Running head: Environmental Factor-Induced Stomatal Closure

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PYR/RCAR Receptors Contribute to Ozone-, Reduced Air Humidity-, Darkness- and CO₂-Induced Stomatal Regulation

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One-sentence summary: Signaling through abscisic acid PYR/RCAR receptors plays a fundamental role in controlling whole-plant stomatal conductance and affects plant stomatal closure in response to low air humidity, darkness, O₃ pulse and elevated CO₂.
Footnotes

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ABSTRACT

Rapid stomatal closure induced by changes in the environment, such as elevation of CO₂, reduction of air humidity, darkness and pulses of air pollutant ozone (O₃), involves the SLAC1 anion channel. SLAC1 is activated by OST1 and Ca²⁺-dependent protein kinases. OST1 activation is controlled through abscisic acid (ABA)-induced inhibition of PP2C protein phosphatases by PYR/RCAR receptor proteins. To address the role of signaling through PYR/RCARs for whole-plant steady-state stomatal conductance (gₛ) and stomatal closure induced by environmental factors, we used a set of *Arabidopsis thaliana* mutants defective in ABA metabolism/signaling. The gₛ values varied several fold 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 O₃. Furthermore, PYR/RCAR proteins seem to function in a dose-dependent manner and there is a functional diversity among them. Although rapid stomatal response to elevated CO₂ was evident in all but slac1 and ost1 mutants, the bicarbonate-induced activation of S-type anion channels was also reduced in dominant active PP2C mutants, abi1-1 and abi2-1. Further experiments with wider range of CO₂ concentrations and analyses of stomatal response kinetics suggested that ABA signalosome affects the CO₂–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 O₃ and are involved in responses to elevated CO₂.
INTRODUCTION

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 CO₂ enrichment. Ozone (O₃), 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 & Littlefield 1969; Vahisalu et al., 2010). The key endogenous factor triggering stomatal closure in response to drought is the plant hormone abscisic acid (ABA). In 1997, Webb and Hetherington suggested that the pathways of ABA- and CO₂- induced closure converge i.e. there is an economy in signaling pathways leading to the promotion of stomatal closure (Webb and Hetherington, 1997). 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, activation and inactivation of guard cell ion channels and transporters are the primary targets of signaling networks controlling stomatal movements (for recent reviews 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 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), e.g. ABI1, ABI2, HAB1 and PP2CA and concomitant activation of Snf1-related subfamily 2 protein kinase SnRK2.6/OST1 (Park et al., 2009; Ma et al., 2009; Umezawa et al., 2009; Vlad et al., 2009; Nishimura et al., 2010; Weiner et al., 2010; Dupeux et al. 2011a; 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, CO₂, humidity and O₃ 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 side by side comparison of whole-plant stomatal responses of plants where various proteins of the ABA signalosome are mutated (Fig. 1; Table 1).

Stomatal responses of several of these mutants to environmental factors have been studied earlier, however, often with different results. For example, initial in vitro studies indicated that OST1 was not involved in CO2-induced stomatal signaling as the stomata of ost1-1 and ost1-2 behaved as wild-type (WT) in response to low CO2 (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 CO2-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, CO2- and light-induced stomatal signaling pathways converge. Stomatal opening induced by light and CO2 removal 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 CO2, whereas the stomatal closure was indistinguishable from WT in ABA-deficient plants.

The involvement of the ABA signalosome in the regulation of stomatal response to reduced air humidity is also disputed. During a genetic screen for mutants involved in 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 WT stomatal responses to reduced humidity (Assmann et al., 2000). Since partial response to low humidity is observed in all studied mutants of ABA signalosome, an 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 epidermes 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 the present study, we used a custom-made gas exchange device with parallel recording of stomatal responses of up to eight Arabidopsis plants to various environmental factors (Kollist et al., 2007; Vahisalu et al., 2008; Vahisalu et al., 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 CO₂ and O₃ concentrations. We found that the ABA signaling through PYR/RCARs is clearly required for rapid stomatal closure in response to darkness, O₃ and reduced air humidity, while it is also involved in stomatal responses to elevated CO₂. 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 CO₂.

RESULTS

To study the role of the ABA signalosome in the regulation of stomatal responses to darkness, CO₂, reduced humidity and O₃, 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, Col-0 and Ler, were used in parallel (Table 1). Representative photos of plants used for gas-exchange measurements (Supplementary Fig. S1) show that even mutants with high gs were healthy and non-wilted in our growth conditions.

