Low relative air humidity and increased stomatal density independently hamper growth in young Arabidopsis

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Abstract

Stomatal pores in plant leaves mediate CO_2 uptake for photosynthesis and water loss via transpiration. Altered stomatal density can affect plant photosynthetic capacity, water use efficiency, and growth, potentially providing either benefits or drawbacks depending on the environment. Here we explore, at different air humidity regimes, gas exchange, stomatal anatomy, and growth of Arabidopsis lines designed to combine increased stomatal density (*epf1*, *epf2*) with high stomatal sensitivity (*ht1-2*, *cyp707a1/a3*). We show that the stomatal density and sensitivity traits combine as expected: higher stomatal density increases stomatal conductance, whereas the effect is smaller in the high stomatal sensitivity mutant backgrounds than in the *epf1epf2* double mutant. Growth under low air humidity increases plant stomatal ratio with relatively more stomata allocated to the adaxial epidermis. Low relative air humidity and high stomatal density both independently impair plant growth. Higher evaporative demand did not punish increased stomatal density, nor did inherently low stomatal conductance provide any protection against low relative humidity. We propose that the detrimental effects of high stomatal density on plant growth at a young age are related with the cost of producing stomata; future experiments need to test if high stomatal densities might pay off in later life stages.

Significance statement

This study delves into the relationship between stomatal density, sensitivity, and environment in Arabidopsis. These findings not only enhance our comprehension of plant responses to humidity but also lay the groundwork for future studies aimed at optimising plant adaptability to varying environmental conditions.

Introduction

Vascular plant stomata have evolved to balance CO_2 supply for photosynthesis and water loss via transpiration (Lawson and Matthews, 2020). To perform photosynthesis efficiently, plants need a sufficient supply of CO_2. Stomata open to allow CO_2 to enter the leaf, which inevitably allows water vapour to escape through transpiration. While transpiration serves important functions like nutrient transport and leaf cooling, excessive transpiration can lead to water stress, especially in water-limited conditions.

Plant water loss is regulated via adjustments of stomatal conductance, determined by stomatal density and aperture. Higher stomatal density generally correlates with higher stomatal conductance (Muchow and Sinclair, 1989; Anderson and Briske, 1990; Pearce *et*

43 *al.*, 2006; Carlson *et al.*, 2016), although it can largely be counteracted with stomatal
44 closure (Büssis *et al.*, 2006; Dow *et al.*, 2014; Hepworth *et al.*, 2015).

45 Stomatal differentiation occurs very early in leaf development, when some epidermal cells
46 enter stomatal lineage and develop into stomata (Lee and Bergmann, 2019; Torii, 2021).
47 During subsequent leaf expansion, stomatal density decreases (Gay and Hurd, 1975).
48 Stomatal differentiation is governed by the sequential activation of the transcription factors
49 SPEECHLESS (SPCH) (MacAlister *et al.*, 2007), MUTE (Pillitteri *et al.*, 2007), and FAMA
50 (Ohashi-Ito and Bergmann, 2006) that is regulated by intercellular signalling mediated by
51 epidermal patterning factors EPF2 and EPF1 (Hara *et al.*, 2007; Hunt and Gray, 2009;
52 Ohki *et al.*, 2011; Zoulias *et al.*, 2018). Loss-of-function mutations in the *EPF1* and *EPF2*
53 genes lead to increased stomatal densities.

54 The plant hormone abscisic acid (ABA) is well known to mediate stomatal closure in
55 response to a wide range of environmental stimuli (Seo and Koshiba, 2011; Merilo *et al.*,
56 2013; Chater *et al.*, 2015; Hsu *et al.*, 2021). Arabidopsis (*Arabidopsis thaliana*) mutants
57 with inherently high ABA levels, such as those lacking the function of some
58 CYTOCHROME P450, FAMILY 707, SUBFAMILY A (CYP707A) proteins, exhibit
59 increased stomatal sensitivity manifested in smaller stomatal apertures and lower stomatal
60 conductance (Okamoto *et al.*, 2006; Okamoto *et al.*, 2009). CO_2 largely regulates plant
61 stomatal apertures via the HIGH LEAF TEMPERATURE 1 (HT1) kinase; the *ht1-2* mutants
62 have constitutively activated CO_2 signalling, resulting in small stomatal apertures and
63 lower stomatal conductance (Hashimoto *et al.*, 2006).

64 Larger stomatal densities and apertures are often accompanied by a reduced water use
65 efficiency (Franks *et al.*, 2015; Kimura *et al.*, 2020). High stomatal conductance may lead
66 to water stress, potentially impairing plant growth. Indeed, Doheny-Adams et al. (2012)
67 demonstrate a negative correlation between stomatal density (and therefore conductance)
68 and plant size. More recently, a series of articles have demonstrated that decreased
69 stomatal density in Arabidopsis (Hepworth *et al.*, 2015), barley (Hughes *et al.*, 2017),
70 wheat (Dunn *et al.*, 2019), and rice (Caine *et al.*, 2019) improves drought tolerance with no
71 adverse effect on plant growth or carbon assimilation.

72 On the other hand, there is a wealth of studies that report positive relationships between
73 stomatal density and photosynthetic assimilation or plant growth. Schlüter *et al.* (2003)
74 demonstrated that low-light adapted Arabidopsis mutant *sdd1-1* with increased stomatal
75 density achieved 30% higher CO_2 assimilation rates after transfer to high light conditions

than wild type. Xu and Zhou (2008) report increased net CO_2 assimilation and even instantaneous water use efficiency with increasing stomatal density in *Leymus*. Tanaka *et al.* (2013) achieved a significant enhancement of steady-state photosynthetic capacity by overexpressing the stomata-inducing peptide STOMAGEN in Arabidopsis, and smaller biomass in a STOMAGEN-RNAi line with decreased stomatal density. Sakoda *et al.* (2020) describe 26% increase in plant biomass gain in the *epf1* mutant compared with wild-type under fluctuating light and no difference under constant light. Similarly, an increase of biomass under fluctuating, but not constant light was found in Arabidopsis mutants with large stomatal apertures (Kimura *et al.*, 2020).

Combining the considerations that suggest both potential benefits and drawbacks of increased stomatal density, one might expect there to be an optimal range of stomatal conductance, subject to environmental conditions, where further increase in water loss would begin to counteract CO_2 availability and turn detrimental to plant growth. Experiments addressing the effects of low air humidity help to address the role of increased evaporative demand on plant growth. Low night-time relative humidity did not affect biomass production in Arabidopsis (Christman *et al.*, 2009), whereas we recently found that low air humidity applied both during the day and night hampered plant growth in both wild-type plants and mutants with increased stomatal conductance due to larger stomatal apertures (Tulva *et al.*, 2023).

Relatively little success has been achieved in improving plant water use efficiency and productivity, and this lack of success has been ascribed to limitations of single-gene or single-trait modification approaches (Flexas, 2016). Here, we combine mutations affecting stomatal density and sensitivity to test i) whether simultaneous manipulation of these traits leads to independent effects on stomatal conductance and could hence have potential for multi-gene applications in engineering stomatal conductance and water use efficiency, and ii) how does growth under low relative humidity affect plants with altered stomatal conductance via modification of stomatal density, stomatal sensitivity or both these traits.

In this experiment, we created Arabidopsis lines that combined mutations in *EPF2* and/or *EPF1* (leading to increased stomatal density (Hara *et al.*, 2007; Hunt and Gray, 2009)) and in *HT1* or *CYP707A1* and *CYP707A3* (leading to increased stomatal sensitivity - here defined as hypersensitive stomatal closure manifested in constantly smaller stomatal apertures (Hashimoto *et al.*, 2006; Okamoto *et al.*, 2006; Okamoto *et al.*, 2009)) and studied them for their gas exchange, stomatal anatomy, and growth under different air

humidity regimes. Stomatal conductance was increased by density-altering mutations both in wild-type background and in plant lines carrying mutations that lead to smaller stomatal apertures. Moderately reduced air humidity was consistently detrimental to the size of all studied plant lines irrespective of stomatal traits. Plant lines with increased stomatal density were hampered in growth, manifesting as a negative correlation between stomatal density and plant size, whereas this reduction was not due to stomatal water loss and might instead be explained by a developmental cost.

Materials and methods

Plant material

The studied *Arabidopsis thaliana* mutant lines were all in Col-0 background; the single and double mutant lines were *epf1-1* (SALK_137549; Alonso *et al.*, 2003; Hara *et al.*, 2009), *epf2-2* (GABI_673E01; Rosso *et al.*, 2003; Hunt & Gray, 2009), *epf1-1epf2-2* (Hunt and Gray, 2009), *ht1-2* (Hashimoto *et al.*, 2006), and *cyp707a1-1cyp707a3* (SALK_069127 and SALK_101566, respectively; Kushiro *et al.*, 2004; Okamoto *et al.*, 2006); higher order mutants were obtained by crossing the single and double mutants. All plant lines used in experiments are listed in Table 1. Primers used for verifying mutant genotypes are shown in Table S1. Age-matched seed stocks were used for experiments (grown under 50% RH, 23°C; 10:14 h day:night until bolting, then 16:8 h day:night).

Gas exchange experiment

Gas exchange was measured using a thermostated 8-chamber Arabidopsis gas exchange system (Plantinvent OÜ, https://www.plantinvent.com/) that allows measurement of whole-rosette leaf conductance and net assimilation rate (Kollist *et al.*, 2007; Hõrak *et al.*, 2017). Six plant lines were studied: Col-0, *ht1-2*, *cyp707a1/a3*, *epf1/2*, *ht1-2epf1/2*, and *cyp707a1/a3epf1/2*. The soil was a 2:1 v:v mixture of peat (OPM 025 W, Kekkilä Oy, Vantaa, Finland) and vermiculite (Vermikuliit Medium 0-4 mm, TopGreen, Vahi, Estonia). The plants were sown into pots where the soil was separated from the aboveground part by a glass, which would later form the bottom of the measuring cuvette. The plants were divided between two Percival AR-22L (Percival Scientific, IA, USA) growth cabinets, which were set to keep a 10:14 hour day:night cycle, with relative humidity (RH) of 60%:80% and air temperature of 23:19ºC day:night, respectively, and daytime photosynthetic photon flux density (PPFD) of 250 µmol m^{-2} s^{-1} in both for the first 14 days, then one cabinet was switched to the low RH conditions (40%:50% day:night air humidity, otherwise identical to

143 the control). The growth cabinets stood in a ventilated room, ensuring CO_2 concentration
144 of about 420 ppm around the plants. Plants were watered every few days by soaking the
145 pots in a tray filled with shallow water to take the soil water content to ~70-80%. Soil
146 moisture in the dry air cabinets decreased faster than that in the control treatment; this
147 was compensated for by more frequent watering, but no precisely defined watering regime
148 was applied. The experiment was performed twice, for total sample size per line per
149 growth condition of 9-15.
150
151 Gas exchange was measured in 22-27 days old plants. Measurement cuvette was
152 thermostated to keep the air temperature at 23ºC; incident PPFD inside the cuvette was
153 250 µmol m^{-2} s^{-1}. Stabilised outside air with [CO_2] about 420 ppm was used to supply air
154 into the chambers, with only RH and temperature altered. The gas exchange system
155 recorded one data point each minute, cycling between eight cuvettes, yielding one data
156 point per plant every eight minutes. Plants were at first kept at ~60% RH for 1.5 h, after
157 which air humidity was lowered to ~40%. Points measured between -24 to 0 minutes
158 before humidity lowering (3-4 data points per plant) were averaged to yield steady-state
159 60% humidity gas exchange, and points recorded between 36 and 60 minutes after
160 humidity lowering likewise averaged to yield 40% RH gas exchange.
161
162 The variables presented are leaf conductance (stomatal+cuticular conductance) g_l and net
163 CO_2 assimilation A_{net}. Cuvette boundary layer conductance was 4.5 cm s^{-1} as measured
164 with wet filter paper plant models during prior calibration of the system - note that this
165 value represents both leaf sides of a typical Arabidopsis plant in this chamber, while in
166 nature, boundary layer conductances may differ between leaf sides.
167
168 After the gas exchange measurement, the sixth leaf was collected, cut in half after
169 weighing, and a surface imprint was taken from each half with dental silicone as in
170 (Casson *et al.*, 2009). Abaxial surface impression was collected from one half of the leaf
171 (oranwash L, Zhermack; www.zhermack.com) and adaxial surface impression from the
172 other (Speedex light body, Coltene/Whaledent AG; Alstätte, Switzerland). Secondary
173 imprints were taken from the silicone with nail varnish and transferred to microscopy
174 slides. One location (0.0675 mm^2) from each imprint was photographed under microscope
175 (Kern OBF 133; Kern & Sohn GmbH; www.kern-sohn.com) at 400X magnification, to be
176 analysed for stomatal density (SD) with ImageJ software (National Institutes of Health,
177 USA; (Schneider *et al.*, 2012)).

