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

Kaspar Koolmeister, Ebe Merilo, Hanna Hõrak, Hannes Kollist

Plant Physiology®

Plant Physiology, 2024, 00, 1–13
https://doi.org/10.1093/plphys/kiae320
Advance access publication 4 June 2024
Research Article

Stomatal CO_2 responses at sub- and above-ambient CO_2 levels employ different pathways in Arabidopsis

Kaspar Koolmeister,[1,2,†] Ebe Merilo,[1] Hanna Hõrak,[1,*,†] and Hannes Kollist[2,3]

[1]Institute of Technology, University of Tartu, Nooruse 1, Tartu 50411, Estonia
[2]Institute of Bioengineering, University of Tartu, Nooruse 1, Tartu 50411, Estonia
[3]Université Paris-Saclay, CNRS, INRAE, Université Evry, Université Paris Cité, Institute of Plant Sciences Paris-Saclay (IPS2), 91190 Gif sur Yvette, France
*Author for correspondence: hanna.horak@ut.ee (H.H.)
†These authors contributed equally.

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.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/General-Instructions) is Hanna Hõrak.

Abstract

Stomatal pores that control plant CO_2 uptake and water loss affect global carbon and water cycles. In the era of increasing atmospheric CO_2 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 CO_2 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 CO_2 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 CO_2-induced stomatal closure have different roles in the sub-ambient and above-ambient CO_2 levels. The CO_2-specific regulators prominently affected sub-ambient CO_2 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 CO_2-induced stomatal closure. Elevated VPD caused lower stomatal conductance in all studied genotypes and CO_2 transitions, as well as faster CO_2-responsiveness in some studied genotypes and CO_2 transitions. Our results highlight the importance of experimental setups in interpreting stomatal CO_2-responsiveness, as stomatal movements under different CO_2 concentration ranges are controlled by distinct mechanisms. Elevated CO_2 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 CO_2 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 CO_2 uptake for photosynthesis. Understanding how stomata respond to changing CO_2 concentrations and increasing VPD is needed for breeding climate-ready crops.

Plant stomata close in response to elevated CO_2 concentration and open in response to decreased CO_2 concentration. Ultimately, elevated CO_2 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). CO_2 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 CO_2 to bicarbonate (HCO_3^-), which can act as a second messenger (Hu et al. 2010). In guard cells, CO_2/HCO_3^- 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 CO_2 (Hashimoto et al. 2006; Hashimoto-Sugimoto et al. 2016; Hõrak 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 (Hõrak et al. 2016; Hiyama et al. 2017; Hayashi et al. 2020). Stomata in HT1-deficient plants do not respond to CO_2 concentration changes while carbonic anhydrase, MPK12 and SLAC1 mutants exhibit impaired stomatal CO_2 responses (Hashimoto et al. 2006; Vahisalu et al. 2008; Hu et al. 2010; Hashimoto-Sugimoto et al. 2016; Hõrak et al. 2016; Jakobson et al. 2016).

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

Received August 29, 2023. Accepted May 12, 2024.

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 CO_2-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 CO_2-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 CO_2 regulation is affected by elevated VPD levels. Previous work in grasses suggests that elevated VPD levels reduce both stomatal conductance and stomatal sensitivity to CO_2 concentration changes (Morison and Gifford 1983) but the interactions of CO_2 and humidity responses in dicots remain poorly understood.

Under light, CO_2 concentration inside the leaf is usually below ambient levels and this causes stomatal opening. Stomata close when CO_2 concentration inside the leaf increases to ambient levels, and an additional rise in CO_2 concentration to above-ambient levels causes further stomatal closure (Brodribb et al. 2009; Hõrak et al. 2017). Thus, stomatal closure response exists within sub-ambient as well as in above-ambient CO_2 levels; however, it is not clear whether these responses are controlled by the same regulators. In some studies, CO_2-induced stomatal closure is defined as the response to an increase in CO_2 concentration from ambient to above-ambient levels (Franks and Britton-Harper 2016; Hõrak et al. 2017), while some studies use a $[CO_2]$ change from sub-ambient to above-ambient levels (Azoulay-Shemer et al. 2015; Chater et al. 2015). Data from previous studies comparing CO_2 responses in ferns and angiosperms suggest that stomatal responses to CO_2 are different, when changing CO_2 levels in the sub-ambient or above-ambient ranges (Brodribb et al. 2009; Hõrak et al. 2017). To address the underlying mechanisms of CO_2-induced stomatal closure at different CO_2 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 CO_2-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 CO_2-induced stomatal closure in sub-ambient and above-ambient CO_2 levels and are also differently affected by VPD.

Results

Stomatal closure kinetics are different between sub-ambient to ambient and ambient to above-ambient $[CO_2]$ transitions

We analyzed stomatal responses to CO_2 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 CO_2 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 CO_2 treatments (Fig. 1, A and E). Experiments were started with ambient (400 ppm) CO_2 concentration and each consecutive CO_2 treatment lasted for 2 h. This approach allowed us to investigate plant stomatal response to different CO_2 transitions—stomatal opening in response to CO_2 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 CO_2 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 CO_2 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 CO_2-induced stomatal closure responses suggest that they could be regulated by different components. Stomatal opening responses to sub-ambient CO_2 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 CO_2 levels, had lower response range and faster 75% response time (Fig. 1, C and D). This suggests that stomatal opening to sub-ambient CO_2 concentrations is a much stronger, albeit slower, response than stomatal opening during the above-ambient to ambient $[CO_2]$ change.

High VPD but not the order of CO_2 transitions affects stomatal CO_2-response kinetics

To study whether high VPD affects CO_2 responses, we conducted the 400–100–400–800 and the 400–800–100 CO_2 transitions under conditions where plants were first acclimatized to increased VPD (2.3 kPa) for ~3 h and then subjected to changes in CO_2 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 CO_2 concentration range was also enhanced under high VPD conditions, resulting in a shorter stomatal 75% response time during the 100–400 ppm $[CO_2]$ 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 $[CO_2]$ 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 CO_2 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 CO_2 levels had an effect on stomatal CO_2-responsiveness, we also applied the CO_2 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 CO_2 concentration transitions on stomatal CO_2-response kinetics.

