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Reactive oxygen species, photosynthesis and environment in the regulation of stomata

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ABSTRACT

Significance: Stomata sense the intercellular CO_2 concentration (C_i) and water availability under changing environmental conditions and adjust their aperture to maintain optimal cellular conditions for photosynthesis. Stomatal movements are regulated by a complex network of signaling cascades where reactive oxygen species (ROS) play a key role as signaling molecules. *Recent Advances*: Recent research has uncovered several new signaling components involved in CO_2 and ABA-triggered guard cell signaling pathways. In addition, we are beginning to understand the complex interactions between different signaling pathways. *Critical Issues*: Plants close their stomata in reaction to stress-conditions, such as drought, and the subsequent decrease in C_i leads to ROS production through photorespiration and over-reduction of the chloroplast electron transport chain. This reduces plant growth and thus drought may cause severe yield losses for agriculture especially in arid areas. *Future Directions*: The focus of future research should be drawn towards understanding the interplay between various different signaling pathway,s and how ROS, redox and hormonal balance changes in space and time. Translating this knowledge from model species to crop plants will help in the development of new drought resistant crop species with high yields.

Introduction

Stomata are tiny pores formed by a pair of guard cells on the surfaces of plant leaves and stems. Their primary role is to maintain an adequate supply of carbon dioxide (CO_2) for photosynthesis while limiting water loss through transpiration. In order to adapt to ever-changing environmental conditions, plants are constantly adjusting their stomatal apertures to control leaf CO_2 and water content.

Guard cells sense the concentration of CO_2 in the sub-stomatal cavity (C_i) and are able to respond rapidly to changes in C_i (31, 98). When conditions are optimal for photosynthesis in C3 plants, CO_2 is consumed by carboxylation reactions in the chloroplasts of mesophyll cells. This leads to a decrease in C_i below the ambient CO_2 concentration (~400 ppm) and triggers stomatal opening to maintain CO_2 supply for the Calvin-Benson cycle in mesophyll chloroplasts. In contrast, an increase in C_i leads to stomatal closure; this helps to conserve water but can also lead to increased leaf temperature and reduced uptake of nutrients by the transpiration stream. Such regulation may occur within minutes and is achieved by controlled transport of osmoregulatory ions, mainly potassium (K^+), chloride (Cl^-) and malate through different types of ion channels in the guard cell membranes (45, 65). Under conditions that limit photosynthesis, such as darkness, C_i increases due to reduced CO_2 fixation through the Calvin-Benson cycle and stomatal aperture decreases (Fig. 1). Conversion of CO_2 into bicarbonate (HCO_3^-) mediates the stomatal response to changes in C_i (28, 52, 53). CO_2 is spontaneously dissolved in water with formation of bicarbonate. Inside plant cells, the rate of this reaction is accelerated by carbonic anhydrases (CAs) (26). A Raf-like protein kinase, HT1 (HIGH LEAF TEMPERATURE 1) is a highly CO_2-specific stomatal regulator (44, 45, 52) which is involved in controlling the activity of the SLOW ANION CHANNEL 1 (SLAC1) (49). Stomatal opening occurs via activation of the guard cell plasma membrane H^+ATPase, which causes hyperpolarization

of the membrane and subsequent uptake of K^+ through polarization-dependent inward rectifying K^+ channels. Stomatal closure occurs through inactivation of the H^+ATPase and activation of the guard cell anion channels, and this leads to depolarization of the membrane and activation of outward rectifying K^+ channels (65).

During drought, water uptake by roots is limited and in order to avoid water loss by transpiration plants close their stomata. This is mostly regulated by the stress hormone abscisic acid (ABA). The first steps in ABA signaling leading to stomatal closure are well characterized. The key genetic components in the ABA signaling pathway include: ABA receptors PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENT OF ABA RECEPTORS (RCAR) (76, 112), a group of type 2C protein phosphatases (PP2Cs), such as ABA-INSENSITIVE1 (ABI1) and ABI2 (140, 143), the protein kinase OPEN STOMATA1 (OST1/SnRK2.6) and calcium dependent protein kinases (CDPKs, in Arabidopsis CPKs). In the absence of ABA, PP2Cs are active and function as constitutive inhibitors of OST1 and CDPKs. Binding of ABA to its receptors inactivates the PP2Cs, and OST1 is activated either by autophosphorylation (11) or phosphorylation by some other protein kinase. Once activated, OST1 is involved in the activation of the guard cell anion channels SLAC1 and QUICK ANION CHANNEL 1 (QUAC1) (57, 73) and inactivation of the inward rectifying Shaker family K^+ channel KAT1 (122). Activation of anion channels leads to an efflux of anions and small metabolites, such as malate, and in combination with the deactivation of the plasma membrane H^+ATPase AHA1 (88) cause plasma membrane depolarization and the consequent activation of voltage-dependent K^+ efflux channels (2). The resulting efflux of anions and K^+ leads to the loss of guard cell turgor and the closure of stomatal pores.

In addition to adjustments of stomatal aperture, plants also react to long-term changes in environmental conditions by adapting the stomatal density in newly developed leaves. Mechanisms underlying the regulation of stomatal density in response to environmental changes have been recently reviewed (18, 28) and will not be discussed here. Here, we address how stomata sense the changes in CO_2 concentration and water availability in C3 plants, how drought-induced stomatal closure leads to increased production of reactive oxygen species, ROS, and how ROS signals regulate stomatal movement. The major forms of ROS, singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) are formed in different subcellular compartments of plants. This occurs mostly through photorespiration-related reactions in peroxisomes, in mitochondrial electron transport chains, by over reduction of the chloroplastic electron transport chain, and by specific ROS-producing enzymes (15, 92, 106). This review covers the current knowledge of how signaling cascades relating to ROS, redox and changing environments are involved in the adjustment in stomatal aperture.

Coordination of mesophyll photosynthetic processes with stomatal aperture

Stomatal responsiveness to light and CO_2 is suppressed when the epidermis is detached from the leaf, whereas re-establishment of the contact between mesophyll and detached epidermis restores stomatal responsiveness. This suggests the existence of diffusible chemical or vapor phase signals released from the mesophyll (71, 99, 121). The nature of the mesophyll-driven signals has, however, remained elusive but a broad range of substances, including sucrose and malate, have been considered (44, 71, 121). There is evidence that, CO_2 concentration inside the leaf rather than outside the leaf influences stomatal aperture (98; Fig. 2). This notion is supported by several studies implying that red light-induced stomatal opening is mediated by the reduction of C_i which is in turn caused by the increased photosynthetic activity of mesophyll cells (118, 119). Guard cells are also

known to have signaling components specific to CO_2-responses. Plants carrying mutations in the highly CO_2-specific protein kinase HT1 (42, 43, 49) showed severely suppressed stomatal opening in response to red light induced decrease in C_i (80). However, Ci may not be the only signal through which guard cells get information on mesophyll processes. Functional red light-induced stomatal opening under artificially sustained C_i suggested that mesophyll photosynthesis could coordinate stomatal regulation by C_i-independent mechanisms (68, 89). The existence of a C_i independent signal was further supported by unaffected stomatal conductance in plants, which had high C_i due to suppressed Rubisco activity (145). In addition, Blue light, a factor affecting photosynthesis and stomatal opening is directly perceived by guard cells (126, 127, 159). In addition, over-reduction of the plastoquinone pool in the mesophyll cell chloroplasts was recently suggested to induce ROS-mediated stomatal closure (147). Taken together, it seems that guard cells are able to recognize both changes in environmental conditions, such as light quality, and inner C_i-dependent and C_i-independent signals from mesophyll. Although the influence of mesophyll cells on stomatal aperture has been demonstrated by several studies, many details of this interplay remain unknown and should be further elucidated in the future.

The role of guard cell chloroplasts in stomatal signaling

In most species, guard cell chloroplasts are smaller, present in a lower number, and have a less developed thylakoid structure with reduced granal stacking than mesophyll cell chloroplasts (3, 154). Despite these differences, guard cell chloroplasts still have functional photosystems I and II as well as Calvin-Benson cycle activity (10, 69, 70, 83, 117) and they can significantly contribute to guard cell metabolism (67). Similarly, regulation of photosynthesis in guard cells and in mesophyll by environmental factors can provide an indirect sensing mechanism coordinating stomatal behavior with mesophyll demands for CO_2 (121, 133). There exist, however, some plant species such

as the orchid genus *Paphiopedilum* (21, 104) that do not have chloroplasts in their guard cells but still display stomatal responses to high CO_2 concentration and changes in light conditions (104), independent of photosynthesis in guard cells.

Guard cell chloroplasts are thought to be involved in osmoregulation of stomatal movements through photosynthetic carbon fixation, which produces osmotically active sugars. However, estimations of photosynthesis-derived osmotica in guard cells vary from 2% to 40% of the total pool of osmotically active substances depending on plant species and experimental approaches (67). Starch degradation in guard cell chloroplasts, which can be initiated by blue light and low CO_2 (50, 111), can also contribute to the formation of guard cell turgor by releasing monosaccharides and/or provide phosphoenolpyruvate for CO_2 fixation by cytosolic phosphoenolpyruvate carboxylase, leading to the formation of malate (50, 67, 121). Plants with impaired starch synthesis, both in mesophyll and in guard cells, demonstrated reduced stomatal responsiveness to elevated CO_2, indicating that conversion of osmotically active carbohydrates has a role in the reduction of osmotic pressure during stomatal closure (8).