ABA signaling through PYR/RCAR receptors plays a fundamental role in controlling plant steady-state stomatal conductance

Whole-rosette stomatal conductance (gs) varied several fold among the studied mutants, ranging from 74 mmol m⁻²s⁻¹ in a triple loss-of-function mutant of ABI1, HAB1 and PP2CA, abi1-2hab1-1pp2ca-1, to 683 mmol m⁻²s⁻¹ in sextuple PYR/RCAR loss-of-function mutant,
pyr1pyl1pyl2pyl4pyl5pyl8, abbreviated as 112458 (Fig. 2A). The difference in g_st between two WTs, Ler and Col-0, was 1.4-fold. The g_st values of 112458, PYR/RCAR pentuple pyr1pyl1pyl2pyl4pyl5pyl8 (12458) and dominant active PP2C mutants (abi1-1L, abi2-1L in Ler background and abi1-1C, hab1G246D in Col-0 background) were the highest, followed by ABA-deficient aba1-1 and aba3-1 (Fig. 2A). The hab1G246D 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., 2011a). 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 WT ABA in stressed leaves, respectively (Rock and Zeevart 1991; Leon-Klooosterziel 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, pyr1pyl1pyl2pyl4pyl5pyl8 (1458) and pyr1pyl1pyl2pyl4 (note that we present data for this mutant in Col-0 and in Ler background, abbreviated as 1124C and 1124L, respectively) also exhibited significantly higher g_st than the corresponding WTs. 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 serine 120 in SLAC1 is critical for channel activation in oocytes (Geiger et al., 2009) and in O3-induced plant stomatal response (Vahisalu et al., 2010). However, the g_st of slac1-7, with a S120F point-mutation, was similar to that of Col-0. CPK21 and CPK23 are other kinases shown to activate SLAC1 in oocytes (Geiger et al., 2010), however g_st of cpk21 and cpk23 did not differ from that of WT plants (Fig. 2A). Together these results unequivocally demonstrate that mutations in ABA signalosome have a major effect on the whole-plant stomatal conductivity and loss of water. 

To address whether the differences in stomatal conductance persisted after application of 1hr darkness, elevated CO2 and reduced humidity, we combined g_st values of all mutants into one Principal Component Analysis (PCA) axis. This PCA axis describes stomatal conductance 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 pre-stimuli 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 stomatal conductance as \((g_{st2}-g_{st1})/(t_2-t_1)\), as shown in Fig. 3A-C. Since \(t_2-t_1\) is similar time interval for all mutants (Fig. 3A-C), this number describes 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 WT 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 calculated (Fig. 3D). However, several mutants either did not have clear stomatal response to applied stimuli or their stomatal responses did not reach stable phase within the timeframe experiments were carried out; i.e. slac1-3 and ost1-3 in Fig. 4A and C as an 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. Results summarizing the results of exponential fitting are shown in Table 2 and Supplemental Fig. 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 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 stomatal conductance were significantly lower than in WT (Fig. 4; Supplemental Fig. S2). In all genotypes, an initial sudden increase in stomatal conductance was detected after transition from humid to dry air. This is caused by a rapid increase in water evaporation from the epidermal cells and concomitant decrease of their pressure on guard cells (Ivanoff 1928). Due to this effect and extremely slow closing response, the \(g_{st}\) of OST1 loss-of-function mutants remained higher after one hour in dry air compared to 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. 4F 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 S120 was important for SLAC1 activation (Fig. 4) (Geiger
et al., 2009; Vahisalu et al., 2010). However, both the WT-like absolute stomatal conductance
(Fig. 2A) and clearly weaker phenotype in CO₂- and O₃-induced stomatal responses of slac1-7
compared to those observed in slac1-3 (Fig. 4, E and H) suggest that phosphorylation of S120
does not fully explain the activation mechanism of SLAC1. The maximum stomatal closure rate
of slac1-7, derived by fitting the kinetics of CO₂-induced stomatal closure with exponential
function, yielded values lower than in WT (Supplemental Fig. S3B). These results confirm that in
addition to O₃-induced stomatal closure (Vahisalu et al., 2010), SLAC1 serine 120 is the target
for OST1 in CO₂-, reduced humidity- and darkness-induced responses as well, but also suggest
that for a full SLAC1 activation, phosphorylation of multiple serines either by OST1 or in
combination with other protein kinases shown to activate SLAC1 in Xenopus 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, Ca²⁺-related protein kinases shown to activate
SLAC1 in oocytes (Geiger et al., 2010), cpk21 and cpk23 plants were used. The stomata of
cpk21 responded to the studied stimuli like WT, whereas the initial changes of stomatal
conductance 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 2
and 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.

