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Feature Review

CO_2 Sensing and CO_2 Regulation of Stomatal Conductance: Advances and Open Questions

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Guard cells form epidermal stomatal gas-exchange valves in plants and regulate the aperture of stomatal pores in response to changes in the carbon dioxide (CO_2) concentration ($[CO_2]$) in leaves. Moreover, the development of stomata is repressed by elevated CO_2 in diverse plant species. Evidence suggests that plants can sense $[CO_2]$ changes via guard cells and via mesophyll tissues in mediating stomatal movements. We review new discoveries and open questions on mechanisms mediating CO_2-regulated stomatal movements and CO_2 modulation of stomatal development, which together function in the CO_2 regulation of stomatal conductance and gas exchange in plants. Research in this area is timely in light of the necessity of selecting and developing crop cultivars that perform better in a shifting climate.

Importance of CO_2 Regulation of Stomatal Conductance

Higher-than-ambient $[CO_2]$ mediates closure of stomatal pores in plants and, conversely, low $[CO_2]$ triggers the opening of stomatal pores. Respiration in plant leaves in the night (dark) causes a rapid rise in the intercellular $[CO_2]$ (Ci) in leaves and measurements indicate that CO_2 levels can exceed 600 ppm (Figure 1A) CO_2 [1]. Moreover, Ci can rapidly drop to below 200 ppm in the light (165 $\pm$ 58 ppm) [1]. In parallel with the diurnal oscillation in Ci, global CO_2 levels have risen exponentially [2,3] since the advent of the Industrial Revolution (Figure 1B). April 2014 was the first month in recorded history to have consistently had CO_2 levels above 400 ppm. This increase in atmospheric CO_2 causes a rise in leaf Ci. Stomatal pore apertures respond to these changes in Ci [4,5]. A longer-term effect of the continuing $[CO_2]$ rise is the downregulation of stomatal development in the leaf epidermis [6]. This developmental response was first discovered almost three decades ago [6] and has also been subsequently verified by evidence in the fossil record [7,8]. While a preponderance of species exhibit this response, some species show either an opposite effect or are unresponsive to elevated $[CO_2]$ [9,10]. Despite the prime importance of these responses, little has been known about the underlying genetic mechanisms.

The combined effect of the above two CO_2 responses negatively impacts overall stomatal conductance on a global scale by reducing both stomatal apertures and the total numbers of stomata per unit leaf area. While decreased stomatal conductance is beneficial for limiting water loss from plant leaves [11] through reduced evapotranspiration, there is also a potential cost to

Trends

Research on how plant guard cells, which form epidermal stomatal gas-exchange valves, regulate the aperture of stomatal pores in response to changes in $[CO_2]$ is of current interest given the necessity of selecting and developing crop cultivars that perform better in a shifting global climate. Understanding of the underlying CO_2 response mechanisms is also needed for modeling efforts to better understand plant responses to rising atmospheric CO_2 levels.

Recent discoveries in guard cell CO_2 and secondary messenger signaling, the contributions of the subcellular localization of the CO_2-binding carbonic anhydrases, the interplay with the stress hormone ABA, and the role of photosynthesis in stomatal responses to the CO_2 stimulus indicate new models and raise new open questions in CO_2 signal transduction.

Elucidation of the molecular mechanisms controlling stomatal development and the identification of initial mechanisms mediating elevated CO_2 repression of stomatal development points to a signaling model and to new avenues for further research on this pathway.

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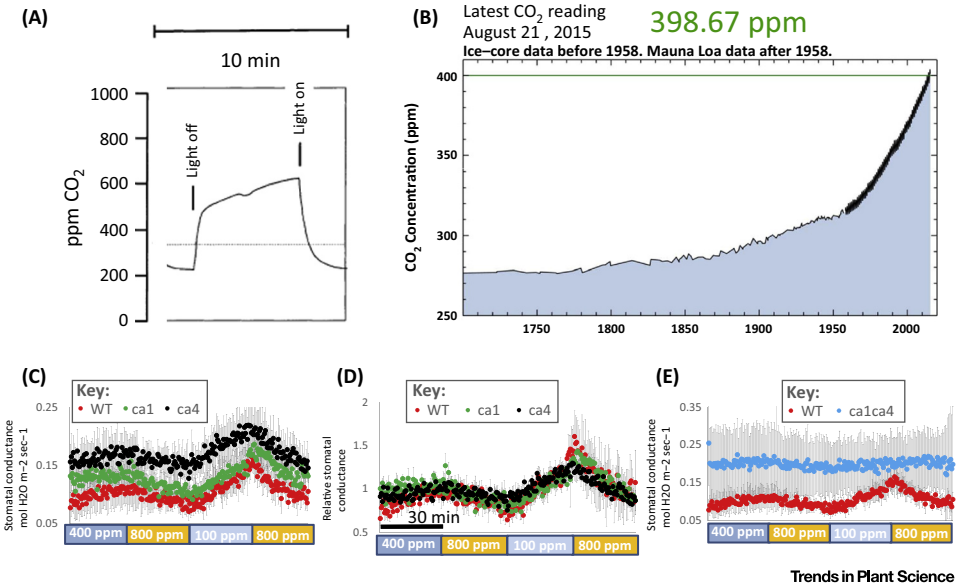