178

179 **Growth experiment**

180 Plants were grown in 50x29 cm trays filled with 6 cm deep soil. Water was added to the
181 outer tray and absorbed by the soil in the inner tray through 40 3 mm holes drilled into the
182 inner tray's bottom.

183

184 Soil was added to the trays in two stages. First, 1200 g of 2:1 peat and vermiculite mixture
185 (v/v; same materials as in the gas exchange measurement) was weighed into each tray.
186 ~50 g soil samples were taken after each 1-2 trays, dried at 70ºC for two days, and
187 weighed to determine mass based water content in the soil mixture, which was 44±1%.
188 Based on this knowledge, 220 g of the same mixture was added to each tray, to take the
189 total amount of estimated dry soil in a tray to 800 g, and 3 litres of water was gradually
190 added to the soil. After the final filling, the soil in the trays was covered with black textile
191 mulch blankets (Baltic Agro AS, Lehmja, Estonia). The mulch sheets had 32 holes (5 mm
192 diameter, 65 mm distance between hole centres) for plants.

193

194 One plant of each Arabidopsis line to be studied was sown into a hole in the central part of
195 the tray, randomly chosen for each tray (Figure 1). Other plants grown in those trays were
196 not part of this study. After sowing, the trays were wrapped in cling film for 4 days to keep
197 saturating air humidity for germination, then transferred to two Percival AR-22L growth
198 cabinets. Conditions in the chambers were the "control" conditions as described in the gas
199 exchange experiment. The trays were rotated and shuffled inside and between the
200 cabinets daily.

201

202 On the 14th day after sowing, one of the growth cabinets was set to the "dry" regime. From
203 that moment onwards, the trays were rotated and shuffled around within each cabinet.
204 Every day in the late afternoon, the trays were weighed for their gross mass to determine
205 soil water content (SWC). When SWC in any tray was below 70%, water was added to the
206 lower tray to take SWC to 75%. This was to compensate for the faster drying of soil in the
207 low RH treatment, establishing a SWC pattern in each tray that would systematically
208 fluctuate between 70% and 75% regardless of RH.

209

210 The trays were photographed daily with a Nikon D3100 camera positioned about 1 m
211 directly above the centre of the tray. Photos were stored in Nikon RAW format, converted

212 to Windows Bitmap with IrfanView batch conversion feature, and analysed with custom-
213 made software for the projected area of each studied plant.
214
215 After 14 days of differential treatment, all studied plants were collected at the age of 4
216 weeks. The sixth leaf was separated, this leaf and the rest of the rosette were weighed for
217 wet mass and photographed for (gently flattened) area. The remaining rosette was dried
218 for 5 days at +60℃ and weighed for dry mass, which was then scaled for whole rosette dry
219 mass based on the ratio of wet masses of the sixth leaf and the remaining rosette. Mass
220 loss of the rosette (without the separated sixth leaf) during oven drying gave water
221 content, and its dry mass divided by its harvested area gave leaf mass per area (LMA).
222
223 The sixth leaf was studied for its stomatal anatomy as described above. Additionally, from
224 each imprint, five typical mature stomata were measured for their length (length of the
225 whole stomatal complex); median of which was used to represent a given leaf side in
226 stomatal size analysis. There were issues with imprint quality; only about half of the leaves
227 could be analysed for their stomatal anatomy on both sides. Since this loss of data was
228 purely technical and did not correlate with plant line or treatment, we regard the obtained
229 sample as representative of the whole studied material. Multiplying SD with the area of the
230 sixth leaf yielded the number of stomata per leaf (stomatal count, SC).
231 The experiment was repeated with the growth cabinets swapped, resulting in an overall
232 sample size of 14-16 plants per treatment and genotype.
233
234 **Statistical analysis**
235 Comparisons between measured variables were done with analysis of variance using R
236 scripting language version 4.2.1. Type 3 sums of squares were ensured with the use of the
237 function Anova(,type="III") from the library car (https://r-forge.r-project.org/projects/car/) for
238 obtaining ANOVA results. For post-hoc tests, Tukey's HSD for unbalanced samples was
239 used, employing library agricolae (https://myaseen208.github.io/agricolae/). Level of
240 significance was always set to 0.05.
241 For analysis of variance, studied genotypes were used as factors as follows (Table 1). In
242 the growth experiment dataset, "epf1" and "epf2" were separate binary variables. In the
243 gas exchange dataset, one variable "epf1/2" indicated whether both *EPF1* and *EPF2* were
244 mutant or wild-type (no single mutants in these genes were studied). The other genes
245 involved, *HT1*, *CYP707A1*, and *CYP707A3* (the latter two always at the same state) were
246 encoded into a three-state variable "sensitivity" with values of "wt" (all three intact),"ht1"

247 (*ht1-2* mutation), or “cyp707a1a3” (*cyp707a1/a3* double mutant) - note that the design
248 lacks the combination of ht1-2 and cyp707a1a3 mutations and is thus not fully factorial.
249 Growth RH was a two-state variable with values “control” or “dry” (60%:80% or 40%:50%
250 day:night RH, respectively).
251

252 ## Accession numbers

253 - *CYP707A1* – AT4G19230
254 - *CYP707A3* – AT5G45340
255 - *EPF1* – AT2G20875
256 - *EPF2* – AT1G34245
257 - *HT1 – AT1G62400*

258

259 ## Results

260

261 ***High SD and high stomatal sensitivity affect leaf conductance largely independently***
262 To test whether mutations that alter stomatal density and sensitivity affect plant gas-
263 exchange independently, we carried out experiments with mutants expected to combine
264 high stomatal density (*epf1/2*) with small stomatal apertures (*ht1-2*, *cyp707a1/a3*). Gas
265 exchange measurements were consistent with the expected behaviour of the studied lines:
266 under both normal and low RH, plants deficient in both *EPF1* and *EPF2* had significantly
267 higher leaf conductance than the corresponding lines with intact *EPF*s (Figure 2a; Figure
268 S1; Table 2, significant main effect of epf1/2). Leaf conductance of *ht1-2* was significantly
269 lower than wild-type under normal RH, whereas both *ht1-2* and *cyp707a1a3* had lower
270 than wild-type g_l under low RH conditions. In combination with the *epf1* and *epf2* alleles
271 these mutations expected to decrease stomatal apertures led to lower leaf conductance
272 compared with the *epf1/2* double mutant under normal RH (Figure 2a). Low RH generally
273 decreased leaf conductance (Table 2, significant main effect of RH), with the most
274 prominent effect in the lines with the *epf1/2* mutations (Figure 2a). Growth conditions had
275 little lasting effect on stomatal behaviour: only the *epf1/2* plants grown under low RH had
276 decreased leaf conductance under low cuvette RH compared to the same genotype grown
277 under normal RH (Figure S2).
278

279 Under normal humidity, area-based net CO_2 assimilation of the *epf1/2* mutant was higher
280 than most other plant lines (Figure 2b). Under low RH, the *cyp707a1/a3epf1/2* line had

281 significantly higher A_{net} than the *cyp707a1/a3* (Figure 2b). Due to decreased g_l, the
282 intrinsic water use efficiency (iWUE=A_{net}/g_l) generally increased under low RH (Figure 3a).
283 Under low RH, water use efficiency was higher than in wild-type in plant lines with
284 increased stomatal sensitivity (*ht1-2* and *cyp707a1/a3*) and about two times lower in the
285 plant lines with the *epf1/2* double mutation than the corresponding lines with intact EPFs.
286 Stomatal density was not affected by the *ht1-2* and *cyp707a1/a3* mutations: the *epf1/2*
287 mutations increased both adaxial and abaxial stomatal density in all genetic backgrounds
288 (Figure 4). Growth under low RH also increased both adaxial and abaxial stomatal density
289 (Table 2, significant main RH effect; Figure 4), with the most prominent effect in the *epf1/2*
290 mutant (significant RH*epf interaction in Table 2; Figure 4). Across all plant lines, the
291 relationship between total stomatal density and leaf conductance remained positive under
292 both growth conditions, but had a steeper slope under normal humidity (Figure 3b).
293
294 ***No effect of epf1 mutation on SD in plant lines with high stomatal sensitivity***
295 To dissect the role of different stomatal density and sensitivity in plant growth under
296 different RH environments, we carried out a growth experiment. The positive effects of the
297 *epf1/2* and low RH on stomatal density were similar as found in the gas-exchange
298 experiment, although low RH significantly increased only adaxial SD in the growth
299 experiment (Figure 5; Table 3, significant RH effect). The *epf1/2* double mutants had larger
300 stomatal density on both leaf sides than the respective lines with intact EPFs, and the *ht1-*
301 *2* or *cyp707a1/a3* mutations alone did not affect stomatal density. In the Col-0 background,
302 both *epf1* and *epf2* single mutations increased stomatal density on both leaf sides under
303 normal RH (Figure 5), but there was a significant interaction between *epf1* mutation and
304 stomatal sensitivity (Table 3), manifested in no increase of SD in lines combining the
305 *cyp707a1/a3* or *ht1-2* mutations with the *epf1* single mutation (Figure 5).
306
307 Stomatal count was affected by plant genetics similarly to stomatal density: the *epf2*
308 mutation had an overall increasing effect, while the impact of the *epf1* mutation was
309 mediated by the state of the *cyp707a1/a3* and *ht1-2* genes (Table 3, significant
310 epf1*sensitivity interaction). This resulted in increased stomatal numbers in the *epf1-1* line
311 compared to the wild-type, but no significant difference in the *cyp707a1/a3epf1* or *ht1-*
312 *2epf1* compared to the *cyp707a1/a3* or *ht1-2*, respectively (Figure 6). Overall, the main
313 effect of the growth environment on the stomatal count was generally weaker than on
314 stomatal density; only the abaxial sides of leaves had fewer stomata in plants grown under

