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Update

Deep dive into CO_2-dependent molecular mechanisms driving stomatal responses in plants

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Overview

Plants are fixed organisms that need to tightly integrate signals from their environment in order to survive. Among those signals, carbon dioxide (CO_2) is a main substrate for photosynthesis. Since the industrial revolution, CO_2 levels in the atmosphere have risen by 50% to over 415 parts per million (ppm) through 2020 (https://keelingcurve.ucsd.edu). On the surface of their leaves, plants have small pores, the stomata that are responsible for gas exchange (Pillitteri and Torii, 2012). CO_2 enters the leaves through stomata while water exits through the same pores (Hetherington and Woodward, 2003). This process has to be fine-tuned in order to appropriately respond to rapidly changing exterior conditions (Hetherington and Woodward, 2003). When plants face drought, they produce abscisic acid (ABA), a well-known signal that triggers stomatal closure (Hetherington and Woodward, 2003; Hsu et al., 2020). By closing their stomata, plants can store water inside their leaves, slowing down the evapotranspiration process (Hetherington and Woodward, 2003; Franks and Farquhar, 2007). Light is a well-characterized signal that induces stomatal opening (Darwin, 1898; Shimazaki et al., 2007). Stomata close in darkness (Darwin, 1898; Costa et al., 2015). Stomatal opening allows for proper gas exchange during the day to favor carbon access for photosynthesis, while closure in response to

ADVANCES

- Additional signaling components, including MPK4/12, GHR1, CBC1/2, and KIN7, together with added roles for previously described proteins have been uncovered in the past few years.
- Recent studies have uncovered a role for basal ABA signaling that facilitates elevated CO_2-mediated stomatal closure, providing evidence that CO_2 and ABA signals converge downstream of OST1/SnRK2.6 kinases in guard cells and that CO_2 does not activate ABA receptor signaling.
- CBC1 and CBC2 protein kinases are at the convergence between blue light and CO_2 signaling pathways.
- Subsidiary cells provide a mechanical advantage that allows grasses to use less water for generating turgor pressure changes. While CO_2 regulates stomatal movements in the grass *B. distachyon*, CO_2 does not regulate stomatal development in this grass.

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darkness reduces unnecessary water loss during the night.When exposed to low concentrations of CO_2 in the intercellular spaces of leaves, stomata open (Hetherington and Woodward, 2003). Elevated CO_2 concentrations, that occur at night due to respiration in leaves, is a well-known signal that triggers stomatal closure (Darwin, 1898; Hetherington and Woodward, 2003; Zhang et al., 2018a). Furthermore, the continuing rise in the atmospheric CO_2 concentration is narrowing stomatal apertures and reducing stomatal conductance on a global scale (Medlyn et al., 2001). Guard cells respond to CO_2 concentration changes via signal transduction mechanisms (Zhang et al., 2018a). It has also been shown in independent labs that the mesophyll contributes to stomatal responses to CO_2 in several plant species, including *Vicia faba* and *Commelina communis* (Mott et al., 2008; Fujita et al., 2013). In the context of climate change, associated with an increase of atmospheric CO_2 in the coming decades, it is crucial to understand how plants sense CO_2 signals and transduce the CO_2 signal that controls stomatal movements (Zhang et al., 2018a). Here, we review recent advances in understanding CO_2 signal transduction mechanisms in guard cells and mesophyll cells. We also review recent findings examining how CO_2 signal transduction converges with other signals in guard cells, including blue light and ABA. We also dedicate a section to stomatal movement modeling, and the importance of modeling to help formulate new hypotheses regarding the complex stomatal signal transduction network.

Additionally, one of the main limitations that we are facing as of today is that research is mostly conducted using Arabidopsis (*Arabidopsis thaliana*), a dicotyledonous plant, while many agronomic species are monocotyledons. These grasses such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and maize (*Zea mays*), make up two-thirds of the calories humans consume per day (Nunes et al., 2019). Thus, it is essential to focus some efforts on understanding how monocotyledonous plants cope with elevated CO_2. The search for a representative monocotyledon has led to a very promising model grass species, *Brachypodium distachyon* from the Pooideae family, due to its relatively small genome size, short life-cycle and its relationship to major agricultural grain species (Brkljacic et al., 2011; McKown and Bergmann, 2020). With the rising global temperatures and atmospheric CO_2 levels, drought tolerance and water use efficiency are desirable crop plant traits (McKown and Bergmann, 2020). Therefore, understanding the difference of stomatal architecture and movements between monocotyledons and dicotyledons, both physically and molecularly, is of vital importance. Finally, we review recent advances at illuminating the long-term effect of elevated CO_2 and its impact on plant growth.