Although guard cell photosynthesis is important for the energization of stomatal opening (8, 133), it does not seem to be directly involved in the regulation of stomatal aperture as stomata of plants without chlorophyll in guard cells still remained responsive to CO_2 and ABA (9, 104). However, numerous studies have highlighted the importance of guard cell chloroplast in stomatal regulation through chloroplast-dependent ROS accumulation (128, 149), Ca^{2+} release (107, 153), and retrograde signaling (113) (see the corresponding sections below). In conclusion, the function of guard cell chloroplasts may not be compulsory for CO_2 and ABA triggered stomatal regulation but

166 appear to be important for amplifying and fine tuning processes through light-derived control of
167 other signals.
168
169 **Mechanism of stomatal opening induced by lower than ambient concentrations of CO_2**
170 Decrease of CO_2 concentration in the leaf intercellular air spaces is a powerful stimulus for the
171 regulation of stomatal aperture since it can induce stomatal opening even under conditions that
172 normally promote stomatal closure, such as darkness when photosynthesis is not possible and low
173 air humidity, which poses the risk of wilting (85). Accordingly, mechanisms of low CO_2-induced
174 stomatal opening are likely to have an early evolutionary origin as stomata of ancient vascular
175 plants, lycophytes and ferns, displayed rapid stomatal opening in CO_2-free air but only a weak
176 response to high CO_2 concentrations (13, 65). The stomata of the lycophyte *Selaginella* responded
177 to both elevated and reduced CO_2 concentrations as well as to ABA (120) and stomatal closure in
178 response to elevated CO_2 and ABA were present in some fern species and had been possibly lost in
179 others (48).
180
181 Although there are major gaps in our understanding of how low CO_2 triggers stomatal opening, it is
182 obvious that it must involve signaling systems that control the activity of H^+ATPases (118). This can
183 be achieved either by enhanced translocation of H^+ATPases from internal membranes into the
184 plasma membrane or by the regulation of the H^+ATPase activity (Fig. 3A). The importance of
185 H^+ATPase translocation was demonstrated by impaired stomatal opening in response to low CO_2 in
186 the Arabidopsis mutant *patrol1*, which has a mutation in the endosome-localized PROTON ATPASE
187 TRANSLOCATION CONTROL 1 (PATROL1), a protein involved in the translocation of the major guard
188 cell H^+ATPase, AHA1, into the plasma membrane (41). The mechanisms controlling AHA1 activation
189 however are not known. Other transporters also contribute to the production of osmotic pressure

190 in guard cells during low CO_2-induced stomatal opening. For example, plants with defects in the
191 NITRATE TRANSPORTER 1.1. demonstrated decreased stomatal opening in CO_2-free air,
192 accompanied by reduced nitrate accumulation in guard cells (40). Malate transporter ATP-BINDING
193 CASSETTE B14 (ABCB14) can also promote stomatal opening by uptake of malate from the apoplast
194 (72). The involvement of other transporters and regulatory pathways in guard cells activated by
195 reduced CO_2 concentration still await identification.
196
197 **Mechanism of high CO_2 concentration-induced stomatal closure**
198 Stomatal closure is triggered by an increased concentration of CO_2 and hence, elevated C_i, induces
199 anion efflux through anion channels in the guard cell plasma membrane, followed by K^+ efflux, and
200 subsequent water outflow and a reduction of guard cell volume (Fig. 3B). Plants with defective S-
201 type anion channel SLAC1 or with defects in the mechanisms that control SLAC1 activation display
202 severely impaired stomatal closure in response to an increase in CO_2 concentration (49, 86, 103,
203 141, 157). The role of apoplastic malate for high CO_2-induced stomatal closure was demonstrated
204 already in 1993 (44). Malate can be transported from mesophyll to guard cells and could act as a
205 mesophyll-driven signal linking mesophyll metabolism with stomatal regulation (6, 109). The R-type
206 anion channel QUAC1 can be activated by apoplastic malate. Accordingly, plants lacking QUAC1 in
207 their guard cells demonstrated partially impaired stomatal response to high CO_2 (57, 90). Guard cells
208 can also control the level of apoplastic malate by its uptake via ABCB14 activity (72). ABCB14 acts as
209 a negative regulator in high CO_2-induced stomatal closure as demonstrated by accelerated and
210 delayed stomatal responses to high CO_2 concentration in the *abcb14* mutants and ABCB14
211 overexpressors, respectively (72).
212

213 It has been suggested that calcium ions play a role as a second messenger in high CO_2-induced
214 stomatal closure. This has been concluded based on experiments where Ca^{2+} accumulation in guard
215 cells subjected to higher than ambient CO_2 concentration was observed, and from the impaired high
216 CO_2-induced stomatal closure in the presence of Ca^{2+} chelators, such as BAPTA or EDTA (55, 124,
217 152). Genetically-encoded Ca^{2+} sensors revealed that guard cells displayed oscillations of cytosolic
218 Ca^{2+} concentration $[Ca^{2+}]_{cyt}$ and these patterns were often associated with changes in stomatal
219 aperture (4). However, unexpectedly, guard cells exposed to reduced CO_2 concentration
220 demonstrated more $[Ca^{2+}]_{cyt}$ transients than those under elevated CO_2 concentration (161). As
221 guard cells produced 'spontaneous' cytoplasmic Ca^{2+}-transients and Ca^{2+} is required for high CO_2-
222 induced stomatal closure, it was suggested that elevated CO_2 concentration enhances sensitivity of
223 stomatal closing mechanisms to $[Ca^{2+}]_{cyt}$. In agreement with this hypothesis, CO_2-derived
224 bicarbonate enhanced Ca^{2+} sensitivity of the S-type anion channel activation in guard cells (157).
225
226 **Bicarbonate as a signaling molecule in CO_2-controlled stomatal movements**
227 A reduction or an increase of C_i should be sensed and translated into activation of corresponding
228 signaling pathways in guard cells. The CO_2 permeability of biological membranes in relation with
229 direct diffusion of CO_2 through the membranes vs the role of CO_2-permeable aquaporin channels
230 has been addresses in several papers and there are indications that specific aquaporins can have a
231 significant role in CO_2 uptake (see 38 for a review). Recently, a plant aquaporin, PLASMA
232 MEMBRANE INTRINSIC PROTEIN 2;1 (PIP2;1), was shown to function as a channel for CO_2 diffusion
233 in *Xenopus laevis* oocytes (145). However, knocking out PIP2;1 was not sufficient to impair stomatal
234 CO_2 responses (145). This could be explained by functional redundancy among guard cell
235 aquaporins; there are 35 AQP homologs in Arabidopsis. Transport of CO_2/bicarbonate to
236 chloroplasts also depends at least partly on aquaporins, including the PIP1;2 that is located in

chloroplast envelope (138). In addition to the proposed role for PIP2;1 in CO_2 transport, it was also involved in ABA-triggered stomatal closure (37).

Conversion of CO_2 into bicarbonate (HCO_3^-) is an important step that mediates stomatal responses to changes in ambient CO_2 and C_i (28, 52, 53). Although CO_2 is spontaneously dissolved in water with formation of bicarbonate, in cells the rate of this reaction is accelerated by carbonic anhydrases (CAs) (26). Among CAs expressed in Arabidopsis, the function of βCA1 and βCA4, localized in chloroplasts and in plasma membrane, respectively, was important for the rapid stomatal response to changes in CO_2 levels (52; Fig. 3). While single βCA mutants did not display clearly altered CO_2 sensitivity, the double knockout of both βCA1 and βCA4 significantly delayed stomatal responses to CO_2 (28, 52, 53). Interestingly, PIP2;1 physically interacted with β-carbonic anhydrase 4 (βCA4) and this connection has been suggested to enable the generation of CO_2 concentration gradient and thus enhance transport of CO_2 into guard cells (145). The importance of HCO_3^- is further supported by the experiments showing that the concentration of cytosolic bicarbonate, rather than CO_2, activated S-type anion channels in guard cell protoplasts (157). The role of bicarbonate as a small signaling molecule in guard cells was also confirmed by reconstitution of CO_2 signaling pathway in *X. laevis* oocytes co-expressing PIP2;1, βCA4, SLAC1 and CPK6/23 or OST1. In these experiments, the presence of these proteins was enough to confer bicarbonate-induced activation of SLAC1 anion currents in oocytes (145).

Despite the established connection between cytosolic bicarbonate and anion channels in guard cells, our knowledge about CO_2 signaling in guard cells has still major gaps. As an example, it has not been resolved which proteins can bind and/or sense the changes in bicarbonate concentration in guard cells to transmit the signal that eventually leads to changes in ion channel activities.

Mitogen activated protein kinases MPK4 and MPK12 and HT1 - a new pathway controlling SLAC1 activation in response to changes in CO_2

Mutant screens with different approaches have led to the identification of important components in guard cell CO_2 signaling (41, 43, 103, 141). The Raf-like protein kinase HT1 was identified by using thermal imaging of mutagenized plants subjected to low CO_2. HT1 is expressed in guard cells and is highly CO_2-specific regulator, since plant lines carrying mutations in HT1 displayed stomata completely insensitive to changes in CO_2 concentration, but remained responsive to other stimuli such as light, ABA, and air humidity (42, 43; Fig. 4). HT1 plays a role in controlling the activation of SLAC1 anion channel as a response to changes in CO_2 concentration (Fig. 3). Experiments carried out in heterologous system, *X. laevis* oocytes, demonstrated that SLAC1 activation by OST1 and by receptor like protein kinase GUARD CELL HYDROGEN PEROXIDE-RESISTANT1 (GHR1) was suppressed by HT1 (49, 137). However, the mechanism how HT1 affects SLAC1 activation remains controversial. Some experiments have suggested that HT1 could phosphorylate OST1 and by that suppress SLAC1 phosphorylation by OST1 (137), however, these experiments were not confirmed in another study (49). Despite of using various versions of the HT1 protein, no inhibition on OST1-induced phosphorylation of SLAC1 was observed in the presence of HT1. Instead, HT1 showed phosphorylation activity towards GHR1 and the N-terminus of SLAC1 *in vitro*; the functional outcome of these reactions, however, remained unclear (49). Thus, mechanism by which HT1 controls anion channel activation during stomatal closure in response to elevated CO_2 requires further research. Furthermore, one should remember that results obtained in *in vitro* experiments and heterologous systems, such as *X. laevis* oocytes, do not necessarily reflect the regulatory interactions *in planta* due to missing components, and the models predicted in these artificial experimental systems need to be confirmed in plants before constructing regulatory models.

Studies with MPK inhibitors and work focusing on the natural variation of water-use efficiency and ozone sensitivity among Arabidopsis natural accessions revealed that MPK12 is an important component of stomatal regulation (24, 58, 59). Further work showed that MPK4 and MPK12 are essential for CO_2-dependent stomatal regulation (Fig. 3). Both of these MPKs inhibited HT1 activity i*n vitro*, whereas experiments in *X. laevis* oocytes indicated that MPK12 was able to restore SLAC1 activation by GHR1 in the presence of HT1 (49, 58). It is noteworthy that CO_2-induced stomatal responses in plants lacking MPK12 and MPK4 in their guard cells were fully abolished, similar to that observed for strong HT1 mutants. Thus, these MPKs seem to play a central role in controlling HT1 in CO_2-induced stomatal regulation, however, the mechanism that relays changes in bicarbonate concentration to MPKs in guard cells remains to be addressed. A MATE-type transporter, RESISTANT TO HIGH CO_2 (RHC1) was also suggested to act as an upstream regulator of HT1. Its abundancy is high in guard cell plasma membranes and its activity was essential for stomatal response to high CO_2 concentration (137). Phenotype of the *rhc1 ht1-2* double mutant and oocyte experiments implied that RHC1 could act as a bicarbonate sensing element upstream of HT1, although its exact mechanism remained unknown (137). In contrast to these results, another study (149) showed that RHC1 alone was able to cause bicarbonate-insensitive ion currents in *X. laevis* oocytes, making the role of RHC1 in CO_2/bicarbonate sensing unresolved.

