also stimulates S-type anion channel activity in guard cells, resulting in stomatal closure in *Hordeum vulgare* (90). The stomatal closure is completely abolished by the peroxidase inhibitor SHAM (86), suggesting that SHAM-sensitive peroxidases are involved in chitosan-induced stomatal closure. These peroxidases are likely type III peroxidases.

Yeast elicitor contains mainly mannan, a yeast cell wall component (158). Not only yeast elicitor but also live yeasts induce stomatal closure and ROS accumulation in guard cells (45, 88). ROS accumulation is not impaired in the *AtrbohD AtrbohF* mutant, but ROS production is slightly inhibited by DPI and completely inhibited by SHAM (45, 83). These results suggest that SHAM-sensitive peroxidases are essential for stomatal closure and H_2O_2 production induced by yeast elicitor (83).

Like ABA and MeJA, yeast elicitor activates hyperpolarization-activated calcium current (I_{Ca}) channels localized in the guard cell membrane to initiate $[Ca^{2+}]_{cyt}$ elevation (88, 183). A mutant study showed that a CDPK, CPK6, activates S-type channels and inhibits K^+_{in} channels to mediate stomatal closure induced by yeast elicitor (183). This result suggests that CPK6 is an important Ca^{2+} sensor in stomatal closure induced by yeast elicitor, but little is known about the roles of CDPKs in MAMP-induced stomatal closure. Moreover, the *cpk6* loss-of-function mutation does not affect ABA- and MeJA-induced H_2O_2 accumulation in guard cells but does inhibit yeast elicitor–induced H_2O_2 accumulation in guard cells and in the apoplasts of leaf tissues (127, 183). These results suggest that CPK6 downregulates certain H_2O_2-scavenging systems in *Arabidopsis* in response to yeast elicitor.

3. REACTIVE METABOLITES

3.1. Carbonyl Compounds

A variety of stresses in plants induce disturbances of metabolism and peroxidation of lipids and proteins through ROS production, leading to the production of reactive carbonyl compounds such as methylglyoxal (CH_3COCHO) and 4-hydroxy-2-nonenal through enzymatic and nonenzymatic reactions (11, 159). Hence, the reactive carbonyl compounds are likely to play important roles in stress responses (107, 154). Both methylglyoxal and 4-hydroxy-2-nonenal induce stomatal closure, and methylglyoxal additionally induces ROS production mediated by SHAM-sensitive peroxidases and $[Ca^{2+}]_{cyt}$ elevation (58, 122). Moreover, carbonyl compounds such as methylglyoxal and acrolein inhibit K^+_{in} channels and light-induced stomatal opening (57, 70). The reactive carbonyl compounds are likely to be closely involved in stomatal movements.

3.2. Isothiocyanates

Isothiocyanates (ITCs) are produced by myrosinase-mediated conversion of metabolites called glucosinolates. They have a repellent effect on insects, pathogens, and herbivores, which is a chemical herbivory defense system found in members of the Brassicaceae. Allyl isothiocyanate (AITC), a hydrolysis product of sinigrin, induces stomatal closure in *Arabidopsis* (84). AITC-induced stomatal closure is accompanied by ROS production, which is mediated mainly by SHAM-insensitive peroxidases (59, 84). There is genetic evidence that AITC-induced stomatal closure and ROS production require endogenous MeJA but not endogenous ABA (84). Plants such as crucifers may produce ITCs to induce stomatal closure, leading to suppression of water loss and invasion of fungi through stomata.

The myrosinase-glucosinolate defense system evolved in members of the Brassicaceae. Myrosinase catalyzes the hydrolysis of a class of compounds called glucosinolates to produce toxic chemicals such as ITCs, thiocyanates, and nitriles. Myrosinases are reported to be involved in stomatal movements of *Arabidopsis*. THIOGLUCOSIDE GLUCOHYDROLASE 1 (TGG1) is involved in light-induced stomatal opening (189), and both TGG1 and TGG2 are involved in ABA-induced stomatal closure (69). Some myrosinase hydrolysis products of glucosinolates inhibit K^+_{in} channels (189), which is favorable to inhibition of stomatal opening.