CO_2 signal transduction in guard cells

Over the past decade, significant advances were made, and important components of CO_2-induced stomatal movements were uncovered (Figure 1). Briefly, CO_2 enters guard cells either via transmembrane diffusion or through PLASMA MEMBRANE INTRINSIC PROTEIN 2 (PIP2) aquaporins (Katsuhara and Hanba, 2008; Wang et al., 2016). Once inside the cell, CO_2 is catalyzed into bicarbonate through the action of two enzymes: ß-CARBONIC ANHYDRASE 1 and 4 (ßCA1 and ßCA4; Hu et al., 2010; Hu et al., 2015; Wang et al., 2016; Chen et al., 2017; Kolbe et al., 2018; Figure 1). In Arabidopsis, ßCA1 and ßCA4 function in and accelerate CO_2-mediated stomatal closure, using HCO_3^- via accelerated conversion from CO_2 (Hu et al., 2010). Similarly, in maize and rice, mutating ßCAs led to an increase in steady-state stomatal conductance and decreased sensitivity and slower stomatal conductance responses to CO_2 (Chen et al., 2017; Kolbe et al., 2018).

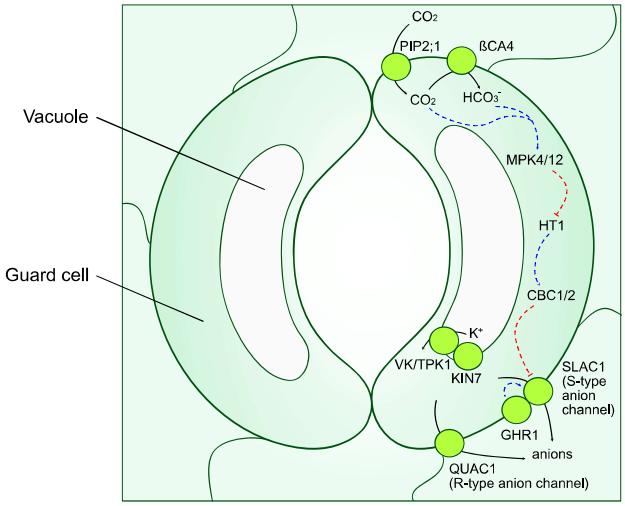

Figure 1 Simplified model for CO_2-induced stomatal movements in Arabidopsis. CO_2 enters guard cells through PIP2;1, and likely other aquaporins and perhaps also passively. It is then converted into bicarbonate (HCO_3^-) via the action of ßCA4 at the plasma membrane and ßCA1. MPK4/12, HT1, and CBC1/2 are proposed to function in a signaling pathway, but the detailed mechanisms and whether they act in the same pathway remain unknown (dashed lines indicate need for more research and possible intermediate steps). CBC1 and CBC2 function at the convergence of blue light signaling and low CO_2-induced stomatal opening pathways in guard cells. By interacting and phosphorylating the VK channel TPK1 at the tonoplast, KIN7 favors the exit of K^+ cations that are crucial for stomatal closure. GHR1 interacts with SLAC1 at the plasma membrane, thereby positively triggering the export of anions. It is hypothesized that GHR1 could act as a scaffolding protein, potentially bringing important components around SLAC1. Anion export via the action of both S-type (SLAC1) and R-type (QUAC1) anion channels is required for stomatal closure in response to elevated CO_2. Red arrows = inhibition; blue arrows = activation.

MITOGEN ACTIVATED PROTEIN KINASE 4 and 12 (MPK4 and MPK12) are the presently known next components downstream of carbonic anhydrases and CO_2/bicarbonate (HCO_3^-) and are essential to CO_2-mediated stomatal responses (Marten et al., 2008; Hõrak et al., 2016; Jakobson et al., 2016; Tõldsepp et al., 2018). Initially, the role of MPK4 in CO_2 responses was observed in *Nicotiana tabacum* leaves (Marten et al., 2008). Using RNA interference,

NtMPK4-silenced plants displayed a higher steady-state stomatal conductance than wild type (WT) (Marten et al., 2008). Such plants were also impaired in CO_2-triggered stomatal closure but showed a functional ABA response (Marten et al., 2008). In Arabidopsis, *mpk12* was isolated via mapping of stomatal conductance and water use efficiency QTL in two different screens and through guard cell expression analyses (Jammes et al., 2009; Marais et al., 2014; Jakobson et al., 2016). The *mpk12* single mutant was shown to be partially defective to CO_2-triggered stomatal movements but not to other signals such as ABA-mediated stomatal closure or light-induced stomatal opening (Hõrak et al., 2016; Jakobson et al., 2016; Tõldsepp et al., 2018). Double mutant alleles in *mpk12 mpk4GC* were generated, in which *MPK4* was specifically silenced in guard cells due to pleiotropic functions of MPK4 in other tissues. The *mpk12 mpk4GC* alleles were severely impaired in response to [CO_2] shifts, but were able to fully respond to ABA, indicating that MPK4 and 12 synergistically/redundantly regulate CO_2-induced stomatal movements in Arabidopsis (Tõldsepp et al., 2018; Figure 1).