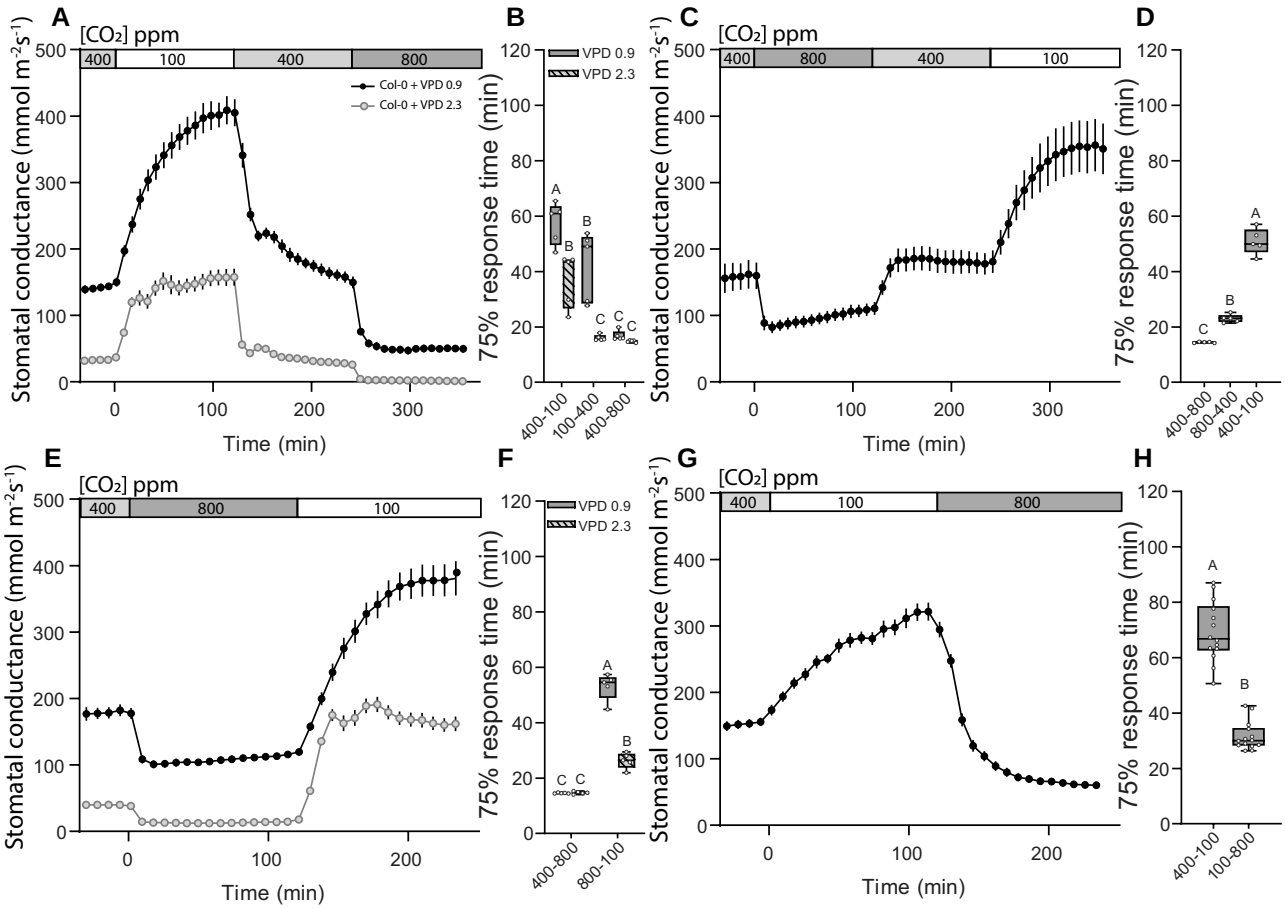

Figure 1. Kinetics of CO_2-induced stomatal responses in wild-type Arabidopsis with regular and elevated vapor pressure deficit (VPD). Col-0 wild-type Arabidopsis stomatal response to sequential changes in CO_2 concentration under regular **(A, C, E, G)** and elevated **(A, E)** VPD conditions. Mean stomatal conductance ± standard error of the mean (SEM) is shown. **B, D, F, H)** Boxplot of 75% response time (min) of stomatal response to CO_2 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 CO_2-specific pathway components MPK12 and carbonic anhydrases are more involved in sub-ambient to ambient than in above-ambient CO_2-induced stomatal closure

To better understand the role of the CO_2-signalling module comprising MPK12, HT1 and carbonic anhydrases CA1 and CA4 in stomatal CO_2 responses at different CO_2 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 CO_2-signalling mutants showed very little stomatal closure in magnitude compared with wild-type plants, whereas 75% response time was significantly reduced only in the *ca1ca4* 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 CO_2 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 *ca1ca4* 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 CO_2 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 CO_2 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 CO_2 concentrations.

Elevated VPD led to lower stomatal conductance in all studied mutants (Fig. 2, A and B), and thus smaller magnitudes of CO_2-induced changes in stomatal conductance (Fig. 2, C and D). Elevated VPD had some effects on the patterns of CO_2-responsiveness in the CO_2-signalling mutants (Fig. 2, A to F). The *ca1ca4* 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 CO_2 ranges was more pronounced under elevated VPD conditions.

Stomatal opening response to 400–100 transition in all of the studied CO_2-signaling 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

