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2 **FORUM REVIEW ARTICLE**

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

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25 **ABSTRACT**

26 *Significance:* Stomata sense the intercellular CO₂ concentration (C_i) and water availability under
27 changing environmental conditions and adjust their aperture to maintain optimal cellular conditions
28 for photosynthesis. Stomatal movements are regulated by a complex network of signaling cascades
29 where reactive oxygen species (ROS) play a key role as signaling molecules. *Recent Advances:* Recent
30 research has uncovered several new signaling components involved in CO₂ and ABA-triggered guard
31 cell signaling pathways. In addition, we are beginning to understand the complex interactions
32 between different signaling pathways. *Critical Issues:* Plants close their stomata in reaction to stress-
33 conditions, such as drought, and the subsequent decrease in C_i leads to ROS production through
34 photorespiration and over-reduction of the chloroplast electron transport chain. This reduces plant
35 growth and thus drought may cause severe yield losses for agriculture especially in arid areas. *Future*
36 *Directions:* The focus of future research should be drawn towards understanding the interplay
37 between various different signaling pathways and how ROS, redox and hormonal balance changes
38 in space and time. Translating this knowledge from model species to crop plants will help in the
39 development of new drought resistant crop species with high yields.

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48 **Introduction**

49 Stomata are tiny pores formed by a pair of guard cells on the surfaces of plant leaves and stems.
50 Their primary role is to maintain an adequate supply of carbon dioxide (CO₂) for photosynthesis
51 while limiting water loss through transpiration. In order to adapt to ever-changing environmental
52 conditions, plants are constantly adjusting their stomatal apertures to control leaf CO₂ and water
53 content.

54

55 Guard cells sense the concentration of CO₂ in the sub-stomatal cavity (C_i) and are able to respond
56 rapidly to changes in C_i (31, 98). When conditions are optimal for photosynthesis in C₃ plants, CO₂
57 is consumed by carboxylation reactions in the chloroplasts of mesophyll cells. This leads to a
58 decrease in C_i below the ambient CO₂ concentration (~400 ppm) and triggers stomatal opening to
59 maintain CO₂ supply for the Calvin-Benson cycle in mesophyll chloroplasts. In contrast, an increase
60 in C_i leads to stomatal closure; this helps to conserve water but can also lead to increased leaf
61 temperature and reduced uptake of nutrients by the transpiration stream. Such regulation may
62 occur within minutes and is achieved by controlled transport of osmoregulatory ions, mainly
63 potassium (K⁺), chloride (Cl⁻) and malate through different types of ion channels in the guard cell
64 membranes (45, 65). Under conditions that limit photosynthesis, such as darkness, C_i increases due
65 to reduced CO₂ fixation through the Calvin-Benson cycle and stomatal aperture decreases (Fig. 1).
66 Conversion of CO₂ into bicarbonate (HCO₃⁻) mediates the stomatal response to changes in C_i (28, 52,
67 53). CO₂ is spontaneously dissolved in water with formation of bicarbonate. Inside plant cells, the
68 rate of this reaction is accelerated by carbonic anhydrases (CAs) (26). A Raf-like protein kinase, HT1
69 (HIGH LEAF TEMPERATURE 1) is a highly CO₂-specific stomatal regulator (44, 45, 52) which is
70 involved in controlling the activity of the SLOW ANION CHANNEL 1 (SLAC1) (49). Stomatal opening
71 occurs via activation of the guard cell plasma membrane H⁺ATPase, which causes hyperpolarization

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

76

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

94

95 In addition to adjustments of stomatal aperture, plants also react to long-term changes in
96 environmental conditions by adapting the stomatal density in newly developed leaves. Mechanisms
97 underlying the regulation of stomatal density in response to environmental changes have been
98 recently reviewed (18, 28) and will not be discussed here. Here, we address how stomata sense the
99 changes in CO₂ concentration and water availability in C3 plants, how drought-induced stomatal
100 closure leads to increased production of reactive oxygen species, ROS, and how ROS signals regulate
101 stomatal movement. The major forms of ROS, singlet oxygen (¹O₂), superoxide anion (O₂^{•-}),
102 hydrogen peroxide (H₂O₂) and hydroxyl radical (HO[•]) are formed in different subcellular
103 compartments of plants. This occurs mostly through photorespiration-related reactions in
104 peroxisomes, in mitochondrial electron transport chains, by over reduction of the chloroplastic
105 electron transport chain, and by specific ROS-producing enzymes (15, 92, 106). This review covers
106 the current knowledge of how signaling cascades relating to ROS, redox and changing environments
107 are involved in the adjustment in stomatal aperture.