ROS production and sensing in guard cell signaling during drought

Under certain conditions, stomata must close despite the mesophyll CO_2 demands and low C_i. This type of stomatal closure can be induced, for example, by limited water availability, salt/osmotic stress, air pollution, or by pathogen attack, which is often referred to as stomatal immunity. Stomatal closure is one of the earliest responses of plants to water deficit. This rapid response is

orchestrated by a complex network of signaling pathways where the main player, ABA, operates together with second messengers Ca^{2+} and ROS (23, 100) and overrides the stomatal regulation by CO_2. The participation of ABA in stomatal responses to drought is well known (35) and ROS and Ca^{2+} are important mediators in ABA signaling.

Stomatal closure is accompanied by increased ROS formation in the guard cell apoplast and chloroplasts in response to various treatments (128, 129; Fig. 5). Apoplastic ROS are generated mainly by two different types of enzymes: plasma membrane NADPH oxidases (RESPIRATORY BURST OXIDASE HOMOLOGS, RBOHs) and cell wall peroxidases. In Arabidopsis guard cells, there are two main isoforms of NADPH oxidases, AtRBOHF and AtRBOHD, which among other signals can also be regulated by ABA-depended processes (66). ABA-triggered stomatal response was significantly reduced in the *atrbohF* mutant and the phenotype was enhanced in the *atrbohD atrbohF* double mutant when the *atrbohD* single mutant did not differ from the wild type (66). Due to its obvious role in pathogen-triggered ROS burst, RBOHD is more commonly recognized for its function in plant immune defense (77). However, recently both these NADPH oxidases were shown to be involved also in the guard cell CO_2 responses, and the CO_2-induced of ROS burst required ABA (17). In addition to NADPH oxidases, also the cell wall bound salicylhydroxamic acid (SHAM)-sensitive peroxidases take part in apoplastic ROS production around guard cells (61, 97). These peroxidases are involved in the pathogen triggered ROS burst (61), but they may also be involved in the response to abiotic stress (93, 110). Apoplastic ROS are also produced by other oxidases such as, di- and polyamine oxidases (114). Copper amine oxidase and polyamine oxidases contribute to the H_2O_2 production involved in the stomatal closure induced by ABA and ethylene in *Vicia faba* and *Arabidopsis thaliana*, respectively (5, 51). However, the evidence for the involvement of peroxidases and amine oxidases in apoplastic ROS production has come from inhibitor studies and further research is needed in

order to understand specific function, molecular identities, and significance of these proteins in ROS induced stomatal regulation.

Apoplastic ROS production initiates the activation of plasma membrane Ca^{2+} channels leading to an increase in cytosolic Ca^{2+} levels. The molecular identity of these inducible plasma membrane Ca^{2+} channels is still not clear. In the cytosol, Ca^{2+} stimulates the activation of NADPH oxidases either directly by binding to their cytoplasmic EF-hands (63) or indirectly by affecting their phosphorylation by CPKs (27). Upon Ca^{2+}-binding, CALCINEURIN-B LIKE PROTEINS (CBLs) interact with the CPKs and CBL-interacting PROTEIN KINASES (CIPKs) (131) and a particular complex formed by CBL1/CBL9-CIPK26 phosphorylated and activated RBOHF (27). The increase in cytoplasmic Ca^{2+} is sensed also in the chloroplasts where a thylakoid membrane-associated Ca^{2+} -binding protein, CALCIUM SENSING RECEPTOR (CAS), is activated through yet unidentified mechanism. The activation of CAS was responsible for the release of Ca^{2+} from thylakoids and a chloroplastic ROS burst (107, 108, 142, 153), both of which contribute to the cytoplasmic Ca^{2+} oscillations, apoplastic Ca^{2+} induced stomatal closure as well as retrograde signaling during plant immune defense (108). Moreover, the drought sensitivity of the Arabidopsis *cas* mutant is caused by the improper closure of stomata (148), which further highlights the importance of chloroplastic Ca^{2+} signaling in stomatal regulation.

The role and ability of OST1 in direct activation SLAC1 has been recently discussed (128). First, phosphorylation of SLAC1 by OST1 has only been detected *in vitro* and second, multiple mutants of CPKs that showed stomatal phenotype still have an active OST1, which nevertheless can not activate SLAC1-mediated ion currents *in vivo* in the absence of specific CPKs. Furthermore, plants with impaired OST1 were shown to have wild type like stomatal closure in response to Ca^{2+} (102), possibly via activation of CPKs. This poses a question whether *in vivo* OST1 would actually be involved in the

activation of guard cell anion channels indirectly through controlling the activation of CPKs, possibly by phosphorylation of RBOHF. The resulting ROS burst would activate Ca^{2+}-channels, followed by CPK-dependent activation of SLAC1 (123, 128). In this model OST1 would function upstream of ROS production and be negatively regulated by the PP2C ABI1, as has been shown (60, 75, 101). Furthermore, GHR1, and its negative regulator ABI2, another PP2C, would be involved in the downstream activation of plasma membrane Ca^{2+}-channels and subsequent stomatal closure (54, 101; Fig. 5).

Although there is clear evidence for the involvement of ROS in the regulation of stomatal aperture, it is still not known how the ROS signals are sensed in the guard cell apoplast. Identification of the ROS and redox sensors has been one of the major challenges in plant ROS research during recent years. In guard cells, only a few ROS sensing mechanisms are known to be involved in the stomatal regulation. These are the redox regulation of the GHR1 apoplastic domain (54) and the redox regulation of OST1 (146) and CPK1 (139). GHR1 is a plasma membrane associated atypical *Leucine-rich repeat* receptor-like protein kinase that has been proposed to be involved in apoplastic ROS perception. The apoplastic C-terminal domain of GHR1 has two conserved cysteines (C-57 and C-66) that are necessary for the correct function of the protein (54). As discussed earlier, GHR1 has been implicated as a central regulator of guard cell CO_2 and early ABA responses but the molecular mechanism for its function is still unclear. GHR1 has been shown to interact with SLAC1 (54) but it is not likely to activate SLAC1 by phosphorylation as its cytoplasmic kinase domain lacks the conserved amino acids that are required for kinase activity (M. Sierla, H. Hõrak, K. Overmyer, H. Kollist, and J. Kangasjärvi, unpublished data). Therefore, it is likely that there are other unidentified proteins involved in the GHR1 mediated SLAC1 activation.

381 The protein phosphatases ABI1 and ABI2 have also been shown to be inactivated in the presence of
382 H_2O_2 (81, 82) but the mechanism for this redox regulation is still unknown. Another example for
383 redox regulation of ABI1 and ABI2 in guard cell is a glutathione peroxidase like enzyme, GPXL3, in
384 H_2O_2 scavenging and cytosolic redox-regulation in response to ABA and drought stress (91). GPXL3
385 was suggested to interact with ABI1 and ABI2. In addition, similarly as H_2O_2 (81, 82), oxidized GPXL3
386 decreased the phosphatase activity of ABI2 by affecting its redox status *in vitro*. However, both
387 proteomic data and subcellular localization of GPXLs as GFP-fusions (7) suggest that GPXL3 is in fact
388 a type II transmembrane protein anchored to the endoplastic reticulum and/or Golgi so that the
389 catalytic side remains in the lumen and would not be able to interact with the PP2Cs *in vivo*. In the
390 light of these results, the molecular basis for the drought sensitive and resistant phenotypes of the
391 *gpxl3* null mutant and GPXL3 overexpressor lines, respectively, (91) and the possible mechanisms of
392 the redox regulation of the guard cell PP2Cs remain unknown.
393
394 **The role of sulfate in drought sensing and the emergence of a new pathway**
395 A number of studies with different plant species have shown that low soil water potential decreases
396 stomatal conductance even before any measurable change in leaf water potential can be observed
397 (34, 36). These results suggest that roots can sense low soil water potential and transmit a signal to
398 guard cells initiating stomatal closure. The earlier hypothesis that root borne ABA acted as a drought
399 signal to leaves has now been questioned since stomatal closure appears to be dependent on foliar
400 ABA production (19, 47, 87). In addition, the ABA that accumulates in roots during long-term
401 drought conditions appears to be derived from the shoots (79). Other signals, such as chemical,
402 electrical and hydraulic, have been suggested to play a role in root to shoot signaling (56) and they
403 all are likely to contribute to the long distance signaling through various signaling networks.
404

405 The role of sulfate in root to shoot signaling and stomatal regulation has been recently highlighted
406 by several studies. The need for sulfate during drought is known to increase as many sulfur-
407 containing compounds, such as glutathione, are involved in plant abiotic stress responses (1). Once
408 taken up from the soil and transported to chloroplast, sulfate is converted into cysteine or 3'-
409 phosphoadenosine-5'-phosphosulfate (PAPS) which are then used for the synthesis of sulfur
410 containing compounds and production of sulfated compounds (33). Cysteine plays an important role
411 in a plant defense against abiotic stress as it is a precursor for glutathione biosynthesis (105) and it
412 is required for the sulfuration of Molybdenum cofactor, which at its sulfated form is required for
413 the final step of ABA biosynthesis. Intriguingly, significant co-regulation of ABA biosynthesis and
414 sulfur metabolism takes place under stress conditions in order to ensure adequate cysteine supply
415 needed for the final step in ABA biosynthesis (14). Sulfate concentration in xylem sap was increased
416 in response to drought and this enhanced the effect of ABA on stomatal regulation during early
417 stage of water stress in maize (29). Similarly, xylem derived sulfate promoted stomatal closure by
418 direct activation of the R-type anion channel QUAC1 and enhanced ABA biosynthesis (78).
419
420 Several sulfated compounds accumulate in plant leaves under drought. These are sulfated in the
421 cytoplasm by a family of enzymes called sulfotransferases (SOTs) that catalyze the transfer of
422 sulfuryl group from PAPS to several different compounds, such as glucosinolates, flavonoids,
423 brassinosteroids and salicylic acid (46). However, the role sulfation of these compounds in drought
424 resistance is not well understood. Instead, the by-product of SOT catalyzed sulfation, 3'-
425 phosphoadenosine-5'-phosphate (PAP), has been implicated in drought and high light signaling (30).
426 Once produced in the cytosol, PAP is transported to chloroplasts where it is detoxified by
427 dephoshorylation to adenosine monophosphate (AMP) by the adenosine bisphosphate
428 phosphatase SAL1 (115). High light and drought inactivated SAL1 by redox-regulated dimerization

