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SPECIAL ISSUE ARTICLE

Signaling mechanisms in abscisic acid-mediated stomatal closure

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SUMMARY

The plant hormone abscisic acid (ABA) plays a central role in the regulation of stomatal movements under water-deficit conditions. The identification of ABA receptors and the ABA signaling core consisting of PYR/PYL/RCAR ABA receptors, PP2C protein phosphatases and SnRK2 protein kinases has led to studies that have greatly advanced our knowledge of the molecular mechanisms mediating ABA-induced stomatal closure in the past decade. This review focuses on recent progress in illuminating the regulatory mechanisms of ABA signal transduction, and the physiological importance of basal ABA signaling in stomatal regulation by CO_2 and, as hypothesized here, vapor-pressure deficit. Furthermore, advances in understanding the interactions of ABA and other stomatal signaling pathways are reviewed here. We also review recent studies investigating the use of ABA signaling mechanisms for the manipulation of stomatal conductance and the enhancement of drought tolerance and water-use efficiency of plants.

Keywords: guard cell, abscisic acid (ABA), basal ABA, drought tolerance, water-use efficiency.

INTRODUCTION

The plant hormone abscisic acid (ABA) plays critical roles in plant survival. Mutant plants lacking ABA biosynthesis or ABA signal transduction components show severe drought-susceptible phenotypes. During the last decade since the discovery of the ABA receptor genes (Ma *et al.*, 2009; Park *et al.*, 2009), our understanding of ABA signal transduction has advanced based on many studies employing genetic, physiological, biochemical, chemical biology and evolutionary approaches. In addition, recent studies have provided evidence for a key function of ABA signaling under non-stress conditions, designated here as basal ABA signal transduction, in modulating stomatal regulation, plant growth and metabolic pathways (Hsu *et al.*, 2018; Yoshida *et al.*, 2019a).

Abscisic acid-induced stomatal closure is mediated by ion efflux from guard cells. Ion efflux in turn leads to osmotic water efflux and a reduction in the turgor and volume of the guard cells, resulting in stomatal closure. During stomatal closure, the activation of S-type anion channels and R-type anion channels plays a lead role (Keller *et al.*, 1989; Schroeder and Hagiwara, 1989). These anion channels: (i) mediate anion efflux from guard cells; and (ii) cause a depolarization of the plasma membrane, which activates depolarization-activated potassium efflux channels. As both S-type anion channels and potassium efflux channels can remain activated for minutes or longer in plants, this enables effective solute release from guard cells and stomatal closure (Schroeder and Hagiwara, 1989).

The PYRABACTIN RESISTANCE/PYRABACTIN RESISTANCE-LIKE/REGULATORY COMPONENTS OF THE ABSCISIC ACID RECEPTOR (PYR/PYL/RCAR) protein family was identified as a family of ABA receptors (Ma *et al.*, 2009; Park *et al.*, 2009; Cutler *et al.*, 2010; Raghavendra *et al.*, 2010). PYR/PYL/RCAR receptors trigger ABA signal transduction through protein phosphorylation reactions in plant cells. PYR/PYL/RCAR receptors inhibit clade-A type-2C protein phosphatases (PP2Cs; with nine members in Arabidopsis) through direct binding in the presence of ABA (Ma *et al.*, 2009; Park *et al.*, 2009; Tischer *et al.*, 2017). This PP2C inhibition leads to the activation of SNF1-related protein kinase 2 (SnRK2; with 10 members in Arabidopsis, three of which are mainly activated by ABA). These SnRK2 kinases are inhibited by PP2C-dependent dephosphorylation in the absence of ABA (Umezawa *et al.*, 2009; Vlad *et al.*, 2009). This reaction is rapidly initiated (within

approx. 1 min) in stomatal guard cells in response to ABA (Li and Assmann, 1996; Mori and Muto, 1997; Takahashi *et al.*, 2017; Zhang *et al.*, 2020). Activated SnRK2s in guard cells, especially OPEN STOMATA 1 (OST1/SnRK2.6) (Mustilli *et al.*, 2002; Yoshida *et al.*, 2002), phosphorylate substrate proteins such as transcription factors, the S-type anion channel SLAC1 and many other targets, thereby inducing stomatal closure in response to ABA (Geiger *et al.*, 2009; Lee *et al.*, 2009). A simplified ABA signal transduction model in stomatal regulation is summarized in Figure 1, which shows many of the recently identified signal transduction and regulatory mechanisms discussed in this review.

The Arabidopsis *PYR/PYL/RCAR* ABA receptor gene family has 14 members, and these members have overlapping (partially 'redundant') physiological functions. Among these genes, four genes encode for PYR/RCAR ABA receptors that are thermodynamically more likely to prevail as receptor dimers in the absence of abscisic acid (Melcher *et al.*, 2009; Nishimura *et al.*, 2009). These 'dimeric' receptors have been shown to have a lower affinity for ABA (Dupeux *et al.*, 2011; Hao *et al.*, 2011). ABA-induced stomatal closure is impaired in higher order mutant plants of *PYR/PYL/RCAR* receptor genes (Nishimura *et al.*, 2010; Gonzalez-Guzman *et al.*, 2012; Zhao *et al.*, 2018). These higher order *pyr/pyl/rcar* mutants show impaired growth phenotypes and lose more water than wild-type plants (Gonzalez-Guzman *et al.*, 2012; Zhao *et al.*, 2018). These mutant plants further show steady-state widely open stomata (Gonzalez-Guzman *et al.*, 2012; Zhao *et al.*, 2018; Zhang *et al.*, 2020), which correlates with the model that basal ABA signal transduction is essential for maintaining proper intermediate stomatal apertures under normal non-stress conditions, as discussed later in this review. In the following, we review diverse advances made in understanding abscisic acid signal transduction during the regulation of stomatal apertures since the discovery of ABA receptor genes/proteins. We also review the literature investigating

