Reactive Oxygen Species in the Regulation of Stomatal Movements[1][OPEN]

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Guard cells form stomatal pores that optimize photosynthetic carbon dioxide uptake with minimal water loss. Stomatal movements are controlled by complex signaling networks that respond to environmental and endogenous signals. Regulation of stomatal aperture requires coordinated activity of reactive oxygen species (ROS)-generating enzymes, signaling proteins, and downstream executors such as ion pumps, transporters, and plasma membrane channels that control guard cell turgor pressure. Accumulation of ROS in the apoplast and chloroplasts is among the earliest hallmarks of stomatal closure. Subsequent increase in cytoplasmic Ca^{2+} concentration governs the activity of multiple kinases that regulate the activity of ROS-producing enzymes and ion channels. In parallel, ROS directly regulate the activity of multiple proteins via oxidative posttranslational modifications to fine-tune guard cell signaling. In this review, we summarize recent advances in the role of ROS in stomatal closure and discuss the importance of ROS in regulation of signal amplification and specificity in guard cells.

Stomata are microscopic pores in plant epidermis surrounded by a pair of guard cells. Opening and closure of the stomatal pore is regulated by changes in guard cell turgor pressure. Environmental factors, such as light intensity and quality, soil and air water content, CO_2, and air pollutants, regulate stomatal aperture (for review, see Sun et al., 2014; Murata et al., 2015). Rapid stomatal movements are initiated within minutes from recognition of a trigger. Mature guard cells lack plasmodesmata; thus, efflux of osmotically active ions and metabolites causing the changes in turgor takes place through ion channels and transporters. Guard cell plasma membrane anion channels are activated by several stimuli, e.g. by abscisic acid (ABA) and leaf intercellular CO_2 concentration (for review, see Roelfsema et al., 2012). Anion fluxes across the plasma membrane lead to membrane depolarization that activates the voltage-gated channels regulating K^+ transport across the guard cell plasma membrane, which in turn causes water efflux and turgor loss resulting in stomatal closure (Ache et al., 2000; Murata et al., 2015).

Reactive oxygen species (ROS) are important signals involved in the regulation of stomatal closure (Song et al., 2014; Murata et al., 2015). ROS are ubiquitous metabolites in all aerobic organisms. They include hydroxyl radical ($HO^{\bullet}$), superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$). In the regulation of stomatal closure, ROS are produced first in guard cell apoplast, followed by sensing and signaling leading into activation of anion channels. In this review, we discuss recent advances and present a model for the interaction of ROS with other components during stomatal closure.

RBOH-GENERATED ROS IN STOMATAL CLOSURE

Plasma membrane NADPH oxidases (Respiratory Burst Oxidase Homologs [RBOHs]) mediate apoplastic ROS production in plants, and their role in the control of developmental and stress-induced responses is well established (Suzuki et al., 2011; Sierla et al., 2013). NADPH oxidases are evolutionarily conserved and present across kingdoms. Similar to their animal counterparts, plant RBOHs have six transmembrane domains and a cytosolic C-terminal part harboring the NADPH- and FAD-binding sites. The hydrophobic region binds two heme irons for transfer of electrons from NADPH to extracellular O_2 to form $O_2^{\bullet-}$, which is subsequently dismutated to H_2O_2. Plant RBOHs contain also an extended N-terminal region with two Ca^{2+}-binding elongation factor (EF)-hand motifs (Suzuki et al., 2011).

[1] The authors were supported by the Academy of Finland Centre of Excellence program (2014-19). T.V. was supported by an Academy of Finland postdoctoral fellowship (decision no. 266793).

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Jaakko Kangasjärvi (jaakko.kangasjarvi@helsinki.fi).

J.K. conceived the review with input from M.S., C.W., and T.V.; M.S., C.W., and T.V. wrote draft sections in their areas of expertise; M.S., C.W., and T.V. prepared the figures with comments from J.K.; J.K. compiled and edited the review; all authors commented, discussed, and revised the review.

[OPEN] Articles can be viewed without a subscription.
www.plantphysiol.org/cgi/doi/10.1104/pp.16.00328

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RBOHs are the most studied ROS-producing enzymes in plants, and they are involved in stomatal regulation. The Arabidopsis (*Arabidopsis thaliana*) isoforms *AtRBOHF* and *AtRBOHD* are expressed in guard cells and regulated by ABA (Kwak et al., 2003). Loss of AtRBOHF leads to partial impairment of ABA-induced stomatal closure. Stomatal closure is further reduced and ROS production is abolished in the *atrbohD atrbohF* double mutant, while exogenously applied ROS rescues stomatal responses. The role of ROS in CO_2-induced stomatal regulation was demonstrated only recently by showing that CO_2-induced stomatal closure was absent in the *atrbohD atrbohF* double mutant (Chater et al., 2015).

ROS also function in stomatal immunity. Pathogen-associated molecular pattern (PAMP)-mediated responses are often studied using peptides representing minimal epitopes of proteins causing immune response, such as flg22 and elf18 corresponding to bacterial flagellin and elongation factor Tu (EF-Tu). The stomata of *atrbohD* were unresponsive to flg22 and elf18, whereas *atrbohF* responded like the wild type (Mersmann et al., 2010; Macho et al., 2012). Despite the mounting evidence for a role of RBOHs in stomatal regulation (Song et al., 2014), novel approaches have presented new questions on their role. With noninvasive nanoinfusion, molecules can be delivered to the intercellular space through a microcapillary inserted into an open stoma (Guzel Deger et al., 2015). Nanoinfusion of ABA and flg22 induced a rapid closure of the stomatal pore. Although the number of stomata responding to the stimuli was slightly lower in the *atrbohD atrbohF* double mutant, they remained largely responsive (Guzel Deger et al., 2015) in contrast to previous reports. Such differences could at least partly be explained by differences in experimental conditions. Experiments with the use of epidermal peels do not take into account the possible involvement of mesophyll-derived signals in stomatal closure and may also be affected by wounding caused by peeling. Additionally, the timescales of the responses measured can have a significant and functionally relevant effect on the observed results. While data accumulated over a decade support the role for RBOH generated ROS in stomatal closure, these results indicate that further research is necessary to gain more detailed understanding of the mechanisms involved.

RBOH REGULATORY MECHANISMS DETERMINED IN HEK CELLS: A ROLE FOR Ca^{2+} AND PHOSPHORYLATION

Detailed molecular studies on the function and regulation of NADPH oxidases in planta are technically challenging; thus, heterologous expression of plant RBOHs in human embryonic kidney (HEK) cells lacking endogenous NADPH oxidases have been used to investigate their regulation. Ca^{2+} binding and phosphorylation have emerged as common themes in RBOH regulation. Treatment with ionomycin, a Ca^{2+} ionophore that induces Ca^{2+} influx, stimulated ROS production in cells transfected with several RBOH isoforms (Ogasawara et al., 2008; Takeda et al., 2008; Kimura et al., 2012). Furthermore, mutations in the EF-hands impaired Ca^{2+}-induced ROS production, suggesting that RBOHs possess Ca^{2+}-activated NADPH oxidase activity mediated by the EF-hand motifs.