108

109 **Coordination of mesophyll photosynthetic processes with stomatal aperture**

110 Stomatal responsiveness to light and CO₂ is suppressed when the epidermis is detached from the
111 leaf, whereas re-establishment of the contact between mesophyll and detached epidermis restores
112 stomatal responsiveness. This suggests the existence of diffusible chemical or vapor phase signals
113 released from the mesophyll (71, 99, 121). The nature of the mesophyll-driven signals has, however,
114 remained elusive but a broad range of substances, including sucrose and malate, have been
115 considered (44, 71, 121). There is evidence that, CO₂ concentration inside the leaf rather than
116 outside the leaf influences stomatal aperture (98; Fig. 2). This notion is supported by several studies
117 implying that red light-induced stomatal opening is mediated by the reduction of C_i which is in turn
118 caused by the increased photosynthetic activity of mesophyll cells (118, 119). Guard cells are also

119 known to have signaling components specific to CO₂-responses. Plants carrying mutations in the
120 highly CO₂-specific protein kinase HT1 (42, 43, 49) showed severely suppressed stomatal opening in
121 response to red light induced decrease in C_i (80). However, C_i may not be the only signal through
122 which guard cells get information on mesophyll processes. Functional red light-induced stomatal
123 opening under artificially sustained C_i suggested that mesophyll photosynthesis could coordinate
124 stomatal regulation by C_i-independent mechanisms (68, 89). The existence of a C_i independent
125 signal was further supported by unaffected stomatal conductance in plants, which had high C_i due
126 to suppressed Rubisco activity (145). In addition, Blue light, a factor affecting photosynthesis and
127 stomatal opening is directly perceived by guard cells (126, 127, 159). In addition, over-reduction of
128 the plastoquinone pool in the mesophyll cell chloroplasts was recently suggested to induce ROS-
129 mediated stomatal closure (147). Taken together, it seems that guard cells are able to recognize
130 both changes in environmental conditions, such as light quality, and inner C_i-dependent and C_i-
131 independent signals from mesophyll. Although the influence of mesophyll cells on stomatal
132 aperture has been demonstrated by several studies, many details of this interplay remain unknown
133 and should be further elucidated in the future.

134

135 **The role of guard cell chloroplasts in stomatal signaling**

136 In most species, guard cell chloroplasts are smaller, present in a lower number, and have a less
137 developed thylakoid structure with reduced granal stacking than mesophyll cell chloroplasts (3,
138 154). Despite these differences, guard cell chloroplasts still have functional photosystems I and II as
139 well as Calvin-Benson cycle activity (10, 69, 70, 83, 117) and they can significantly contribute to
140 guard cell metabolism (67). Similarly, regulation of photosynthesis in guard cells and in mesophyll
141 by environmental factors can provide an indirect sensing mechanism coordinating stomatal
142 behavior with mesophyll demands for CO₂ (121, 133). There exist, however, some plant species such

143 as the orchid genus *Paphiopedilum* (21, 104) that do not have chloroplasts in their guard cells but
144 still display stomatal responses to high CO₂ concentration and changes in light conditions (104),
145 independent of photosynthesis in guard cells.

146

147 Guard cell chloroplasts are thought to be involved in osmoregulation of stomatal movements
148 through photosynthetic carbon fixation, which produces osmotically active sugars. However,
149 estimations of photosynthesis-derived osmotica in guard cells vary from 2% to 40% of the total pool
150 of osmotically active substances depending on plant species and experimental approaches (67).
151 Starch degradation in guard cell chloroplasts, which can be initiated by blue light and low CO₂ (50,
152 111), can also contribute to the formation of guard cell turgor by releasing monosaccharides and/or
153 provide phosphoenolpyruvate for CO₂ fixation by cytosolic phosphoenolpyruvate carboxylase,
154 leading to the formation of malate (50, 67, 121). Plants with impaired starch synthesis, both in
155 mesophyll and in guard cells, demonstrated reduced stomatal responsiveness to elevated CO₂,
156 indicating that conversion of osmotically active carbohydrates has a role in the reduction of osmotic
157 pressure during stomatal closure (8).

158

159 Although guard cell photosynthesis is important for the energization of stomatal opening (8, 133),
160 it does not seem to be directly involved in the regulation of stomatal aperture as stomata of plants
161 without chlorophyll in guard cells still remained responsive to CO₂ and ABA (9, 104). However,
162 numerous studies have highlighted the importance of guard cell chloroplast in stomatal regulation
163 through chloroplast-dependent ROS accumulation (128, 149), Ca²⁺ release (107, 153), and
164 retrograde signaling (113) (see the corresponding sections below). In conclusion, the function of
165 guard cell chloroplasts may not be compulsory for CO₂ 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 CO₂**

170 Decrease of CO₂ 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 CO₂-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 CO₂-free air but only a weak
176 response to high CO₂ concentrations (13, 65). The stomata of the lycophyte *Selaginella* responded
177 to both elevated and reduced CO₂ concentrations as well as to ABA (120) and stomatal closure in
178 response to elevated CO₂ 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 CO₂ 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 CO₂ 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 CO₂-induced stomatal opening. For example, plants with defects in the
191 NITRATE TRANSPORTER 1.1. demonstrated decreased stomatal opening in CO₂-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 CO₂ concentration still await identification.

196

197 **Mechanism of high CO₂ concentration-induced stomatal closure**

198 Stomatal closure is triggered by an increased concentration of CO₂ 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 CO₂ concentration (49, 86, 103,
203 141, 157). The role of apoplastic malate for high CO₂-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 CO₂ (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 CO₂-induced stomatal closure as demonstrated by accelerated and
210 delayed stomatal responses to high CO₂ 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 CO₂-induced
214 stomatal closure. This has been concluded based on experiments where Ca²⁺ accumulation in guard
215 cells subjected to higher than ambient CO₂ concentration was observed, and from the impaired high
216 CO₂-induced stomatal closure in the presence of Ca²⁺ chelators, such as BAPTA or EDTA (55, 124,
217 152). Genetically-encoded Ca²⁺ sensors revealed that guard cells displayed oscillations of cytosolic
218 Ca²⁺ concentration [Ca²⁺]_{cyt} and these patterns were often associated with changes in stomatal
219 aperture (4). However, unexpectedly, guard cells exposed to reduced CO₂ concentration
220 demonstrated more [Ca²⁺]_{cyt} transients than those under elevated CO₂ concentration (161). As
221 guard cells produced 'spontaneous' cytoplasmic Ca²⁺-transients and Ca²⁺ is required for high CO₂-
222 induced stomatal closure, it was suggested that elevated CO₂ concentration enhances sensitivity of
223 stomatal closing mechanisms to [Ca²⁺]_{cyt}. In agreement with this hypothesis, CO₂-derived
224 bicarbonate enhanced Ca²⁺ sensitivity of the S-type anion channel activation in guard cells (157).