Figure 1. Changes of Leaf Carbon Dioxide (CO_2) Concentration (Ci) in Response to Light and Darkness, Atmospheric CO_2 Rise, and Stomatal Gas-exchange Response to Ambient [CO_2] Changes. (A) Effects of light on Ci in the *Vicia faba* substomatal cavity at an external ambient [CO_2] of 350 ppm. Substomatal cavity [CO_2] was measured with a potentiometric CO_2-biosensor microprobe inserted into leaves through open stomata [1]. Reprinted with permission from Elsevier Publishing Group. (B) [CO_2] in the Earth's atmosphere [2,146]. Reprinted with permission from https://scripps.ucsd.edu/programs/keelingcurve/wp-content/plugins/sio-bluemoon/graphs/co2_800k_zoom.png. (C–E) Effects of CO_2 on gas exchange in carbonic anhydrase mutant leaves. Raw (C,E) and normalized (D) stomatal conductance values for *ca1* and *ca4* single carbonic anhydrase mutants and wild type (WT). Individual ambient CO_2 treatments were each 30 min in duration. One leaf from each of three separate plants per genotype were analyzed over a period of 3 weeks and averaged. Errors represent the standard error of the mean.

such water savings. Fewer and more closed stomata in response to elevated CO_2 levels also reduce the evapotranspirative cooling ability of leaves, which in turn adds to leaf heat stress [12–14] under water-limited growth regimens. Heat stress, combined with drought and rising temperatures, can reduce plant health [15] and crop productivity globally, thus impacting agricultural practices and possibly nutrient content [16,17] and supply [18]. Furthermore, a large stomatal conductance can correlate with improved crop yield [19–21], and thus the downregulation of stomatal conductance by CO_2 may contribute to suboptimal yields when sufficient water is available. Additionally, [CO_2] also affects leaf development and leaf area in some species and these responses have a direct bearing on water conservation and improving biomass in plants. These varied responses to changes in atmospheric CO_2 levels complicate efforts to model and predict the effects of rising CO_2 levels on global gas exchange in forests and ecosystems. Future research and attempts to adapt crop production to climate change would benefit significantly from a molecular understanding of CO_2-regulated plant gas exchange. Here we summarize recent discoveries of molecular and cellular mechanisms mediating CO_2 regulation of stomatal movements. Interested readers are directed to reviews summarizing previous metabolic and physiological aspects of stomatal function [22–24]. We also review a recently uncovered initial framework [25] for mechanisms mediating elevated CO_2 repression of stomatal development in plants and point to open questions for further inquiry into this pathway. Predicting plant performance and plant responses to the combination of rising CO_2 and other environmental stresses such as heat and drought is reviewed elsewhere [12–14,26–29].

Convergence of CO_2 and Abscisic Acid (ABA) Signaling

Pioneering research has shown that the plant hormone ABA enhances the stomatal CO_2 response [30]. However, the precise signaling convergence point remains to be resolved

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[31–33]. The first genetic stomatal CO_2 signaling components discovered were the dominant ABA-insensitive PP2C protein phosphatase mutations abi1-1 and abi2-1, which cause conditional CO_2 insensitivity [31,34]. Molecular genetic components of the ABA and stomatal movement regulatory signal transduction machinery have been shown to be conserved over evolutionary timescales [35–37]. Interestingly, ABA signaling through ABA receptors, named PYR/RCARs, partially affects stomatal responses to elevated CO_2 [33]. A present model proposes that these ABA receptors may not directly mediate guard cell CO_2 signaling, but disruption slows CO_2 responses. These results suggest that CO_2 and ABA responses are mediated by converging pathways, such that the CO_2 response is weakened in the complete absence of an ABA response. Thus two models could be considered: (i) ABA and ABA receptors function upstream of the convergence point of CO_2 and ABA signaling, while synergistically amplifying common downstream signaling mechanisms [32,33]; or (ii) CO_2 elevation may rapidly (within 1–3 min) cause an elevation in the guard cell [ABA] and thus mediate stomatal closing. The recent development of real-time ABA reporters enables time-dependent monitoring of [ABA] changes in single cells including guard cells [38,39]. Such Förster resonance energy transfer (FRET) reporters will enable future analyses that address the question of whether [CO_2] elevation causes a rapid [ABA] increase in guard cells.

Roles for Carbonic Anhydrase Enzymes in CO_2 Signaling

The cellular sensing mechanisms for changes in [CO_2] and/or bicarbonate concentration that mediate rapid high-CO_2-induced stomatal movements are key to understanding the underlying response. Carbonic anhydrase enzymes functioning in guard cells have been shown to be important for the rapid wild-type (WT) stomatal movement response to changes in CO_2 levels [40]. These enzymes are beta carbonic anhydrases [40] and the CO_2 response of the alpha and gamma classes of carbonic anhydrases [41] has not yet been determined. Interestingly, complementation of double-mutant plants by expressing a structurally unrelated mammalian carbonic anhydrase preferentially in guard cells restores the WT response [40]. These data indicate that the catalytic activity of the carbonic anhydrase enzymes is involved in 'transponding' [42] the CO_2 stimulus in this pathway and point to bicarbonate and/or protons as possible second messengers involved in signal transduction. The importance of the role of bicarbonate in stomatal aperture regulation was found in patch-clamp studies. Elevation in the cytoplasmic bicarbonate concentration activates anion channels in guard cells required for stomatal closing [32]. A recent study has suggested that a MATE transporter-like protein, RHC1, functions as a bicarbonate sensor in this pathway [43].

A key question in stomatal CO_2 signaling is the subcellular sites of CO_2 perception. CO_2 perception in animals is currently believed to be a vestigial sensory mechanism [42] and studies in animals have linked carbonic anhydrases to the perception of carbonation [44]. Adenylyl cyclase enzymes have been shown to play important roles in CO_2 sensing in mammalian and fungal systems [45,46], but plant orthologs for these proteins have not been identified. Another area of scientific inquiry is whether photosynthesis plays a direct role in rapid CO_2-regulated stomatal movements. Of the six *Arabidopsis thaliana* beta carbonic anhydrases, CA4 is localized at the plasma membrane while CA1 is mainly targeted to chloroplasts on transient expression in tobacco (*Nicotiana benthamiana*) cells [40,47]. This expression pattern raises the question: how does this expression pattern of carbonic anhydrases function in mediating stomatal responses to CO_2?