A role for protein phosphorylation in RBOH activation has been demonstrated using a Ser/Thr protein phosphatase inhibitor calyculin A (CA), which was sufficient to induce ROS production in *RBOH*-transfected cells (Ogasawara et al., 2008; Takeda et al., 2008; Kimura et al., 2012). However, CA-induced ROS production was lower than that induced by ionomycin. Phosphorylation-induced activation of AtRBOHF and AtRBOHD was independent of Ca^{2+} binding as EF-hand mutants, which were unresponsive to ionomycin, remained responsive to CA (Ogasawara et al., 2008; Kimura et al., 2012). On the contrary, application of the kinase inhibitor K252a inhibited ionomycin-induced ROS production, indicating that phosphorylation was necessary for Ca^{2+}-induced activation of AtRBOHD and AtRBOHF (Kimura et al., 2012). Synergistic activation by phosphorylation and Ca^{2+} seems necessary for full RBOH activity as CA significantly enhanced ionomycin-induced ROS production (Ogasawara et al., 2008).

REGULATION OF RBOHs BY PHOSPHORYLATION IN PLANTA

The use of HEK cells to study the regulation of RBOH revealed that their activity can be modulated by phosphorylation. Since HEK cells were transfected only with plant *RBOH*s, the phosphatases and kinases targeted by the inhibitors, and thus phosphorylating/dephosphorylating RBOHs, originated from HEK cells. Consequently, results obtained with the use of (any) heterologous system need to be treated with caution, and in planta validation is necessary before data interpretation. Identification of kinases responsible for RBOH activation in planta as well as mapping of their target phosphorylation sites will contribute to a better understanding of early signaling events leading to stomatal closure.

The protein kinase OPEN STOMATA1 (OST1/SnRK2.6) is a crucial signaling component in stomatal closure induced by ABA (Ng et al., 2011) and a broad range of environmental and endogenous stimuli. Accordingly, numerous OST1 phosphorylation targets have been identified. The molecular components acting upstream of OST1 in ABA signaling are well characterized and include the PYROBACTIN RESISTANCE1 (PYR1)/PYR1-LIKE/REGULATORY COMPONENT OF ABA RECEPTORS receptor complex (Ma et al., 2009; Park et al., 2009) and a group of type 2C protein phosphatases (PP2Cs), including ABA-INSENSITIVE1 (ABI1) and ABI2 (Umezawa et al., 2009; Vlad et al., 2009), which act as constitutive OST1 inhibitors. Binding of ABA induces a conformational change in the receptor complex leading to inhibition of PP2Cs and consequent

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release of OST1 from inhibition. OST1 subsequently autophosphorylates Ser-175 in the activation loop resulting in autoactivation (Belin et al., 2006). As inferred from genetic evidence, patch-clamp studies in guard cells, in vitro kinase assays, and channel activity assays upon coexpression of the components in *Xenopus laevis* oocytes, OST1 is then thought to bind and activate by phosphorylation the guard cell anion channels SLOW ANION CHANNEL-ASSOCIATED1 (SLAC1) and QUICK-ACTIVATING ANION CHANNEL1 (QUAC1) leading to stomatal closure (Figs. 1 and 2; Lee et al., 2009; Imes et al., 2013). It should be noted that phosphorylation of the N terminus of SLAC1 by OST1 has been detected only in vitro. Moreover, in contrast to a OST1:split-YFP fusion protein (Geiger et al., 2009; Lee et al., 2009; and all following studies where SLAC1 activation by OST1 has been seen in *X. laevis* oocytes), untagged OST1 did not activate SLAC1 in oocytes (Vahisalu et al., 2008), indicating that the split-YFP tag might stabilize OST1 and/or its interaction with SLAC1.

OST1 is required for ABA-triggered ROS production in guard cells, indicating that it acts upstream of RBOHs (Mustilli et al., 2002; Shang et al., 2016). AtRBOHF is the main isoform involved in ABA-related stomatal responses (Kwak et al., 2003). Sirichandra et al. (2009) showed that OST1 phosphorylated the N terminus of AtRBOHF in vitro (Figs. 1 and 2A), and a Ser-174Ala substitution resulted in 40% reduction in phosphorylation. These phosphorylation events have not been

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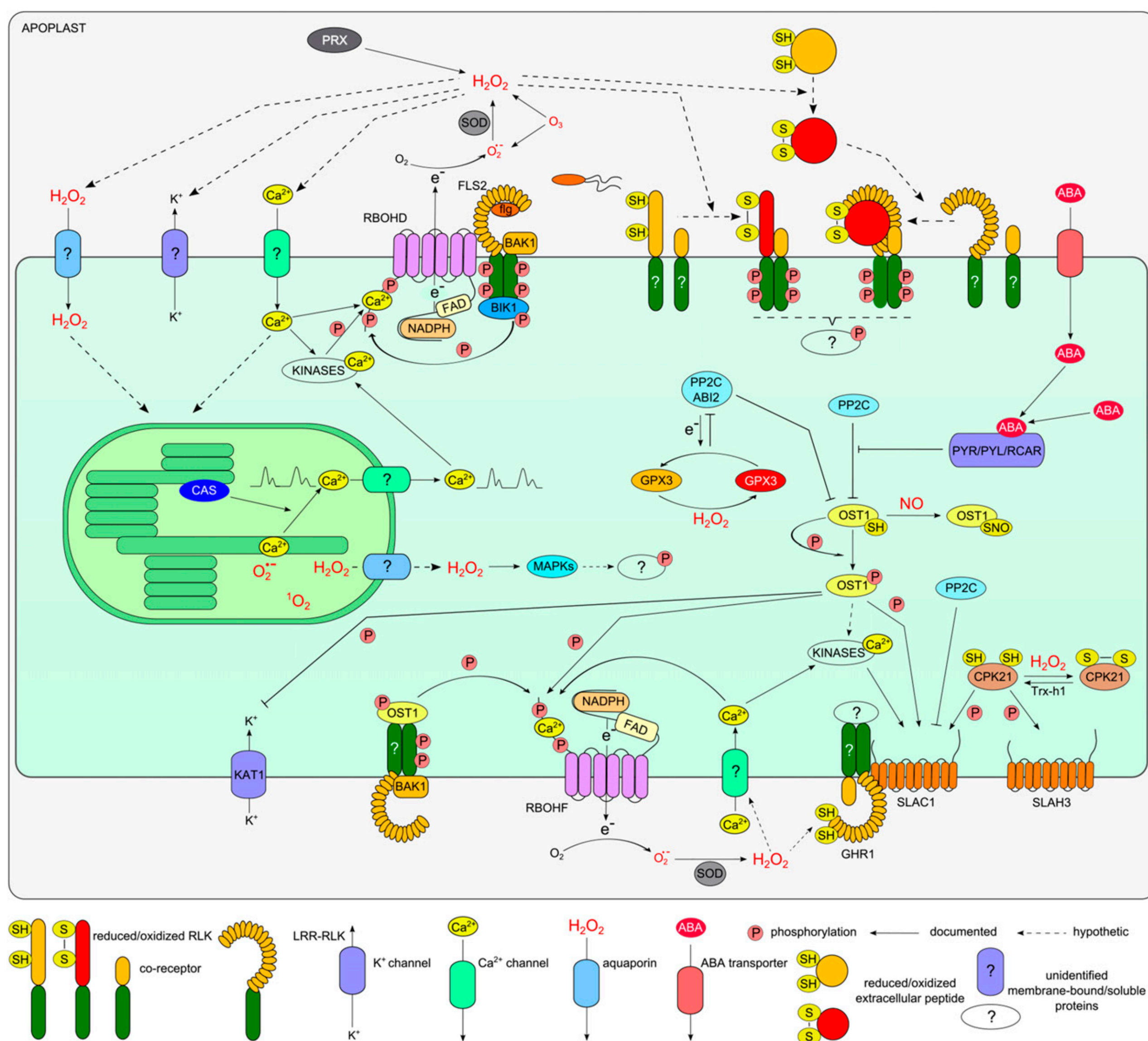

