PYR/RCAR Receptors Contribute to Ozone-, Reduced Air Humidity-, Darkness-, and CO_2-Induced Stomatal Regulation[1][C][W][OA]

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Rapid stomatal closure induced by changes in the environment, such as elevation of CO_2, reduction of air humidity, darkness, and pulses of the air pollutant ozone (O_3), involves the SLOW ANION CHANNEL1 (SLAC1). SLAC1 is activated by OPEN STOMATA1 (OST1) and Ca^{2+}-dependent protein kinases. OST1 activation is controlled through abscisic acid (ABA)-induced inhibition of type 2 protein phosphatases (PP2C) by PYRABACTIN RESISTANCE/REGULATORY COMPONENTS OF ABA RECEPTOR (PYR/RCAR) receptor proteins. To address the role of signaling through PYR/RCARs for whole-plant steady-state stomatal conductance and stomatal closure induced by environmental factors, we used a set of Arabidopsis (*Arabidopsis thaliana*) mutants defective in ABA metabolism/signaling. The stomatal conductance values varied severalfold among the studied mutants, indicating that basal ABA signaling through PYR/RCAR receptors plays a fundamental role in controlling whole-plant water loss through stomata. PYR/RCAR-dependent inhibition of PP2Cs was clearly required for rapid stomatal regulation in response to darkness, reduced air humidity, and O_3. Furthermore, PYR/RCAR proteins seem to function in a dose-dependent manner, and there is a functional diversity among them. Although a rapid stomatal response to elevated CO_2 was evident in all but *slac1* and *ost1* mutants, the bicarbonate-induced activation of S-type anion channels was reduced in the dominant active PP2C mutants *abi1-1* and *abi2-1*. Further experiments with a wider range of CO_2 concentrations and analyses of stomatal response kinetics suggested that the ABA signalosome partially affects the CO_2-induced stomatal response. Thus, we show that PYR/RCAR receptors play an important role for the whole-plant stomatal adjustments and responses to low humidity, darkness, and O_3 and are involved in responses to elevated CO_2.

Stomata, small pores in the leaf epidermis, are formed by a pair of guard cells that have developed mechanisms to sense and respond to various endogenous and environmental stimuli. Stomata close in response to reduction in air humidity, darkness, and CO_2 enrichment. Ozone (O_3), a major secondary air pollutant with adverse impacts on global vegetation (Ashmore, 2005) and climate change (Sitch et al., 2007), has also been shown to cause rapid stomatal closure (Hill and Littlefield, 1969; Vahisalu et al., 2010). The key endogenous factor triggering stomatal closure in response to drought is the plant hormone abscisic acid (ABA). Webb and Hetherington (1997) suggested that the pathways of ABA- and CO_2-induced closure converge (i.e. that there is an economy in signaling pathways leading to the promotion of stomatal closure). However, the location of this convergence point is still under debate. Opening and closure of stomatal pores is achieved by the uptake and release of osmotically active ions, leading to expanding and shrinking of guard cells. Thus, the activation and inactivation of guard cell ion channels and transporters are the primary targets of signaling networks controlling stomatal

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movements (for review, see Kim et al., 2010; Kollist et al., 2011; Roelfsema et al., 2012).

In 2009, independent groups simultaneously discovered the functional and structural mechanisms of ABA sensing by cytosolic PYRABACTIN RESISTANCE1 (PYR1)/PYR1-like (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) receptor proteins (Ma et al., 2009; Park et al., 2009). Identification of the guard cell slow-type anion channel gene, *SLAC1*, is another substantial finding in stomatal research (Negi et al., 2008; Vahisalu et al., 2008). ABA-induced stomatal closure involves the activation of SLAC1, which is controlled by PYR/RCAR-dependent sequestration of type 2 protein phosphatases (PP2Cs), such as ABI1, ABI2, HAB1, and PP2CA, and the concomitant activation of the Snf1-related subfamily 2 protein kinase SnRK2.6/OST1 (Ma et al., 2009; Nishimura et al., 2010; Park et al., 2009; Umezawa et al., 2009; Vlad et al., 2009; Weiner et al., 2010; Dupeux et al., 2011; Soon et al., 2012). Phosphorylation by OST1 activates several proteins, including SLAC1 (Geiger et al., 2009; Lee et al., 2009; Vahisalu et al., 2010). SLAC1 can also be activated by different calcium-dependent protein kinases (CPKs; Geiger et al., 2010, Brandt et al., 2012; Scherzer et al., 2012). Thus, several molecular details are known for the general activation mechanism of SLAC1 and subsequent stomatal closure. The importance of SLAC1 and OST1 in rapid stomatal responses to environmental factors such as darkness, CO_2, humidity, and O_3 is also established (Negi et al., 2008; Vahisalu et al., 2008; Ache et al., 2010; Xue et al., 2011). However, whether the ABA- and PYR/RCAR-dependent inhibition of PP2Cs that ultimately results in the activation of OST1 and anion channels like SLAC1 (herein defined as the ABA signalosome) is also required for rapid stomatal responses to important environmental factors is not fully resolved. One approach to address this question is to perform a side-by-side comparison of whole-plant stomatal responses of plants where various proteins of the ABA signalosome are mutated (Fig. 1; Table I).

Stomatal responses of several of these mutants to environmental factors have been studied previously, although often with different results. For example, initial in vitro studies indicated that OST1 was not involved in CO_2-induced stomatal signaling, as the stomata of *ost1-1* and *ost1-2* behaved like the wild type in response to low CO_2 (Mustilli et al., 2002). Similarly, mutations in OST1 did not affect stomatal regulation by light, leading to the suggestion that OST1 is specifically involved in ABA signaling (Mustilli et al., 2002). However, recently, it was shown that OST1 is a major regulator of CO_2-induced stomatal closing and activation of the S-type anion channels in guard cells (Xue et al., 2011). Furthermore, dominant hypermorphic *abi1-1* and *abi2-1* mutations, which generate mutant PP2Cs that are refractory to inhibition by PYR/RCAR receptors (Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009), have been used to address whether ABA-, CO_2-, and light-induced stomatal signaling pathways converge. Stomatal opening induced by light and CO_2 removal

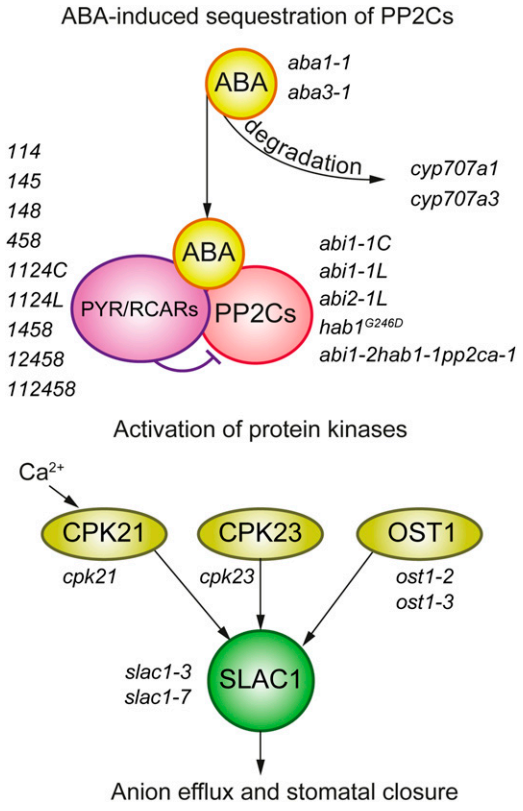


Figure 1. Schematic overview of ABA-induced signaling by PYR/RCAR receptors leading to sequestration of PP2Cs, activation of protein kinases OST1, CPK21, and CPK23, and subsequent phosphorylation/activation of the SLAC1 anion channel that is essential for anion efflux and stomatal closure. Mutants selected for this study are shown. Detailed descriptions of the mutants are provided in Table I. [See online article for color version of this figure.]

was 50% reduced in *abi1-1* and *abi2-1* plants; however, it was concluded that this might have been caused by constitutively more open stomata of these mutants (Leymarie et al., 1998a, 1998b). Other studies showed that light-induced stomatal opening was not disrupted in *abi1-1* and *abi2-1* mutants (Roelfsema and Prins, 1995; Eckert and Kaldenhoff, 2000). In contrast, Webb and Hetherington (1997) found that *abi1-1* and *abi2-1* did not respond clearly to elevated CO_2, whereas the stomatal closure was indistinguishable from the wild type in ABA-deficient plants.

The involvement of the ABA signalosome in the regulation of the stomatal response to reduced air humidity is also disputed. During a genetic screen for mutants involved in the stomatal response to reduced air humidity, new alleles for OST1 and ABA2, an enzyme involved in ABA biosynthesis, were identified, suggesting that OST1 activity and ABA biosynthesis are essential for low-humidity-induced stomatal closure (Xie et al., 2006). Furthermore, activation of OST1 is induced by low-humidity stress (Yoshida et al., 2006). Contrarily, *aba1*, *abi1-1*, and *abi2-1* mutants had wild-type stomatal responses to reduced humidity (Assmann et al., 2000). Since partial

Table I. *Descriptions of the mutants used in the study*
EMS, Ethyl methanesulfonate; T-DNA, transfer DNA.