In a recent study, the intracellular targeting of the beta carbonic anhydrases CA1 and CA4 in guard cells was characterized in relation to their roles in CO_2 control of stomatal movements. Expression of fluorescently tagged carbonic anhydrases in guard cells of *ca1ca4* double-mutant plants showed that expression of CA4 at the plasma membrane or CA1 in guard cell

chloroplasts can restore CO_2-induced stomatal responses [48]. Mathematical modeling and experiments have revealed that spatial gradients of intercellular second messengers can play a role in signaling events [49]. In addition, recent computational and experimental studies in *Xenopus laevis* oocytes have demonstrated that increases or decreases in the external [CO_2] can lead to significant and transient spatial intracellular gradients of CO_2 and its reaction products including bicarbonate [50]. In this mathematical model, the plasma membrane was assumed to be permeable to CO_2 and carbonic anhydrases were assumed to be in the cytoplasm. Although the reaction-kinetic parameters of carbonic anhydrases may be similar in plants, the size of guard cells is significantly smaller than *X laevis* oocytes: ≈5 μm compared with ≈1000 μm. This smaller dimension leads to a much smaller diffusional timescale, which can be estimated by taking the ratio of the square of the cell size and the diffusion constant. Taking the diffusion constant of CO_2 to be of the same order as in oocytes, $D \approx 1000\ \mu m^2/s$, the diffusion time in guard cells is less than 1 s [48], which is faster than the typical timescale of stomatal responses. Thus, CO_2 gradients are unlikely to occur in guard cells or play a significant role in regulating stomatal opening and closing. Consistent with this analysis, recent mathematical modeling addressing the spatial localization of carbonic anhydrases showed virtually identical dynamics of [HCO_3^-] changes at any depth in modeled guard cells [48]. This modeling study predicts that carbonic anhydrases that are localized to the plasma membrane and to the cytoplasm produce effective intracellular [HCO_3^-] changes in guard cells, while the participation of chloroplast-localized carbonic anhydrases, in this process, is less likely [48]. This simplified model, together with functional analyses of CA1 and CA4, predicted that intracellular [HCO_3^-] in guard cells, driven by carbonic anhydrases at the plasma membrane or in the cytosol, can regulate stomatal CO_2 responses. In addition, this research also suggests that, in guard cells, chloroplast-localized carbonic anhydrases contribute to CO_2 control of stomatal closing via an unknown plastidial mechanism that will require further investigation [48].

Based on the above model and findings, new gas-exchange analyses of single carbonic anhydrase mutants support the model that the plasma membrane-localized CA4 plays an important role in CO_2 signal transduction as the *ca4* mutant can show a slightly altered sensitivity, even if not strictly significant, to the CO_2 stimulus (Figure 1C,D). The *ca1ca4* double mutant shows a clearly more-pronounced slowed CO_2 response [40,48] (Figure 1E). One possible model to explain the enhanced phenotype in *ca1ca4* could be that either mutation alone causes a largely subthreshold impairment in the CO_2 response. However, overexpression of either carbonic anhydrase is sufficient for the recovery of a more WT-like CO_2 response by overcoming this threshold effect [40,48]. This threshold model is also consistent with the finding that the catalytic activity of carbonic anhydrases mediates the stomatal CO_2 response [40] and that CAs accelerate the rate of $CO_2/HCO_3^- + H^+$ equilibration.

A. thaliana βCA1 is targeted to the chloroplast stroma [47]. Furthermore, YFP-tagged βCA1 in guard cell chloroplasts restored stomatal responses to CO_2 [48]. These data raised the question of whether guard cell photosynthesis functions directly in the stomatal CO_2 response. Transgenic plants in which chlorophyll was directly removed from guard cell chloroplasts, but not from mesophyll chloroplasts, showed that chlorophyll-lacking guard cells continue to show CO_2-induced stomatal closing [51]. Together with pharmacological studies using norflurazon [52], these findings suggest that the role of βCA1 in CO_2 signaling does not lie in photosynthesis and imply an unknown function of guard cell chloroplasts in the CO_2 response that needs to be identified.

Mesophyll and Guard Cell Mechanisms of CO_2 Signaling and a Role for Photosynthesis

Photosynthesis plays a well-documented role in CO_2 regulation of stomatal apertures by lowering Ci in leaves [4] (Figure 1A). In addition to this indirect function of photosynthesis via

lowering of Ci, present studies have led to differing models for the site of direct CO_2 sensing in the published literature (for reviews see [53,54]). Some studies have shown a role for guard cells in CO_2 sensing [52,55–58]. However, other studies indicate that mesophyll cells play a key role by directly sensing CO_2 [59,60]. Presently it is likely that the two pathways (mesophyll and guard cell) converge. Genetic evidence for CO_2-insensitive mutants in both pathways is needed. We review the two pathways separately.

CO_2 Sensing in the Mesophyll

Stomatal conductance was demonstrated to respond to Ci rather than external leaf surface [CO_2] [4]. Based on results from experiments showing that stomata in isolated epidermal tissues of *Tradescantia pallida* and *Pisum sativum* exhibited a limited response to CO_2 and that mesophyll tissue enhanced the response, it has been proposed that the mesophyll tissue in leaves plays an important role in CO_2 sensing [60,61]. Studies revealed that an enhanced and reversible stomatal response to CO_2 occurs when mesophyll tissues are placed on an excised leaf epidermis [60]. Studies suggest that this CO_2 response involves a diffusible substance [60,62]. Adding a polyethylene (diffusion-disruption) barrier impairs mesophyll amplification of the stomatal response [62]. This result was proposed to show that mesophyll-derived signals could be small, diffusible molecules [62]. While the precise nature of the mesophyll-to-stomata signal is unknown, possible candidates include sucrose [53] and malate [63]. ABA should also be considered as a possible diffusible small molecule, as ABA plays a synergistic role in the CO_2 response [30,33,34,64]. Notably, ABA was shown to amplify the CO_2 response in *Vicia faba* guard cells, which typically show a much more sluggish response to CO_2 without the addition of ABA [30,65].