How myrosinases function in ABA-induced stomatal closure in *Arabidopsis* is unknown. This function is not common to all land plants because the myrosinase-glucosinolate defense system is not present in all plants, but ITCs may induce stomatal closure using certain common signal pathways because of their electrophilicity.

AITC induces stomatal closure, which is inhibited by treatment with glutathione (GSH; γ-glutamylcysteinylglycine) monoethylester (84), suggesting that GSH is closely involved in AITC-induced stomatal closure. GSH deficiency enhances ABA- and MeJA-induced stomatal closure (4, 73), and AITC-induced stomatal closure is disrupted in the MeJA-deficient mutant *allene oxide synthase* (*aos*) (84). These results suggest that endogenous MeJA plays a role in AITC-induced stomatal closure.

Tubulin is reported to be a major in vivo binding target for ITCs in animal cells (116), but little is known about the binding target for ITCs in plant cells. However, microtubules are essential for guard cell function in *Vicia* and *Arabidopsis* (38, 39). In plant cells, microtubules may be one of the major binding targets for ITCs.

3.3. Glutathione

GSH is the most abundant nonprotein thiol compound in plants and is a key regulator of cellular redox homeostasis. It is involved in various physiological processes, including growth, development, and (in particular) defense responses to abiotic and biotic stresses (108, 134).

Monochlorobimane staining indicates that guard cells accumulate larger amounts of GSH than other epidermal cells (73). Both ABA and MeJA induce stomatal closure and coinstantaneously decrease the GSH contents of guard cells (4, 140). ABA- and MeJA-induced stomatal closure was enhanced in the *Arabidopsis* GSH-deficient mutants *chlorinal 1-1* (*ch1-1*) and *cadmium-sensitive 2-1* (*cad2-1*) (3–5, 73, 140) and in *Arabidopsis* wild-type plants treated with the GSH-depletion chemicals 1-chloro-2,4-dinitrobenzene, *p*-nitrobenzyl chloride, iodomethane, and ethacrynic acid (5, 73). Application of GSH monoethylester, a membrane-permeable derivative of GSH, restores the stomatal phenotype of *ch1-1* and *cad2-1* mutants (3–5, 73, 140), whereas application of GSH does not affect stomatal movements (73). These results suggest that intracellular GSH functions as a negative regulator of ABA and MeJA signaling in guard cells.

Arabidopsis GLUTATHIONE PEROXIDASE 3 (AtGPX3) is involved in scavenging H_2O_2, and stomata of *atgpx3* plants are less sensitive to ABA than those of wild-type plants (117). In animal cells, GPXs scavenge oxyradicals using GSH as an electron donor (9). However, *Arabidopsis* GPXs (AtGPX1, -2, -3, -5, and -6) employ thioredoxin rather than GSH as an electron donor to scavenge H_2O_2 and organic hydroperoxides (66, 117). Therefore, GPXs cannot be involved in the negative regulation by GSH of ABA- and MeJA-induced stomatal closure. In addition, although GSH can nonenzymatically react with various oxidants, GSH and ascorbate react much more slowly with H_2O_2 than with hydroxyl radicals, singlet oxygen, and superoxide (147). Thus, GSH cannot be expected to scavenge H_2O_2 nonenzymatically in plants.

Genetic depletion of intracellular GSH does not affect ABA-induced ROS production in guard cells (73) but does significantly increase ROS production in whole leaves (129). The depletion of GSH by the *cad2* mutation may be involved in apoplastic and mesophyll cell ROS production rather than guard cell ROS production. Moreover, intracellular GSH does not affect cytosolic alkalization or $[Ca^{2+}]_{cyt}$ elevation elicited by ABA (140), but GSH depletion enhances H_2O_2-induced stomatal closure (such as ABA- and MeJA-induced stomatal closure), and activation of I_{Ca} channels by H_2O_2 is potentiated in the *cad2* mutant plant (129). The negative regulation by GSH of ABA-induced stomatal closure is likely attributable in part to an increased accumulation of extracellular ROS and modulation of I_{Ca} by GSH. The exact mechanism by which GSH modulates the responses of guard cells to ABA and MeJA is still unclear.