315 low RH (Table 3, significant RH main effect), and this effect was not significant in any
316 particular genotype (Figure 6).
317
318 Growth under low RH resulted in shorter stomatal complexes on both leaf sides (Table 3,
319 significant main effect of RH; Figure 7). Stomatal size correlated negatively but weakly
320 with stomatal density on the adaxial side, while no significant relationship was found on
321 the abaxial side (Figure S3).
322
323 ***Growth at low RH increases stomatal ratio***
324 Growth under low RH slightly but consistently increased adaxial to abaxial stomatal ratio in
325 all studied plant lines (Table 3, significant main RH effect; Figure 8; Figure S4). This effect
326 primarily stemmed from generally increased adaxial SD under low RH, with significant
327 increase in adaxial SD in response to low RH in the *epf1epf2* and *cyp707a1/a3epf1/2*
328 mutants (Figure 5a; Table 3, significant RH main effect). The increase in stomatal ratio in
329 response to low RH was strongest in plants with the *epf1* mutant allele (Figure 8).
330
331 ***Growth at low RH and high stomatal density independently decrease plant size***
332
333 Plants grown under low RH showed mild signs of xeromorphy, with slight but consistent
334 increase in LMA and substantial decrease in water content (Figure 9; Table 4 RH main
335 effects). A subtle increase in LMA and decrease in water content was also associated
336 independently with both *epf1* and *epf2* mutations (Table 4, respective main effects),
337 leading to overall positive relationship between LMA and stomatal density (Table S2,
338 Figure S5a).
339
340 Plant growth under low RH was significantly impaired. All plant lines showed a relatively
341 steady decrease in dry mass at harvest time of about 25% - significantly so in the Col-0,
342 *epf1*, *cyp707a1/a3,* and *cyp707a1/a3epf1* lines, but the trend was similar in all genotypes
343 (Figure 10a; Table 4, significant main RH effect). The projected rosette area at the end of
344 the experiment (28 days after sowing) decreased even more (Figure 10b), owing to the
345 general increase in leaf mass to area ratio (Figure 9; Table 4, significant main RH effect)
346 under low RH. Across the plant line and treatment combinations, there was a negative
347 relationship between LMA and plant mass under low RH but not under control conditions
348 (Figure S5b).
349

350 Both plant area and dry mass at the end of the experiment were markedly smaller in the
351 lines with the *epf1/2* double mutation; in case of mass, this was due to the main effect of
352 the *epf2* mutation and there was no main effect of *epf1* (Figure 10a; Table 4), while the
353 final area was affected by both *epf1* and *epf2* mutations (Table 4). At the onset of the RH
354 treatment 14 days after sowing, the plant lines with the *epf2* mutations had slightly smaller
355 projected area, a small enough effect to only appear in the pooled data (Table 4, epf2
356 main effect; Figure S6). During the differential treatment in weeks 3 and 4, growth under
357 low RH resulted in a smaller relative growth rate in all plant lines, with no significant
358 difference associated with any genetic features (Table S3, Figure S7).
359
360 As a result of the size reduction associated with the *epf1* and *epf2* mutations, particularly
361 that of *epf1/2* double mutants, there was a significant negative correlation between total
362 stomatal density and plant size (Figure 11a, Table S2). The slope of this relationship
363 remained the same under both growth humidities, with the normal RH-grown plants
364 consistently around 3.5 cm^2 larger in their projected area, or 10 mg heavier in their dry
365 mass. Whereas the stomatal count and plant size relationship had a similar downward
366 trend, it was not statistically significant (Figure 11b; Table S2). Notably, the enhanced
367 photosynthesis of *epf1/2* mutants (Figure 2b, Table 2) and overall strong correlation
368 between stomatal density and net photosynthesis (Table S2) did not translate into
369 improved growth but instead led to a strongly negative correlation between net
370 photosynthesis and plant size (Table S2).
371

372 ## Discussion

373 Both altered stomatal density and sensitivity impact plant water use efficiency and
374 productivity (Doheny-Adams *et al.*, 2012; Franks *et al.*, 2015; Kimura *et al.*, 2020; Sakoda
375 *et al.*, 2020), but the potential interaction of signalling pathways governing these traits and
376 their combined effects on plant stomatal conductance, water use efficiency, and growth
377 have not been addressed. Here we combined mutations in *EPF1* and *EPF2* that affect
378 stomatal density and in *HT1* and *CYP707A1/A3* that affect stomatal sensitivity under
379 normal and low RH conditions to address these questions. While the studied mutations
380 that affect stomatal density and sensitivity mostly acted independently (Figures 2, 4, 5,
381 Tables 2, 3), mutations in the *EPF1* gene stood out, as in the high stomatal sensitivity
382 backgrounds *epf1* alone did not increase stomatal density (Figure 5, Table 3). EPF2 can
383 partially replace EPF1 function but not vice versa (Hara *et al.*, 2009); it is possible that the

384 high stomatal sensitivity background stimulates *EPF2* expression or activity in the *epf1*
385 single mutants, leading to normal stomatal densities. It has been proposed that a
386 combination of multiple traits could be necessary for engineering more water use efficient
387 and productive crops (Flexas, 2016). Our results suggest that combining traits that affect
388 stomatal density and sensitivity could be one approach, as these traits affect stomatal
389 conductance independently. Given the negative relationship between stomatal density and
390 plant growth (Figure 11a), optimisation of stomatal density and sensitivity trait
391 combinations is needed for practical applications.

392 Our experiments showed an increase in stomatal ratio in response to low air humidity
393 (Figure 8, Table 3). Allocating more stomata to the adaxial epidermis under dry air
394 conditions may seem counterintuitive, as excessive water loss via adaxial stomata has
395 been proposed as one of the costs of adaxial stomata in plants (Buckley *et al.*, 2015;
396 Drake *et al.*, 2019). Yet, a similar increase in stomatal ratio under dry air was found also in
397 several cotton varieties (Devi and Reddy, 2018), whereas amphistomatous plant species
398 are common in dry habitats (Wood, 1934; Muir, 2015; Bucher *et al.*, 2017) and tend to
399 have faster stomatal responses than hypostomatous plants (Haworth *et al.*, 2018). It is
400 possible that high stomatal ratios allow plants to make better use of favourable conditions
401 when they arise, by increasing CO_2 uptake capacity (Mott *et al.*, 1982; Muir, 2018; Xiong
402 and Flexas, 2020). At the same time, densely packed stomata are typically smaller and
403 faster than sparse stomata (Drake *et al.*, 2013), ensuring fast and efficient restriction of
404 water loss via speedy stomatal closure upon water stress. In line with this, we found
405 smaller stomata with potential for faster stomatal responses in plants grown under low RH
406 (Table 3, Figure 7). In our experiments, the negative relationship between stomatal density
407 and size that is typically found in plants (Franks and Beerling, 2009; Caine *et al.*, 2023)
408 was weakly present only in the adaxial leaf surface that had lower stomatal density than
409 abaxial leaf surface, where no such relationship was found (Figure S3, Figure 6). These
410 data suggest that the often observed negative relationship between stomatal density and
411 size is not due to physical limitations of packing stomata into the epidermis but may have
412 other underlying causes.

413 How plants adjust stomatal ratio in response to changes in environmental conditions is not
414 clear. The pronounced increase in stomatal ratio under low RH in the *epf1* single mutant
415 backgrounds suggests that EPF1 as a negative regulator of stomatal development
416 preferentially suppresses stomatal production in the adaxial epidermis in response to a
417 decrease in relative air humidity (Figure 8). EPF2 peptide acts on an earlier step in the

418 stomatal differentiation pathway compared with EPF1; it is possible that its absence in the
419 *epf1epf2* double mutant suppresses the effects of *epf1* mutation, leading to no significant
420 increase in stomatal ratio under low RH in the *epf1epf2* double mutant (Figure 8). On the
421 other hand, the *epf1* and *epf2* mutations are additive (Figure 5), indicating that they can
422 largely replace the function of each other. The leaf-side specific behaviour of EPFs and
423 other stomatal regulators has not been addressed, but given that silencing of *SPCH*
424 expression or overexpression of a *SPCH* repressor led to preferential loss of abaxial
425 stomata (Dow *et al.*, 2014; Qi *et al.*, 2019), it is likely that signalling events that govern
426 adaxial and abaxial stomatal formation are regulated independently and at least partly via
427 different mechanisms. The latter hypothesis is also supported by our recent study of
428 adaxial and abaxial stomatal development patterns in plants deficient in known stomatal
429 regulators (Jalakas *et al.*, 2024). Indeed, across-species studies indicate independent
430 evolution of adaxial and abaxial stomatal densities with a greater flexibility of adaxial SD
431 (Muir *et al.*, 2023), in line with more prominent increases in adaxial SD in response to low
432 air humidity (Figure 5). Further studies of stomatal ratio and its responses to changes in
433 environment in different mutants of stomatal patterning, environmental and hormonal
434 signalling pathways are needed to understand how stomatal ratio adjustments occur in
435 plant development.

436 In our experiments we found that relative air humidity and high stomatal density
437 independently impair plant growth (Table 4, Figure 10, Figure 11). We anticipated a larger
438 negative effect of low RH in plant lines with increased stomatal conductance that we
439 presumed would suffer from water stress. It is well known that drought induces preferential
440 resource allocation into roots, leading to reduced above-ground growth (Eziz *et al.*, 2017)
441 and it is possible that dry air would trigger a similar outcome. In our experiment, low RH
442 was uniformly detrimental to all genotypes, despite compensation of high stomatal
443 conductance caused by high stomatal density of the *epf1epf2* mutations by the increased
444 stomatal sensitivity mutations *ht1-2* and *cyp707a1/a3* (Figures 2, 4, 5). It is possible that
445 efficient stomatal closure under low RH compensated for increased stomatal densities in
446 our experiment (Figure 3b). Indeed, mutants with high stomatal density can efficiently
447 close stomata in response to changes in environmental conditions (Dow *et al.*, 2014;
448 Hepworth *et al.*, 2015). However, in our recent study, growth under low RH was not more
449 impaired in the *ost1* mutant with large stomatal conductance than in wild-type plants (Tulva
450 *et al.*, 2023). As the *ost1* mutant is deficient in stomatal closure in response to low
451 humidity, the lack of stronger growth impairment than in wild-type plants cannot be

452 explained by stomatal closure in response to low RH. Thus, hampered plant growth under
453 low RH seems to occur independently of stomatal traits.

454 Plants with high stomatal densities were stunted in growth irrespective of conditions
455 (Figures 10, 11, Table 4), indicating that excess stomatal development impairs plant
456 growth during the first 4 weeks in Arabidopsis. Similar impairments have been found
457 before in mutants with high SD (Doheny-Adams *et al.*, 2012; Lawson *et al.*, 2014),
458 although other studies have reported increased photosynthesis, carbon gain or biomass
459 production in high stomatal density mutants (Schlüter *et al.*, 2003; Tanaka *et al.*, 2013;
460 Sakoda *et al.*, 2020). In this study, we saw that higher stomatal density was indeed
461 associated with increased net photosynthesis across plant lines, but resulted in reduced
462 above-ground growth nonetheless (Table S2), indicating stronger investment of
463 photosynthetic products into processes other than leaf growth in plant lines with higher
464 stomatal density. Hepworth *et al*. (2016) showed a strong positive correlation between
465 stomatal density and root growth, possibly associated with increased water demand due to
466 high stomatal conductance. An increased investment into root production in early
467 development in high stomatal density mutants could potentially lead to delayed above-
468 ground growth. This mechanism, however, is not easy to reconcile with the absence of
469 similar growth retardation in the high stomatal conductance *ost1-3* mutant in Tulva *et al*.
470 (2023).