Further research is needed to determine whether the level of the unknown mesophyll-derived amplifying factor is itself regulated by [CO_2] changes or whether such a factor is constitutively present independent of CO_2 and thus amplifies CO_2-sensing mechanisms in guard cells. Identification of the mesophyll-derived signaling molecule is needed to elucidate the CO_2 signaling mechanisms. Isolation of genetic mutants that show CO_2 insensitivity in the regulation of stomatal movements and for which the underlying proteins function in mesophyll cells would also help to advance understanding of the underlying mechanisms.

Photosynthesis and CO_2-controlled Movements

Studies have proposed that leaf photosynthesis directly mediates stomatal function during CO_2 regulation of stomatal movements [59,60]. However, other studies suggest that these CO_2-sensing mechanisms do not depend on leaf photosynthesis [52,57,66]. Altering the photosynthetic capacity via Rubisco antisense plant lines did not affect stomatal movement rates or conductance responses at ambient CO_2 compared with the WT [57,66]. Norflurazon-treated chlorophyll-less 'albino' leaves showed functional CO_2 regulation of stomatal movements [40,52]. Studies with phosphoenolpyruvate carboxylase (PEPC) antisense construct lines showed that, in C4 plants, PEPC activity is required for stomatal aperture opening under high light fluxes and low Ci [67].

Photosynthesis can partake in red-light-induced stomatal opening by the photosynthesis-derived decrease in leaf CO_2 (Ci) [68–70]. Similarly, increased Ci in *Xanthium strumarium* leaves induces a decline of their stomatal conductance [59]. The slope of the stomatal conductance change was gradual until the transition of photosynthesis from Rubisco limitation to electron transport limitation, after which point the slope steeply declined with high values of Ci. A shift in the slope of stomatal conductance with Ci in concert with this type of photosynthesis limitation was observed under different [O_2] and light intensities, thus implicating a role of photosynthesis in determining stomatal aperture [59]. However, when the calculated Ci was kept stable by adjusting ambient CO_2, most red-light-induced stomatal opening was not dependent on

mesophyll-induced reductions in intercellular CO_2. Therefore, this study also indicated a Ci-independent component of red-light-induced stomatal opening. In addition, leaves treated with the photosystem II inhibitor DCMU still responded to CO_2 [59]. Taken together, these studies point to the question of whether red-light-induced stomatal opening occurs via more than one pathway, with one pathway mediated by low Ci signaling. A recent genetic study of the High Temperature *ht1* mutant supports a model of a Ci-dependent and a Ci-independent component of red-light-induced stomatal opening [70]. Another recent study showed that carbonic anhydrase *ca1ca4* double-mutant plants did not affect whole-leaf assimilation rates at a wide range of Ci from <50 to >1200 ppm CO_2 [51], further supporting the model that the *ca1ca4* mutant does not affect the CO_2 response via impairing whole-leaf photosynthesis.

The function of guard cell photosynthesis in CO_2-induced stomatal responses was genetically investigated by studying transgenic plants that lack chlorophyll specifically in guard cells [51]. Gas-exchange analyses and stomatal movement analysis showed that CO_2-induced stomatal closure is not directly mediated by guard cell photosynthesis or electron transport. Interestingly, ~45% of the stomata in these lines showed a deflated, 'thin-shaped' stomatal morphology, suggesting a different but key role of guard cell photosynthesis in energization and turgor production in guard cells. The role of photosynthesis in the direct response to CO_2-mediated stomatal conductance regulation remains a subject of debate.

CO_2 Sensing in Guard Cells

Several studies have shown the ability of isolated guard cells to respond to environmental stimuli (and specifically [CO_2] changes) suggesting that sensing and signaling pathway components for these reside in guard cells [40,55,56,71–73]. One of the first reported instances is the classical work by J.D.B. Weyers and colleagues, which showed that, in response to changes in [CO_2], isolated guard cell protoplasts swell and shrink [56,73]. The presently identified genetic mutants that impair CO_2 control of stomatal movements encode proteins that exert their function in guard cells, including the carbonic anhydrases CA1 and CA4 [40], the HT1 protein kinase [74], the SLAC1 anion channel [75,76], the OST1 protein kinase [32,77], the PATROL1 Munc13-like protein [78], the AtALMT12/QUAC1 R-type anion channel [79], and the RHC1 MATE transporter [43].

Role for Protein Kinases

Thermal imaging of plant leaves is a convenient way to predict their transpiration and stomatal conductance [80,81]. The *high leaf temperature 1*; *ht1-1* and *ht1-2* (*ht1*) mutant was the first CO_2-response mutant selected through a thermal imaging screen in *A. thaliana* [74]. HT1 encodes a protein kinase and *ht1-1* mutation leads to decreased HT1 kinase activity resulting in a reduced stomatal CO_2 response. The *ht1-2* mutant abolishes measureable HT1 kinase activity. Leaf stomata of *ht1-2* mutant plants show a constitutively 'high' CO_2 response resulting in low stomatal conductance and thus elevated leaf temperature. Nonetheless, *ht1-2* leaf stomata retain responses to blue light, fusicoccin, and ABA. To date, at least five *ht1* alleles have been isolated by the same strategy (M. Hashimoto-Sugimoto, unpublished). HT1 may be a central negative regulator of stomatal high CO_2 signaling [74] (Figure 2A). The open stomata 1 (OST1) protein kinase is required for CO_2-induced stomatal closing [32]. A recent study has proposed that the HT1 protein kinase phosphorylates the OST1 protein kinase, thereby deactivating OST1 [43]. However, another study has found HT1 to be epistatic to OST1 in a high-CO_2-induced stomatal closing assay [70]. Further research is warranted to fully understand the roles of OST1 and HT1 in stomatal movement control.

