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

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Running head: CO_2 concentration-dependent stomatal CO_2 responses

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. It is unknown whether stomatal responses to sub-ambient and above-ambient CO_2 levels are governed by the same regulators and whether these responses depend on VPD. We studied stomatal conductance responses in Arabidopsis 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 SLAC1 more strongly affected the speed of above-ambient CO_2-induced stomatal closure. Elevated VPD caused lower stomatal conductance in all and faster CO_2-responsiveness in some studied genotypes and CO_2-transitions. Our results highlight the importance of experimental set-ups in interpreting stomatal CO_2-responsiveness, as stomatal movements under different CO_2 concentration

ranges are controlled by distinct mechanisms. Sometimes elevated CO_2 and VPD responses also interact. Hence, multi-factor treatments are needed to understand plant behavior under future climate conditions.

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 S-type anion channel SLAC1, which causes rapid stomatal closure (Vahisalu et al., 2008; Negi et al., 2008). CO_2 can enter guard cells through the PIP2 plasma membrane channel or by transmembrane diffusion (Katsuhara and Hanba, 2008; Wang et al., 2016). Carbonic anhydrases CA1 and 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 MPK4/MPK12 and the Raf-like kinase 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 CBC1/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

62 impaired stomatal CO_2 responses (Hashimoto et al., 2006; Vahisalu et al., 2008; Hu et
63 al., 2010; Hashimoto-Sugimoto et al., 2016; Hõrak et al., 2016; Jakobson et al., 2016).

64 Elevated VPD increases transpiration and reduces epidermal turgor that due to
65 mechanical interactions between guard and epidermal cells in angiosperms leads to
66 faster light-induced stomatal opening (Mott et al., 1999; Pichaco et al., 2024). To
67 prevent wilting, stomata close under elevated VPD. Abscisic acid (ABA) is a drought-
68 induced plant stress hormone and an important stomatal regulator. VPD has a direct
69 effect on ABA concentration as increased ABA levels in angiosperms were observed
70 20 minutes after increasing VPD from 0.7 to 1.5 kPa (McAdam and Brodribb, 2015).
71 Considering that ABA has a major role in angiosperm stomatal closure (Cutler et al.,
72 2010), elevated VPD might prime plant stomata for fast closure via increased ABA
73 concentration. The protein kinase OST1 and the leucine-rich receptor-like
74 pseudokinase GHR1 are activated in the presence of ABA and trigger anion efflux
75 through the major guard cell slow-type anion channel SLAC1 (Brandt et al., 2012; Hua
76 et al., 2012; Sierla et al., 2018). OST1 and GHR1 are both involved in elevated VPD-
77 and CO_2-induced stomatal closure response (Xue et al., 2011; Merilo et al., 2018;
78 Sierla et al., 2018; Hsu et al., 2021; Jalakas et al., 2021b).

79 Understanding CO_2-induced plant stomatal closure responses is essential for future
80 plant breeding. Due to changing climate conditions, it is also important to understand
81 if and how stomatal CO_2 regulation is affected by elevated VPD levels. Previous work
82 in grasses suggests that elevated VPD levels reduce both stomatal conductance and
83 stomatal sensitivity to CO_2 concentration changes (Morison and Gifford, 1983) but the
84 interactions of CO_2 and humidity responses in dicots remain poorly understood.

85 Under light, CO_2 concentration inside the leaf is usually below ambient levels and this
86 causes stomatal opening. Stomata close when CO_2 concentration inside the leaf
87 increases to ambient levels, and an additional rise in CO_2 concentration to above-
88 ambient levels causes further stomatal closure (Brodribb et al., 2009; Hõrak et al.,
89 2017). Thus, stomatal closure response exists within sub-ambient as well as in above-
90 ambient CO_2 levels, however it is not clear whether these responses are controlled by
91 the same regulators. In some studies, CO_2-induced stomatal closure is defined as the
92 response to an increase in CO_2 concentration from ambient to above-ambient levels
93 (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 suggests 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*, *ost1-3*) or in the CO_2-specific stomatal signaling branch regulated by MPK12 and HT1 kinases (*mpk12-4*, *ht1-2*, *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.

Materials and Methods

Plant lines and growth conditions

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), *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 night-time 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 1-2 hours 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

124 Fig. 1. The first CO_2 treatment was applied approximately at noon (11:30 to 12:30),
125 stomatal conductance was always followed for 2 hours under each treatment (Fig. 1).

126 ***Data analysis***

127 Magnitude of stomatal closure response was calculated as the absolute difference in
128 stomatal conductance between last time point before treatment and at the end of 2
129 hours of treatment at a given CO_2 level (displayed as 'Change in g_s'). To describe the
130 kinetic characteristics of stomatal responses, we calculated the 75% response time by
131 scaling the two-hour stomatal responses to a range from 0% to 100% and by
132 calculating the time when 75% of the total stomatal response was achieved. One-way
133 ANOVA with Tukey *post hoc* test was used for statistical analysis as indicated in the
134 figure legends, $p < 0.05$ were considered statistically significant. Statistical analyses
135 were carried out with Past 4.0 (Hammer et al., 2001) and Statistica 7.1 (Stat. Soft. Inc).

136 **Results**

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

139 We analyzed stomatal responses to CO_2 in the sub-ambient and above-ambient
140 concentration ranges in the model plant *Arabidopsis thaliana* to clarify whether these
141 responses are controlled by the same or by different regulators. Four different CO_2
142 transition sequences were used (Fig. 1A,C,E,G); in two set-ups we applied high VPD
143 (2.3 kPa) as an additional factor before and throughout CO_2 treatments (Fig. 1A,E).
144 Experiments were started with ambient (400ppm) CO_2 concentration and each
145 consecutive CO_2 treatment lasted for 2 hours. This approach allowed to investigate
146 plant stomatal response to different CO_2 transitions – stomatal opening in response to
147 CO_2 transition from 400 to 100 ppm (from here on referred to as 400-100) (Fig.
148 1A,C,G), from 800 to 400 ppm (800-400) (Fig. 1C), or from 800 to 100 ppm (800-100)
149 (Fig. 1E), and stomatal closure in response to CO_2 transition from 100 to 400 ppm
150 (100-400) (Fig. 1A), from 400 to 800 ppm (400-800) (Figure 1A,C,E), or from 100 to
151 800 ppm (100-800) (Fig. 1G).

152 Stomatal closure responses had clearly different 75% response time in Col-0 wild-type
153 plants at different CO_2 transitions: the 400-800 stomatal closure was faster than 100-
154 400 closure under normal VPD (Fig. 1A,B). The rapid 400-800 response was

155 consistent throughout all experimental setups (Fig. 1A-F). The observed different
156 kinetics of these CO_2-induced stomatal closure responses suggest that they could be
157 regulated by different components. Stomatal opening responses to sub-ambient CO_2
158 levels, 400-100 (Fig. 1A-D,G,H) and 800-100 (Fig. 1E,F) had relatively slow 75%
159 response times, whereas the 800-400 response, opening from above-ambient to
160 ambient CO_2 levels, had lower response range and faster 75% response time (Fig.
161 1C,D). This suggests that stomatal opening to sub-ambient CO_2 concentrations is a
162 much stronger, albeit slower, response than stomatal opening during the above-
163 ambient to ambient [CO_2] change.