471 The negative relationship between stomatal density and plant growth may be explained by
472 increased porosity due to higher numbers of substomatal cavities and decreased amounts
473 of photosynthetic tissue in high density mutants. Dow et al. (2017) showed that mesophyll
474 porosity increases together with stomatal density in Arabidopsis lines; similar coupling
475 between stomatal and mesophyll anatomy was shown in wheat (Lundgren *et al.*, 2019).
476 Given the negative relationship between stomatal density and plant growth, but not overall
477 leaf stomatal numbers and plant growth (Figure 11), it is possible that the proportion of leaf
478 tissue dedicated to non-stomatal structures that is larger in plant lines with lower stomatal
479 density contributes to their improved growth. Alternatively, the lack of significant correlation
480 between stomatal count and plant size (Figure 11b) may reflect different expansion growth
481 due to differences in energy allocation into cell divisions, leading to a negative relationship
482 between stomatal density and plant size (Figure 11a). Leaf construction cost is strongly
483 positively related with LMA (Mizokami *et al.*, 2022), hence leaves with higher LMA values
484 should be more expensive for the plant to build. Indeed, we found a positive relationship
485 between LMA and stomatal density (Figure S5a), potentially suggesting larger construction

costs of leaves with high stomatal densities. At the same time, LMA was negatively related with dry mass only under low RH but not under normal conditions (Figure S5b), suggesting that LMA as a proxy for leaf construction costs alone does not explain the reduced growth of plants with higher stomatal numbers irrespective of growth humidity (Figure 11a). More detailed analyses of leaf anatomy, including mesophyll characteristics, are needed to understand the mechanism of growth reduction in high stomatal density mutants.

It is worth noting that studies that find adverse effects of high stomatal density, e.g. Doheny-Adams et al. (2012) and this study, work with constant artificial lighting, as is common in indoor growth chambers. On the other hand, studies reporting positive relationship between plant growth and stomatal density or conductance often find such effects under fluctuating light conditions (Kimura *et al.*, 2020; Sakoda *et al.*, 2020), where the growth improvement probably results from immediately high stomatal conductance upon step increase in light availability, thereby reducing resistance to CO_2.

The formation of stomata has been associated with developmental and metabolic costs, whereas cell division costs have been considered larger than cell expansion and differentiation costs (Dow *et al.*, 2014; Franks *et al.*, 2015; Bowers, 2018). It is possible that a stoma, as a derivative of a protodermal cell through multiple divisions and differentiations, has a greater construction cost associated with it than pavement cells. Indeed, in this study, the high-SD associated size differences were already present in day 0 when stomata must have been differentiated, and there was no difference in relative growth rate throughout 3rd and 4th growth week. The energy-consuming process of stomatal formation might lead to growth impairment in high stomatal density mutants at a young age, whereas this deficiency may well be compensated by leaf expansion in later growth stages. Then, high stomatal numbers that potentially mediate increased CO_2 uptake may pay off in improved photosynthesis and biomass production and/or yield. For example, high stomatal conductance in Arabidopsis cauline leaves was associated with higher seed yield, similar to flag leaves in rice (Ding *et al.*, 2023). Further studies addressing the effects of altered stomatal densities on plant growth, biomass production and yield in later developmental stages are needed to assess the potential benefits of increased stomatal density.

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727

728 **Table 1.** Plant lines studied, their short names used in this article, and levels of the factors
729 used in ANOVA.

Line	Short name	sensitivity	epf1	epf2
Col-0	Col-0	wt	0	0
ht1-2	*ht1-2*	ht1	0	0
cyp707a1-1 cyp707a3	*cyp707a1/a3*	cyp	0	0
epf1-1	*epf1*	wt	1	0
ht1-2 epf1-1	*ht1-2epf1*	ht1	1	0
cyp707a1-1 cyp707a3 epf1-1	*cyp707a1/a3epf1*	cyp	1	0
epf2-2	*epf2*	wt	0	1
ht1-2 epf2-2	*ht1-2epf2*	ht1	0	1
cyp707a1-1 cyp707a3 epf2-2	*cyp707a1/a3epf2*	cyp	0	1
epf1-1 epf2-2	*epf1/2*	wt	1	1
ht1-2 epf1-1 epf2-2	*ht1-2epf1/2*	ht1	1	1
cyp707a1-1 cyp707a3 epf1-1 epf2-2	*cyp707a1/a3epf1/2*	cyp	1	1

730

731

732 **Table 2**. P-values of ANOVA on steady-state gas exchange experiment. g_l, leaf
733 conductance; A_{net}, net assimilation; WUE, intrinsic water use efficiency (=A_{net}/g_l); SD,
734 stomatal density. Single EPF mutants were not included in this experiment; the factor
735 epf1/2 encodes double mutant in *EPF1* and *EPF2* genes vs wild-type. RH, growth air
736 humidity (60 % or 40 % at daytime); sensitivity encodes the state of stomatal reaction
737 genes (*ht1-2* mutant, *cyp707a1/a3* double mutant, or wild-type). P-values < 0.05 shown in
738 bold.
739

	g_l (growth RH)	A_n (growth RH)	intrWUE (growth RH)	SD abaxial	SD adaxial
RH	**0.00**	0.08	**0.00**	**0.00**	**0.00**
epf1/2	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
sensitivity	**0.00**	**0.02**	**0.00**	**0.00**	0.34
RH*epf1/2	**0.00**	0.92	0.72	0.22	**0.02**
RH*sensitivity	**0.01**	0.93	0.69	0.23	0.18
epf1/2*sensitivity	0.11	0.65	**0.00**	**0.00**	0.66
RH*epf1/2*sensitivity	**0.01**	**0.00**	0.19	0.17	0.24

740

741 **Table 3**. P-values of ANOVA on the growth experiment stomatal data (collected on days 15-16 after treatment start = 29-30 after sowing). P-
742 values < 0.05 shown in bold. SD, stomatal density; RH, growth air humidity; epf1, state of the *EPF1* gene (wild-type or mutant), epf2 likewise;
743 sensitivity encodes the state of stomatal reaction genes (*ht1-2* mutant, *cyp707a1/a3* double mutant, or wild-type).

Factor	SD abaxial	SD adaxial	SD sum	Stomatal count abaxial	Stomatal count adaxial	Stomatal count sum	Stomatal ratio	Stomatal length abaxial	Stomatal length adaxial
RH	0.06	**0.00**	**0.00**	**0.00**	0.14	**0.04**	**0.00**	**0.00**	**0.00**
epf1	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.31	0.77	0.05
epf2	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.69	0.07	0.20
sensitivity	**0.00**	0.16	**0.00**	**0.00**	**0.00**	**0.00**	0.23	0.58	**0.01**
RH*epf1	0.79	**0.02**	0.67	0.28	0.10	0.77	0.11	0.20	0.60
RH*epf2	0.38	0.22	0.29	0.63	0.70	0.58	0.87	0.94	0.62
epf1*epf2	**0.00**	**0.02**	**0.00**	0.27	0.12	0.82	**0.01**	0.92	0.46
RH*sensitivity	0.49	0.53	0.97	0.49	0.84	0.81	0.37	**0.03**	0.51
epf1*sensitivity	**0.00**	**0.03**	**0.00**	**0.00**	**0.00**	**0.00**	0.29	0.15	0.20
epf2*sensitivity	0.20	0.28	0.11	0.34	0.15	0.05	0.36	0.70	0.19
RH*epf1*epf2	0.47	0.38	0.50	0.74	0.52	0.71	0.92	0.81	0.54
RH*epf1*sensitivity	0.19	0.82	0.41	0.22	0.66	0.47	0.22	0.37	**0.03**
RH*epf2*sensitivity	0.57	0.29	0.93	0.99	0.11	0.72	0.12	0.88	0.76
epf1*epf2*sensitivity	0.98	0.09	0.66	0.89	**0.01**	0.31	0.45	0.13	0.09
RH*epf1*epf2*sensitivity	0.16	0.90	0.40	0.17	0.93	0.35	0.58	0.36	0.91

744

745 **Table 4**. P-values of ANOVA on the growth experiment harvest data (collected on days 15-16 after
746 treatment start = 29-30 after sowing), as well as projected plant areas on the day treatment started
747 (day 0) and the last growth day (day 14; 14 and 28 days after sowing resp.). P-values < 0.05
748 shown in bold. LMA, leaf mass per area; RH, growth air humidity; epf1, state of the *EPF1* gene
749 (wild-type or mutant), epf2 likewise; sensitivity encodes the state of stomatal reaction genes (*ht1-2*
750 mutant, *cyp707a1/a3* double mutant, or wild-type).
751

Factor	area day 0	area day 14	dry mass	LMA	water content
RH	0.06	**0.00**	**0.00**	**0.00**	**0.00**
epf1	0.96	**0.00**	0.13	**0.01**	**0.00**
epf2	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
sensitivity	0.05	**0.00**	**0.00**	0.18	0.11
RH*epf1	0.59	0.89	0.76	0.89	0.25
RH*epf2	0.98	0.97	0.41	0.53	0.58
epf1*epf2	**0.01**	**0.00**	**0.01**	0.30	0.41
RH*sensitivity	0.77	**0.03**	0.48	0.34	0.36
epf1*sensitivity	0.26	**0.03**	0.15	0.96	0.09
epf2*sensitivity	0.78	0.06	0.74	0.09	0.55
RH*epf1*epf2	0.39	0.53	0.52	0.53	0.82
RH*epf1*sensitivity	0.69	0.16	0.63	0.98	0.86
RH*epf2*sensitivity	0.09	0.07	0.13	0.73	0.72
epf1*epf2*sensitivity	0.11	0.36	0.15	**0.04**	0.97
RH*epf1*epf2*sensitivity	0.72	0.42	0.88	0.61	0.77