Genotype	Mutation	Description	Reference
ABA biosynthesis and catabolism			
aba3-1 (Col-0)	EMS, G to A at position 3,707	Defective in the conversion of ABA-aldehyde to ABA, ABA deficient	Léon-Kloosterziel et al. (1996); Nambara and Marion-Poll (2005)
aba1-1 (L*er*)	EMS, G to A at position 2,139 (premature stop codon)	Defective in the ABA biosynthetic enzyme zeaxanthin epoxidase, strongly ABA deficient	Rock and Zeevaart (1991); Nambara and Marion-Poll (2005)
cyp707a1 (Col-0)	SALK_069127	Defective in ABA 8′-hydroxylase, responsible for ABA catabolism in guard cells	Okamoto et al. (2009)
cyp707a3 (Col-0)	SALK_078173	Defective in ABA 8′-hydroxylase, responsible for ABA catabolism in vascular tissues	Okamoto et al. (2009)
Core ABA signaling			
abi1-1C (Col-0)	EMS, Gly-180 to Asp	Dominant point mutation in ABI1 resulting in the loss of PYR/RCAR binding and ABA insensitivity	Nishimura et al. (2004); Umezawa et al. (2009)
abi1-1L (L*er*)	EMS, Gly-180 to Asp	See *abi1-1C*	Leung et al. (1997); Ma et al. (2009)
abi2-1C (Col-0)	EMS, Gly-168 to Asp	Dominant point mutation in ABI2, resulting in the loss of PYR/RCAR binding and ABA insensitivity	Nishimura et al. (2004)
abi2-1L (L*er*)	EMS, Gly-168 to Asp	See *abi2-1C*	Leung et al. (1997); Ma et al. (2009)
abi1-2 hab1-1 pp2ca-1 (Col-0)	SALK_072009, SALK_002104, SALK_028132	Triple knockout mutant of PP2Cs ABI1, HAB1, and PP2CA	Rubio et al. (2009)
hab1^{G246D} (Col-0)	Transgenic line	Overexpression of HAB1 carrying the G246D mutation that prevents the binding to PYR/PYL and ABA insensitivity	Robert et al. (2006); Dupeux et al. (2011)
ost1-2 (L*er*)	EMS, G to A at position 97	Point mutation in ABA-activated protein kinase OST1	Mustilli et al. (2002)
ost1-3 (Col-0)	SALK_008068	= srk2e = snrk2.6, T-DNA knockout mutation of ABA-activated protein kinase OST1	Yoshida et al. (2002)
pyr1pyl1pyl4 (114) (Col-0)	EMS + T-DNA	Triple mutant of ABA receptor proteins	Park et al. (2009)
pyr1pyl4pyl5 (145) (Col-0)	EMS + T-DNA + transposon	Triple mutant of ABA receptor proteins	Gonzalez-Guzman et al. (2012)
pyr1pyl4pyl8 (148) (Col-0)	EMS + T-DNA	Triple mutant of ABA receptor proteins	Gonzalez-Guzman et al. (2012)
pyl4pyl5pyl8 (458) (Col-0)	T-DNA + transposon	Triple mutant of ABA receptor proteins	Gonzalez-Guzman et al. (2012)
pyr1pyl1pyl2pyl4(1124C) (Col-0)	EMS + T-DNA	Quadruple mutant of ABA receptor proteins	Park et al. (2009)
pyr1pyl1pyl2pyl4 (1124L) (L*er*)	EMS + T-DNA	Quadruple mutant of ABA receptor PYR/PYL/RCAR proteins	Park et al. (2009)
pyr1pyl4pyl5pyl8(1458) (Col-0)	EMS + T-DNA + transposon	Quadruple mutant of ABA receptor proteins	Gonzalez-Guzman et al. (2012)
pyr1pyl2pyl4pyl5pyl8(12458) (Col-0)	EMS + T-DNA + transposon	Pentuple mutant of ABA receptor proteins	Gonzalez-Guzman et al. (2012)
pyr1pyl1pyl2pyl4pyl5pyl8 (112458) (Col-0)	EMS + T-DNA + transposon	Sextuple mutant of ABA receptor proteins	Gonzalez-Guzman et al. (2012)
Other mutants			
cpk21 (Col-0)	GABI_322A03	Defective in CPK21	This study (Supplemental Fig. S9)
cpk23 (Col-0)	SALK_007958	Defective in CPK23	Geiger et al. (2010)
slac1-3 (Col-0)	SALK_099139	T-DNA insertion in SLAC1	Vahisalu et al. (2008)
slac1-7 (Col-0)	C to T at position 527	Point mutation of Ser-120 to Phe in SLAC1	Vahisalu et al. (2010)

response to low humidity is observed in all studied mutants of the ABA signalosome, the existence of a separate ABA-independent pathway mediating low-humidity-induced stomatal closure has been proposed (Xie et al., 2006).

A large amount of data for stomatal signaling is collected by using isolated leaf epidermises or guard cell protoplasts, whereas the significance of these results is not always tested in intact plants. Thus, gas-exchange

analysis of whole-plant and leaf stomatal responses is important in verifying the stomatal responsiveness to environmental stimuli. In this study, we used a custom-made gas-exchange device with parallel recording of stomatal responses of up to eight Arabidopsis (*Arabidopsis thaliana*) plants to various environmental factors (Kollist et al., 2007; Vahisalu et al., 2008, 2010). We chose mutants where different components of the ABA signalosome are affected (Fig. 1) to address the role of ABA signaling through PYR/RCAR proteins in stomatal responses to reduced air humidity, darkness, and elevated CO_2 and O_3 concentrations. We found that ABA signaling through PYR/RCARs is clearly required for rapid stomatal closure in response to darkness, O_3, and reduced air humidity, while it is also partially involved in stomatal responses to elevated CO_2. Since functional OST1 and SLAC1 were important in response to all stimuli, we discuss the possibility that other signaling elements besides ABA and its signalosome are able to activate OST1 in response to changes in CO_2.

RESULTS

To study the role of the ABA signalosome in the regulation of stomatal responses to darkness, CO_2, reduced humidity, and O_3, we used mutants where different proteins of the ABA signalosome were affected (Fig. 1). If possible, at least two mutants for each protein were analyzed, and in many cases, mutants from different genetic backgrounds, Columbia (Col-0) and Landsberg *erecta* (L*er*), were used in parallel (Table I). Representative photographs of plants used for gas-exchange measurements (Supplemental Fig. S1) show that even mutants with high stomatal conductance (g_{st}) were healthy and nonwilted in our growth conditions.

ABA Signaling through PYR/RCAR Receptors Plays a Fundamental Role in Controlling Plant Steady-State g_{st}

Whole-rosette g_{st} varied severalfold among the studied mutants, ranging from 74 mmol m^{-2} s^{-1} in a triple loss-of-function mutant of ABI1, HAB1, and PP2CA, *abi1-2hab1-1pp2ca-1*, to 683 mmol m^{-2} s^{-1} in the sextuple PYR/RCAR loss-of-function mutant *pyr1pyl1pyl2pyl4pyl5pyl8*, abbreviated as *112458* (Fig. 2A). The difference in g_{st} between two wild types, L*er* and Col-0, was 1.4-fold. The g_{st} values of *112458*, the PYR/RCAR pentuple mutant *pyr1pyl2pyl4pyl5pyl8* (*12458*), and dominant active PP2C mutants (*abi1-1L* and *abi2-1L* in L*er* and *abi1-1C* and $hab1^{G246D}$ in Col-0) were highest, followed by ABA-deficient *aba1-1* and *aba3-1* (Fig. 2A). The $hab1^{G246D}$ mutation is analogous to *abi1-1* and *abi2-1* mutations and causes resistance to ABA-dependent inhibition by PYR/RCAR receptors, leading to strong ABA insensitivity (Robert et al., 2006; Dupeux et al., 2011). It should be noted that ABA biosynthesis mutants have reduced, but not abolished, ABA synthesis, with *aba1-1* and *aba3-1* still having 3% and 10% of the wild type ABA in stressed leaves, respectively (Rock and Zeevaart, 1991; Léon-Kloosterziel et al., 1996). Plants carrying loss-of-function mutations in SLAC1 and OST1 (*slac1-3*, *ost1-2*, and *ost1-3*) as well as in four PYR/RCAR receptors, *pyr1pyl4pyl5pyl8* (*1458*) and *pyr1pyl1pyl2pyl4* (note that we present data for this mutant in the Col-0 and L*er* backgrounds, abbreviated as *1124C* and *1124L*, respectively), also exhibited significantly higher g_{st} than the corresponding wild types. Gradual removal of PYR/RCAR receptor proteins had an increasing effect on g_{st}. In two mutants with elevated ABA concentrations due to defective ABA catabolism (*cyp707a1* and *cyp707a3*; Okamoto et al., 2009), only *cyp707a1* had significantly reduced g_{st}. OST1-dependent phosphorylation of Ser-120 in SLAC1 is critical for channel activation in *Xenopus laevis* oocytes (Geiger et al., 2009) and in O_3-induced plant stomatal response (Vahisalu et al., 2010). However, the g_{st} of *slac1-7*, with an S120F point mutation, was similar to that of Col-0. CPK21 and CPK23 are other kinases shown to activate SLAC1 in *X. laevis* oocytes (Geiger et al., 2010); however, g_{st} of *cpk21* and *cpk23* did not differ from that of wild-type plants (Fig. 2A). Together, these results unequivocally demonstrate that mutations in the ABA signalosome have a major effect on the whole-plant stomatal conductivity and loss of water.

To address whether the differences in g_{st} persisted after the application of 1 h of darkness, elevated CO_2, and reduced humidity, we combined g_{st} values of all mutants into one principal component analysis (PCA) axis. This PCA axis describes g_{st} in stimuli-affected conditions. The ranking of genotypes by g_{st} in stimuli-affected conditions (Fig. 2B) did not reveal any major differences from g_{st} values in normal prestimuli conditions (Fig. 2A): although there were some relocations (e.g. *ost1-3* and *1124C*), the groups of statistical significance remained unchanged. The result that plants with more open stomata remain more open even after receiving a signal to close may indicate either that stomatal closure is impaired due to the given mutation or that the absolute extent of stomatal closure induced by these stimuli is generally limited.

Data Analysis

Since the number of studied mutants was high, we determined two characteristics describing stomatal closure and enabling quantitative comparisons of different genotypes. First, we calculated changes of g_{st} as $(g_{st2} - g_{st1})/(t_2 - t_1)$, as shown in Figure 3, A to C. Since $t_2 - t_1$ is a similar time interval for all mutants (Fig. 3, A–C), this number describes the initial stomatal response to the applied factor (i.e. the magnitude of change in g_{st}). This characteristic can be effectively applied to all mutants. Stomatal closure in response to darkness, elevated CO_2, and reduced air humidity of wild-type plants followed an exponential function (Fig. 3D). Thus, to provide a value describing the stomatal response kinetics of different genotypes, an exponential function was fitted to their stomatal closure responses and the maximum stomatal closure rate was