GSH-depletion reagents such as ethacrynic acid can function as an inhibitor of glutathione *S*-transferase (GST) (106). The genetic and chemical depletion of GSH, a substrate of GST, results in a reduction of GST activities. Hence, the effect of GSH depletion on stomatal responses can be both a decrease in GSH contents and an indirect inhibition of GST. Therefore, the involvement of GST in ABA- and MeJA-induced stomatal closure should also be considered.

Other low-molecular-weight thiols, such as cysteine, cystine, γ-glutamylcysteine, cysteinylglycine, and phytochelatins, are also highly reactive compounds involved in maintaining cellular redox homeostasis and regulating cellular metabolism in plants under abiotic and biotic stresses (7, 54, 184). However, it remains to be clarified whether such thiols negatively regulate stomatal closure induced by ABA and MeJA or other stimuli.

4. SIGNAL INTEGRATION MECHANISM

In this section, we focus on a proposed integration of signal pathways in guard cells.

4.1. Reactive Oxygen Species: Metabolism

Abiotic and biotic stresses trigger ROS generation related to stomatal movements. ROS generation is catalyzed mainly by two types of enzymes: plasma membrane NAD(P)H oxidases and cell wall peroxidases (**Figure 2**). Other ROS-generating enzymes, such as apoplastic amine oxidases and oxalate oxidases, may also be involved in the ROS generation responsible for stomatal closure as well as oxidative bursts (8, 94). Here, we discuss plasma membrane NAD(P)H oxidases and cell wall peroxidases, as both of these enzymes are well studied and such knowledge allows us to build an integrated model of guard cell signaling.

Genetic and pharmacological studies using *Arabidopsis* have revealed that NAD(P)H oxidases, including AtRBOHD and AtRBOHF, mediate ROS generation in guard cells in response to ABA (93), MeJA (165), ethylene (36), flg22 (114), and elf18 (103). These NAD(P)H oxidases are regulated by direct binding of Ca^{2+} (139), phosphatidic acid (187), and Rac GTPases (181) and by phosphorylation by OST1/SUCROSE NON-FERMENTING 1–RELATED KINASE 2.6

(SnRK2.6) (162), CDPKs (19, 89), and BIK1 (80) in plants. Therefore, NAP(D)H oxidases likely integrate multiple upstream signal transductions that lead to stomatal closure.

Cytosolic alkalization is a major step in signal transduction for ABA, MeJA, and elicitors in guard cells (10, 48, 52, 67). It has been proposed that cytosolic alkalization occurs upstream of NAD(P)H oxidase–mediated ROS production in signal transduction (52, 165). However, the mechanism by which cytosolic alkalization triggers activation of NAD(P)H oxidases remains to be clarified. It has also been reported that cytosolic alkalization occurs downstream of ROS production (68, 186).

Stomatal closures induced by SA (125), chitosan (86), yeast elicitor (83), methylglyoxal (58), and ITC (85) are accompanied by SHAM-sensitive, peroxidase-mediated extracellular ROS generation. Although the molecular identities of such peroxidases are yet to be revealed, these findings suggest a critical role for peroxidases at a node integrating multiple signals in ROS generation and stomatal closure. Oxidative bursts mediated by apoplastic class III peroxidases are involved in MAMP-elicited defense responses (31, 138). It has been suggested that production of H_2O_2 by apoplastic peroxidases preferentially occurs at high pH in the presence of an excess of reductants (13, 138). However, how apoplastic pH and reductant amount are regulated during stomatal closure is unknown.

Catalase, an enzyme that catalyzes the decomposition of H_2O_2, is predominantly located in the peroxisomes of plant cells (81, 180). The peroxisome is considered a major ROS source (28, 32, 135). Disruption of catalase genes and treatment with the catalase inhibitor 3-amino-1,2,4-triazole enhanced ABA- and MeJA-induced stomatal closure in *Arabidopsis* (75–77). Consequently, in addition to the apoplast and plasma membrane, intracellular organelles such as peroxisomes might be ROS sources involved in regulating stomatal movements (**Figure 2**). Expression of catalase genes is tightly regulated by the circadian clock (190), suggesting that catalase might function as a coordinator of stomatal sensitivity to environmental stresses and the circadian clock.