752

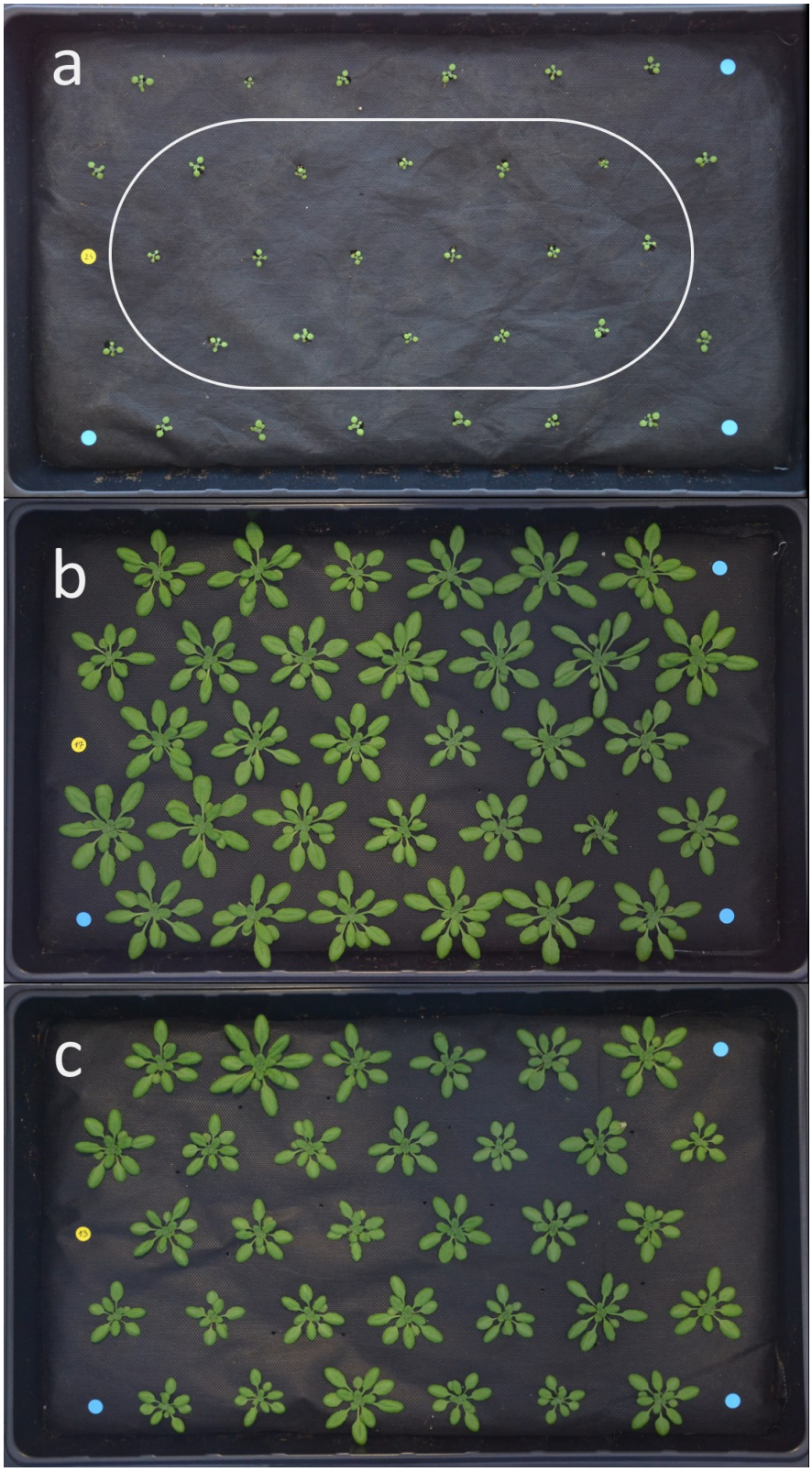
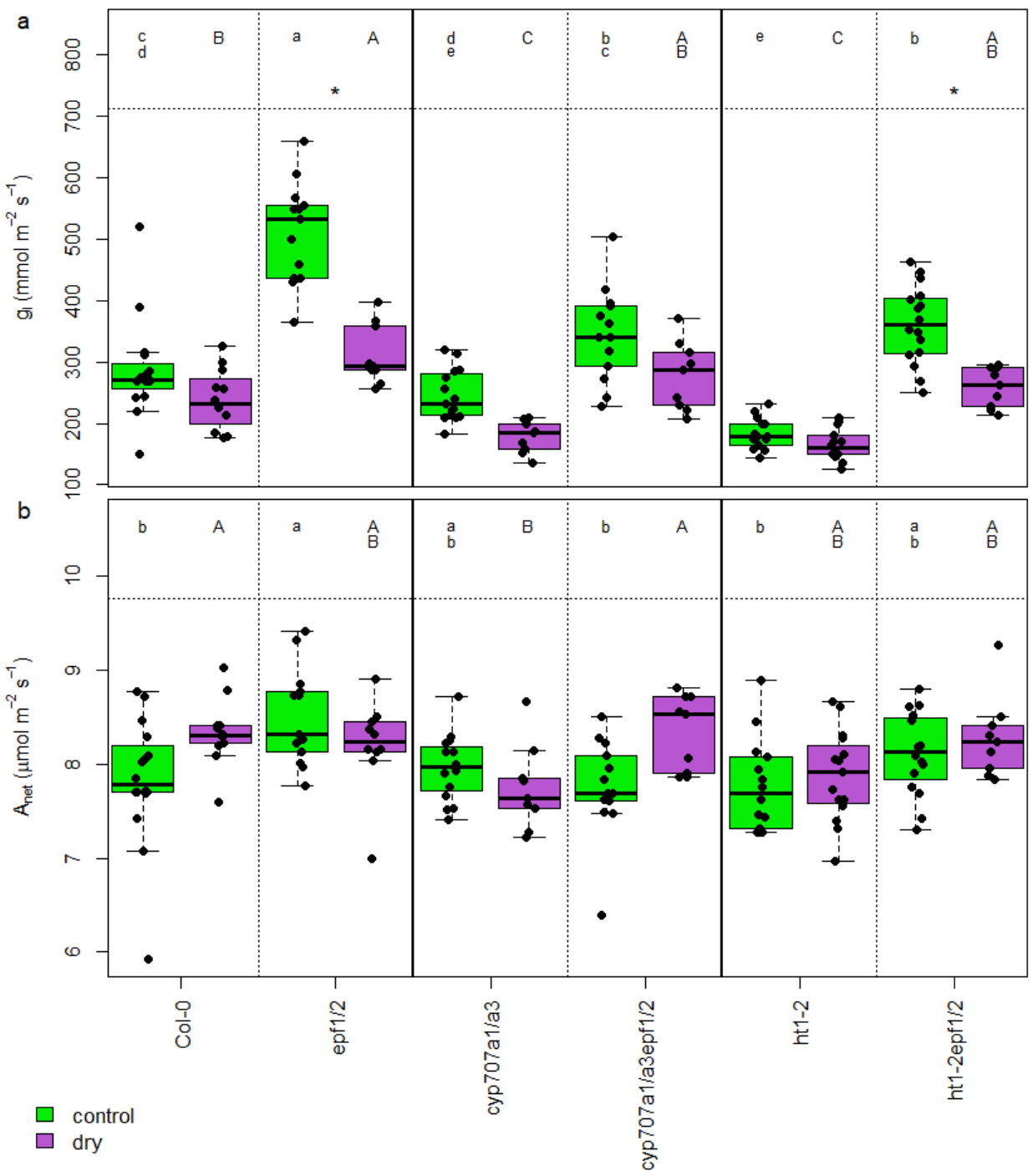


Figure 1. Layout of the trays in the growth experiment. (a), an example tray on Day 0 (14th day after sowing). The region surrounded by the oval included the studied plants in randomised positions, others outside were Col-0. (b), an example control tray on Day 14; (c), an example low-RH tray on Day 14.



759
760 **Figure 2**. Leaf conductance to water vapour (a) and net photosynthesis (b), measured with RH in
761 the cuvette equal to the growth RH for each plant. Green boxes, normal growth RH; magenta
762 boxes, low RH. The boxes span from the first to the third quartile, with median indicated with the
763 horizontal line; the whiskers span the non-outlier range. Individual plants shown as solid dots.
764 Asterisk above a pair of boxes indicates significant difference within that genotype according to
765 Tukey's HSD (ANOVA involving all 24 treatment and genotype combinations). Shared lower case
766 letters above the normal RH boxes indicate no significant difference according to Tukey HSD test
767 (ANOVA involving only normal RH treatment); shared capital letters above the low RH boxes
768 likewise for low RH treatment.

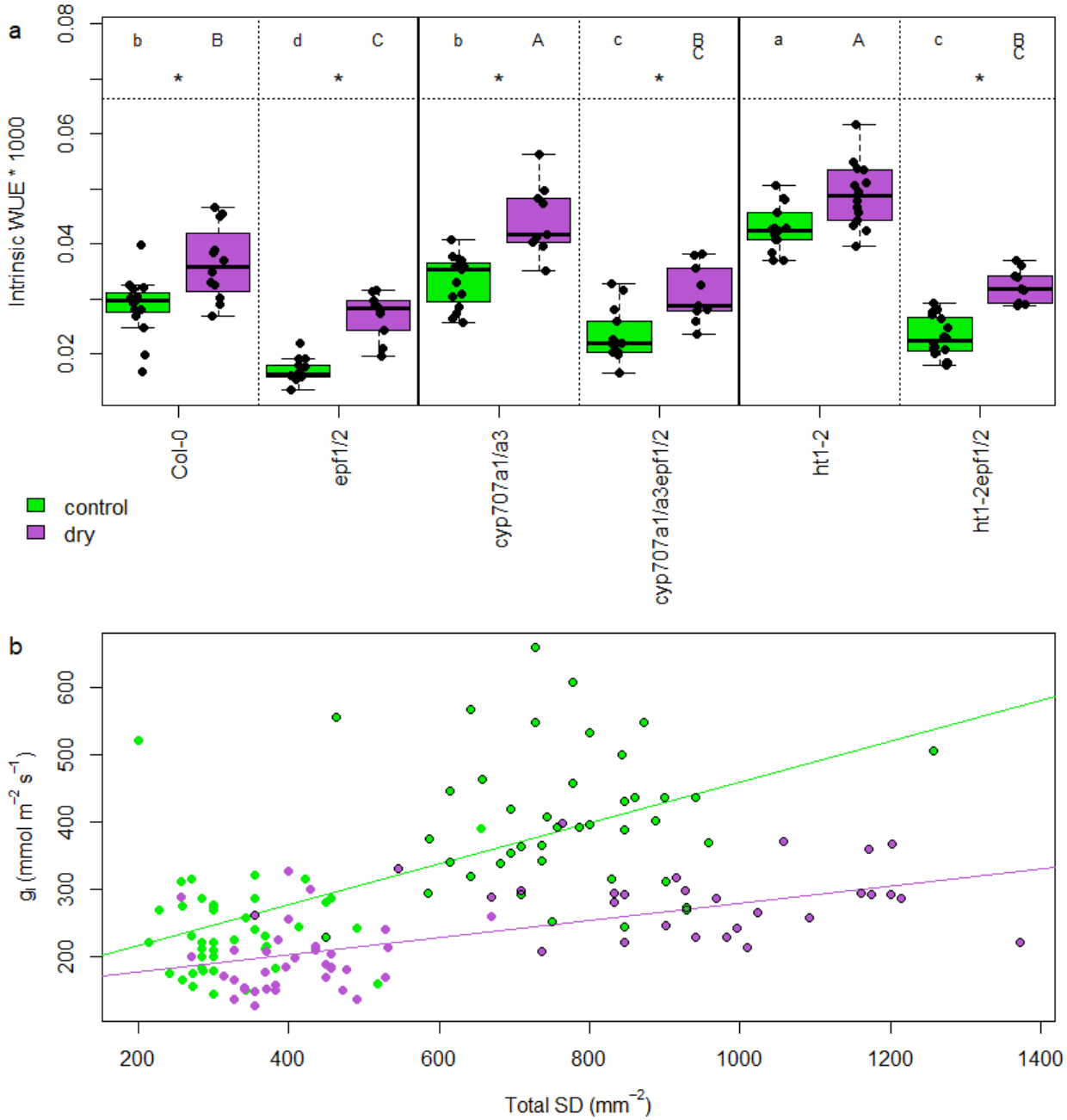


Figure 3. (A) Intrinsic water use efficiency in the gas exchange experiment, measured with RH in the cuvette equal to the growth RH for each plant. Green boxes, normal growth RH; magenta boxes, low RH. The boxes span from the first to the third quartile, with median indicated with the horizontal line; the whiskers span the non-outlier range. Individual plants shown as solid dots. Asterisk above a pair of boxes indicates significant difference within that genotype according to Tukey's HSD (ANOVA involving all 24 treatment and genotype combinations). Shared lower case letters above the normal RH boxes indicate no significant difference according to Tukey HSD test (ANOVA involving only normal RH treatment); shared capital letters above the low RH boxes

780 likewise for that treatment. (b) Relationship between stomatal density and leaf conductance at
781 growth conditions. Each dot is one plant; the plants with the *epf1/2* double mutation are circled.
782 Slopes of the linear regressions differ significantly from zero ($p<0.05$) and from each other
783 ($p<0.001$).