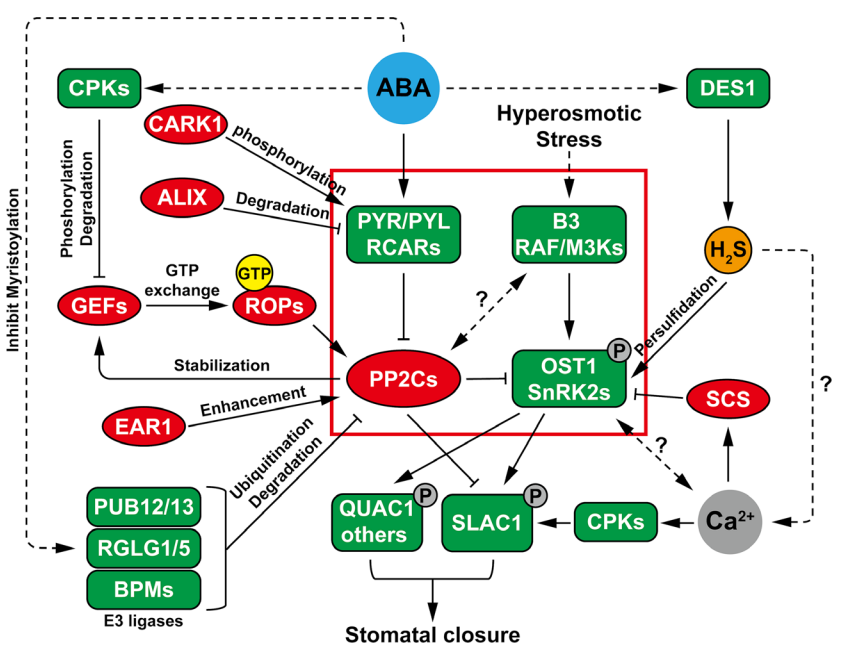

Figure 1. Simplified model of abscisic acid (ABA) signal transduction and regulatory mechanisms in stomatal closure discussed herein.
A reconstitutable ABA signaling module is enclosed in a red rectangle. Positive regulators of ABA signaling are presented in green boxes. Negative regulators of ABA signal transduction are presented in red ovals. Arrows indicate activation and bars indicate downregulation. Solid lines represent regulation pathways predicted to be direct, and dashed lines represent some of the unknown pathways that remain to be investigated further. ABA-bound PYR/PYL receptors inhibit PP2C phosphatase activity leading OST1/SnRK2.6 kinase activation by clade-B3 Raf-like M3Ks in guard cells. The S-type anion channel SLAC1, the R-type anion channel QUAC1/AtALMT12 and other diverse targets not shown here are activated via phosphorylation by the OST1/SnRK2.6, SnRK2.2 and SnRK2.3 protein kinases, thus triggering stomatal closure. Note that additional phosphorylation targets of SnRK2 protein kinases are required for stomatal closure and many of these remain to be identified. PP2Cs downregulate SnRK2 protein kinases and also additional targets (not shown), including SLAC1, thus preventing non-specific Ca^{2+}-induced S-type anion channel activation by calcium-dependent protein kinase (CPKs). L-CYSTEINE DESULFHYDRASE 1 (DES1) mediates ABA-induced hydrogen sulfide (H_2S) production, and then H_2S persulfidates OST1/SnRK2.6 on cysteine residues to enhance OST1 kinase activity. OST1/SnRK2.6 kinase is inhibited by the SnRK2-interacting calcium sensor (SCS). CARK1 phosphorylates PYR/PYL/RCAR receptors to enhance ABA signal transduction. The stability of PYR/PYL/RCARs receptors is regulated by ALIX-mediated protein degradation. A PP2C–GEF–ROP control loop can ensure the off/on regulation of ABA signal transduction, with ABA triggering GEF trafficking to the prevacuolar compartment for degradation through CPK phosphorylation of GEF, thus removing ROP activity. EAR1 enhances PP2C phosphatase activity to repress ABA signal transduction, and PUB12/13, RGLG1/5 and BPMs regulate PP2C stability through the ubiquitin–*26S* proteasome pathway. ABA inhibits RGLG1 myristoylation through an unknown mechanism to promote its nuclear localization and PP2CA degradation.

ABA signal transduction interactions with other stimuli that function in the regulation of stomatal movements.

RECENT ADVANCES IN UNDERSTANDING EARLY ABA SIGNAL TRANSDUCTION MECHANISMS

Evolutionary aspects of stomatal ABA signaling

Recent remarkable advances in genomic sequencing analyses have contributed to our understanding of the evolutionary aspects of ABA-dependent stomatal movement (Hauser *et al.*, 2011; Cai *et al.*, 2017). Recent studies have reported that the streptophyte algae *Zygnema circumcarinatum* genome has a canonical ABA receptor Zc*PYL8* (de Vries *et al.*, 2018), and that ZcPYL8 inhibits PP2C protein phosphatase activity in the absence of ABA (Sun *et al.*, 2019). This study suggests that an early form of PYR/PYL/RCAR proteins in algae functioned in PP2C phosphatase regulation. Core ABA signaling genes involved in stomatal closure, including PYR/PYL/RCAR ABA receptors, clade-A PP2C protein phosphatases, SnRK2 protein kinases and the SLAC1 S-type anion channel, have been identified and coexist in some liverwort and moss species (Komatsu *et al.*, 2013; Cai *et al.*, 2017; Jahan *et al.*, 2019; Shinozawa *et al.*, 2019). The moss *Physcomitrella patens* shows ABA-dependent stomatal closure (Chater *et al.*, 2011). This observation is also supported by genomic approaches (Lind *et al.*, 2015). *Physcomitrella patens* PpOST1 activates PpSLAC1 in *Xenopus* oocytes and rescues the Arabidopsis *ost1/snrk2.6* mutant plant stomatal response, indicating that these mechanisms may function in *P. patens* (Lind *et al.*, 2015). Further direct analyses of stomatal ABA responses in *P. patens* would be needed to ascertain functions derived from the *Xenopus* oocyte system, however. Although providing a potent reconstitution system for developing signaling models, the oocyte system has been shown to not necessarily reflect ABA responses *in planta* (Brandt *et al.*, 2015), as expected for any simplified reconstitution system.

Although stomata have essential roles in most land plants, through controlling gas exchange between plants and the atmosphere, a large part of our knowledge of stomatal ABA signal transduction has been derived from research using angiosperms, including Arabidopsis. Information is more limited in other plant species, however. For example, it has been controversial when land plants acquired ABA-dependent stomatal movements. It was reported that endogenous ABA does not close stomata in fern plants (McAdam and Brodribb, 2012; Cardoso and McAdam, 2019), but other research has shown ABA responses in ferns (Ruszala *et al.*, 2011). Recent studies have provided important information demonstrating that stomata in intact fern plants could unequivocally close in response to exogenous ABA (Cai *et al.*, 2017; Horak *et al.*, 2017). Presently, the debate about whether fern stomata do not respond to ABA (Brodribb and McAdam, 2011; McAdam and Brodribb, 2012) or do respond to ABA (Chater *et al.*, 2011; Ruszala *et al.*, 2011; Cai *et al.*, 2017) may have been resolved by findings that some fern species have weaker or no ABA responses, whereas other fern species have "medium strength" ABA responses (Horak *et al.*, 2017). Furthermore, as in other land plant species, growth conditions can affect the strength of the stomatal ABA responses in fern species (Horak *et al.*, 2017).

Activation of SnRK2: a key step in ABA signal transduction

Upon ABA activation, SnRK2 protein kinases orchestrate downstream ABA signal transduction. The activation of SnRK2 protein kinases is a key step for ABA signal transduction, as demonstrated by in-gel kinase assays from plant extracts, including from guard cell protoplasts (Li and Assmann, 1996; Mori and Muto, 1997; Mustilli *et al.*, 2002; Takahashi *et al.*, 2017). Three SnRK2 kinase genes, *SnRK2.2*, *SnRK2.3* and *OST1/SnRK2.6*, out of 10 *SnRK2* gene family members in Arabidopsis are strongly activated in response to ABA in plant cells (Boudsocq *et al.*, 2004), and triple mutant plants in these *SnRK2* genes show severe ABA insensitivity (Fujii and Zhu, 2009; Umezawa *et al.*, 2009). These three SnRK2 protein kinases are considered to have overlapping functions in mediating ABA responses through the phosphorylation of diverse substrate proteins, including OST1/SnRK2.6 phosphorylation of SLAC1 at a specific residue, serine 120 (Geiger *et al.*, 2009). Additional phosphorylation sites are found in SLAC1 that function in ABA signaling (Brandt *et al.*, 2012, 2015). In stomatal guard cells, SnRK2.2 and SnRK2.3 have been proposed to have a role in the long-term stomatal response to drought conditions (Virlouvet and Fromm, 2015), but are also rapidly activated by ABA and contribute to rapid ABA responses in guard cells (Brandt *et al.*, 2015; Zhang *et al.*, 2020).

It remains unclear how these SnRK2 protein kinases recognize various substrate proteins while maintaining substrate specificities. It has been suggested that a conserved C-terminal 10 amino acid sequence in SLAC1 S-type anion channels may be required for recognition by OST1/SnRK2.6 (Lind *et al.*, 2015). SnRK2 protein kinases also phosphorylate themselves (Belin *et al.*, 2006), and autophosphorylation at the activation loop has been considered to activate SnRK2 kinases. Recent research has shown that SnRK2 protein kinases require additional protein kinases to activate and mediate signal transduction in guard cells, however.