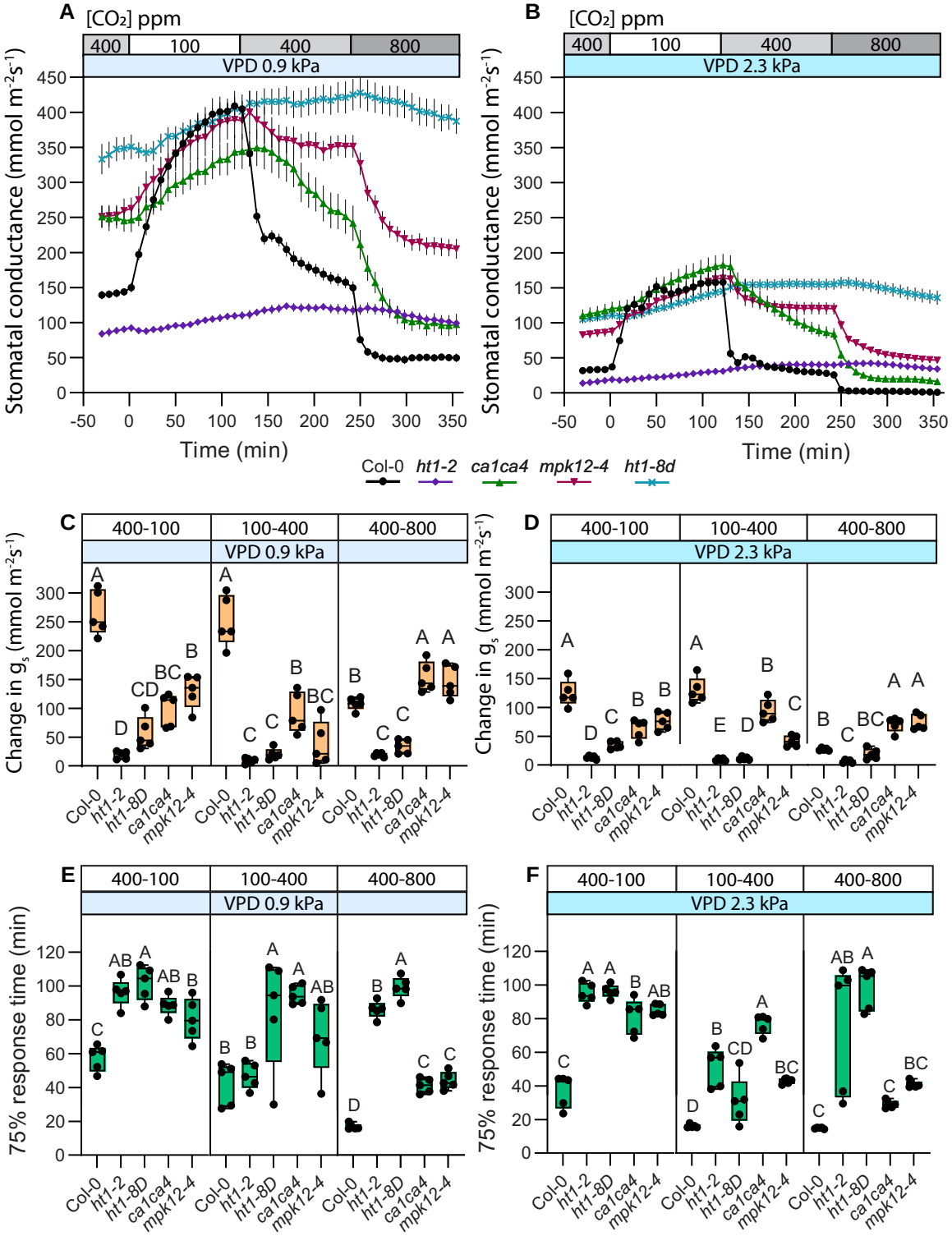

Figure 2. CO_2 pathway mutants retain response patterns under regular and elevated vapor pressure deficit (VPD) conditions. **A and B)** Stomatal response to CO_2 concentration changes from 400 to 100 parts per million (ppm), 100 to 400 ppm, and 400 to 800 ppm under regular **(A)** and elevated **(B)** VPD conditions, mean stomatal conductance ± SEM is shown. **C, D)** Boxplot of stomatal conductance (g_s) change (mmol m^{-2} s^{-1}) in response to CO_2 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 CO_2 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 [CO_2] involves all of the CO_2-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 CO_2-induced stomatal closure

The SLAC1 anion channel is a major component in the activation of stomatal closure response. Thus, we examined CO_2 responses across different CO_2 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 CO_2 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 [CO_2] 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 CO_2-induced stomatal closure.

Plants deficient in SLAC1 activation via OST1 or GHR1 also showed impaired CO_2-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 CO_2 responses in all tested concentration ranges, but GHR1, like SLAC1, appears to contribute more toward above-ambient CO_2-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 CO_2-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 CO_2-induced stomatal opening than the guard cell CO_2-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 CO_2 and VPD signaling in stomatal opening responses.

Shifting CO_2 levels from 100 to 800 ppm masks the differences between stomatal behavior in 100–400 and 400–800 ppm [CO_2] transitions

To further study how stomatal movements differ depending on CO_2 concentrations, we did additional gas-exchange measurements, during which CO_2 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 CO_2 transitions for different mutants that we failed to detect during the 100–800 CO_2 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 CO_2 concentrations that are used for experiments.

Similar to CO_2-induced stomatal closure experiments, [CO_2] 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 CO_2 transitions.

We combined information from previously analyzed CO_2 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 CO_2 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 CO_2 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 CO_2 concentration, which occurs both at sub-ambient and at above-ambient CO_2 concentration ranges, is regulated by both the CO_2-specific and SLAC1-related pathways under all CO_2 ranges, but these pathways have a different degree of importance under different CO_2 concentration ranges. Stomatal closure in response to the 400–800 CO_2 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 CO_2 levels,