225

226 **Bicarbonate as a signaling molecule in CO₂-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 CO₂ permeability of biological membranes in relation with
229 direct diffusion of CO₂ through the membranes vs the role of CO₂-permeable aquaporin channels
230 has been addresses in several papers and there are indications that specific aquaporins can have a
231 significant role in CO₂ 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 CO₂ diffusion
233 in *Xenopus laevis* oocytes (145). However, knocking out PIP2;1 was not sufficient to impair stomatal
234 CO₂ responses (145). This could be explained by functional redundancy among guard cell
235 aquaporins; there are 35 AQP homologs in Arabidopsis. Transport of CO₂/bicarbonate to
236 chloroplasts also depends at least partly on aquaporins, including the PIP1;2 that is located in

237 chloroplast envelope (138). In addition to the proposed role for PIP2;1 in CO₂ transport, it was also
238 involved in ABA-triggered stomatal closure (37).

239

240 Conversion of CO₂ into bicarbonate (HCO₃⁻) is an important step that mediates stomatal responses
241 to changes in ambient CO₂ and C_i (28, 52, 53). Although CO₂ is spontaneously dissolved in water with
242 formation of bicarbonate, in cells the rate of this reaction is accelerated by carbonic anhydrases
243 (CAs) (26). Among CAs expressed in Arabidopsis, the function of βCA1 and βCA4, localized in
244 chloroplasts and in plasma membrane, respectively, was important for the rapid stomatal response
245 to changes in CO₂ levels (52; Fig. 3). While single βCA mutants did not display clearly altered CO₂
246 sensitivity, the double knockout of both βCA1 and βCA4 significantly delayed stomatal responses to
247 CO₂ (28, 52, 53). Interestingly, PIP2;1 physically interacted with β-carbonic anhydrase 4 (βCA4) and
248 this connection has been suggested to enable the generation of CO₂ concentration gradient and
249 thus enhance transport of CO₂ into guard cells (145). The importance of HCO₃⁻ is further supported
250 by the experiments showing that the concentration of cytosolic bicarbonate, rather than CO₂,
251 activated S-type anion channels in guard cell protoplasts (157). The role of bicarbonate as a small
252 signaling molecule in guard cells was also confirmed by reconstitution of CO₂ signaling pathway in
253 *X. laevis* oocytes co-expressing PIP2;1, βCA4, SLAC1 and CPK6/23 or OST1. In these experiments, the
254 presence of these proteins was enough to confer bicarbonate-induced activation of SLAC1 anion
255 currents in oocytes (145).

256

257 Despite the established connection between cytosolic bicarbonate and anion channels in guard
258 cells, our knowledge about CO₂ signaling in guard cells has still major gaps. As an example, it has not
259 been resolved which proteins can bind and/or sense the changes in bicarbonate concentration in
260 guard cells to transmit the signal that eventually leads to changes in ion channel activities.

261

262 **Mitogen activated protein kinases MPK4 and MPK12 and HT1 - a new pathway controlling SLAC1**
263 **activation in response to changes in CO₂**

264 Mutant screens with different approaches have led to the identification of important components
265 in guard cell CO₂ signaling (41, 43, 103, 141). The Raf-like protein kinase HT1 was identified by using
266 thermal imaging of mutagenized plants subjected to low CO₂. HT1 is expressed in guard cells and is
267 highly CO₂-specific regulator, since plant lines carrying mutations in HT1 displayed stomata
268 completely insensitive to changes in CO₂ concentration, but remained responsive to other stimuli
269 such as light, ABA, and air humidity (42, 43; Fig. 4). HT1 plays a role in controlling the activation of
270 SLAC1 anion channel as a response to changes in CO₂ concentration (Fig. 3). Experiments carried out
271 in heterologous system, *X. laevis* oocytes, demonstrated that SLAC1 activation by OST1 and by
272 receptor like protein kinase GUARD CELL HYDROGEN PEROXIDE-RESISTANT1 (GHR1) was suppressed
273 by HT1 (49, 137). However, the mechanism how HT1 affects SLAC1 activation remains controversial.
274 Some experiments have suggested that HT1 could phosphorylate OST1 and by that suppress SLAC1
275 phosphorylation by OST1 (137), however, these experiments were not confirmed in another study
276 (49). Despite of using various versions of the HT1 protein, no inhibition on OST1-induced
277 phosphorylation of SLAC1 was observed in the presence of HT1. Instead, HT1 showed
278 phosphorylation activity towards GHR1 and the N-terminus of SLAC1 *in vitro*; the functional
279 outcome of these reactions, however, remained unclear (49). Thus, mechanism by which HT1
280 controls anion channel activation during stomatal closure in response to elevated CO₂ requires
281 further research. Furthermore, one should remember that results obtained in *in vitro* experiments
282 and heterologous systems, such as *X. laevis* oocytes, do not necessarily reflect the regulatory
283 interactions *in planta* due to missing components, and the models predicted in these artificial
284 experimental systems need to be confirmed in plants before constructing regulatory models.

