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FORUM REVIEW ARTICLE

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

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ABSTRACT

Significance: Stomata sense the intercellular CO₂ 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 CO₂ 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 pathways 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.

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

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 CO₂ 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 (¹O₂), superoxide anion (O₂^{•-}), hydrogen peroxide (H₂O₂) and hydroxyl radical (HO[•]) 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 CO₂ 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, CO₂ 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 CO₂-responses. Plants carrying mutations in the highly CO₂-specific protein kinase HT1 (42, 43, 49) showed severely suppressed stomatal opening in response to red light induced decrease in C_i (80). However, C_i 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 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

in guard cells during low CO₂-induced stomatal opening. For example, plants with defects in the NITRATE TRANSPORTER 1.1. demonstrated decreased stomatal opening in CO₂-free air, accompanied by reduced nitrate accumulation in guard cells (40). Malate transporter ATP-BINDING CASSETTE B14 (ABCB14) can also promote stomatal opening by uptake of malate from the apoplast (72). The involvement of other transporters and regulatory pathways in guard cells activated by reduced CO₂ concentration still await identification.

Mechanism of high CO₂ concentration-induced stomatal closure

Stomatal closure is triggered by an increased concentration of CO₂ and hence, elevated C_i, induces anion efflux through anion channels in the guard cell plasma membrane, followed by K⁺ efflux, and subsequent water outflow and a reduction of guard cell volume (Fig. 3B). Plants with defective S-type anion channel SLAC1 or with defects in the mechanisms that control SLAC1 activation display severely impaired stomatal closure in response to an increase in CO₂ concentration (49, 86, 103, 141, 157). The role of apoplastic malate for high CO₂-induced stomatal closure was demonstrated already in 1993 (44). Malate can be transported from mesophyll to guard cells and could act as a mesophyll-driven signal linking mesophyll metabolism with stomatal regulation (6, 109). The R-type anion channel QUAC1 can be activated by apoplastic malate. Accordingly, plants lacking QUAC1 in their guard cells demonstrated partially impaired stomatal response to high CO₂ (57, 90). Guard cells can also control the level of apoplastic malate by its uptake via ABCB14 activity (72). ABCB14 acts as a negative regulator in high CO₂-induced stomatal closure as demonstrated by accelerated and delayed stomatal responses to high CO₂ concentration in the *abcb14* mutants and ABCB14 overexpressors, respectively (72).

It has been suggested that calcium ions play a role as a second messenger in high CO₂-induced stomatal closure. This has been concluded based on experiments where Ca²⁺ accumulation in guard cells subjected to higher than ambient CO₂ concentration was observed, and from the impaired high CO₂-induced stomatal closure in the presence of Ca²⁺ chelators, such as BAPTA or EDTA (55, 124, 152). Genetically-encoded Ca²⁺ sensors revealed that guard cells displayed oscillations of cytosolic Ca²⁺ concentration [Ca²⁺]_{cyt} and these patterns were often associated with changes in stomatal aperture (4). However, unexpectedly, guard cells exposed to reduced CO₂ concentration demonstrated more [Ca²⁺]_{cyt} transients than those under elevated CO₂ concentration (161). As guard cells produced 'spontaneous' cytoplasmic Ca²⁺-transients and Ca²⁺ is required for high CO₂-induced stomatal closure, it was suggested that elevated CO₂ concentration enhances sensitivity of stomatal closing mechanisms to [Ca²⁺]_{cyt}. In agreement with this hypothesis, CO₂-derived bicarbonate enhanced Ca²⁺ sensitivity of the S-type anion channel activation in guard cells (157).

Bicarbonate as a signaling molecule in CO₂-controlled stomatal movements

A reduction or an increase of C_i should be sensed and translated into activation of corresponding signaling pathways in guard cells. The CO₂ permeability of biological membranes in relation with direct diffusion of CO₂ through the membranes vs the role of CO₂-permeable aquaporin channels has been addressed in several papers and there are indications that specific aquaporins can have a significant role in CO₂ uptake (see 38 for a review). Recently, a plant aquaporin, PLASMA MEMBRANE INTRINSIC PROTEIN 2;1 (PIP2;1), was shown to function as a channel for CO₂ diffusion in *Xenopus laevis* oocytes (145). However, knocking out PIP2;1 was not sufficient to impair stomatal CO₂ responses (145). This could be explained by functional redundancy among guard cell aquaporins; there are 35 AQP homologs in Arabidopsis. Transport of CO₂/bicarbonate to 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 CO₂ transport, it was also involved in ABA-triggered stomatal closure (37).

Conversion of CO₂ into bicarbonate (HCO₃⁻) is an important step that mediates stomatal responses to changes in ambient CO₂ and C_i (28, 52, 53). Although CO₂ 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 CO₂ levels (52; Fig. 3). While single βCA mutants did not display clearly altered CO₂ sensitivity, the double knockout of both βCA1 and βCA4 significantly delayed stomatal responses to CO₂ (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 CO₂ concentration gradient and thus enhance transport of CO₂ into guard cells (145). The importance of HCO₃⁻ is further supported by the experiments showing that the concentration of cytosolic bicarbonate, rather than CO₂, 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 CO₂ 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 CO₂ 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.