HIGH LEAF TEMPERATURE 1 (HT1) is a RAF-like MAP kinase kinase kinase that controls CO_2-induced stomatal movements (Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2016); the precise location in the cascade remains to be determined. Recessive *ht1* mutant alleles showed that HT1 is a negative regulator of high CO_2-induced stomatal closing, while remaining responsive to ABA and blue light signals (Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2016). HT1 is highly expressed in Arabidopsis guard cells and found at the plasma membrane (Hashimoto-Sugimoto et al., 2016). HT1 was further reported to regulate the OPEN STOMATA1 (OST1)/SnRK2.6 protein kinase (Tian et al., 2015). RESISTANT TO HIGH CO_2 (RHC1) is a MATE-type transporter that was proposed to function as a CO_2 sensor in guard cells. Recent studies have, however, found no stomatal CO_2 response phenotypes in the original two *rhc1* mutant alleles (Tõldsepp et al., 2018) and a regulation of OST1/SnRK2.6 by HT1 could not be confirmed (Hõrak et al., 2016), suggesting that models of a RHC1 function in stomatal CO_2 signaling would require further research. HT1 was also shown to downregulate S-type anion channel activity in Arabidopsis guard cells (Xue et al., 2011; Tian et al., 2015). Consequently, HT1 is a central component of CO_2-triggered signal transduction (Figure 1).

Recently, two kinases named CONVERGENCE OF BLUE LIGHT AND CO_2 1 and 2 (CBC1 and 2) were identified and were described as required for proper guard cell signaling and stomatal opening in response to blue light and also to low CO_2 (Hiyama et al., 2017). CBC1 and 2 interact with and are phosphorylated by HT1 in vitro (Hiyama et al., 2017). A *cbc1 cbc2* double mutant is not able to respond to both blue light-induced stomatal opening and low CO_2-induced stomatal opening, thereby providing evidence that CBC1 and CBC2 are central elements of both signaling pathways (Hiyama et al., 2017; Figure 1).

Classical studies have shown a role for Ca^{2+} in CO_2 regulation of stomatal apertures (Schwartz et al., 1988; Webb et al., 1996). Recent research has provided evidence that CDPKs play a role in amplifying and accelerating the stomatal CO_2 response (Schulze et al., 2021). The guard cell vacuole is central for proper stomatal movements. It was recently reported that a receptor-like kinase named KIN7 could interact with the Vacuolar K+ channel (VK channel) encoded by the *TPK1* gene at the tonoplast (Isner et al., 2018). By phosphorylating and activating the VK channel TPK1, KIN7 favors the vacuolar release of potassium cations, which is crucial for proper stomatal closure (Isner et al., 2018; Figure 1).

GUARD CELL HYDROGEN PEROXIDE RESISTANT1 (*GHR1*) encodes a receptor-like (pseudo)kinase localized at the plasma membrane in Arabidopsis (Hua et al., 2012; Sierla et al., 2018). GHR1 is an essential component required for stomatal closure (Hua et al., 2012; Hõrak et al., 2016; Sierla et al., 2018). GHR1 was initially identified as required for H_2O_2 and ABA-induced stomatal closure in aperture assays from Arabidopsis epidermal peels (Hua et al., 2012). GHR1 mutation impairs high CO_2-induced stomatal closure (Hõrak et al., 2016). In 2012, it was demonstrated that GHR1 could interact with SLOW ANION CHANNEL-ASSOCIATED1 (SLAC1) in vivo and in vitro, and it was proposed that GHR1 could activate the channel through phosphorylation (Hua et al., 2012). *SLAC1* encodes a slow (S-type) anion channel, which mediates ion efflux from guard cells (Negi et al., 2008; Vahisalu et al., 2008; Geiger et al., 2009; Lee et al., 2009). However, no GHR1-induced phosphorylation of SLAC1 in response to ABA or H_2O_2 treatments was found in vitro (Hua et al., 2012). More recently, findings have led to the model that GHR1 may not have a kinase activity based on Xenopus oocyte expression experiments and in vitro experiments (Sierla et al., 2018). GHR1 was proposed to act as a scaffolding component bridging the gap between essential proteins required for proper signaling and activation of SLAC1 to trigger stomatal closure (Sierla et al., 2018; Figure 1). Further research into the biochemical function of GHR1 will be of interest.

Both slow-type (S-type) and rapid-type (R-type) anion channels mediate anion efflux from guard cells and are required for proper stomatal movements in Arabidopsis (Negi et al., 2008; Vahisalu et al., 2008; Meyer et al., 2010; Figure 1). *ALMT12/QUAC1* encodes an R-type voltage-dependent anion channel and contributes to stomatal closure in response to elevated CO_2 (Meyer et al., 2010). *SLAC1* is required for S-type anion channel activity in guard cells, is also crucial for high CO_2-induced stomatal closure (Negi et al., 2008; Vahisalu et al., 2008).

CO_2 sensing: what do we know so far?

While CO_2 elevation is a sufficient signal to trigger stomatal closure, the primary CO_2 sensory mechanism that controls stomatal movements in plant leaves remains unknown. As described further above, in previous work, both guard cells

located on the leaf surface and mesophyll cells are able to respond to CO_2 (Mott et al., 2008; Fujita et al., 2013; Zhang et al., 2018a). It has been hypothesized that both cell types could contribute to CO_2-induced stomatal movements.