Figure 1. Schematic representation of ROS production and signaling in guard cells. Detailed description of specific regulatory events and abbreviations are in the main text.

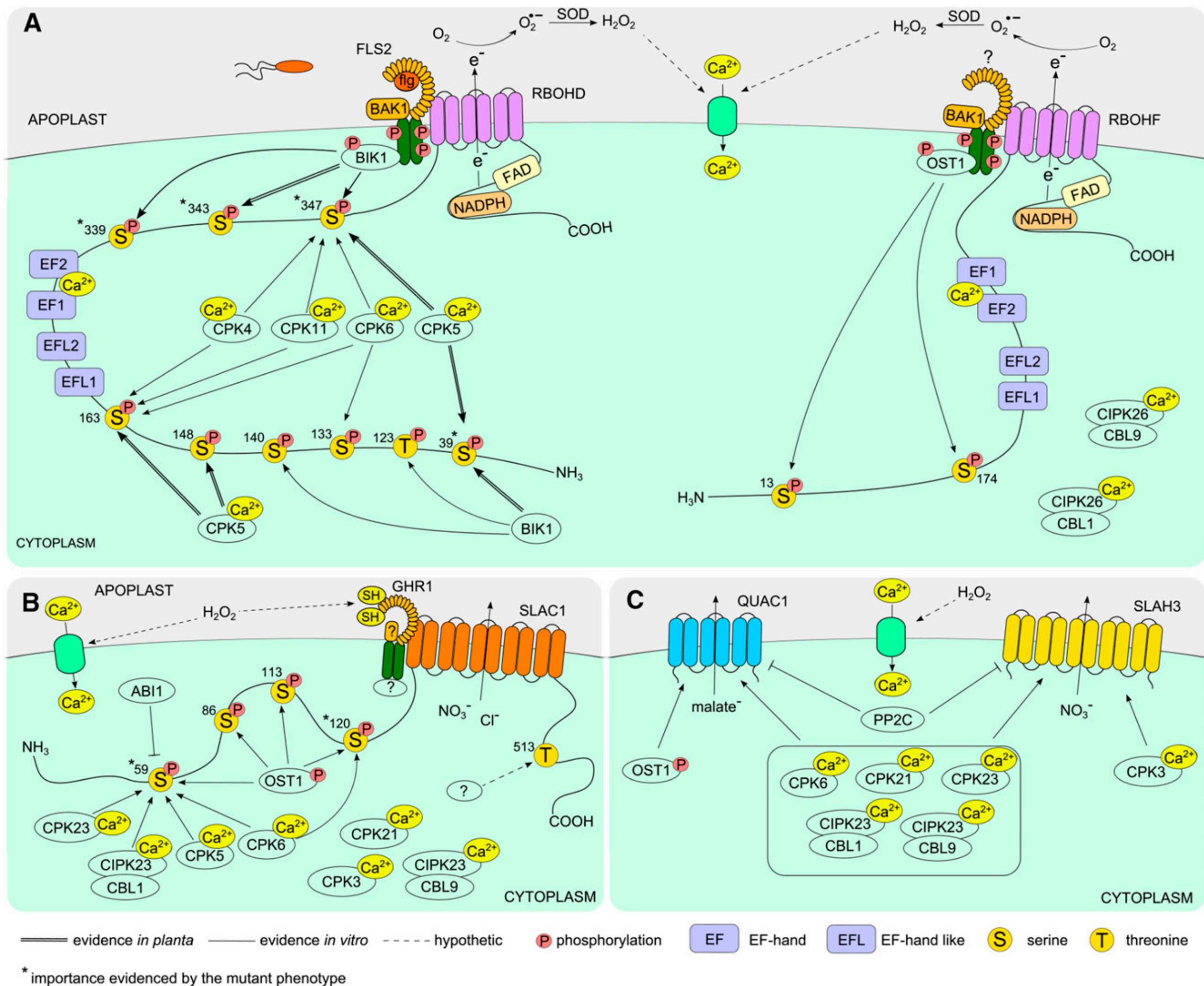

Figure 2. Schematic representation of regulatory events governing the activity of RBOHs and anion channels in guard cell signaling. H_2O_2-driven rise in cytoplasmic Ca^{2+} concentration leads to activation of multiple Ca^{2+}-regulated protein kinases that phosphorylate the indicated Ser/Thr residues within the cytoplasmic N-termini of AtRBOHD, AtRBOHF (A) and SLAC1 (B). The phosphorylated residues of QUAC1 and SLAH3 (C) are yet unidentified; hence, only the contributions of specific regulatory proteins to channel activation/deactivation are indicated. For description of abbreviations, see the main text.

verified in vivo, but interaction of OST1 and AtRBOHF in planta supported their regulatory function. Therefore, OST1 is likely involved in regulation of stomatal ROS production through direct phosphorylation and activation of AtRBOHF.