Role for Calcium

Calcium ions have been shown to act as guard cell second messengers in CO_2 signal transduction in *Commelina communis* [72,82] and *A. thaliana* [58,83]. An ABA-insensitive

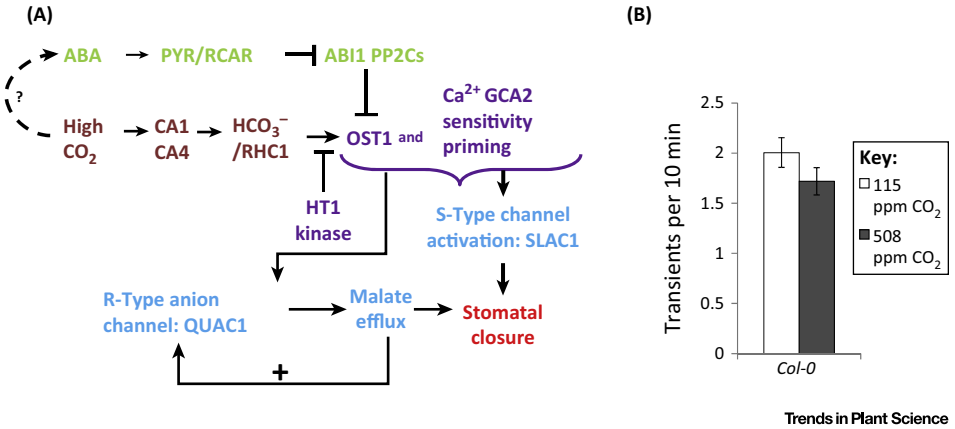

Figure 2. Carbon dioxide (CO_2) Signaling Pathway in Stomatal Movement Regulation and Putative Convergence Points with Abscisic Acid (ABA) Signaling. (A) A simplified model for CO_2 and ABA signal transduction pathways in guard cells that mediate stomatal closure. The current model involves the enzymatic function of the carbonic anhydrases CA1 and CA4 with bicarbonate ions as intermediary signaling molecules. Downstream calcium, protein kinases, and ion channels are required for the stomatal closure response to CO_2. Note that amplifying signals from the mesophyll [60,62] are not shown here for simplicity (see text for details). Different pathway components are color coded for ease of viewing: ABA genes, green; CO_2 genes, brown; kinases, purple; channels, blue. PYR/RCAR, ABA receptors; CA, carbonic anhydrase; HCO_3^-, bicarbonate; Ca, calcium; ABI1, protein phosphatase 2C. (B) The rate of cytosolic calcium transient production in guard cells of the *Arabidopsis thaliana* Col-0 ecotype are not significantly modulated by the [CO_2], in contrast to the *Ler* ecotype. Cytosolic calcium transients in Col-0 guard cells pre-exposed to low CO_2, switched to high CO_2, at the indicated concentrations. Buffer composition was 10 mM KCl/50 μM $CaCl_2$/10 mM MES-Tris, pH 6.15. Data are the means of $n = 27$ guard cells ± standard error.

mutant, *growth controlled by abscisic acid* (*gca2*) shows a similar $[Ca^{2+}]_{cyt}$ transient rate at low and elevated CO_2 and exhibits strongly attenuated stomatal closure in response to elevated CO_2 in leaves [58]. The *gca2* mutant also exhibits an altered ABA-induced $[Ca^{2+}]_{cyt}$ pattern in guard cells [148], suggesting that GCA2 functions downstream or at the convergence point of CO_2 and ABA signaling. Research suggests that the increased Ca^{2+} spiking rate of *gca2* mutant guard cells results from a more-negative plasma membrane voltage in guard cells due to CO_2 and ABA insensitivity [58]. Consistent with this model, more-negative membrane potentials cause an increased Ca^{2+} spike rate in guard cells [84–86]. Cloning of the *GCA2* gene may help us better understand the synergistic mechanisms in CO_2 and ABA signal transduction.

WT guard cells of the *A. thaliana* accession *Landsberg erecta* show dampening of the cytosolic Ca^{2+} transient rate in response to elevated CO_2 and ABA [58,85] similar to *V. faba* and *C. communis* guard cells exposed to ABA [84,87]. CO_2-induced dampening of the Ca^{2+} transient rate was not as clearly resolved in guard cells of the *A. thaliana* Columbia accession compared with the *Landsberg* accession (Figure 2B). These findings may be linked to a weaker stomatal closing response in the *A. thaliana* Columbia guard cells relative to those in the *L. erecta* accession. The findings that guard cells show such 'spontaneous' Ca^{2+} transients without ABA or CO_2, but that CO_2 signaling requires cytosolic Ca^{2+}, led to the formulation of a 'Ca^{2+} sensitivity priming' hypothesis [58]. This model postulates that the stomatal closing stimuli, CO_2 and ABA, enhance (prime) the Ca^{2+} sensitivity of stomatal closing mechanisms, as has been found for ABA [86,88] and for CO_2/HCO_3^- activation of slow (S-type) anion channels in guard cells [32]. Initial molecular, genetic, biochemical, and cellular mechanisms mediating stimulus-induced Ca^{2+} sensitivity priming have been identified for ABA signal transduction, showing that type 2C protein phosphatases control the ABA-mediated Ca^{2+} signal specificity [89].