A role of SLAC1 as a contributing bicarbonate sensor in guard cells was investigated (Zhang et al., 2018b). Using time-resolved gas exchange experiments on Arabidopsis leaves, it was shown that *slac1* mutant alleles were severely impaired in CO_2-driven stomatal closure compared to WT controls. This phenotype was rescued by the expression of SLAC1 under the control of its native promoter. Molecular dynamics modeling predicted putative bicarbonate interaction sites of SLAC1. Interestingly, a point mutation in the arginine residue 256 into alanine, but not other examined mutations, partially impaired the CO_2-triggered, but not ABA-dependent stomatal closure. This same mutation impaired CO_2-activation, but not ABA-activation, of S-type anion channels in guard cells, showing the relevance of this residue, predicted to interact with bicarbonate, in CO_2 responses. However, since stomata showed partial CO_2 responses in four independent SLAC1 mutant lines expressing the SLAC1-R256A variant and since stomatal closing requires activation of protein phosphorylation mechanisms, it was proposed that this direct and partial upregulation of SLAC1 by bicarbonate represents a secondary bicarbonate sensor in guard cells (Zhang et al., 2018b). Indeed, several knockout mutants with dramatic phenotypes were uncovered in the past decade, providing evidence for CO_2 sensory mechanisms upstream of SLAC1. Thus, the primary CO_2/bicarbonate sensor that drives protein phosphorylation-triggered CO_2 signaling has yet to be discovered and represents an important area of investigation (see "Outstanding questions").

Several studies have described a role of the mesophyll in CO_2-controlled stomatal movements (Lee and Bowling, 1995; Mott et al., 2008; Fujita et al., 2013, 2019). When the leaf epidermis was isolated from the mesophyll in *Tradescantia pallida* and *Commelina communis*, stomatal movements were significantly impaired (Mott et al., 2008; Fujita et al., 2013; Mott and Peak, 2018). Interestingly, the stomatal movements were restored when mesophyll tissues were placed back onto the epidermis (Mott et al., 2008; Fujita et al., 2013). However, the nature of this signal is still unclear. Data suggest that the diffusion of soluble molecules or a vapor-phase ion from the mesophyll to guard cells mediate this response (Fujita et al., 2013; Mott et al., 2014).

Interactions with other stimuli

Interaction between ABA and CO_2 during stomatal closure

Stomatal closure is rapidly induced by the plant hormone ABA and CO_2 elevation. Classic studies on different plant species have debated how ABA and CO_2 interact with each other to affect the sensitivity of stomatal closure (Raschke, 1975; Mansfield, 1976; Raschke et al., 1976; Eamus and Narayan, 1989). Recent research has added to early observations (Lahr and Raschke, 1988) that guard cells maintain basal ABA concentrations even in non-stressed leaves (Waadt et al., 2015; Hsu et al., 2018; Zhang et al., 2020). The involvement of ABA signal transduction in CO_2-induced stomatal closure has been investigated in ABA biosynthesis and ABA signaling mutants, but some conclusions have been controversial. Although it was reported that a severe ABA biosynthesis *nced3 nced5* double mutant and an ABA receptor *pyr1 pyl1 pyl2 pyl4* quadruple mutant could not close their stomata in response to high CO_2 (Chater et al., 2015), gas exchange experiments with intact plants in several studies showed that severe ABA biosynthesis *aba1*, *aba2*, *aba3* single mutants, and *nced3 nced5* double mutants rapidly respond to CO_2 elevation while *pyr1 pyl1 pyl2 pyl4* quadruple mutant only exhibited slightly slower elevated CO_2-induced stomatal closure (Xue et al., 2011; Merilo et al., 2013, 2015; Hsu et al., 2018).

A recent study reported that *pyr1 pyl1 pyl4 pyl5 pyl8* and *pyr1 pyl2 pyl4 pyl5 pyl8* quintuple mutants displayed a largely abrogated elevated CO_2-induced stomatal closure response (Dittrich et al., 2019). However, two other studies that each involved independent experiments in two laboratories indicated that the same mutant alleles respond well to CO_2 elevation, albeit with a slowed stomatal closing response (Hsu et al., 2018; Zhang et al., 2020). Moreover, an ABA receptor *pyr1 pyl1 pyl2 pyl4 pyl5 pyl8* hextuple mutant and an *ost1-3* mutant that abolished ABA-induced stomatal closing exhibited a dramatic slowing of the response to elevated CO_2-induced stomatal closure (Xue et al., 2011; Merilo et al., 2013, 2018; Hsu et al., 2018). Based on these results, different models were proposed that the elevated CO_2 signal activates ABA signal transduction and/or more directly the downstream protein kinase OST1/SnRK2.6 to trigger stomatal closure (Merilo et al., 2013; Chater et al., 2015; Dittrich et al., 2019). However, several pieces of evidence reject either of these hypotheses. First, no ABA concentration increase was detected in guard cells in response to CO_2 elevation using in vivo FRET-based ABA reporters (Hsu et al., 2018; Zhang et al., 2020). Second, in contrast to ABA, CO_2 elevation did not increase OST1/SnRK2.6 protein kinase activities in guard cells analyzed by in-gel kinase assays and in time-resolved analyses in intact guard cells using a real-time FRET-based SnRK2 activity sensor (Hsu et al., 2018; Zhang et al., 2020). Third, no noticeable increase of ABA downstream gene expression by high CO_2 treatments was observed in planta in guard cells by an ABA-signaling responsive reporter, pRAB18::GFP (Hsu et al., 2018). A recently developed SnRK2 protein kinase activity sensor, named "SNACS", further provided evidence for a basal SnRK2 activity in non-stressed guard cells. This basal SnRK2 protein kinase activity, together with basal ABA levels in guard cells of nonstressed plants, support a role for basal ABA signaling in guard cells (Hsu et al., 2018; Zhang et al., 2020).