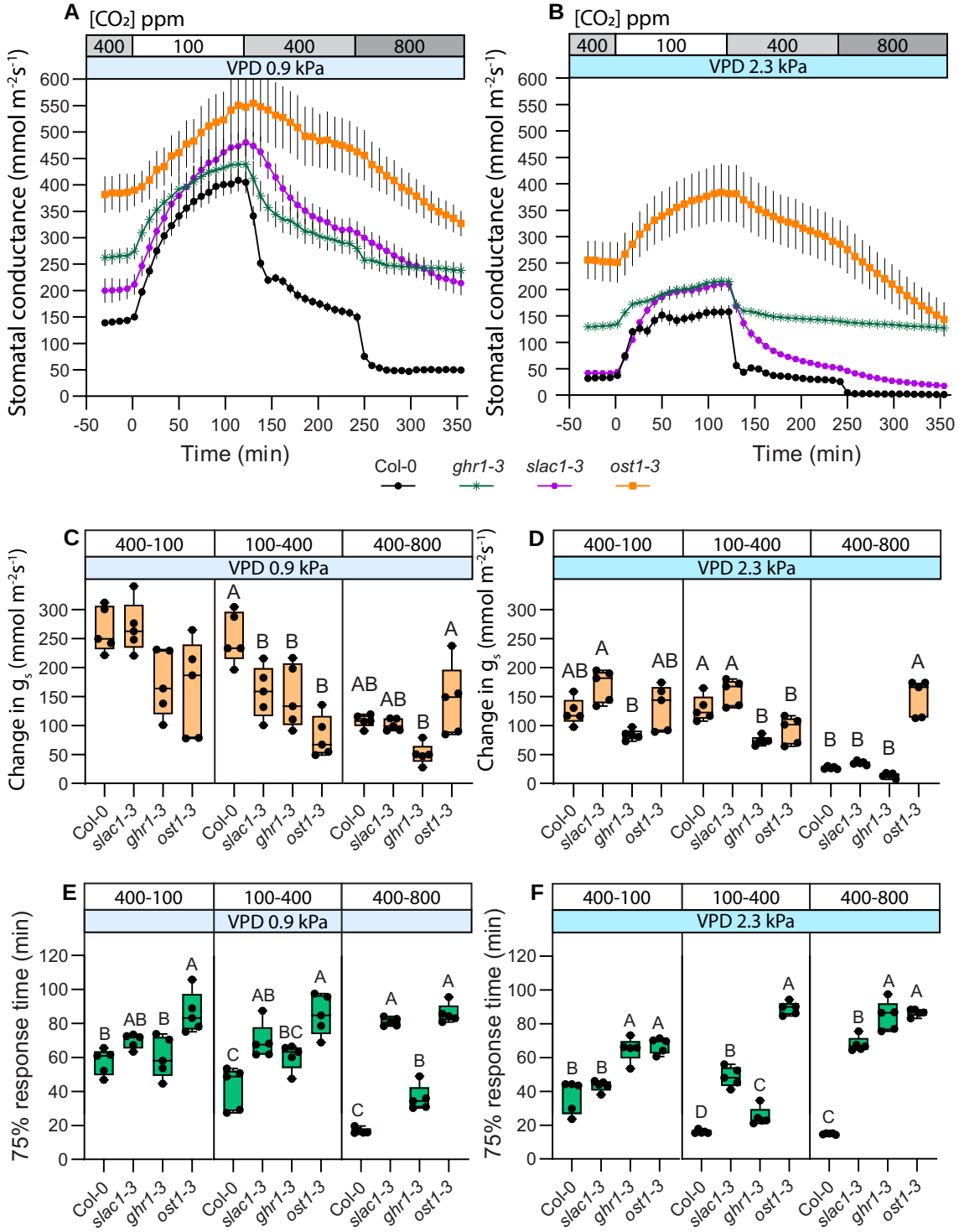

Figure 3. CO_2-response patterns in anion channel activation mutants are affected by vapor pressure deficit (VPD) conditions. **A** and **B)** Stomatal response to CO_2 concentration changes from 400 to 100 parts per million (ppm), 100 to 400 ppm, and 400 to 800 ppm in regular **(A)** and elevated **(B)** VPD conditions, mean stomatal conductance ± SEM is shown. **C, D)** Boxplot of stomatal conductance (g_s) change (mmol m^{-2} s^{-1}) in response to CO_2 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 CO_2 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 CO_2-signalling is also different in the 400–800 and 100–400 stomatal closure responses (Figs. 2 to 3). Increasing CO_2 abruptly from 100 to 800 masked the presence of two

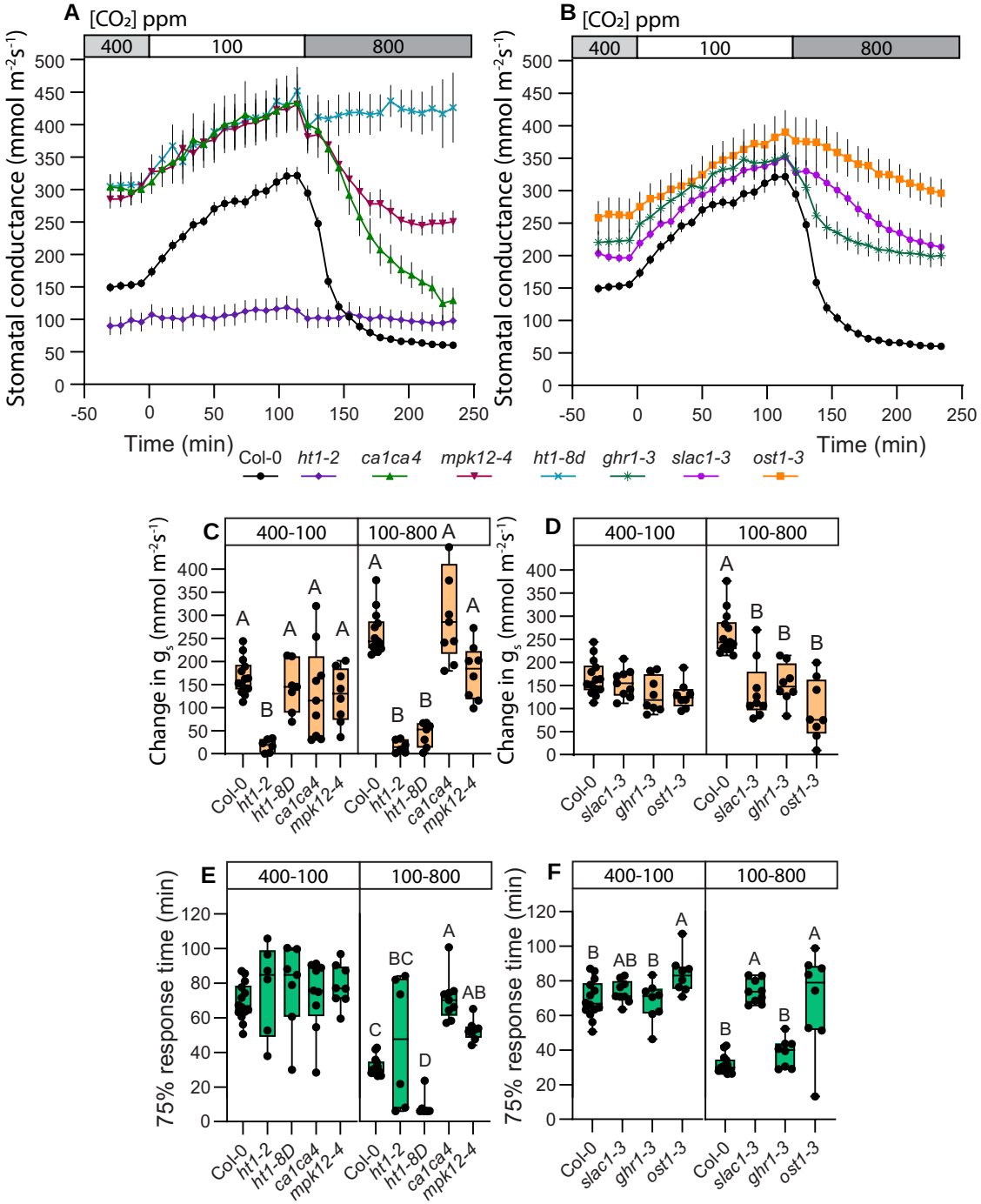

Figure 4. CO_2 transition from 100 to 800 parts per million (ppm) masks different responses present in 100 to 400 and 400 to 800 ppm CO_2 transitions. **A** and **B)** Stomatal response to CO_2 concentration changes from 400 to 100 ppm and 100 to 800 ppm, mean stomatal conductance ± SEM is shown. **C, D)** Boxplot of stomatal conductance (g_s) change (mmol m^{-2} s^{-1}) in response to CO_2 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 CO_2 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 CO_2 levels separately in the ambient to above-ambient and sub-ambient to ambient CO_2 concentration ranges.

The HT1 kinase is required for plant stomatal CO_2 signaling; plants with impaired HT1 function are nearly insensitive to all CO_2 concentration changes (Hashimoto et al. 2006; Hashimoto-Sugimoto et al. 2016; Hõrak et al. 2016). HT1 together with MPK12 or MPK4 forms a primary CO_2 sensing complex, where CO_2/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