429 causing the accumulation of its substrate, PAP (16, 30). It was suggested that PAP moves into
430 nucleus (32) where it is thought to inhibit the post-transcriptional gene silencing of stress responsive
431 genes by 5'-3' exoribonucleases (XRNs) (30). However, it is not clear whether chloroplastic or
432 cytoplasmic PAP is responsible for gene regulation since the PAPS/PAP antiporter transports PAPS
433 out and PAP into the chloroplast according to a concentration gradient (33, 34), which implies that
434 inactivation of SAL1 results in increase of cytoplasmic PAP due to decrease of the concentration
435 gradient-driven transport of PAP to chloroplast.
436
437 The involvement of PAP in ABA-dependent stomatal closure was also shown recently (113). The
438 sensitivity of the guard cells of *abi1-1* and *ost1-3* for ABA was restored in mutant plants by
439 genetically, or exogenously increasing PAP levels. In addition, PAP upregulated the expression of
440 many ABA and Ca^{2+} responsive genes, including several CPKs. It was suggested (113) that because
441 of the transcriptional regulation, PAP-mediated chloroplast signaling could bypass the canonical
442 ABA signaling pathway and activate SLAC1. However, PAP-induced stomatal closure required
443 sufficient concentrations of Ca^{2+} and apoplastic ROS production by NADPH oxidases, but did not
444 affect the activity of SLAC1 or the highly selective inward-rectifying potassium channels KAT1 or
445 KAT2 in *X. laevis* oocytes. This suggests that PAP is dependent on ABA-mediated processes and
446 works rather as a second messenger in ABA signaling. Intriguingly, exogenous application of PAP on
447 Arabidopsis and barley leaf peels was able to trigger stomatal closure within a few minutes and the
448 kinetics of this reaction was almost identical to that of exogenous ABA application (113). It is highly
449 unlikely that stomatal closure through transcriptional regulation would occur as fast as by ABA
450 triggered post-transcriptional regulation. Therefore, PAP may also regulate SLAC1 activity through
451 direct post-transcriptional regulation of other kinases such as CPKs or MAPKs (Fig. 5).
452

The role of other plant hormones in guard cell drought response

In addition to ABA, also other plant hormones and low-molecular-weight compounds have a role in the induction of stomatal responses to drought and in the mediation of ROS-related or -dependent signal transduction leading to stomatal closure. Jasmonic acid (JA) and it its methyl ester (Methyl jasmonate, MeJa) induce ROS production and stomatal closure through the activation of RBOHD and/or RBOHF (134). MeJa-induced stomatal closure, ROS production, and cytosolic alkalization were unaffected in the *pyr1 pyl1 pyl2 pyl4* quadruple mutant, but was impaired in the SnRK protein kinase OST1 loss of function mutants, *ost1* and *srk2e*, and in the ABA deficient, *aba2-2* mutant (160). This suggest that the MeJa activation of RBOHD and/or RBOHF requires ABA priming (as also implied by previous studies; Hou *et al.*, 2013; Murata *et al.*, 2015) and OST1 function, but does not activate OST1 through the canonical ABA signaling pathway in guard cells. JA and MeJa have been suggested to regulate stomatal closure through transcriptional regulation of MeJa responsive genes and through ROS and nitric oxide (NO) -triggered, Ca^{2+}-dependent activation of CPK6 and its downstream target SLAC1 (23).

Salicylic acid (SA) accumulates in plant leaves during drought stress and pathogen invasion and induces stomatal closure in response to apoplastic superoxide production (84, 94). SA-induced apoplastic ROS accumulation around guard cells was inhibited by the application of the peroxidase inhibitor SHAM but not by the NADPH oxidase inhibitor diphenyle iodonium (DPI) (61, 97). This suggests that the SA-induced apoplastic ROS production is mediated through the cell wall bound peroxidases. However, it must be noted that salicylhydroxamid acid (SHAM) is not a specific inhibitor of peroxidases but has been more commonly used as an inhibitor of the mitochondrial alternative oxidase (AOX), which is activated under conditions involving increased mitochondrial ROS production (96). Furthermore, low (1-5 mM) concentrations of SHAM act actually as peroxidase

activators, when only higher concentrations (20 mM) inhibit peroxidases (130); in some published studies the use of low SHAM concentrations has been interpreted as an inhibitory effect. Accordingly, it has been suggested that AOX helps to maintain the NO homeostasis in guard cell mitochondria by preventing the over-reduction of the electron transport chain, particularly during stomatal closure when NO concentration increases in cytosol (20). Therefore, the mechanism of SA-induced peroxidase activation remains to be verified by further studies. The SA accumulating mutants *siz1* (93) *cpr5* (12) and *acd6* (116) have constitutively decreased stomatal aperture and show drought tolerance. The application of peroxidase inhibitors SHAM and azide compromised the narrow stomatal phenotypes of the mutants while the application of the NADPH oxidase DPI had no effect (93, 110). These results imply that peroxidase-facilitated ROS production is involved in the SA-mediated, drought-induced stomatal closure.

In contrast to ABA, JAs, and SA, all of which positively regulate stomatal closure, ethylene can promote both stomatal opening and closure, although the reaction seems to be highly species dependent (23, 100). In general, there is great inconsistence in the results from different studies on the effect of ethylene on stomatal regulation. One possible explanation to the differences could be that these studies have mainly been performed with leaf disks, epidermal peals, or detached leaves and experiments on these samples do not always reflect the real response to studied stimuli. In addition, the effect of ethylene on stomatal aperture seems to be dependent on the hormonal homeostasis and the detachment of leaves will disrupt the cellular balance. Two independent studies have shown that in the absence of ABA, ethylene promoted stomatal closure whereas in the presence of ABA it inhibited stomatal closure in Arabidopsis (25, 135). In addition, auxin and cytokinin, the major plant hormones involved in various aspects of plant growth and development, inhibited the ABA induced stomatal closure by enhancing ethylene biosynthesis (136). Ethylene-

induced stomatal closure was also depend on the RBOHF-mediated ROS production (25), whereas opening or inhibition of ROS-induced stomatal closure could be promoted by the ethylene-induced accumulation of flavonols (150). Flavonols are plant metabolites with antioxidant properties and they accumulate in guard cells reducing ROS levels and consequently suppress stomatal closure (150). Taken together, ethylene seems to affect guard cell signaling mainly by controlling ROS homeostasis in the guard cells and its function is controlled by other hormones.

MPKs play multiple roles in the regulation of stomatal movement

In addition to CO_2 signaling, MPKs are also suggested to have a role in guard cell ABA and pathogen signaling (22, 74). Whereas MPK9 and MPK12 were involved in the stomatal responses to ABA, MPK3 and MPK6 mediated pathogen signaling in guard cells (95). As discussed earlier, ROS are produced by RBOHs in response to both ABA and pathogen signaling, but while RBOHF is mainly responsible for the ROS production in response to ABA, RBOHD is involved in stomatal closure in response to recognition of potentially pathogenic microorganisms (60, 77). It would be tempting to speculate that the two NADPH oxidases, RBOHD and RBOHF are regulating two separate MPK pathways but recent research has indicated that the reality is more complicated.

The activation of RBOHD was not required for the activation of MPK3 and MPK6 in response to bacterial pathogens (156). Moreover, it has been suggested that the rapid ROS burst and the activation of MPK3/MPK6 are two independent early signaling events during stomatal immune response in Arabidopsis. More recently, these two signaling events were shown to belong to separate but interdependent signaling cascades that control stomatal movements (Fig. 6), and the loss of function of both MPK3 and MPK6 impaired pathogen-triggered stomatal closure (132). The activation of MPK3 and MPK6 was independent of the ABA, SLAC1, and RBOHD-mediated ROS burst.

Instead of regulating anion channels, the two kinases controlled the metabolism of osmotically active organic acids such as malate and citrate. Under pathogen attack, the level of osmotically active metabolites in the cytosol decreased and the guard cell turgor was lost promoting stomatal closure. However, at the same time the ABA-induced ROS production by RBOHD activated ABA signaling, leading to SLAC1 activation and stomatal closure (132). To what extent these interdependent signaling cascades interact and whether they share common mediators remains to be elucidated. To further complicate the story, MPK3 and MPK6 have been suggested to regulate stomatal closure also through an ABA-independent oxylipin pathway (95). MPK3 and MPK6 activated guard cell specific lipoxygenase, LOX1, and SA was needed for the downstream signaling events leading to stomatal closure.

Both MPK9 and MPK12 are also involved in SA mediated stomatal signaling in guard cells as SA activated S-type anion channels and elicited stomatal closure in wild type Arabidopsis but not in the *mpk9 mpk12* double mutant (62). It was suggested that the two kinases could be involved in the same signaling cascade through LOX1. However, the studies on MPK9 and MPK12 on ABA and SA mediated stomatal regulation have been performed mainly with TILLING mutants of *mpk9-1* and *mpk12-1* (containing, in addition to the mutations in *MPK9* and *MPK12*, an undetermined number of point mutations elsewhere in the genome) and epidermal peels or guard cell protoplasts (59, 62). Point mutations can affect the protein function in different ways when compared to loss of function mutants. Similarly, experiments performed with epidermal peels or protoplasts are missing the mesophyll contact, as discussed earlier in the text. Therefore, the involvement of MPK9 and MPK12 in stomatal regulation by ABA and SA would require experiments with especially loss of function alleles and with intact plants to evaluate their role in stomatal processes.