Role for Intracellular Membrane Trafficking

Stomatal opening is initiated by the activation of H^+-ATPases in the guard cell plasma membrane. Low [CO_2] triggers a more negative plasma membrane voltage (hyperpolarization), and elevated [CO_2] is likely to inhibit proton efflux by plasma membrane H^+-ATPases [55]. However, a direct link between CO_2 and H^+-ATPase regulation remains to be characterized. The *A. thaliana patrol1* mutant, isolated in a thermal imaging screen, is impaired in stomatal opening in response to low [CO_2] and light [78]. The *PATROL1* gene encodes a protein with a MUN domain [90]. Munc13 has been reported to be involved in the membrane trafficking of neurotransmitter release in animals and the MUN domain is the minimal Munc13 region sufficient for the activity of Munc13-1 in synaptic vesicle priming [90]. This suggests a molecular role of PATROL1 in intracellular membrane traffic. PATROL1 is expressed throughout plant tissues, including guard cells, and is located in endosomes. Intriguingly, environmental conditions affect the intracellular distribution of the PATROL1 protein. The PATROL1 protein is detected in close proximity to the plasma membrane of guard cells under stomatal opening conditions (well-watered plants and light) and is observed inside guard cells as numerous punctate structures under stomata-closure conditions (dark or desiccation). Loss-of-function mutation of *patrol1* disturbs the normal plasma membrane targeting of the *A. thaliana* H^+-ATPase AHA1 but does not affect that of the S-type anion channel SLAC1 or the inward rectifying potassium channel KAT1 [78]. This indicates that PATROL1 is target selective, and it has been proposed to function in targeting or tethering the AHA1 H^+-ATPase to the plasma membrane during stomatal opening. Overexpression of *PATROL1* in plants (PATROL1-OX) leads to a rapid and enhanced rise in stomatal conductance in response to low [CO_2] or light, eventually resulting in higher CO_2 assimilation and increased biomass production compared with WT plants [78]. Furthermore, PATROL1-OX plants show normal stomatal closure in response to high [CO_2], darkness, and desiccation. Whether the increased biomass of PATROL1-OX plants is solely due to stomatal conductance changes or additional roles in enhancing photosynthesis as well as cellular expansion will be interesting for future analyses.

Plasma Membrane Regulators

Increased [CO_2] has been shown to enhance anion channel activity in guard cells [40,69,71,91]. S-type anion channels in the plasma membrane of guard cells were proposed to provide a central control mechanism for stomatal closing [92]. Genetic evidence for this model was obtained in two studies. Thermography was used to isolate the *A. thaliana* mutant *carbon dioxide insensitive 3* (*cdi3*), which is impaired in CO_2-dependent leaf temperature change [75]. The CDI3 protein is a distant homolog of bacterial and fungal C4 dicarboxylate transporters and is localized specifically in the plasma membrane of guard cells. *cdi3* guard cell protoplasts exhibited a higher content of K^+, Cl^-, and malate^{2-} [75]. Parallel with this research, the characterization of the ozone signaling mutant *radical induced cell death* (*rcd3*) in *A. thaliana* revealed that *rcd3 A. thaliana* leaves are more sensitive to the ozone radical because stomatal closing is impaired in response to ozone, ABA, -, Ca^{2+} and CO_2 [76]. The *RCD3* gene was independently mapped to the same C4 dicarboxylate transporter homologous gene [76]. Patch-clamp analyses of *rcd3* mutant alleles showed that S-type anion channel currents were greatly impaired, whereas the R-type anion channel and Ca^{2+} channel currents were intact in mutant guard cells [76], providing genetic evidence for findings suggesting that S-type and R-type anion channels are distinct from one another [93]. These results supported the idea that CDI3/RCD3 encodes an essential anion-transporting subunit of the S-type anion channels in plants and it was renamed SLAC1 [75,76]. S-type anion channels in guard cells are activated by phosphorylation events [94,95]. Electrophysiological experiments in *X. laevis* oocytes demonstrated that, in the presence of the protein kinase OST1 [77,96], SLAC1 generates S-type anion channel activity [97,98]. These studies provided evidence that SLAC1 encodes a S-type anion channel that is activated by OST1-mediated phosphorylation. Recent research has focused on the regulatory events that trigger anion channel activation. The calcium-dependent protein kinases

CPK21 and CPK23 phosphorylate and activate SLAC1 [99]. CPK6 with CPK3 was found to participate in the ABA- and Ca^{2+}-dependent regulation of guard cell S-type anion channels and stomatal closure [100]. The functional reconstitution of ABA activation of SLAC1 was shown using either the CPK6 or the OST1 protein kinase in *X. laevis* oocytes [101].

Several regulators involved in the ABA-induced activation of SLAC1 have been identified. An example is the receptor-like kinase GHR1, which is mainly localized in the guard cell plasma membrane and activates SLAC1 anion currents in *X. laevis* oocytes [102]. However, the relative functions of the Ca^{2+}-dependent and Ca^{2+}-independent CO_2 signaling pathways, which are key for models of guard cell signaling *in planta*, remain unknown. It remains to be genetically dissected whether these two pathways function independently of one another. Furthermore, the molecular mechanism by which CO_2 activates S-type anion channels is a matter of present interest. OST1 is a positive regulator of CO_2-induced stomatal closing and activation of the S-type anion channels in guard cells [32]. Tian *et al.* recently reported [43] that a MATE-type transporter, RHC1, is activated by bicarbonate and functions upstream of HT1, and furthermore that HT1 directly phosphorylates OST1 and inhibits OST1-induced activation of SLAC1 (Figure 2A).

Various mechanisms have been suggested that couple a rise in [CO_2] to changes in the activity of plasma membrane ion channels. The apoplastic malate concentration rises from 1.00 ± 0.60 to 3.10 ± 2.30 mM in response to high [CO_2], which can activate R-type anion channels in guard cells [103]. This rise in malate may result from malate efflux from guard cells, as stomatal closing has been shown to be accompanied by malate efflux [104]. Furthermore, R-type anion channels mediate malate efflux [79,105]. An alternative model has also been proposed in which CO_2 causes malate release from mesophyll cells [103], but direct evidence or mechanisms for this model have not yet been reported. The *AtALMT12/QUAC1* gene was shown to encode the anion-transporting activity of R-type anion channels in guard cells [79,106]. AtALMT12/QUAC1, a member of the aluminum-activated malate transporter family in *A. thaliana*, is highly expressed in guard cells and is targeted to the plasma membrane. Plants lacking AtALMT12/QUAC1 are impaired in CO_2-induced stomatal closure as well as in ABA responses. Electrophysiological studies of loss-of-function mutant guard cells and *X. laevis* oocytes expressing the protein revealed that AtALMT12/QUAC1 represents the malate-sensitive R-type anion channel [79].