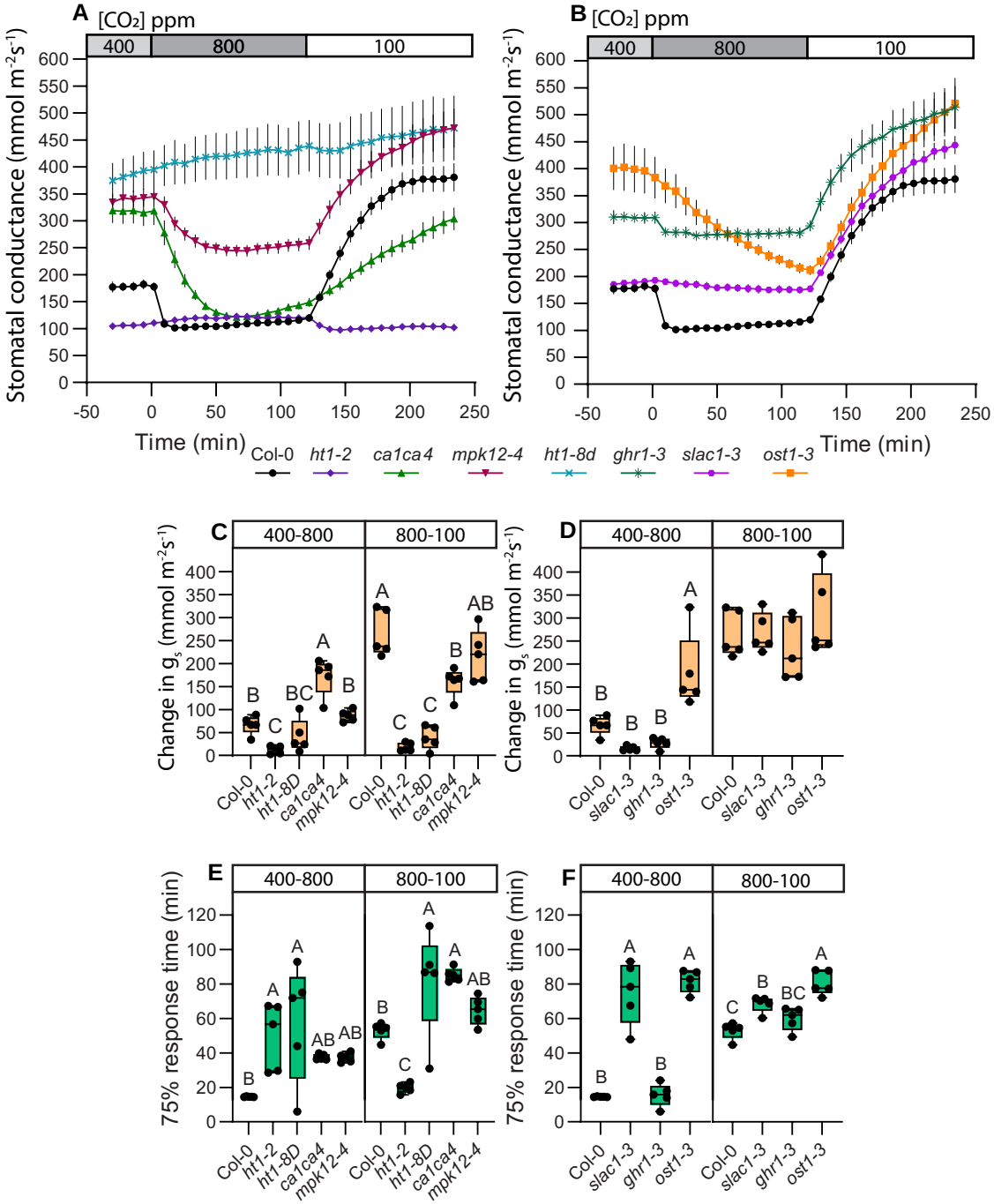

Figure 5. Stomatal opening in 800 to 100 parts per million (ppm) CO_2 transition masks different responses present in sub-ambient and above-ambient CO_2 concentrations. **A and B)** Stomatal response to CO_2 concentration changes from 400 to 800 ppm and 800 to 100 ppm, mean stomatal conductance ± SEM is shown. **C, D)** Boxplot of stomatal conductance (g_s) change (mmol m^{-2} s^{-1}) in response to CO_2 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 CO_2 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 CO_2 transition, while the 400–800 amplitude was unaffected (Fig. 2, A and C). In Tõldsepp et al (2018) *mpk12 mpk4GC* double-mutants, where MPK4 expression is suppressed only in guard cells, had no 400–800 CO_2 response, yet *mpk4GC* single-mutants responded to the 400–800 CO_2 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 (Tõldsepp et al. 2018), but losing only MPK12 preferentially affects the 100–400 CO_2 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 CO_2 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]; Hõrak 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 CO_2 levels. This could mean that while MPK12 and MPK4 both can form a CO_2/bicarbonate sensing complex with HT1, their affinity for CO_2/bicarbonate may be different.

CA1 and CA4 also affected stomatal responsiveness more in the 100–400 CO_2 range, with slow and shallow stomatal response in the *ca1ca4* mutant (Fig. 2). The strong stomatal closure in above-ambient 400–800 CO_2 transition in this mutant (Fig. 2, Supplementary Fig. S2, A, C and E) might be related to increased autonomous CO_2 conversion to HCO_3^- in the elevated CO_2 environment due to shifting of the reaction balance toward bicarbonate production under above-ambient CO_2 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 CO_2 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 CO_2 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 CO_2 concentration changes. In the 100–400 CO_2 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 CO_2 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 CO_2 response, although it does not affect the 400–800 stomatal CO_2 response (Jalakas et al. 2021a). Future studies should address the 100–400 CO_2 response in mutants deficient in major S- and R-type anion channels to better understand their potential role in the 100–400 CO_2 response.

Stomatal opening triggered by decreased CO_2 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 CO_2 concentration had a consistently large amplitude and slow response rate in different experimental setups and were not affected by the order of CO_2 treatments. Thus, stomatal opening in response to sub-ambient CO_2 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 CO_2, or low CO_2 and elevated VPD. In such combinations, Arabidopsis stomata always opened in response to sub-ambient CO_2 levels, further indicating that reduction of CO_2 is a strong and prevailing signal. The sub-ambient CO_2-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 CO_2 levels.

Elevated VPD triggers ABA biosynthesis (McAdam et al. 2016), which can potentially increase stomatal responsiveness to CO_2 due to interactions of CO_2 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 CO_2, 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 CO_2 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 [CO_2] under elevated VPD are in line with this (Fig. 1, B and E). Additionally, elevated VPD also accelerated stomatal closure responses to elevated CO_2 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). CO_2-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 CO_2 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 CO_2 (Hõrak et al. 2016; Sierla et al. 2018). Thus, CO_2 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 CO_2 elevation was triggered.

Here we have focused on guard cell-specific stomatal CO_2-signaling components and their different contribution to stomatal closure responses in the sub-ambient to ambient and ambient to above-ambient CO_2 concentration transitions. In addition to these components, it is likely that signal mediators outside guard cells also contribute to different stomatal CO_2-response characteristics under different CO_2 levels. While guard cells in isolated epidermis can respond to elevated CO_2 levels by a decrease in stomatal aperture (Webb et al. 1996; Chater et al. 2015), signals from mesophyll are needed for strong CO_2-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 CO_2-response patterns under different CO_2 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 CO_2 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 CO_2-responsiveness. Nevertheless, in some genetic backgrounds, we found an interaction between CO_2 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 CO_2 has different kinetics under sub-ambient and above-ambient CO_2 concentration ranges and its known regulators contribute to a different degree under different CO_2 concentration transitions. Thus, to better understand stomatal responses to CO_2 it is necessary to carefully consider CO_2 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* (Hõrak 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 μmol m^{-2} 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; Hõrak 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 CO_2. Experiments with various CO_2 transitions were carried out and in some cases both under normal and at high VPD conditions, as shown in Fig. 1. The first CO_2 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 CO_2 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 CO_2 stomatal responses as an example.