Based on the above findings, an updated model suggests that (1) the CO_2-induced signal transduction pathway merges with ABA signal transduction downstream of OST1/

SnRK2.6 kinase activation and (2) in guard cells, basal ABA levels, basal ABA signal transduction and basal OST1/SnRK2.6 activities are important for amplifying and/or accelerating CO_2-induced stomatal closing (Hsu et al., 2018; Zhang et al., 2020) Most of the published classical and recent observations can be reconciled with this model.

CO_2 signaling mutants including, mitogen-activated protein kinase *mpk12 mpk4GC* double mutant, *ht1-2* mutant, dominant *ht1-3* (R102K) and *ht1-8D* (A109V) mutants, completely lack CO_2-induced stomatal responses but present intact ABA-induced stomatal closure (Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2016; Hõrak et al., 2016; Tõldsepp et al., 2018), providing further evidence for an ABA-independent early stomatal CO_2 signal transduction pathway. Truncation and point mutation analyses on the SLAC1 anion channel indicated that the transmembrane region and two tyrosine residues 243 and 462 are involved in the CO_2 response via an ABA-independent pathway (Yamamoto et al., 2016).

Light-mediated stomatal opening and convergence with the CO_2 signaling pathway in guard cells

Light is a well-known signal that causes stomatal opening (Shimazaki et al., 2007). Red light and blue light differ in their action. Red light activates photosynthesis, which reduces inter-cellular [CO_2] (Ci), thereby resulting in stomatal opening (Farquhar et al., 1982; Roelfsema et al., 2002). A study also reported a role for HT1 in red light-induced stomatal opening. Indeed, Matrosova et al. (2015) showed that *ht1-1* and *ht1-2* single mutants were impaired in red light-induced stomatal opening while blue light signals were able to induce stomatal opening for both mutants. In addition, a non-CO_2 dependent red light response exists in guard cells (Baroli et al., 2008; Matthews et al., 2019). Blue light has a more direct role at low light intensities and acts through PHOT1 and PHOT2 photoreceptors, which regulate activation of proton pumps via BLUE LIGHT SIGNALING 1 (BLUS1) and the inhibition of anion channels at the plasma membrane (Kinoshita et al., 2001; Marten et al., 2007; Takemiya et al., 2013; Hiyama et al., 2017). BLUS1 was shown to be phosphorylated by PHOT1 in vitro, which is required for activation of H^+-ATPase and proper blue light signaling (Takemiya et al., 2013). More recently, it was revealed that the action of BLUS1 together with a decrease in Ci are required for induction of stomatal opening in response to blue light (Hosotani et al., 2021). Also and as mentioned previously, CBC1 and CBC2 are RAF-like protein kinases required for proper signaling in response to blue light and low CO_2 (Hiyama et al., 2017). It was shown that both CBC1 and CBC2 could interact with the photoreceptor PHOT1 (Hiyama et al., 2017). CBC1, but not CBC2, was phosphorylated by PHOT1 in response to blue light in vitro and in vivo (Hiyama et al., 2017).

Interestingly, CBC1 and CBC2 both interact with HT1 and can be phosphorylated by the HT1 kinase in vitro, although the effect of this phosphorylation on the CBC kinase is unknown (Hiyama et al., 2017). While *cbc1 cbc2* double mutant lines do not respond to blue light and low CO_2 signals, such plants are able to further close their stomata upon ABA treatment (Hiyama et al., 2017). With their central role in both low CO_2- and blue light-induced stomatal opening, future research will be of the utmost importance to identify putative target proteins of both CBC kinases and the HT1 kinase (see "Outstanding questions").