549 The above studies on MAPK3/MAPK6 signaling cascades were focusing on pathogen triggered
550 stomatal closure. However, MPK3 and MPK6 are activated by both biotic and abiotic stresses, as
551 well as by ABA (22). Decreased expression of MPK3 by guard cell specific gene silencing resulted in
552 impaired ABA-mediated inhibition of stomatal opening and H_2O_2-induced stomatal closure, but did
553 not affect the ABA-induced stomatal closure (39). In addition, the *mpk6* mutant guard cell were
554 impaired in ABA-induced H_2O_2 accumulation (155). Taken together, it seems likely that MPK3/MPK6-
555 regulated organic acid metabolism would also have a role in stomatal responses to abiotic stresses
556 such as drought. However, this needs to be verified by testing the stomatal responses of the *mpk3*
557 *mpk6* double mutant to abiotic stresses

558

559 **Negative regulation of ABA signaling**

560 Stomata are generally considered to respond to abiotic and biotic stresses by decreasing their
561 aperture. However, it is important to note that during the day C3 and C4 plants rarely close their
562 stomata completely. Instead, they have developed negative regulatory mechanisms to ensure
563 minimal carbon dioxide supply for photosynthesis by keeping stomata open during stress as well.
564 As discussed earlier, ethylene negatively regulates ABA signaling in guard cells. In addition to
565 hormonal regulation, cytoplasmic nitrosylation reactions are involved in the negative regulation of
566 ABA signaling. The ABA-dependent rapid accumulation of NO negatively regulated the OST1
567 function by S-nitrosylation of Cys137 near the catalytic site of the kinase (146). The S-nitrosylation
568 of OST1 was observed as a late event in the ABA signaling, thus, it has been suggested that this
569 mechanism helps to reset ABA signaling. Considering the role of OST1 in the activation of RBOHF, it
570 has been further suggested (128) that inhibition of OST1 by NO might also restrict ROS formation.
571 Cytoplasmic ROS participate also in the negative feedback regulation of CPK21 (139). Oxidation of
572 CPK21 by H_2O_2 resulted in the formation of intramolecular disulfide bond that reduced the kinase

activity. Conversely, CPK21 was activated by a THIOREDOXIN H-TYPE1 (Trx-h). Thioredoxins are small proteins that catalyse the thiol to disulfide exchange reaction in their target proteins. Incubation of the oxidized CPK21 together with the Trx-h rescued the kinase activity suggesting that CPK21 could be subjected to redox regulation under changing conditions (139). Furthermore, during stress the inactivation of CPK21 by H_2O_2 could act as a negative feedback regulation of ABA-induced stomatal closure. It would be interesting to see if other CPKs are regulated in similar manner.

Connection between CO_2 and ABA signaling in guard cells

Since both ABA- and high CO_2-induced stomatal closure involve activation of SLAC1 in guard cells, one could expect that ABA- and CO_2-signaling converge. Indeed, several mutations causing stomatal ABA-insensitivity, such as *abi1-1* and *abi2-1*, (86, 151) as well as *ost1* and *ghr1* (49, 86, 157; Fig. 4) display impaired stomatal responses to high CO_2 concentrations. Stomata of the GROWTH CONTROLLED BY ABSCISSIC ACID 2 (GCA2) mutant, *gca2*, which is related to CPKs were insensitive to both ABA and high CO_2 concentration. Furthermore, *gca2* displayed altered patterns of cytoplasmic Ca^{2+} transients in response to these stimuli and was suggested as a convergence point between CO_2 and ABA signaling (4, 161).

ABA receptors, PYR/PYL/RCARs, are also involved in CO_2 signaling, as inactivation of several of these proteins impaired stomatal closure in elevated CO_2 concentrations (17, 86). Due to a large number of the PYR/PYL /RCAR proteins and a functional redundancy between them, further research is required to identify which combination of PYR/PYL /RCARs function in the regulation of CO_2-induced stomatal closure (17, 86, 157). Furthermore, recently developed fluorescent probes that enable real time in vivo monitoring of ABA in plant cells (144) should be used in addressing the interplay between CO_2 and accumulation of ABA in guard cells during changes in CO_2 concentration (28).

Although several key-components of ABA signaling are also connected with stomatal responses to high CO_2 concentration, also ABA-independent components exist. ABA-induced stomatal closure was completely functional in the mutants of HT1 and MPK12, whereas these plants were deficient in CO_2-controlled stomatal movements (49, 58). Moreover, experiments aimed to dissect which parts of the SLAC1 anion channel are important for ABA- and which for high CO_2-induced stomatal closure showed that transgenic plants expressing SLAC1 anion channel without both C- and N-terminal regions were still able to respond to changes in CO_2 concentration, but remained ABA-insensitive. Thus, ABA-induced activation of SLAC1 seems to involve C- and N-terminal regions of the SLAC1, whereas CO_2-induced stomatal closure seems to rely only on the transmembrane region (158).

The overlap between CO_2 and ABA signaling suggests that ROS production in guard cells can increase in response to high CO_2 concentration, similar to ABA-induced stomatal closure (Fig. 3B). Using a fluorescent probe H2DCF-DA, ROS accumulation was indeed observed in guard cells treated with bicarbonate or high CO_2 concentration (17, 64, 125). Moreover, ROS scavengers impaired stomatal closure induced by CO_2 (17, 64). A connection between CO_2 and ABA signaling was further proved by the absence of ROS accumulation in stomata under elevated CO_2 concentration in the ABA-deficient double mutant *nced3 nced5*, as well as in the triple *pyr1 pyl1 pyl4* and the quadruple *pyr1 pyl1 pyl2 pyl4* mutants (17). Similar to ABA, elevated CO_2 induced ROS formation by NADPH oxidases (17, 125; Fig. 3). Thus, the *rbohD rbohF* double mutant demonstrated insensitivity of guard cells to bicarbonate/high CO_2 concentration. These mutants also failed to produce ROS in guard cells in response to elevated CO_2 (17, 64). Impaired accumulation of ROS in guard cells and decreased

620 stomatal closure in response to high CO_2 concentration were also observed in the tomato mutant
621 *rboh1* (125).
622
623 The current knowledge about high CO_2-induced stomatal closure suggests at least three partially
624 overlapping pathways: 1) Signaling through HT1/MPKs, which is ABA-independent and is triggered
625 by increased bicarbonate in guard cells (49, 58). 2) Direct perception of bicarbonate by SLAC1 in the
626 presence of protein kinases that activate SLAC1 (145). 3) An ABA-dependent component which
627 partially mediates high CO_2-controlled stomatal closure (17, 86, 157). ABA signaling that activates
628 OST1 and CPKs by suppression of PP2Cs could enhance SLAC1 sensitivity to bicarbonate, as well as
629 directly trigger SLAC1 anion currents, although this hypothesis should be verified in the future. It is
630 possible that plants under water stress should react to increased C_i faster and stronger than plants
631 with satisfactory water supply in order to save water in leaves when CO_2 supply for mesophyll cells
632 is sufficient. This could explain the importance of ABA signaling for CO_2-controlled stomatal
633 movements, which would allow plant to adapt changing environmental conditions.
634
635 **Future perspectives**
636 Recent research has highlighted the complex interplay between apoplastic, cytoplasmic and
637 chloroplastic redox/ROS signaling, as well as hormonal regulation in the control of stomatal
638 aperture. However, major gaps remain in the understanding of the complex interactions within the
639 guard cell signaling networks in response to changes in CO_2 and water availability. Considerable
640 efforts are needed for understanding how guard cells regulate, and are regulated by mesophyll
641 photosynthesis. The outstanding key questions are related to how guard cells perceive and transmit
642 signals from the surrounding environment and mesophyll cells. Furthermore, identification of
643 proteins that can sense changes in bicarbonate and ROS in guard cells is also needed. In the future

644 major breakthroughs will most likely come from the development of tools that enable real time
645 imaging of the cellular localization of ROS in guard cells in response to various stimuli. The focus of
646 future research should be directed to understand the complex interactions between various guard
647 cell signaling pathways, and how the guard cell hormones, ROS and Ca^{2+} homeostasis modulate
648 these interactions. In addition, the translation of such knowledge from model plants to important
649 crop species, especially to those grown in arid areas, will be increasingly important in the near
650 future.

651

652 **Innovation**

653 Stomata are essential for the survival of land plants in the changing environment as they control
654 water loss and CO_2 flow for photosynthesis. During recent years, several key molecular components in
655 guard cell CO_2 and drought induced ABA signaling have been identified and we are beginning to
656 understand complex interplay between the two signaling pathways. Future research should focus
657 on translating the knowledge from model species to agricultural crops in order to develop cultivars
658 that are more resistant to the stresses caused by environmental change.

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672 **Abbreviations**

673	ABA	Abscisic acid
674	ABA2	ABA DEFICIENT 2
675	ABCB14	ATP-BINDING CASSETTE B14
676	ABI1	ABA-INSENSITIVE 1
677	ABI2	ABA-INSENSITIVE 2
678	AHA1	H+ATPase 1
679	AMP	adenosine monophosphate
680	AOX	Alternative oxidase
681	ATP	Adenosine-5´-triphosphate
682	CA	Carbonic anhydrase
683	CAS	CALCIUM SENSING RECEPTOR
684	CBL	CALCINEURIN-B LIKE PROTEINS
685	CDPK/CPK	CALCIUM-DEPENDENT PROTEIN KINASE
686	Ci	intercellular CO_2 concentration
687	CIPK	CBL-interacting PROTEIN KINASES
688	CO_2	Carbon dioxide
689	DPI	NADPH inhibitor diphenyle iodonium
690	GCA2	GROWTH CONTROLLED BY ABSCISSIC ACID 2
691	GHR1	GUARD CELL HYDROGEN PEROXIDE-RESISTANT 1
692	GPXL3	GLUTATHIONE PEROXIDASE LIKE 3
693	HCO_3^-	Bicarbonate
694	HT1	HIGH LEAF TEMPERATURE 1
695	H^+ATPase	HYDROGEN ATPase

696	$HO^{\cdot}$	Hydroxyl radical
697	H2DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
698	H_2O_2	Hydrogen peroxide
699	JA	jasmonic acid
700	KAT1	Inward rectifying Shaker family K^+ channel
701	K^+	Potassium
702	LOX1	LIPOXYGENASE 1
703	MeJA	methyl jasmonate
704	NADPH	Nicotinamide adenine dinucleotide phosphate
705	MPK/MAPK	MITOGEN ACTIVATED PROTEIN KINASES
706	NO	Nitrogen oxide
707	OST1	OPEN STOMATA 1
708	$^1O_2$	Singlet oxygen
709	$O_2^{\cdot}$	Superoxide anion
710	PAP	3'-phosphoadenosine-5'-phosphate
711	PAPS	3'-phosphoadenosine-5'-phosphosulfate
712	PATROL1	PROTON ATPase TRANSLOCATION CONTROL 1
713	PIP2;1	PLASMA MEMBRANE INTRINSIC PROTEIN 2;1
714	PP2C	type 2C protein phosphatases
715	PYR	PYRABACTIN
716	PYL	PYR-LIKE
717	RBOH	RESPIRATORY BURST OXIDASE HOMOLOG
718	RCAR	REGULATORY COMPONENTS OF ABA RECEPTOR
719	RHC1	RESISTANT TO HIGH CO2

ROS	Reactive oxygen species
SA	Salicylic acid
SAL1	ADENOSINE BISPHOSPHATE PHOSPHATASE 1
SHAM	Salicylhydroxamid acid
SLAC1	SLOW ANION CHANNEL 1
SOT	sulfotransferases
Trx-h	THIOREDOXIN H-TYPE1
QUAC1	QUICK ANION CHANNEL 1
XRN	5'-3' exoribonuclease

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Figure 1. A simplified overview on how stomata control gas-exchange between leaves and the surrounding atmosphere. Guard cells react to changes in the environment as well as inside the plant. In response to light and decrease in CO_2 concentration, guard cells accumulate osmotically active potassium ions and anions (A^-), leading to water (H_2O) influx and an increase of guard cell volume. Open stomata allow CO_2 influx into the leaf with simultaneous efflux of water and release of oxygen (O_2). Stomata close in response to darkness, increase in CO_2 concentration, and drought. A phytohormone abscisic acid (ABA) accumulates in plants during drought and triggers stomatal closure. Efflux of osmotically active ions and water leads to reduced guard cell volume and stomatal closure. This process involves burst of reactive oxygen species (ROS) and elevation of calcium ion (Ca^{2+}) concentration. To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars.