In addition to catalase, ascorbate peroxidase functions as an H_2O_2 scavenger involved in regulating stomatal movement. It catalyzes the conversion of H_2O_2 into H_2O using ascorbate as an electron donor. Disruption of a cytosolic ascorbate peroxidase (APX1) in *Arabidopsis* altered the stomatal response to light but not to ABA (145). In tobacco plants, overexpression of dehydroascorbate reductase increased the ratio of ascorbate to dehydroascorbate in guard cells, reducing stomatal responsiveness to H_2O_2 and ABA (24). The involvement of ascorbate in stomatal responses to biotic stress has not yet been reported.

As discussed above, the myrosinases TGG1 and TGG2 are involved in ABA and MeJA signaling in guard cells (69, 189). Myrosinases are activated by ascorbate (20), and cleavage of indol-3-ylmethyl glucosinolate by myrosinase in the presence of ascorbic acid produces ascorbigen, a condensation product of ascorbic acid with 3-hydroxymethylindole, implying that the myrosinase system could provide the storage pool of ascorbate through its inactivation (14). Further work is needed to reveal the roles of ascorbate and myrosinases in guard cell signal integration.

4.2. Reactive Oxygen Species: Sensing

Biochemical data have shown that the PP2Cs ABI1 and ABI2 are downregulated by H_2O_2 in vitro (110, 111). In budding yeast, the GPX-like enzyme ORP1 functions as an H_2O_2 receptor that oxidizes and activates the bZIP transcription factor YAP1 (33). Miao et al. (117) proposed that, similar to the yeast ROS-sensing mechanism, AtGPX3 mediates ROS perception in guard cell ABA signaling. They also reported that AtGPX3 downregulates and interacts with ABI2 and (to a lesser extent) ABI1 in vitro. Therefore, a possible mechanism is that the ROS-sensing module ABI2-GPX3 functions as a signal decoder of guard cell signaling.

The mitogen-activated protein kinases (MAPKs) MPK3, MPK9, and MPK12 are activated by ABA and H_2O_2 (74, 99). *MPK3* antisense plants show attenuated ABA- and H_2O_2-induced stomatal closure (55). Disruption of two MAPK genes, *MPK9* and *MPK12*, impairs stomatal closure responses to cold, ABA, chitosan, and yeast elicitor as well as H_2O_2 (74, 152, 153). Montillet et al. (122) recently reported that MPK3 and MPK6 are involved in flagellin but not ABA guard cell signaling. These findings highlight the importance of ROS-MAPK pathways in guard cell signal integration. *Arabidopsis* MAPKs are activated not only by phosphorylation but also by calmodulin binding (166), suggesting that Ca^{2+} modulates the signal integration pathway.

Hua et al. (61) recently showed that a leucine-rich-repeat receptor-like kinase, GUARD CELL HYDROGEN PEROXIDE–RESISTANT 1 (GHR1), acts downstream of ROS and PP2Cs and is required for ABA-, MeJA-, SA-, and flg22-induced stomatal closure. Like CPKs and SnRK2s, GHR1 directly phosphorylates and activates an S-type anion channel protein, SLOW ANION CHANNEL–ASSOCIATED 1 (SLAC1), in *Xenopus laevis* oocytes. Moreover, GHR1 interacts with ABI2 but not ABI1, and GHR1 activation of SLAC1 is inhibited by ABI2 but not ABI1 (61). Murata et al. (131) reported that the *abi2-1* mutation disrupts ABA signaling downstream of ROS production, whereas the *abi1-1* mutation does not. These findings, together with the GPX3 findings discussed above (117), suggest a dominant role of ABI2 over other PP2Cs in redox sensing in guard cells. Desikan et al. (35) have also reported that an *Arabidopsis* ethylene receptor, ETR1, mediates H_2O_2 signaling in guard cells and might serve as a sensor of H_2O_2, but the downstream event is entirely unknown.