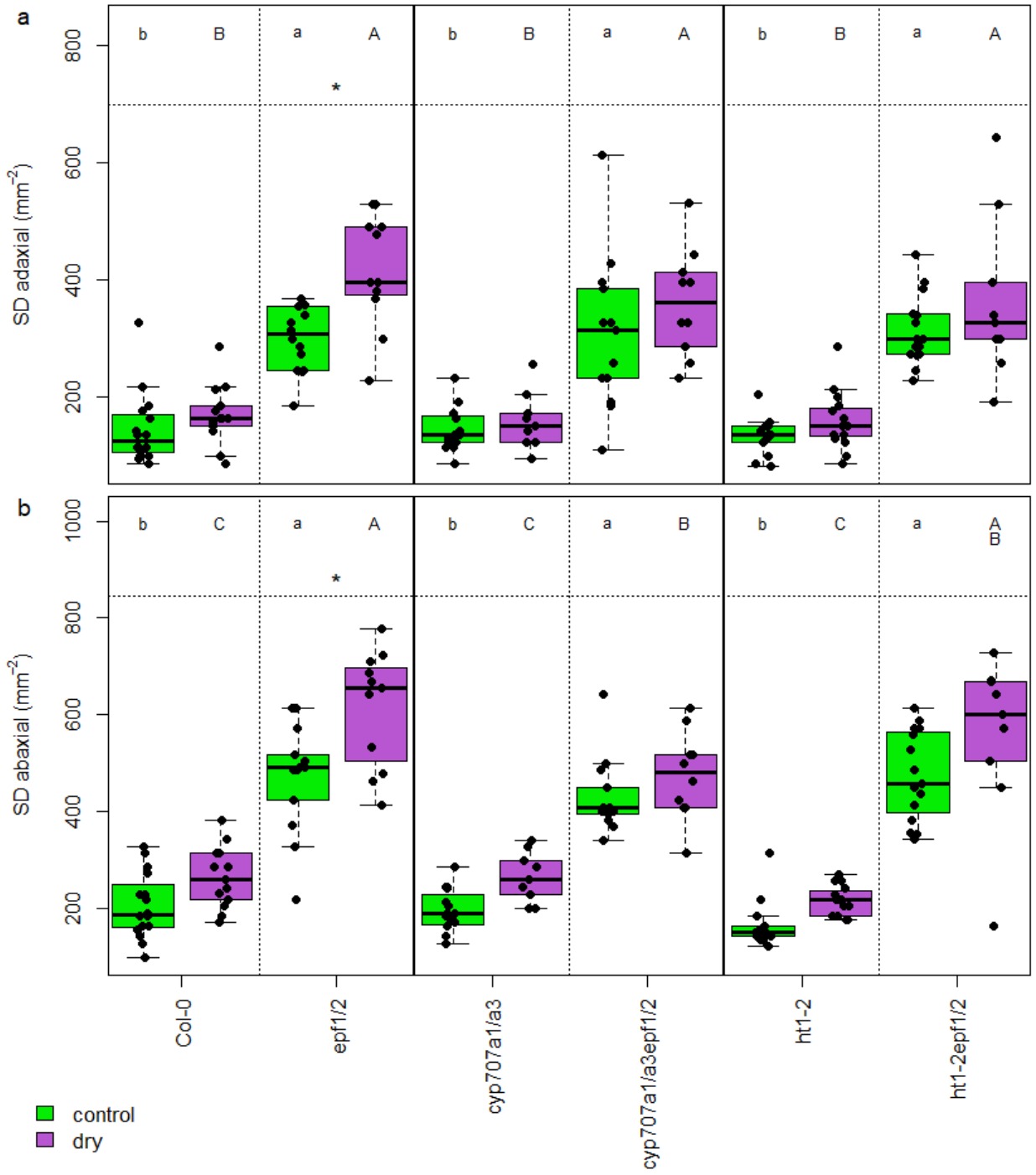


Figure 4. Adaxial (a) and abaxial (b) stomatal densities in the gas exchange experiment. Green boxes, normal growth RH; magenta boxes, low RH. The boxes span from the first to the third quartile, with median indicated with the horizontal line; the whiskers span the non-outlier range. Individual plants shown as solid dots. Asterisk above a pair of boxes indicates significant difference within that genotype according to Tukey's HSD (ANOVA involving all 24 treatment and genotype combinations). Shared lower case letters above the normal RH boxes indicate no significant difference according to Tukey HSD test (ANOVA involving only normal RH treatment); shared capital letters above the low RH boxes likewise for that treatment.

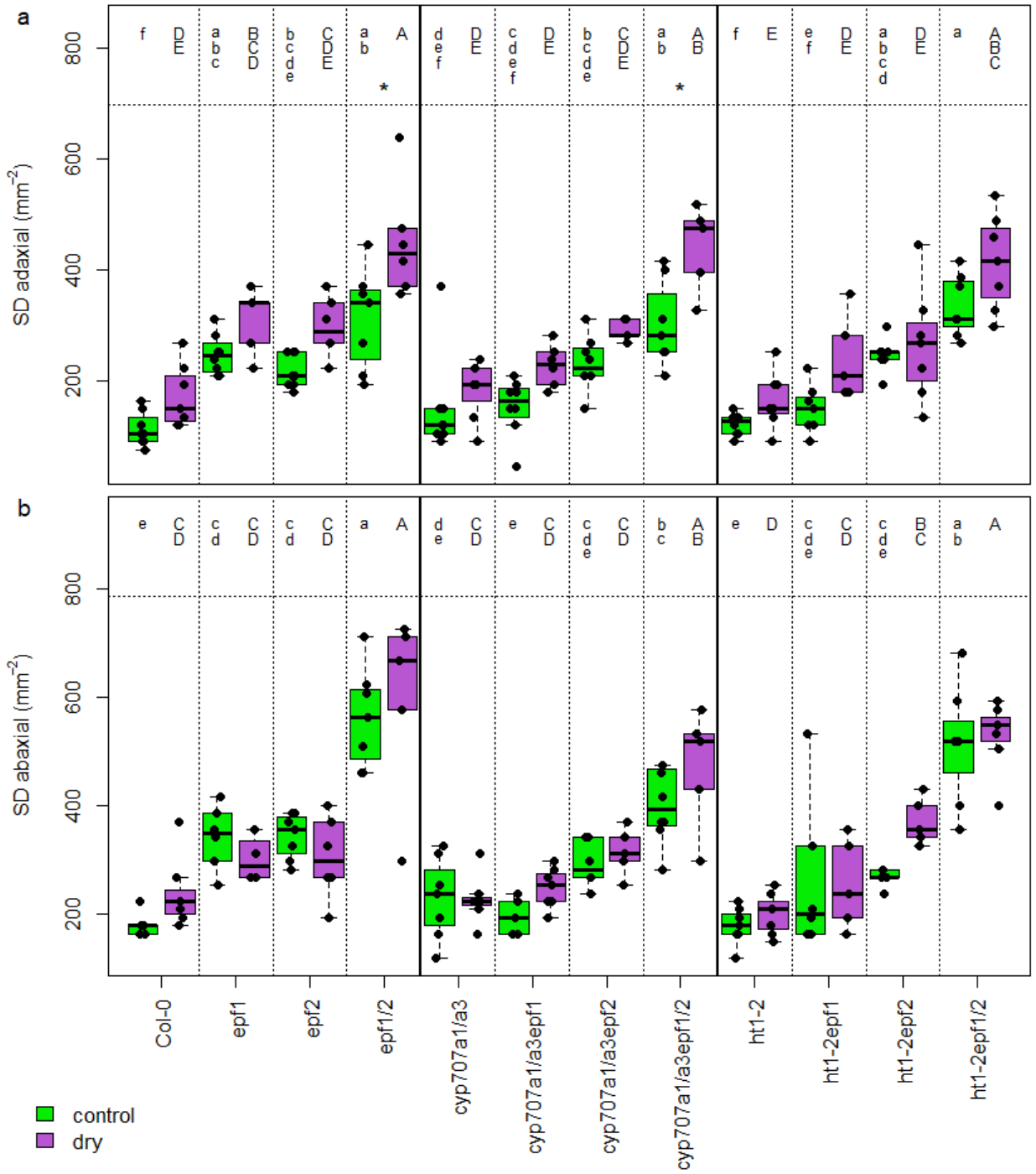


Figure 5. Stomatal density at the adaxial (a) and abaxial (b) leaf surface in the growth experiment. Green boxes, normal growth RH; magenta boxes, low RH. The boxes span from the first to the third quartile, with median indicated with the horizontal line; the whiskers span the non-outlier range. Individual plants shown as solid dots. Asterisk above a pair of boxes indicates significant difference within that genotype according to Tukey's HSD (ANOVA involving all 24 treatment and genotype combinations). Shared lower case letters above the normal RH boxes indicate no significant difference according to Tukey HSD test (ANOVA involving only normal RH treatment); shared capital letters above the low RH boxes likewise for that treatment.

