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Stomatal CO₂ responses at sub- and above-ambient CO₂ levels employ different pathways in Arabidopsis

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H.H. designed the study, H.H., K.K., and E.M. performed experiments, H.H., K.K., E.M., and H.K. analyzed data, H.H., K.K., and H.K. wrote the manuscript, all authors commented, edited and approved the final manuscript.

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Abstract

Stomatal pores that control plant CO₂ uptake and water loss affect global carbon and water cycles. In the era of increasing atmospheric CO₂ levels and vapor pressure deficit (VPD), it is essential to understand how these stimuli affect stomatal behavior. Whether stomatal responses to sub-ambient and above-ambient CO₂ levels are governed by the same regulators and depend on VPD remains unknown. We studied stomatal conductance responses in Arabidopsis (*Arabidopsis thaliana*) stomatal signaling mutants under conditions where CO₂ levels were either increased from sub-ambient to ambient (400 ppm) or from ambient to above-ambient levels under normal or elevated VPD. We found that guard cell signaling components involved in CO₂-induced stomatal closure have different roles in the sub-ambient and above-ambient CO₂ levels. The CO₂-specific regulators prominently affected sub-ambient CO₂ responses, whereas the lack of guard cell slow-type anion channel SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) more strongly affected the speed of above-ambient CO₂-induced stomatal closure. Elevated VPD caused lower stomatal conductance in all studied genotypes and CO₂ transitions, as well as faster CO₂-responsiveness in some studied genotypes and CO₂ transitions. Our results highlight the importance of experimental setups in interpreting stomatal CO₂-responsiveness, as stomatal movements under different CO₂ concentration ranges are controlled by distinct mechanisms. Elevated CO₂ and VPD responses may also interact. Hence, multi-factor treatments are needed to understand how plants integrate different environmental signals and translate them into stomatal responses.

Introduction

Atmospheric CO₂ concentration has nearly doubled within the past 150 years. As a result, the global average temperature has increased by 1.5 °C and relative air humidity decreased across many vegetated areas (Yuan et al. 2019). Increased air temperature and decreased air humidity lead to rising values of vapor pressure deficit (VPD), the difference between actual and saturated air vapor pressures, and this increases transpiration from plants. In order to survive such conditions, plants need to adjust their water management by regulating stomatal conductance. Each stoma is composed of two guard cells and an opening formed between them; the size of the opening is controlled by increasing or decreasing guard cell turgor pressure. Guard cells adjust their turgor pressure in response to various abiotic stimuli to balance water loss and CO₂ uptake for photosynthesis. Understanding how stomata respond to changing CO₂ concentrations and increasing VPD is needed for breeding climate-ready crops.

Plant stomata close in response to elevated CO₂ concentration and open in response to decreased CO₂ concentration. Ultimately, elevated CO₂ activates the S-type anion channel SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1), which causes rapid stomatal closure (Negi et al. 2008; Vahisalu et al. 2008). CO₂ can enter guard cells through the PLASMA MEMBRANE INTRINSIC PROTEIN 2 (PIP2) plasma membrane channel or by transmembrane diffusion

(Katsuhara and Hanba 2008; Wang et al. 2016). Carbonic anhydrases BETA CARBONIC ANHYDRASE 1 (CA1) and BETA CARBONIC ANHYDRASE 4 (CA4) accelerate the conversion of intracellular CO₂ to bicarbonate (HCO₃⁻), which can act as a second messenger (Hu et al. 2010). In guard cells, CO₂/HCO₃⁻ promotes interaction between the protein kinases MITOGEN-ACTIVATED PROTEIN KINASE 4 (MPK4)/MITOGEN-ACTIVATED PROTEIN KINASE 12 (MPK12), and the Raf-like kinase HIGH LEAF TEMPERATURE 1 (HT1), leading to HT1 inhibition, which is an essential step in the regulation of stomatal responses to CO₂ (Hashimoto et al. 2006; Hashimoto-Sugimoto et al. 2016; Hörak et al. 2016; Jakobson et al. 2016; Takahashi et al. 2022; Yeh et al. 2023). HT1 phosphorylates the CONVERGENCE OF BLUE LIGHT AND CO₂ (CBC1)/CONVERGENCE OF BLUE LIGHT AND CO₂ 2 (CBC2) Raf-like kinases that function downstream of HT1 and these Raf kinases can inhibit the S-type anion channel activation via a currently unknown mechanism (Hörak et al. 2016; Hiyama et al. 2017; Hayashi et al. 2020). Stomata in HT1-deficient plants do not respond to CO₂ concentration changes while carbonic anhydrase, MPK12 and SLAC1 mutants exhibit impaired stomatal CO₂ responses (Hashimoto et al. 2006; Vahisalu et al. 2008; Hu et al. 2010; Hashimoto-Sugimoto et al. 2016; Hörak et al. 2016; Jakobson et al. 2016).

Elevated VPD increases transpiration and reduces epidermal turgor that due to mechanical interactions between guard and

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epidermal cells in angiosperms leads to faster light-induced stomatal opening (Mott et al. 1999; Pichaco et al. 2024). To prevent wilting, stomata close under elevated VPD. Abscisic acid (ABA) is a drought-induced plant stress hormone and an important stomatal regulator (Cutler et al. 2010). VPD has a direct effect on ABA concentration as increased ABA levels in angiosperms were observed 20 min after increasing VPD from 0.7 to 1.5 kPa (McAdam and Brodribb 2015), promoting the conclusion that ABA may be involved in elevated VPD-induced stomatal closure in angiosperms (McAdam et al. 2016). The protein kinase OPEN STOMATA 1 (OST1) and the leucine-rich receptor-like pseudokinase GUARD CELL HYDROGEN PEROXIDE-RESISTANT 1 (GHR1) are activated in the presence of ABA and trigger anion efflux through the major guard cell slow-type anion channel SLAC1 (Brandt et al. 2012; Hua et al. 2012; Sierla et al. 2018). OST1 and GHR1 are both involved in elevated VPD- and CO₂-induced stomatal closure response (Xue et al. 2011; Merilo et al. 2018; Sierla et al. 2018; Hsu et al. 2021; Jalakas et al. 2021b).

Understanding CO₂-induced plant stomatal closure responses is essential for future plant breeding. Due to changing climate conditions, it is also important to understand if and how stomatal CO₂ regulation is affected by elevated VPD levels. Previous work in grasses suggests that elevated VPD levels reduce both stomatal conductance and stomatal sensitivity to CO₂ concentration changes (Morison and Gifford 1983) but the interactions of CO₂ and humidity responses in dicots remain poorly understood.

Under light, CO₂ concentration inside the leaf is usually below ambient levels and this causes stomatal opening. Stomata close when CO₂ concentration inside the leaf increases to ambient levels, and an additional rise in CO₂ concentration to above-ambient levels causes further stomatal closure (Brodribb et al. 2009; Hörak et al. 2017). Thus, stomatal closure response exists within sub-ambient as well as in above-ambient CO₂ levels; however, it is not clear whether these responses are controlled by the same regulators. In some studies, CO₂-induced stomatal closure is defined as the response to an increase in CO₂ concentration from ambient to above-ambient levels (Franks and Britton-Harper 2016; Hörak et al. 2017), while some studies use a [CO₂] change from sub-ambient to above-ambient levels (Azoulay-Shemer et al. 2015; Chater et al. 2015). Data from previous studies comparing CO₂ responses in ferns and angiosperms suggest that stomatal responses to CO₂ are different, when changing CO₂ levels in the sub-ambient or above-ambient ranges (Brodribb et al. 2009; Hörak et al. 2017). To address the underlying mechanisms of CO₂-induced stomatal closure at different CO₂ transitions under normal and elevated VPD conditions, we studied plants deficient either in guard cell anion channel SLAC1 and its activation (*slac1-3*, *ghr1-3*, and *ost1-3*) or in the CO₂-specific stomatal signaling branch regulated by MPK12 and HT1 kinases (*mpk12-4*, *ht1-2*, and *ht1-8D*) and carbonic anhydrases CA1 and CA4 (*ca1ca4*). Our results show that different stomatal regulators have a different degree of importance in CO₂-induced stomatal closure in sub-ambient and above-ambient CO₂ levels and are also differently affected by VPD.