164 **High VPD but not the order of CO_2 transitions affects stomatal CO_2 response**
165 **kinetics**

166 To study whether high VPD affects CO_2 responses, we conducted the 400-100-400-
167 800 and the 400-800-100 CO_2 transitions under conditions where plants were first
168 acclimatized to increased VPD (2.3 kPa) for approximately three hours and then
169 subjected to changes in CO_2 levels under the elevated VPD conditions. Under such
170 conditions, both the 400-100 and 800-100 stomatal opening responses were
171 significantly faster than under normal VPD (Fig. 1A,B E,F). Stomatal closure in sub-
172 ambient to ambient CO_2 concentration range was also enhanced under high VPD
173 conditions, resulting in shorter stomatal 75% response time during the 100-400 ppm
174 [CO_2] transition compared with normal VPD (Fig. 1A,B), whereas 400-800 ppm [CO_2]
175 transition 75% response time was not affected by high VPD (Fig. 1A,B,E,F). These
176 results suggest that during high VPD stress, plant stomata could be primed for faster
177 movements in the sub-ambient to ambient CO_2 concentration range, but VPD has a
178 minor role in above-ambient CO_2-induced stomatal closure.

179 In our first experiments, the 400-100 opening stimulus was applied before the 400-800
180 closure. To test whether the exposure to sub-ambient CO_2 levels had an effect on
181 stomatal CO_2-responsiveness, we also applied the CO_2 transitions in reverse order
182 (400-800-400-100, Fig. 1C). Stomatal 75% response times during the 400-100
183 transition appeared slightly shorter when it was the last transition (Fig. 1C,D) than when
184 it was the first (Fig. 1A,B,G,H), whereas the 400-800 transition response speed was
185 unaffected by previous treatments (Fig. 1A,B,C-F). Thus, there were no major effects
186 of the order of CO_2 concentration transitions on stomatal CO_2-response kinetics.

187 **The CO_2-specific pathway components MPK12 and carbonic anhydrases are**
188 **more involved in sub-ambient to ambient than in above-ambient CO_2-induced**
189 **stomatal closure**

190 To better understand the role of the CO_2-signalling module comprising MPK12, HT1
191 and carbonic anhydrases CA1 and CA4 in stomatal CO_2 responses at different CO_2
192 levels, we analyzed the *mpk12-4*, *ht1-2*, *ht1-8D*, and *ca1a4* in a similar experimental
193 set-up as described for wild-type plants in Fig. 1A. All the CO_2-signalling mutants
194 showed very little stomatal closure in magnitude compared with wild-type plants,
195 whereas 75% response time was significantly reduced only in the *ca1ca4* mutant in
196 response to the 100-400 transition (Fig. 2A,C,E). The 75% response times for the *ht1-*
197 *2* and *ht1-8D* mutants in this CO_2 transition are not informative due to hardly any
198 stomatal response in these mutants (Fig 2A,C,E). The response of *mpk12-4* to the 100-
199 400 transition was as fast as in wild-type, but significantly smaller in magnitude (Fig.
200 2A,C,E). In response to the 400-800 transition, the HT1 mutants had very weak
201 stomatal response with small magnitude and slow speed, whereas stomatal closure in
202 the *mpk12-4* and *ca1ca4* plants was slower than in WT, but larger in magnitude (Fig.
203 2A,C,E). Very weak responses of the HT1 mutants suggest that HT1 is necessary to
204 initiate stomatal responses to CO_2 concentration changes in both ambient and sub-
205 ambient levels. MPK12 and carbonic anhydrase mutants display stronger stomatal
206 responses in the above-ambient than sub-ambient CO_2 range (Fig. 2A,C,E),
207 suggesting that respective signaling components have a more important role at sub-
208 ambient compared with above-ambient CO_2 concentrations.

209 Elevated VPD led to lower stomatal conductance in all studied mutants (Fig. 2A,B),
210 and thus smaller magnitudes of CO_2-induced changes in stomatal conductance (Fig.
211 2C,D). Elevated VPD had some effects on the patterns of CO_2-responsiveness in the
212 CO_2-signalling mutants (Fig 2A-F). The *ca1ca4* and *mpk12-4* stomatal response times
213 were similar to WT in the 400-800 transition under elevated VPD (Fig. 2F), and the
214 *mpk12-4* mutant had a significantly slower than wild-type response to the 100-400
215 transition only under elevated VPD. Thus, the relatively larger degree of importance of
216 MPK12 and carbonic anhydrases in regulating stomatal responses in the sub-ambient
217 CO_2 ranges was more pronounced under elevated VPD conditions.

218 Stomatal opening response to 400-100 transition in all of the studied CO_2-signalling
219 mutants was lower in magnitude and slower in response time, irrespective of VPD,
220 whereas the difference in response time between wild-type and mutants was larger
221 under elevated VPD (Fig. 2A-F). Thus, stomatal opening response to sub-ambient
222 [CO_2] is affected by all of the CO_2 signaling pathway components represented in this
223 study.

224 **SLAC1 and GHR1 are more important for above-ambient CO_2-induced stomatal**
225 **closure**

226 The SLAC1 anion channel is a major component in the activation of stomatal closure
227 response. Thus, we examined CO_2 responses across different CO_2 concentration
228 ranges in plants deficient in the SLAC1 anion channel (*slac1-3*) and in OST1 or GHR1:
229 proteins involved in SLAC1 activation (*ost1-3*, *ghr1-3*, Fig. 3). Stomatal 75% response
230 time of *slac1-3* plants was longer in both, 100-400 and 400-800 CO_2 transitions (Fig.
231 3A,E) but the latter was more affected as the 75% response time difference compared
232 with wild-type plants was notably larger in the 400-800 transition. Yet, the magnitude
233 of stomatal closure between wild-type Col-0 and *slac1-3* was only different in the 100-
234 400 transition (Fig. 3C), while their 400-800 response magnitude was similar. Under
235 elevated VPD, *slac1-3* stomatal response amplitude was similar to wild-type both
236 during the 100-400 and 400-800 transitions, although stomatal response times of
237 *slac1-3* were still significantly longer than wild-type on both transitions (Fig. 2D,F).
238 Together, these results show that SLAC1 is important in stomatal closure in both sub-
239 ambient and above-ambient [CO_2] ranges, but response speed tends to be more
240 severely impacted in the 400-800 transition, suggesting a more prominent role for
241 SLAC1 in ensuring fast above-ambient CO_2-induced stomatal closure.

242 Plants deficient in SLAC1 activation via OST1 or GHR1 also showed impaired CO_2-
243 induced stomatal closure both in the 100-400 and 400 to 800 transitions (Fig. 3A,C,E).
244 The *ost1-3* mutant had long stomatal response times in both the 100-400 and 400-800
245 transitions, whereas the *ghr1-3* response was similar to wild-type in the 100-400
246 transition, but slower and very small in magnitude during the 400-800 transition (Fig.
247 3A,C,E). Thus, both SLAC1-activating proteins are involved in CO_2 responses in all
248 tested concentration ranges, but GHR1, like SLAC1, appears to contribute more
249 towards above-ambient CO_2-induced stomatal closure.

250 As in other mutants (Fig. 2), elevated VPD lowered stomatal conductance and stomatal
251 response magnitude, but tended to increase stomatal opening speed (Fig. 3). The
252 *ost1-3* 75% response times and magnitude remained similar in both VPD conditions,
253 in line with its VPD-insensitivity (Fig 3C-F, Merilo et al., 2013). In *ghr1-3*, stomatal
254 response to 400-800 was either absent or extremely weak under elevated VPD (Fig.
255 3B,D).

256 Sub-ambient CO_2-induced stomatal opening response magnitude was similar to wild-
257 type in all of the studied anion channel activation mutants, irrespective of VPD (Fig
258 3C,D). Stomatal 75% response time under regular VPD was similar to wild-type in all
259 but *ost1-3* (Fig. 3E), whereas under elevated VPD conditions both *ghr1*-3 and *ost1*-3
260 had longer stomatal opening 75% response times (Fig. 3F). Therefore, regulation of
261 SLAC1 is less important for sub-ambient CO_2-induced stomatal opening than the guard
262 cell CO_2-specific signaling pathway. Only under elevated VPD, the 400-100 transition
263 response was slower compared to wild-type in *ghr1-3* (Fig. 3F), indicating an
264 interaction between CO_2 and VPD signaling in stomatal opening responses.