Phosphoproteomic profiling of Arabidopsis suspension cells identified differential phosphorylation of AtRBOHD upon elicitor treatment (Benschop et al., 2007; Nühse et al., 2007). Transformation of *atrbohD* with $AtRBOHD^{S343/347A}$ phosphosite mutant did not restore the flg22-triggered ROS burst, indicating functional significance of these residues (Nühse et al., 2007). During immune responses, perception of PAMPs by the cell surface pattern recognition receptors (PRRs) leads to interaction with coreceptors, phosphorylation of the complexes, and subsequent signaling events that include rapid ROS production and intracellular Ca^{2+} fluxes, and result in PRR-triggered immunity. FLAGELLIN-SENSITIVE2 (FLS2) and EF-TU RECEPTOR, which recognize flg22 and elf18, associate in a complex with the Leu-rich repeat receptor-like kinase (LRR-RLK) BRASSINOSTEROID INSENSITIVE1-ASSOCIATED RECEPTOR KINASE (BAK1) and receptor-like cytoplasmic kinases such as BOTRYTIS-INDUCED KINASE1 (BIK1; Fig. 1; for review, see Kadota et al., 2015).

BAK1 is a coreceptor for a number of LRR-RLKs. A role for BAK1 in PAMP-induced stomatal closure mediated by BIK1-induced phosphorylation of AtRBOHD has been demonstrated (Kadota et al., 2014; Li et al., 2014). AtRBOHD interacted with FLS2 and BIK1 in planta, and BIK1 phosphorylated AtRBOHD upon

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PAMP perception in Arabidopsis seedlings. BIK1 phosphorylated several residues of AtRBOHD (Fig. 2A), and while the phosphorylation of some residues appeared BIK1-specific, Ser-347 was also phosphorylated by Ca^{2+}-dependent protein kinases (CPKs; Fig. 2A). Several PAMPs, including flg22, elf18, and chitin, induced phosphorylation of AtRBOHD within 1 to 2 min, leading to ROS production 2 to 5 min after PAMP treatment.

PAMPs triggered phosphorylation of AtRBOHD also in the absence of Ca^{2+}, since PAMP-induced Ser-39 phosphorylation was not affected by treatment with Ca^{2+} chelator EGTA or by Ca^{2+}-channel blocker $LaCl_3$. Further, PAMP-induced Ser-39 phosphorylation was unaffected in the *cpk5 cpk6 cpk11* triple mutant. Together, the results suggested Ca^{2+}-independent phosphorylation of this site. Thus, the authors suggested that BIK1-mediated, Ca^{2+}-independent phosphorylation occurred upstream of Ca^{2+} and primed AtRBOHD for Ca^{2+} regulation (Kadota et al., 2014, 2015). This model is also in agreement with the observation that in HEK cells Ca^{2+} stimulation did not lead to RBOH activation in the presence of a kinase inhibitor (Kimura et al., 2012).

The relevance of BIK1-mediated phosphorylation of AtRBOHD to PAMP-induced stomatal closure was addressed by Kadota et al. (2014) and Li et al. (2014). Treatment of wild-type plants with flg22 and elf18 triggered stomatal closure, while the stomata of *atrbohD* mutant and the mutant complemented with phospho-dead $RBOHD^{S39A/S339A/S343A}$ remained unresponsive. Importantly, ABA-induced stomatal closure was not affected in the phospho-dead AtRBOHD line (Kadota et al., 2014). Furthermore, expression of the phosphosite mutants $AtRBOHD^{S343A/S347A}$ and $AtRBOHD^{S39A/S343A/S347A}$ did not restore flg22-induced stomatal closure in *atrbohD* (Li et al., 2014). Moreover, the stomata of *bik1* were unresponsive to flg22, but fully responsive to ABA. Taken together, BIK1 and the identified phosphosites of AtRBOHD play a specific role in stomatal immunity but are not involved in ABA-regulated stomatal movements. It would be of great interest to generate corresponding constructs for AtRBOHF and test them for ABA-related functions.

A role for BAK1 in ABA-induced stomatal closure was demonstrated by Shang et al. (2016). ABA-induced ROS production and stomatal closure was absent in *bak1*, while application of H_2O_2 rescued the phenotype, suggesting that BAK1 functions upstream of ROS production in stomatal closure. Although RBOH activation was not directly addressed, the results suggest its involvement. BAK1 formed a complex with OST1 in the cell periphery and the complex formation in planta was promoted by ABA treatment. Phosphorylation of OST1 was oppositely regulated by BAK1 and ABI1 in vitro. Furthermore, ABI1 interacted with BAK1 in vivo and inhibited the interaction of BAK1 and OST1. This suggests that BAK1 regulates ABA-induced stomatal closure by affecting OST1 activation. As OST1 interacted with AtRBOHF in planta and phosphorylated AtRBOHF in vitro (Sirichandra et al., 2009), it could be envisaged that BAK1-induced activation of OST1 would mediate stomatal closure through activation of AtRBOHF. However, this does not rule out the existence of additional targets for the BAK1-OST1 signal relay, such as anion channels.

Ca^{2+}-DEPENDENT REGULATION OF RBOHs

Stomatal movements are associated with changes in guard cell cytosolic Ca^{2+} concentrations (McAinsh et al., 1996; Pei et al., 2000; Thor and Peiter, 2014). Two classes of Ca^{2+}-regulated kinases govern plant development and stress responses: the calcium-dependent protein kinases (CDPKs; in Arabidopsis, CPKs) and CBL-interacting protein kinases (CIPKs), activated upon interaction with Calcineurin B-like (CBL) Ca^{2+} sensor proteins (Steinhorst and Kudla, 2013).

CBL/CIPK complexes have been implicated as potential regulators of stomatal function. CIPK23 interacted with SLAC1 in planta, and coexpression of CBL1 or CBL9 together with CIPK23 in *X. laevis* oocytes induced SLAC1 and SLAC1 HOMOLOG3 (SLAH3) currents (Maierhofer et al., 2014). CBL/CIPK complexes are emerging as candidates linking ROS production and subsequent signaling leading to guard cell anion channel activation as the activation of AtRBOHF by CBL1/CBL9-CIPK26 complexes has also been recently described (Drerup et al., 2013). CIPK26 interacted with AtRBOHF in planta and phosphorylated its N-terminal domain in vitro (Fig. 2A). Coexpression of CBL1 or CBL9 with CIPK26 enhanced ROS production by AtRBOHF in HEK cells (Drerup et al., 2013). The biological relevance for the CBL1/CBL9-CIPK26 signaling module has not been described, but it could be involved in guard cell ABA signaling, given the role of CIPK26 in ABA signaling (Lyzenga et al., 2013) and the role of AtRBOHF in ABA-induced stomatal closure (Kwak et al., 2003). Such a potential role would not be restricted to the CBL1/CBL9-CIPK26 complex as CIPKs and CBLs are encoded by multigene families. CBL-CIPK complexes regulate a multitude of signaling pathways in plants and apart from AtRBOHF, SLAC1, and SLAH3, their targets include other ion channels and transporters (for review, see Yu et al., 2014).