Supplementary Figure S2. Plant stomatal responses to 400–800–400–100 parts per million (ppm) CO_2 concentration changes.

Funding

This work was supported by the Estonian Research Council grant PSG404 to H.H., Basic funding from the Institute of Technology, PRG719, and PRG1620 to E.M. and PRG433 and PRG2004 to H.K.; and Estonian Research Infrastructure Roadmap project TAIM;

and European Regional Development Fund via Center of Excellence in Molecular Cell Engineering.

Conflict of interest statement. None declared.

Data availability

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

References

Azoulay-Shemer T, Palomares A, Bagheri A, Israelsson-Nordstrom M, Engineer CB, Bargmann BOR, Stephan AB, Schroeder JI. Guard cell photosynthesis is critical for stomatal turgor production, yet does not directly mediate CO_2- and ABA-induced stomatal closing. *Plant J*. 2015:83(4):567–581. https://doi.org/10.1111/tpj.12916

Brandt B, Brodsky DE, Xue S, Negi J, Iba K, Kangasjärvi J, Ghassemian M, Stephan AB, Hu H, Schroeder JI. Reconstitution of abscisic acid activation of SLAC1 anion channel by CPK6 and OST1 kinases and branched ABI1 PP2C phosphatase action. *Proc Natl Acad Sci U S A*. 2012:109(26):10593–10598. https://doi.org/10.1073/pnas.1116590109

Brodribb TJ, McAdam SAM, Jordan GJ, Feild TS. Evolution of stomatal responsiveness to CO_2 and optimization of water-use efficiency among land plants. *New Phytol*. 2009:183(3):839–847. https://doi.org/10.1111/j.1469-8137.2009.02844.x

Bunce JA. Effects of humidity on short-term responses of stomatal conductance to an increase in carbon dioxide concentration. *Plant Cell Environ*. 1998:21(1):115–120. https://doi.org/10.1046/j.1365-3040.1998.00253.x

Chater C, Peng K, Movahedi M, Dunn JA, Walker HJ, Liang Y-K, McLachlan DH, Casson S, Isner JC, Wilson I, *et al*. Elevated CO_2-induced responses in stomata require ABA and ABA signaling. *Curr Biol*. 2015:25(20):2709–2716. https://doi.org/10.1016/j.cub.2015.09.013

Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR. Abscisic acid: emergence of a core signaling network. *Annu Rev Plant Biol*. 2010:61(1):651–679. https://doi.org/10.1146/annurev-arplant-042809-112122

Drake PL, Froend RH, Franks PJ. Smaller, faster stomata: scaling of stomatal size, rate of response, and stomatal conductance. *J Exp Bot*. 2013:64(2):495–505. https://doi.org/10.1093/jxb/ers347

Flütsch S, Santelia D. Mesophyll-derived sugars are positive regulators of light-driven stomatal opening. *New Phytol*. 2021:230(5):1754–1760. https://doi.org/10.1111/nph.17322

Franks PJ, Britton-Harper ZJ. No evidence of general CO_2 insensitivity in ferns: one stomatal control mechanism for all land plants? *New Phytol*. 2016:211(3):819–827. https://doi.org/10.1111/nph.14020

Fujita T, Noguchi K, Terashima I. Apoplastic mesophyll signals induce rapid stomatal responses to CO_2 in *Commelina communis*. *New Phytol*. 2013:199(2):395–406. https://doi.org/10.1111/nph.12261

Grossiord C, Buckley TN, Cernusak LA, Novick KA, Poulter B, Siegwolf RTW, Sperry JS, McDowell NG. Plant responses to rising vapor pressure deficit. *New Phytol*. 2020:226(6):1550–1566. https://doi.org/10.1111/nph.16485

Hammer Ø, Harper DAT, Ryan PD. PAST: paleontological statistics software package for education and data analysis. *Palaeontol Electron*. 2001:4(1):9pp. http://palaeo-electronica.org/2001_1/past/issue1_01.htm

Hashimoto-Sugimoto M, Negi J, Monda K, Higaki T, Isogai Y, Nakano T, Hasezawa S, Iba K. Dominant and recessive mutations in the Raf-like kinase HT1 gene completely disrupt stomatal responses to CO_2 in Arabidopsis. *J Exp Bot*. 2016:67(11):3251–3261. https://doi.org/10.1093/jxb/erw134

Hashimoto M, Negi J, Young J, Israelsson M, Schroeder JI, Iba K. Arabidopsis HT1 kinase controls stomatal movements in response to CO_2. *Nat Cell Biol*. 2006:8(4):391–397. https://doi.org/10.1038/ncb1387

Hayashi M, Sugimoto H, Takahashi H, Seki M, Shinozaki K, Sawasaki T, Kinoshita T, Inoue S. Raf-like kinases CBC1 and CBC2 negatively regulate stomatal opening by negatively regulating plasma membrane H^+-ATPase phosphorylation in Arabidopsis. *Photochem Photobiol Sci*. 2020:19(1):88–98. https://doi.org/10.1039/c9pp00329k

Hiyama A, Takemiya A, Munemasa S, Okuma E, Sugiyama N, Tada Y, Murata Y, Shimazaki K. Blue light and CO_2 signals converge to regulate light-induced stomatal opening. *Nat Commun*. 2017:8(1):1284. https://doi.org/10.1038/s41467-017-01237-5

Hõrak H, Kollist H, Merilo E. Fern stomatal responses to ABA and CO_2 depend on species and growth conditions. *Plant Physiol*. 2017:174(2):672–679. https://doi.org/10.1104/pp.17.00120

Hõrak H, Sierla M, Tõldsepp K, Wang C, Wang Y-S, Nuhkat M, Valk E, Pechter P, Merilo E, Salojärvi J, *et al*. A dominant mutation in the HT1 kinase uncovers roles of MAP kinases and GHR1 in CO_2-induced stomatal closure. *Plant Cell*. 2016:28(10):2493–2509. https://doi.org/10.1105/tpc.16.00131

Hsu P-K, Takahashi Y, Merilo E, Costa A, Zhang L, Kernig K, Lee KH, Schroeder JI. Raf-like kinases and receptor-like (pseudo)kinase GHR1 are required for stomatal vapor pressure difference response. *Proc Natl Acad Sci U S A*. 2021:118(47):e2107280118. https://doi.org/10.1073/pnas.2107280118