Modeling stomatal movement's responses

Several different aspects of the CO_2 response in guard cells have been modeled in recent years. At the signaling levels, models have incorporated many signal transduction components involved in the ABA signaling network (Albert et al., 2017; Maheshwari et al., 2019, 2020). Other models have incorporated, kinetics of pumps, transporters and ion channels their voltage dependency and regulation mechanisms (Hills et al., 2012; Vialet-Chabrand et al., 2017). Some models have addressed a specific mechanism within the CO_2 signal transduction cascade, for example examining the relevance of catalytic carbonic anhydrase activity in guard cell compartments (Hu et al., 2015). Additionally, molecular dynamics modeling has been used to predict testable bicarbonate interaction sites in the SLAC1 anion channel (Zhang et al., 2018b). Finally, a number of studies have addressed the mechanical aspects of stomatal closure, focusing on the deformation of the guard cell morphology (Woolfenden et al., 2018). The common aim of these modeling efforts is to investigate potential biological mechanisms and to generate experimentally testable predictions. Clearly, a thorough review of all modeling is beyond the scope of this review, as most models have focused on ABA responses. We will therefore limit ourselves to brief overviews of some aspects that have been investigated with respect to CO_2 responses.

Spatial models of cellular components are required to address how the localization of components to specific compartments within guard cells affects the dynamics of the CO_2 response. An example of such a model examined the potential importance of the subcellular localization of carbonic anhydrases in guard cells (Hu et al., 2015). Using a reaction-diffusion model, the dynamics of CO_2 and HCO_3^- after an increase in external CO_2 in the presence of membrane-attached carbonic anhydrases, cytosolic carbonic anhydrases, and carbonic anhydrases in internal organelles was modeled. The simulations predicted that HCO_3^- increases were not highly sensitive to the precise location of carbonic anhydrases, at the plasma membrane or in the cytoplasm (Hu et al., 2015). This model predicted that any spatial separation of bicarbonate at the inner leaflet of the plasma membrane or in the bulk cytosol will equilibrate rapidly, which was validated in experimental analyses and could explain the role of the accelerated CO_2 to bicarbonate and protons catalysis mediated by carbonic anhydrases in the stomatal CO_2 response (Hu et al., 2015).

Guard cells change shape as a result of changes in turgor pressure, the hydrostatic pressure generated within a cell by

osmotic uptake of water. The resulting closing and opening of the stomata is a mechanical process, and the morphological changes of the stomata therefore depend on the material properties of the guard cells and the surrounding cells (Woolfenden et al., 2018). Advances in 3D imaging and material property measurements have enabled the construction of detailed, 3D finite element models that are able to simulate shape changes of guard cells (Carter et al., 2017; Yi et al., 2018). These models have highlighted the critical role of cell wall anisotropy in the deformations of guard cells. Furthermore, they were able to predict the stress and strain distributions and revealed large increases in strain at the pore-facing sides and at the tips. Future work can potentially incorporate some of the abovementioned signaling models to create multilevel computational tools that can address stomatal opening and closing in response to CO_2 at different spatial and temporal scales.

Studying monocotyledons to fully grasp how crops will be affected by climate change

The terms monocotyledon and dicotyledon delineate plants by their difference in structure and functions. Monocotyledons have one cotyledon while dicotyledons have two (Chandler, 2008). Stomatal morphology and development also differ between them. Arabidopsis, the prominent dicotyledon model, has stomata consisting of two guard cells and no specialized subsidiary cells (Nunes et al., 2019; Figure 2). *Brachypodium distachyon*, a well-developed model in grasses, displays a graminoid stoma with four cells: two dumbbell-like guard cells flanked by two dome-like subsidiary cells (Brkljacic et al., 2011; Nunes et al., 2019; Figure 2).

Dicotyledons feature randomly oriented stomatal complexes, with multiple precursor cells and stomata dotted throughout the leaf epithelium in many stages of development simultaneously (McKown and Bergmann, 2020). Monocotyledons follow a longitudinal development, with stomatal complexes being polarized in the same orientation as their neighbors (McKown and Bergmann, 2020). There are proliferating stomatal rows which are flanked by veins, repeating this alternation in parallel for the entire width of the leaf (Figure 2). This divergence is of great interest and suggests that more research should be conducted directly in grasses.

This stomatal morphology of grasses is of particular interest to scientists because of the implications it has on stomatal movements and thus water use efficiency (Nunes et al., 2019). The four-cell stomatal structure of grasses allows larger pore apertures and a faster response to stimuli (Franks and Farquhar, 2007; Nunes et al., 2019). Stomatal movements are driven by osmotic gradients which, therefore, rely on efficient exchange of ions in and out of guard cells (Raschke and Fellows, 1971; Kollist et al., 2014; Jezek and Blatt, 2017). As mentioned above, stomata in grasses are composed of guard cells and subsidiary cells, suggesting the need for an efficient relationship between the two cell types to have a functional stomatal complex (Figure 2). Interestingly, the alternating turgor pressure between guard cells and subsidiary cells provides a mechanical advantage that allows grasses to use less water and ions to undergo turgor pressure changes (Franks and Farquhar, 2007; Nunes et al., 2019; Gray et al., 2020). The reciprocity between guard cells and subsidiary cells is thought to be responsible for the rapid stomatal response in grasses (Nunes et al., 2019). The shape of the four-celled stomatal graminoid complex is also larger because the pore is elongated and thus, there is decreased lateral guard cell displacement (Nunes et al., 2019).