Results

Stomatal closure kinetics are different between sub-ambient to ambient and ambient to above-ambient [CO₂] transitions

We analyzed stomatal responses to CO₂ in the sub-ambient and above-ambient concentration ranges in the model plant *Arabidopsis* (*Arabidopsis thaliana*) to clarify whether these responses are

controlled by the same or by different regulators. Four different CO₂ transition sequences were used (Fig. 1, A, C, E, and G); in two setups, we applied high VPD (2.3 kPa) as an additional factor before and throughout CO₂ treatments (Fig. 1, A and E). Experiments were started with ambient (400 ppm) CO₂ concentration and each consecutive CO₂ treatment lasted for 2 h. This approach allowed us to investigate plant stomatal response to different CO₂ transitions—stomatal opening in response to CO₂ transition from 400 to 100 ppm (from here on referred to as 400–100; Fig. 1, A, C, and G), from 800 to 400 ppm (800–400; Fig. 1C), or from 800 to 100 ppm (800–100; Fig. 1E), and stomatal closure in response to CO₂ transition from 100 to 400 ppm (100–400; Fig. 1A), from 400 to 800 ppm (400–800; Fig. 1, A, C, and E), or from 100 to 800 ppm (100–800; Fig. 1G). We analyzed the amplitude and speed of stomatal responses, whereas the speed was defined here as stomatal 75% response time (see Methods and Supplementary Fig. S1).

Stomatal closure responses had clearly different 75% response times in Col-0 wild-type plants at different CO₂ transitions: the 400–800 stomatal closure was faster than 100–400 closure under normal VPD (Fig. 1, A and B). The rapid 400–800 response was consistent throughout all experimental setups (Fig. 1, A to F). The observed different kinetics of these CO₂-induced stomatal closure responses suggest that they could be regulated by different components. Stomatal opening responses to sub-ambient CO₂ levels, 400–100 (Fig. 1, A to D, G, and H) and 800–100 (Fig. 1, E and F) had relatively slow 75% response times, whereas the 800–400 response, opening from above-ambient to ambient CO₂ levels, had lower response range and faster 75% response time (Fig. 1, C and D). This suggests that stomatal opening to sub-ambient CO₂ concentrations is a much stronger, albeit slower, response than stomatal opening during the above-ambient to ambient [CO₂] change.

High VPD but not the order of CO₂ transitions affects stomatal CO₂-response kinetics

To study whether high VPD affects CO₂ responses, we conducted the 400–100–400–800 and the 400–800–100 CO₂ transitions under conditions where plants were first acclimatized to increased VPD (2.3 kPa) for ~3 h and then subjected to changes in CO₂ levels under the elevated VPD conditions. Under such conditions, both the 400–100 and 800–100 stomatal opening responses were significantly faster than under normal VPD (Fig. 1, A, B, E, and F). Stomatal closure in sub-ambient to ambient CO₂ concentration range was also enhanced under high VPD conditions, resulting in a shorter stomatal 75% response time during the 100–400 ppm [CO₂] transition compared with normal VPD (Fig. 1, A and B), whereas we were not able to detect an effect of high VPD on the 400–800 ppm [CO₂] transition 75% response time (Fig. 1, A, B, E, and F). These results suggest that during high VPD stress, plant stomata could be primed for faster movements in the sub-ambient to ambient CO₂ concentration range.

In our first experiments, the 400–100 opening stimulus was applied before the 400–800 closure. To test whether the exposure to sub-ambient CO₂ levels had an effect on stomatal CO₂-responsiveness, we also applied the CO₂ transitions in reverse order (400–800–400–100, Fig. 1C). Stomatal 75% response times during the 400–100 transition appeared slightly shorter when it was the last transition (Fig. 1, C and D) than when it was the first (Fig. 1, A, B, G, and H), whereas the 400–800 transition response speed was unaffected by previous treatments (Fig. 1, A, B, and C to F). Thus, there were no major effects of the order of CO₂ concentration transitions on stomatal CO₂-response kinetics.

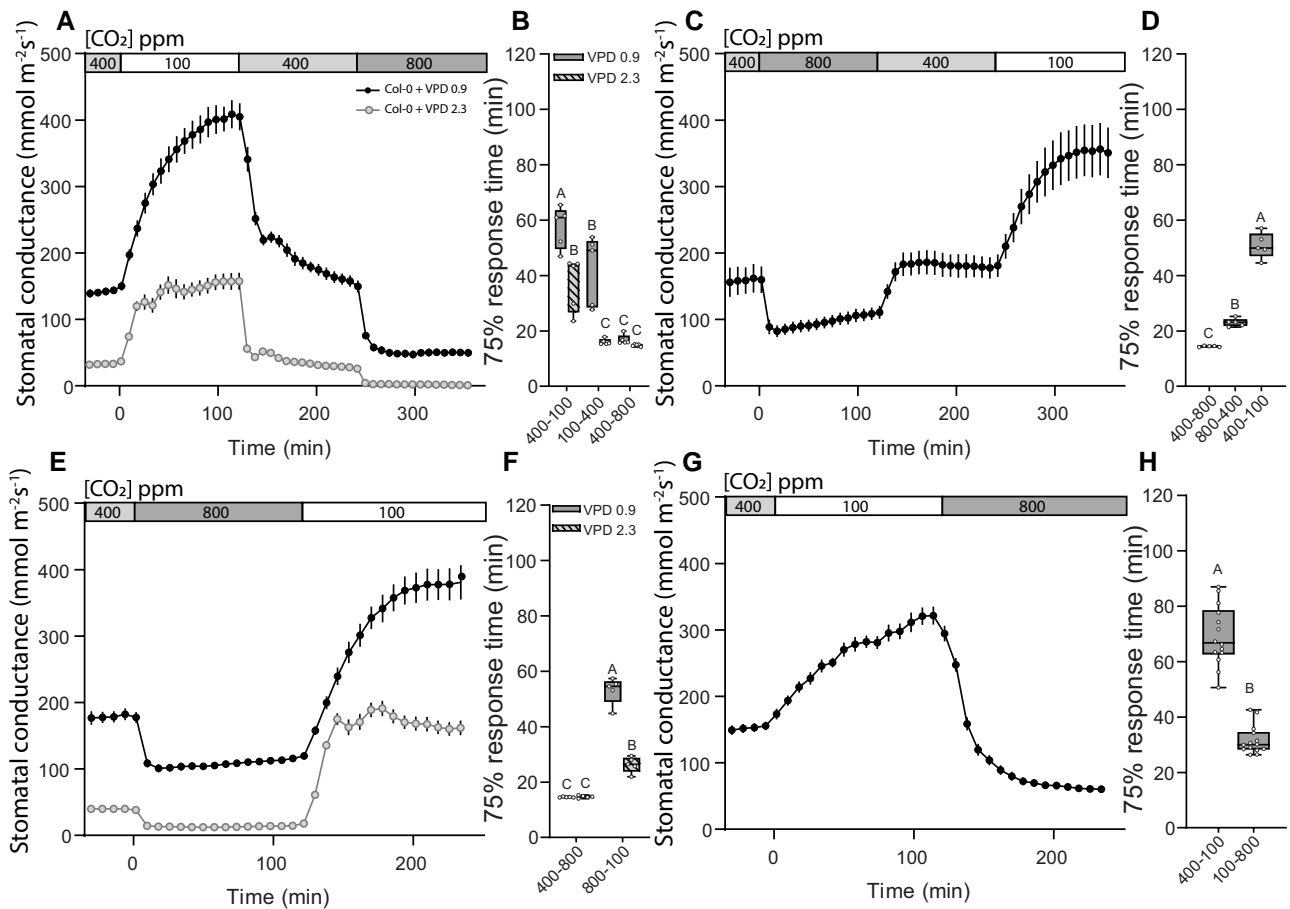


Figure 1. Kinetics of CO₂-induced stomatal responses in wild-type Arabidopsis with regular and elevated vapor pressure deficit (VPD). Col-0 wild-type Arabidopsis stomatal response to sequential changes in CO₂ concentration under regular (A, C, E, G) and elevated (A, E) VPD conditions. Mean stomatal conductance \pm standard error of the mean (SEM) is shown. B, D, F, H) Boxplot of 75% response time (min) of stomatal response to CO₂ concentration changes. Boxes represent 25% to 75% quartiles and median as the horizontal lines, whiskers indicate the smallest and largest values, and points show individual plant values. B, F) Statistically significantly different groups are indicated with different letters (Two-way ANOVA with Tukey post hoc test, $P < 0.05$). D, H) Statistically significantly different groups are indicated with different letters (One-way ANOVA with Tukey post hoc test, $P < 0.05$). Sample size was 5 in (A, B, C, D, E, F) and 14 in (G, H). Start of the first treatment was between 11:30 and 12:30.