CPKs are encoded by a family of 34 genes in Arabidopsis, and several CPKs regulate stomatal function, including CPK3 (Mori et al., 2006), CPK4 and CPK11 (Zhu et al., 2007), CPK6 (Mori et al., 2006; Munemasa et al., 2011), and CPK23 (Merilo et al., 2013). Several CPK isoforms have been shown to phosphorylate SLAC1 and SLAH3 in vitro (Fig. 2, B and C) and activate anion channels in *X. laevis* oocytes. However, the ability of a single CPK to activate anion currents in oocytes does not always reflect their function in planta. For example, CPK21 activated SLAC1 and SLAH3 currents in oocytes (Geiger et al., 2010, 2011), but the *cpk21* mutant did not display impaired stomatal regulation (Merilo et al., 2013). Similarly, CPK5 activated SLAC1 currents in oocytes,

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but whole-cell patch-clamp studies showed that loss of CPK5 alone did not disrupt ABA activation of S-type anion channels (Brandt et al., 2015). However, channel activation and ABA-induced stomatal closure were reduced in the *cpk5 cpk6 cpk11 cpk23* quadruple mutant, suggesting functional redundancy or combinatorial requirement within the CPK family.

CPKs were demonstrated to control PAMP-induced ROS burst through RBOH activation. The flg22-induced ROS burst was gradually diminished in Arabidopsis *cpk5 cpk6* double, *cpk5 cpk6 cpk11* triple, and *cpk5 cpk6 cpk11 cpk4*^{VIGS} quadruple mutants (Boudsocq et al., 2010) possibly through reduced phosphorylation of RBOHs. Potato (*Solanum tuberosum*) StCDPK5 phosphorylated StRBOHB in planta, resulting in increased ROS production and ectopic expression of a constitutively active StCDPK5 provoked ROS accumulation (Kobayashi et al., 2007). Dubiella et al. (2013) identified AtRBOHD as an in vivo target for CPK5, which became phosphorylated and activated upon flg22 and elf18 treatment, and flg22-induced ROS production was attenuated in the *cpk5* mutant. PAMP treatment stimulated CPK5-dependent in vivo phosphorylation of AtRBOHD (Fig. 2A). Ser-39 and Ser-148 were also phosphorylated by CPK5 upon H_2O_2 stimulation. Two reports subsequently identified Ser-39 of AtRBOHD as a Ca^{2+}-independent phosphosite phosphorylated by BIK1 (Kadota et al., 2014; Li et al., 2014); this difference needs further elucidation. In vitro quantitative proteomic analysis revealed overlapping and additional CPK-mediated phosphosites on AtRBOHD (Fig. 2A; Kadota et al., 2014).

PEROXIDASE- AND AMINE OXIDASE-GENERATED ROS IN STOMATAL CLOSURE

Apart from RBOHs, peroxidases and amine oxidases can also produce ROS in the apoplast. Inhibitor studies have suggested a role for peroxidase-dependent but RBOH-independent ROS production in yeast elicitor-, chitosan-, salicylic acid-, and methylglyoxal-induced stomatal closure in Arabidopsis (Khokon et al., 2010a, 2010b, 2011; Hoque et al., 2012) and for salicylic acid- and UV-B-induced stomatal closure in *Vicia faba* (Mori et al., 2001; He et al., 2011). These results imply that in stomatal closure, peroxidase- and RBOH-generated ROS are not functionally equivalent, similar to microbe-associated molecular pattern-induced ROS production in Arabidopsis defense responses (Daudi et al., 2012). Apoplastic ROS are also produced upon catabolization of polyamines by di- and polyamine oxidases (Pottosin et al., 2014). Application of diamine oxidase and polyamine oxidase inhibitors reduced guard cell ROS production and stomatal closure induced by ABA in *V. faba* and by ethylene in Arabidopsis (An et al., 2008; Hou et al., 2013). Thus, both peroxidases and amine oxidases add to the complexity of apoplastic ROS signaling in guard cells. Further studies are necessary to characterize the molecular identity, precise function, and importance of these ROS-producing enzymes to stomatal movements.

THE INTERPLAY BETWEEN APOPLASTIC AND INTRACELLULAR ROS PRODUCTION

Numerous studies have demonstrated chloroplastic ROS accumulation following ABA treatment (Pei et al., 2000; Zhang et al., 2001b), extracellular Ca^{2+} (Nomura et al., 2008; Wang et al., 2012), ozone (Joo et al., 2005; Vahisalu et al., 2010), and other stimuli leading to stomatal closure. Following these discoveries, hypotheses of the regulatory role of chloroplast-dependent ROS accumulation in stomatal movements have been drawn (Sierla et al., 2013). Zhang et al. (2001b) described ABA-induced cytoplasmic ROS accumulation in the vicinity of guard cell chloroplasts significantly earlier than in other parts of the cell, followed by an increase in ROS signal in adjacent epidermal cells. Application of diphenyleneiodonium, a NADPH oxidase inhibitor, partly abolished but was not sufficient to fully inhibit the chloroplastic ROS accumulation.

The use of ozone (O_3) as an apoplastic ROS donor (Vainonen and Kangasjärvi, 2015) triggered a biphasic H_2O_2 production with the first peak observed 1 to 1.5 h after the onset of exposure, followed by a late peak between 12 and 24 h (Joo et al., 2005). ROS signal was first observed in guard cell chloroplasts, later spreading to adjacent epidermal cells and finally creating groups of fluorescent epidermal cells. In agreement with Zhang et al. (2001b), diphenyleneiodonium inhibited the early ROS accumulation partially, while the late ROS accumulation was completely abolished. Inhibition of photosynthetic electron transport prevented both H_2O_2 peaks as well as O_3-dependent lesion formation, suggesting a significant role for the chloroplastic ROS production in the execution of cell death. Application of high temporal resolution (Vahisalu et al., 2010) identified an early ROS burst 3 min after the onset of O_3 exposure, followed by a late peak (after 90 min), possibly correlating with the first peak observed by Joo et al. (2005). Similarly, ROS accumulation started in guard cell chloroplasts. The late ROS peak was markedly lower in the *atrbohD atrbohF* double mutant, suggesting an involvement of RBOH-derived ROS generation during O_3 treatment in initiating chloroplastic/cytoplasmic ROS accumulation. Together, these data indicate a link between apoplastic and chloroplastic ROS production and suggest a role for chloroplasts in guard cell signaling (Fig. 1). It can be anticipated that the progress in the development of methods for detection of specific forms of ROS (Mattila et al., 2015; Noctor et al., 2016) will facilitate the research on the contribution of respective ROS-generating systems to the guard cell signaling.