Figure 2. Schematic diagram showing possible interactions between mesophyll cells (MC) and guard cells (GC) in epidermis (Ep) of leaves with open stomata (A) and with closed stomata (B). CO_2 enters the sub-stomatal cavity, where its concentration (C_i) regulates CO_2-dependent signaling in guard cells. Photosynthesis in mesophyll consumes CO_2 and by that reduces C_i and promotes stomatal opening. The flow of water from vascular bundles, formed by xylem (Xc) a phloem (Ph), transports a number of substances regulating stomatal apertures, including phytohormones, such as abscisic acid (ABA), and mesophyll-driven signals, such as malate (Mal) and sucrose (Suc). ABA can be also synthesized directly in guard cells. Stomatal closure induced by drought, salt/osmotic stress or by pathogen attack decrease the flow of CO_2 into leaves and

leads to a significant decrease in C_i. It also enhances photorespiration in mesophyll due to Rubisco oxygenase activity. To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars.

Figure 3. Schematic representation of CO_2 signaling events in guard cells. Aquaporines, including PLASMA MEMBRANE INTRINSIC PROTEIN 2;1 (PIP2;1), play a role in uptake of CO_2 by guard cells where it is converted into bicarbonate (HCO_3^-) by B-CARBONIC ANHYDRASEs 1 and 4 (βCA1, βCA4). Low (A) and high (B) CO_2 concentrations lead to fluctuations of the cytosolic bicarbonate, activating and inactivating downstream signaling components. A - Low CO_2-induced stomatal opening is initiated by proton extrusion via H^+-ATPases such as AHA1 whose translocation from inner membranes to plasma membrane is controlled by PROTON ATPASE TRANSLOCATION CONTROL 1 (PATROL1). Protein kinases HIGH LEAF TEMPERATURE 1 (HT1) and mitogen-activated protein kinases MPK4 and MPK12 are also involved in the activation of AHA1 but this mechanism is not defined yet. Protein kinases GUARD CELL HYDROGEN PEROXIDE RESISTANCE 1 (GHR1), and OPEN STOMATA 1 (OST1) are kept inactive by protein phosphatases PP2Cs and protein kinase HT1 during stomatal opening. B – High CO_2-induced stomatal closure is triggered by accumulation of cytosolic bicarbonate that leads to suppression of HT1 by MPK4, MPK12, and RESISTANT TO HIGH CO2 (RHC1) as well as inactivation of proton pumping by AHA1. Proteins sensing changes in cytosolic concentration of bicarbonate are not known although it has been suggested that SLAC1 could have a role in this. Stomatal closure in response to elevated CO_2 concentration involves components of ABA signaling including ABA binding by

PYR/PYL/RCAR proteins that leads to PP2Cs inactivation and activation of OST1 which is involved in the activation of anion channels SLAC1 and QUICK-ACTIVATING ANION CHANNEL 1 (QUAC1) as well as superoxide anion ($O_2^{\cdot-}$) production by RESPIRATORY BURST OXIDASE HOMOLOG F (RBOH F). Superoxide anion is further converted into hydrogen peroxide (H_2O_2) that can enter guard cells though aquaporins (PIPs). QUAC1 is also activated by increased concentration of apoplastic malate (Mal) acting as mesophyll-driven signal. Solid lines denote interactions that are supported by experimental data, and dotted lines indicate signaling events that still require further verification. Question marks show unknown components in signaling pathways. CBC – the Calvin-Benson cycle, G3P - glyceraldehyde-3-phosphate. To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars.

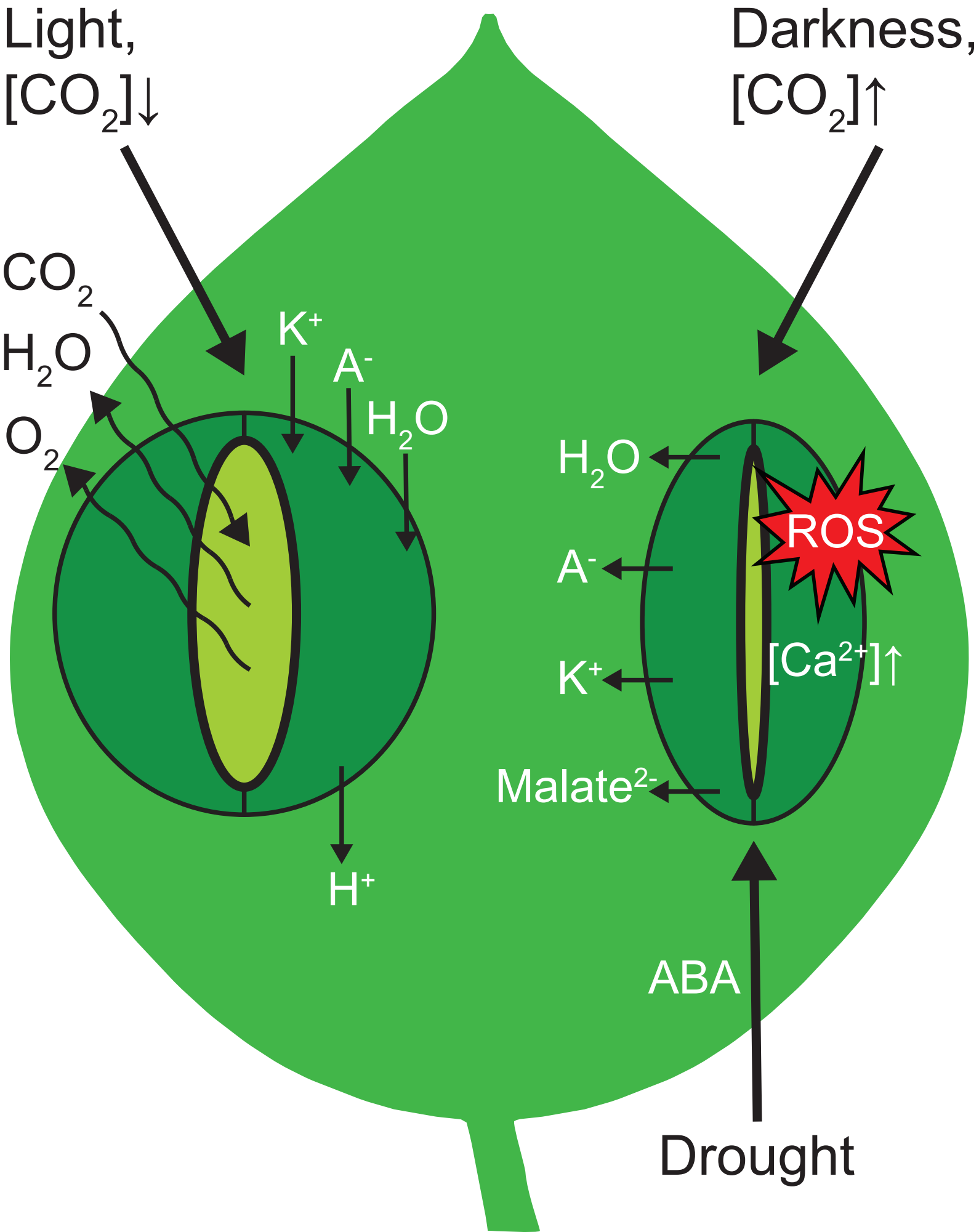
Figure 4. Gas-exchange experiments demonstrate the central roles for HIGH LEAF TEMPERATURE 1 (HT1) and OPEN STOMATA 1 (OST1) in stomatal responses to high CO_2 and abscisic acid (ABA), respectively. The four-weeks-old Arabidopsis plants were incubated in the gas-exchange cuvette until their stomatal conductance was stabilized. Subsequently plants were treated either with increased concentration of CO_2 or sprayed with 5 μM of ABA. Stomata of the HT1 mutants were completely insensitive to changes in CO_2 concentration, but displayed the unaffected response to ABA. The recessive *ht1-2* mutant has no HT1 protein kinase activity (45) and demonstrates reduced stomatal conductance indicating constantly activated stomatal closure. On the contrary, stomatal conductance is constitutively higher in plants carrying the dominant A109V mutation in HT1 that eliminates MPK4 and MPK12-dependent suppression of