Key function of Raf-like MAPKK kinases in the ABA signaling core and potential roles in osmotic stress signaling

The PYR/PYL/RCAR–PP2C–SnRK2 ABA signaling core is required for most if not all ABA responses, including ABA inhibition of seed germination and root elongation. Since

the discovery of PYR/PYL/RCAR receptors, this signaling module has been widely accepted as a 'core' mechanism underlying early ABA signal transduction (Cutler *et al.*, 2010; Raghavendra *et al.*, 2010). Interestingly, recently a B3 family of Raf-like MAP kinase kinase kinases (M3Ks) was reported as another essential signaling component (Takahashi *et al.*, 2020). The Arabidopsis M3K family consists of 80 gene members and contains important genes in a wide variety of plant signal transduction pathways (Ichimura *et al.*, 2002; de Zelicourt *et al.*, 2016). In stomatal guard cells, for example, the Raf-like M3Ks include HIGH LEAF TEMPERATURE 1 (HT1), CONVERGENCE OF BLUE LIGHT AND CO_2 1/2 (CBC1/2) and BLUE LIGHT-DEPENDENT H^+-ATPASE PHOSPHORYLATION (BHP). These protein kinases have been shown to function in stomatal CO_2 and blue light signal transduction pathways (Hashimoto *et al.*, 2006; Hayashi *et al.*, 2017; Hiyama *et al.*, 2017).

Initially, clade-B Raf-like M3Ks were identified in a redundancy-circumventing forward genetic artificial microRNA (amiRNA) screen as candidate genes involved in ABA signaling (Hauser *et al.*, 2013). In *P. patens*, an ortholog of B3 Raf-like M3Ks, designated *PpARK* (ABA and abiotic stress-responsive Raf-like kinase), was identified in a mutant showing reduced ABA sensitivity and reduced hyperosmotic tolerance (Saruhashi *et al.*, 2015). Recently, additional forward genetic amiRNA screening identified a mutant that targeted overlapping B3 M3K family members, and at least three out of seven genes from the B3 Raf-like M3K family, M3Kδ1 (At5g11850), M3Kδ6 (At1g73660) and M3Kδ7 (At1g18160), were shown to be required for OST1 reactivation after PP2C-mediated dephosphorylation and inhibition (Takahashi *et al.*, 2020). Independent amiRNA lines targeting these genes and knockout lines in these *M3K* genes show ABA-insensitive phenotypes in several responses, including in ABA-induced stomatal closure and ABA inhibition of seed germination (Takahashi *et al.*, 2020). In independent research, combinations of B2, B3 and B4 M3Ks were identified by phosphoproteomics and shown to contribute to the activation of SnRK2s in rapid ABA-independent osmotic stress and ABA responses (Lin *et al.*, 2020). In addition, analyses of the orthologs of the osmotic stress-linked *M3K* gene, *PpARK*, in *P. patens* showed that an *m3kd5 m3kd6 m3kd7* (*atark3*; At4g24480 *atark2 atark1*) triple mutant in Arabidopsis demonstrates impairment in ABA-induced stomatal closure (Katsuta *et al.*, 2020).

In vitro reconstitution of signal transduction pathways provides an approach to test putative ABA signal transduction mechanisms that need to be investigated further *in planta*. PYR1, PP2C, ABI1 and OST1/SnRK2.6 recombinant proteins produced by *Escherichia coli* were used for *in vitro* reconstitution analyses (Fujii *et al.*, 2009). Using this system, it was shown that the protein kinase activity of SnRK2 recombinant proteins was dependent on the presence of ABA, suggesting that these three components are sufficient for ABA-induced SnRK2 activation (Fujii *et al.*, 2009). Moreover, by injecting capped sense RNA (cRNA) into *Xenopus* oocytes, ABA-induced S-type anion channel activation was reconstituted using *PYR1*, *ABI1*, *OST1/SnRK2.6* and *SLAC1* (Brandt *et al.*, 2012); however, robust activation of SLAC1 by OST1 in oocytes required the artificial linking of these two proteins using bimolecular fluorescence complementation (BiFC) constructs (Geiger *et al.*, 2009).

Type-2C protein phosphatases (PP2Cs) directly shut off the SnRK2.2, SnRK2.3 and OST1 protein kinase activities via the dephosphorylation of SnRK2s. Two theories prevail: SnRK2 protein kinases could be reactivated either via autophosphorylation or via phosphorylation by unknown protein kinases in response to ABA. A recent study revealed that if recombinant OST1/SnRK2.6 protein was initially dephosphorylated by PP2Cs, surprisingly OST1/SnRK2.6 could not reactivate *in vitro*, even in the presence of ABA and ABA receptor and PP2C proteins (Takahashi *et al.*, 2020). Thus, these findings provide evidence that dephosphorylated SnRK2 protein kinases cannot reactivate themselves solely by autophosphorylation. The B3 Raf-like M3Ks were shown to be sufficient for ABA-dependent OST1/SnRK2.6 reactivation *in vitro* and by BiFC-free SLAC1 activation by OST1 in oocytes (Takahashi *et al.*, 2020). These findings and the above reviewed genetic and biochemical evidence in plants suggest that the B3 Raf-like M3Ks are components required for the ABA signaling module (Figure 1).

Recently, ABA-induced expression of an ABA-responsive luciferase (LUC) reporter gene was reconstituted in yeast (Ruschhaupt *et al.*, 2019). The absence of endogenous ABA and signaling components in yeast provides a potent research tool that allows combinatorial ABA signaling analyses. Using this system, it was found that SnRK2.4 kinase, which is activated by osmotic stress in plant cells, can mediate ABA-dependent gene expression in yeast (Ruschhaupt *et al.*, 2019). Osmotic stress activates at least nine Arabidopsis SnRK2s in plant tissues in the absence of an increase in ABA concentration (Boudsocq *et al.*, 2004, 2007; Yoshida *et al.*, 2006). Interestingly, Raf-like M3Ks are also important for osmotic stress-induced SnRK2 activation (Katsuta *et al.*, 2020; Lin *et al.*, 2020; Soma *et al.*, 2020; Takahashi *et al.*, 2020). Members of a distinct clade, the B4 clade of M3Ks, were identified as playing a key role in ABA-independent osmotic stress-activated SnRK2s, including SnRK2.1, 2.4, 2.5, 2.9 and 2.10 (Lin *et al.*, 2020; Soma *et al.*, 2020). Moreover, the ABA signaling M3K B3 family members δ1, δ6 and δ7 are required for osmotic stress activation of the ABA signaling SnRK2.2, 2.3 and OST1 kinases (Takahashi *et al.*, 2020). Furthermore, osmotic stress in seedlings induces post-translational modification(s), including mobility shifts and phosphorylation of the M3Ks

(Stecker *et al.*, 2014; Lin *et al.*, 2020; Takahashi *et al.*, 2020). How the M3Ks receive osmotic stress signaling remains an open question. Moreover, further research is needed to illuminate whether ABA modifies Raf-like M3Ks during ABA signaling and stomatal closure. Furthermore, it remains unclear how M3Ks can override and interface with the PP2C inhibition of SnRK2s during the rapid osmotic stress response (Figure 1).