Hu H, Boisson-Dernier A, Israelsson-Nordström M, Böhmer M, Xue S, Ries A, Godoski J, Kuhn JM, Schroeder JI. Carbonic anhydrases are upstream regulators of CO_2-controlled stomatal movements in guard cells. *Nat Cell Biol*. 2010:12(1):87–93. https://doi.org/10.1038/ncb2009

Hu H, Rappel W-J, Occhipinti R, Ries A, Böhmer M, You L, Xiao C, Engineer CB, Boron WF, Schroeder JI. Distinct cellular locations of carbonic anhydrases mediate carbon dioxide control of stomatal movements. *Plant Physiol*. 2015:169(2):1168–1178. https://doi.org/10.1104/pp.15.00646

Hua D, Wang C, He J, Liao H, Duan Y, Zhu Z, Guo Y, Chen Z, Gong Z. A plasma membrane receptor kinase, GHR1, mediates abscisic acid- and hydrogen peroxide-regulated stomatal movement in *Arabidopsis*. *Plant Cell Online*. 2012:24(6):2546–2561. https://doi.org/10.1105/tpc.112.100107

Imes D, Mumm P, Böhm J, Al-Rasheid KAS, Marten I, Geiger D, Hedrich R. Open stomata 1 (OST1) kinase controls R–type anion channel QUAC1 in Arabidopsis guard cells. *Plant J*. 2013:74(3):372–382. https://doi.org/10.1111/tpj.12133

Inoue S, Kinoshita T. Blue light regulation of stomatal opening and the plasma membrane H^+-ATPase. *Plant Physiol*. 2017:174(2):531–538. https://doi.org/10.1104/pp.17.00166

Jakobson L, Vaahtera L, Tõldsepp K, Nuhkat M, Wang C, Wang Y-S, Hõrak H, Valk E, Pechter P, Sindarovska Y, *et al*. Natural variation in *Arabidopsis* Cvi-0 accession reveals an important role of MPK12 in guard cell CO_2 signaling. *PLoS Biol*. 2016:14(12):e2000322. https://doi.org/10.1371/journal.pbio.2000322

Jalakas P, Nuhkat M, Vahisalu T, Merilo E, Brosché M, Kollist H. Combined action of guard cell plasma membrane rapid- and slow-type anion channels in stomatal regulation. *Plant Physiol*. 2021a:187(4):2126–2133. https://doi.org/10.1093/plphys/kiab202

Jalakas P, Takahashi Y, Waadt R, Schroeder JI, Merilo E. Molecular mechanisms of stomatal closure in response to rising vapour pressure deficit. *New Phytol.* 2021b:232(2):468–475. https://doi.org/10.1111/nph.17592

Katsuhara M, Hanba YT. Barley plasma membrane intrinsic proteins (PIP aquaporins) as water and CO_2 transporters. *Pflüg Arch—Eur J Physiol.* 2008:456(4):687–691. https://doi.org/10.1007/s00424-007-0434-9

Kollist T, Moldau H, Rasulov B, Oja V, Rämma H, Hüve K, Jaspers P, Kangasjärvi J, Kollist H. A novel device detects a rapid ozone-induced transient stomatal closure in intact Arabidopsis and its absence in abi2 mutant. *Physiol Plant.* 2007:129(4):796–803. https://doi.org/10.1111/j.1399-3054.2006.00851.x

Kübarsepp L, Laanisto L, Niinemets Ü, Talts E, Tosens T. Are stomata in ferns and allies sluggish? Stomatal responses to CO_2, humidity and light and their scaling with size and density. *New Phytol.* 2020:225(1):183–195. https://doi.org/10.1111/nph.16159

Laanemets K, Wang Y-F, Lindgren O, Wu J, Nishimura N, Lee S, Caddell D, Merilo E, Brosche M, Kilk K, *et al.* Mutations in the SLAC1 anion channel slow stomatal opening and severely reduce K^+ uptake channel activity via enhanced cytosolic $[Ca^{2+}]$ and increased Ca^{2+} sensitivity of K^+ uptake channels. *New Phytol.* 2013:197(1):88–98. https://doi.org/10.1111/nph.12008

Lawson T, Matthews J. Guard cell metabolism and stomatal function. *Annu Rev Plant Biol.* 2020:71(1):273–302. https://doi.org/10.1146/annurev-arplant-050718-100251

López J, Way DA, Sadok W. Systemic effects of rising atmospheric vapor pressure deficit on plant physiology and productivity. *Glob Change Biol.* 2021:27(9):1704–1720. https://doi.org/10.1111/gcb.15548

Marten H, Hedrich R, Roelfsema MRG. Blue light inhibits guard cell plasma membrane anion channels in a phototropin-dependent manner. *Plant J.* 2007:50(1):29–39. https://doi.org/10.1111/j.1365-313X.2006.03026.x

Matrosova A, Bogireddi H, Mateo-Peñas A, Hashimoto-Sugimoto M, Iba K, Schroeder JI, Israelsson-Nordström M. The HT1 protein kinase is essential for red light-induced stomatal opening and genetically interacts with OST1 in red light and CO_2 -induced stomatal movement responses. *New Phytol.* 2015:208(4):1126–1137. https://doi.org/10.1111/nph.13566

McAdam SAM, Brodribb TJ. The evolution of mechanisms driving the stomatal response to vapor pressure deficit. *Plant Physiol.* 2015:167(3):833–843. https://doi.org/10.1104/pp.114.252940

McAdam SAM, Sussmilch FC, Brodribb TJ. Stomatal responses to vapour pressure deficit are regulated by high speed gene expression in angiosperms. *Plant Cell Environ.* 2016:39(3):485–491. https://doi.org/10.1111/pce.12633

Merilo E, Jõesaar I, Brosché M, Kollist H. To open or to close: species-specific stomatal responses to simultaneously applied opposing environmental factors. *New Phytol.* 2014:202(2):499–508. https://doi.org/10.1111/nph.12667

Merilo E, Laanemets K, Hu H, Xue S, Jakobson L, Tulva I, Gonzalez-Guzman M, Rodriguez PL, Schroeder JI, Broschè M, *et al.* PYR/RCAR receptors contribute to ozone-, reduced air humidity-, darkness-, and CO_2-induced stomatal regulation. *Plant Physiol.* 2013:162(3):1652–1668. https://doi.org/10.1104/pp.113.220608

Merilo E, Yarmolinsky D, Jalakas P, Parik H, Tulva I, Rasulov B, Kilk K, Kollist H. Stomatal VPD response: there is more to the story than ABA. *Plant Physiol.* 2018:176(1):851–864. https://doi.org/10.1104/pp.17.00912

Meyer S, Mumm P, Imes D, Endler A, Weder B, Al-Rasheid KAS, Geiger D, Marten I, Martinoia E, Hedrich R. AtALMT12 represents an R-type anion channel required for stomatal movement in Arabidopsis guard cells. *Plant J.* 2010:63(6):1054–1062. https://doi.org/10.1111/j.1365-313X.2010.04302.x