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

267 To further study how stomatal movements differ depending on CO_2 concentrations, we
268 did additional gas exchange measurements, during which CO_2 concentration was
269 changed directly from sub-ambient (100 ppm) to above-ambient (800 ppm). Previously,
270 in Fig. 2 and 3 we saw different stomatal response characteristics for 100-400 and 400-
271 800 CO_2 transitions for different mutants that we failed to detect during the 100-800
272 CO_2 transition (Fig. 4). For example, *mpk12-4* stomatal response was small in
273 amplitude in the 100-400 transition, but much larger in the 400-800 transition (Fig.
274 2A,C), whereas in the 100-800 transition the *mpk12-4* stomatal response amplitude
275 was similar to wild-type (Fig. 4A). Similarly, in the *ca1ca4* plants 100-400 stomatal
276 response was smaller and 400-800 response larger than in wild-type in amplitude (Fig.
277 2C), yet the 100-800 response magnitude was similar to wild-type (Fig. 4C). The *ghr1-*
278 *3* mutant had slower response to the 400-800 transition (Fig. 3E), whereas its 75%
279 response time was similar to wild-type in the 100-800 transition (Fig. 4F). These results
280 demonstrate that differences in stomatal responses between plant lines can remain
281 undiscovered depending on CO_2 concentrations that are used for experiments.

282 Similar to CO_2-induced stomatal closure experiments, [CO_2] ranges were important
283 also in stomatal opening assays (Fig. 2-3 vs Fig. 5). For example, response to the 400-
284 100 transition in *mpk12-4* was slower and smaller in magnitude compared with wild-
285 type plants (Fig 2C,E), but in the 800-100 experiments, *mpk12-4* had normal response
286 amplitude and wild-type-like 75% response time (Fig. 5C,E). The *slac1-3* plants had
287 similar to wild-type 400-100 stomatal opening speed and magnitude (Fig. 3C,E), but
288 were slower in the 800-100 response (Fig. 5F). We also observed a significantly slower
289 opening response to the 800-400 transition in *slac1-3* (Supplementary Fig. 1F), which
290 indicates that the reduced stomatal opening speed of the 800-100 response in this
291 mutant is caused by slower opening in the 800-400 range. Thus, although wild-type
292 plants have no discernible differences between the 400-100 and the 800-100 stomatal
293 opening responses (Fig. 1A,B,E,F), the molecular mechanisms are at least partly
294 different for these CO_2 transitions.

295 We combined information from previously analyzed CO_2 transitions in the mutants
296 used in our study with our results (Table 1). Our findings mostly confirm previous
297 results, where available, with the exception of some differences in response amplitude
298 in the *slac1-3*, *ost1-3,* and *ca1ca4* mutants that can be explained by longer treatment
299 duration in this work that allowed the slower stomatal responses of these mutants to
300 reach amplitudes similar to wild-type plants. We also found faster stomatal response
301 to sub-ambient to above-ambient CO_2 transition in the *ht1-2*, potentially explained by
302 different parameters used to describe response speed in different studies. Our
303 experiments add new information on the ambient to sub-ambient, above-ambient to
304 ambient, sub-ambient to above-ambient, above-ambient to sub-ambient and sub-
305 ambient to ambient CO_2 transitions that have not been systematically addressed
306 before in all the mutants analyzed here.

307 **Discussion**

308 Here we show that stomatal closure in response to an increase in CO_2 concentration,
309 which occurs both at sub-ambient and above-ambient CO_2 concentration ranges, is
310 regulated by both the CO_2-specific and SLAC1-related pathways under all CO_2 ranges,
311 but these pathways have a different degree of importance under different CO_2
312 concentration ranges. Stomatal closure in response to the 400-800 CO_2 concentration
313 range is faster (Fig. 1B), while the 100-400 response has greater amplitude (Fig. 1A,

314 2C). These differences may be explained by higher guard cell volume and larger
315 stomatal apertures under lower CO_2 levels, leading to slower responses with larger
316 amplitudes, similar to slower responses of larger stomata (Drake et al., 2013;
317 Kübarsepp et al., 2020). However, the relative contribution of the components involved
318 in stomatal CO_2-signalling is also different in the 400-800 and 100 to 400 stomatal
319 closure responses (Figures 2-3). Increasing CO_2 abruptly from 100 to 800 masked the
320 presence of two processes with different kinetics (Figures 2, 3 and 4), indicating the
321 necessity to analyze stomatal closure responses to elevated CO_2 levels separately in
322 the ambient to above-ambient and sub-ambient to ambient CO_2 concentration ranges.

323 The HT1 kinase is required for plant stomatal CO_2 signaling; plants with impaired HT1
324 function are nearly insensitive to all CO_2 concentration changes (Hashimoto et al.,
325 2006; Hashimoto-Sugimoto et al., 2016; Hõrak et al., 2016). HT1 together with MPK12
326 or MPK4 forms a primary CO_2 sensing complex, where CO_2/bicarbonate triggers
327 interaction of MPKs with HT1 and this leads to inhibition of the HT1 kinase activity
328 (Takahashi *et al.*, 2022). In our experiments, *mpk12-4* plants had disrupted stomatal
329 response amplitude to the 100-400 CO_2 transition, while the 400-800 amplitude was
330 unaffected (Fig. 2A,C). In Tõldsepp *et al,* (2018) *mpk12 mpk4GC* double-mutants,
331 where MPK4 expression is suppressed only in guard cells, had no 400-800 CO_2
332 response, yet *mpk4GC* single-mutants responded to the 400-800 CO_2 transition similar
333 to wild-type plants. Takahashi *et al.* (2022) showed MPK12 mutants to have the 400-
334 800 response similar to current study, although neither of these studies tested the 100-
335 400 response. Thus, it seems that the MPK12/MPK4-HT1 complex largely loses
336 functionality if HT1 is impaired (Fig. 2), same happens if both MPK12 and MPK4 are
337 missing from guard cells (Tõldsepp et al., 2018), but losing only MPK12 preferentially
338 affects the 100-400 CO_2 transition (Fig. 2). Therefore, while in the 400-800 transition
339 the lack of MPK12 is likely compensated by MPK4, MPK4 cannot effectively replace
340 the function of MPK12 at sub-ambient CO_2 levels. This could mean that while MPK12
341 and MPK4 both can form CO_2/bicarbonate sensing complex with HT1, their affinity for
342 CO_2/bicarbonate may be different.

343 CA1 and CA4 also affected stomatal responsiveness more in the 100-400 CO_2 range,
344 with slow and shallow stomatal response in the *ca1ca4* mutant (Fig. 2). The strong
345 stomatal closure in above-ambient 400-800 CO_2 transition in this mutant (Fig. 2) might
346 be related to increased autonomous CO_2 conversion to HCO_3^- in the elevated CO_2

347 environment due to shifting of the reaction balance towards bicarbonate production
348 under above-ambient CO_2 levels; or by involvement of other carbonic anhydrases, as
349 recently demonstrated (Sun et al., 2022).