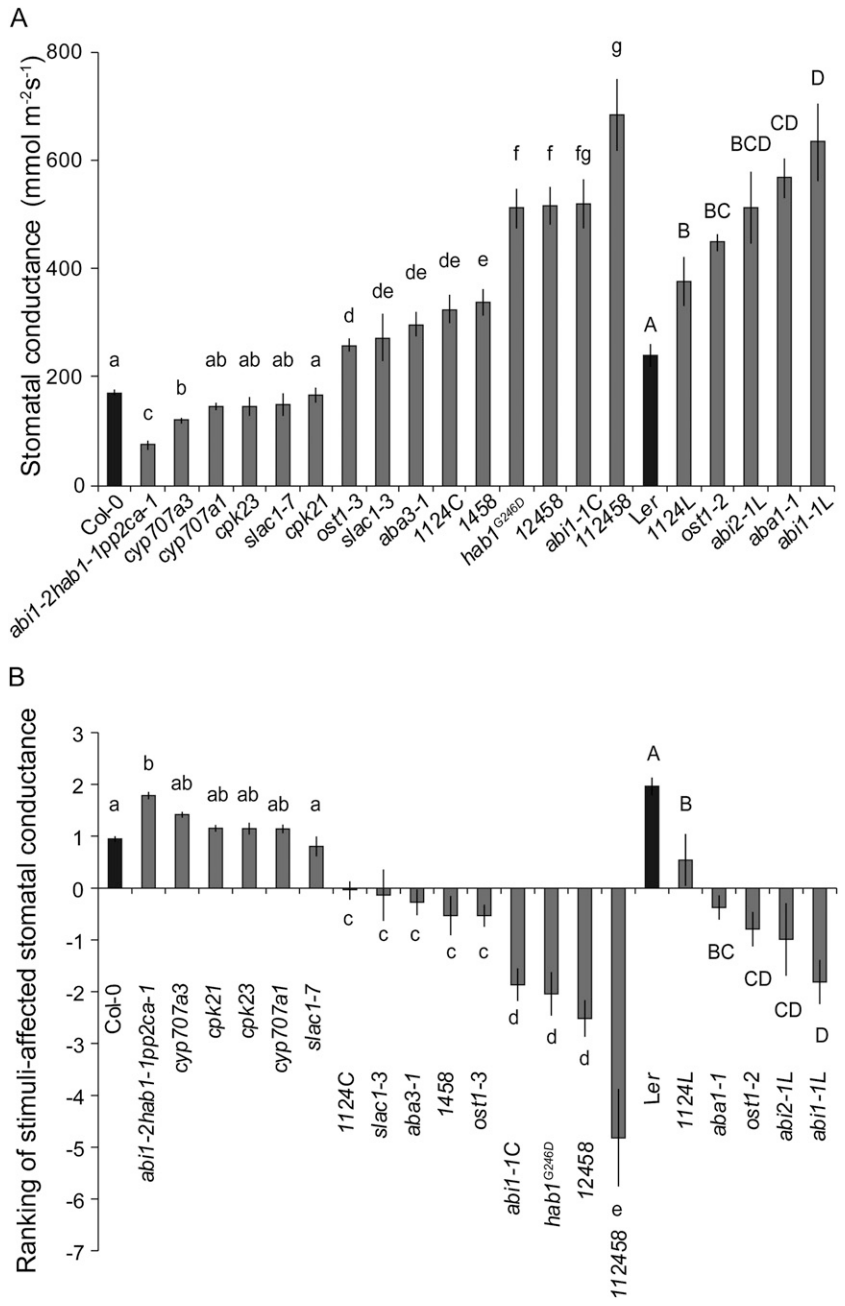


Figure 2. A, There is a large variation in whole-plant steady-state stomatal conductance of plants with mutations in ABA signalosome. The average stomatal conductance values of three- to four-week-old mutants and corresponding wild types. Significant differences ($P < 0.05$, n = 6-46) are denoted with different small and capital letters for Col-0- and *Ler*-based mutants, respectively. B, Ranking of genotypes by their stomatal conductance after application of 1 h darkness, elevated CO_2 and reduced humidity derived from principal component analysis. Significant differences ($P < 0.05$, n = 6–46) are denoted with different small and capital letters for Col-0 and *Ler*-based mutants, respectively.

calculated (Fig. 3D). However, several mutants either did not have clear stomatal responses to applied stimuli or their stomatal responses did not reach a stable phase within the time frame in which the experiments were carried out (e.g. *slac1-3* and *ost1-3* in Fig. 4, A and C, as examples, respectively). In such cases, fitting stomatal response to exponential function was not possible, and we interpreted this as an indication that the stomatal closure was affected due to the respective mutation. Data summarizing the results of exponential fitting are shown in Table II and Supplemental Figure S3.

For clarity and to indicate at which step CO_2-, darkness-, reduced humidity-, and O_3-induced stomatal closures diverge, we present the mutants of ABA signaling and SLAC1 activation from the bottom up, starting from SLAC1 anion channel regulation and moving stepwise to mutants defective in PP2C phosphatases, PYR/PYL proteins, and, finally, ABA biosynthesis and catabolism.

SLAC1 and OST1 Are Required for Rapid Stomatal Closure in Response to All Studied Stimuli

Loss-of-function mutations in the SLAC1 anion channel and its main regulator, the protein kinase OST1, led to significantly impaired stomatal responses to darkness, high CO_2, reduced air humidity, and O_3, and the corresponding initial changes of g_{st} were significantly lower

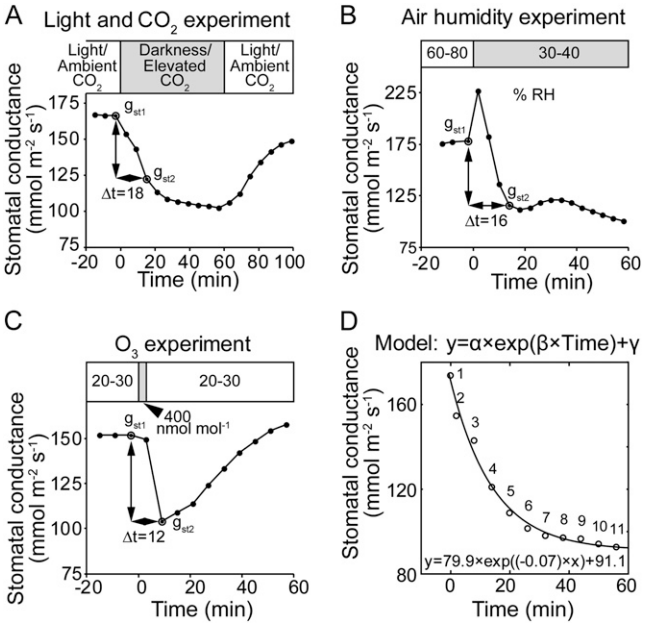


Figure 3. A to C, Representative time courses of g_{st} in response to darkness and CO_2 enrichment (A), decreased air humidity (B), and O_3 pulse (C) are given to illustrate the calculation of initial changes in stomatal conductance. D, An example of fitting the kinetics of stomatal closure with exponential function, where α describes the overall stomatal response and γ is the value at which g_{st} stabilized in response to stimuli. In case the stomatal closure significantly followed an exponential function, maximum stomatal closure rate was calculated as $\alpha \times \beta$. RH, Relative humidity.

than in the wild type (Fig. 4; Supplemental Fig. S2). In all genotypes, an initial sudden increase in g_{st} was detected after the transition from humid to dry air. This is caused by a rapid increase in water evaporation from the epidermal cells and a concomitant decrease of their pressure on guard cells (Ivanoff, 1928). Due to this effect and the extremely slow closing response, the g_{st} of OST1 loss-of-function mutants remained higher after 1 h in dry air compared with humid air (Fig. 4B; Supplemental Fig. S2B). Humidity- and darkness-induced stomatal closures were the only responses where significant differences between SLAC1 and OST1 loss-of-function plants were detected (Fig. 4, F and G).

The stomata of *slac1-7* closed significantly less than those of Col-0 in response to all stimuli, confirming that the phosphorylation of Ser-120 was important for SLAC1 activation (Fig. 4; Geiger et al., 2009; Vahisalu et al., 2010). However, both the wild-type-like absolute g_{st} (Fig. 2A) and the clearly weaker phenotype in CO_2- and O_3-induced stomatal responses of *slac1-7* compared with those observed in *slac1-3* (Fig. 4, E and H) suggest that the phosphorylation of Ser-120 does not fully explain the activation mechanism of SLAC1. The maximum stomatal closure rate of *slac1-7*, derived by fitting the kinetics of CO_2-induced stomatal closure with exponential function, yielded values lower than in the wild type (Supplemental Fig. S3B). These results confirm that, in addition to O_3-induced stomatal closure (Vahisalu et al., 2010), SLAC1 Ser-120 is the target for OST1 in CO_2-, reduced humidity-, and darkness-induced responses as well, but they also suggest that for full SLAC1 activation, phosphorylation of multiple Ser residues either by OST1 or in combination with other protein kinases shown to activate SLAC1 in *X. laevis* oocytes (Geiger et al., 2010; Brandt et al., 2012; Hua et al., 2012; Scherzer et al., 2012) is needed.

To address the role of CPK21 and CPK23, Ca^{2+}-related protein kinases shown to activate SLAC1 in *X. laevis* oocytes (Geiger et al., 2010), *cpk21* and *cpk23* plants were used. The stomata of *cpk21* responded to the studied stimuli like the wild type, whereas the initial changes of g_{st} of *cpk23* were significantly reduced (Fig. 4, E–H), although much less than those of *slac1-3* and *ost1-3*. Fitting the kinetics of stomatal closure with exponential function further confirmed that darkness- and humidity-induced stomatal closure is impaired in *cpk23* (Table II; Supplemental Fig. S3A).

In conclusion, our results indicate that OST1-induced phosphorylation of SLAC1 is needed for rapid stomatal closure in response to all studied stimuli. Additionally, CPK23 is required, although to a minor extent.

PP2Cs Are Important for Rapid O_3- and Humidity-Induced Stomatal Closure But Less Important for Darkness- and CO_2-Induced Closure

PP2Cs function as negative regulators in ABA-induced stomatal closure by inhibiting the OST1- and CPK-induced activation of SLAC1 (Geiger et al., 2009, 2010; Brandt et al., 2012). In the presence of ABA, their activity is suppressed by PYR/RCAR receptors. We used plants carrying dominant active *abi1-1*, *abi2-1*, and *hab1*^{G246D} mutations that prevent ABA-dependent inhibition of PP2Cs by PYR/RCAR receptors. Humidity- and O_3-induced stomatal responses and initial changes of g_{st} were reduced in *abi1-1C* (Fig. 5, B, F, D, and H) and *abi1-1L* and *abi2-1L* (Supplemental Fig. S4, B, F, D, and H) as compared with their wild types, whereas in *hab1*^{G246D}, only the O_3 response was reduced (Fig. 5, D and H). Thus, for O_3- and humidity-induced stomatal closure, the inhibition of PP2C activity is important.

The role of PP2Cs in CO_2- and darkness-induced stomatal closure was less clear. The initial changes in g_{st} of dominant *abi1-1C* and *hab1*^{G246D} mutants in the Col-0 background (Fig. 5, A, E, C, and G) and similarly those of *abi1-1L* and *abi2-1L* in the L*er* background (Supplemental Fig. S4, A, E, C, and G) were generally wild type like. Similar results were obtained for plants with *abi1-1* and *abi2-1* mutations in the Col-0 and L*er* backgrounds when analyzing elevated CO_2-induced stomatal closure with a leaf gas-exchange analyzer (Supplemental Fig. S5). However, obtained patterns of stomatal closure could not be described with an exponential function in several cases, such as the darkness responses of *abi1-1L* and *abi2-1L* and the CO_2 responses of *abi1-1C* and *abi2-1L* (Table II), suggesting that the closure responses of these mutants were different from their wild types.