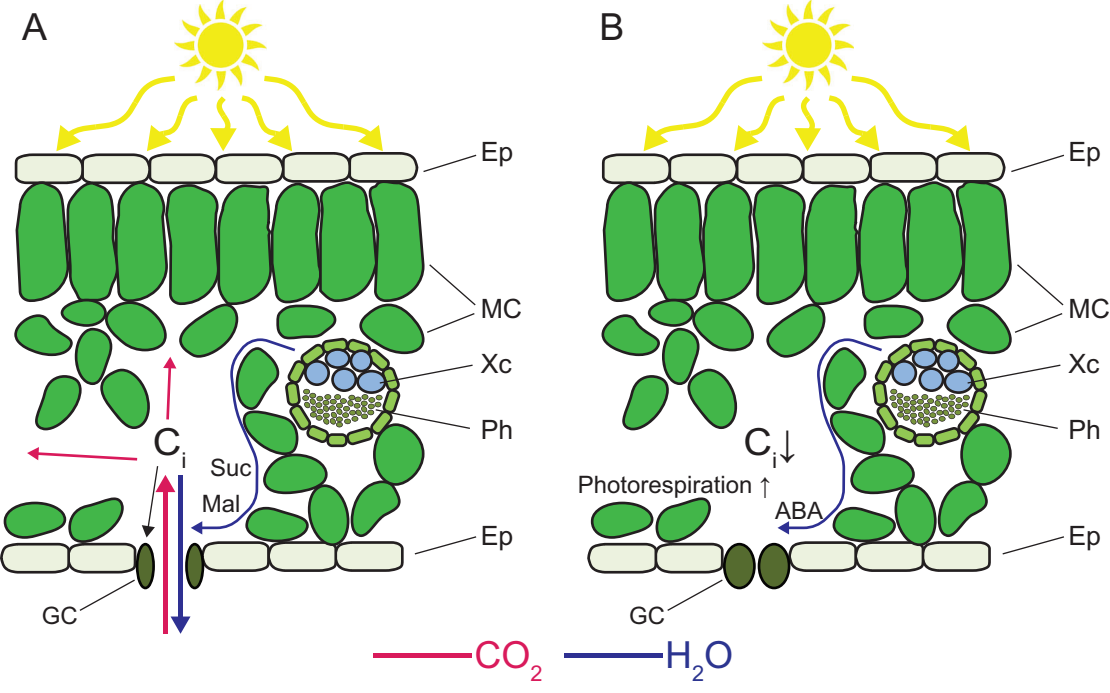
this protein (52) and shows that HT1 promotes stomatal opening through an unknown mechanism. Completely impaired ABA-induced stomatal closure in the *ost1-3* mutant demonstrates that OST1 is an important player in stomatal response to ABA. Stomata of this mutant responded to high CO_2 only partially, suggesting a role of OST1 for high CO_2 signaling in guard cells. Plant growth conditions and the used gas exchange system are described in (52, 62). The time of the sprays with 5 µM ABA is shown by the arrow. The values are the averages ± SE (n=4 for Col-0, *ht2-1*, n=3 for *ost1-3*, and $HT1^{A109V}$).

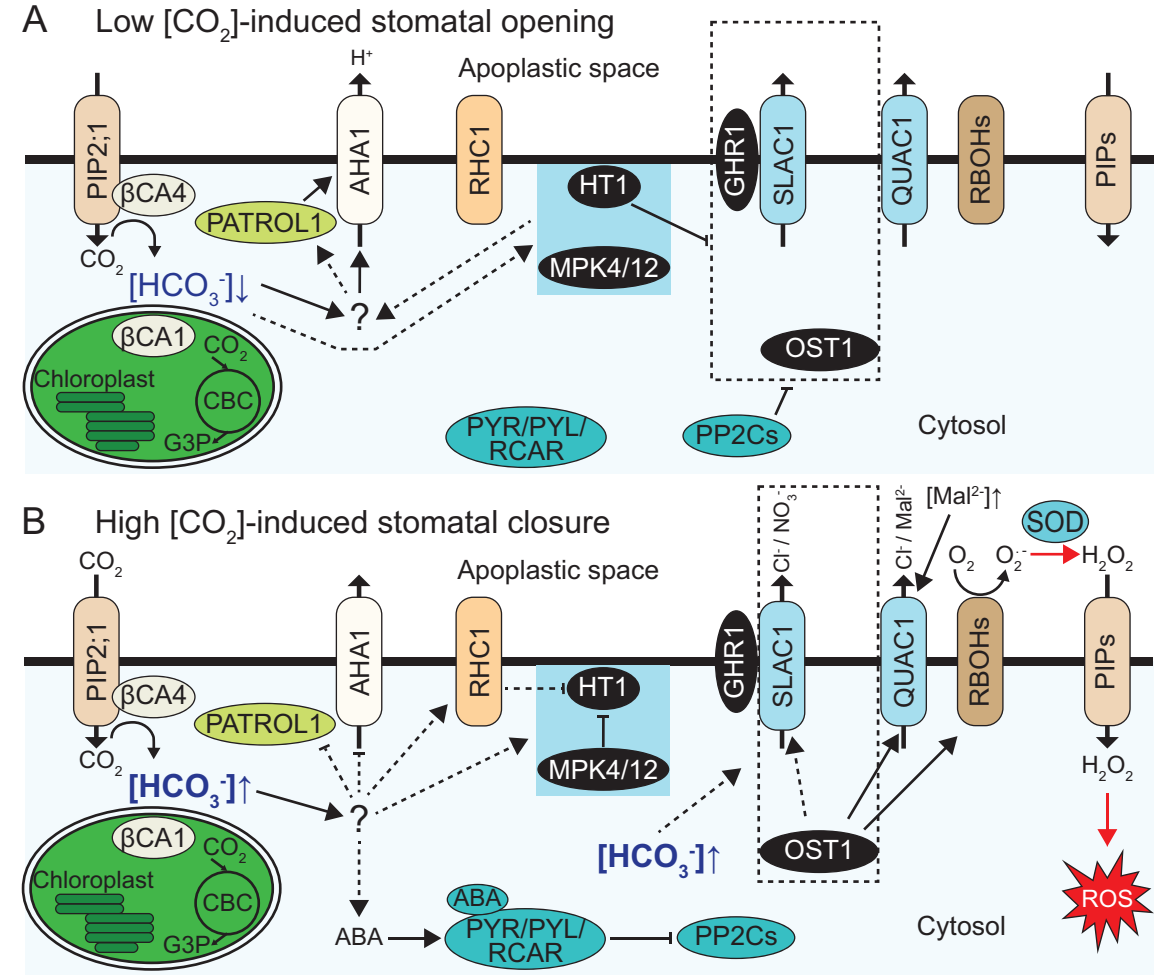
Figure 5. Schematic representation of signaling events regulating stomatal aperture in response to drought. Abscisic acid (ABA) is produced in guard cells and/or transported from apoplast by transporters, such as ABCG40. In the absence of ABA, TYPE 2C PROTEIN PHOSPHATASEs (PP2Cs) are active and function as inhibitors of OPEN STOMATA 1 (OST1) and CALCIUM-DEPENDENT PROTEIN KINASES (CPKs). During stress, binding of ABA to its receptor, PYR/PYL/RCAR, inactivates PP2Cs and OST1 is activated. OST1 is involved in the activation of SLOW ANION CHANNEL 1 (SLAC1), QUICK-ACTIVATING ANION CHANNEL 1 (QUAC1) and NADPH oxidase RESPIRATORY BURST OXIDASE HOMOLOG F (RBOHF) as well as inactivation of the potassium-inward channel KAT1. Apoplastic hydrogen peroxide (H_2O_2) is produced cell wall peroxidases (PRX) or by the conversion of superoxide anion ($O_2^{\cdot-}$) to hydrogen peroxide by SUPEROXIDE DISMUTASE (SOD). H_2O_2 enters guard cells through aquaporins (PIPs). Accumulation of reactive oxygen species (ROS) in guard cells leads to the activation of unknown inward rectifying calcium channels. GROWTH CONTROLLED BY ABSCISIC ACID 2 (GCA2) is involved in the formation of

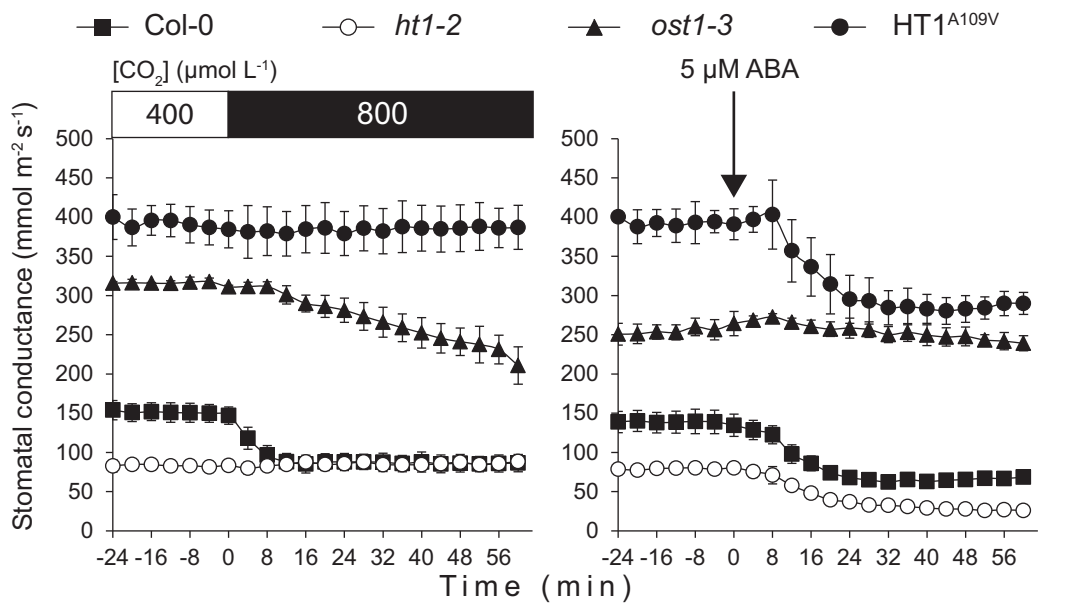
cytoplasmic calcium transients. ROS and Ca^{2+} are involved in the regulation of many different ABA signaling components. Degradation of 3'-phosphoadenosine 5'- phosphate (PAP) to adenosine-monophosphate (AMP) and phosphate (P_i) is catalyzed by adenosine bisphosphate phosphatase (SAL1) which activity is suppressed by chloroplastic redox state. CALCIUM SENSING RECEPTOR (CAS) is involved in the release of Ca^{2+} from thylakoids. Mal- malate. Solid lines indicate verified interactions; dashed lines indicate hypothetical/indirect interactions. Question marks denote unknown components. To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars.

