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.

Introduction

Vascular plant stomata have evolved to balance CO_2 supply for photosynthesis and water loss via transpiration (Lawson & 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 & Sinclair 1989; Anderson & Briske 1990; Pearce, Millard, Bray & Rood 2006; Carlson, Adams & Holsinger 2016), although it can largely be counteracted with stomatal closure (Büssis *et al.* 2006; Dow, Berry & Bergmann 2014; Hepworth, Doheny-Adams, Hunt, Cameron & Gray 2015).

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

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

Larger stomatal densities and apertures are often accompanied by a reduced water use efficiency (Franks, W. Doheny-Adams, Britton-Harper & Gray 2015; Kimura, Hashimoto-Sugimoto, Iba, Terashima & Yamori 2020). High stomatal conductance may lead to water stress, potentially impairing plant growth. Indeed, Doheny-Adams et al. (2012) demonstrate a negative correlation between stomatal density (and therefore conductance) and plant size. More recently, a series of articles have demonstrated that decreased stomatal density in Arabidopsis (Hepworth *et al.* 2015), barley (Hughes *et al.*

2017), wheat (Dunn *et al.* 2019), and rice (Caine *et al.* 2019) improves drought tolerance with no adverse effect on plant growth or carbon assimilation.

On the other hand, there is a wealth of studies that report positive relationships between stomatal density and photosynthetic assimilation or plant growth. Schlüter *et al.* (2003) demonstrated that low-light adapted Arabidopsis mutant *sdd1-1* with increased stomatal density achieved 30% higher CO_2 assimilation rates after transfer to high light conditions than wild type. Xu & 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, Donovan & Richards 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, Välbe & Merilo 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 & 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, 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 & 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 an 8-chamber Arabidopsis gas exchange system (Kollist *et al.* 2007; Hõrak, Kollist & Merilo 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 the control). The experiment was performed twice, for total sample size per line per growth condition of 9-15.

Gas exchange was measured in 22-27 days old plants. Measurement cuvette was thermostated to keep the air temperature at 23ºC; incident PPFD inside the cuvette was 250 µmol m^{-2} s^{-1}. Each plant was at first kept at ~60% RH for 1.5 h, after which its gas exchange was registered during 24 minutes (4 readings per plant); the air humidity was then lowered to ~40% and gas exchange registered after a 1.5 h stabilisation period. The variables presented are leaf conductance (stomatal+cuticular conductance) g_l and net CO_2 assimilation A_{net}.

After the gas exchange measurement, the sixth leaf was collected, cut in half after weighing, and a surface imprint was taken from each half with dental silicone as in (Casson *et al.* 2009). Abaxial surface impression was collected from one half of the leaf (oranwash L, Zhermack; www.zhermack.com) and adaxial surface impression from the other (Speedex light body, Coltene/Whaledent AG; Alstätte, Switzerland). Secondary imprints were taken from the silicone with nail varnish and transferred to microscopy slides. One location (0.0675 mm^2) from each imprint was photographed under microscope (Kern OBF 133; Kern & Sohn GmbH; www.kern-sohn.com) at 400X magnification, to be analysed for stomatal density (SD) with ImageJ software (National Institutes of Health, USA; Schneider et al. 2012).

Growth experiment

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

Soil was added to the trays in two stages. First, 1200 g of 2:1 peat and vermiculite mixture (v/v; same materials as in the gas exchange measurement) was weighed into each tray. ~50 g soil samples were taken after each 1-2 trays, dried at 70ºC for two days, and weighed to determine mass based water content in the soil mixture, which was 44±1%.

Based on this knowledge, 220 g of the same mixture was added to each tray, to take the total amount of estimated dry soil in a tray to 800 g, and 3 litres of water was gradually added to the soil. After the final filling, the soil in the trays was covered with black textile mulch blankets (Baltic Agro AS, Lehmja, Estonia). The mulch sheets had 32 holes (5 mm diameter, 65 mm distance between hole centres) for plants.