4.3. Calcium: Transport

The calcium ion Ca^{2+} is an important second messenger in both plants and animals. In guard cells, changes in $[Ca^{2+}]_{cyt}$ are sensed by Ca^{2+}-binding proteins (63, 92); here, we focus on CDPKs and CALCIUM-SENSING RECEPTOR (CAS). Cytosolic Ca^{2+} is positively involved in stomatal closure through activation of S-type anion channels (23, 124, 156, 160) and negatively involved in stomatal opening through downregulation of K^+_{in} channels (53, 82, 156).

Plasma membrane Ca^{2+}-permeable I_{Ca} channels of guard cells are activated by H_2O_2 (144) and protein phosphorylation (91). Disruption of *ABI2*, *GPX3*, or *GHR1* impairs the activation of I_{Ca} channels by H_2O_2 (61, 117, 131). The Ca^{2+}-dependent protein kinases CPK3 and CPK6 and the Ca^{2+}-independent protein kinase OST1 are also involved in ABA activation of I_{Ca} channels (2, 124). The I_{Ca} channel activation is required for stomatal closure induced by MeJA (127, 130), chitosan (88), and yeast elicitor (88, 183).

Nitric oxide (NO) promotes intracellular Ca^{2+} release from the endomembrane via a cGMP- and cADPR-dependent pathway, leading to inactivation of K^+_{in} channels and activation of anion channels (47). Unlike application of ROS, application of NO does not activate plasma membrane Ca^{2+} channels (47). Joudoi et al. (79) recently identified a nitrated derivative of cGMP, 8-nitro-cGMP (8-nitroguanosine 3′,5′-cyclic monophosphate), as an important signal molecule that acts upstream of cADPR-mediated Ca^{2+} release in guard cells. ROS molecules function upstream of NO production in guard cells (19, 79), suggesting that they are responsible for both Ca^{2+} influx through plasma membrane I_{Ca} channels and NO-mediated Ca^{2+} release from the endomembrane in guard cells.

4.4. Calcium: Sensing by CDPKs

The genes encoding CDPKs constitute a large family of 34 members in *Arabidopsis* (60). CDPKs contain EF hands in their C-terminal domains and are activated by Ca^{2+} binding with distinct

affinities (15, 50). They can therefore sense changes in $[Ca^{2+}]$, and some of them are known to be involved in guard cell signaling (19, 87, 92, 157).

Two *Arabidopsis* CDPKs, CPK3 and CPK6, are involved in ABA regulation of S-type anion channels and I_{Ca} channels, resulting in stomatal closure (124), although CPK6 rather than CPK3 is involved in MeJA- and MAMP-induced stomatal closure (127, 183). These results suggest that distinct Ca^{2+} input pathways may be triggered during abiotic and biotic stresses in guard cells. In a heterologous expression system using *X. laevis* oocytes, CPK6 directly phosphorylated and activated the S-type anion channel protein SLAC1 (18). Regulation of K^+_{in} channels by yeast elicitor is impaired in the *cpk6-1* mutant, suggesting that CPK6 phosphorylates substrates other than SLAC1 in guard cells (183). The possible CPK6 substrates are K^+_{in} channel proteins such as POTASSIUM CHANNEL IN *ARABIDOPSIS THALIANA* 1 (KAT1) (150), the NAD(P)H oxidases AtRBOHD and AtRBOHF (37), and unidentified I_{Ca} channels, activities of which are strongly related to CPK6 (124, 127, 183). More work will be needed to understand the predominant role of CPK6 in Ca^{2+} signaling in guard cells. Although CPK3 can be activated by cold, salt, heat, H_2O_2, and MAMPs in transient overexpression system using *Arabidopsis* mesophyll protoplasts, the endogenous kinase appears to be constitutively active in roots and leaves in planta (109). Thus, the possible role of CPK3 as a signal decoder remains to be clarified.