To further address the roles of ABI1 and ABI2 phosphatases for CO_2-induced stomatal regulation, two

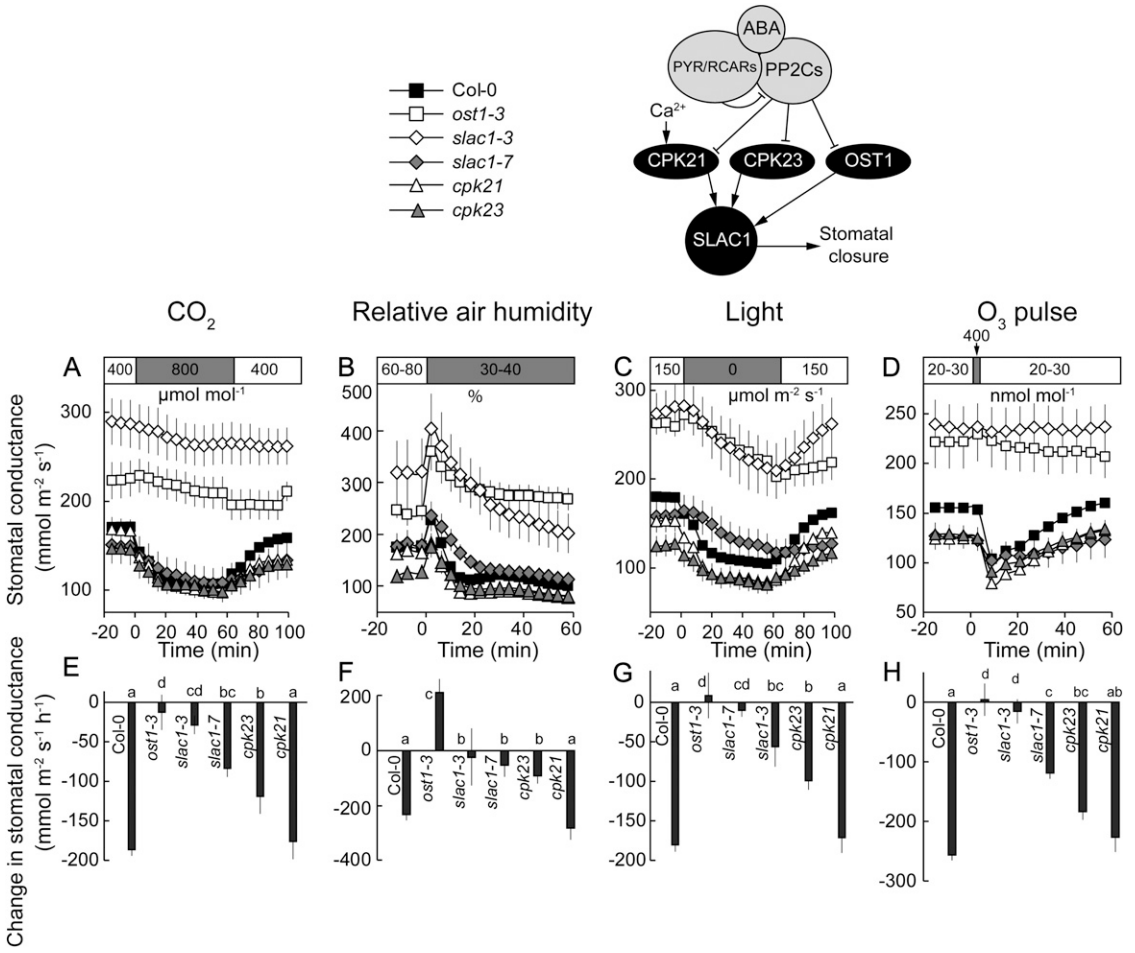


Figure 4. Time courses of g_{st} in response to elevated CO_2 (A), reduced air humidity (B), darkness (C), and O_3 pulse (D) together with corresponding changes in g_{st} values (E–H) of the plants carrying mutations in the SLAC1 anion channel and protein kinases (OST1, CPK21, and CPK23) shown to activate SLAC1. Changes in g_{st} (E–H) were calculated as shown in Figure 3, A to C. Significant differences ($P < 0.05$; $n = 5$–50) are denoted with different lowercase letters.

additional experiments were performed. First, in a separate gas-exchange experiment, *abi1-1L*, *abi2-1L*, and L*er* stomatal responses were tested within a wider range of CO_2 concentrations. Plants were first acclimatized under 50 μL L^{-1} CO_2 until stable g_{st} values were reached (75–100 min). Thereafter, CO_2 concentration was increased stepwise to 100, 200, 400, and 800 μL L^{-1} in 30-min intervals. Such treatment induced clear stomatal closure in all three genotypes (Fig. 6A); however, when the stomatal closures caused by each additional step in CO_2 concentration were determined, differences between the genotypes emerged (Fig. 6B). There was no reduction in g_{st} within the CO_2 range from 50 to 100 μL L^{-1}. Furthermore, from 50 to 200 μL L^{-1}, a decrease in g_{st} was observed only in L*er*. Change from 50 to 400 μL L^{-1} CO_2 caused a closure response in all genotypes; however, the decrease in g_{st} was significantly smaller in *abi2-1L* than in *abi1-1L*, and this difference between *abi1-1L* and *abi2-1L* remained at 800 μL L^{-1}, where the CO_2-induced decrease in g_{st} was similar in L*er* and *abi1-1L* but significantly lower in *abi2-1L*. Second, recent research showed that β-carbonic acid anhydrases function early in CO_2-induced stomatal closure (Hu et al., 2010) and that bicarbonate (HCO_3^-) is an important intracellular signal that triggers the activation of S-type anion channels in Arabidopsis guard cells (Xue et al., 2011). To further address the roles of ABI1 and ABI2 in CO_2-induced stomatal signaling, HCO_3^--induced activation of S-type anion currents was measured in *abi1-1L* and *abi2-1L*. Guard cell protoplasts from *abi1-1L* and *abi2-1L* displayed clearly reduced but still functional HCO_3^--induced activation of anion currents (Fig. 6, C–K).

In conclusion, PP2Cs are important for stomatal closure in response to reduced humidity and O_3, and they also participate in darkness- and CO_2-induced responses.

Removal of Six PYR/RCAR Receptor Proteins Impairs Plant Stomatal Responsiveness to O_3, Reduced Humidity, Elevated CO_2, and Darkness

Gradual removal of PYR/RCAR receptor proteins increasingly impaired the whole-plant stomatal

Table II. *Results of fitting exponential functions to stomatal closure patterns in response to darkness, elevated CO_2, and reduced air humidity*

The first number shows how many closure responses could be described with an exponential function, whereas the second number shows the total number of experiments. Boldface values indicate cases where 50% of experiments did not follow an exponential function.

Genotype	Darkness Response	Humidity Response	CO_2 Response
Col-0	34/34	21/27	45/46
slac1-3	**3/7**	**2/6**	**3/7**
ost1-3	**1/6**	**0/6**	**2/6**
112458	**1/5**	**2/8**	**3/7**
abi1-1C	3/5	5/7	**1/5**
slac1-7	**0/6**	4/7	4/6
1458	**3/6**	5/5	6/8
12458	**2/7**	6/6	5/8
148	**2/5**	**3/6**	5/5
1124C	6/6	**4/8**	8/8
cpk23	5/6	**3/7**	6/7
abi1-2hab1-1pp2c-1	5/5	**2/5**	7/7
hab1^{G246D}	3/5	3/5	9/10
458	5/5	6/6	6/6
114	5/5	5/5	5/5
145	4/5	6/6	6/6
aba3-1	4/6	5/6	6/6
cpk21	6/6	5/5	7/7
cyp707a1	8/8	5/6	7/7
cyp707a3	6/6	5/6	6/6
L*er*	12/12	9/10	11/12
ost1-2	**0/6**	**2/6**	**2/8**
abi2-1L	**2/6**	**2/6**	**2/7**
aba1-1	**1/7**	6/6	**3/6**
abi1-1L	**2/6**	**2/6**	6/8
1124L	**3/6**	**1/6**	6/7

responsiveness to environmental factors; the sextuple *112458* PYR/RCAR mutant showed the strongest effect and displayed significantly impaired stomatal responses (Fig. 7, A–D) and reduced initial changes of g_{st} due to all stimuli, except for elevated CO_2 (Fig. 7, E–H). Additionally, patterns of *112458* stomatal closure did not follow an exponential function in darkness, CO_2, and humidity experiments (Table II), suggesting that the lack of these proteins modified the fast kinetics of stomatal closure in response to these stimuli. A separate gas-exchange experiment with stepwise increases in CO_2 concentration also revealed that CO_2-induced stomatal closure is reduced in *112458* compared with the wild type, particularly at lower CO_2 concentrations (Fig. 7, I and J). Thus, the lack of six PYR/PYL proteins significantly impaired stomatal closure due to all studied factors. Quadruple PYR/RCAR mutants displayed impaired stomatal responsiveness as well; however, here, the differences from the wild type depended on the applied stimuli and the combination of PYR/RCAR mutations. For example, O_3- and humidity-induced initial changes of g_{st} in quadruple *1124C* (Fig. 7, F and H) and *1124L* (Supplemental Fig. S6, F and H) mutants were significantly reduced, whereas *1458* quadruple and even *12458* pentuple mutants showed similar or even higher than wild-type closures in response to reduced humidity and O_3 (Fig. 7, F and H). These data indicate a functional diversity among PYR/RCAR proteins and suggest the importance of PYL1 for stomatal functioning. Recently, it was demonstrated that PYL1 played an important role in ABA-induced transcriptional response as well (Gonzalez-Guzman et al., 2012). Various combinations of triple loss-of-function PYR/PYL mutants, including *pyr1pyl1pyl4*, previously found to have higher steady-state g_{st} values than the wild type (Gonzalez-Guzman et al., 2012), generally showed initial changes of g_{st} that were wild type like or even larger than in the wild type (Supplemental Fig. S7). This suggests that a certain threshold of PYR/RCAR receptors is required in guard cells to trigger stomatal closure in response to environmental factors. Furthermore, the compensatory changes in the concentration/activity of other PYR/RCARs in triple loss-of-function PYR/RCAR mutants can explain why their initial rates of stomatal closure were sometimes higher than in the wild type.