ADVANCES IN UNDERSTANDING REGULATORY MECHANISMS OF ABA SIGNAL TRANSDUCTION

Phosphorylation of PYL/RCAR receptors

It has been suggested that PYR/PYL/RCAR abscisic acid receptors are regulated by protein phosphorylation of these receptors. Recent phosphoproteomic analyses suggest that ABA decreases the level of phosphorylated PYL4 proteins in Arabidopsis (Wang *et al.*, 2018c). Further analyses suggest that PYL4 is phosphorylated and inhibited by the TARGET OF RAPAMYCIN (TOR) protein kinase, which plays an important role in plant growth regulation (Wang *et al.*, 2018c). It was also suggested that ABA, in turn, inhibits TOR kinase activity through SnRK2-mediated protein phosphorylation of the REGULATORY-ASSOCIATED PROTEIN OF TOR 1B (RAPTOR1B). This competitive phosphorylation loop mechanism has been proposed to optimize plant stress tolerance and growth recovery under changing water-stress conditions (Wang *et al.*, 2018c). In contrast to the above inhibitory phosphorylation of ABA receptors, some PYR/PYL/RCAR members from subfamily III were shown to be activated by phosphorylation mediated by a putative receptor-like cytoplasmic kinase, CARK1 (Zhang *et al.*, 2018; Li *et al.*, 2019). The *cark1* mutant exhibited wider stomatal apertures after ABA treatment, whereas overexpression of *CARK1* demonstrated the opposite phenotype to that observed in the *cark1* mutant (Zhang *et al.*, 2018).

Newly identified regulatory components of PP2C phosphatases

The clade-A PP2Cs are central negative regulators of ABA signaling by dephosphorylating SnRK2 protein kinases and also by directly dephosphorylating downstream targets of SnRK2 protein kinases (Brandt *et al.*, 2015; Peirats-Llobet *et al.*, 2016). Members of the small GTP-binding protein Rho-like GTPase from plant (ROP) family and of the GTP exchange factor RopGEF protein family have been found to form a signaling loop that can regulate PP2C activity (Figure 1) (Li *et al.*, 2016b). The ABI1 PP2C interacts with and stabilizes RopGEF1 protein in the absence of ABA (Li *et al.*, 2016b). RopGEF1, in turn, activates the plant ROP small GTP-binding proteins (Berken *et al.*, 2005; Gu *et al.*, 2006). The active ROP10 and ROP11 proteins, in turn, interact with PP2Cs and either stabilize (Li *et al.*, 2012) or increase (Yu *et al.*, 2012) PP2C activity. Thus, the two closely related ROP10 and ROP11 proteins act as negative regulators of ABA signaling (Zheng *et al.*, 2002; Li *et al.*, 2012; Yu *et al.*, 2012) and may further help in shutting off 'leaky' ABA receptor signaling by monomeric PYR/PYL/RCAR receptors that can respond to very low basal ABA concentrations or potentially under ABA-free conditions (Dupeux *et al.*, 2011; Hao *et al.*, 2011). The ROP–GEF pathway negatively regulates ABA-induced stomatal closure (Zheng *et al.*, 2002; Li *et al.*, 2012; Li and Liu, 2012; Yu *et al.*, 2012).

Abscisic acid (ABA) was found to cause the rapid trafficking and degradation of RopGEF1 and additional members of the RopGEF family (Li *et al.*, 2016b). This, in turn, would remove the negative hold on ABA signaling by ROP10 and ROP11. A recent study showed that protein phosphorylation of the N-terminal region of the GTP exchange factor RopGEF1 mediated by calcium-dependent protein kinases causes ABA-induced trafficking and degradation of RopGEF1 in Arabidopsis (Figure 1) (Li *et al.*, 2018).

Six members of Arabidopsis clade A PP2Cs, ABI1, ABI2, HAB1, HAB2, AHG1 and AHG3, have an N-terminal autoinhibitory domain, and this domain interacts with a recently characterized protein ENHANCER OF ABA CO-RECEPTOR 1 (EAR1) (Wang *et al.*, 2018b). EAR1 functions as a negative regulator of ABA responses, including ABA-induced stomatal closure, by enhancing PP2C activities through direct interaction with PP2Cs (Figure 1) (Wang *et al.*, 2018b).

Possible SnRK2 regulation by other protein phosphatases and Ca^{2+}-related signaling

The SnRK2 kinases may be regulated by other protein phosphatases in addition to the clade-A PP2Cs. TYPE ONE PROTEIN PHOSPHATASE 1 (TOPP1) and its regulatory protein At Inhibitor-2 (Atl-2) were reported to interact with SnRK2s and PYL/RCAR ABA receptors, and to negatively regulate OST1/SnRKs kinase activities and ABA signal transduction, using similar mechanisms as clade-A PP2Cs (Hou *et al.*, 2016). OST1/SnRK2s also interact with a singleton PP2C homolog KINASE-ASSOCIATED PROTEIN PHOSPHATASE (KAPP) (Lu *et al.*, 2020). It was suggested that KAPP negatively regulates ABA inhibition of seed germination but not ABA-mediated stomatal closure (Lu *et al.*, 2020); however, it remains unclear whether these phosphatases regulate OST1/SnRK2 activity and/or whether ABA affects the activity of these phosphatases.

The SnRK2 kinases are considered to be Ca^{2+}-independent protein kinases. Interestingly, an EF-hand-containing protein SNRK2-INTERACTING CALCIUM SENSOR (SCS) has been reported to directly bind to SnRK2s and to negatively regulate drought tolerance in Arabidopsis (Figure 1) (Tarnowski *et al.*, 2020). SCS may inhibit SnRK2 kinase activity, and SCS overexpression in Arabidopsis decreases ABA-induced SnRK2 activation (Tarnowski *et al.*, 2020).

Possible interaction between SnRK2s and Ca^{2+} signaling can shed light on how Ca^{2+} signaling contributes to stomatal ABA signal transduction (McAinsh *et al.*, 1990). Abscisic acid enhances the Ca^{2+} sensitivity of S-type anion channel activation in Arabidopsis and *Vicia faba* guard cells (Siegel *et al.*, 2009; Chen *et al.*, 2010). The Ca^{2+} activation of S-type anion channels requires ABA inhibition of PP2Cs, as these PP2Cs can dephosphorylate and deactivate SLAC1 (Brandt *et al.*, 2015). Moreover, in *pp2c* quadruple mutant guard cells, Ca^{2+} activation of S-type anion channels no longer requires the co-application of ABA (Brandt *et al.*, 2015). These findings provide a mechanism for specificity in Ca^{2+} signaling in which clade-A PP2Cs ensure against uncontrolled Ca^{2+} activation of S-type anion channels and stomatal closure (Figure 1). ABA primes guard cells for a Ca^{2+} response by inhibiting PP2Cs (Brandt *et al.*, 2015). At the same time, Ca^{2+}-activation of S-type anion channels in guard cells requires an intact Ca^{2+}-independent signaling branch via the Ca^{2+}-independent SnRK2.2, SnRK2.3 and OST1/SnRK2.6 protein kinases (Brandt *et al.*, 2015). A recent systems biology approach suggests an important role of Ca^{2+} signaling in enhancing stomatal ABA signal transduction (Albert *et al.*, 2017), as discussed later.

Transcriptional regulation of ABA signaling core components

Several studies have provided evidence for how the ABA signaling core itself is regulated by gene expression. Recent discoveries in the primitive streptophyte algae *Z. circumcarinatum* show that *ZcPYL8* has ABA-independent activity and that its expression level is repressed by cold stress, suggesting the potential importance of gene expression in the regulation of PYR/PYL/RCAR ABA receptors (de Vries *et al.*, 2018; Sun *et al.*, 2019). Arabidopsis *PYL10*, which has an ABA-independent PP2C inhibitory activity, and has been proposed to function as a 'leaky' ABA receptor (Hao *et al.*, 2011), is expressed at very low levels. This low expression level of *PYL10* is controlled by its genomic 3′ region (Sun *et al.*, 2019). Interestingly, transgenic (over)expression of the *PYL10* gene without this 3′ region reduces the stomatal conductance of the ABA-deficient mutant *aba2-1* (Sun *et al.*, 2019). Note that *aba2-1* plants continue to synthesize ABA, albeit at reduced levels.