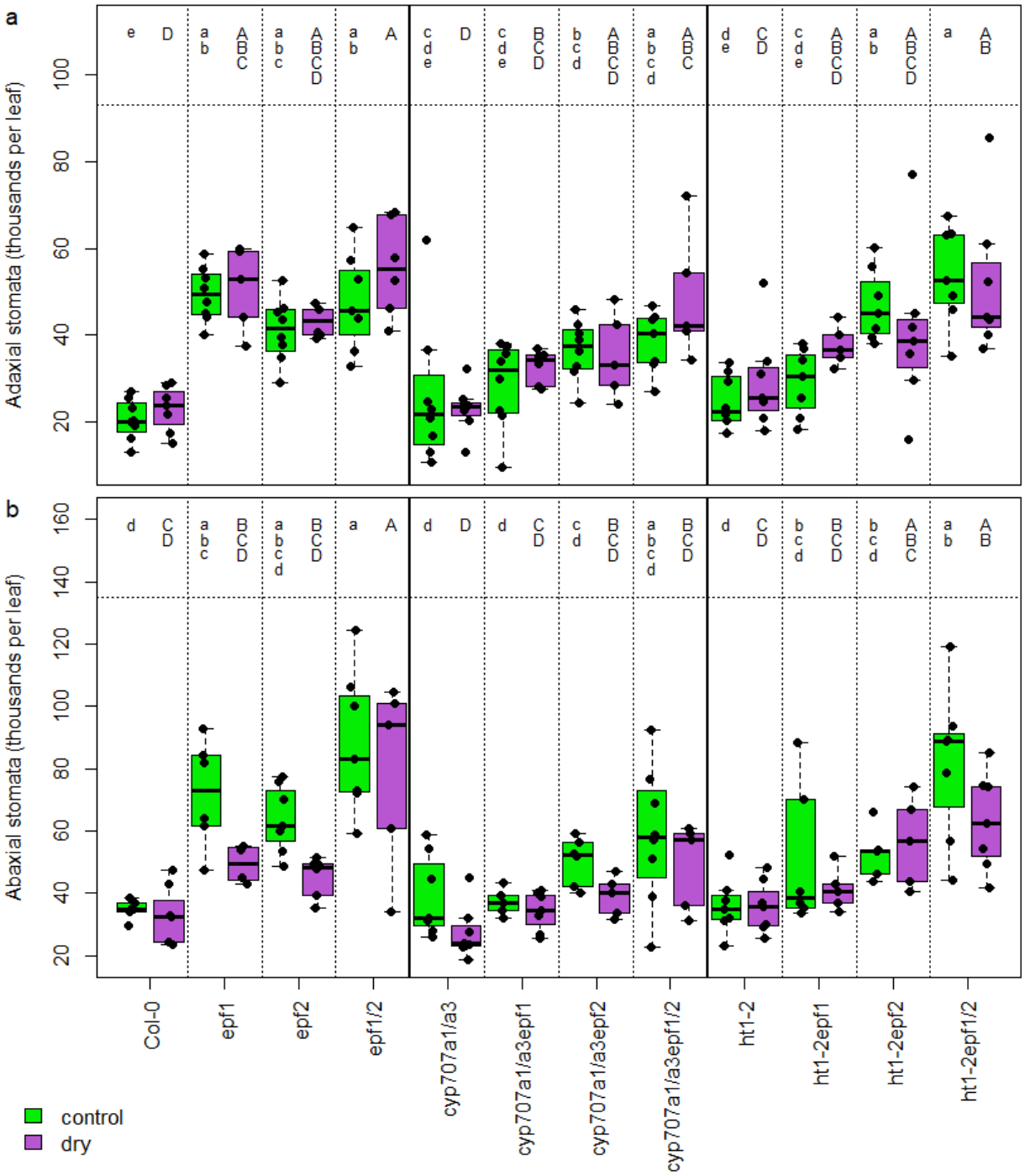


Figure 6. Stomatal count on the adaxial (a) and abaxial (b) leaf surface in the growth experiment. Green boxes, normal growth RH; magenta boxes, low RH. The boxes span from the first to the third quartile, with median indicated with the horizontal line; the whiskers span the non-outlier range. Individual plants shown as solid dots. Asterisk above a pair of boxes indicates significant difference within that genotype according to Tukey's HSD (ANOVA involving all 24 treatment and genotype combinations). Shared lower case letters above the normal RH boxes indicate no significant difference according to Tukey HSD test (ANOVA involving only normal RH treatment); shared capital letters above the low RH boxes likewise for that treatment.

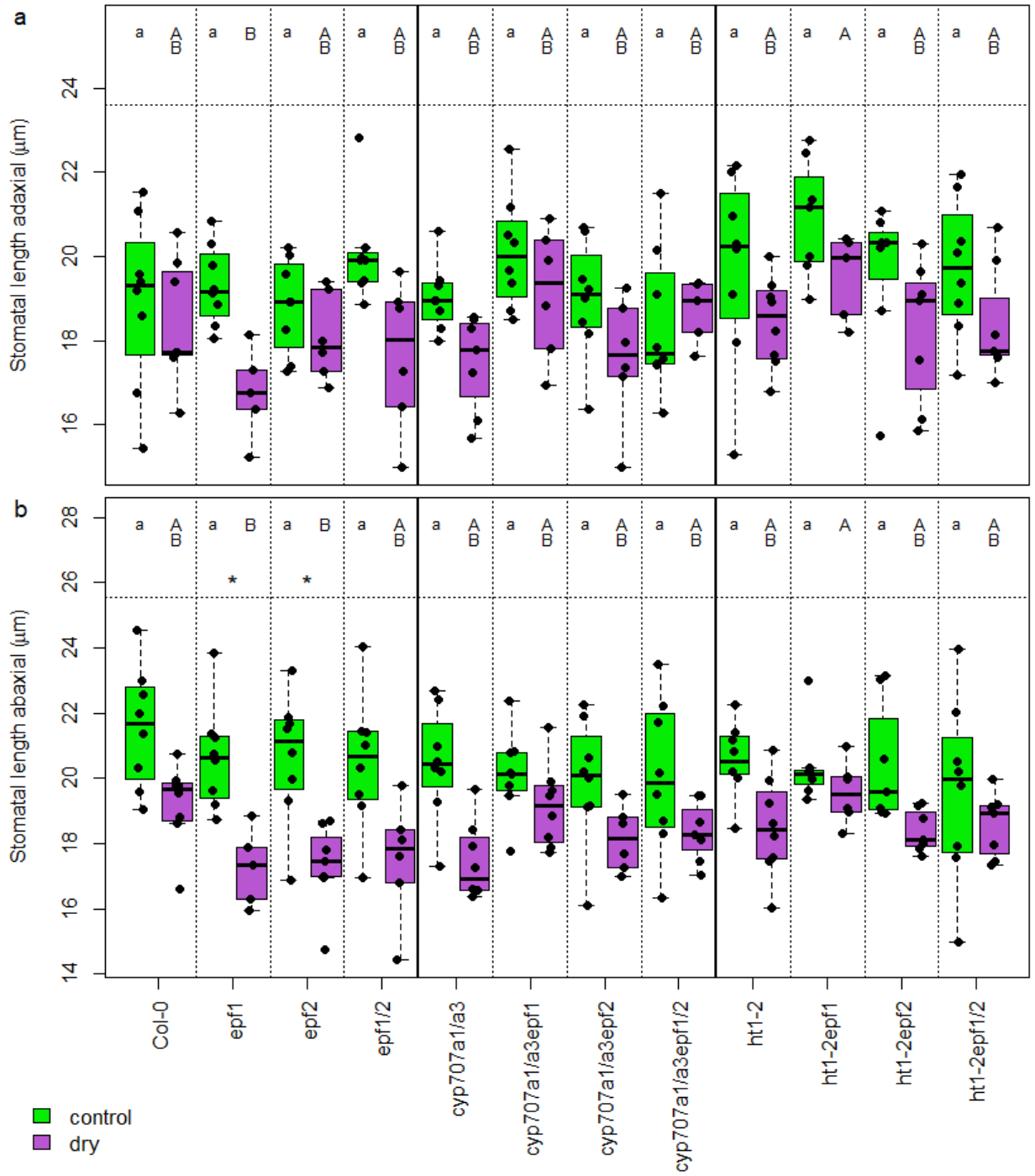


Figure 7. Stomatal length on the adaxial (a) and abaxial (b) leaf surface in the growth experiment. Green boxes, normal growth RH; magenta boxes, low RH. The boxes span from the first to the third quartile, with median indicated with the horizontal line; the whiskers span the non-outlier range. Individual plants shown as solid dots. Asterisk above a pair of boxes indicates significant difference within that genotype according to Tukey's HSD (ANOVA involving all 24 treatment and genotype combinations). Shared lower case letters above the normal RH boxes indicate no significant difference according to Tukey HSD test (ANOVA involving only normal RH treatment); shared capital letters above the low RH boxes likewise for that treatment.

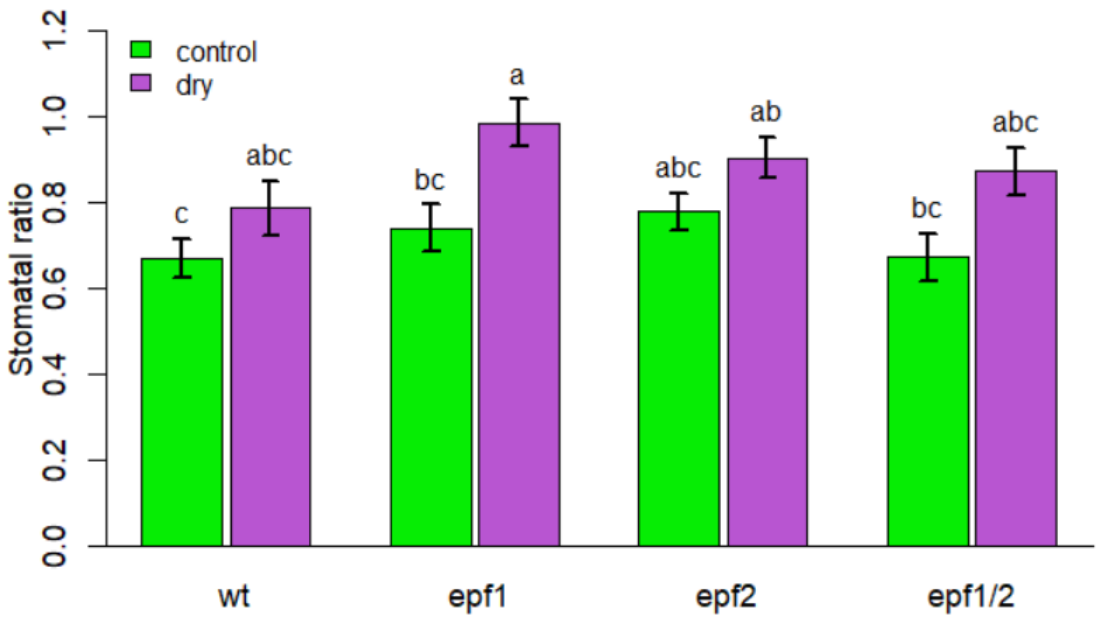


Figure 8. Comparison of stomatal ratio (adaxial/abaxial) in the growth experiment depending on the state of *EPF1* or *EPF2.* Stomatal sensitivity mutations, showing no significant effect on stomatal ratio (Table 3), are pooled in this data - see Figure S4 for detailed dissection. Green bars, normal growth RH; magenta bars, low RH. Shared letter(s) above the bars denote no significant difference according to Tukey HSD test (α=0.05).