Elevated CO_2, Reduced Air Humidity, Darkness, and O_3-Induced Stomatal Closure of ABA Biosynthesis and Catabolism Mutants

Plants with mutations in ABA biosynthesis had weaker impairments of stomatal responses (Fig. 8) than those observed for plants impaired in ABA signaling (Figs. 4–7). The initial changes of g_{st} in *aba1-1* and *aba3-1* did not differ from the L*er* and Col-0 wild types, respectively (Fig. 8, E, G, and H). The only exception was significantly lower darkness-induced stomatal closure in *aba1-1* than in its L*er* wild type (Fig. 8G). Furthermore, the stomatal kinetics of *aba1-1* did not follow an exponential function in darkness and CO_2 experiments. ABA is most likely essential for proper plant development; hence, no null mutants for ABA biosynthesis have been isolated. Accordingly, residual amounts of ABA in *aba1-1* and *aba3-1* (Rock and Zeevaart, 1991; Léon-Kloosterziel et al., 1996; Xie et al., 2006) could be sufficient to activate ABA signaling, since half-maximal inhibition of PP2Cs takes place at nanomolar ABA concentrations in the presence of PYR/RCARs (Szostkiewicz et al., 2010).

Surprisingly, the initial reductions in g_{st} due to the reduced humidity of ABA biosynthesis mutants were significantly larger than those of the respective wild types (Fig. 8F). This result is confirmed by significantly larger maximum stomatal closure rates derived from exponential curve fitting of *aba1-1* and *aba3-1* (Supplemental Fig. S3C). This was an unexpected but not unique result: Assmann et al. (2000) found that *aba1-1* plants showed a greater than wild-type stomatal response to an increase in leaf-air vapor pressure difference from 0.4 to 0.7 kPa. Since submission of the original version of this manuscript, it was reported that guard cell-autonomous ABA biosynthesis is important for low humidity-induced stomatal responses (Bauer et al., 2013). However, our study suggests the alternative model where basal low ABA in *aba3-1* and

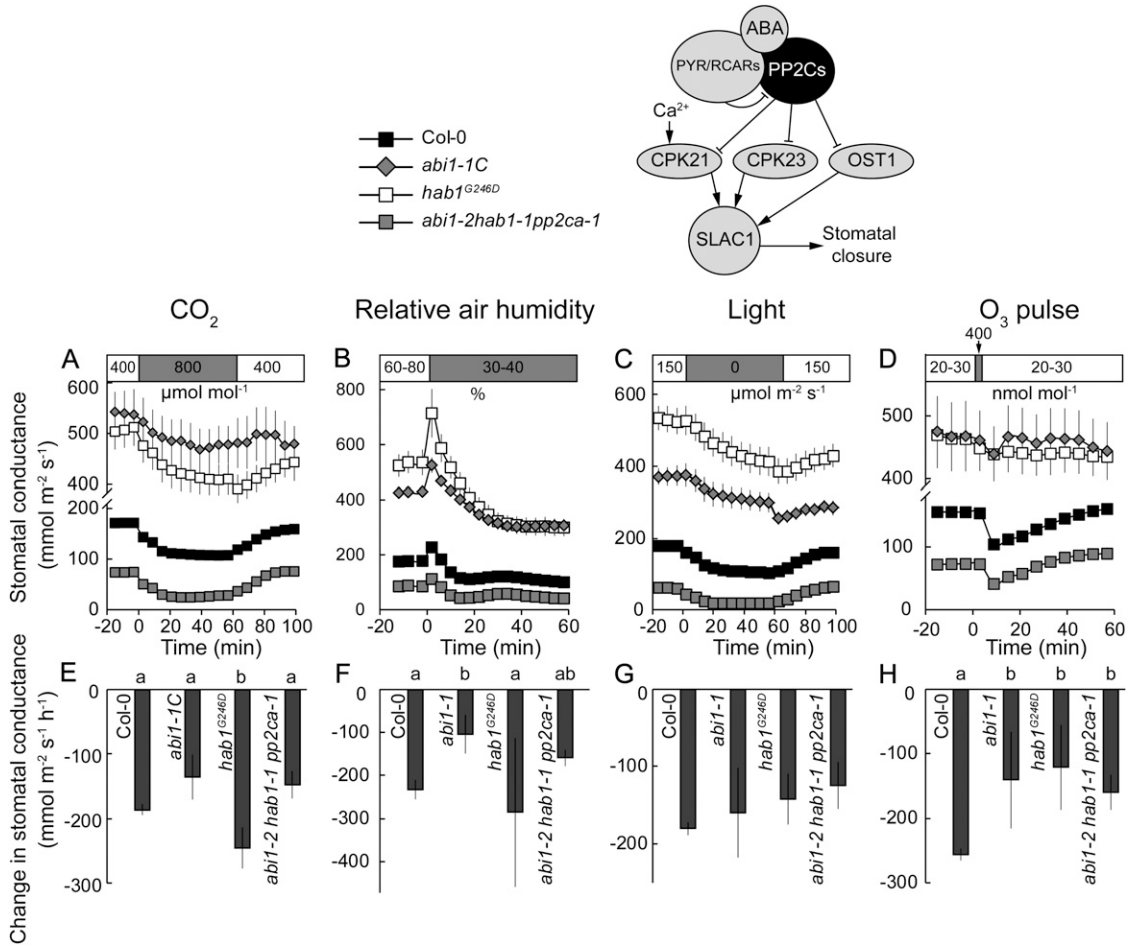


Figure 5. Time courses of g_{st} in response to elevated CO_2 (A), reduced air humidity (B), darkness (C), and O_3 pulse (D) together with corresponding changes in g_{st} values (E–H) in dominant mutants of protein phosphatases ABI1 and HAB1 (*abi1-1C* and *hab1*^{G246D}) and in the triple knockout mutant of ABI1, HAB1, and PP2CA (*abi1-2hab1-1pp2ca-1*). Changes in g_{st} values (E–H) were calculated as shown in Figure 3, A to C. Significant differences ($P < 0.05$; $n = 5–50$) are denoted with different lowercase letters.

aba1-1 may enhance the low humidity-sensitivity of guard cells; possibly through increasing the sensitivity of ABA-signalosome. As compared with ABA-deficient mutants, mutants defective in ABA catabolism contain higher concentrations of ABA and could be predicted to respond more strongly to environmental stimuli. However, the initial changes in g_{st} (Fig. 8) and maximum stomatal closure rates (Supplemental Fig. S3, A–C) of *cyp707a1* and *cyp707a3* were wild type like.

DISCUSSION

Rapid stomatal closure is one of the fastest responses in plant adaptation to sudden changes in environmental conditions. The finding that ABA perception by PYR/RCAR receptors leads to the inhibition of PP2C phosphatases has been a major breakthrough in plant science to understand ABA signaling and the regulation of stomatal aperture by ABA (Ma et al., 2009; Park et al., 2009). With these recent advances, the key question arises whether rapid changes in g_{st} caused by physiological stimuli are affected by defined steps within the ABA signalosome in intact whole plants. Here, we have addressed the relevance of the ABA signalosome (Fig. 1) for the whole-plant steady-state g_{st} and stomatal responses to darkness, reduced air humidity, elevated CO_2, and O_3 (i.e. environmental factors). As this study used a large number of mutants and four different factors, we have summarized the descriptions of mutants into one table (Table I) and their stomatal responses to studied environmental factors into a simplified figure (Fig. 9B) to help readers follow the main results of the study.

Fundamental Role of the ABA Signalosome in Controlling Whole-Plant Steady-State g_{st}

The g_{st} values of the studied mutants varied severalfold. The whole-plant g_{st} values were altered in accordance with the proposed functioning of the ABA

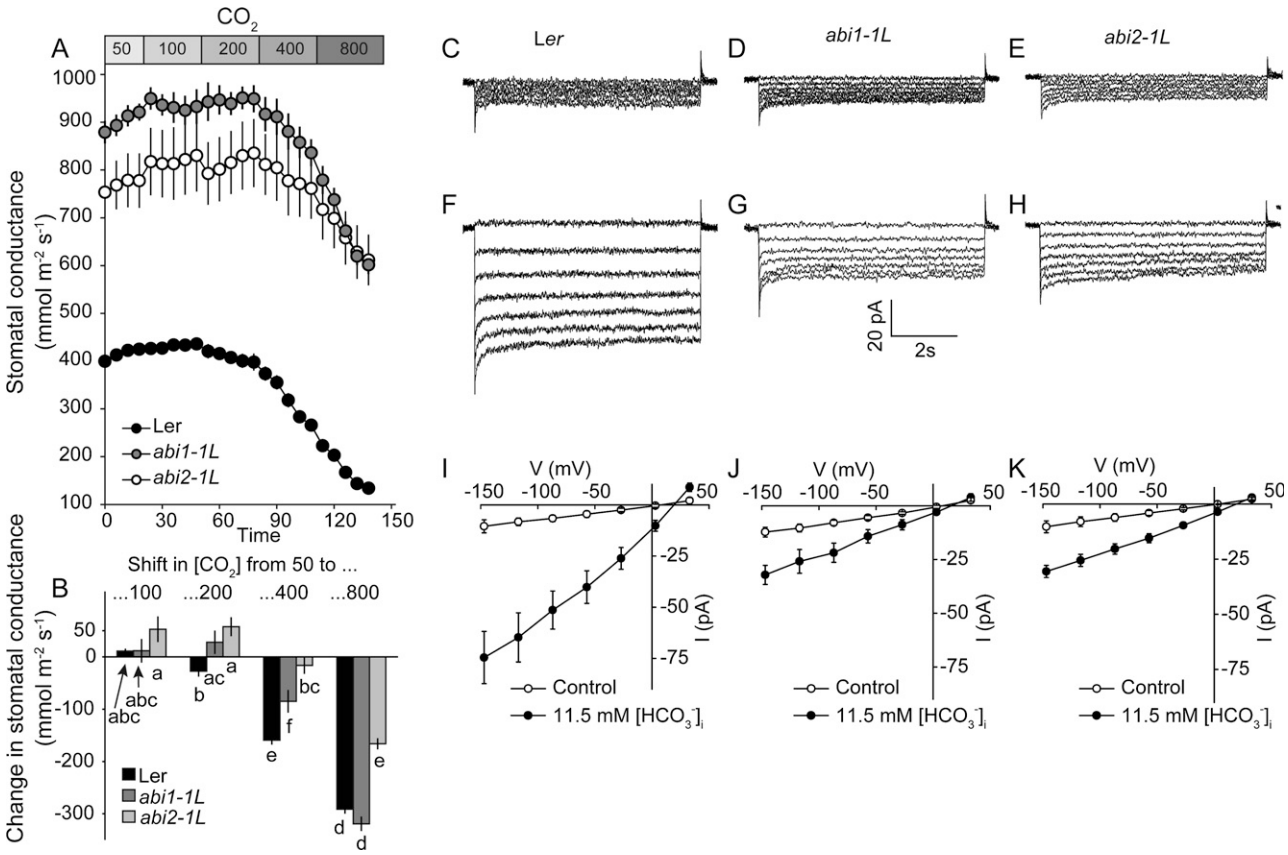


Figure 6. Dominant mutations in ABI1 and particularly in ABI2 phosphatase cause partial impairment of CO_2-induced stomatal responses, whereas bicarbonate-induced activation of S-type anion channels is reduced in both *abi1-1L* and *abi2-1L* guard cell protoplasts. A, Time courses of stomatal conductances in response to stepwise change of CO_2 from 50 to 800 μL L^{-1} in *abi1-1L*, *abi2-1L* and L*er* plants ($n = 6$). B, Changes in stomatal conductance induced by each step of [CO_2]. C, D, E, Typical whole-cell recording without bicarbonate and F, G, H, with 11.5 mM free bicarbonate added to the pipette solution in the guard cell protoplasts of L*er* wild type and *abi1-1L* and *abi2-1L*. Average steady-state current-voltage relationships for L*er* (open circles, $n = 6$; filled circles, $n = 7$), *abi1-1L* (open circles, $n = 6$; filled circles, $n = 7$) and *abi2-1L* (open circles, $n = 5$; filled circles, $n = 8$) guard cell protoplasts are shown in I, J, and K, respectively.