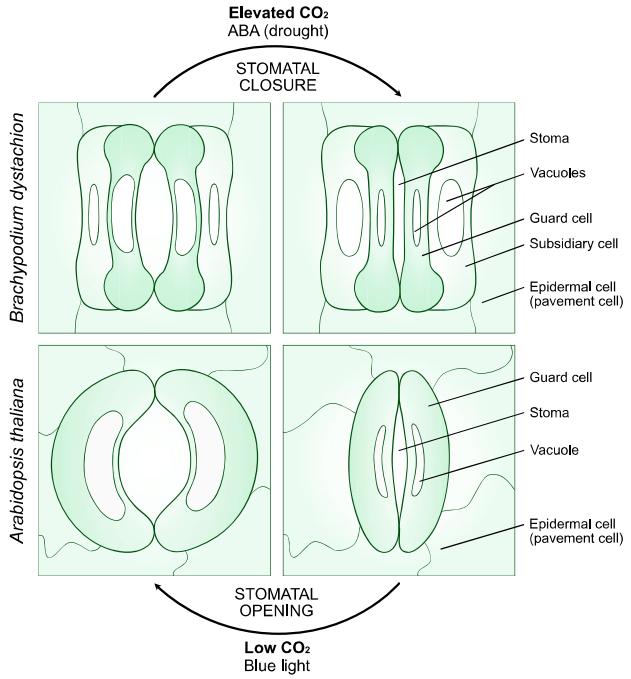

Figure 2 Stomatal movements in Brachypodium and Arabidopsis are faster in the former due to the presence of subsidiary cells. In both *B. distachyon* (top) and *A. thaliana* (bottom), stomatal closure is induced by elevated CO_2 and ABA while stomatal opening is triggered by low CO_2 and blue and red-light signals. The presence of subsidiary cells surrounding dumbbell-shaped guard cells allows for faster stomatal movements in Brachypodium and other grasses. In both Arabidopsis and Brachypodium, the turgor pressure associated with vacuole swelling/shrinking plays a crucial part in stomatal opening/closure, respectively. In Brachypodium, the regulation of the turgor pressure in subsidiary cells enhances the speed of stomatal movements.

CO_2 affects both stomatal development and movements. As mentioned previously, carbonic anhydrases in dicots, AtβCA1 and AtβCA4, have been found to play a role in CO_2-mediated stomatal closure (Hu et al., 2010). In the grass maize, mutating ZmβCA1 and ZmβCA2 led to an increase in stomatal conductance and decreased sensitivity to high [CO_2] (Kolbe et al., 2018). Mutating the rice homolog, OsβCA1, had the same effects (Chen et al., 2017). Notably, in Arabidopsis, stomatal development is additionally affected in β*ca1* β*ca4* double mutants and CO_2 control of stomatal development is impaired (Engineer et al., 2014).

There is considerable variation in the literature about the magnitude of stomatal density responses to CO_2 in different

plant species (Xu et al., 2016). Figure 3 shows long-term effects of CO_2 on stomatal development in *B. distachyon*. We observed that Brachypodium had similar stomatal indices when grown under various CO_2 conditions (Figure 3A). Similarly, plants grown under ambient, low, or high CO_2 concentrations had similar stomatal densities (Figure 3B). These data suggest that *B. distachyon* does not alter stomatal development in response to CO_2 elevation, unlike Arabidopsis (Gray et al., 2000). It is conceivable that the unique stomatal complexes of *Brachypodium* are responsible for accommodating high CO_2 concentration levels without lowering the stomatal density of the plant.

Long-term impact of elevated CO_2: the effect on plant growth

Approximately 85%–90% of land plants are estimated to use C3 photosynthesis. CO_2 elevation in the atmosphere enhances the rate of photosynthesis in C3 plants and can also enhance plant growth in C3 plants due to the increased efficiency of the Calvin cycle catalyzed by the critical enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo; Taylor et al., 1994; Drake et al., 1997; Makino and Mae, 1999; Kirschbaum, 2011). Plant growth stimulated by elevated CO_2 is also determined by nitrogen and phosphorus availability (Sun et al., 2002; Reich et al., 2006). Indeed, large-scale free-air CO_2 enrichment experiments support that CO_2 elevation enhances plant growth and increases above-ground biomass and crop yield in C3 plant species under nonstress conditions (Ainsworth and Long, 2005, 2020). Some studies, however, suggest that the stimulation of biomass and crop yield by rising CO_2 concentration may not compensate for the probable losses caused by climate change (Long et al., 2006). Moreover, reductions in the zinc, iron, and protein content in major crops grown under CO_2 elevation can be detrimental to human nutrition and health (Taub et al., 2008; Loladze, 2014; Myers et al., 2014). Understanding the detailed molecular, biochemical, and cellular mechanisms in response to CO_2 elevation in plants will help develop strategies for food security in the future (see "Outstanding questions").