350 SLAC1 is important for stomatal closure responses to both elevated CO_2 transitions,
351 but compared with wild-type, the response speed of *slac1-3* in the 400-800 transition
352 was more affected than in the 100-400 transition (Fig. 3A,E). In *slac1-3* plants there
353 was small in magnitude and slow stomatal closure and opening in the 400-800-400
354 CO_2 transitions, yet stomatal opening in response to the 400-100 transition was strong
355 and similar in speed to wild-type plants (Supplementary Fig. 1B,D,F), further
356 supporting the major role of SLAC1 in stomatal responses under ambient to above-
357 ambient CO_2 concentration changes. In the 100-400 CO_2 transition, stomatal
358 responsiveness may be partly compensated by other ion channels that are functional
359 in *slac1-3*. In addition to the S-type anion channels like SLAC1, stomatal closure is
360 also affected by R-type anion channels, such as QUAC1 (Meyer et al., 2010; Imes et
361 al., 2013). Jalakas *et al.* (2021a) demonstrated that the *quac1-1 slac1-3* double mutant
362 and *quac1-1 slac1-3 slah1-3* triple-mutant had no response to the 400-800 CO_2
363 transition, while individually *slac1-3* had a weak response and *quac1-1* stomatal
364 response was similar to wild-type plants. SLAH3 is another S-type anion channel
365 contributing to stomatal closure (Zhang et al., 2016) and could potentially compensate
366 for the lack of SLAC1 in the 100-400 CO_2 response, although it does not affect the
367 400-800 stomatal CO_2 response (Jalakas et al., 2021a). Future studies should address
368 the 100-400 CO_2 response in mutants deficient in major S- and R-type anion channels
369 to better understand their potential role in the 100-400 CO_2 response.

370 Stomatal opening triggered by decreased CO_2 levels, from 400 to 100 and from 800 to
371 100, had larger magnitude than the 800-400 response (Fig. 1A,C,E,G). Responses to
372 100 ppm final CO_2 concentration had a consistently large amplitude and slow response
373 rate in different experimental set-ups and were not affected by the order of CO_2
374 treatments. Thus, stomatal opening in response to sub-ambient CO_2 concentrations is
375 a very prominent response. This is in line with Merilo *et al*., (2014), where two stimuli
376 with an opposing effect on stomata were simultaneously applied, such as darkness
377 and low CO_2, or low CO_2 and elevated VPD. In such combinations, Arabidopsis
378 stomata always opened in response to sub-ambient CO_2 levels, further indicating that
379 reduction of CO_2 is a strong and prevailing signal. The sub-ambient CO_2-induced

380 stomatal opening, similar to light-induced opening, likely involves H^+ATPase activation
381 (Inoue and Kinoshita, 2017), changes in sugar and starch metabolism (Flütsch and
382 Santelia, 2021), and suppression of stomatal closure via e.g. inhibiting anion channel
383 activation (Marten et al., 2007). The combined activation of all these processes may
384 explain the slow stomatal opening rate in response to sub-ambient CO_2 levels.

385 Elevated VPD triggers ABA biosynthesis (McAdam et al., 2016), that can potentially
386 increase stomatal responsiveness to CO_2 due to interactions of CO_2 and ABA signaling
387 pathways (Raschke, 1975; Merilo et al., 2013; Chater et al., 2015). In previous
388 experiments, elevated VPD has been shown to either increase stomatal
389 responsiveness to elevated CO_2, potentially via enhanced ABA levels (Bunce, 1998),
390 or decrease it, potentially due to reduced stomatal apertures under elevated VPD
391 (Morison and Gifford, 1983; Talbott et al., 2003). Our results showed an enhanced
392 stomatal response speed in the 400-100, 100-400 and 800-100 CO_2 concentration
393 transitions but not in the already faster 400-800 transition (Fig. 1A,B,E,F). Elevated
394 VPD has been shown to accelerate stomatal opening in light in angiosperms due to
395 reduced back-pressure of epidermal cells on guard cells (Mott et al., 1999; Pichaco et
396 al., 2024), our results of faster stomatal opening in response to sub-ambient [CO_2]
397 under elevated VPD are in line with this (Fig. 1B,E). Additionally, elevated VPD also
398 accelerated stomatal closure responses to elevated CO_2 levels in the sub-ambient to
399 ambient concentration ranges in wild-type Arabidopsis (Fig. 1B). Faster stomatal
400 closure may be caused by increased ABA levels under elevated VPD conditions
401 (McAdam and Brodribb, 2015) or may be explained by the smaller steady-state
402 stomatal conductance caused by smaller stomatal apertures under elevated VPD that
403 can adjust faster in response to environmental changes.

404 Steady-states of stomatal conductance of all plant lines were decreased by elevated
405 VPD (Fig. 2A,B, 3A,B), confirming that VPD is an important factor for steady-state
406 stomatal conductance (Grossiord et al., 2020; López et al., 2021). Mutants with
407 disrupted stomatal ABA response, *ghr1-3*, *ost1-3* and *slac1-3*, also had lower steady-
408 state stomatal conductances under elevated VPD (Fig. 3A,B). Their high VPD-induced
409 decrease in steady-state stomatal conductance could be caused by ABA-independent
410 active processes (e.g. ABA-independent OST1 activation, whose contribution to
411 stomatal closure under high VPD increases in time according to Jalakas et al.,
412 (2021b)), or hydropassive stomatal closure. These data indicate that elevated ABA

413 levels alone are not sufficient to explain the decrease of stomatal conductance under
414 elevated VPD conditions. CO_2-induced stomatal responses under elevated VPD were
415 sometimes faster and mostly had a smaller amplitude (Fig. 2B,D,F and 3B,D,F).
416 However, response to the 400-800 CO_2 transition disappeared completely in the *ghr1-*
417 *3* plants under elevated VPD conditions (Fig. 3B,D). GHR1 contributes to stomatal
418 closure in response to both elevated VPD (Hsu et al., 2021) and CO_2 (Hõrak et al.,
419 2016; Sierla et al., 2018). Thus, CO_2 and VPD responses may interact in *ghr1-3*: if the
420 relatively small elevated VPD-induced stomatal closure already occurred in the *ghr1-3*
421 plants subjected to elevated VPD (Sierla et al., 2018; Hsu et al., 2021), no further
422 response to CO_2 elevation was triggered.

423 Here we have focused on guard cell-specific stomatal CO_2 signaling components and
424 their different contribution to stomatal closure responses in the sub-ambient to ambient
425 and ambient to above-ambient CO_2 concentration transitions. In addition to these
426 components, it is likely that signal mediators outside guard cells also contribute to
427 different stomatal CO_2 response characteristics under different CO_2 levels. While guard
428 cells in isolated epidermis can respond to elevated CO_2 levels by a decrease in
429 stomatal aperture (Webb et al., 1996; Chater et al., 2015), signals from mesophyll are
430 needed for strong CO_2-induced stomatal closure (Mott et al., 2008; Fujita et al., 2013).
431 Mesophyll processes, e.g. photosynthesis and sugar metabolism, are known to impact
432 on stomatal behavior (Lawson and Matthews, 2020), and their contribution to different
433 stomatal CO_2 response patterns under different CO_2 concentration ranges merits
434 further study.

435 Elevated temperatures caused by climate change increase evaporative demand of the
436 atmosphere manifested as higher VPD levels, which increases transpiration, and
437 triggers stomatal closure to avoid wilting. Together, changes in atmospheric VPD and
438 CO_2 levels are perhaps the greatest agricultural challenges of the future, yet how both
439 these stimuli together affect plant stomatal behavior is poorly understood. Here we
440 show that while elevated VPD negatively affects steady-state stomatal conductances,
441 it has little effect on stomatal CO_2-responsiveness. Nevertheless, in some genetic
442 backgrounds, we found an interaction between CO_2 and VPD treatments, indicating
443 that the simultaneous effects of these factors on stomatal behavior merit further study.
444 We also show that stomatal response to elevated CO_2 has different kinetics under sub-
445 ambient and above-ambient CO_2 concentration ranges and its known regulators

446 contribute to a different degree under different CO_2 concentration transitions. Thus, to
447 better understand stomatal responses to CO_2 it is necessary to carefully consider CO_2
448 levels and experimental set-ups.