signalosome: reduced ABA concentration, gradually reduced levels of functional PYR/RCAR proteins, the presence of dominant active PP2Cs, and the lack of functional OST1 and SLAC1 resulted in higher g_{st}. Contrarily, reduced levels of functional PP2Cs in *abi1-2hab1-1pp2ca-1* and higher ABA concentrations in *cyp707a3* resulted in lower g_{st} (Fig. 2). Previous gas-exchange experiments have also found that the mutants of the ABA signaling module have high g_{st} (*abi1-1* and *abi1-2* [Leymarie et al., 1998a, 1998b]; *aba1*, *abi1-1*, and *abi2-1* [Assmann et al., 2000]; and *abi1-1*, *abi1-2*, and *ost1-3* [Xue et al., 2011]), whereas the reduced g_{st} of triple loss-of-function PP2C mutants (*hab1-1abi1-2pp2ca-1* and *hab1-1abi1-2abi2-2*) and ABA catabolism mutants (*cyp707a1* and *cyp707a3*) was detected previously by Rubio et al. (2009) and Okamoto et al. (2009), respectively. However, when the stomatal apertures from extracted epidermal fragments have been measured, a larger aperture of open stomatal mutants is not always evident (*ost1-1* and *ost1-2* [Mustilli et al., 2002]; *hab1*^{G246D} [Robert et al., 2006]; *ost1-2* and *abi1-1* [Siegel et al., 2009]; *pyr1pyl1pyl2pyl4* [Nishimura et al., 2010]; and *ost1-3* [Xue et al., 2011]). Earlier and more recent studies indicated that at least part of the stomatal responses to CO_2 and light depend on signals generated by the mesophyll (Lee and Bowling, 1992; Mott et al., 2008). Thus, it is expected that mesophyll or signals from the mesophyll may also play a role in determining the plant steady-state g_{st}.

Nevertheless, it is obvious that plant steady-state g_{st} is not determined only by the signal flow from ABA to OST1 and the SLAC1 anion channel. For example, the g_{st} values of dominant active PP2C mutants were almost two times higher than those of OST1 and SLAC1 loss-of-function mutants. Guard cell plasma membrane H^+-ATPases, activated by phosphorylation, provide the driving force for stomatal opening (for review, see Shimazaki et al., 2007). The basal and blue light-induced phosphorylation of the guard cell H^+-ATPase was higher in *abi1-1* and *abi2-1* than in the wild type (Hayashi et al., 2011),

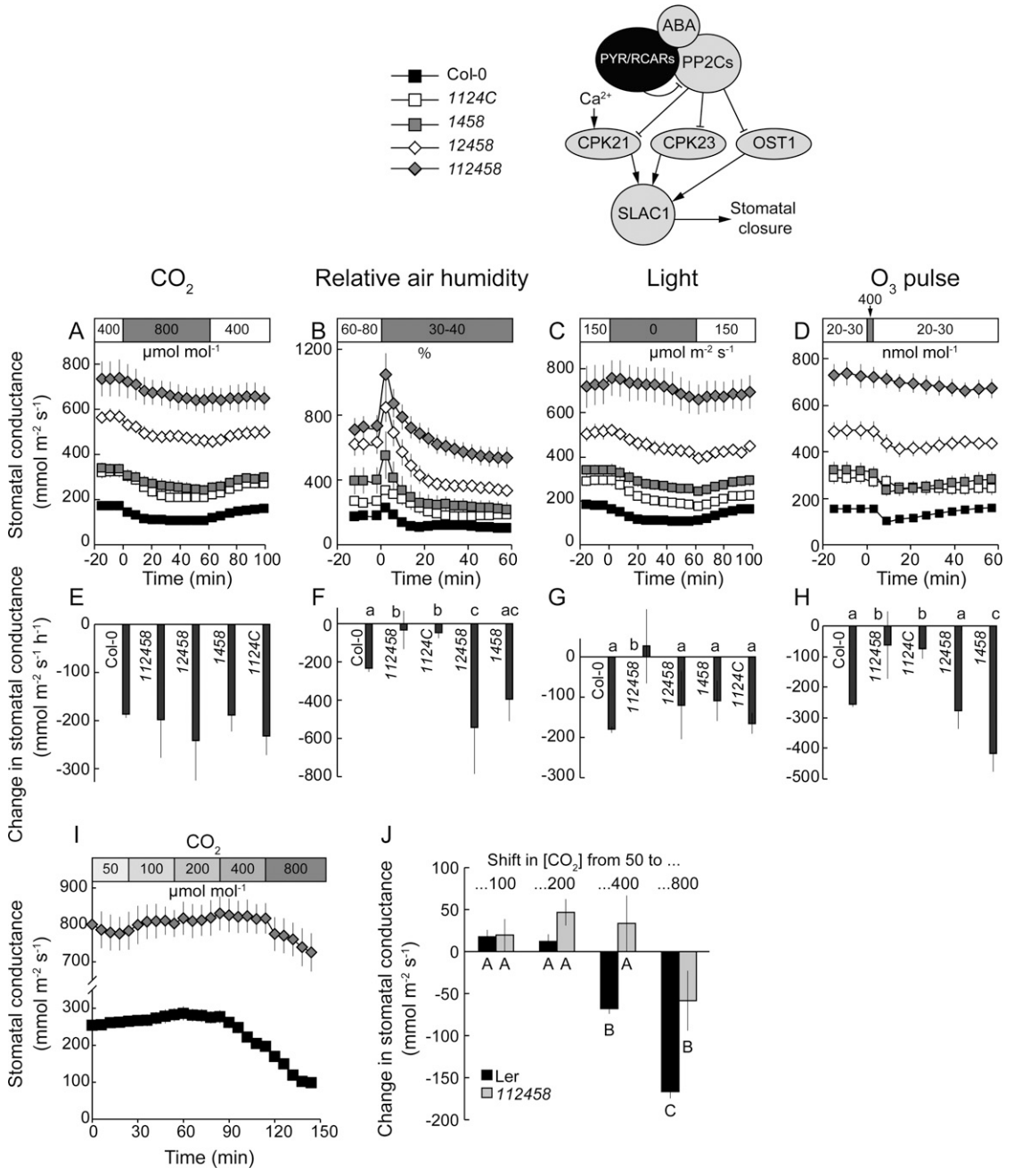


Figure 7. A to H, Time courses of g_{st} in response to elevated CO_2 (A), reduced air humidity (B), darkness (C), and O_3 pulse (D) together with corresponding changes in g_{st} values (E–H) in the loss-of-function mutants of PYR/RCAR receptors. Changes in g_{st} values (E–H) were calculated as shown in Figure 3, A to C. Significant differences ($P < 0.05$; $n = 5–50$) are denoted with different letters. I, Time courses of g_{st} in response to stepwise changes in CO_2 from 50 to 800 μL L^{-1} in *112458* and Col-0 plants ($n = 6$). J, Changes in g_{st} induced by each step of CO_2.

indicating that the higher g_{st} of dominant active PP2C mutants could be caused by their higher H^+-ATPase activity. It remains to be established whether high H^+-ATPase activity also explains the highest g_{st} of plants lacking six PYR/RCAR receptors and the mechanistic connection between the ABA signalosome and the phosphorylation of guard cell H^+-ATPases.

PYR/RCARs and PP2Cs Are Important for O_3-, Humidity-, and Darkness-Induced Rapid Stomatal Regulation and Are Involved in CO_2-Induced Rapid Stomatal Regulation

The *112458* sextuple mutant of PYR/RCAR proteins is one of the most ABA-insensitive mutants described so far, being able to germinate and grow in the presence of 100 μM ABA (Gonzalez-Guzman et al., 2012).