The signals that trigger chloroplastic ROS burst are not well understood; however, multiple lines of evidence indicate a role for Ca^{2+}. The AtRBOHD and/or AtRBOHF-dependent increase in cytoplasmic Ca^{2+} upon ABA treatment is well documented (Pei et al., 2000; Kwak et al., 2003), and elevated extracellular Ca^{2+} led to stomatal closure (McAinsh et al., 1996). Moreover, rapid Ca^{2+} influx was activated upon O_3 treatment (Clayton et al., 1999; Evans et al., 2005),

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further strengthening the link between apoplastic ROS and increase in cytosolic Ca^{2+}. The responses of chloroplasts to extracellular and cytoplasmic Ca^{2+} transients are controlled by the chloroplast thylakoid membrane-localized CALCIUM SENSING RECEPTOR (CAS; Nomura et al., 2008; Vainonen et al., 2008; Weinl et al., 2008). The exact molecular function of CAS is not understood, but CAS plays a role in cytosolic Ca^{2+} accumulation and controls stomatal closure in response to extracellular Ca^{2+} (Nomura et al., 2008; Weinl et al., 2008). Moreover, the Ca^{2+}-induced chloroplastic ROS burst was diminished in CAS antisense lines, suggesting Ca^{2+} dependence (Wang et al., 2012). Nomura et al. (2012) demonstrated that flg22 induced a rapid Ca^{2+} transient in the cytoplasm, followed by an increase in the Ca^{2+} level in the chloroplast stroma. This suggests that Ca^{2+} might serve as a signal linking extracellular PAMP perception with chloroplasts. Interestingly, flg22- and dark-induced stromal transients were reduced in the *cas-1* mutant, and flg22-induced stomatal closure was suppressed. Treatment with H_2O_2 was sufficient to induce stomatal closure in the *cas-1* mutant indicating that CAS might work upstream of chloroplastic ROS signaling in PAMP-induced stomatal closure (Nomura et al., 2012).

ROS SENSING IN GUARD CELL SIGNALING

A major challenge in plant ROS and redox biology is the identification of ROS/redox sensors. The apoplastic ROS-sensing components remain still elusive (Fig. 1). Proposed models (Møller and Sweetlove, 2010; Sierla et al., 2013; Wrzaczek et al., 2013) assume the existence of compartment-specific components that continuously monitor redox status. Another model for recognition of apoplastic ROS signal assumes intracellular perception of apoplastic H_2O_2 upon import through aquaporins. Indeed, the H_2O_2 transport by plant aquaporins has been demonstrated in yeast cells (Bienert et al., 2007; Hooijmaijers et al., 2012), and a recent study by Tian et al. (2016) provided evidence for occurrence of such events in planta. Finally, the apoplastic ROS could directly affect the activity of ion channels leading to stomatal closure.

These models are not mutually exclusive and are likely to coexist. However, the following evidence supports direct apoplastic ROS perception: (1) ROS are highly reactive and unstable molecules and among other ROS molecules H_2O_2 is the most stable form (Giorgio et al., 2007), with estimated half-life from 1 ms (most sources) to seconds. As inferred from the measurements utilizing the genetically encoded HyPer probe, the half-life of H_2O_2 in Arabidopsis guard cells, including the effect of influx from the apoplast and degradation in the symplasm, is in the range of seconds (Costa et al., 2010), while in animal cells H_2O_2 appears more stable (Weller et al., 2014). The half-life of $O_2^{\bullet -}$ in biological systems is several orders of magnitude shorter (Giorgio et al., 2007), thus, the transport of ROS other than H_2O_2 seems unlikely. (2) ROS formation leads to Ca^{2+} transients and transcriptomic responses specific to the nature and subcellular localization of the molecules, implying the existence of separate signaling pathways relying on specific ROS sensors (Vaahtera et al., 2014). Because of multiple intracellular ROS sources present within the plant cells, it is unlikely that the signal specificity could originate solely from the import of H_2O_2 to the cytoplasm. In animal cells, localized perception of apoplastic H_2O_2 signal involves localized coregulation of intracellular H_2O_2 scavenging capacity. Activation of cell surface receptors triggers phosphorylation and inhibition of Peroxiredoxin I, thereby allowing localized increase in the H_2O_2 concentration and oxidation of receptor proteins (Woo et al., 2010). However, thus far, such mechanisms have not been described in plants. (3) Due to the low redox-buffering capacity, apoplast is an excellent medium for ROS signal propagation. In the context of long-distance signaling, the transport/diffusion of ROS through the symplast network would be potentially damaging to cytoplasmic environment and hampered by intracellular antioxidants. (4) Apoplast harbors a large number of Cys-rich kinases that could possibly participate in ROS sensing mechanisms (Bourdais et al., 2015).

One of the largest subgroups of RLKs, the Cys-rich receptor-like kinases (CRKs) have been suggested to function in mediating the effects of ROS (Wrzaczek et al., 2010; Bourdais et al., 2015). A prominent feature of the CRKs is the presence of two Cys-rich DUF26 domains (C-X8-C-X2-C-motifs) within the extracellular region. It has been suggested that these cysteines could undergo redox modifications leading to conformational changes and/or affect complex formation. In agreement with their proposed function, specific CRKs were found to control the basal stomatal aperture and stomatal responses to environmental stimuli (Bourdais et al., 2015); however, their ROS-sensing capabilities are yet to be demonstrated.

Regulation of stomatal movements requires coordinated activity of ion pumps, transporters, and plasma membrane channels (Hedrich, 2012). The activity of ion channels can be modified by H_2O_2, suggesting that they could be involved in H_2O_2 perception. H_2O_2 inhibited both inward- and outward-rectifying K^+ channels in *V. faba* guard cells (Zhang et al., 2001a; Köhler et al., 2003) and activated Ca^{2+}-permeable cation channels in Arabidopsis guard cells (Pei et al., 2000). Analogously, O_3 induced a biphasic cytosolic Ca^{2+} elevation within seconds of exposure (Clayton et al., 1999; Evans et al., 2005), and a recovery period was needed before the Ca^{2+} elevation could be elicited again (Evans et al., 2005). Furthermore, Arabidopsis seedlings distinguished H_2O_2 from ozone; plants still produced the Ca^{2+} signal in response to ozone after becoming refractory to H_2O_2, and vice versa, suggesting specificity to different forms of ROS (Evans et al., 2005). Analogically to Ca^{2+} responses, guard cells were desensitized to ozone during the recovery phase (Vahisalu et al., 2010).