**Type 2C Protein phosphatases (PP2Cs) are important for rapid O₂- and humidity-induced
stomatal closure, but less so for darkness- and CO₂-induced closure**

Type 2C protein phosphatases function as negative regulators in ABA-induced stomatal closure
by inhibiting the OST1- and CPKs-induced activation of SLAC1 (Geiger et al., 2009, Geiger et
al., 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 hab1G246D
mutations that prevent ABA-dependent inhibition of PP2Cs by PYR/RCAR receptors. Humidity- and O₃-induced stomatal responses and initial changes of stomatal conductance were reduced in abi1-1C, (Fig. 5B,F,D,H), abi1-1L and abi2-1L (Supplemental Fig. S4B,F,D,H) as compared to their WTs, whereas in hab1G246D, only the O₃-response was reduced (Fig. 5, D and H). Thus, for O₃- and humidity-induced stomatal closure, the inhibition of PP2C activity is important.

The role of PP2Cs in CO₂- and darkness-induced stomatal closure was less clear. The initial changes in stomatal conductance of dominant abi1-1C, hab1G246D mutants in Col-0 background (Fig. 5A,E,C,G) and similarly those of abi1-1L and abi2-1L in Ler background (Fig. S4A,E,C,G) were generally WT-like. Similar results were obtained for plants with abi1-1 and abi2-1 mutations in Col-0 and in Ler background when analyzing elevated CO₂-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 darkness responses of abi1-1L and abi2-1L and CO₂-responses of abi1-1C and abi2-1L (Table 2), suggesting that the closure responses of these mutants were altered/different from their WTs.

To further address the role of ABI1 and ABI2 phosphatases for CO₂-induced stomatal regulation, two additional experiments were performed. First, in a separate gas-exchange experiment, abi1-1L, abi2-1L and Ler stomatal responses were tested within a wider range of CO₂ concentrations. Plants were first acclimatized under 50 μL L⁻¹ of CO₂ until stable gₛ values were reached (75-100 min). Thereafter CO₂ concentration was increased stepwise to 100, 200, 400 and 800 μL L⁻¹ by 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 CO₂ concentration were determined, differences between the genotypes emerged (Fig. 6B). There was no reduction in stomatal conductance within CO₂ range from 50 to 100 μL L⁻¹. Furthermore, from 50 to 200 μL L⁻¹, a decrease in stomatal conductance was observed only in Ler. Change from 50 to 400 μL L⁻¹ of CO₂ caused a closure response in all genotypes, however, the decrease in gₛ 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 CO₂-induced decrease in stomatal conductance was similar in Ler and in abi1-1L, but significantly lower in abi2-1L. Secondly, recent research showed that β-carbonic acid anhydrases function early in CO₂-induced stomatal closure (Hu et al. 2010) and that bicarbonate (HCO₃⁻) 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 role of ABI1 and ABI2 in CO₂–induced stomatal signaling, HCO₃⁻-induced activation of S-type anion currents

11
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. 6C-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 responsiveness to environmental factors; sextuple *112458* PYR/RCAR mutant showed the strongest effect and displayed significantly impaired stomatal responses (Fig. 7, A-D) and reduced initial changes of stomatal conductance 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 2) suggesting that the lack of these proteins modified 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 to WT particularly at lower CO$_2$ concentrations (Fig. 7I-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 WT depended on the applied stimuli and the combination of PYR/RCAR mutations. For example, O$_3$- and humidity-induced initial changes of stomatal conductance in quadruple, *1124C* (Fig. 7, F and H) and *1124L* mutants (Supplemental Fig. S6, F and H), were significantly reduced, whereas *1458* quadruple and even *12458* pentuple mutants showed similar or even higher than WT 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 WT (Gonzales-Guzman et al. 2012), generally showed initial changes of stomatal conductance that were WT-like or even larger than in WT (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 WT.