The ABC transporter AtABCB14 was suggested to encode a malate uptake transporter in the guard cell plasma membrane [107]. Plants lacking the AtABCB14 transporter were reported to show slightly more rapid high-CO_2-induced stomatal closure compared with WT controls. However, in isolated epidermal strips that contained guard cells, no difference in stomatal CO_2 responses was observed between WT and *atabcb14* mutants. The authors suggested that AtABCB14 removes extracellular malate, which is known to activate anion channels [103], and, consequently, that part of the CO_2 response is mediated by malate secreted into the apoplast. Recent research has also proposed a role for AtABCB14 in auxin transport [108]. Further research on AtABCB14 functions is needed to determine the range of transported substrates.

CO_2 Modulation of Stomatal Development

[CO_2] regulates the development of stomata [6,109,110] and WT *A. thaliana* plants of the Columbia ecotype show slight repression of stomatal development at elevated CO_2 [111]. Responses of stomatal density to long-term CO_2 doubling are varied in different accessions of *A. thaliana*; however, most plant species exhibit reduced stomatal development [7,109]. The mechanisms by which CO_2 levels can modulate stomatal development mechanisms [112–118] are of interest given the continuing rise in atmospheric CO_2 levels.

Research has suggested that CO_2 control of stomatal development is mediated by Ci in leaves [119]. A cell-wall wax biosynthesis mutant, *hic*, was shown to disrupt this response and

exhibited an inverted developmental response, with more stomata being produced at elevated CO_2 in the *hic* mutant [120]. While the precise mechanism of action is unknown, *HIC* encodes a putative 3-keto acyl coenzyme A synthase that is involved in the synthesis of very-long-chain fatty acids. Interestingly, other genetic mutations that affect cell-wall wax deposition in plants also show changes in stomatal development [120–123]. Presently unknown systemic signals that travel from mature to young leaves can function in CO_2-mediated changes of stomatal density in emerging young leaves [124] and thus cuticular waxes may serve as candidates that affect the movement of diffusible signals. Recent research found that carbonic anhydrase mutant plants exhibit an inversion in their stomatal development response to elevated CO_2, showing increased stomatal development in plants grown at elevated [CO_2] (500 ppm or 1000 ppm) compared with low [CO_2] (150 ppm) [25]. These effects were observed in both cotyledons and mature rosette leaves [25]. The findings that elevated CO_2 causes enhanced stomatal development in cotyledons of *ca1ca4* double-mutant plants provides evidence that CO_2 can also exert a developmental response without long-distance leaf-to-leaf signaling [25]. The epidermal patterning factor (EPF) gene EPF2 [125,126] has been shown to bind the ERECTA receptor kinase [127–129]. The ERECTA receptor kinase [130,131] and transgenic modulation of EPF2 expression [132] have also been shown to play roles in determining the transpiration and water-use efficiency of plants via regulation of the stomatal index. Interestingly, *epf2* mutant alleles also show an inverted stomatal development phenotype at elevated CO_2 [25]. Activation of the EPF2 propeptide requires EPF2 cleavage. Cell-wall proteome analyses led to the identification of CO_2-responsive secreted protease (CRSP), which can cleave EPF2 *in vitro* and is involved in EPF2 function *in vivo*. Mutations in the CA1 and CA4 carbonic anhydrase *EPF2* and *CRSP* genes show an inverted stomatal development response to elevated CO_2 [25]. Carbonic anhydrases are required for high-CO_2 upregulation of EPF2 and CRSP mRNAs based on qPCR analyses [25]. The mechanisms by which and whether carbonic anhydrases modulate EPF2 and CRSP activity remain to be determined. Several new questions emerge from this framework model (Figure 3); chief among them are the following. (i) Is CO_2 regulation of EPF2 and CRSP transcripts due to feedback modulation or is the CO_2 response transcriptional, and, if so, what are the transcriptional regulators mediating the elevated CO_2 response? (ii) What are the downstream signaling components beyond the EPF2 peptide and ERECTA receptor? (iii) Analyses of CRSP protease insertion-mutant alleles have led to the suggestion that additional proteases may act in the CO_2 response [25]; hence, are there other proteases that cleave and activate EPF2? (iv) How is excess EPF2 degraded or sequestered? (v) Why do the described mutants show an increase in stomatal density and index at elevated CO_2 rather than a decrease?

While initial experiments have explored aspects of the effects of environmental stress on stomatal development [109,118,124,133,134], future genetic studies involving the specific effects of abiotic factors such as CO_2, light, temperature, and humidity [124,133] will reveal whether and how these signaling cascades overlap and interact. A recent study showed that ABA plays an important role in stomatal cell-lineage specification [135] by regulating the initiation of stomatal development and inducing the expansion of epidermal pavement cells. Another area of future research that appears promising is the response of plants at lower-than-ambient Ci that occurs in daylight [1]. The carbonic anhydrase mutants exhibit WT-like stomatal development at low CO_2 [6,25]. These findings indicate the possibility of a separate mechanism regulating stomatal development at these lower-than-ambient CO_2 levels. One possibility is that other carbonic anhydrases may play key roles at such low [CO_2] and remain to be identified. Alternatively, if the enzymatic activity of carbonic anhydrases transmits the developmental CO_2 signal, it is plausible that at a low [CO_2] of 150 ppm, non-accelerated catalysis of CO_2 to bicarbonate and protons may be sufficient for this CO_2 response. The specific mechanisms of EPF2 peptide stability and abundance modulation are open questions and lend themselves to several avenues of future research that could target the precise timing of CO_2-mediated stomatal developmental repression and the temporal scale of repressive inhibition.