The CO₂-specific pathway components MPK12 and carbonic anhydrases are more involved in sub-ambient to ambient than in above-ambient CO₂-induced stomatal closure

To better understand the role of the CO₂-signalling module comprising MPK12, HT1 and carbonic anhydrases CA1 and CA4 in stomatal CO₂ responses at different CO₂ levels, we analyzed the *mpk12-4*, *ht1-2*, *ht1-8D*, and *ca1a4* in a similar experimental set-up as described for wild-type plants in Fig. 1A. All the CO₂-signalling mutants showed very little stomatal closure in magnitude compared with wild-type plants, whereas 75% response time was significantly reduced only in the *ca1a4* mutant in response to the 100–400 transition (Fig. 2, A, C, and E). The 75% response times for the *ht1-2* and *ht1-8D* mutants in this CO₂ transition are not informative due to hardly any stomatal response in these mutants (Fig. 2, A, C, and E). The response of *mpk12-4* to the 100–400 transition was as fast as in wild-type, but significantly smaller in magnitude (Fig. 2, A, C, and E). In response to the 400–800 transition, the HT1 mutants had a very weak stomatal response with small magnitude and slow speed, whereas stomatal closure in the *mpk12-4* and *ca1a4* plants was slower than in WT, but larger in magnitude (Fig. 2, A, C, and E). Very weak responses of the HT1 mutants suggest that HT1 is necessary to initiate stomatal

responses to CO₂ concentration changes in both ambient and sub-ambient levels. MPK12 and carbonic anhydrase mutants display stronger stomatal responses in the above-ambient than sub-ambient CO₂ range (Fig. 2, A, C, and E), suggesting that respective signaling components have a more important role in sub-ambient compared with the above-ambient CO₂ concentrations.

Elevated VPD led to lower stomatal conductance in all studied mutants (Fig. 2, A and B), and thus smaller magnitudes of CO₂-induced changes in stomatal conductance (Fig. 2, C and D). Elevated VPD had some effects on the patterns of CO₂-responsiveness in the CO₂-signalling mutants (Fig. 2, A to F). The *ca1a4* and *mpk12-4* stomatal response times were similar to WT in the 400–800 transition under elevated VPD (Fig. 2F), and the *mpk12-4* mutant had a significantly slower than wild-type response to the 100–400 transition only under elevated VPD. Thus, the relatively larger degree of importance of MPK12 and carbonic anhydrases in regulating stomatal responses in the sub-ambient CO₂ ranges was more pronounced under elevated VPD conditions.

Stomatal opening response to 400–100 transition in all of the studied CO₂-signalling mutants was lower in magnitude and slower in response time, irrespective of VPD, whereas the difference in response time between wild-type and mutants was larger under elevated VPD (Fig. 2, A to F). Thus, stomatal opening response to

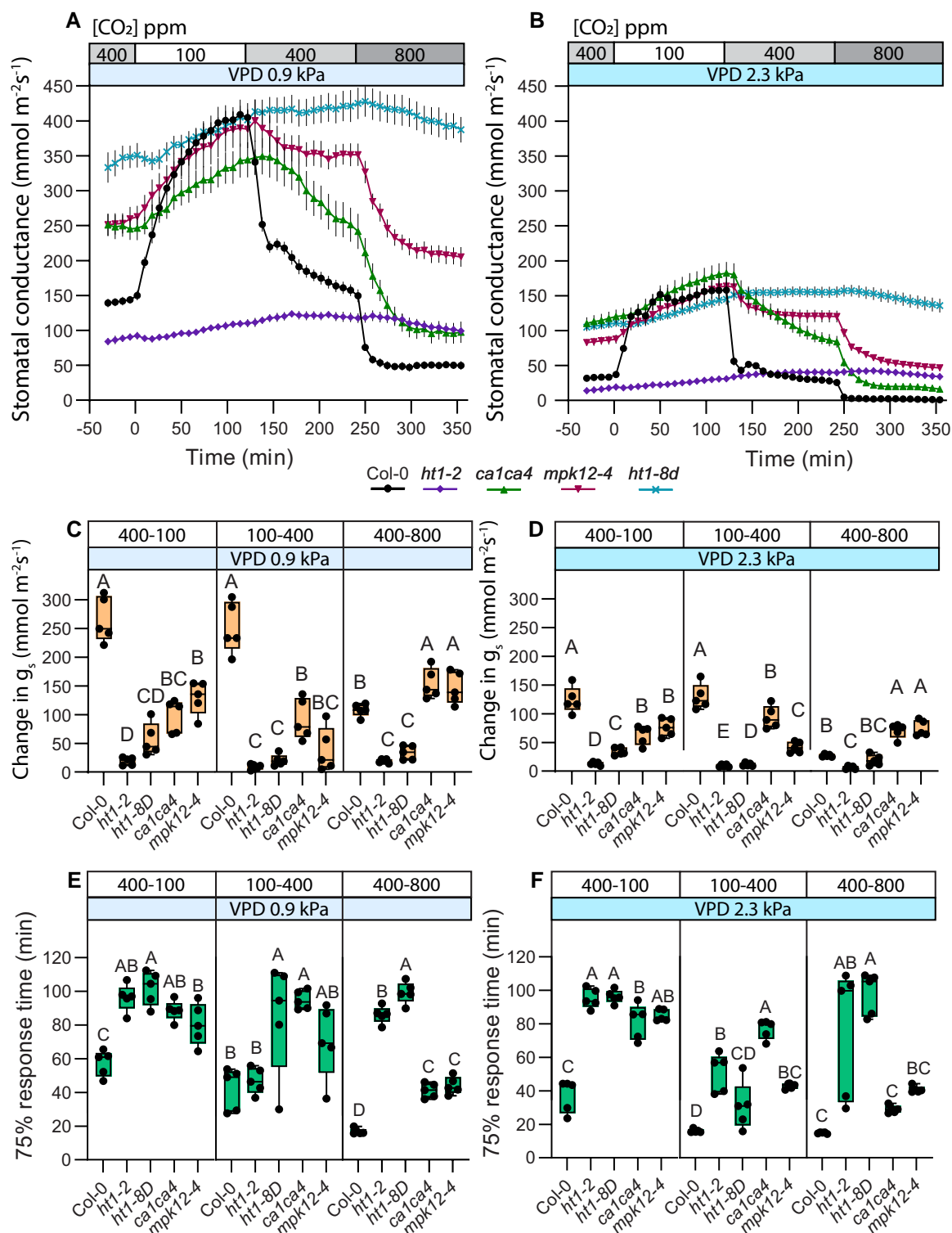


Figure 2. CO₂ pathway mutants retain response patterns under regular and elevated vapor pressure deficit (VPD) conditions. **A and B**) Stomatal response to CO₂ concentration changes from 400 to 100 parts per million (ppm), 100 to 400 ppm, and 400 to 800 ppm under regular (**A**) and elevated (**B**) VPD conditions, mean stomatal conductance \pm SEM is shown. **C, D**) Boxplot of stomatal conductance (*g_s*) change (mmol m⁻² s⁻¹) in response to CO₂ concentration changes from 400 to 100 ppm, 100 to 400 ppm, and 400 to 800 ppm, respectively. **E, F**) Boxplot of 75% response time (min) of stomatal response to CO₂ concentration changes from 400 to 100 ppm, 100 to 400 ppm, and 400 to 800 ppm, respectively. **C to F**) Boxes represent 25% to 75% quartiles and median as the horizontal lines, whiskers indicate the smallest and largest values, and points show individual plant values. Statistically significantly different groups are marked with different letters (One-way ANOVA with Tukey post hoc test, *P* < 0.05). **A to F**) Sample size was 5 for all plant lines. VPD during experiments was 0.9 kPa in (**A, C, E**) and 2.3 kPa in (**B, D, F**). Start of the first treatment was between 11:30 and 12:30. Col-0 data are the same as used in Fig. 1, and experiments with Col-0 and mutant lines were done together.

sub-ambient [CO₂] involves all of the CO₂-signaling pathway components represented in this study, including the carbonic anhydrases CA1 and CA4, and the HT1 and MPK12 kinases.