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454 **Author Contributions**

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

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588

589 **Figure Legends**

590 **Figure 1. Kinetics of CO_2-induced stomatal responses in wild-type Arabidopsis**
591 **with regular and elevated VPD.**

592 Col-0 wild-type Arabidopsis stomatal response to sequential changes in CO_2
593 concentration under regular **(A,C,E,G)** and elevated **(A,E)** VPD conditions. Mean
594 stomatal conductance ± SEM is shown. **(B,D,F,H)** Boxplot of 75% response time
595 (minutes) of stomatal response to CO_2 concentration changes. Boxes represent 25-75

596 % quartiles and median as the horizontal lines, whiskers indicate the smallest and
597 largest values, points show individual plant values. **(B,F)** Statistically significantly
598 different groups are indicated with different letters (Two-way ANOVA with Tukey *post-*
599 *hoc* test). **(D,H)** Statistically significantly different groups are indicated with different
600 letters (One-way ANOVA with Tukey *post-hoc* test). Sample size was 5 in
601 **(A,B,C,D,E,F)** and 14 in **(G,H)**. Start of first treatment was between 11:30 to 12:30.

602

603 **Figure 2. CO_2 pathway mutants retain response patterns under regular and**
604 **elevated VPD conditions**

605 **(A)** and **(B)** Stomatal response to CO_2 concentration changes from 400 to 100 ppm,
606 100 to 400 ppm and 400 to 800 ppm under regular **(A)** and elevated **(B)** VPD
607 conditions, mean stomatal conductance ± SEM is shown. **(C,D)** Boxplot of stomatal
608 conductance (g_s) change (mmol m^{-2} s^{-1}) in response to CO_2 concentration changes
609 from 400 to 100 ppm, 100 to 400 ppm and 400 to 800 ppm, respectively. **(E,F)** Boxplot
610 of 75% response time (minutes) of stomatal response to CO_2 concentration changes
611 from 400 to 100 ppm, 100 to 400 ppm and 400 to 800 ppm, respectively. **(C-F)** Boxes
612 represent 25-75 % quartiles and median as the horizontal lines, whiskers indicate the
613 smallest and largest values, points show individual plant values. Statistically
614 significantly different groups are marked with different letters (One-way ANOVA with
615 Tukey *post hoc* test). **(A-F)** Sample size was 5 for all plant lines. VPD during
616 experiments was 0.9 kPa in **(A,C,E)** and 2.3 kPa in **(B,D,F)**. Start of first treatment was
617 between 11:30 to 12:30. Col-0 data is same as used in figure 1, experiments with Col-
618 0 and mutant lines were done together.

619 **Figure 3. CO_2 response patterns in anion channel activation mutants are affected**
620 **by VPD conditions**

621 **(A)** and **(B)** Stomatal response to CO_2 concentration changes from 400 to 100 ppm,
622 100 to 400 ppm and 400 to 800 ppm in regular **(A)** and elevated **(B)** VPD conditions,
623 mean stomatal conductance ± SEM is shown. **(C,D)** Boxplot of stomatal conductance
624 (g_s) change (mmol m^{-2} s^{-1}) in response to CO_2 concentration changes from 400 to 100
625 ppm, 100 to 400 ppm and 400 to 800 ppm, respectively. **(E,F)** Boxplot of 75% response
626 time (minutes) of stomatal response to CO_2 concentration changes from 400 to 100

627 ppm, 100 to 400 ppm and 400 to 800 ppm, respectively. **(C-F)** Boxes represent 25-75
628 % quartiles and median as the horizontal lines, whiskers indicate the smallest and
629 largest values, points show individual plant values. Statistically significantly different
630 groups are marked with different letters (One-way ANOVA with Tukey *post hoc* test).
631 **(A-F)** Sample size was 5 for all plant lines. VPD during experiments was 0.9 kPa in
632 **(A,C,E)** and 2.3 kPa in **(B,D,F)**. Start of first treatment was between 11:30 to 12:30.
633 Col-0 data is same as used in figure 1, experiments with Col-0 and mutant lines were
634 done together.

635 **Figure 4. CO_2 transition from 100 to 800 ppm masks different responses present**
636 **in 100 to 400 and 400 to 800 ppm CO_2 transitions**

637 **(A)** and **(B)** Stomatal response to CO_2 concentration changes from 400 to 100 ppm
638 and 100 to 800 ppm, mean stomatal conductance ± SEM is shown. **(C,D)** Boxplot of
639 stomatal conductance (g_s) change (mmol m^{-2} s^{-1}) in response to CO_2 concentration
640 changes from 400 to 100 ppm and 100 to 800 ppm, respectively. **(E,F)** Boxplot of 75%
641 response time (minutes) of stomatal response to CO_2 concentration changes from 400
642 to 100 ppm and 100 to 800 ppm, respectively. **(C-F)** Boxes represent 25-75 % quartiles
643 and median as the horizontal lines, whiskers indicate the smallest and largest values,
644 points show individual plant values. Statistically significantly different groups are
645 marked with different letters (One-way ANOVA with Tukey *post hoc* test). **(A-F)** Sample
646 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
647 *slac1-3* and *ca1ca4*. VPD during experiments was 0.9 kPa in **(A-F)**. Start of first
648 treatment was between 11:30 to 12:30. Col-0 data is same as used in figure 1,
649 experiments with Col-0 and mutant lines were done together.

650 **Figure 5. Stomatal opening in 800 to 100 ppm CO_2 transition masks different**
651 **responses present in sub-ambient and above ambient CO_2 concentrations**

652 **(A)** and **(B)** Stomatal response to CO_2 concentration changes from 400 to 800 ppm
653 and 800 to 100 ppm, mean stomatal conductance ± SEM is shown. **(C,D)** Boxplot of
654 stomatal conductance (g_s) change (mmol m^{-2} s^{-1}) in response to CO_2 concentration
655 changes from 400 to 800 ppm and 800 to 100 ppm, respectively. **(E,F)** Boxplot of 75%
656 response time (minutes) of stomatal response to CO_2 concentration changes from 400
657 to 800 ppm and 800 to 100 ppm, respectively. **(C-F)** Boxes represent 25-75 % quartiles
658 and median as the horizontal lines, whiskers indicate the smallest and largest values,

659 points show individual plant values. Statistically significantly different groups are
660 marked with different letters (One-way ANOVA with Tukey *post hoc* test). **(A-F)** Sample
661 size was 5 for all plant lines. VPD during experiments was 0.9 kPa in **(A-F)**. Start of
662 first treatment was between 11:30 to 12:30. Col-0 data is same as used in figure 1,
663 experiments with Col-0 and mutant lines were done together.

664

665 **Supplementary figure 1**

666 **(A)** and **(B)** Stomatal response to CO_2 concentration changes from 400 to 800 ppm,
667 800 to 400 ppm and 400 to 100 ppm, mean stomatal conductance ± SEM is shown.
668 **(C,D)** Boxplot of stomatal conductance (g_s) change (mmol m^{-2} s^{-1}) in response to CO_2
669 concentration changes from 400 to 800 ppm, 800 to 400 ppm and 400 to 100 ppm,
670 respectively. **(E,F)** Boxplot of 75% response time (minutes) of stomatal response to
671 CO_2 concentration changes from 400 to 800 ppm, 800 to 400 ppm and 400 to 100
672 ppm, respectively. **(C-F)** Boxes represent 25-75 % quartiles and median as the
673 horizontal lines, whiskers indicate the smallest and largest values, points show
674 individual plant values. Statistically significantly different groups are marked with
675 different letters (One-way ANOVA with Tukey *post hoc* test). **(A-F)** Sample size was 5
676 for all plant lines. VPD during experiments was 0.9 kPa in **(A-F)**. Start of first treatment
677 was between 11:30 to 12:30. Col-0 data is same as used in figure 1, experiments with
678 Col-0 and mutant lines were done together.