The expression of PP2C can be regulated by alternative splicing. A putative PWI and RRM motif-containing protein RBM25 is suggested to be required for proper ABA responses in Arabidopsis (Zhan *et al.*, 2015). *rbm25* knockout mutants are hypersensitive to ABA in seedling greening but are not defective in transpirational water loss, and RNA-seq analyses revealed that the splicing pattern of *HAB1* is affected in *rbm25* mutants, suggesting that RBM25 is required for the expression of mature *HAB1* transcript.

Roles of protein degradation in ABA signaling core components

Protein concentrations of PYR/PYL/RCAR receptors and PP2Cs can be regulated by protein degradation through the *26S* proteasome. The Arabidopsis DET1- AND DDB1-ASSOCIATED 1 (DDA1) protein is a component of COP10–DET1–DDB1 (CDD)-related complexes, which works as an adapter to facilitate the interaction of PYL4, PYL8 and PYL9 proteins with CULLIN4–RING E3 ubiquitin ligases, thereby triggering the proteasomal degradation of those ABA receptors (Irigoyen *et al.*, 2014). Overexpression of DDA1 reduces ABA sensitivity in Arabidopsis plants. PYR/PYL/RCAR receptors are also degraded through the late endosome route and vacuolar degradation (Garcia-Leon *et al.*, 2019). Arabidopsis ALIX weakens ABA-induced stomatal closure by directly binding to ABA receptors in late endosomes and mediating their degradation (Figure 1).

Two U-Box E3 ligases, PUB12 and PUB13, interact with the ABI1 PP2C and promote ABA-dependent ABI1 degradation through the *26S* proteasome (Figure 1) (Kong *et al.*, 2015). In addition, two Ring-type E3 ligases, RGLG1 and RGLG5, are involved in the ABA-dependent degradation of PP2CA (Figure 1) (Wu *et al.*, 2016). ABA inhibits the myristoylation of RGLG1 and promotes the nuclear translocation of RGLG1, which could lead to the nuclear degradation of PP2CA (Figure 1) (Belda-Palazon *et al.*, 2019). Furthermore, BTB/POZ AND MATH DOMAIN proteins (BPMs), which are substrate adaptors of the multimeric CULLIN3 (CUL3)-RING-based E3 ligases (CRL3s), were identified to promote the degradation of PP2Cs in response to ABA (Figure 1) (Julian *et al.*, 2019). Mutants in these E3 ligases exhibited a reduction in ABA sensitivity in seed germination and stomatal movements (Kong *et al.*, 2015; Wu *et al.*, 2016; Julian *et al.*, 2019).

ROLES OF BASAL ABA CONCENTRATIONS IN GUARD CELLS AND BASAL ABA SIGNALING

Plants maintain higher basal ABA concentrations and ABA signaling in guard cells

Classical research suggested that guard cell protoplasts maintain higher ABA concentrations than mesophyll protoplasts isolated from well-watered plants (Lahr and Raschke, 1988). Recent studies using several approaches have provided evidence that non-stressed stomatal guard cells have clear basal ABA and basal SnRK2 kinase activities. The FRET-based *in vivo* ABA reporters, ABAleons, showed higher basal ABA concentrations in guard cells compared with other leaf tissues *in vivo* (Waadt *et al.*, 2014, 2015). In an independent approach using the ABA-responsive reporter pRAB18-GFP (Kim *et al.*, 2011), higher basal ABA signaling in guard cells than other leaf cells was detected under non-stress conditions (Waadt *et al.*, 2014,

2015; Hsu *et al.*, 2018). Recently, basal OST1/SnRK2 kinase activities in guard cells were revealed by a genetically encoded FRET-based SnRK2 activity sensor (SNACS) (Zhang *et al.*, 2020). Genetic evidence further supports the model that basal ABA levels and signaling are crucial for maintaining steady-state stomatal conductance, with knockout mutants in ABA receptors showing higher steady-state stomatal conductances in non-stressed plants (Gonzalez-Guzman *et al.*, 2012; Merilo *et al.*, 2013, 2018; Hsu *et al.*, 2018; Zhang *et al.*, 2020). It is still not fully understood how plants maintain higher ABA concentrations in the guard cells of non-stressed and stressed plants. ABA could be directly synthesized in guard cells (Bauer *et al.*, 2013), and/or transported from other synthesis sites into guard cells (Kang *et al.*, 2010; Kuromori *et al.*, 2010, 2011; Merilo *et al.*, 2015).

Basal ABA signaling contributes to other stomatal movement stimuli

Genetic evidence showed that ABA signal transduction plays a key role in elevated CO_2-induced stomatal closure (Xue *et al.*, 2011; Chater *et al.*, 2015; Hsu *et al.*, 2018; Dittrich *et al.*, 2019; Zhang *et al.*, 2020). Time-resolved stomatal conductance assays in the ABA receptor *pyr1 pyl1 pyl2 pyl4 pyl5 pyl8* hextuple mutant and *ost1-3* mutant suggest that ABA signaling enhances rapid CO_2-induced stomatal closure (Hsu *et al.*, 2018; Zhang *et al.*, 2020). A recent study suggested that CO_2 can activate ABA receptor signaling (Dittrich *et al.*, 2019). Surprisingly, several independent in-depth studies could not confirm this model. First, no noticeable increases in ABA concentrations in guard cells were observed in response to CO_2 elevation using *in vivo* FRET-based ABA reporters (Hsu *et al.*, 2018; Zhang *et al.*, 2020). Second, OST1/SnRK2.6 protein kinase activities in guard cells were not activated by CO_2 elevation in in-gel kinase assays and in time-resolved analyses in intact guard cells using a real-time FRET-based SnRK2 activity sensor (Hsu *et al.*, 2018; Zhang *et al.*, 2020). At the same time, however, ABA receptors and the OST1/SnRK2.6 kinase were required for a wild-type-like CO_2 response (Hsu *et al.*, 2018). Taken together, these diverse lines of evidence support a model in which basal ABA receptor signaling contributes in parallel with CO_2 signaling, but in which CO_2 does not rapidly increase ABA concentrations, ABA receptor activation or SnRK2 kinase activity in guard cells (Hsu *et al.*, 2018; Zhang *et al.*, 2020). Findings showing significant basal ABA concentrations and signaling in guard cells (Lahr and Raschke, 1988; Waadt *et al.*, 2015; Hsu *et al.*, 2018; Zhang *et al.*, 2020) are also likely to contribute to observations showing reduced light-induced stomatal opening in the farnesyltransferase β-subunit mutant *enhanced response to ABA1* (*era1*) plants (Jalakas *et al.*, 2017). In addition, farnesylation functions in brassinosteroid synthesis, which in turn counteracts ABA signaling (Northey *et al.*, 2016). Note that there is only a single *ERA1* gene encoding a β subunit of farnesyltransferases in the Arabidopsis genome and the *era1* mutant plants thus have a number of phenotypes. Therefore, farnesylation of distinct targets and processes in guard cells may hypothetically be occurring in *era1* mutants.

Methyl jasmonate (MeJA)-induced stomatal closure was reported to be impaired in the *ost1/snrk2.6* mutant (Yin *et al.*, 2016), but OST1/SnRK2.6 protein kinase activities in guard cells were not activated by MeJA (Zhang *et al.*, 2020). Jasmonate is required for wounding-induced stomatal closure, which includes CBL1-CIPK5-mediated GORK K^+ channel activation (Förster *et al.*, 2019). Possibly similar to CO_2 signaling, stomatal MeJA responses may not directly activate ABA signaling, but they may rely on basal ABA signaling to transduce stomatal movement responses effectively. Further research would be needed to investigate this model.