285

286 Studies with MPK inhibitors and work focusing on the natural variation of water-use efficiency and
287 ozone sensitivity among *Arabidopsis* natural accessions revealed that MPK12 is an important
288 component of stomatal regulation (24, 58, 59). Further work showed that MPK4 and MPK12 are
289 essential for CO₂-dependent stomatal regulation (Fig. 3). Both of these MPKs inhibited HT1 activity
290 *in vitro*, whereas experiments in *X. laevis* oocytes indicated that MPK12 was able to restore SLAC1
291 activation by GHR1 in the presence of HT1 (49, 58). It is noteworthy that CO₂-induced stomatal
292 responses in plants lacking MPK12 and MPK4 in their guard cells were fully abolished, similar to that
293 observed for strong HT1 mutants. Thus, these MPKs seem to play a central role in controlling HT1
294 in CO₂-induced stomatal regulation, however, the mechanism that relays changes in bicarbonate
295 concentration to MPKs in guard cells remains to be addressed. A MATE-type transporter, RESISTANT
296 TO HIGH CO₂ (RHC1) was also suggested to act as an upstream regulator of HT1. Its abundance is
297 high in guard cell plasma membranes and its activity was essential for stomatal response to high
298 CO₂ concentration (137). Phenotype of the *rhc1 ht1-2* double mutant and oocyte experiments
299 implied that RHC1 could act as a bicarbonate sensing element upstream of HT1, although its exact
300 mechanism remained unknown (137). In contrast to these results, another study (149) showed that
301 RHC1 alone was able to cause bicarbonate-insensitive ion currents in *X. laevis* oocytes, making the
302 role of RHC1 in CO₂/bicarbonate sensing unresolved.

303

304 **ROS production and sensing in guard cell signaling during drought**

305 Under certain conditions, stomata must close despite the mesophyll CO₂ demands and low C_i. This
306 type of stomatal closure can be induced, for example, by limited water availability, salt/osmotic
307 stress, air pollution, or by pathogen attack, which is often referred to as stomatal immunity.
308 Stomatal closure is one of the earliest responses of plants to water deficit. This rapid response is

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

313

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

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

335

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

350

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

357 activation of guard cell anion channels indirectly through controlling the activation of CPKs, possibly
358 by phosphorylation of RBOHF. The resulting ROS burst would activate Ca²⁺-channels, followed by
359 CPK-dependent activation of SLAC1 (123, 128). In this model OST1 would function upstream of ROS
360 production and be negatively regulated by the PP2C ABI1, as has been shown (60, 75, 101).
361 Furthermore, GHR1, and its negative regulator ABI2, another PP2C, would be involved in the
362 downstream activation of plasma membrane Ca²⁺-channels and subsequent stomatal closure (54,
363 101; Fig. 5).

364

365 Although there is clear evidence for the involvement of ROS in the regulation of stomatal aperture,
366 it is still not known how the ROS signals are sensed in the guard cell apoplast. Identification of the
367 ROS and redox sensors has been one of the major challenges in plant ROS research during recent
368 years. In guard cells, only a few ROS sensing mechanisms are known to be involved in the stomatal
369 regulation. These are the redox regulation of the GHR1 apoplastic domain (54) and the redox
370 regulation of OST1 (146) and CPK1 (139). GHR1 is a plasma membrane associated atypical *Leucine-*
371 *rich repeat* receptor-like protein kinase that has been proposed to be involved in apoplastic ROS
372 perception. The apoplastic C-terminal domain of GHR1 has two conserved cysteines (C-57 and C-66)
373 that are necessary for the correct function of the protein (54). As discussed earlier, GHR1 has been
374 implicated as a central regulator of guard cell CO₂ and early ABA responses but the molecular
375 mechanism for its function is still unclear. GHR1 has been shown to interact with SLAC1 (54) but it
376 is not likely to activate SLAC1 by phosphorylation as its cytoplasmic kinase domain lacks the
377 conserved amino acids that are required for kinase activity (M. Sierla, H. Hörak, K. Overmyer, H.
378 Kollist, and J. Kangasjärvi, unpublished data). Therefore, it is likely that there are other unidentified
379 proteins involved in the GHR1 mediated SLAC1 activation.