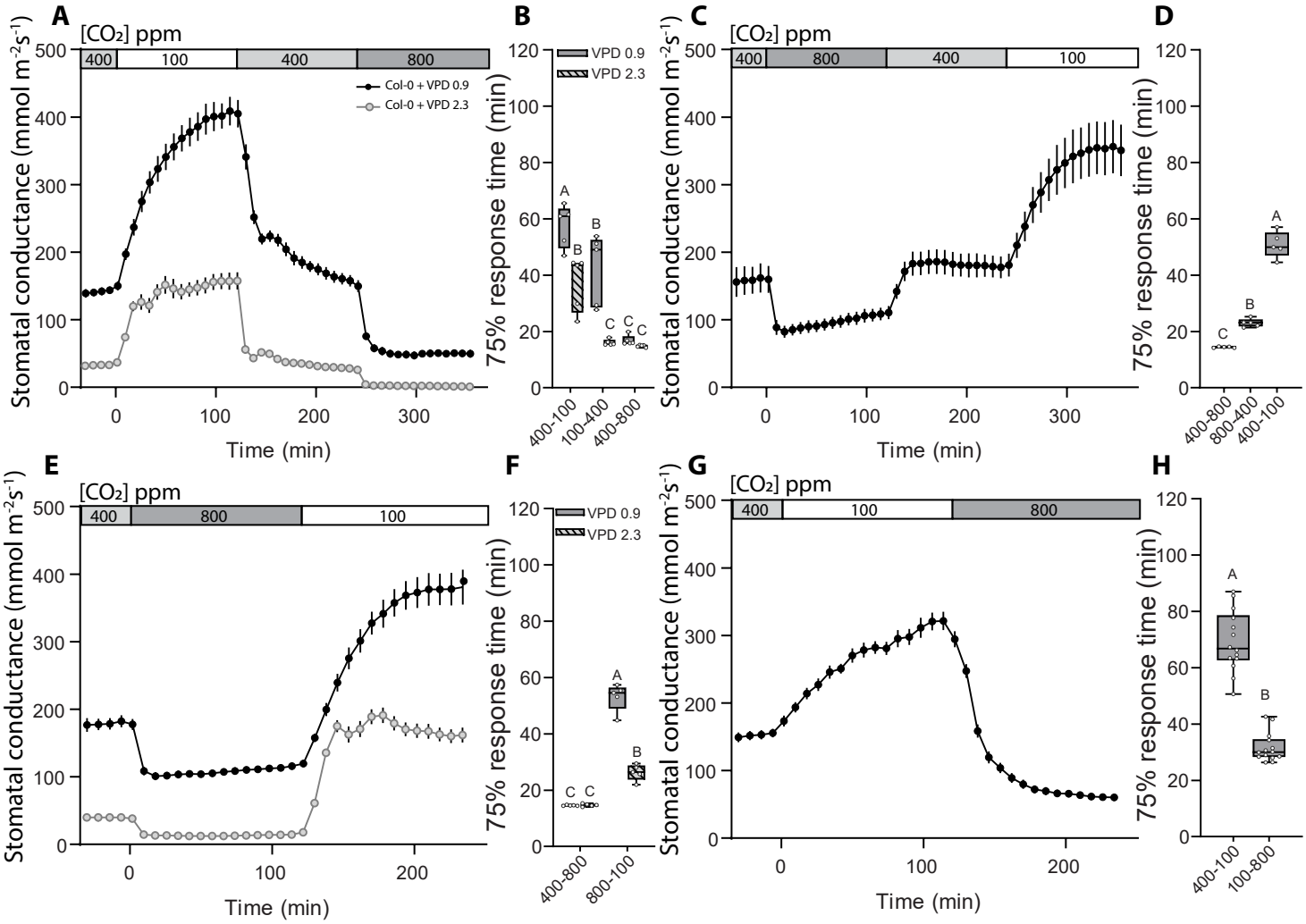

Figure 1. Kinetics of CO_2-induced stomatal responses in wild-type Arabidopsis with regular and elevated VPD.

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 ± SEM is shown. **(B,D,F,H)** Boxplot of 75% response time (minutes) of stomatal response to CO_2 concentration changes. Boxes represent 25-75 % quartiles and median as the horizontal lines, whiskers indicate the smallest and largest values, points show individual plant values. **(B,F)** Statistically significantly different groups are indicated with different letters (Two-way ANOVA with Tukey post-hoc test). **(D,H)** Statistically significantly different groups are indicated with different letters (One-way ANOVA with Tukey *post-hoc* test). Sample size was 5 in **(A,B,C,D,E,F)** and 14 in **(G,H)**. Start of first treatment was between 11:30 to 12:30.

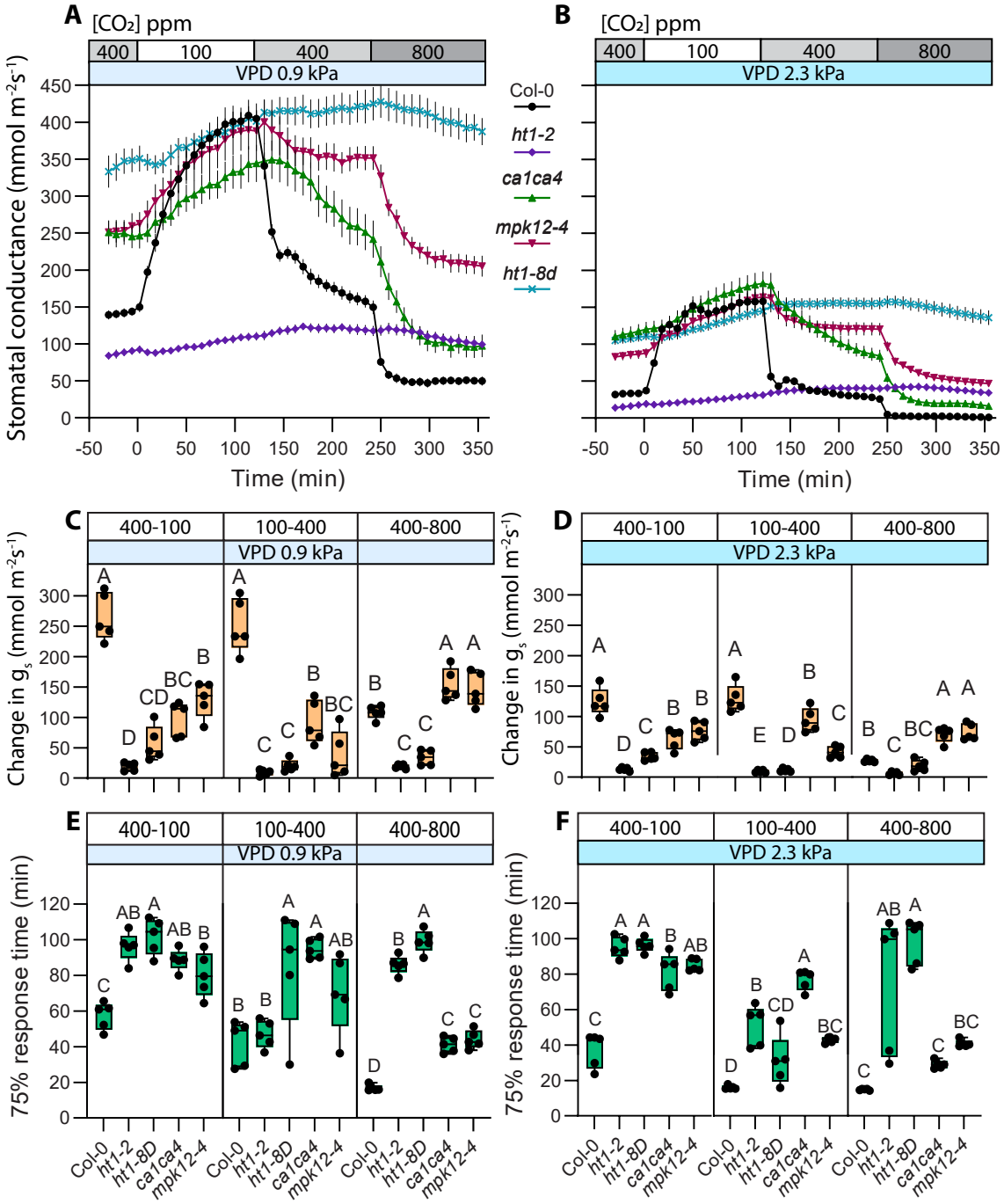

Figure 2. CO_2 pathway mutants retain response patterns under regular and elevated VPD conditions