Two other CPKs, CPK4 and CPK11, are activated by ABA and phosphorylate two ABA-responsive transcription factors, ABA-RESPONSIVE ELEMENT BINDING FACTOR 1 (ABF1) and ABF4 (191). Disruption of CPK4 and CPK11 impairs stomatal closure induced by ABA (191) and by imposed Ca^{2+} oscillation (63) but not stomatal closure induced by MeJA (127). In addition, interaction between CPK11 and ABF3 has been observed (100). Boudsocq et al. (17) reported that CPK4, -5, -6, and -11 function as crucial positive regulators in flagellin signaling, and Gao et al. (46) reported that they phosphorylate WRKY transcription factors in bifurcated immune responses activated by the nucleotide-binding-domain leucine-rich-repeat proteins RESISTANT TO *PSEUDOMONAS SYRINGAE* 2 (RPS2) and RESISTANCE TO *PSEUDOMONAS SYRINGAE* PV. *MACULICOLA* 1 (RPM1). These results suggest that CPK4, -5, -6, and -11 provide hubs for signal crosstalk between abiotic and biotic stresses via transcriptional regulation of downstream target genes (16, 157). Another CPK, CPK12, phosphorylates ABF1 and ABF4 in vitro and phosphorylates ABI2 to increase phosphatase activity of ABI2 in vitro; *CPK12*–RNA interference lines do not show any ABA-related stomatal phenotype (188).

CPK21 and CPK23 directly phosphorylate and activate SLAC1 and SLAH3 in *X. laevis* oocytes (49, 50), and SLAC1 activation results in rapid stomatal closure (133, 172). However, CPK21 and CPK23 in planta function as negative regulators of drought and osmotic stress responses (44, 101). The challenge for future research is to reveal the environmental stimuli that trigger CPK21/CPK23-mediated SLAC1 activation in planta.

Another CPK, CPK10, interacts with a heat shock protein, HSP1, which is facilitated by Ca^{2+}, and these proteins are involved in the regulation of K^+_{in} channels by ABA (194). In addition to ABA-induced stomatal closure, CPK10 is involved in stomatal closure induced by high extracellular Ca^{2+} (194) and by imposed Ca^{2+} oscillation (63). CPK4 and CPK11 also interact with HSP1 (171).

In *Arabidopsis*, CPK1 localizes to oil bodies and peroxisomes. This protein is a regulator of the innate immune system (27) that phosphorylates and downregulates a calmodulin-stimulated Ca^{2+} pump, *ARABIDOPSIS* CA^{2+}-ATPASE ISOFORM 2 (ACA2), in the endoplasmic reticulum membrane (64). Both CPK1 and CPK2 phosphorylate the NAD(P)H oxidases AtRBOHD and AtRBOHF (46), implying a possible role in regulating stomatal movement.

A noncanonical CDPK, CPK13, phosphorylates and inhibits the guard cell–expressed K^+_{in} channel proteins KAT1 and KAT2 in *X. laevis* oocytes (150). A single disruption of *CPK13* does

not cause a measurable stomatal phenotype, but *CPK13* overexpression suppresses light-induced stomatal opening (150).

Like CPK12, CPK32 phosphorylates ABF4 in vitro (26). And like *cpk3 cpk6* and *cpk4 cpk11* double mutants, the *cpk7 cpk8 cpk32* triple mutant shows impaired Ca^{2+} oscillation–induced stomatal closure (63).

4.5. Calcium: Sensing by CAS

CAS was originally identified as a plasma membrane–localized extracellular Ca^{2+}-sensing receptor that exhibits low-affinity/high-capacity Ca^{2+} binding (56). Subsequent reports revealed that it is in fact a chloroplast-localized protein involved in extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ elevation (136, 179). Nonetheless, it is involved in stomatal closure in response to high extracellular Ca^{2+} (56) and water stress (176). A recent study demonstrated that CAS mediates flg22-induced stomatal closure (137). These results suggest that CAS functions as a Ca^{2+} signal decoder under abiotic and biotic stresses.