380

381 The protein phosphatases ABI1 and ABI2 have also been shown to be inactivated in the presence of
382 H₂O₂ (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 H₂O₂ 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 H₂O₂ (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 endoplasmic 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 dephosphorylation 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 Ca²⁺ 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 Ca²⁺ 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

453 The role of other plant hormones in guard cell drought response

454 In addition to ABA, also other plant hormones and low-molecular-weight compounds have a role in
455 the induction of stomatal responses to drought and in the mediation of ROS-related or -dependent
456 signal transduction leading to stomatal closure. Jasmonic acid (JA) and its methyl ester (Methyl
457 jasmonate, MeJa) induce ROS production and stomatal closure through the activation of RBOHD
458 and/or RBOHF (134). MeJa-induced stomatal closure, ROS production, and cytosolic alkalization
459 were unaffected in the *pyr1 pyl1 pyl2 pyl4* quadruple mutant, but was impaired in the SnRK protein
460 kinase OST1 loss of function mutants, *ost1* and *srk2e*, and in the ABA deficient, *aba2-2* mutant (160).
461 This suggests that the MeJa activation of RBOHD and/or RBOHF requires ABA priming (as also implied
462 by previous studies; Hou *et al.*, 2013; Murata *et al.*, 2015) and OST1 function, but does not activate
463 OST1 through the canonical ABA signaling pathway in guard cells. JA and MeJa have been suggested
464 to regulate stomatal closure through transcriptional regulation of MeJa responsive genes and
465 through ROS and nitric oxide (NO) -triggered, Ca²⁺-dependent activation of CPK6 and its downstream
466 target SLAC1 (23).

467

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

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

488

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

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

507

508 **MPKs play multiple roles in the regulation of stomatal movement**

509 In addition to CO₂ signaling, MPKs are also suggested to have a role in guard cell ABA and pathogen
510 signaling (22, 74). Whereas MPK9 and MPK12 were involved in the stomatal responses to ABA,
511 MPK3 and MPK6 mediated pathogen signaling in guard cells (95). As discussed earlier, ROS are
512 produced by RBOHs in response to both ABA and pathogen signaling, but while RBOHF is mainly
513 responsible for the ROS production in response to ABA, RBOHD is involved in stomatal closure in
514 response to recognition of potentially pathogenic microorganisms (60, 77). It would be tempting to
515 speculate that the two NADPH oxidases, RBOHD and RBOHF are regulating two separate MPK
516 pathways but recent research has indicated that the reality is more complicated.

517

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

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

535

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

548

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 H₂O₂-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 H₂O₂ 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 H₂O₂ resulted in the formation of intramolecular disulfide bond that reduced the kinase

573 activity. Conversely, CPK21 was activated by a THIOREDOXIN H-TYPE1 (Trx-h). Thioredoxins are small
574 proteins that catalyse the thiol to disulfide exchange reaction in their target proteins. Incubation of
575 the oxidized CPK21 together with the Trx-h rescued the kinase activity suggesting that CPK21 could
576 be subjected to redox regulation under changing conditions (139). Furthermore, during stress the
577 inactivation of CPK21 by H₂O₂ could act as a negative feedback regulation of ABA-induced stomatal
578 closure. It would be interesting to see if other CPKs are regulated in similar manner.

579

580 **Connection between CO₂ and ABA signaling in guard cells**

581 Since both ABA- and high CO₂-induced stomatal closure involve activation of SLAC1 in guard cells,
582 one could expect that ABA- and CO₂-signaling converge. Indeed, several mutations causing stomatal
583 ABA-insensitivity, such as *abi1-1* and *abi2-1*, (86, 151) as well as *ost1* and *ghr1* (49, 86, 157; Fig. 4)
584 display impaired stomatal responses to high CO₂ concentrations. Stomata of the GROWTH
585 CONTROLLED BY ABSCISSIC ACID 2 (GCA2) mutant, *gca2*, which is related to CPKs were insensitive
586 to both ABA and high CO₂ concentration. Furthermore, *gca2* displayed altered patterns of
587 cytoplasmic Ca²⁺ transients in response to these stimuli and was suggested as a convergence point
588 between CO₂ and ABA signaling (4, 161).

589

590 ABA receptors, PYR/PYL/RCARs, are also involved in CO₂ signaling, as inactivation of several of these
591 proteins impaired stomatal closure in elevated CO₂ concentrations (17, 86). Due to a large number
592 of the PYR/PYL /RCAR proteins and a functional redundancy between them, further research is
593 required to identify which combination of PYR/PYL /RCARs function in the regulation of CO₂-induced
594 stomatal closure (17, 86, 157). Furthermore, recently developed fluorescent probes that enable real
595 time in vivo monitoring of ABA in plant cells (144) should be used in addressing the interplay
596 between CO₂ and accumulation of ABA in guard cells during changes in CO₂ concentration (28).

597

598 Although several key-components of ABA signaling are also connected with stomatal responses to
599 high CO₂ concentration, also ABA-independent components exist. ABA-induced stomatal closure
600 was completely functional in the mutants of HT1 and MPK12, whereas these plants were deficient
601 in CO₂-controlled stomatal movements (49, 58). Moreover, experiments aimed to dissect which
602 parts of the SLAC1 anion channel are important for ABA- and which for high CO₂-induced stomatal
603 closure showed that transgenic plants expressing SLAC1 anion channel without both C- and N-
604 terminal regions were still able to respond to changes in CO₂ concentration, but remained ABA-
605 insensitive. Thus, ABA-induced activation of SLAC1 seems to involve C- and N-terminal regions of
606 the SLAC1, whereas CO₂-induced stomatal closure seems to rely only on the transmembrane region
607 (158).