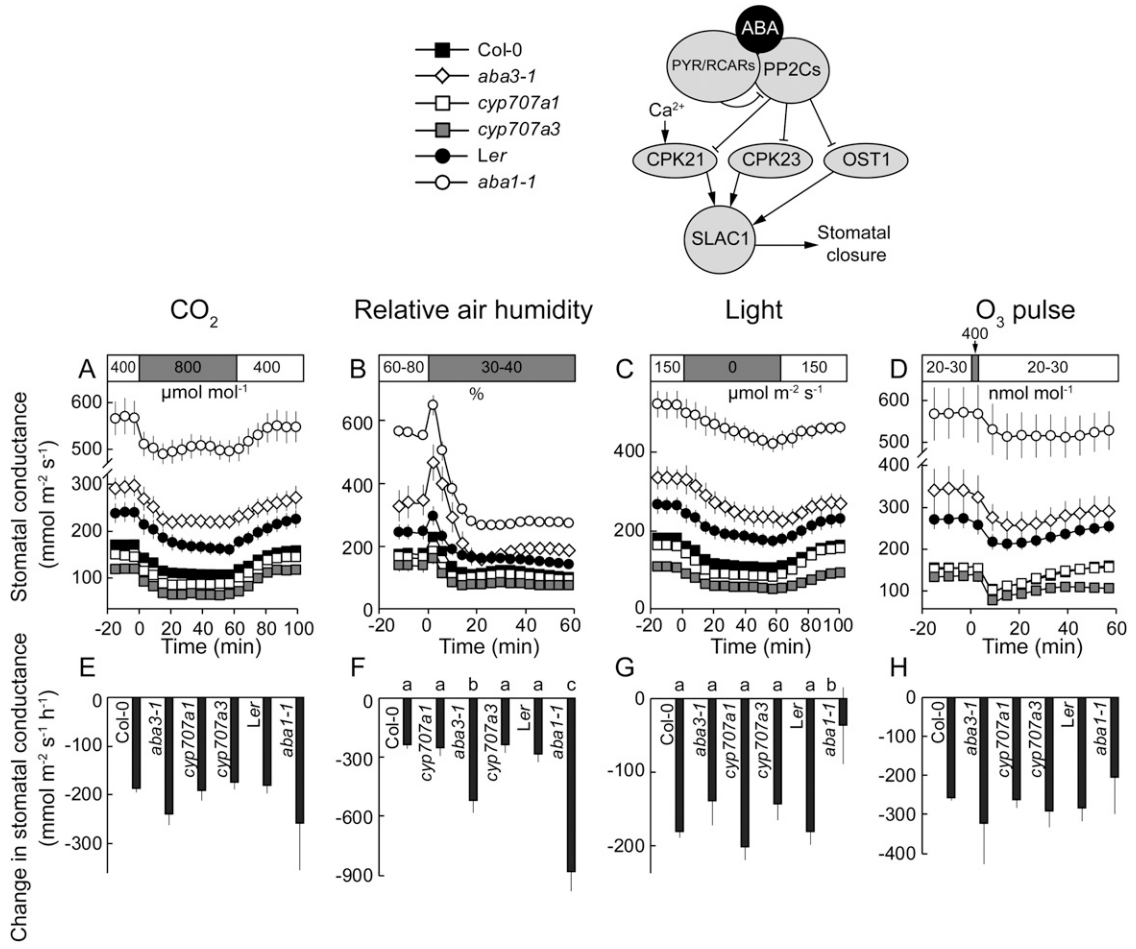
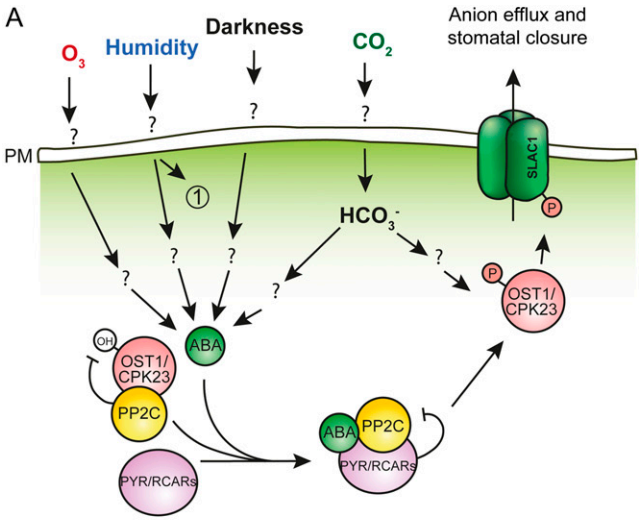


Figure 8. Time courses of g_{st} in response to elevated CO_2 (A), reduced air humidity (B), darkness (C), and O_3 pulse (D) together with corresponding changes in g_{st} values (E–H) in mutants of ABA biosynthesis (*aba1-1* and *aba3-1*) and catabolism (*cyp707a1* and *cyp707a3*). Changes in g_{st} (E–H) were calculated as shown in Figure 3, A to C. Significant differences ($P < 0.05$; $n = 5–50$) are denoted with different lowercase letters.

Strongly reduced darkness-, reduced air humidity-, and O_3-induced stomatal closure of *112458* together with its altered CO_2 response kinetics indicate that PYR/RCAR receptors influence the rapid initiation of stomatal closure by these stimuli. Furthermore, downstream components of the ABA signalosome (ABI2, OST1, and SLAC1) were required, since stomatal responses of these mutants were reduced (Fig. 9B). Only ABA biosynthesis mutants did not fit in, generally showing wild-type-like stomatal closure, but this can be explained by their residual ABA concentrations. Thus, rapid stomatal closure induced by reduced air humidity, darkness, elevated CO_2, and O_3 involves PYR/RCAR-dependent inhibition of PP2Cs, leading to the activation of OST1 and SLAC1.

For O_3 and reduced humidity, the impairment of responses is clear in all key mutants (*slac1-3*, *ost1-3*, *ost1-2*, *abi1-1C*, *abi1-1L*, *abi2-1L*, *112458*, *1124C*, and *1124L*). One possible mechanism contributing to the O_3 response is the direct inhibition of ABI1 (Meinhard and Grill, 2001) and ABI2 (Meinhard et al., 2002) by hydrogen peroxide. However, in darkness and particularly CO_2, stomatal closure was often evident in key mutants (except *slac1-3*, *ost1-2*, and *ost1-3*), and only further experiments and analysis of fast kinetics revealed that the closure response was impaired. The partial response of PYR/RCAR mutants can be explained by redundancy among 14 PYR/RCAR proteins, whereas regulation of the remaining PP2Cs by ABA and PYR/RCARs in *abi1-1C*, *abi2-1C*, *hab1*^{G246D}, *abi1-1L*, and *abi2-1L* (Szostkiewicz et al., 2010) can explain partial CO_2- and darkness-induced stomatal responses of dominant active PP2C mutants. An alternative explanation is that in response to CO_2 and darkness, there might be an ABA-PYR/RCAR-PP2C-independent pathway for OST1 activation. In addition to ABA-dependent activation, ABA-independent activation of OST1 might be induced by osmotic and low-humidity stress (Xie et al., 2006; Yoshida et al., 2006; Boudsocq et al., 2007). Furthermore, many OST1-inducible genes are not responsive to ABA (Zheng et al., 2010).



B

Reduced stomatal closure				Genotype (Col-0)	Enhanced stomatal closure		
D++	H++	CO_2++	O_3+	*slac1-3*			
D++	H++	CO_2++	O_3+	*ost1-3*			
D++	H+	CO_2++	O_3+	*slac1-7*			
D++	H++	CO_2+	O_3+	*112458*			
	H++		O_3+	*1124C*			
D++	H++	CO_2+	O_3+	*cpk23*			
			O_3+	*abi1-2hab1-1pp2c-1*			
	H+	CO_2+	O_3+	*abi1-1C*			
			O_3+	*hab1*^{G246D}		CO_2	
				458	H	CO_2	O_3
				114	D		O_3
				145		CO_2	O_3
				aba3-1	H		
				1458			O_3
				12458	H		
				Genotype (Ler)			
D++	H++	CO_2++	O_3+	*ost1-2*			
D+	H++		O_3+	*abi1-1L*			
D+	H++	CO_2+	O_3+	*abi2-1L*			
D+	H++		O_3+	*1124L*			
D++		CO_2+		*aba1-1*	H		

Figure 9. Schematic model for environmental factor-induced stomatal closure (A) and summary of the stomatal responses of mutants characterized in this study (B). A, The emerging model suggests that in the case of O_3, all components of the ABA signalosome are required to trigger stomatal closure. For reduced air humidity, we show that the ABA signalosome plays an important role; however, the presence of a parallel, ABA-independent pathway (marked as 1) was suggested by Assmann et al. (2000) and Xie et al. (2006). Darkness-induced stomatal closure is mediated by increased intercellular CO_2 concentration that activates anion channels and by inactivation of H^+-ATPase (Roelfsema et al., 2002; Shimazaki et al., 2007). CO_2-induced stomatal closure involves the activation of carbonic acid anhydrases that convert CO_2 to HCO_3^-. The results presented in this study suggest that CO_2-induced stomatal closure and HCO_3^--induced activation of S-type anion channels are partly controlled by the ABA signalosome. Question marks highlight that the nature of signal perception at the plasma membrane and signal transduction in the cytosol leading to the activation of the ABA signalosome remain to be addressed. B, Summary of stomatal responses to closure-inducing stimuli in the studied mutants. Mutants are presented in the order of phenotypic severity. H and D indicate air humidity and darkness, respectively. + indicates that either initial change in g_{st} or curve fitting was different from the wild type, and ++ indicates that initial change in g_{st} and curve fitting were both different from the wild type. The absence of a symbol indicates wild-type-like stomatal closure. Mutants that showed wild-type-like stomatal responses to all stimuli (*148*, *cpk21*, *cyp707a1*, and *cyp707a3*) are not listed here. [See online article for color version of this figure.]

Partial inhibition of HCO_3^--induced activation of S-type anion currents in *abi1-1L* and *abi2-1L* guard cells, together with the result that the obtained CO_2-induced stomatal closure patterns of *abi1-1C* and *abi2-1L* could not be described with an exponential function, indicate that these dominant active phosphatases can affect the stomatal response to CO_2 (Fig. 6; Table II). It is of particular interest that the delay in CO_2-induced stomatal closure was clearly stronger in *abi2-1L* than in *abi1-1L* (Fig. 6B). Recently, a new regulator, GHR1, involved in ABA- and hydrogen peroxide-induced activation of SLAC1 was identified (Hua et al., 2012). GHR1, a receptor-like kinase preferentially localized in guard cell plasma membranes, was shown to activate SLAC1 anion currents in *X. laevis* oocytes (Hua et al., 2012). Interestingly, GHR1 is regulated by ABI2 but not by ABI1 (Hua et al., 2012). Thus, the differential responses of *abi1-1L* and *abi2-1L* could be a result of their different roles in the regulation of GHR1.

OST1 and SLAC1 Are Important for Stomatal Closure by All Four Stimuli, But There Are Additional Components Whose Exact Roles in Plant Stomatal Regulation Remain to Be Clarified

CO_2- and O_3-induced stomatal closures were small, whereas darkness- and humidity-induced closures were delayed but still functional in SLAC1 loss-of-function plants (Negi et al., 2008; Vahisalu et al., 2008; Ache et al., 2010; Xue et al., 2011; this study). There are other anion channels that participate in stomatal closure together with SLAC1, including the membrane voltage-dependent rapid-type anion channel QUAC1 (Meyer et al., 2010) and SLAH3, another slow-type anion channel that is activated in *X. laevis* oocytes via phosphorylation by CPK21 (Geiger et al., 2011). It remains to be determined why, then, SLAC1 has a vital role in regulating CO_2- and O_3-induced stomatal closure (Fig. 4, A and D), whereas in humidity and darkness responses, QUAC1 or possibly SLAH3 could partly replace SLAC1 function (Fig. 4, B and C). Perhaps since darkness and drought, with accompanying decreases in air humidity, are important environmental factors that have affected plants in the evolutionary time scale, plants have developed parallel signaling pathways and ion channels mediating rapid stomatal closure in response to these

factors. Furthermore, darkness-induced stomatal closure is accomplished through two different signaling pathways. First, it is mediated via phototropins and H^+-ATPase (Shimazaki et al., 2007), since blue light, which activates phototropins, is part of visible light spectrum. Second, CO_2 is an intermediate signal in the darkness response; photosynthesis immediately stops in darkness, resulting in increased intercellular CO_2 concentration and activation of anion channels (Roelfsema et al., 2002). Thus, the partial darkness response of SLAC1 and OST1 loss-of-function mutants could be caused by signaling via phototropins and H^+-ATPase, while the CO_2-mediated signaling remains inactive.