Role of basal ABA signaling under non-stress conditions

In addition to acting as a hormone that controls transpiration via stomatal closure, ABA regulates plant growth and development, including seed dormancy and germination, root architecture and stomatal development (Finkelstein *et al.*, 2008; Tanaka *et al.*, 2013; Chater *et al.*, 2014; Harris, 2015; Née *et al.*, 2017; Belda-Palazon *et al.*, 2018; Rosales *et al.*, 2019). Recent studies have addressed how basal ABA signal transduction regulates plant growth and development. A triple mutant in the three ABA-activated subclass-III SnRK2s, *snrk2.2 snk2.3 ost1/snrk2.6*, and an ABA biosynthesis mutant, *aba2-1*, exhibited an increase in leaf numbers under non-stress conditions (Fujita *et al.*, 2009; Yoshida *et al.*, 2019b). Primary metabolite profile analysis of *snrk2.2 snk2.3 ost1/snrk2.6* and *aba2-1* mutants and isotope-labeling experiments in the *snrk2.2 snk2.3 ost1/snrk2.6* mutant suggest that basal ABA signal transduction modulates metabolism through repressing the tricarboxylic acid (TCA) cycle under non-stress conditions (Yoshida *et al.*, 2019b).

An ABA-induced stomatal closure pathway has been proposed in guard cells that functions independently of the dominant *abi1-1* and *abi2-1* PP2C mutants and the *slac1-1* mutant (Pantin *et al.*, 2013); however, a high concentration of 50 μM ABA was applied in this study, which could bypass the dominant *abi1-1* and *abi2-1* mutants, given the many overlapping PP2Cs that function in the stomatal ABA signaling pathway. Furthermore, the R-type anion channel, AtALMT12/QUAC1, functions in parallel with SLAC1 and thus appears a likely candidate for bypassing SLAC1 to mediate stomatal closure at high ABA concentrations (Figure 1) (Meyer *et al.*, 2010; Sasaki *et al.*, 2010). Thus, the proposal that a non-canonical ABA signaling pathway exists that regulates stomatal movements (Pantin *et al.*, 2013) may require further investigation in

stronger higher order mutants than *abi1-1* and *abi2-1*, such as the *snrk2.2 snk2.3 ost1/snrk2.6* triple mutant, which fully disrupts the ABA signaling pathway.

As discussed earlier, ABA signaling has been reported to downregulate signal transduction by the TOR protein kinase (Wang *et al*., 2018c). Further research will be needed to determine whether basal ABA and/or the increase in plant growth and the alteration of metabolism in ABA-deficient mutants and ABA signaling mutants under non-stress conditions (Negin *et al*., 2019; Yoshida *et al*., 2019b) could result from enhanced TOR signaling.

Interaction between ABA and other signal transduction pathways in stomatal movement

Several small molecules have been shown to enhance ABA-mediated stomatal movement. Hydrogen sulfide (H_2S), a small gaseous signaling molecule, can trigger stomatal closure when applied exogenously (Jia *et al*., 2018). More recently, Chen and colleagues shed light on a mechanism describing how H_2S post-translationally regulates OST1 to trigger stomatal closure upon ABA treatment (Chen *et al*., 2020). In this model, ABA initially induces H_2S production via L-CYSTEINE DESULFHYDRASE 1 (DES1). In turn, H_2S persulfidates OST1 on two cysteine residues, Cys131 and Cys137, which are exposed on the protein surface (Figure 1). Interestingly, OST1 persulfidation on Cys131 and Cys137 increases the phosphorylation of the transcription factor ABF2 *in vitro* (Chen *et al*., 2020). Using BiFC analyses and *in vitro* pull-downs, the authors also suggested that OST1 interacts preferentially with ABF2 when Cys131 and Cys137 are post-translationally modified (Chen *et al*., 2020). In an independent study, patch-clamp experiments showed that OST1/SnRK2.6 and Ca^{2+} are required for the H_2S activation of S-type anion currents in guard cells (Wang *et al*., 2016). Altogether, H_2S appears to be a relevant signaling player in ABA-triggered stomatal closure.

Interestingly, H_2S also triggers calcium (Ca^{2+}) waves in guard cells (Figure 1) (Chen *et al*., 2020). Ca^{2+} has been shown to be a regulator during ABA-mediated stomatal closure. In guard cells, ABA application causes Ca^{2+} transients, which appear to be much shorter than H_2S-triggered Ca^{2+} influx (Chen *et al*., 2020). Recently, *AGB1*, which encodes a heterotrimeric G-protein β-subunit, was shown to be required for Ca^{2+} sensing and calcium-induced stomatal closure in Arabidopsis (Jeon *et al*., 2019). Ca^{2+} was also described to increase the efficiency of ABA-triggered stomatal closure (Huang *et al*., 2019), with intracellular Ca^{2+} being monitored in this study using a genetically encoded Ca^{2+} reporter R-GECO-mTurquoise (Waadt *et al*., 2017). Using intracellular double-barreled microelectrodes in intact Arabidopsis leaves, the authors report that Ca^{2+} accelerates stomatal closure (Huang *et al*., 2019). These findings correlate with Boolean modeling predictions (Albert *et al*., 2017). Ca^{2+} ions activate SLAC1 by stimulating calcium-dependent protein kinase (CPK) activity (Brandt *et al*., 2015). ABA-triggered stomatal closure is only partially impaired at low ABA concentrations in *cpk* quadruple mutant plants (Brandt *et al*., 2015); however, higher ABA concentrations appear to bypass *cpk* quadruple mutants in stomatal closure (Brandt *et al*., 2015). Previous research also showed that ABA closes stomata when Ca^{2+} transients are completely inhibited at higher ABA concentrations (Siegel *et al*., 2009). Furthermore, a number of studies, including recent research, showed that ABA can act independently of cytosolic Ca^{2+} elevation (Levchenko *et al*., 2005; Marten *et al*., 2007). Interestingly, the injection of 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), a strong Ca^{2+} chelator, into *V. faba* guard cells abolished the ABA activation of anion channel currents in guard cells (Levchenko *et al*, 2005). These findings, together with findings of weak ABA-induced stomatal closure when Ca^{2+} transients were completely inhibited (Siegel *et al*., 2009), may be explained by a model in which guard cells have a partial response to resting cytosolic Ca^{2+} concentrations (Siegel *et al*., 2009).

The emerging model that Ca^{2+} elevation plays an amplifying effect on ABA signaling in guard cells appears to be most consistent with many independent findings in several laboratories. Research has further indicated that physiological resting Ca^{2+} concentrations can contribute to amplifying ABA responses, via ABA enhancement in (the priming of) the Ca^{2+} sensitivity of S-type anion activation and K^+ uptake channel deactivation in guard cells (Siegel *et al*., 2009; Chen *et al*., 2010; Brandt *et al*., 2015).

Cytosolic anions have also been described as essential players in the activation of S-type anion channels. Physiological concentrations of oxaloacetate and malate facilitate S-type anion channel activation in guard cell patch-clamp experiments (Wang *et al*., 2018a). Additionally, S-type anion channel activation by intracellular malate was shown to require basal (resting) cytosolic Ca^{2+}. However, high concentrations of cytosolic malate inhibit S-type anion channels (Wang and Blatt, 2011; Wang *et al*., 2018a), which correlates with an increase in guard cell malate levels during stomatal opening.