SLAC1 and GHR1 are more important for above-ambient CO₂-induced stomatal closure

The SLAC1 anion channel is a major component in the activation of stomatal closure response. Thus, we examined CO₂ responses across different CO₂ concentration ranges in plants deficient in the SLAC1 anion channel (*slac1-3*) and in OST1 or GHR1: proteins involved in SLAC1 activation (*ost1-3*, *ghr1-3*; Fig. 3). Stomatal 75% response time of *slac1-3* plants was longer in both, 100–400 and 400–800 CO₂ transitions (Fig. 3, A and E) but the latter was more affected as the 75% response time difference compared with wild-type plants was notably larger in the 400–800 transition. Yet, the magnitude of stomatal closure between wild-type Col-0 and *slac1-3* was only different in the 100–400 transition (Fig. 3C), while their 400–800 response magnitude was similar. Under elevated VPD, *slac1-3* stomatal response amplitude was similar to wild-type both during the 100–400 and 400–800 transitions, although stomatal response times of *slac1-3* were still significantly longer than wild-type on both transitions (Fig. 3, D and F). Together, these results show that SLAC1 is important in stomatal closure in both sub-ambient and above-ambient [CO₂] ranges, but response speed tends to be more severely impacted in the 400–800 transition, suggesting a more prominent role for SLAC1 in ensuring fast above-ambient CO₂-induced stomatal closure.

Plants deficient in SLAC1 activation via OST1 or GHR1 also showed impaired CO₂-induced stomatal closure both in the 100–400 and in the 400–800 transitions (Fig. 3, A, C, and E). The *ost1-3* mutant had long stomatal response times in both the 100–400 and 400–800 transitions, whereas the *ghr1-3* response was similar to wild type in the 100–400 transition, but slower and very small in magnitude during the 400–800 transition (Fig. 3, A, C and E). Thus, both SLAC1-activating proteins are involved in CO₂ responses in all tested concentration ranges, but GHR1, like SLAC1, appears to contribute more toward above-ambient CO₂-induced stomatal closure.

As in other mutants (Fig. 2), elevated VPD lowered stomatal conductance and stomatal response magnitude, but tended to increase stomatal opening speed (Fig. 3). The *ost1-3* 75% response times and magnitude remained similar in both VPD conditions, in line with its VPD-insensitivity (Fig. 3, C to F, Merilo et al. 2013). In *ghr1-3*, stomatal response to 400–800 was either absent or extremely weak under elevated VPD (Fig. 3, B and D).

Sub-ambient CO₂-induced stomatal opening response magnitude was similar to wild-type in all of the studied anion channel activation mutants, irrespective of VPD (Fig. 3, C and D). Stomatal 75% response time under regular VPD was similar to wild-type in all but *ost1-3* (Fig. 3E), whereas under elevated VPD conditions, both *ghr1-3* and *ost1-3* had longer stomatal opening 75% response times (Fig. 3F). Therefore, regulation of SLAC1 is less important for sub-ambient CO₂-induced stomatal opening than the guard cell CO₂-specific signaling pathway. Only under elevated VPD, the 400–100 transition response was slower compared to the wild-type in *ghr1-3* (Fig. 3F), indicating an interaction between CO₂ and VPD signaling in stomatal opening responses.

Shifting CO₂ levels from 100 to 800 ppm masks the differences between stomatal behavior in 100–400 and 400–800 ppm [CO₂] transitions

To further study how stomatal movements differ depending on CO₂ concentrations, we did additional gas-exchange measurements,

during which CO₂ concentration was changed directly from sub-ambient (100 ppm) to above-ambient (800 ppm). Previously, in Figs. 2 and 3 we saw different stomatal response characteristics for 100–400 and 400–800 CO₂ transitions for different mutants that we failed to detect during the 100–800 CO₂ transition (Fig. 4). For example, *mpk12-4* stomatal response was small in amplitude in the 100–400 transition, but much larger in the 400–800 transition (Fig. 2, A and C), whereas in the 100–800 transition the *mpk12-4* stomatal response amplitude was similar to wild type (Fig. 4A). Similarly, in the *ca1ca4* plants 100–400 stomatal response was smaller and 400–800 response larger than in wild-type in amplitude (Fig. 2C), yet the 100–800 response magnitude was similar to wild-type (Fig. 4C). The *ghr1-3* mutant had slower response to the 400–800 transition (Fig. 3E), whereas its 75% response time was similar to wild-type in the 100–800 transition (Fig. 4F). These results demonstrate that differences in stomatal responses between plant lines can remain undiscovered depending on CO₂ concentrations that are used for experiments.

Similar to CO₂-induced stomatal closure experiments, [CO₂] ranges were important also in stomatal opening assays (Figs. 2–3 vs Fig. 5). For example, response to the 400–100 transition in *mpk12-4* was slower and smaller in magnitude compared with wild-type plants (Fig. 2, C and E), but in the 800–100 experiments, *mpk12-4* had normal response amplitude and wild-type-like 75% response time (Fig. 5, C and E). The *slac1-3* plants had similar to wild-type 400–100 stomatal opening speed and magnitude (Fig. 3, C and E), but were slower in the 800–100 response (Fig. 5F). We also observed a significantly slower opening response to the 800–400 transition in *slac1-3* (Supplementary Fig. S2F), which indicates that the reduced stomatal opening speed of the 800–100 response in this mutant is caused by slower opening in the 800–400 range. Thus, although wild-type plants have no discernible differences between the 400–100 and the 800–100 stomatal opening responses (Fig. 1, A, B, E, and F), the molecular mechanisms are at least partly different for these CO₂ transitions.

We combined information from previously analyzed CO₂ transitions in the mutants used in our study with our results (Table 1). Our findings mostly confirm previous results, where available, with the exception of some differences in response amplitude in the *slac1-3*, *ost1-3*, and *ca1ca4* mutants that can be explained by longer treatment duration in this work that allowed the slower stomatal responses of these mutants to reach amplitudes similar to wild-type plants. We also found a faster stomatal response to sub-ambient to above-ambient CO₂ transition in the *ht1-2*, potentially explained by different parameters used to describe response speed in different studies. Our experiments add information on the ambient to sub-ambient, above-ambient to ambient, sub-ambient to above-ambient, above-ambient to sub-ambient, and sub-ambient to ambient CO₂ transitions that have not been systematically addressed before in all the mutants analyzed here.