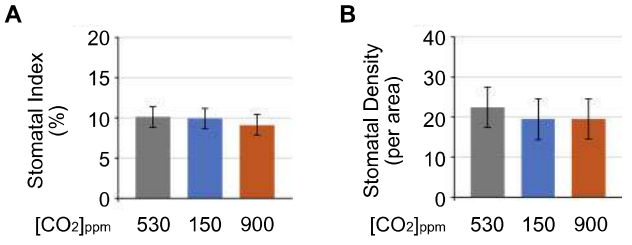

Figure 3 *Brachypodium distachyon* plants grown under distinct CO_2 concentrations have similar stomatal densities and indices. *Brachypodium distachyon* plants do not regulate stomatal development in response to long-term CO_2 exposure (A–B). *Brachypodium distachyon* plants grown under distinct CO_2 concentrations have similar stomatal indices (A) and densities (B). Plants were grown under 530 ppm, low (150 ppm), or high (900 ppm) CO_2 concentrations for 4 weeks and stomatal indices and densities within the same area (18 μm^2) were calculated. Bars represent the average of three leaves from independent plants with three images per leaf per treatment ± SD.

Growth of plants at elevated CO_2 reduces stomatal apertures and, in some species, also reduces stomatal density (Ainsworth and Rogers, 2007). Overall, this reduction of stomatal conductance can be beneficial under water-limiting conditions. Many plants have weak stomatal movement responses to CO_2. Plants grown in water-limited regions could benefit from an enhanced and robust stomatal CO_2 response, given the continuing increase in atmospheric CO_2 fertilization, thus improving water use efficiency (Hu et al., 2010; Busch et al., 2018).

On the other hand, the CO_2-induced reduction in gas exchange counteracts the beneficial effects of the enhanced atmospheric CO_2 fertilization. Thus, for plants grown in regions with sufficient water and soil nutrients, a weaker stomatal closing response to elevated CO_2 or a faster stomatal response to fluctuating light could be beneficial (Lawson and Blatt, 2014; Busch et al., 2018; Kimura et al., 2020).

Long-term elevated CO_2 exposure additionally can lead to physiological acclimation by reducing leaf photosynthesis capacity that limits the enhancement of plant growth (Drake et al., 1997; Moore et al., 1999; Adam et al., 2004). Downregulation of RuBisCo transcript and protein levels by long-term CO_2 elevation has been reported in several plant species (Nie et al., 1995; Cheng et al., 1998; Moore et al., 1998, 1999; Adam et al., 2004). It has been hypothesized that excessive carbohydrate and starch accumulation under CO_2 enrichment is correlated with the downregulation of photosynthesis genes (Nie et al., 1995; Cheng et al., 1998). Another hypothesis suggests that the observed reduction of leaf nitrogen content and nitrate assimilation under elevated CO_2 are due to photosynthetic biochemical limitations (Nakano et al., 1997; Stitt and Krapp, 1999; Warren et al., 2015). Sink capacity and source–sink balance were also proposed to affect the photosynthesis acclimation (Rogers et al., 1998; Paul and Foyer, 2001; Ainsworth et al., 2004; Bloom et al., 2010). In addition, sugar sensing and signaling pathways and altered hormone metabolism are suggested to indirectly regulate plant growth under elevated CO_2 conditions (Thompson et al., 2017; Gamage et al., 2018). Advanced mechanistic knowledge of how plant stomata and photosynthesis adjust to elevated atmospheric CO_2 concentrations is required for developing future strategies aimed at enhancing yields and potentially carbon capture in a high CO_2 world.

Concluding remarks

While progress has been made in the past decade at understanding molecular, biochemical, and biophysical mechanisms that mediate CO_2-controlled stomatal movements, the early signaling network remains fragmented (Figure 1). Improving our understanding of how plants sense CO_2 in guard cells and mesophyll cells represents a central field of investigation in the coming years (see "Outstanding

OUTSTANDING QUESTIONS

- What is the primary CO_2/HCO_3^- sensor in guard cells? Are there distinct perception mechanisms/signaling pathways for dicots and monocots?
- Can GHR1 act as a scaffolding protein around ion channels for proper signaling in response to elevated CO_2?
- What are the targets of CBC1 and CBC2, central proteins in both low CO_2— and blue light-induced stomatal opening?
- What is the signal from mesophyll cells that functions in stomatal responses to changes in CO_2 levels?
- Does high CO_2 concentration affect stomatal development in some grasses? Can grasses respond to CO_2 concentration solely by modulating stomatal aperture?
- How do subsidiary cells in grasses enhance stomatal movement responses to external stimuli? Are there separate signaling pathways in each cell type with opposite outcomes in turgor pressure?
- How will climate change affect stomatal movements, stomatal development, and plant water use efficiency in C3 and C4 plants?

Questions"). Crops are an essential food source for the global population. Therefore, studying the stomata of opportune crop grass models like *B. distachyon* is essential. With the ever-increasing global temperatures and rising CO_2 levels, the global population needs sustainable crop plants that are drought tolerant and water-use efficient. Interestingly, grasses may have a stomatal advantage under elevated CO_2 conditions. Overall, we should focus our efforts on understanding how plants perceive and respond to elevated CO_2 toward increasing water-use efficiency and yield in a world affected by climate change.

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