(A) and **(B)** Stomatal response to CO_2 concentration changes from 400 to 100 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 (minutes) of stomatal response to CO_2 concentration changes from 400 to 100 ppm, 100 to 400 ppm and 400 to 800 ppm, respectively. **(C-F)** Boxes represent 25-75 % quartiles and median as the horizontal lines, whiskers indicate the smallest and largest values, points show individual plant values. Statistically significantly different groups are marked with different letters (One-way ANOVA with Tukey *post hoc* test). **(A-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 first treatment was between 11:30 to 12:30. Col-0 data is same as used in figure 1, experiments with Col-0 and mutant lines were done together.

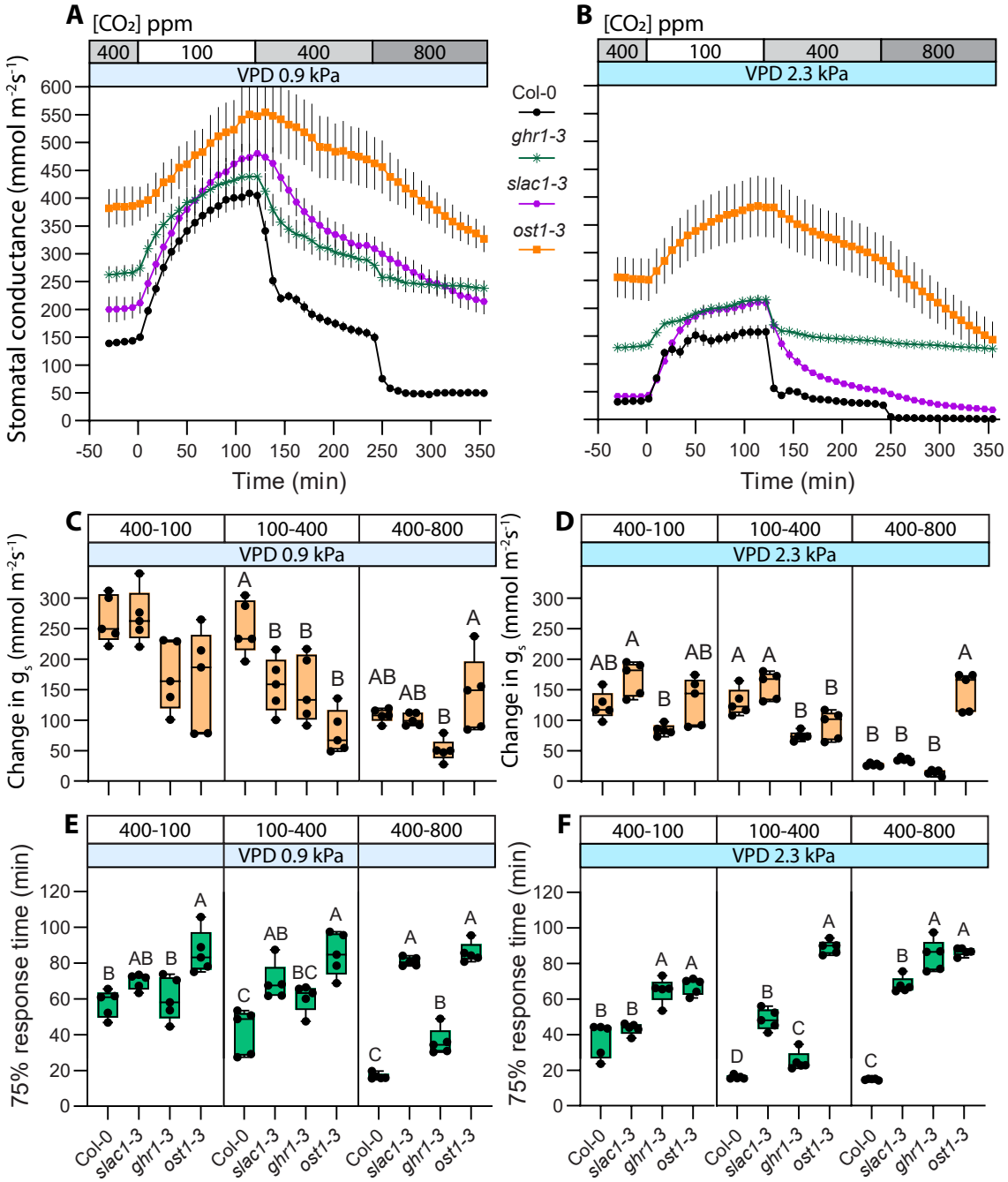

Figure 3. CO_2 response patterns in anion channel activation mutants are affected by VPD conditions

(A) and **(B)** Stomatal response to CO_2 concentration changes from 400 to 100 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 (minutes) of stomatal response to CO_2 concentration changes from 400 to 100 ppm, 100 to 400 ppm and 400 to 800 ppm, respectively. **(C-F)** Boxes represent 25-75 % quartiles and median as the horizontal lines, whiskers indicate the smallest and largest values, points show individual plant values. Statistically significantly different groups are marked with different letters (One-way ANOVA with Tukey *post hoc* test). **(A-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 first treatment was between 11:30 to 12:30. Col-0 data is same as used in figure 1, experiments with Col-0 and mutant lines were done together.