Given the complexity and the myriad of proteins implicated in ABA signaling, independent *in silico* modeling approaches have been applied in recent years to develop hypotheses for roles of Ca^{2+} in ABA-mediated stomatal closure (Albert *et al*., 2017; Waidyarathne and Samarasinghe, 2018; Maheshwari *et al*., 2019). Interestingly, these models suggest a role for Ca^{2+} elevation in accelerating stomatal closure *in silico*, as discussed earlier, and in improving the overall resilience of the system (Waidyarathne and Samarasinghe, 2018). Another (discrete) modeling approach used an 81-node Boolean logic network to develop new guard cell signaling hypotheses (Albert *et al*., 2017). A

condensed network comprising 49 of these nodes predicted a putative role of Ca^{2+} elevation in inhibiting PP2Cs, which was supported by experimental analyses (Maheshwari *et al.*, 2019). These recent articles illustrate how *in silico* modeling combined with wet-lab experiments can help in the understanding of guard cell ABA signaling.

Interaction between peptide hormones and ABA signal transduction

Plant dehydration can be rapidly transmitted from root to leaves via an accompanying hydraulic change (Christmann *et al.*, 2007). This long-distance signal then causes ABA biosynthesis and ABA signaling in shoots (Christmann *et al.*, 2007). Recently, a slower signal has been linked to root-to-shoot drought communication (Takahashi *et al.*, 2018). Under water-deficient conditions, the small peptide CLAVATA 3/EMBRYO-SURROUNDING REGION RELATED 25 (CLE25) is expressed in root vascular tissues and moves to aerial parts of the plant to trigger stomatal closure, by increasing the expression of the *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*) ABA biosynthesis encoding gene in leaves (Takahashi *et al.*, 2018). This recently reported long-distance peptide signaling mechanism is slower than the rapid hydraulic signaling mechanism, and further research could dissect the relative contributions and any interdependencies of these two pathways.

FERONIA (FER) is a central transmembrane receptor-like kinase involved in the perception of short open reading frame RAPID ALKALINIZATION FACTOR (RALF) signaling peptides. FER is implicated in a myriad of processes (Li *et al.*, 2016a; Liao *et al.*, 2017), which points to the question of how FER integrates these different signaling events. The *fer-4* mutant is more sensitive to several abiotic stresses compared with wild-type (WT) plants (Chen *et al.*, 2016; Feng *et al.*, 2018b). FER was shown to mediate [Ca^{2+}] transients in roots that function in maintaining cell-wall integrity in response to salt stress (Feng *et al.*, 2018a). The role of FER in maintaining cell-wall integrity may be linked to several phenotypes caused by *fer* mutations. Interestingly, the extracellular domain of FER was shown to interact with pectin in cell walls and thus protect cell walls from salinity-induced damage. FER is phosphorylated and activated in response to the RALF signaling peptide (Haruta *et al.*, 2014; Chen *et al.*, 2016); however, research has shown that a kinase-dead version of FER can complement some functions in ovules and that overexpression of a kinase-dead isoform can restore stomatal closure mediated by the RALF1 peptide (Chakravorty *et al.*, 2018; Haruta *et al.*, 2018). ABA has also been reported to cause the phosphorylation of FER (Chen *et al.*, 2016). These observations are consistent with the findings that stomatal movements require guard cell-wall integrity (Rui *et al.*, 2017; Wu *et al.*, 2017).

Low-humidity-induced stomatal closure

Low relative humidity in the atmosphere is one of the environmental factors that causes stomatal closure, which was first reported by Francis (Darwin, 1898). A reduction in relative humidity increases the vapor-pressure deficit (VPD), which quantifies the difference between actual vapor pressure and water-saturated vapor pressure at a given temperature. The water vapor pressure in the intercellular air spaces inside leaves is assumed to be nearly saturated (Monteith, 1995; Peak and Mott, 2011). A high VPD from inside leaves to ambient air strengthens the driving force for stomatal transpiration. Therefore, stomatal closure induced by high VPD/low relative humidity prevents excessive water loss by plants and maintains leaf turgor (Buckley, 2016). It is still unclear how VPD is sensed by plants to regulate stomatal movements. Recent studies have suggested that high VPD/low relative humidity rapidly reduces vapor pressure near the inner leaf side (cavity) of stomata, and the water potential of the guard cell wall equilibrates with the vapor phase changes to trigger stomatal closure (Shope *et al.*, 2008; Peak and Mott, 2011; Rockwell *et al.*, 2014; Wang *et al.*, 2017). In response to a steep increase in VPD, stomata are known to initially transiently open before they close. It has been suggested that epidermal cells have a mechanical 'advantage' over guard cells, and that the initial water loss of epidermal cells and the subsequent loss of epidermal cell turgor triggers a guard cell turgor-driven transient stomatal opening in response to a reduction in relative humidity before stomatal closure (Buckley, 2019). The genes and proteins involved in sensing shifts in water potential or the accompanying mechanical signals in guard cells remain unknown. 'Hydroactive' (requiring intracellular signal transduction) and 'hydropassive' models have been proposed for mediating high VPD/low relative humidity-induced stomatal closure (Peak and Mott, 2011; Buckley, 2016), with recent studies discussed here supporting contributions from both mechanisms to the VPD response.

The role of the plant hormone ABA in the high-VPD response has been debated over the past two decades. Previous studies with the ABA-deficient mutant (*aba1-1*) and ABA-insensitive mutants (*abi1-1* and *abi2-1*) suggest that ABA and ABA signaling are not directly required for the low-humidity response (Assmann *et al.*, 2000). In contrast, the ABA biosynthesis mutant (*aba2-13*) and a new OST1 protein kinase allele (*ost1-4*) were isolated based on a defective low-humidity-mediated leaf temperature response, which is affected by the overall stomatal conductance (Xie *et al.*, 2006). In addition, the guard cell potassium content in the ABA biosynthesis mutant *aba3-1* was approximately 80% higher than in WT guard cells under humid conditions, consistent with the more widely open stomata observed in the *aba3-1* mutant (Bauer *et al.*, 2013). Furthermore, a reduction in the relative humidity caused

an approximately 45% reduction in the potassium content of guard cells both in the WT and in the ABA-deficient *aba3-1* mutant (Bauer *et al.*, 2013), which would be consistent with high-VPD-induced stomatal closure in WT and *aba3-1*. These data notwithstanding, a model was proposed for low humidity causing rapid ABA biosynthesis in guard cells for stomatal closure (Bauer *et al.*, 2013), which could be investigated by conditional measurements of guard cell ABA concentrations over short time periods.

The question of whether a rapid ABA increase triggers the low-humidity (high-VPD) stomatal closure response was further investigated. Recent studies using whole-plant stomatal conductance analyses showed that all ABA biosynthesis mutants exhibited significantly higher stomatal conductance (as expected, through reduced basal ABA concentrations). However, these mutants still responded rapidly to high VPD with clearly intact stomatal closure responses (Merilo *et al.*, 2013; Merilo *et al.*, 2018). The ABA biosynthesis proteins NCED3 and NCED5 have been shown to be key mediators of the gradual drought-stress-induced increase in the ABA concentration in leaves (Frey *et al.*, 2012; Sato *et al.*, 2018). Notably, the most severe ABA biosynthesis mutant (*nced3 nced5*) still retained approximately 25% of the steady-state leaf ABA content compared with the WT in well-watered non-stressed conditions (Merilo *et al.*, 2018), suggesting that residual steady-state ABA concentrations in *nced3 nced5* might be sufficient to contribute to the high VPD-induced stomatal closure, rather than a rapid rise in ABA concentration. One study has suggested that the ABA biosynthesis gene *NCED3* is rapidly induced by high VPD within 5 min to synthesize ABA and trigger stomatal closure (Sussmilch *et al.*, 2017). This finding awaits independent analyses of rapid NCED3 protein level increases.