**Elevated CO₂, reduced air humidity, darkness and O₃-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 stomatal conductance in *aba1-1* and *aba3-1* did not differ from *Ler* and Col-0 WTs (Fig. 8 E, G, H). The only exception was significantly lower darkness-induced stomatal closure of *aba1-1* than its *Ler* WT (Fig. 8G). Furthermore, stomatal kinetics of *aba1-1* did not follow an exponential function in darkness and CO₂ experiments. ABA is most likely essential for proper plant development, and hence no null mutants for ABA biosynthesis have been isolated. Accordingly, residual amounts of ABA in *aba1-1* and *aba3-1* (Rock & Zeevaart 1991; Léon-Kloosterziel et al., 1996; Xie et al., 2006) could be sufficient to activate the ABA signaling since physiologically active IC₅₀ values of ABA for PP2C inhibition are in the nanomolar range (Szostkiewicz et al., 2010).

Surprisingly, the initial reductions in stomatal conductance due to reduced humidity of ABA biosynthesis mutants were significantly larger than those of respective WTs (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 greater-than-wild-type stomatal response to an increase in leaf-air vapour pressure difference from 0.4 to 0.7 kPa. As compared with ABA-deficient mutants, mutants defective in ABA catabolism contain higher concentration of ABA and could be predicted to respond more strongly to environmental stimuli. However, the initial changes in stomatal conductance (Fig. 8) and maximum stomatal closure rates (Supplemental Fig. S3A-C) of *cyp707a1* and *cyp707a3* were WT-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 stomatal
conductance 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 stomatal conductance ($g_{st}$) 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 1) and their stomatal responses to studied environmental factors into a simplified figure (Fig. 9B) to help readers to follow the main results of the study.

**Fundamental role of ABA signalosome in controlling whole-plant steady-state stomatal conductance**

The $g_{st}$ values of the studied mutants varied several fold. The whole-plant stomatal conductances were altered in accordance with the proposed functioning of the ABA 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 ABA signaling module have high stomatal conductance (*abi1-1* and *abi1-2*, Leymarie et al. 1998a, 1998b; *aba1*, *abi1-1* and *abi2-1*, Assmann et al. 2000; *abi1-1*, *abi1-2* and *ost1-3*, Xue et al. 2011), whereas reduced stomatal conductance of triple loss-of-function PP2C mutants (*hab1-1abi1-2pp2ca-1*, *hab1-1abi1-2abi2-2*) and ABA catabolism mutants (*cyp707a1*, *cyp707a3*) was previously detected by Rubio et al. (2009) and Okamoto et al. (2009), respectively. However, when the stomatal apertures from extracted epidermal fragments have been measured, larger aperture of open stomatal mutants is often not clearly 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; *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 & Bowling, 1992; Mott et al., 2008). Thus, it is rather expected that mesophyll or signals from the mesophyll may also play a role in determining the plant steady-state stomatal conductance.

Nevertheless, it is obvious that plant steady-state stomatal conductance 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, provides 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 \textit{abi1-1} and \textit{abi2-1} than in WT (Hayashi et al., 2011), indicating that 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 what is the mechanistic connection between ABA signalosome and phosphorylation of guard cell H\(^{+}\)-ATPase.

**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 \textit{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 \(\mu\)M of ABA (Gonzalez-Guzman et al., 2012). Strongly reduced darkness-, reduced air humidity- and O\(_3\)-induced stomatal closure of \textit{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 WT-like stomatal closures, 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 PP2C phosphatases leading to the activation of OST1 and SLAC1.

For O\(_3\) and reduced humidity, the impairment of responses is clear in all key mutants (\textit{slac1-3, ost1-3, ost1-2, abi1-1C, abi1-1L, abi2-1L, 112458, 1124C, 1124L}). One possible mechanism contributing to the ozone response in these mutants is the direct inhibition of ABI1 (Meinhard & 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 \textit{slac1-3, ost1-2} and \textit{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 remaining PP2Cs by ABA and PYR/RCARs in \textit{abi1-1C, abi2-1C, hab1\(^{G246D}\), abi1-1L} and \textit{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 CO₂ 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).