Morison JIL, Gifford RM. Stomatal sensitivity to carbon dioxide and humidity: a comparison of two C3 and two C4 grass Species. *Plant Physiol.* 1983:71(4):789–796. https://doi.org/10.1104/pp.71.4.789

Mott KA, Shope JC, Buckley TN. Effects of humidity on light-induced stomatal opening: evidence for hydraulic coupling among stomata. *J Exp Bot.* 1999:50(336):1207–1213. https://doi.org/10.1093/jxb/50.336.1207

Mott KA, Sibbernsen ED, Shope JC. The role of the mesophyll in stomatal responses to light and CO_2. *Plant Cell Environ.* 2008:31(9):1299–1306. https://doi.org/10.1111/j.1365-3040.2008.01845.x

Negi J, Matsuda O, Nagasawa T, Oba Y, Takahashi H, Kawai-Yamada M, Uchimiya H, Hashimoto M, Iba K. CO_2 regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. *Nature.* 2008:452(7186):483–486. https://doi.org/10.1038/nature06720

Pichaco J, Manandhar A, McAdam SAM. Mechanical advantage makes stomatal opening speed a function of evaporative demand. *Plant Physiol.* 2024:195(1):370–377. https://doi.org/10.1093/plphys/kiae023

Raschke K. Simultaneous requirement of carbon dioxide and abscisic acid for stomatal closing in *Xanthium strumarium* L. *Planta.* 1975:125(3):243–259. https://doi.org/10.1007/BF00385601

Sierla M, Hõrak H, Overmyer K, Waszczak C, Yarmolinsky D, Maierhofer T, Vainonen JP, Salojärvi J, Denessiouk K, Laanemets K, *et al.* The receptor-like pseudokinase GHR1 is required for stomatal closure. *Plant Cell.* 2018:30(11):2813–2837. https://doi.org/10.1105/tpc.18.00441

Sun P, Isner J-C, Coupel-Ledru A, Zhang Q, Pridgeon AJ, He Y, Menguer PK, Miller AJ, Sanders D, Mcgrath SP, *et al.* Countering elevated CO_2 induced Fe and Zn reduction in *Arabidopsis* seeds. *New Phytol.* 2022:235(5):1796–1806. https://doi.org/10.1111/nph.18290

Takahashi Y, Bosmans KC, Hsu P-K, Paul K, Seitz C, Yeh C-Y, Wang Y-S, Yarmolinsky D, Sierla M, Vahisalu T, *et al.* Stomatal CO_2/bicarbonate sensor consists of two interacting protein kinases, Raf-like HT1 and non-kinase-activity activity requiring MPK12/MPK4. *Sci Adv.* 2022:8(49):eabq6161. https://doi.org/10.1126/sciadv.abq6161

Talbott LD, Rahveh E, Zeiger E. Relative humidity is a key factor in the acclimation of the stomatal response to CO_2. *J Exp Bot.* 2003:54-(390):2141–2147. https://doi.org/10.1093/jxb/erg215

Tõldsepp K, Zhang J, Takahashi Y, Sindarovska Y, Hõrak H, Ceciliato PHO, Koolmeister K, Wang Y-S, Vaahtera L, Jakobson L, *et al.* Mitogen-activated protein kinases MPK4 and MPK12 are key components mediating CO_2-induced stomatal movements. *Plant J.* 2018:96(5):1018–1035. https://doi.org/10.1111/tpj.14087

Vahisalu T, Kollist H, Wang Y-F, Nishimura N, Chan W-Y, Valerio G, Lamminmäki A, Brosché M, Moldau H, Desikan R, *et al.* SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. *Nature.* 2008:452(7186):487–491. https://doi.org/10.1038/nature06608

Wang Y, Hills A, Vialet-Chabrand S, Papanatsiou M, Griffiths H, Rogers S, Lawson T, Lew VL, Blatt MR. Unexpected connections between humidity and Ion transport discovered using a model to bridge guard cell-to-leaf scales. *Plant Cell.* 2017:29(11):2921–2939. https://doi.org/10.1105/tpc.17.00694

Wang C, Hu H, Qin X, Zeise B, Xu D, Rappel W-J, Boron WF, Schroeder JI. Reconstitution of CO_2 regulation of SLAC1 anion channel and function of CO_2-permeable PIP2; 1 aquaporin as CARBONIC

ANHYDRASE4 interactor. *Plant Cell*. 2016:28(2):568–582. https://doi.org/10.1105/tpc.15.00637

Webb AAR, McAinsh MR, Mansfield TA, Hetherington AM. Carbon dioxide induces increases in guard cell cytosolic free calcium. *Plant J*. 1996:9(3):297–304. https://doi.org/10.1046/j.1365-313X.1996.09030297.x

Xue S, Hu H, Ries A, Merilo E, Kollist H, Schroeder JI. Central functions of bicarbonate in S-type anion channel activation and OST1 protein kinase in CO_2 signal transduction in guard cell. *EMBO J*. 2011:30(8):1645–1658. https://doi.org/10.1038/emboj.2011.68

Yaaran A, Negin B, Moshelion M. Role of guard-cell ABA in determining steady-state stomatal aperture and prompt vapor-pressure-deficit response. *Plant Sci*. 2019:281:31–40. https://doi.org/10.1016/j.plantsci.2018.12.027

Yeh C-Y, Wang Y-S, Takahashi Y, Kuusk K, Paul K, Arjus T, Yadlos O, Schroeder JI, Ilves I, Garcia-Sosa AT, *et al.* MPK12 in stomatal CO_2 signaling: function beyond its kinase activity. *New Phytol*. 2023:239(1):146–158. https://doi.org/10.1111/nph.18913

Yoshida R, Hobo T, Ichimura K, Mizoguchi T, Takahashi F, Aronso J, Ecker JR, Shinozaki K. ABA-Activated SnRK2 protein kinase is required for dehydration stress signaling in *Arabidopsis*. *Plant Cell Physiol*. 2002:43(12):1473–1483. https://doi.org/10.1093/pcp/pcf188

Yuan W, Zheng Y, Piao S, Ciais P, Lombardozzi D, Wang Y, Ryu Y, Chen G, Dong W, Hu Z, *et al.* Increased atmospheric vapor pressure deficit reduces global vegetation growth. *Sci Adv*. 2019:5(8):eaax1396. https://doi.org/10.1126/sciadv.aax1396

Zhang A, Ren H-M, Tan Y-Q, Qi G-N, Yao F-Y, Wu G-L, Yang L-W, Hussain J, Sun S-J, Wang Y-F. S-Type anion channels SLAC1 and SLAH3 function as essential negative regulators of inward K^+ channels and stomatal opening in *Arabidopsis*. *Plant Cell*. 2016:28(4):949–965. https://doi.org/10.1105/tpc.15.01050