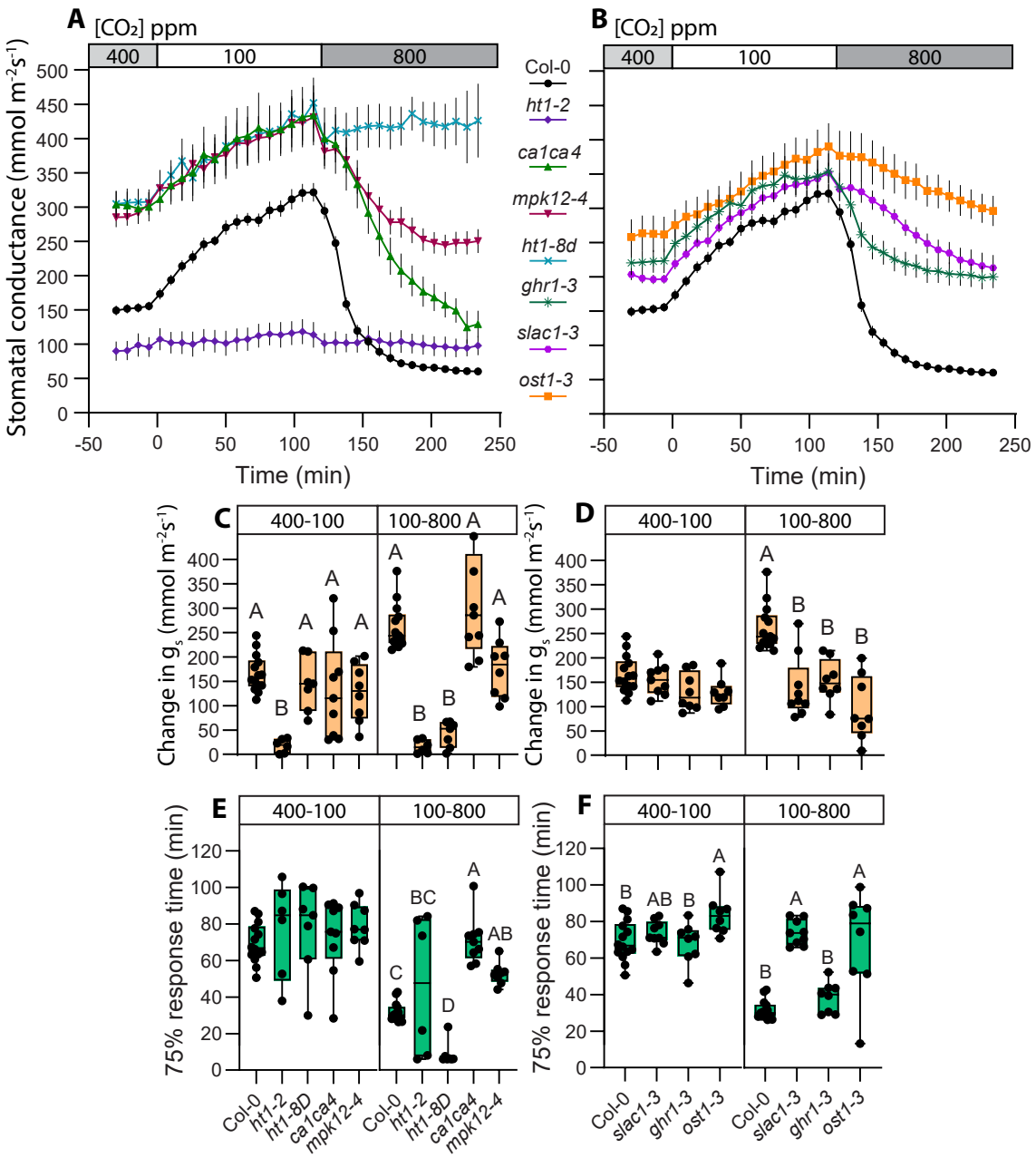

Figure 4. CO_2 transition from 100 to 800 ppm masks different responses present in 100 to 400 and 400 to 800 ppm CO_2 transitions

(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 (minutes) of stomatal response to CO_2 concentration changes from 400 to 100 ppm and 100 to 800 ppm, respectively. **(C-F)** Boxes represent 25-75 % quartiles and median as the horizontal lines, whiskers indicate the smallest and largest values, points show individual plant values. Statistically significantly different groups are marked with different letters (One-way ANOVA with Tukey *post hoc* test). **(A-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-F)**. Start of first treatment was between 11:30 to 12:30. Col-0 data is same as used in figure 1, experiments with Col-0 and mutant lines were done together.

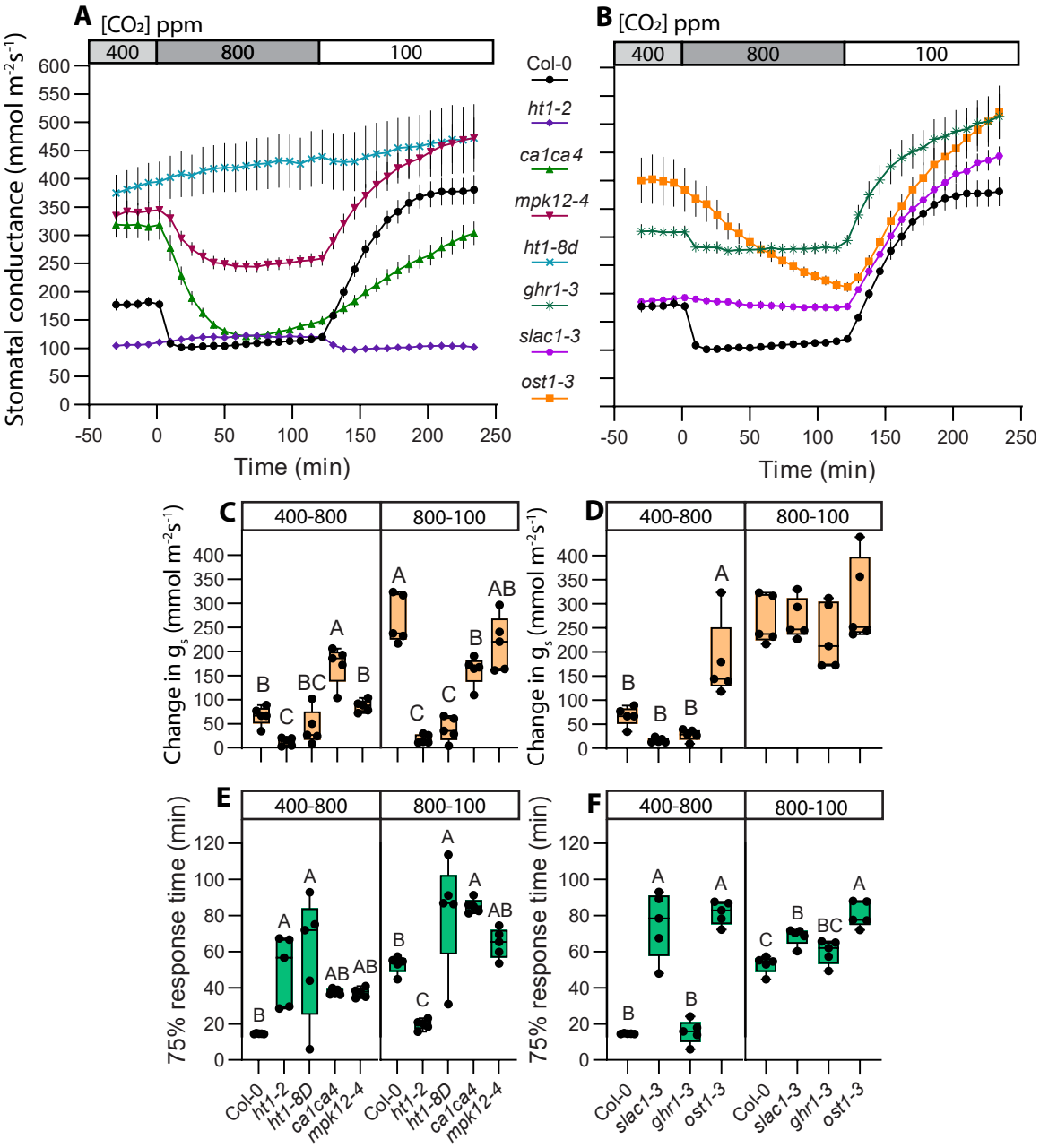

Figure 5. Stomatal opening in 800 to 100 ppm CO_2 transition masks different responses present in sub-ambient and above ambient CO_2 concentrations

(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 (minutes) of stomatal response to CO_2 concentration changes from 400 to 800 ppm and 800 to 100 ppm, respectively. **(C-F)** Boxes represent 25-75 % quartiles and median as the horizontal lines, whiskers indicate the smallest and largest values, points show individual plant values. Statistically significantly different groups are marked with different letters (One-way ANOVA with Tukey *post hoc* test). **(A-F)** Sample size was 5 for all plant lines. VPD during experiments was 0.9 kPa in **(A-F)**. Start of first treatment was between 11:30 to 12:30. Col-0 data is same as used in figure 1, experiments with Col-0 and mutant lines were done together.

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.

	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/-	$+_{[1,8\text{-}10]}$/+	$+_{[1,8\text{-}10]}$/+	$+_{[4]}$/+	$+_{[4]}$/-	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	$+_{[3,7,8]}$/+	$+_{[3,7,8]}$/+	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 += different from WT