Discussion

Here, we show that stomatal closure in response to an increase in CO₂ concentration, which occurs both at sub-ambient and at above-ambient CO₂ concentration ranges, is regulated by both the CO₂-specific and SLAC1-related pathways under all CO₂ ranges, but these pathways have a different degree of importance under different CO₂ concentration ranges. Stomatal closure in response to the 400–800 CO₂ concentration range is faster (Fig. 1B), while the 100–400 response has greater amplitude (Figs. 1A and 2C). These differences may be explained by higher guard cell volume and larger stomatal apertures under lower CO₂ levels,

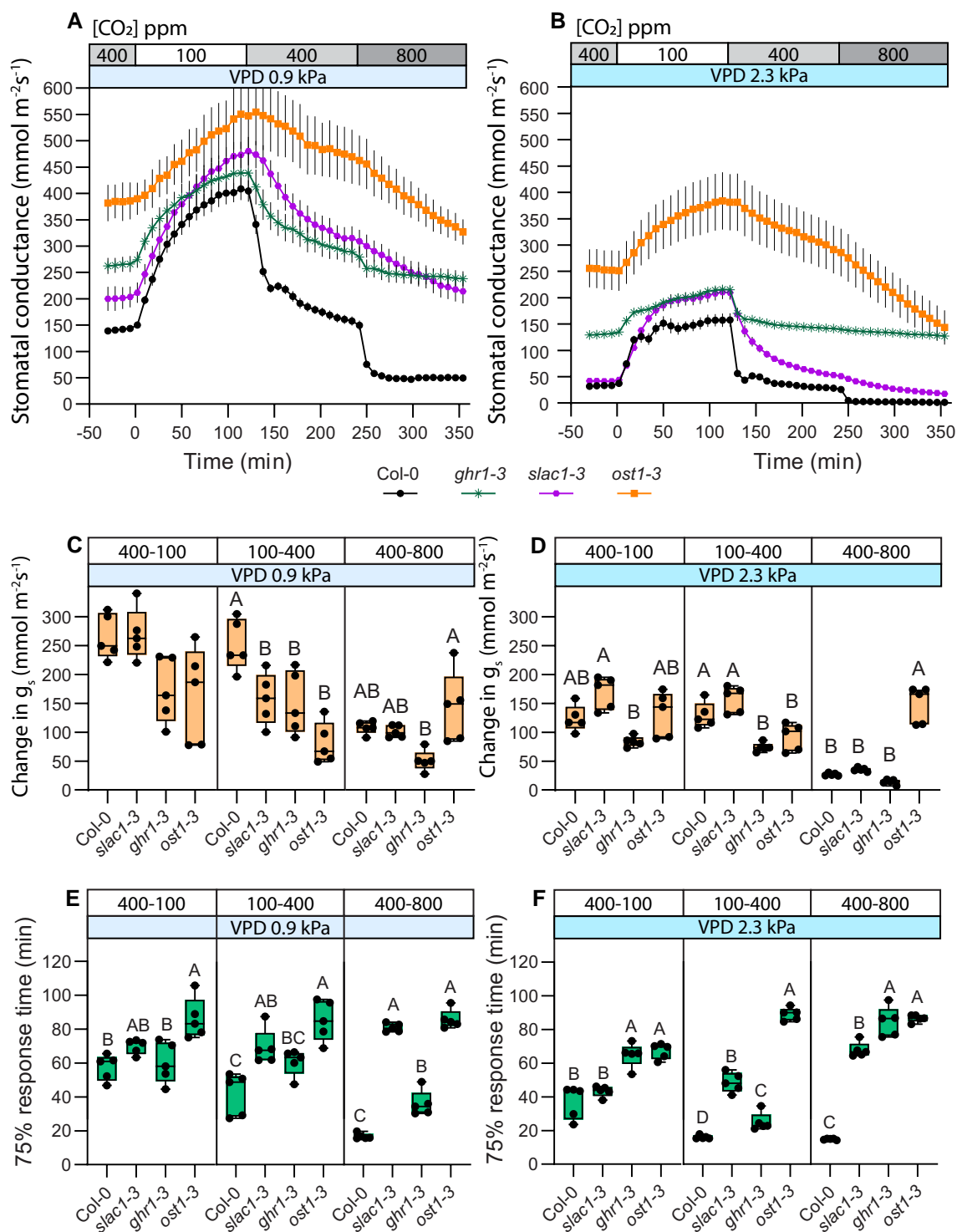


Figure 3. CO₂-response patterns in anion channel activation mutants are affected by vapor pressure deficit (VPD) conditions. **A** and **B** Stomatal response to CO₂ concentration changes from 400 to 100 parts per million (ppm), 100 to 400 ppm, and 400 to 800 ppm in regular (**A**) and elevated (**B**) VPD conditions, mean stomatal conductance \pm SEM is shown. **C**, **D** Boxplot of stomatal conductance (g_s) change (mmol m⁻² s⁻¹) in response to CO₂ concentration changes from 400 to 100 ppm, 100 to 400 ppm, and 400 to 800 ppm, respectively. **E**, **F** Boxplot of 75% response time (min) of stomatal response to CO₂ concentration changes from 400 to 100 ppm, 100 to 400 ppm, and 400 to 800 ppm, respectively. **C** to **F** Boxes represent 25% to 75% quartiles and median as the horizontal lines, whiskers indicate the smallest and largest values, and points show individual plant values. Statistically significantly different groups are marked with different letters (One-way ANOVA with Tukey post hoc test, $P < 0.05$). **A** to **F** Sample size was 5 for all plant lines. VPD during experiments was 0.9 kPa in (**A**, **C**, **E**) and 2.3 kPa in (**B**, **D**, **F**). Start of the first treatment was between 11:30 and 12:30. Col-0 data are the same as used in Fig. 1, and experiments with Col-0 and mutant lines were done together.

leading to slower responses with larger amplitudes, similar to slower responses of larger stomata (Drake et al. 2013; Kübarsepp et al. 2020). However, the relative contribution of the components

involved in stomatal CO₂-signalling is also different in the 400–800 and 100–400 stomatal closure responses (Figs. 2 to 3). Increasing CO₂ abruptly from 100 to 800 masked the presence of two