608

609 The overlap between CO₂ and ABA signaling suggests that ROS production in guard cells can increase
610 in response to high CO₂ concentration, similar to ABA-induced stomatal closure (Fig. 3B). Using a
611 fluorescent probe H₂DCF-DA, ROS accumulation was indeed observed in guard cells treated with
612 bicarbonate or high CO₂ concentration (17, 64, 125). Moreover, ROS scavengers impaired stomatal
613 closure induced by CO₂ (17, 64). A connection between CO₂ and ABA signaling was further proved
614 by the absence of ROS accumulation in stomata under elevated CO₂ concentration in the ABA-
615 deficient double mutant *nced3 nced5*, as well as in the triple *pyr1 pyl1 pyl4* and the quadruple *pyr1*
616 *pyl1 pyl2 pyl4* mutants (17). Similar to ABA, elevated CO₂ induced ROS formation by NADPH oxidases
617 (17, 125; Fig. 3). Thus, the *rbohD rbohF* double mutant demonstrated insensitivity of guard cells to
618 bicarbonate/high CO₂ concentration. These mutants also failed to produce ROS in guard cells in
619 response to elevated CO₂ (17, 64). Impaired accumulation of ROS in guard cells and decreased

620 stomatal closure in response to high CO₂ concentration were also observed in the tomato mutant
621 *rboh1* (125).

622

623 The current knowledge about high CO₂-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 CO₂-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 CO₂ supply for mesophyll cells
632 is sufficient. This could explain the importance of ABA signaling for CO₂-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 CO₂ 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 Ca²⁺ 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 CO₂ flow for photosynthesis. During recent years, several key molecular components in
655 guard cell CO₂ 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.

659

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670

671

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	C _i	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·	Hydroxyl radical
697	H ₂ DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
698	H ₂ O 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	¹ O ₂	Singlet oxygen
709	O ₂ ^{·-}	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 CO ₂

720	ROS	Reactive oxygen species
721	SA	Salicylic acid
722	SAL1	ADENOSINE BISPHOSPHATE PHOSPHATASE 1
723	SHAM	Salicylhydroxamid acid
724	SLAC1	SLOW ANION CHANNEL 1
725	SOT	sulfotransferases
726	Trx-h	THIOREDOXIN H-TYPE1
727	QUAC1	QUICK ANION CHANNEL 1
728	XRN	5'-3' exoribonuclease
729		
730		
731		

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1 **Figure 1.** A simplified overview on how stomata control gas-exchange between leaves and the
2 surrounding atmosphere. Guard cells react to changes in the environment as well as inside the
3 plant. In response to light and decrease in CO₂ concentration, guard cells accumulate
4 osmotically active potassium ions and anions (A⁻), leading to water (H₂O) influx and an increase
5 of guard cell volume. Open stomata allow CO₂ influx into the leaf with simultaneous efflux of
6 water and release of oxygen (O₂). Stomata close in response to darkness, increase in CO₂
7 concentration, and drought. A phytohormone abscisic acid (ABA) accumulates in plants during
8 drought and triggers stomatal closure. Efflux of osmotically active ions and water leads to
9 reduced guard cell volume and stomatal closure. This process involves burst of reactive oxygen
10 species (ROS) and elevation of calcium ion (Ca²⁺) concentration. To see this illustration in color,
11 the reader is referred to the online version of this article at www.liebertpub.com/ars.

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14 **Figure 2.** Schematic diagram showing possible interactions between mesophyll cells (MC) and
15 guard cells (GC) in epidermis (Ep) of leaves with open stomata (A) and with closed stomata (B).
16 CO₂ enters the sub-stomatal cavity, where its concentration (C_i) regulates CO₂-dependent
17 signaling in guard cells. Photosynthesis in mesophyll consumes CO₂ and by that reduces C_i and
18 promotes stomatal opening. The flow of water from vascular bundles, formed by xylem (Xc) a
19 phloem (Ph), transports a number of substances regulating stomatal apertures, including
20 phytohormones, such as abscisic acid (ABA), and mesophyll-driven signals, such as malate (Mal)
21 and sucrose (Suc). ABA can be also synthesized directly in guard cells. Stomatal closure induced
22 by drought, salt/osmotic stress or by pathogen attack decrease the flow of CO₂ into leaves and

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

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27
28 **Figure 3.** Schematic representation of CO₂ signaling events in guard cells. Aquaporines, including
29 PLASMA MEMBRANE INTRINSIC PROTEIN 2;1 (PIP2;1), play a role in uptake of CO₂ by guard cells
30 where it is converted into bicarbonate (HCO₃⁻) by B-CARBONIC ANHYDRASEs 1 and 4 (βCA1,
31 βCA4). Low (A) and high (B) CO₂ concentrations lead to fluctuations of the cytosolic bicarbonate,
32 activating and inactivating downstream signaling components. A - Low CO₂-induced stomatal
33 opening is initiated by proton extrusion via H⁺-ATPases such as AHA1 whose translocation from
34 inner membranes to plasma membrane is controlled by PROTON ATPASE TRANSLOCATION
35 CONTROL 1 (PATROL1). Protein kinases HIGH LEAF TEMPERATURE 1 (HT1) and mitogen-
36 activated protein kinases MPK4 and MPK12 are also involved in the activation of AHA1 but this
37 mechanism is not defined yet. Protein kinases GUARD CELL HYDROGEN PEROXIDE RESISTANCE 1
38 (GHR1), and OPEN STOMATA 1 (OST1) are kept inactive by protein phosphatases PP2Cs and
39 protein kinase HT1 during stomatal opening. B – High CO₂-induced stomatal closure is triggered
40 by accumulation of cytosolic bicarbonate that leads to suppression of HT1 by MPK4, MPK12,
41 and RESISTANT TO HIGH CO₂ (RHC1) as well as inactivation of proton pumping by AHA1.
42 Proteins sensing changes in cytosolic concentration of bicarbonate are not known although it
43 has been suggested that SLAC1 could have a role in this. Stomatal closure in response to
44 elevated CO₂ concentration involves components of ABA signaling including ABA binding by