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Despite the clear effect of H_2O_2 on guard cell ion channels, it is still unclear whether direct modification by ROS is the underlying mechanism. While well documented in mammals, the role of oxidative posttranslational modifications of plant ion channels is only starting to emerge. The direct voltage-dependent activation of heterologously expressed Arabidopsis plasma membrane K^+ channel STELAR K+ OUTWARD RECTIFIER (SKOR) by H_2O_2 has been shown (Garcia-Mata et al., 2010). In SKOR, Cys-168 residing within the S3 α-helix of voltage sensor complex was responsible for its activation by H_2O_2. Cys-168 is exposed to the aqueous phase at the outer membrane surface, suggesting its regulation by apoplastic rather than cytoplasmic ROS. Considering the above, a similar activation mechanism could be envisaged for the guard cell ion channels.

Recent evidence suggests that GUARD CELL HYDROGEN PEROXIDE-RESISTANT1 (GHR1), a plasma membrane-associated atypical LRR RLK, might be involved in perception of apoplastic ROS signal (Hua et al., 2012). GHR1 interacted with SLAC1 anion channel and controlled its activity in *X. laevis* oocytes when connected with split YFP. Consequently, *ghr1* mutants were unable to close stomata in response to multiple stimuli (Hua et al., 2012; M. Sierla, H. Hõrak, K. Overmyer, H. Kollist, and J. Kangasjärvi, unpublished data). The GHR1 protein consists of apoplast-localized C terminus harboring 19 LRRs, a transmembrane domain, and an atypical cytoplasmic kinase domain lacking conserved amino acids required for kinase activity. Two Cys residues localized within the C-terminal domain (Cys-57 and Cys-66) were necessary for proper GHR1 function as $GHR1^{C57A}$ and $GHR1^{C66A}$ did not complement the stomatal phenotype of *ghr1* (Hua et al., 2012). It is not yet clear whether these residues are necessary for correct GHR1 fold or subject to oxidative posttranslational modifications.

The cytoplasmic accumulation of H_2O_2 in guard cells follows the apoplastic ROS burst within minutes. Among other sensors, the cytoplasmic ROS signal can be decoded by GLUTATHIONE PEROXIDASE3-ABI2 H_2O_2-sensing complex. The activity of the PP2C phosphatases ABI1 and ABI2, negative regulators of ABA signaling, was downregulated by H_2O_2 in vitro (Meinhard and Grill, 2001; Meinhard et al., 2002). However in vivo, ABI2, and to a lesser extent ABI1 interacted with GPX3, which regulates ABA- and H_2O_2-induced stomatal closure (Miao et al., 2006). Oxidized GPX3 decreased the phosphatase activity of ABI2 and converted ABI2 from reduced to oxidized form in vitro. Therefore, it might be concluded that GPX3 could function as an intracellular H_2O_2 sensor in guard cell ABA signaling and transduce the oxidative signal to ABI2 via a redox relay. It is not yet clear whether ABI1 and ABI2 are the sole targets of GPX3. It can be expected that the efforts to solve the GPX3 structure will pave the way toward further investigation of this pathway.

Among targets of cytoplasmic H_2O_2 is CPK21 (Ueoka-Nakanishi et al., 2013), which also can activate guard cell-expressed anion channels (Geiger et al., 2010, 2011; Demir et al., 2013). Oxidation of CPK21 led to formation of an intramolecular disulfide bond (Cys-97–Cys-108) and was associated with decrease in kinase activity. Incubation of oxidized CPK21 with THIOREDOXIN H-TYPE1 efficiently restored the activity. The physiological relevance of CPK21 oxidation is not clear, as H_2O_2 would be expected to positively influence CPK21 activity and, thus, SLAC1/SLAH3 currents. It is possible that after the triggering of stomatal closure, high H_2O_2 concentrations would deactivate CPK21 in a negative feedback loop to desensitize ABA signal. Another regulatory event that could serve to restrict this pathway is *S*-nitrosylation of Cys-137 in OST1, which negatively regulates kinase activity (Wang et al., 2015). The ABA-dependent accumulation of NO and associated inhibition of OST1 activity is among the late (30–60 min after ABA treatment) signaling events; therefore, it might be assumed that this modification serves to reset the guard cell ABA signaling (Wang et al., 2015). Considering that OST1 regulates the activity of AtRBOHF, it might be assumed that the decrease in its activity could also restrict ROS formation. However, it is yet not clear which mechanisms serve to restore the guard cell redox balance to the initial preclosure state.

ROS SIGNAL AMPLIFICATION IN ABA-INDUCED STOMATAL CLOSURE

OST1 is a key regulator of guard cell signaling and integrates signals from most, if not all, abiotic and biotic stimuli tested to date (Melotto et al., 2006; Vahisalu et al., 2010; Xue et al., 2011; Merilo et al., 2013; Guzel Deger et al., 2015). The signals are thought to culminate in OST1-mediated phosphorylation and activation of multiple cellular targets, including anion channels. However, these observations are mostly drawn from in vitro evidence and heterologous expression systems and await validation in planta. A core ABA signaling pathway that was reconstituted in *X. laevis* oocytes required only the PYR1 receptor, PP2Cs, and OST1 or CPK6 to activate SLAC1 (Fig. 3A; Brandt et al., 2012). While this simplistic model implies that ROS are not needed for SLAC1 activation and subsequent stomatal closure, evidently, in planta ABA signaling is much more complex. Moreover, as discussed above, it is important to note that although the activation of SLAC1 by OST1 in *X. laevis* oocytes has been demonstrated by several groups, it has always required the presence of split-YFP tag in OST1 to fulfill this function.

Early genetic work addressed the position of ABI1 and ABI2 in ABA signaling leading to I_{Ca} channel activation and stomatal closure (Murata et al., 2001). While ABA-induced activation of I_{Ca} channels was disrupted in *abi1-1* and *abi2-1* mutants, H_2O_2 triggered channel activation and H_2O_2-induced stomatal closure was disrupted only in *abi2-1* and remained functional in *abi1-1*. Further, ABA induced ROS production in *abi2-1*, but not in *abi1-1* guard cells, suggesting that genetically ABI1 and ABI2 function upstream and downstream of ROS