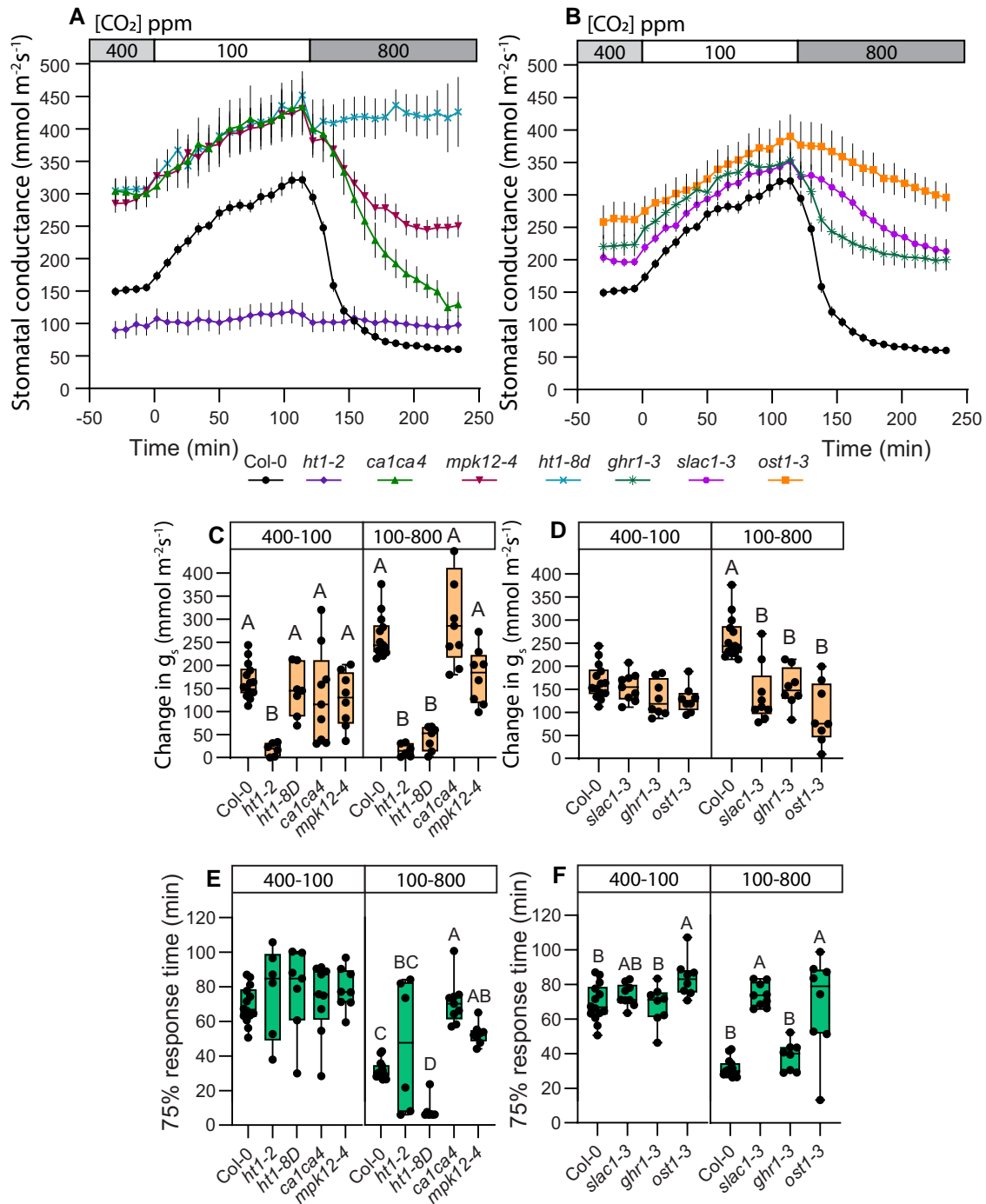


Figure 4. CO₂ transition from 100 to 800 parts per million (ppm) masks different responses present in 100 to 400 and 400 to 800 ppm CO₂ transitions. **A** and **B**) Stomatal response to CO₂ concentration changes from 400 to 100 ppm and 100 to 800 ppm, mean stomatal conductance \pm SEM is shown. **C, D**) Boxplot of stomatal conductance (*g_s*) change (mmol m⁻² s⁻¹) in response to CO₂ concentration changes from 400 to 100 ppm and 100 to 800 ppm, respectively. **E, F**) Boxplot of 75% response time (min) of stomatal response to CO₂ concentration changes from 400 to 100 ppm and 100 to 800 ppm, respectively. **C to F**) Boxes represent 25% to 75% quartiles and median as the horizontal lines, whiskers indicate the smallest and largest values, and points show individual plant values. Statistically significantly different groups are marked with different letters (One-way ANOVA with Tukey post hoc test, $P < 0.05$). **A to F**) Sample size was 14 for Col-0; 6 for *ht1-2*; 7 for *ht1-8D*; 8 for *ghr1-3*, *mpk12-4* and *ost1-3*; 9 for *slac1-3* and *ca1ca4*. VPD during experiments was 0.9 kPa in (**A to F**). Start of the first treatment was between 11:30 and 12:30. Col-0 data are the same as used in Fig. 1, and experiments with Col-0 and mutant lines were done together.

processes with different kinetics (Figs. 2 to 4), indicating the necessity to analyze stomatal closure responses to elevated CO₂ levels separately in the ambient to above-ambient and sub-ambient to ambient CO₂ concentration ranges.

The HT1 kinase is required for plant stomatal CO₂ signaling; plants with impaired HT1 function are nearly insensitive to all CO₂

concentration changes (Hashimoto et al. 2006; Hashimoto-Sugimoto et al. 2016; Hörak et al. 2016). HT1 together with MPK12 or MPK4 forms a primary CO₂ sensing complex, where CO₂/bicarbonate triggers the interaction of MPKs with HT1, and this leads to inhibition of the HT1 kinase activity (Takahashi et al. 2022). In our experiments, *mpk12-4* plants had disrupted stomatal response amplitude to the

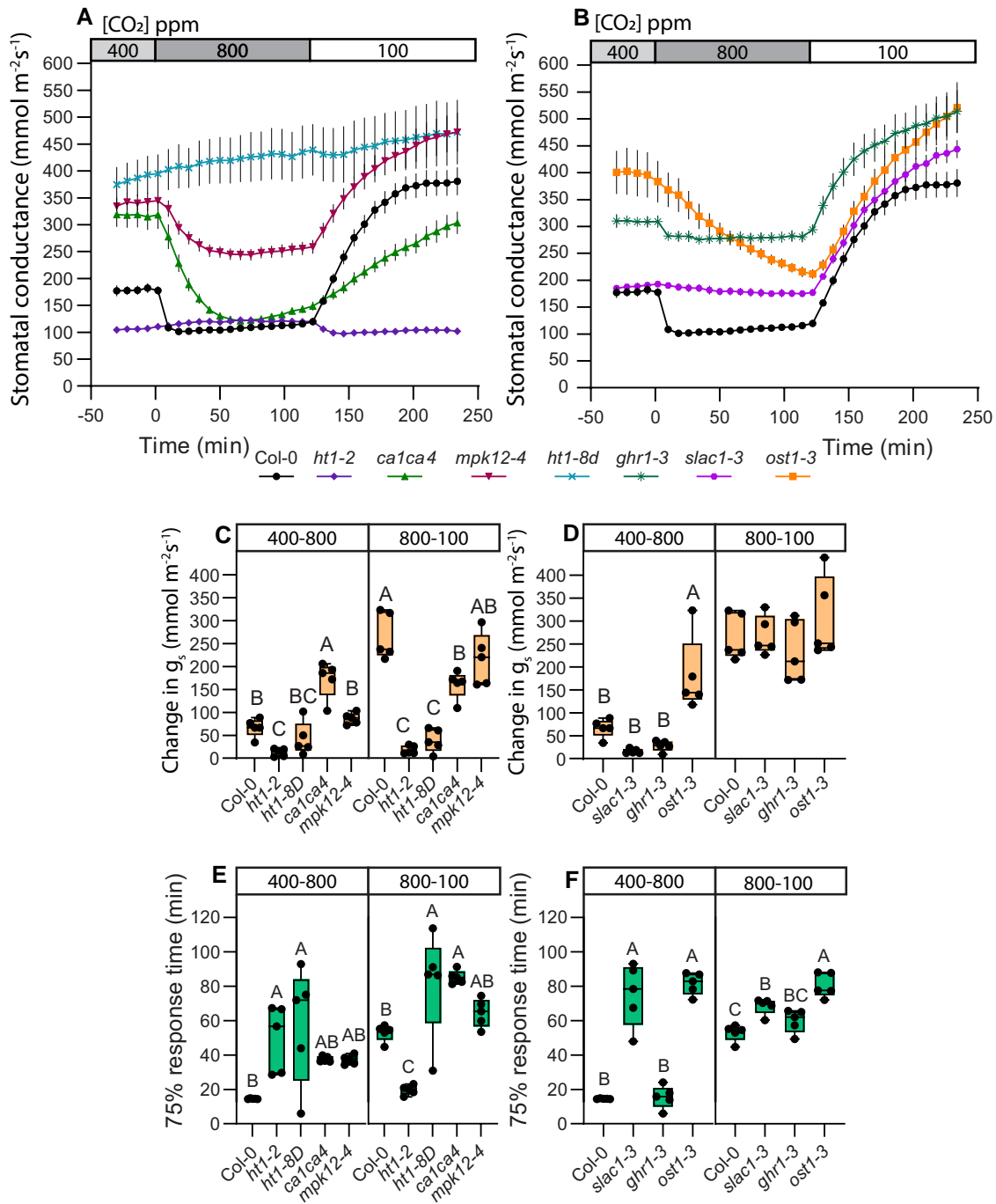


Figure 5. Stomatal opening in 800 to 100 parts per million (ppm) CO₂ transition masks different responses present in sub-ambient and above-ambient CO₂ concentrations. **A and B**) Stomatal response to CO₂ concentration changes from 400 to 800 ppm and 800 to 100 ppm, mean stomatal conductance \pm SEM is shown. **C, D**) Boxplot of stomatal conductance (*g_s*) change (mmol m⁻²s⁻¹) in response to CO₂ concentration changes from 400 to 800 ppm and 800 to 100 ppm, respectively. **E, F**) Boxplot of 75% response time (min) of stomatal response to CO₂ concentration changes from 400 to 800 ppm and 800 to 100 ppm, respectively. **C to F**) Boxes represent 25% to 75% quartiles and median as the horizontal lines, whiskers indicate the smallest and largest values, and points show individual plant values. Statistically significantly different groups are marked with different letters (One-way ANOVA with Tukey post hoc test, *P* < 0.05). **A to F**) Sample size was 5 for all plant lines. VPD during experiments was 0.9 kPa in (**A to F**). Start of the first treatment was between 11:30 and 12:30. Col-0 data are the same as used in Fig. 1, experiments with Col-0 and mutant lines were done together.