Partial inhibition of bicarbonate induced activation of S-type anion currents in abi1-1L and abi2-1L guard cells together with the result that obtained CO₂-induced stomatal closure patterns of abi1-1C and abi2-1L could not be described with an exponential function indicates that these dominant active phosphatases are involved in the stomatal response to CO₂ (Fig. 6, Table 2). It is of particular interest that the delay in CO₂-induced stomatal closure was clearly stronger in abi2-1L than in abi1-1L (Fig. 6B). Very recently, a new regulator GHR1 involved in ABA and H₂O₂-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 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**

CO₂- and O₃-induced stomatal closures were small, whereas darkness and humidity-induced closures were delayed but still functional in SLAC1 loss-of-function plants (present study, Negi et al., 2008; Vahisalu et al., 2008; Ache et al., 2010; Xue et al., 2011). 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 oocytes via phosphorylation by CPK21 (Geiger et al., 2011). It remains to be determined why, then, SLAC1 has a vital role in regulating CO₂- and O₃-induced stomatal closure (Fig. 4 A,D), whereas in humidity and darkness responses QUAC1 or possibly SLAH3 could replace SLAC1 function (Fig. 4 B,C). Perhaps since darkness and drought with accompanying decrease in air humidity are important environmental factors that have affected plants in the evolutionary timescale, 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. Secondly, CO₂ is an
intermediate signal in darkness response; photosynthesis immediately stops in darkness,
resulting in increased intercellular CO₂ concentration and activation of anion channels
(Roelfsema et al., 2002). Thus, partial darkness response of SLAC1 and OST1 loss-of-function
mutants could be caused by the signaling via phototropins and H⁺-ATPase, while the CO₂-
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 been taken
place and affect the whole-plant stomatal response. For example, the regulation of PP2C activity
can become less or more sensitive to ABA, depending on PP2C:PYR/RCAR ratio (Szostkiewicz
et al., 2010), representing compensation within 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 K⁺ in channel activity of slac1 mutants (Laanemets et al., 2013). These unexpected
phenotypes of slac1 turned out to be caused by higher cytosolic pH and Ca²⁺ concentration
(Wang et al., 2012) and increased Ca²⁺-sensitivity of K⁺ in 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 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 & 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 the present study, whole-plant stomatal conductance in the cpk23
mutant was similar to that in WT (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 oocytes and to allow functional reconstitution of ABA activation of SLAC1 (Brandt et al., 2012). Interestingly, serine 59 in the SLAC1 N-terminus that was phosphorylated by CPK6 and this phosphorylation is essential for SLAC1 activation (Brandt et al., 2012). S59 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, 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 parallel, yet to be identified, signaling pathway that activates OST1 is possible.

**Material and Methods**

**Plant material and growth conditions**

*Arabidopsis thaliana* seeds were planted in soil containing 4:3 (v:v) peat:vermiculite and grown through a hole in a glass plate covering pot as described (Kollist et al., 2007). Soil moisture was kept at 60-80% of maximum water capacity. Plants were grown in growth chambers (AR-66LX and AR-22L, Percival Scientific, IA, USA) at 12/12 photoperiod, 23/18°C temperature, 150 µmol m\(^{-2}\) s\(^{-1}\) light and 70-80% relative humidity. For gas-exchange experiments, we used plants with total rosette area between 5 and 15 cm\(^2\). This corresponds to 21-25-d-old plants for most mutant lines. However, some mutants (*aba1-1, aba3-1, abi1-1C, hab1\(^{G246D}\)*) had slower growth rate and, thus, older plants (26-32-d-old) were analyzed. Full list of used mutants is given in Table 1. Mutants were obtained from the European Arabidopsis Stock Centre (www.arabidopsis.info) and from Sean Cutler (114, 1124C and 1124L). The *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 with the primers listed in Supplemental Table S3. The cpk21 knockout was verified to lack full length transcripts for CPK21 using RT-PCR (Supplemental Fig. S8).