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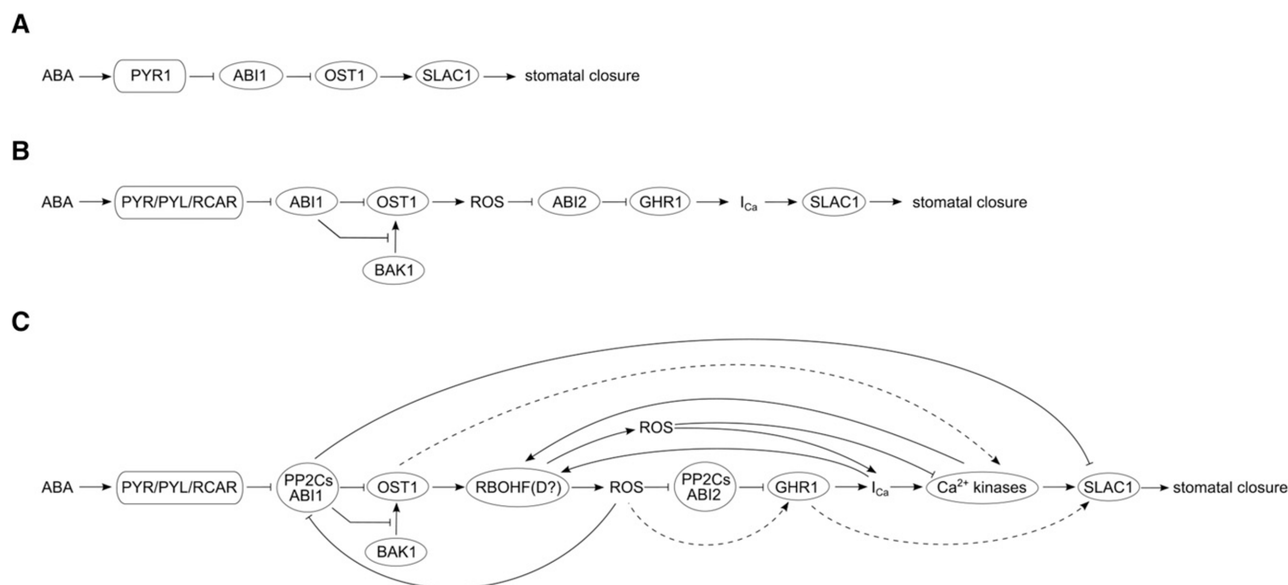

Figure 3. Schematic model for the position of ROS in ABA-induced guard cell signaling. A, Minimal model based on oocyte studies. B, Genetic studies imply nonredundant function for ABI1 and ABI2. C, Role for ROS in signal amplification. For detailed description of proposed pathways and abbreviations, see the main text. Solid lines indicate verified interactions; dashed lines indicate hypothetical/indirect interactions.

production. It was subsequently shown that OST1 functions upstream of ROS production (Mustilli et al., 2002) and that *abi1-1* mutation, but not *abi2-1* mutation, strongly inhibited ABA-dependent OST1 activation (Yoshida et al., 2006).

More recent results suggest that ABI1 inhibits ABA-induced ROS production through at least two mechanisms at the level of OST1. First, ABI1 has been implicated in dephosphorylation of Ser-175 (Vlad et al., 2009), which is autophosphorylated in OST1 activation process. Second, ABI1 dephosphorylated BAK1-mediated transphosphorylation sites of OST1 (Shang et al., 2016). Taken together, this suggests that OST1 and BAK1 are positive regulators of ABA-induced ROS production and ABI1-induced inhibition occurs at the level of OST1. This is particularly intriguing as OST1 has been linked to AtRBOHF activation (Sirichandra et al., 2009). BAK1-dependent and -independent pathways activating OST1 would likely lead to AtRBOHF-derived ROS production and subsequently to I_{Ca} channel activation. While a caveat of this view is the lack of in planta evidence for such a mechanism, the model is indirectly supported by PAMP-mediated stomatal closure involving ROS signal amplification through phosphorylation of AtRBOHD by BIK1 (Kadota et al., 2014; Li et al., 2014).

Characterization of yet another component, the LRR-RLK GHR1, shed light on ABA signaling downstream of ROS production. A mutation in *GHR1* blocked ABA-induced stomatal closure downstream of H_2O_2 production, but upstream of I_{Ca} channel activation (Hua et al., 2012). ABI2, but not ABI1, interacted with GHR1 in planta. ABI2 also inhibited GHR1-induced SLAC1 currents in oocytes. This is consistent with the genetic position of ABI2 downstream of ROS production in ABA-induced stomatal closure (Murata et al., 2001). All the results together allow us to create a model where BAK1, OST1, and ABI1 function upstream of ROS in ABA signaling, whereas GHR1 and ABI2 connect to downstream activation of the I_{Ca} channel and subsequent stomatal closure (Fig. 3B).

However, it is evident that signaling is in fact far more complex in planta than the simplified representation in Figure 3B. For example, while distinct roles for ABI1 and ABI2 were highlighted above, some studies suggest overlapping roles for PP2Cs in OST1 inhibition (Geiger et al., 2009; Vlad et al., 2009). This is also evident from the studies that implement OST1 in direct activation of anion channels. Signal integration and modulation by parallel and interacting pathways might be centrally important in planta. Stomatal closure and ABA activation of SLAC1 in the *cpk5 cpk6 cpk11 cpk23* quadruple mutant was impaired despite intact OST1, thus OST1 alone cannot complement the loss of CPKs (Brandt et al., 2015). Conversely, even though CPKs can activate SLAC1 in *X. laevis* oocytes, they do not substitute the loss of OST1 function in planta (Scherzer et al., 2012).

Taken together, a model is emerging where ROS would be involved in multiple steps during ABA signaling and play a role in signal amplification rather than regulation of any single step (Fig. 3C). Activation of OST1 through BAK1-dependent and -independent mechanisms would lead to increased ROS production, at least in part through direct activation of AtRBOHF by OST1. Subsequently, ROS production and Ca^{2+} channel

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activation could lead to amplification of both ROS and Ca^{2+} signals and anion channel activation in an OST1-, GHR1-, and CPK-dependent manner. OST1 has been suggested to be required for CPK activation, either directly, or more likely indirectly through modulation of cytosolic Ca^{2+} levels (Scherzer et al., 2012). Hence, a primary, but thus far largely overlooked, function of OST1 could be activation and regulation of ROS production and subsequent Ca^{2+} signal amplification (Fig. 3) rather than direct anion channel regulation.

It is likely that several important components of the ABA signal amplification loop are yet to be identified. For example, LRR-RLKs typically function in receptor complexes. Since BAK1 is a coreceptor for a number of LRR-RLKs, it is reasonable to assume that receptors are also present in the BAK1-OST1 complex analogous to a similar complex involving FLS2, BAK1, and BIK1 in flg22-induced stomatal closure (Fig. 1; Kadota et al., 2014; Li et al., 2014). Likewise, it is likely that the atypical LRR-RLK GHR1 requires a coreceptor (Fig. 1). Identification of these likely coreceptors should provide a broader picture of guard cell responses. Finally, knowledge of how a specific subset of components would be employed in constitutive or inducible complexes, in response to a specific stimuli at a given time and location, will be required for full understanding of the mechanisms underlying stomatal responses.

ACKNOWLEDGMENTS

We thank Prof. Alistair Hetherington, Prof. Rainer Hedrich, Dr. Mikael Brosché, and Dr. Michael Wrzaczek for their comments on an earlier version of the manuscript. Dr. Wrzaczek is also acknowledged for contribution to the figures.

Received March 7, 2016; accepted April 20, 2016; published April 21, 2016.

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