It is also important to consider that in all studied ABA signalosome mutants, compensatory changes, either directly or indirectly related to the signalosome itself, could have taken place and affected the whole-plant stomatal response. For example, the regulation of PP2C activity can become less or more sensitive to ABA, depending on the PP2C:PYR/RCAR ratio (Szostkiewicz et al., 2010), representing compensation within the ABA signalosome. Besides guard cell anion channels, the ABA signalosome also regulates the activity of guard cell potassium channels (Armstrong et al., 1995; Sato et al., 2009). Recently, it was shown that plants with impaired SLAC1 have slowed stomatal opening in response to various stimuli and that this is caused by strongly reduced inward-rectifying K^+ channel activity of *slac1* mutants (Laanemets et al., 2013). These unexpected phenotypes of *slac1* turned out to be caused by higher cytosolic pH and Ca^{2+} concentration (Wang et al., 2012) and increased Ca^{2+} sensitivity of inward-rectifying K^+ channels in *slac1* guard cells (Laanemets et al., 2013). These changes represent adaptive changes not directly related to the ABA signalosome and counteract the adverse effects of the *slac1* mutation and allow the plant to maintain control over stomatal openness. Thus, while interpreting the results of this study, it is important to consider that some compensatory changes that also affect stomatal regulation have probably occurred in the studied mutants.

The calcium-dependent protein kinases CPK21 and CPK23 phosphorylate and activate SLAC1 similarly to OST1 (Geiger et al., 2010). The stomata of CPK23 loss-of-function plants showed slightly reduced responses to all environmental factors. Interestingly, Ma and Wu (2007) found that CPK23 acts as a positive regulator for stomatal opening: the stomatal apertures of epidermal peels were significantly decreased in the *cpk23* loss-of-function mutant, resulting in enhanced drought and salt tolerance. In this study, whole-plant g_{st} in the *cpk23* mutant was similar to that in the wild type (Fig. 2A). Phosphorylation by CPK21 was found to activate both SLAC1 (Geiger et al., 2010) and SLAH3 (Geiger et al., 2011). However, no changes in stomatal responses to the studied stimuli were found in the *cpk21* mutant. This may not be not surprising, considering that the family of Ca^{2+}-dependent protein kinases is large, with possible redundancy in their function (Cheng et al., 2002). As an example, CPK3 and CPK6 were found to participate in ABA- and Ca^{2+}-dependent regulation of guard cell S-type anion channels and stomatal closure (Mori et al., 2006). Recently, CPK6 was shown to strongly activate SLAC1-mediated anion currents in *X. laevis* oocytes and to allow the functional reconstitution of ABA activation of SLAC1 (Brandt et al., 2012). Interestingly, Ser-59 in the SLAC1 N terminus was phosphorylated by CPK6, and this phosphorylation is essential for SLAC1 activation (Brandt et al., 2012). Ser-59 was earlier shown to be phosphorylated also by OST1 (Vahisalu et al., 2010). The strong stomatal phenotypes of OST1 loss-of-function plants in the presence of many alternative kinases (CPK3, CPK6, CPK21, and CPK23) activating SLAC1 suggests that interaction of the Ca^{2+}-dependent and Ca^{2+}-independent pathways requires further investigation.

In conclusion, the signaling pathways of different stomatal closure-inducing factors converge at OST1 and SLAC1. In darkness-, O_3-, and reduced air humidity-induced stomatal closure, signaling through PYR/RCAR receptors plays an important role. In response to elevated CO_2, the ABA signalosome was partially involved, and the presence of a parallel, yet to be identified signaling pathway that activates OST1 is possible.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) seeds were planted in soil containing 4:3 (v/v) peat:vermiculite and grown through a hole in a glass plate covering the pot as described (Kollist et al., 2007). Soil moisture was kept at 60% to 80% of maximum water capacity. Plants were grown in growth chambers (AR-66LX and AR-22L; Percival Scientific) in a 12/12-h photoperiod, 23°C/18°C temperature, 150 μmol m^{-2} s^{-1} light, and 70% to 75% relative humidity. For gas-exchange experiments, we used plants with a total rosette area between 5 and 15 cm^2. This corresponds to 21- to 25-d-old plants for most mutant lines. However, some mutants (*aba1-1*, *aba3-1*, *abi1-1C*, and *hab1*^{G246D}) had slower growth rates; thus, older plants (26–32 d old) were analyzed. The full list of the mutants used is given in Table I. Mutants were obtained from the European Arabidopsis Stock Centre (www.arabidopsis.info) and from Sean Cutler (*114*, *1124C*, and *1124L*). The mutants *cpk21* (GABI_322A03), *cpk23* (SALK_007958; Geiger et al., 2010), *cyp707a1* (SALK_069127; Okamoto et al., 2009), and *cyp707a3* (SALK_078173; Okamoto et al., 2009) were confirmed to be homozygous using PCR. The *cpk21* knockout mutant was verified to lack full-length transcripts for *CPK21* using reverse transcription-PCR (Supplemental Fig. S8).

Whole-Rosette g_{st} Measurements

The Arabidopsis whole-rosette rapid-response gas-exchange measurement device was described previously (Kollist et al., 2007; Vahisalu et al., 2008). Plants were inserted into the device, and the treatments started about 2 h later, when g_{st} had stabilized. Photographs of plants were taken before the experiment, and rosette leaf area was calculated using ImageJ 1.37v (National Institutes of Health). g_{st} for water vapor was calculated with a custom-written program as described by Kollist et al., (2007).

In light-dark transition experiments, the g_{st} values were first measured in light, and then darkness was applied for 60 min by covering the measuring cuvettes. In CO_2 enrichment experiments, plants were kept in ambient CO_2 concentration (400 μL L^{-1}) until g_{st} was stable, and then CO_2 concentration was increased to 800 μL L^{-1} for 60 min. In darkness and elevated CO_2 experiments, we also followed the reopening of stomata when light and ambient CO_2 were restored. In O_3 experiments, plants were exposed to 350 to 450 nL L^{-1} O_3 for 3 min and kept in measuring cuvettes for 60 min after exposure. In

reduced humidity experiments, plants were kept in humid air (relative humidity = 60%–80%), air humidity was abruptly reduced about two times (relative humidity = 30%–40%), and g_{st} was followed for the next 56 min.

We present the time-resolved stomatal responses to stimuli in absolute units. Although the course of stomatal reopening in light and ambient CO_2 is not discussed, it is presented in the figures for darkness and CO_2 experiments. In order to provide a quantitative value for the initial stomatal responsiveness to applied stimuli, we calculated changes of g_{st} as described in Figure 3, A–C. Furthermore, we fitted the patterns of g_{st} values in response to darkness, elevated CO_2, and reduced air humidity with exponential functions [$g_{st} = \alpha \times \exp(\beta \times \text{time}) + \gamma$]. Fit was accepted as significant when both α and β were significantly different from 0 at $P < 0.1$; in this case, we calculated maximum stomatal closure rate as $\alpha \times \beta$ (Fig. 3D).

Electrophysiology

Arabidopsis guard cell protoplasts were isolated as according to Siegel et al. (2009). Whole-cell patch-clamp recordings were performed as described previously (Pei et al., 1997). For S-type anion current recordings (Schroeder and Keller, 1992), the pipette solution contained 150 mM CsCl, 2 mM $MgCl_2$, 6.7 mM EGTA, 5.86 mM $CaCl_2$ (2 μM intracellular [Ca^{2+}]), 5 mM Mg-ATP, and 1 mM HEPES-Tris, pH 7.1. The bath solution contained 30 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM MES-Tris, pH 5.6. Osmolalities of all solutions were adjusted to 485 mmol kg^{-1} for bath solution and 500 mmol kg^{-1} for pipette solution by the addition of D-sorbitol. The membrane voltage was stepped from +35 mV to −145 mV with −30-mV decrements, and the holding potential was +30 mV. Liquid junction potential was determined using Clampex 10.0. No leak subtraction was applied for all current-voltage curves. Steady-state currents were the average currents during the last 500 ms of voltage pulses. For HCO_3^- activation of S-type anion currents, 13.5 mM total HCO_3^- (equivalent to 11.5 free intracellular [HCO_3^-] and 2 mM free intracellular [CO_2]) was added freshly in the pipette solution. The details were described previously (Xue et al., 2011).

Statistical Analysis

Statistical analyses were performed with Statistica, version 7.0 (StatSoft). ANOVA (General Linear Models) was used to assess the effect of genotype on g_{st}, initial changes in g_{st}, and maximum stomatal closure rates, and comparisons between individual means were done using Fisher's LSD test. Data were ln transformed when necessary. All effects were considered significant at $P < 0.05$. Exponential fitting of stomatal closure responses due to darkness, elevated CO_2, and reduced air humidity was done with nonlinear least-squares model estimation of Statistica (Gauss-Newton estimation method). PCA was used to combine the values of g_{st} after 1 h in darkness, elevated CO_2, and reduced humidity into one PCA axis describing whole-plant g_{st} after application of given stimuli.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Representative photographs of mutants and wild types.

Supplemental Figure S2. Time-courses of g_{st} in response to stimuli and initial changes in g_{st} values of *ost1-2* and L*er*.

Supplemental Figure S3. Maximum stomatal closure rates derived from exponential fitting of stomatal closure responses.

Supplemental Figure S4. Time-courses of g_{st} in response to stimuli and initial changes in g_{st} values of *abi1-1L*, *abi2-1L* and L*er*.

Supplemental Figure S5. Time-courses of leaf g_{st} in response to elevated CO_2 in *abi1-1* and *abi2-1* in Col-0 and L*er* backgrounds.

Supplemental Figure S6. Time-courses of g_{st} in response to stimuli and initial changes in g_{st} values of *1124L* and L*er*.

Supplemental Figure S7. Time-courses of g_{st} in response to stimuli and initial changes in g_{st} values of triple loss-of-function mutants of PYR/RCARs.

Supplemental Figure S8. The *cpk21* T-DNA mutant is a transcriptional knockout.

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