**Whole-rosette stomatal conductance measurements**

The *Arabidopsis* whole-rosette rapid-response gas exchange measurement device is 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, USA). Stomatal conductance ($g_{st}$) for water vapour was calculated with a custom written program as described in 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 $\mu$L L$^{-1}$) until stomatal conductance was stable and then CO$_2$ concentration was increased to 800 $\mu$L L$^{-1}$ for 60 min. In darkness- and elevated CO$_2$-experiments, we also followed reopening of stomata when light and ambient CO$_2$ were restored. In O$_3$ experiments, plants were exposed to 350-450 nL L$^{-1}$ of ozone for 3 min and kept in measuring cuvettes for 60 min after exposure. In reduced humidity experiment, plants were kept in humid air (RH=60-80%), then air humidity was abruptly reduced about two times (RH=30-40%) and $g_{st}$ followed for 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 present in Figures of darkness and CO$_2$-experiments. In order to provide a quantitative value for the initial stomatal responsiveness to applied stimuli, we calculated changes of stomatal conductance as described in Fig. 3A-B. Furthermore, we fitted the patterns of stomatal conductances 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 $\alpha$ and $\beta$ were significantly different from 0 at the level of significance of $P < 0.1$; in this case, we calculated maximum stomatal closure rate as $\alpha \times \beta$ (see also 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 & Keller, 1992), the pipette solution contained 150 mM CsCl, 2 mM MgCl₂, 6.7 mM EGTA, 5.86 mM CaCl₂ (2 μM [Ca²⁺]), 5 mM Mg-ATP, 1 mM HEPES/Tris, pH 7.1. The bath solution contained 30 mM CsCl, 2 mM MgCl₂, 1 mM CaCl₂ 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 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 bicarbonate activation of S-type anion currents, 13.5 mM total bicarbonate (equivalent 11.5 free [HCO₃⁻] and 2 mM free [CO₂]) were 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 Inc., Tulsa, OK, USA). Analysis of variance (GLM procedure) was used to assess the effect of genotype on gₛ, initial changes in stomatal conductance and maximum stomatal closure rates, comparisons between individual means were done using Fisher 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 CO₂ and reduced air humidity was done with nonlinear least squares model estimation of Statistica (Gauss-Newton estimation method). Principal Component Analysis (PCA) was used to combine the values of gₛ after 1 hr in darkness, elevated CO₂ and reduced humidity into one PCA axis describing whole plant stomatal conductance after application of given stimuli.

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Figure legends

**Figure 1.** A schematic overview of ABA-induced signaling by PYR/RCAR receptors leading to sequestration of type 2C protein phosphatases (PP2Cs), activation of protein kinases OST1, CPK21, CPK23 and subsequent phosphorylation/activation of SLAC1 anion channel that is essential for anion efflux and stomatal closure. Mutants selected for the present study are shown. Detailed description of the mutants is provided in Table 1.

**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 five week old mutants and corresponding WTs. 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 hr 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.

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**Figure 5.** Time courses of stomatal conductances in response to elevated CO$_2$ (A) reduced air humidity (B), darkness (C) and O$_3$ pulse (D) together with corresponding changes in stomatal conductances (E, F, G, H) in dominant mutants of protein phosphatase ABI1 and HAB1 ($abi1^{-1C}$, $hab1^{G246D}$) and in triple knockout mutant of ABI1, HAB1 and PP2CA phosphatases ($abi1^{-}$-
Changes in stomatal conductances (E-H) were calculated as shown in Fig. 3A-C. Significant differences (P<0.05, n = 5-50) are denoted with different small letters.

**Figure 6.** Dominant mutations in ABI1 and particularly in ABI2 phosphatase cause partial impairment of CO₂-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 CO₂ from 50 to 800 μL L⁻¹ in abi1-1L, abi2-1L and Ler plants (n=6). (B) Changes in stomatal conductance induced by each step of [CO₂]. (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 Ler wild type and abi1-1L and abi2-1L. Average steady-state current-voltage relationships for Ler (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.

**Figure 7.** Time courses of stomatal conductances in response to elevated CO₂ (A), reduced air humidity (B), darkness (C) and O₃ pulse (D) together with corresponding change in stomatal conductance (E, F, G, H) in the loss-of-function mutants of PYR/RCAR receptors. Changes in stomatal conductances (E-H) were calculated as shown in Fig. 3A-C. Significant differences (P<0.05, n = 5-50) are denoted with different small letters. (I) Time courses of stomatal conductances in response to stepwise change in CO₂ from 50 to 800 μmol mol⁻¹ in 112458 and Col-0 plants (n=6). (J) Changes in stomatal conductance induced by each step of CO₂.