An ABA receptor *pyr1 pyl1 pyl2 pyl4 pyl5 pyl8* hextuple mutant abrogates ABA-induced stomatal closure (Gonzalez-Guzman *et al.*, 2012; Merilo *et al.*, 2018). Whole intact plant stomatal conductance analyses in this strong *pyr1 pyl1 pyl2 pyl4 pyl5 pyl8* hextuple mutant exhibited functional high-VPD-induced stomatal closure, but a reduced initial response rate and significantly increased half-response time of stomatal closure (Merilo *et al.*, 2018). These data point to the model that a pathway parallel with ABA biosynthesis and ABA signaling plays a key role in the hydroactive VPD response (Merilo *et al.*, 2018). These data further support a model in which steady-state basal ABA in leaves may contribute to the hydroactive VPD response. In addition, similar half-response times, changes in stomatal conductance and the initial closing and opening rate in VPD-regulated stomatal movements in *pyr1 pyl1 pyl2 pyl4 pyl5 pyl8* hextuple mutant plants, compared with WT plants (Merilo *et al.*, 2018), indicate a contribution of the hydropassive VPD response (McAdam and Brodribb, 2015; Merilo *et al.*, 2018).

Unlike ABA receptor hextuple mutant plants, disruption of the OST1 protein kinase strongly impaired both ABA- and high-VPD-induced stomatal closure (Xie *et al.*, 2006; Merilo *et al.*, 2013, 2018), further highlighting a role for guard cell signal transduction in the stomatal closure response. The OST1-mediated hydroactive VPD response may not fully depend on ABA signaling (Merilo *et al.*, 2018). It has been reported that both ABA-dependent and ABA-independent pathways are involved in the activation of OST1 kinase activities under low-humidity stimulation in leaves (Yoshida *et al.*, 2006). Interestingly, the hydropassive effect can also contribute to the VPD response in *ost1-3* mutant plants, when steady-state stomatal conductances are increased by low CO_2 or high light stimulation (Merilo *et al.*, 2018). Wang *et al.* (2017) developed a computational platform to model leaf transpiration influenced by the high-VPD response in guard cells, including signal transduction and a hydropassive response. In the model, intracellular potassium concentrations, as well as calcium concentrations and pH, are predicted to increase as a result of the reduction in guard cell volume, resulting in enhanced K^+ efflux channel currents and in reduced inward-rectifying potassium channel currents, which favors stomatal closure (Wang *et al.*, 2017).

Taken together, the model in which an increase in VPD/low relative humidity causes stomatal closure via a rapid increase in ABA synthesis and the ABA concentration in guard cells (Bauer *et al.*, 2013; Sussmilch *et al.*, 2017) was considered insufficient to explain all of the experimental observations (Merilo *et al.*, 2018). Given the higher stomatal conductance of ABA-deficient and ABA receptor mutants, and the effect of these mutants on the rate of the stomatal VPD response in intact plants (Merilo *et al.*, 2013, 2018), a model in which basal ABA concentrations in guard cells contribute as an amplifier of the hydroactive stomatal signaling VPD response could be further investigated, similar to recent findings for high-CO_2-triggered stomatal closure (Hsu *et al.*, 2018; Zhang *et al.*, 2020). The severely impaired VPD phenotype in the *ost1-3* mutant may further result from a combined defect in both basal ABA-dependent (Zhang *et al.*, 2020) and ABA-independent signaling pathways that make use of OST1 activity (Yoshida *et al.*, 2006; Merilo *et al.*, 2018). In conclusion, a framework in which ABA-independent and basal ABA-dependent hydroactive signaling mechanisms, as well as a contribution of hydropassive mechanisms to the stomatal VPD response, may best explain many of the published observations. Further time-resolved analyses of various contributing mechanisms could further test this model.

Manipulating water use and drought response in plants

In addition to improving drought tolerance, another major challenge for plants in agriculture and forestry is water-use efficiency. In the past years, several teams were able to

increase the water-use efficiency of plants by overexpressing specific PYR/PYL/RCAR ABA receptors (Ma *et al.*, 2009; Yang *et al.*, 2016, 2019; Zhao *et al.*, 2016; Mega *et al.*, 2019; Papacek *et al.*, 2019). For instance, Arabidopsis plants overexpressing RCAR6/PYL12 showed a 40% enhanced water-use efficiency and had similar growth rates compared with the WT (Yang *et al.*, 2016). Overexpression of *Populus canescens PcRCAR10*, but not *PcRCAR9*, in Arabidopsis reduced water consumption and increased biomass under progressive drought, and these plants grew similarly to WT controls under non-stress conditions (Papacek *et al.*, 2019). Similarly, *TaPYL4*-overexpressing *Tritium aestivum* (wheat) plants showed an enhanced ABA sensitivity, which conferred a higher drought tolerance than in the WT (Mega *et al.*, 2019). As a result, the ABA receptor overexpressing wheat lines had larger biomass and higher seed production under drought conditions (Mega *et al.*, 2019). Surprisingly, ABA receptor overexpressing wheat also promoted water conservation without affecting biomass and seed productivity, and showed increased photosynthesis activities under well-watered conditions (Mega *et al.*, 2019).

Another approach for improving the drought responsiveness of plants would be to use a ligand that specifically binds to ABA receptors to increase ABA signaling. Five years ago, Park et al. genetically modified PYR1, an ABA receptor, to bind an agrochemical compound called mandipropamid (Park *et al.*, 2015). Mandipropamid was shown to induce an ABA-like transcriptional response in PYR1-modified expressing plants. Furthermore, treating PYR1-modified Arabidopsis lines and WT plants using the chemical compound conferred a significantly higher drought tolerance to engineered plants than to controls (Park *et al.*, 2015). More recently, the same group discovered and engineered ABA receptor ligands that have a higher affinity to ABA receptors than ABA itself (Vaidya *et al.*, 2019). In this elegant study, Vaidya and colleagues conducted a virtual screening and uncovered multiple ABA receptor agonists (Vaidya *et al.*, 2019). They notably found that 'opabactin', the most promising ligand, had a higher affinity for subfamily-III ABA receptors than ABA in Arabidopsis (Vaidya *et al.*, 2019). Opabactin also activated transcriptional ABA responses in wheat, conferring plants an enhanced drought tolerance compared with non-treated plants (Vaidya *et al.*, 2019). In summary, the engineering of the early ABA signal transduction pathway or genomics-enhanced quantitative trait selection of ABA signaling component variants provide potential approaches for the improvement of crops and trees grown in water-limited regions.

CONCLUSIONS

Since the discovery of ABA receptors, our knowledge of the ABA sensing and signal transduction mechanisms operating during the regulation of stomatal closure and opening has advanced substantially. It remains largely unknown, however, how ABA signaling interacts with environmental stimuli and other signals that regulate stomatal movements. To investigate whether and how basal ABA concentrations and basal OST1/SnRK2 kinase activity play a role in stomatal signal transduction in response to stimuli, in addition to CO_2, may be an interesting testable working hypothesis for future research. Stomatal closure usually proceeds within approximately 15 min of diverse stimuli. Models that propose rapid increases in the ABA concentration of guard cells before stomatal closure should be investigated to determine whether rapid time-dependent ABA concentration changes occur. Recent attempts during elevated CO_2 signaling have not observed ABA concentration increases in guard cells on this short time scale. Recent studies in model plants and initial crop/tree models have demonstrated that using ABA signaling to manipulate stomatal conductance is potentially an efficient strategy to increase drought tolerance, water retention and/or water-use efficiency. One of the significant challenges will be to enhance drought tolerance and water-use efficiency through the control of stomatal conductance without compromising photosynthetic CO_2 assimilation and productivity. As it is predicted that our climate will continue to change in the future, understanding the mechanisms of ABA-mediated stomatal regulation is likely to further inform the engineering or marker-assisted breeding of enhanced drought resilience in crops and trees.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this article. We apologize to the authors whose work we could not include because of space limitations.

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