100–400 CO₂ transition, while the 400–800 amplitude was unaffected (Fig. 2, A and C). In Töldsepp et al (2018) *mpk12 mpk4GC* double-mutants, where MPK4 expression is suppressed only in guard cells, had no 400–800 CO₂ response, yet *mpk4GC* single-mutants responded to the 400–800 CO₂ transition similar to wild-type plants. Takahashi et al. (2022) showed MPK12 mutants to have the 400–800 response similar to the current study, although neither of these studies tested

the 100–400 response. Thus, it seems that the MPK12/MPK4-HT1 complex largely loses functionality if HT1 is impaired (Fig. 2), same happens if both MPK12 and MPK4 are missing from guard cells (Töldsepp et al. 2018), but losing only MPK12 preferentially affects the 100–400 CO₂ transition (Fig. 2). Therefore, while in the 400–800 transition, the lack of MPK12 is likely compensated by MPK4, and MPK4 cannot effectively replace the function of MPK12 at

Table 1. Stomatal CO₂ responses for the studied mutants from the previously published studies (Hashimoto et al. 2006 [1]; Vahisalu et al. 2008 [2]; Hu et al. 2010 [3]; Xue et al. 2011 [4]; Laanemets et al. 2013 [5]; Merilo et al. 2013 [6]; Hu et al. 2015 [7]; Matrosova et al. 2015 [8]; Hashimoto-Sugimoto et al. 2016 [9]; Hōrak et al. 2016 [10]; Jakobson et al. 2016 [11]; Sierla et al. 2018 [12]; Takahashi et al. 2022 [13]; Yeh et al. 2023 [14]) and from this study

Plant line	Ambient to sub-ambient		Sub-ambient to ambient		Ambient to above-ambient		Sub-ambient to above-ambient		Above-ambient to ambient		Above-ambient to sub-ambient	
	Amplitude	Speed	Amplitude	Speed	Amplitude	Speed	Amplitude	Speed	Amplitude	Speed	Amplitude	Speed
ht1-2	+ _[1,10] /+	+ _[1,10] /+	na/+	na/-	+ _[1,8-10] /+	+ _[1,8-10] /+	+ _[4] /+	+ _[4] /-	na/+	na/-	+ _[8,9] /+	+ _[8,9] /+
ht1-8D	+ _[10] /+	+ _[10] /+	na/+	na/+	+ _[10,13] /+	+ _[10,13] /+	na/+	na/+	na/+	na/+	+ _[13] /+	+ _[13] /+
mpk12-4	+ _[14] /+	+ _[14] /+	na/+	na/-	+ _[11,14] /+	+ _[11,14] /+	na/-	na/+	+ _[14] /+	+ _[14] /+	- _[13] /-	- _[13] /-
ca1ca4	+ _[1,7,8] /+	+ _[3,7,8] /+	na/+	na/+	+ _[3,7] /+	+ _[3,7] /+	+ _[3,7] /-	+ _[3,7] /+	na/+	na/+	+ _[3,7] /+	+ _[3,7] /+
slac1-3	- _[5] /-	- _[5] /-	na/+	na/+	+ _[2,6] /-	+ _[2,6] /-	na/+	na/+	+ _[2] /-	+ _[2] /-	na/-	na/+
ghr1-3	na/-	na/-	na/+	na/-	+ _[12] /+	+ _[12] /+	na/+	na/-	na/+	na/-	na/-	na/-
ost1-3	+ _[4] /-	+ _[4] /+	na/+	na/+	+ _[4,6] /-	+ _[4,6] /+	+ _[4] /+	+ _[4] /+	+ _[6] /+	+ _[6] /+	+ _[4] /-	+ _[4] /+

Published results/our results, “+” means different from wild type and “-” means not different from wild type.

sub-ambient CO₂ levels. This could mean that while MPK12 and MPK4 both can form a CO₂/bicarbonate sensing complex with HT1, their affinity for CO₂/bicarbonate may be different.

CA1 and CA4 also affected stomatal responsiveness more in the 100–400 CO₂ range, with slow and shallow stomatal response in the *ca1ca4* mutant (Fig. 2). The strong stomatal closure in above-ambient 400–800 CO₂ transition in this mutant (Fig. 2, Supplementary Fig. S2, A, C and E) might be related to increased autonomous CO₂ conversion to HCO₃⁻ in the elevated CO₂ environment due to shifting of the reaction balance toward bicarbonate production under above-ambient CO₂ levels or by the involvement of other carbonic anhydrases, as recently demonstrated (Sun et al. 2022).

SLAC1 is important for stomatal closure responses to both elevated CO₂ transitions, but compared with wild type, the response speed of *slac1-3* in the 400–800 transition was more affected than in the 100–400 transition (Fig. 3, A and E). In *slac1-3* plants, there was small in magnitude and slow stomatal closure and opening in the 400–800–400 CO₂ transitions, yet stomatal opening in response to the 400–100 transition was strong and similar in speed to wild-type plants (Supplementary Fig. S2, B, D and F), further supporting the major role of SLAC1 in stomatal responses under ambient to above-ambient CO₂ concentration changes. In the 100–400 CO₂ transition, stomatal responsiveness may be partly compensated by other ion channels that are functional in *slac1-3*. In addition to the S-type anion channels like SLAC1, stomatal closure is also affected by R-type anion channels, such as QUICK-ACTIVATING ANION CHANNEL 1 (QUAC1, Meyer et al. 2010; Imes et al. 2013). Jalakas et al. (2021a) demonstrated that the *quac1-1 slac1-3* double mutant and *quac1-1 slac1-3 slah1-3* triple-mutant had no response to the 400–800 CO₂ transition, while individually *slac1-3* had a weak response and *quac1-1* stomatal response was similar to wild-type plants. SLAC1 HOMOLOGUE 3 (SLAH3) is another S-type anion channel contributing to stomatal closure (Zhang et al. 2016) and could potentially compensate for the lack of SLAC1 in the 100–400 CO₂ response, although it does not affect the 400–800 stomatal CO₂ response (Jalakas et al. 2021a). Future studies should address the 100–400 CO₂ response in mutants deficient in major S- and R-type anion channels to better understand their potential role in the 100–400 CO₂ response.

Stomatal opening triggered by decreased CO₂ levels, from 400 to 100 and from 800 to 100, had larger magnitude than the 800–400 response (Fig. 1, A, C, E, and G). Responses to 100 ppm final CO₂ concentration had a consistently large amplitude and slow response rate in different experimental setups and were not affected by the order of CO₂ treatments. Thus, stomatal opening

in response to sub-ambient CO₂ concentrations is a very prominent response. This is in line with Merilo et al. (2014), where two stimuli with an opposing effect on stomata were simultaneously applied, such as darkness and low CO₂, or low CO₂ and elevated VPD. In such combinations, Arabidopsis stomata always opened in response to sub-ambient CO₂ levels, further indicating that reduction of CO₂ is a strong and prevailing signal. The sub-ambient CO₂-induced stomatal opening, similar to the light-induced opening, likely involves H⁺ ATPase activation (Inoue and Kinoshita 2017), changes in sugar and starch metabolism (Flütsch and Santelia 2021), and suppression of stomatal closure via e.g. inhibiting anion channel activation (Marten et al. 2007). The combined activation of all these processes may explain the slow stomatal opening rate in response to sub-ambient CO₂ levels.