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

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

67 this protein (52) and shows that HT1 promotes stomatal opening through an unknown
68 mechanism. Completely impaired ABA-induced stomatal closure in the *ost1-3* mutant
69 demonstrates that OST1 is an important player in stomatal response to ABA. Stomata of this
70 mutant responded to high CO₂ only partially, suggesting a role of OST1 for high CO₂ signaling in
71 guard cells. Plant growth conditions and the used gas exchange system are described in (52, 62).
72 The time of the sprays with 5 μM ABA is shown by the arrow. The values are the averages ± SE
73 (n=4 for Col-0, *ht2-1*, n=3 for *ost1-3*, and HT1^{A109V}).

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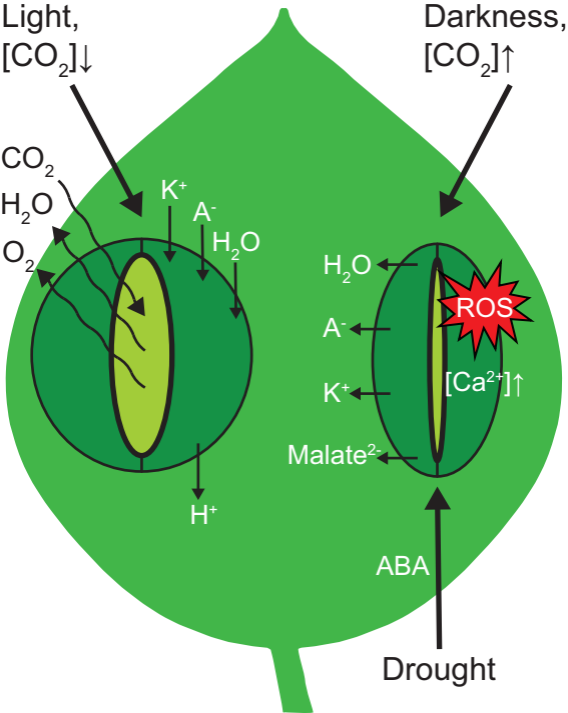
76 **Figure 5.** Schematic representation of signaling events regulating stomatal aperture in response
77 to drought. Abscisic acid (ABA) is produced in guard cells and/or transported from apoplast by
78 transporters, such as ABCG40. In the absence of ABA, TYPE 2C PROTEIN PHOSPHATASEs (PP2Cs)
79 are active and function as inhibitors of OPEN STOMATA 1 (OST1) and CALCIUM-DEPENDENT
80 PROTEIN KINASES (CPKs). During stress, binding of ABA to its receptor, PYR/PYL/RCAR,
81 inactivates PP2Cs and OST1 is activated. OST1 is involved in the activation of SLOW ANION
82 CHANNEL 1 (SLAC1), QUICK-ACTIVATING ANION CHANNEL 1 (QUAC1) and NADPH oxidase
83 RESPIRATORY BURST OXIDASE HOMOLOG F (RBOHF) as well as inactivation of the potassium-
84 inward channel KAT1. Apoplastic hydrogen peroxide (H₂O₂) is produced cell wall peroxidases
85 (PRX) or by the conversion of superoxide anion (O₂^{•-}) to hydrogen peroxide by SUPEROXIDE
86 DISMUTASE (SOD). H₂O₂ enters guard cells through aquaporins (PIPs). Accumulation of reactive
87 oxygen species (ROS) in guard cells leads to the activation of unknown inward rectifying calcium
88 channels. GROWTH CONTROLLED BY ABSCISIC ACID 2 (GCA2) is involved in the formation of

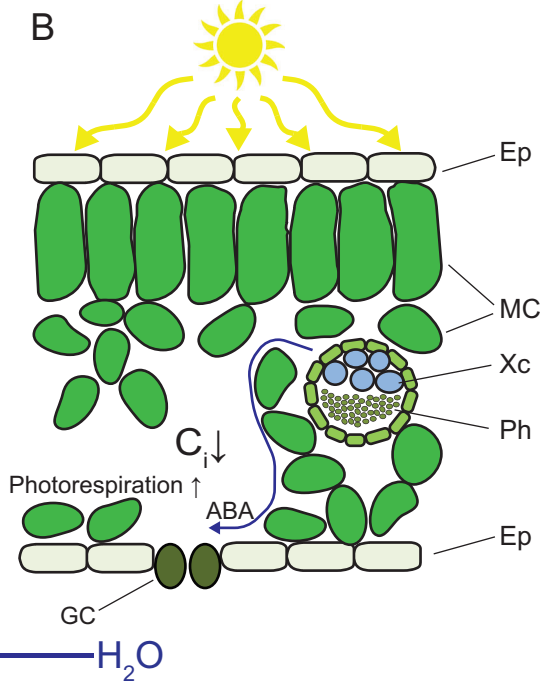
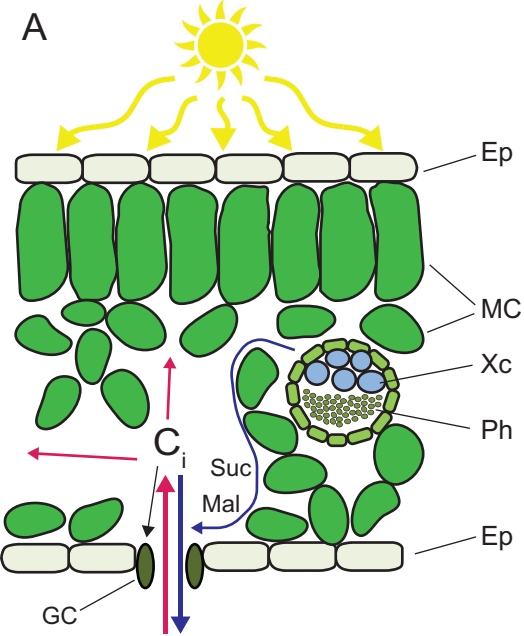
89 cytoplasmic calcium transients. ROS and Ca²⁺ are involved in the regulation of many different
90 ABA signaling components. Degradation of 3'-phosphoadenosine 5'- phosphate (PAP) to
91 adenosine-monophosphate (AMP) and phosphate (P_i) is catalyzed by adenosine bisphosphate
92 phosphatase (SAL1) which activity is suppressed by chloroplastic redox state. CALCIUM SENSING
93 RECEPTOR (CAS) is involved in the release of Ca²⁺ from thylakoids. Mal- malate. Solid lines
94 indicate verified interactions; dashed lines indicate hypothetical/indirect interactions. Question
95 marks denote unknown components. To see this illustration in color, the reader is referred to
96 the online version of this article at www.liebertpub.com/ars.