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

On the 14th day after sowing, one of the growth cabinets was set to the “dry” regime. From that moment onwards, the trays were rotated and shuffled around within each cabinet. Every day in the late afternoon, the trays were weighed for their gross mass to determine soil water content (SWC). When SWC in any tray was below 70%, water was added to the lower tray to take SWC to 75%.

The trays were photographed daily with a Nikon D3100 camera positioned about 1 m directly above the centre of the tray. Photos were stored in Nikon RAW format, converted to Windows Bitmap with IrfanView batch conversion feature, and analysed with custom-made software for the projected area of each studied plant.

After 14 days of differential treatment, all studied plants were collected at the age of 4 weeks. The sixth leaf was separated, this leaf and the rest of the rosette were weighed for wet mass and photographed for (gently flattened) area. The remaining rosette was dried for 5 days at +60℃ and weighed for dry mass, which was then scaled for whole rosette dry mass based on the ratio of wet masses of the sixth leaf and the remaining rosette.

The sixth leaf was studied for its stomatal anatomy as described above. There were issues with imprint quality; only about half of the leaves could be analysed for their stomatal anatomy on both sides. Since this loss of data was purely technical and did not correlate with plant line or treatment, we regard the obtained sample as representative of the whole

studied material. Multiplying SD with the area of the sixth leaf yielded the number of stomata per leaf (stomatal count, SC).
The experiment was repeated with the growth cabinets swapped, resulting in an overall sample size of 14-16 plants per treatment and genotype.

Statistical analysis

Comparisons between measured variables were done with analysis of variance using R scripting language version 4.2.1. Type 3 sums of squares were ensured with the use of the function Anova(,type="III") from the library car (https://r-forge.r-project.org/projects/car/) for obtaining ANOVA results. For post-hoc tests, Tukey's HSD for unbalanced samples was used, employing library agricolae (https://myaseen208.github.io/agricolae/). Level of significance was always set to 0.05.
For analysis of variance, studied genotypes were used as factors as follows (Table 1). In the growth experiment dataset, "epf1" and "epf2" were separate binary variables. In the gas exchange dataset, one variable "epf1/2" indicated whether both *EPF1* and *EPF2* were mutant or wild-type (no single mutants in these genes were studied). The other genes involved, *HT1*, *CYP707A1*, and *CYP707A3* (the latter two always at the same state) were encoded into a three-state variable "sensitivity" with values of "wt" (all three intact),"ht1" (*ht1-2* mutation), or "cyp707a1a3" (*cyp707a1/a3* double mutant). Growth RH was a two-state variable with values "control" or "dry" (60%:80% or 40%:50% day:night RH, respectively).

Results

High SD and high stomatal sensitivity affect leaf conductance largely independently

To test whether mutations that alter stomatal density and sensitivity affect plant gas-exchange independently, we carried out experiments with mutants expected to combine high stomatal density (*epf1/2*) with small stomatal apertures (*ht1-2*, *cyp707a1/a3*). Gas exchange measurements were consistent with the expected behaviour of the studied lines: under both normal and low RH, plants deficient in both *EPF1* and *EPF2* had significantly higher leaf conductance than the corresponding lines with intact *EPF*s (Figure 2a, Table 2). Leaf conductance of *ht1-2* was significantly lower than wild-type under normal RH, whereas both *ht1-2* and *cyp707a1a3* had lower than wild-type g_l under low RH conditions. In combination with the *epf1* and *epf2* alleles these mutations expected to decrease stomatal apertures led to lower leaf conductance compared with the

epf1/2 double mutant under normal RH (Figure 2a). Low RH generally decreased leaf conductance (Table 2), with the most prominent effect in the lines with the *epf1/2* mutations (Figure 2a). Growth conditions had little lasting effect on stomatal behaviour: only the *epf1/2* plants grown under low RH had decreased leaf conductance under low cuvette RH compared to the same genotype grown under normal RH (Figure S1).