Elevated VPD triggers ABA biosynthesis (McAdam et al. 2016), which can potentially increase stomatal responsiveness to CO₂ due to interactions of CO₂ and ABA signaling pathways (Raschke 1975; Merilo et al. 2013; Chater et al. 2015). In previous experiments, elevated VPD has been shown to either increase stomatal responsiveness to elevated CO₂, potentially via enhanced ABA levels (Bunce 1998), or decrease it, potentially due to reduced stomatal apertures under elevated VPD (Morison and Gifford 1983; Talbott et al. 2003). Our results showed an enhanced stomatal response speed in the 400–100, 100–400, and 800–100 CO₂ concentration transitions but not in the already faster 400–800 transition (Fig. 1, A, B, E, and F). Elevated VPD has been shown to accelerate stomatal opening in light in angiosperms due to reduced back-pressure of epidermal cells on guard cells (Mott et al. 1999; Pichaco et al. 2024), our results of faster stomatal opening in response to sub-ambient [CO₂] under elevated VPD are in line with this (Fig. 1, B and E). Additionally, elevated VPD also accelerated stomatal closure responses to elevated CO₂ levels in the sub-ambient to ambient concentration ranges in wild-type Arabidopsis (Fig. 1B). Faster stomatal closure may be caused by increased ABA levels under elevated VPD conditions (McAdam and Brodribb 2015) or may be explained by the smaller steady-state stomatal conductance caused by smaller stomatal apertures under elevated VPD that can adjust faster in response to environmental changes.

Steady-states of stomatal conductance of all plant lines were decreased by elevated VPD (Figs. 2, A and B, 3, A and B), confirming that VPD is an important factor for steady-state stomatal conductance (Grossiord et al. 2020; López et al. 2021). Mutants with disrupted stomatal ABA response, *ghr1-3*, *ost1-3*, and *slac1-3*, also had lower steady-state stomatal conductances under elevated VPD (Fig. 3, A and B). Their high VPD-induced decrease in steady-

state stomatal conductance could be caused by ABA-independent active processes (e.g. ABA-independent OST1 activation, whose contribution to stomatal closure under high VPD increases in time according to Jalakas et al. (2021b)), or hydropassive stomatal closure. These data indicate that elevated ABA levels alone are not sufficient to explain the decrease of stomatal conductance under elevated VPD conditions, as discussed before (Wang et al. 2017; Merilo et al. 2018; Yaaran et al. 2019). CO₂-induced stomatal responses under elevated VPD were sometimes faster and mostly had a smaller amplitude (Figs. 2, B, D and F, 3, B, D and F). However, the response to the 400–800 CO₂ transition disappeared completely in the *ghr1-3* plants under elevated VPD conditions (Fig. 3, B and D). GHR1 contributes to stomatal closure in response to both elevated VPD (Hsu et al. 2021) and CO₂ (Hörak et al. 2016; Sierla et al. 2018). Thus, CO₂ and VPD responses may interact in *ghr1-3*: if the relatively small elevated VPD-induced stomatal closure already occurred in the *ghr1-3* plants subjected to elevated VPD (Sierla et al. 2018; Hsu et al. 2021), no further response to CO₂ elevation was triggered.

Here we have focused on guard cell-specific stomatal CO₂-signaling components and their different contribution to stomatal closure responses in the sub-ambient to ambient and ambient to above-ambient CO₂ concentration transitions. In addition to these components, it is likely that signal mediators outside guard cells also contribute to different stomatal CO₂-response characteristics under different CO₂ levels. While guard cells in isolated epidermis can respond to elevated CO₂ levels by a decrease in stomatal aperture (Webb et al. 1996; Chater et al. 2015), signals from mesophyll are needed for strong CO₂-induced stomatal closure (Mott et al. 2008; Fujita et al. 2013). Mesophyll processes, e.g. photosynthesis and sugar metabolism, are known to impact stomatal behavior (Lawson and Matthews 2020), and their contribution to different stomatal CO₂-response patterns under different CO₂ concentration ranges merits further study.

Elevated temperatures caused by climate change increase the evaporative demand of the atmosphere manifested as higher VPD levels, which increases transpiration and triggers stomatal closure to avoid wilting. Together, changes in atmospheric VPD and CO₂ levels are among the greatest agricultural challenges of the future, yet how these stimuli together affect plant stomatal behavior is poorly understood. Here, we show that while elevated VPD negatively affects steady-state stomatal conductances, it has little effect on stomatal CO₂-responsiveness. Nevertheless, in some genetic backgrounds, we found an interaction between CO₂ and VPD treatments, indicating that the simultaneous effects of these factors on stomatal behavior merit further study. We also show that stomatal response to elevated CO₂ has different kinetics under sub-ambient and above-ambient CO₂ concentration ranges and its known regulators contribute to a different degree under different CO₂ concentration transitions. Thus, to better understand stomatal responses to CO₂ it is necessary to carefully consider CO₂ levels and experimental setups.

Materials and methods

Plant lines and growth conditions

Arabidopsis (*Arabidopsis thaliana*) accession Col-0 and the following mutants in the same genetic background were used for experiments: *slac1-3* (Vahisalu et al. 2008), *ost1-3* (Yoshida et al. 2002), *ghr1-3* (Sierla et al. 2018), *ht1-2* (Hashimoto et al. 2006), *ht1-8D* (Hörak et al. 2016), *mpk12-4* (Jakobson et al. 2016), and *ca1ca4* (Hu et al. 2010). Plants were grown in 4:2:3 v/v peat:vermiculite:water mixture at 12/12 photoperiod with 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light

in controlled-environment growth cabinets (AR-66LX; Percival Scientific; MCA1600, Snijders Scientific) at 70% relative humidity and day-time temperature of 23 °C (VPD 0.84 kPa) and nighttime temperature 18 °C (VPD 0.62 kPa). Plant age at experiment time was ~25 days.

Gas-exchange measurements

Measurements of stomatal conductance were carried out with a temperature-controlled custom-built gas-exchange device (Kollist et al. 2007; Hörak et al. 2017). Plants were inserted into measurement cuvettes and allowed to acclimate for 1 to 2 h at normal air humidity (VPD, 0.9 kPa) or at lower air humidity (VPD, 2.3 kPa), 24 °C air temperature and 400 ppm CO₂. Experiments with various CO₂ transitions were carried out and in some cases both under normal and at high VPD conditions, as shown in Fig. 1. The first CO₂ treatment was applied approximately at noon (11:30 to 12:30), stomatal conductance was always followed for 2 h under each treatment (Fig. 1).

Data analysis

Magnitude of stomatal closure response was calculated as the absolute difference in stomatal conductance between last time point before treatment and at the end of 2 h of treatment at a given CO₂ level (displayed as “Change in g_s ”). To describe the kinetic characteristics of stomatal responses, we identified the difference between stomatal conductance at the last time point before treatment and at the time point when maximal change in stomatal conductance had occurred for any given response as the total 100% response and calculated the time when 75% of the total stomatal response was achieved (see also illustration in Supplementary Fig. S1). The 75% response time describes the overall response speed across the 2-h treatment time but not the initial kinetics of the stomatal responses. One-way ANOVA with Tukey post hoc test was used for statistical analyses as indicated in the figure legends, and $P < 0.05$ was considered statistically significant. Statistical analyses were carried out using Past 4.0 (Hammer et al. 2001) and Statistica 7.1 (Stat. Soft. Inc).

Accession numbers

AGI accession numbers for genes studied in this article are AT1G12480 (SLAC1), AT3G01500 (CA1), AT1G70410 (CA4), AT2G46070 (MPK12), AT1G62400 (HT1), AT4G33950 (OST1), and AT4G20940 (GHR1).

Supplementary data

The following materials are available in the online version of this article.

Supplementary Figure S1. Scheme explaining the calculation process for 75% stomatal response time using Col-0 and *ghr1-3* ambient to above-ambient CO₂ stomatal responses as an example.

Supplementary Figure S2. Plant stomatal responses to 400–800–400–100 parts per million (ppm) CO₂ concentration changes.

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Conflict of interest statement. None declared.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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