CAS mediates production of inositol 1,4,5-trisphosphate (169), ROS, and NO (177) in guard cells in response to high extracellular Ca^{2+}. Nomura et al. (137) recently demonstrated that CAS mediates flg22-induced Ca^{2+} transients in guard cells and stomatal closure. Although flg22 induces stomatal closure via AtRBOHD activation (80, 103, 114), the NAD(P)H oxidase inhibitor DPI does not inhibit flg22-induced stromal Ca^{2+} transients, and the *cas-1* mutant showed intact flg22-induced ROS generation (137), suggesting that stromal Ca^{2+} transients occur parallel to AtRBOHD activation in guard cell flg22 signaling. Moreover, phosphorylation of CAS is dependent on Ca^{2+} (164). However, the functional roles of the CAS phosphorylation in guard cell signaling are unknown.

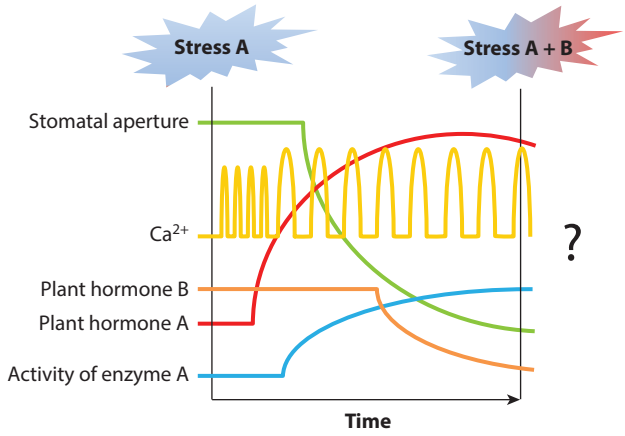

Figure 3

Real-time imaging for an array of responses involved in stress signal integration in guard cells. In this example model, stress A causes $[Ca^{2+}]_{cyt}$ oscillation, resulting in a concentration increase of plant hormone A. Plant hormone A then induces $[Ca^{2+}]_{cyt}$ oscillation and activates enzyme A, leading to stomatal closure. In response to stomatal closure, the concentration of plant hormone B decreases. The question then is, if stress B happens at this point, what will we see? The development of sensors for in vivo detection of second messengers, plant hormones, and enzyme activities will allow high-resolution analysis of plant stress integration responses to help answer this and similar questions.

5. CONCLUSION

The emerging principle is that Ca^{2+} and ROS are key to signal integration in plants. The cellular mechanisms of ROS sensing remain unclear. It has been reported that stomatal aperture is programmed by defined $[Ca^{2+}]_{cyt}$ signatures. Recent studies have revealed that plant Ca^{2+}-binding proteins have a wide range of Ca^{2+} affinities and that the subcellular localization is tightly controlled. However, how the defined Ca^{2+} messages are decoded in plant cells remains elusive.

Guard cells rapidly transduce environmental input signals into activation of plasma membrane ion channels in order to respond to environmental stimuli, in most cases within a few minutes. Recently, techniques have been developed for directly visualizing plant hormones in vivo using genetically encoded biosensors (78, 175). In the future, simultaneous time-resolved live imaging of guard cells for second messengers, plant hormones, and enzyme activities will advance our understanding of how guard cells achieve their fine-tuned integration to ensure growth optimization (**Figure 3**).

SUMMARY POINTS

1. Guard cells employ signal transduction mechanisms that integrate diverse environmental signals with reactive oxygen species (ROS) and Ca^{2+} signals in order to optimize stomatal aperture. How environmental signals are integrated into ROS and Ca^{2+} signatures remains to be clarified.
2. ROS and Ca^{2+} are versatile second messengers and play crucial roles in signal integration.
3. The Ca^{2+} signature is decoded by Ca^{2+}-binding proteins, including calcium-dependent protein kinase (CDPKs) and CALCIUM-SENSING RECEPTOR (CAS). The mechanisms by which the Ca^{2+} signature is decoded to yield stimulus-specific stomatal responses need investigation. Moreover, plasma membrane Ca^{2+}-permeable cation channels in guard cells need to be identified.
4. ROS molecules are produced in several systems in the signal integration. The temporal-spatial dynamics of ROS generation, which are likely to be associated with the Ca^{2+} signature, need to be clarified.

DISCLOSURE STATEMENT

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