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 Ca^{2+} -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 Ca^{2+} -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 CO_2 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

The protein phosphatases ABI1 and ABI2 have also been shown to be inactivated in the presence of H₂O₂ (81, 82) but the mechanism for this redox regulation is still unknown. Another example for redox regulation of ABI1 and ABI2 in guard cell is a glutathione peroxidase like enzyme, GPXL3, in H₂O₂ scavenging and cytosolic redox-regulation in response to ABA and drought stress (91). GPXL3 was suggested to interact with ABI1 and ABI2. In addition, similarly as H₂O₂ (81, 82), oxidized GPXL3 decreased the phosphatase activity of ABI2 by affecting its redox status *in vitro*. However, both proteomic data and subcellular localization of GPXLs as GFP-fusions (7) suggest that GPXL3 is in fact a type II transmembrane protein anchored to the endoplasmic reticulum and/or Golgi so that the catalytic side remains in the lumen and would not be able to interact with the PP2Cs *in vivo*. In the light of these results, the molecular basis for the drought sensitive and resistant phenotypes of the *gpxl3* null mutant and GPXL3 overexpressor lines, respectively, (91) and the possible mechanisms of the redox regulation of the guard cell PP2Cs remain unknown.

The role of sulfate in drought sensing and the emergence of a new pathway

A number of studies with different plant species have shown that low soil water potential decreases stomatal conductance even before any measurable change in leaf water potential can be observed (34, 36). These results suggest that roots can sense low soil water potential and transmit a signal to guard cells initiating stomatal closure. The earlier hypothesis that root borne ABA acted as a drought signal to leaves has now been questioned since stomatal closure appears to be dependent on foliar ABA production (19, 47, 87). In addition, the ABA that accumulates in roots during long-term drought conditions appears to be derived from the shoots (79). Other signals, such as chemical, electrical and hydraulic, have been suggested to play a role in root to shoot signaling (56) and they all are likely to contribute to the long distance signaling through various signaling networks.

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 sulfonyl 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

causing the accumulation of its substrate, PAP (16, 30). It was suggested that PAP moves into nucleus (32) where it is thought to inhibit the post-transcriptional gene silencing of stress responsive genes by 5'-3' exoribonucleases (XRNs) (30). However, it is not clear whether chloroplastic or cytoplasmic PAP is responsible for gene regulation since the PAPS/PAP antiporter transports PAPS out and PAP into the chloroplast according to a concentration gradient (33, 34), which implies that inactivation of SAL1 results in increase of cytoplasmic PAP due to decrease of the concentration gradient-driven transport of PAP to chloroplast.

The involvement of PAP in ABA-dependent stomatal closure was also shown recently (113). The sensitivity of the guard cells of *abi1-1* and *ost1-3* for ABA was restored in mutant plants by genetically, or exogenously increasing PAP levels. In addition, PAP upregulated the expression of many ABA and Ca^{2+} responsive genes, including several CPKs. It was suggested (113) that because of the transcriptional regulation, PAP-mediated chloroplast signaling could bypass the canonical ABA signaling pathway and activate SLAC1. However, PAP-induced stomatal closure required sufficient concentrations of Ca^{2+} and apoplastic ROS production by NADPH oxidases, but did not affect the activity of SLAC1 or the highly selective inward-rectifying potassium channels KAT1 or KAT2 in *X. laevis* oocytes. This suggests that PAP is dependent on ABA-mediated processes and works rather as a second messenger in ABA signaling. Intriguingly, exogenous application of PAP on Arabidopsis and barley leaf peels was able to trigger stomatal closure within a few minutes and the kinetics of this reaction was almost identical to that of exogenous ABA application (113). It is highly unlikely that stomatal closure through transcriptional regulation would occur as fast as by ABA triggered post-transcriptional regulation. Therefore, PAP may also regulate SLAC1 activity through direct post-transcriptional regulation of other kinases such as CPKs or MAPKs (Fig. 5).

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 diphenyl 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-

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 CO₂ 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.

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

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 H₂O₂ 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 CO₂ and ABA signaling in guard cells

Since both ABA- and high CO₂-induced stomatal closure involve activation of SLAC1 in guard cells, one could expect that ABA- and CO₂-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 CO₂ 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 CO₂ concentration. Furthermore, *gca2* displayed altered patterns of cytoplasmic Ca²⁺ transients in response to these stimuli and was suggested as a convergence point between CO₂ and ABA signaling (4, 161).

ABA receptors, PYR/PYL/RCARs, are also involved in CO₂ signaling, as inactivation of several of these proteins impaired stomatal closure in elevated CO₂ 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 CO₂-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 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

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

Innovation

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

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668

669

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	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·	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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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 CO₂ concentration, guard cells accumulate osmotically active potassium ions and anions (A⁻), leading to water (H₂O) influx and an increase of guard cell volume. Open stomata allow CO₂ influx into the leaf with simultaneous efflux of water and release of oxygen (O₂). Stomata close in response to darkness, increase in CO₂ 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 (Ca²⁺) 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). CO₂ enters the sub-stomatal cavity, where its concentration (C_i) regulates CO₂-dependent signaling in guard cells. Photosynthesis in mesophyll consumes CO₂ and by that reduces C_i and promotes stomatal opening. The flow of water from vascular bundles, formed by xylem (Xc) and 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 CO₂ 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 CO₂ signaling events in guard cells. Aquaporines, including PLASMA MEMBRANE INTRINSIC PROTEIN 2;1 (PIP2;1), play a role in uptake of CO₂ by guard cells where it is converted into bicarbonate (HCO₃⁻) by B-CARBONIC ANHYDRASEs 1 and 4 (βCA1, βCA4). Low (A) and high (B) CO₂ concentrations lead to fluctuations of the cytosolic bicarbonate, activating and inactivating downstream signaling components. A - Low CO₂-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 CO₂-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 CO₂ 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 through 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 CO₂ only partially, suggesting a role of OST1 for high CO₂ 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. Absciscic 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 (H₂O₂) is produced cell wall peroxidases (PRX) or by the conversion of superoxide anion (O₂^{•-}) to hydrogen peroxide by SUPEROXIDE DISMUTASE (SOD). H₂O₂ 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.

— CO_2 — H_2O

A Low $[CO_2]$ -induced stomatal opening

B High $[CO_2]$ -induced stomatal closure