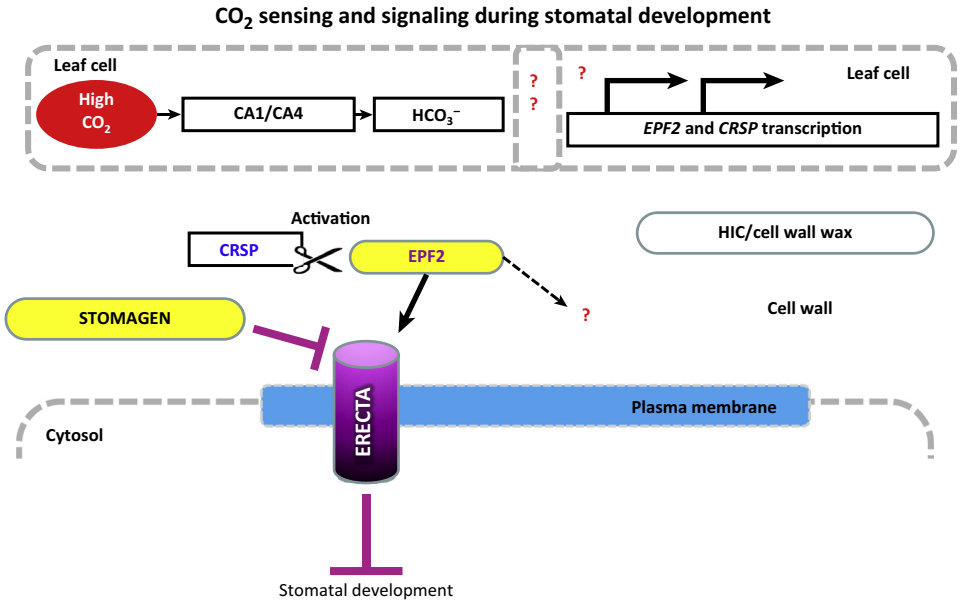

Figure 3. Carbon dioxide (CO_2) Control of Stomatal Development. A current framework [25,120] includes epidermal patterning factor 2 (EPF2), CO_2-responsive secreted protease (CRSP), the carbonic anhydrases CA1 and CA4, and the cell-wall wax biosynthesis mutant *hic* [120]. The secreted extracellular protease CRSP cleaves and activates EPF2, which is involved in extracellular communication, resulting in modulatory repression of stomatal development in response to elevated CO_2. Experiments have shown that the ERECTA receptor kinase has specific EPF2-binding activity [128,129]. Downstream signaling components include key stomatal-fate transcriptional regulators (see [113,147]). Unknown signaling components and mechanisms are indicated by question marks (see text for details). Abbreviations: CA, carbonic anhydrase; HIC, high CO_2 mutant; HCO_3^- bicarbonate.

Concluding Remarks and Future Directions

The observed transcriptional response to a CO_2 stimulus of hundreds of genes [25,136], including photosynthesis-linked genes, indicates that unidentified mechanisms for transcriptional control exist in this pathway as well as in CO_2-regulated photosynthesis. Future research aimed at isolating these players could involve bioinformatics mining of published data on stomatal gene networks and guard cell responses to environmental triggers [25,136–142]. Systems and network biology tools could be employed to probe transcriptomic and proteomic resources to identify not only putative transcription factors but also candidate response elements of known transcriptional regulators of the stomatal development and movement pathways. Such candidates may also aid efforts in engineering plants that maximize stomatal conductance under well-watered conditions and more water-use-efficient plants capable of adjusting to elevated CO_2 in a more desirable fashion than present cultivars. Another area of future research is the need to study the stomatal and physiological responses of plants to the atmospheric CO_2 levels predicted to be present in the next decade [132,143]. Results from such studies could assist breeders and biotechnological choices for plant and crop cultivars and germplasm better capable of adapting to those CO_2 levels. As an example, experiments exploring natural variation in stomatal density [144], which impacts plant water use and productivity, could be conducted. Along these lines, a recent comprehensive study of 374 *A. thaliana* accessions [145] has unveiled a wealth of and diversity in stomatal responses to stimuli of light and CO_2. As a starting point, these varied responses (see Outstanding Questions) to the same stimulus may indicate a plethora of underlying mechanisms that mediate the responses of plants to abiotic stress and the environment.

Outstanding Questions

Stomatal guard cells simultaneously perceive various cues from the environment and are sensory organs that process this information in ways that most benefit the plant. Thus for sessile plants, stomata play key roles in avoiding and mitigating environmental and biotic stress. However, many aspects of the molecular mechanisms and network underlying stomatal function remain unknown. A few important questions in stomatal CO_2 responses follow.

How does the chloroplastic localization of CA1 in guard cells function in transducing the CO_2 stimulus into stomatal movement responses?

These recent findings suggest an important role for guard cell chloroplasts in CO_2 signaling for stomatal movements that however does not require guard cell photosynthesis. Hence, what are the underlying plastidial mechanisms that transmit the CO_2 signal in guard cells and how do these converge with the cytosolic bicarbonate signaling branch?

What are the guard cell bicarbonate sensors and what is the biochemical protein basis by which intracellular bicarbonate functions as a second messenger in guard cells?

What is the mechanism by which mesophyll cells function in the stomatal CO_2 response?

What is the major CO_2-responsive mechanism and what are the transcription factors involved in the CO_2-regulated stomatal development response?

When such necessary advanced information becomes available, can we robustly manipulate stomatal gas exchange and plant responses to the continuing rise in atmospheric CO_2 levels?

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