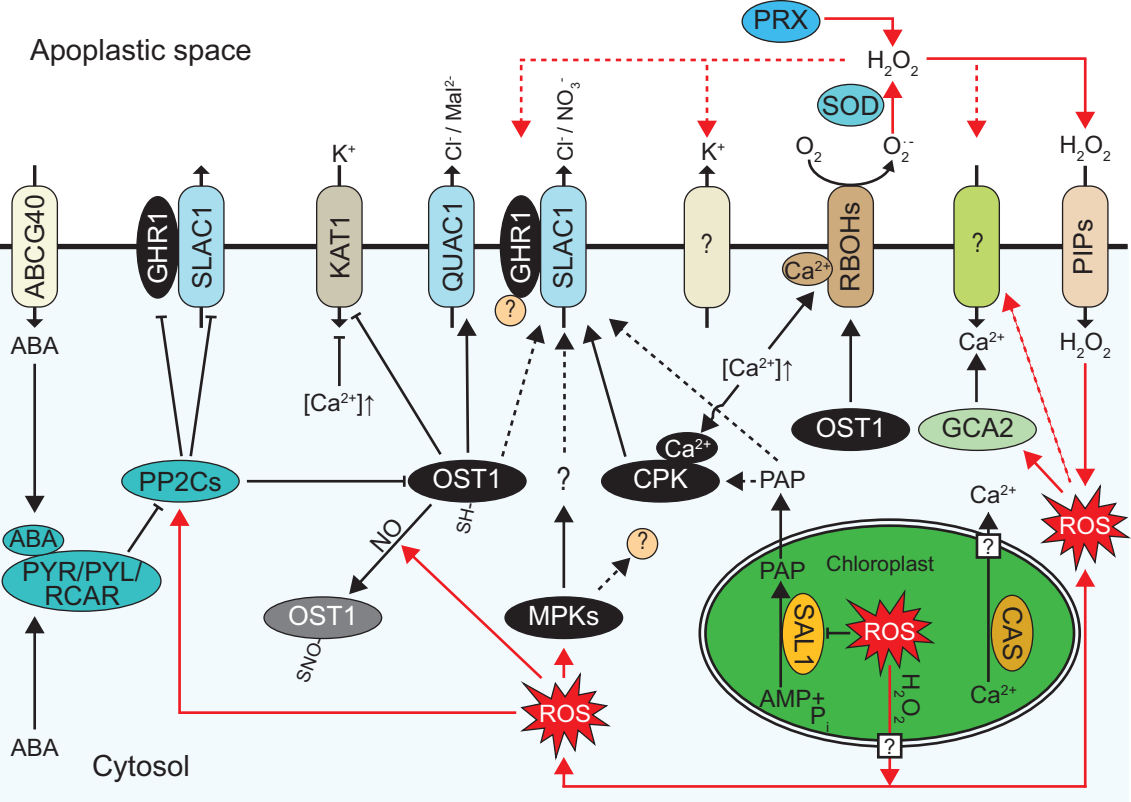
Figure 6. Overview of MITOGEN ACTIVATED PROTEIN KINASE (MPK) pathways involved in stomatal regulation. Pathogen associated molecular patterns (PAMPs), abscisic acid (ABA), and methyl jasmonate (MeJA) trigger activation of MPK pathways. MPK9 and MPK12 induce stomatal closure through SLOW ANION CHANNEL 1 (SLAC1) activation in response to ABA and MeJA induced production of reactive oxygen species (ROS) by RESPIRATORY BURST OXIDASE HOMOLOGs (RBOHs). MPK3 and MPK6 also contribute to the stomatal closure by increasing the metabolism of osmotically active organic acids, such as malate (Mal). In addition, MPK3 and MPK6 may activate QUICK-ACTIVATING ANION CHANNEL 1 (QUAC1) and the subsequent malate efflux would contribute to the decrease in cytosolic osmolyte concentration. The MPK3/6 contribute to the MPK9/12 activation through LIPOXYGENASE 1 (LOX1)-dependent stomatal pathway that requires salicylic acid (SA). SA is able to activate MPK9/12 and ROS is required in this process. Solid lines indicate verified interactions; dashed lines indicate hypothetical/indirect interactions. Pyr^- – pyruvate. To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars.

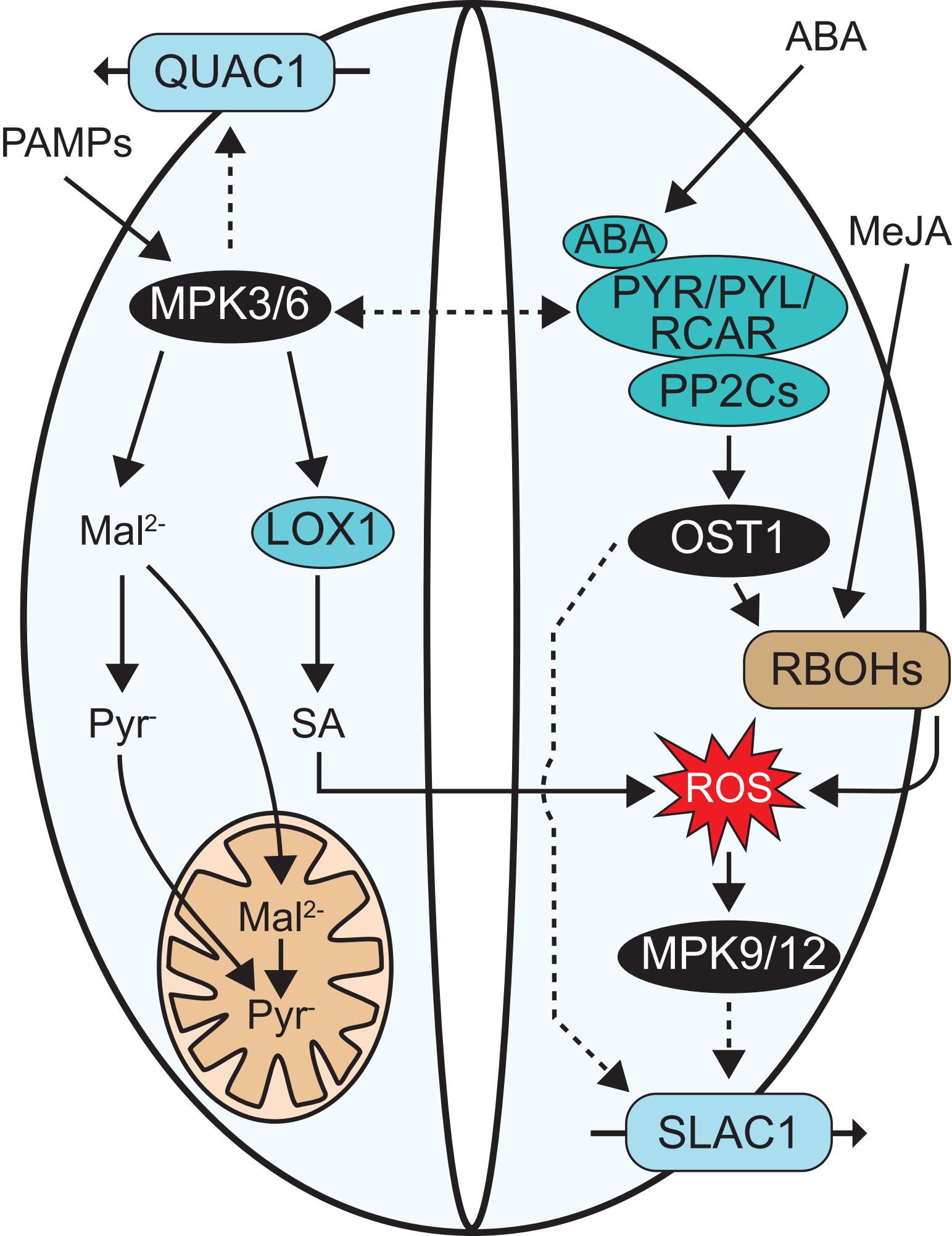

Light,
[CO2]↓
Darkness,
[CO2]↑
CO2
H2O
O2
K+
A-
H2O
H+
H2O
A-
K+
Malate2-
ROS
[Ca2+]↑
ABA
Drought


A
B
Ep
MC
Xc
Ph
Ep
C_i
Suc
Mal
GC
$C_i\downarrow$
Photorespiration ↑
ABA
CO_2
H_2O


A
Low [CO2]-induced stomatal opening
H+
Apoplastic space
PIP2;1
βCA4
CO2
PATROL1
AHA1
RHC1
HT1
MPK4/12
GHR1
SLAC1
QUAC1
RBOHs
PIPs
[HCO3-]↓
?
βCA1
CO2
Chloroplast
CBC
G3P
OST1
PYR/PYL/
RCAR
PP2Cs
Cytosol
B
High [CO2]-induced stomatal closure
CO2
Apoplastic space
Cl- / NO3-
Cl- / Mal2-
[Mal2-]↑
SOD
O2
O2-
H2O2
PIP2;1
βCA4
CO2
PATROL1
AHA1
RHC1
HT1
MPK4/12
GHR1
SLAC1
QUAC1
RBOHs
PIPs
H2O2
[HCO3-]↑
?
βCA1
CO2
Chloroplast
CBC
G3P
[HCO3-]↑
OST1
ROS
ABA
ABA
PYR/PYL/
RCAR
PP2Cs
Cytosol


Col-0
ht1-2
ost1-3
HT1A109V
[CO2] (μmol L-1)
400
800
5 μM ABA
Stomatal conductance (mmol m-2 s-1)
0
50
100
150
200
250
300
350
400
450
500
-24 -16 -8 0 8 16 24 32 40 48 56
Time (min)


Apoplastic space
PRX
H_2O_2
SOD
O_2
$O_2^{\cdot-}$
H_2O_2
ABCG40
GHR1
SLAC1
KAT1
QUAC1
GHR1
SLAC1
?
RBOHs
?
PIPs
K^+
Cl^- / Mal^{2-}
Cl^- / NO_3^-
K^+
Ca^{2+}
Ca^{2+}
H_2O_2
ABA
$[Ca^{2+}]\uparrow$
$[Ca^{2+}]\uparrow$
OST1
GCA2
Ca^{2+}
PP2Cs
OST1
?
CPK
PAP
ROS
ABA
PYR/PYL/
RCAR
NO
SH
Ca^{2+}
?
?
Chloroplast
PAP
SAL1
ROS
CAS
OST1
SNO
MPKs
?
AMP+P_i
H_2O_2
Ca^{2+}
ROS
ABA
Cytosol
?


QUAC1
ABA
PAMPs
ABA
MeJA
MPK3/6
PYR/PYL/
RCAR
PP2Cs
Mal2-
LOX1
OST1
RBOHs
Pyr-
SA
ROS
Mal2-
Pyr-
MPK9/12
SLAC1