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**Figure 9.** Schematic model for environmental factors-induced stomatal closure (A) and summary of stomatal responses of mutants characterized in the present study (B). (A) Emerging model suggests that in case of O₃, all components of ABA signalosome are required to trigger stomatal closure. For reduced air humidity, we show that 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 CO₂ concentration that activates anion channels and by inactivation of H⁺-ATPase (Roelfsema et al., 2002; Roelfsema & Hedrich, 2005). CO₂-induced stomatal closure involves activation of carbonic acid anhydrases that convert CO₂ to bicarbonate (HCO₃⁻). Results presented in this study suggest that CO₂-induced stomatal closure and bicarbonate-induced activation of S-type anion channels is partly controlled by 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 ABA signalosome remain to be addressed. (B) Summary of stomatal responses to closure-inducing stimuli in studied mutants. Mutants are presented in the order of phenotypic severity. O₃, H, D and CO₂ indicate environmental factors ozone, air humidity, darkness and CO₂, respectively. “+” indicates that either initial change in stomatal conductance or curve fitting was different from WT; “++” indicates that initial change in stomatal conductance and curve fitting were both different from WT. Absence of symbol indicates WT-like stomatal closure. Mutants that showed WT-like stomatal responses to all stimuli (148, cpk21, cyp707a1, cyp707a3) are not listed in the table.
Table 1. Description of the mutants used in the study.

| Genotype | Mutation | Description | Reference |
|----------|----------|-------------|-----------|
| **ABA biosynthesis and catabolism** | | | |
| aba3-1 (Col-0) | EMS, G-to-A at position 3707 | Defective in the conversion of ABA-aldehyde to ABA, ABA-deficient | Leon-Kloosterziel et al., 1996; Nambara & Marion-Poll 2005 |
| aba1-1 (Ler) | EMS, G to A at position 2139 | Defective in ABA biosynthetic enzyme zeaxanthin epoxidase, strongly ABA-deficient | Rock & Zeevaart 1991; Nambara & Marion-Poll 2005 |
| cyp707a1 (Col-0) | SALK_069127 | Defective in ABA 8'-hydroxylase, responsible for ABA catabolism in guard cells | Okamoto et al., 2006; Okamoto et al., 2009 |
| cyp707a3 (Col-0) | SALK_078173 | Defective in ABA 8'-hydroxylase responsible for ABA catabolism in vascular tissues | Okamoto et al., 2006; 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 (Ler) | 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 (Ler) | 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 |
| hab1G246D (Col-0) | Transgenic line | Overexpression of the HAB1 carrying G246D mutation that prevents binding to PYR/PYL and ABA-insensitivity | Robert et al., 2006; Dupeux et al. 2011a |
| ost1-2 (Ler) | EMS, G to A at position 97 | Point mutation in ABA-activated protein kinase OST1 = srk2e = srk2.6, T-DNA knockout mutation of ABA-activated protein kinase OST1 | Yoshida et al., 2002 |
| ost1-3 (Col-0) | SALK_008068 | | |
| **pyr1pyl4 (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 |
| **pyr4pyl5pyl8 (458) (Col-0)** | T-DNA + Transposon | Triple mutant of ABA receptor proteins | Gonzalez-Guzman et al., 2012 |
| **pyr1pyl1pyl4 (1124C) (Col-0)** | EMS + T-DNA | Quadruple mutant of ABA receptor proteins | Park et al., 2009 |
| **pyr1pyl1pyl4 (1124L) (Ler)** | 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 calcium dependent protein kinase CPK21 | This study (Supplemental Fig. S9) |
| cpk23 (Col-0) | SALK_007958 | Defective in calcium dependent protein kinase CPK23 | Geiger et al., 2010 |
| slac1-3 (Col-0) | SALK_099139 | T-DNA insertion in SLAC1 protein | Vahisalu et al., 2008 |
| slac1-7 (Col-0) | C to T at position 527 | Point mutation of Serine 120 to Phenylalanine in SLAC1 protein | Vahisalu et al., 2010 |
Table 2. The results of fitting exponential functions to stomatal closure patterns in response to darkness, elevated CO2 and reduced air humidity. The first number shows how many closure responses could be described with an exponential function, whereas the second shows the total number of experiments. Bold values indicate cases where 50% of experiments did not follow an exponential function.

| Genotype | Darkness-response | Humidity-response | CO2-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          |
| hab1G246D-U | 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          |
| Ler      | 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          |
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