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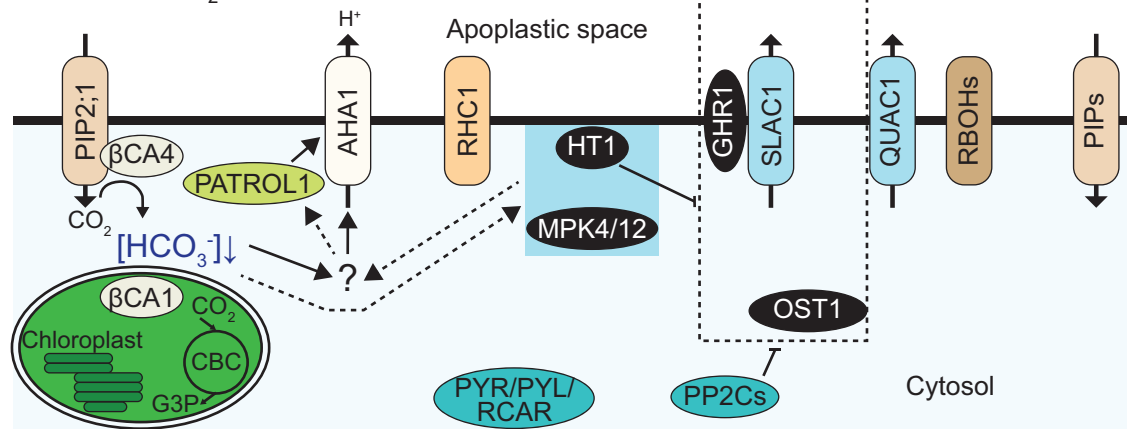
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99 **Figure 6.** Overview of MITOGEN ACTIVATED PROTEIN KINASE (MPK) pathways involved in stomatal
100 regulation. Pathogen associated molecular patterns (PAMPs), abscisic acid (ABA), and methyl jasmonate
101 (MeJA) trigger activation of MPK pathways.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

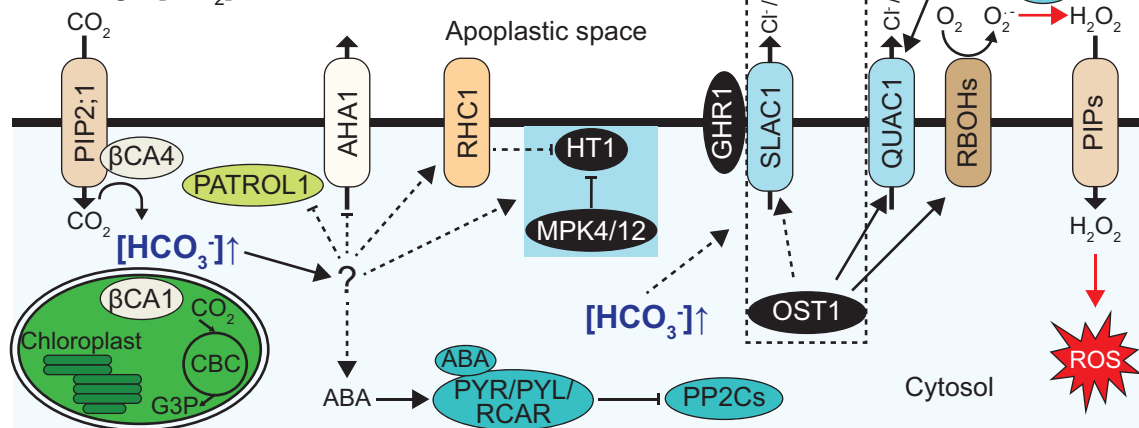




A Low [CO₂]-induced stomatal opening



B High [CO₂]-induced stomatal closure



■ Col-0 ○ *ht1-2* ▲ *ost1-3* ● HT1^{A109V}