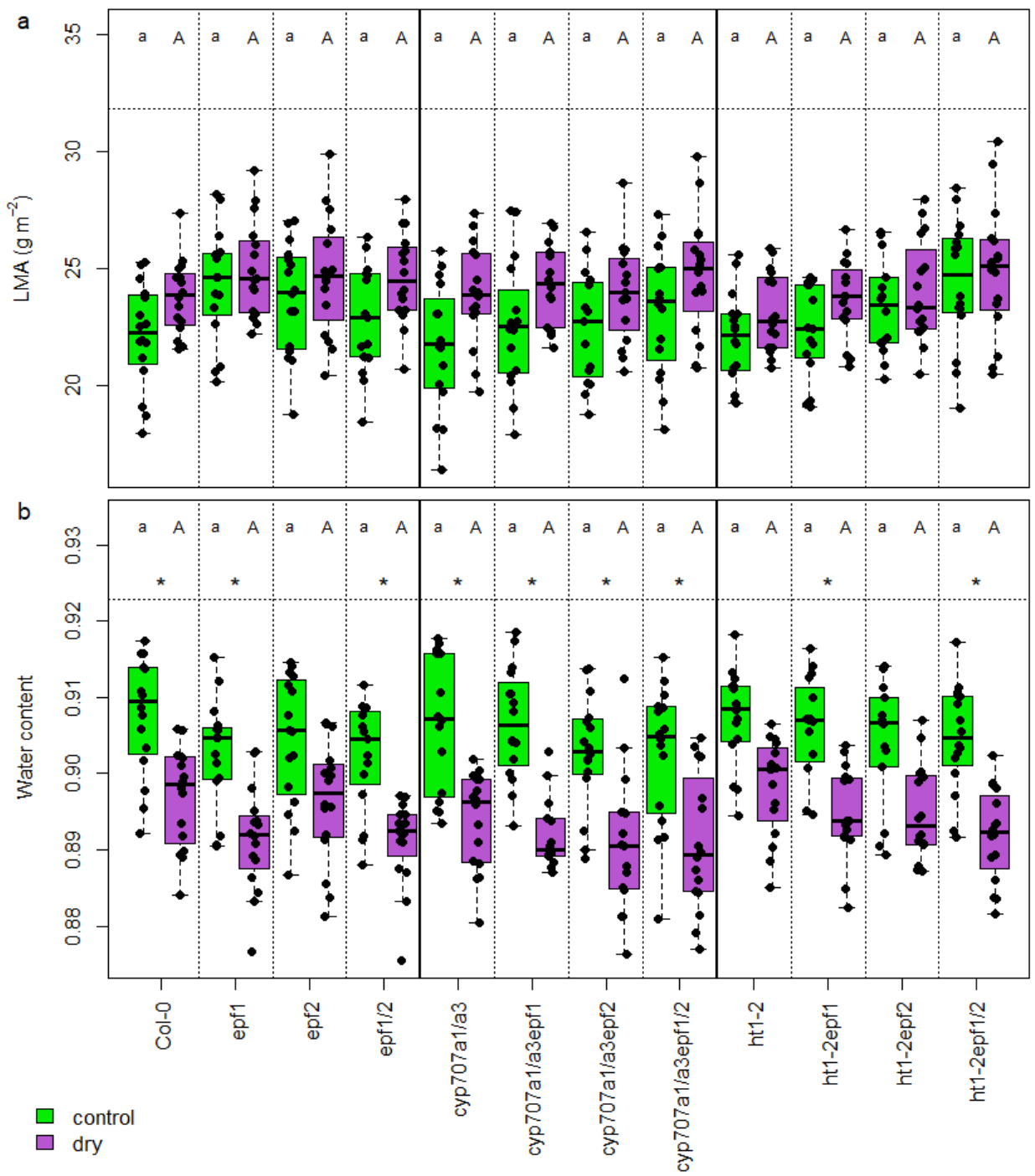


Figure 9. Harvest-time leaf mass / area ratio (a) and water content (b). Green boxes, normal growth RH; magenta boxes, low RH. The boxes span from the first to the third quartile, with median indicated with the horizontal line; the whiskers span the non-outlier range. Individual plants shown as solid dots. Asterisk above a pair of boxes indicates significant difference within that genotype according to Tukey's HSD (ANOVA involving all 24 treatment and genotype combinations). Shared lower case letters above the normal RH boxes indicate no significant difference according to Tukey HSD test (ANOVA involving only normal RH treatment); shared capital letters above the low RH boxes likewise for that treatment.

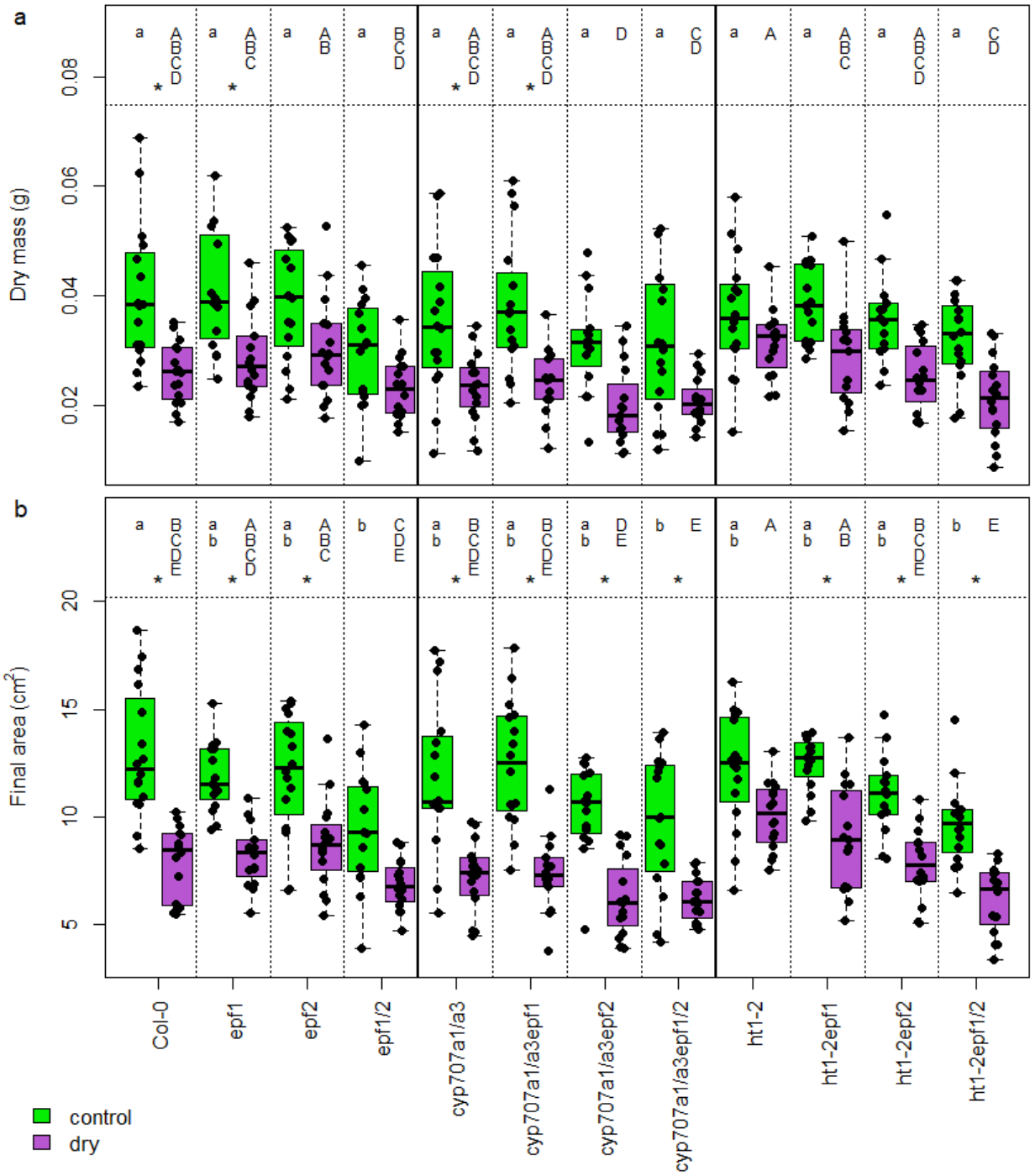


Figure 10. Plant above-ground dry mass (29-30 days old; a) and projected area (28 days old; b). Green boxes, normal growth RH; magenta boxes, low RH. The boxes span from the first to the third quartile, with median indicated with the horizontal line; the whiskers span the non-outlier range. Individual plants shown as solid dots. Asterisk above a pair of boxes indicates significant difference within that genotype according to Tukey's HSD (ANOVA involving all 24 treatment and genotype combinations). Shared lower case letters above the normal RH boxes indicate no significant difference according to Tukey HSD test (ANOVA involving only normal RH treatment); shared capital letters above the low RH boxes likewise for that treatment.

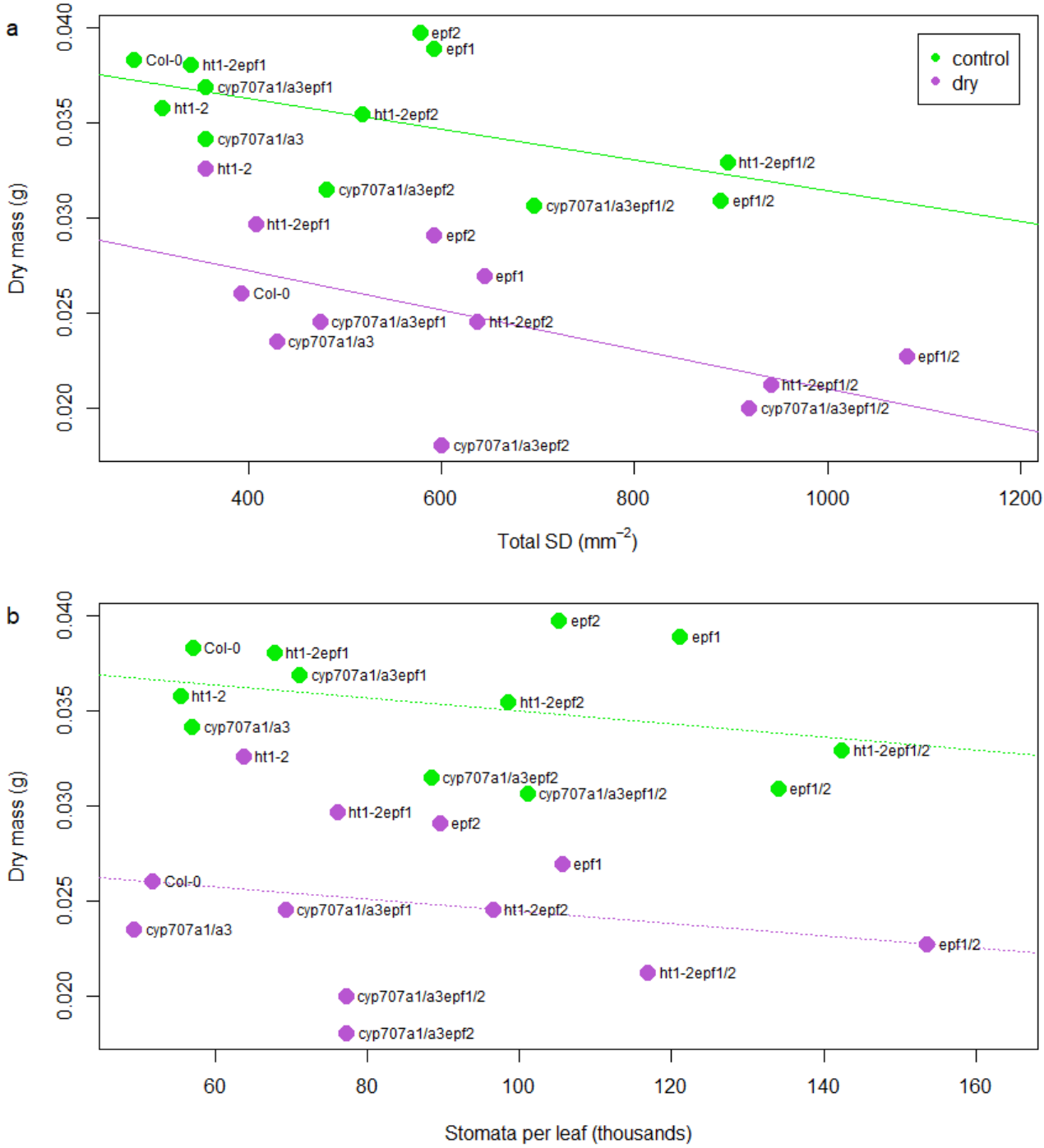


Figure 11. Dependence of above-ground dry mass (29-30 days old) on total stomatal density of leaf 6 (sum of both leaf sides; a) or estimated total number of stomata on leaf 6 (b). Green markers, normal growth RH; magenta markers, low RH. Each dot indicates the median values (in both dimensions) of the shown plant line in given growth conditions. The slopes of the linear regression lines in (a), themselves all significantly different from zero at P<0.01, do not differ significantly from each other between growth conditions. The slopes in (b) do not differ significantly from 0.