Under normal humidity, area-based net CO_2 assimilation of the *epf1/2* mutant was higher than most other plant lines (Figure 2b). Under low RH, the *cyp707a1/a3epf1/2* line had significantly higher A_{net} than the *cyp707a1/a3* (Figure 2b). Due to decreased g_l, the intrinsic water use efficiency (iWUE=A_{net}/g_l) generally increased under low RH (Figure 3a). Water use efficiency was higher than in wild-type in plant lines with increased stomatal sensitivity (*ht1-2* and *cyp707a1/a3*) and about two times lower in the plant lines with the *epf1/2* double mutation than the corresponding lines with intact EPFs. Stomatal density was not affected by the *ht1-2* and *cyp707a1/a3* mutations: the *epf1/2* mutations increased both adaxial and abaxial stomatal density in all genetic backgrounds (Figure 4). Growth under low RH also increased both adaxial and abaxial stomatal density (Table 2), with the most prominent effect in the *epf1/2* mutant (Figure 4). Across all plant lines, the relationship between total stomatal density and leaf conductance remained positive under both growth conditions, but had a steeper slope under normal humidity (Figure 3b).

No effect of epf1 mutation on SD in plant lines with high stomatal sensitivity

To dissect the role of different stomatal density and sensitivity in plant growth under different RH environments, we carried out a growth experiment. The positive effects of the *epf1/2* double mutation and RH on stomatal density described above were also found in the growth experiment (Figure 5; Table 3). The *epf1/2* double mutants had larger stomatal density on both leaf sides than the respective lines with intact EPFs, and the *ht1-2* or *cyp707a1/a3* mutations alone did not affect stomatal density. In the Col-0 background, both *epf1* and *epf2* single mutations increased stomatal density on both leaf sides under normal RH (Figure 5), but there was a significant interaction between *epf1* mutation and stomatal sensitivity (Table 3), manifested in no increase of SD in lines combining the *cyp707a1/a3* or *ht1-2* mutations with the *epf1* single mutation (Figure 5).

Stomatal count was affected by plant genetics similarly to stomatal density: the *epf2* mutation had an overall increasing effect, while the impact of the *epf1* mutation was mediated by the state of the *cyp707a1/a3* and *ht1-2* genes (Table 3, significant

epf1*sensitivity interaction). This resulted in increased stomatal numbers in the *epf1-1* line compared to the wild-type, but no significant difference in the *cyp707a1/a3epf1* or *ht1-2epf1* compared to the *cyp707a1/a3* or *ht1-2*, respectively (Figure 6). Overall, the main effect of the growth environment on the stomatal count was generally weaker than on stomatal density; only the abaxial sides of leaves had fewer stomata in plants grown under low RH (Table 3), and this effect was not significant in any particular genotype (Figure 6).

Growth at low RH increases stomatal ratio with the most prominent effect in epf1

Growth under low RH slightly but consistently increased adaxial to abaxial stomatal ratio in all studied plant lines (Table 3, Figure 7). This effect primarily stemmed from generally increased adaxial SD under low RH, with significant increase in adaxial SD in response to low RH in the *epf1epf2* and *cyp707a1/a3epf1/2* mutants (Figure 5a, Table 3). The increase in stomatal ratio in response to low RH was strongest in plants with the *epf1* mutant allele (Figure 7).

Growth at low RH and high stomatal density independently decrease plant size

Plant growth under low RH was significantly impaired. All plant lines showed a relatively steady decrease in dry mass at harvest time of about 25% - significantly so in the Col-0, *epf1*, *cyp707a1/a3,* and *cyp707a1/a3epf1* lines, but the trend was similar in all genotypes (Figure 8a; Table 3). The projected rosette area at the end of the experiment (28 days after sowing) decreased even more (Figure 8b), owing to the general increase in leaf mass to area ratio (Figure S2; Table 3) under low RH.

Impairment in the epidermal patterning factors, causing excessive stomatal density, resulted in slight but consistent decrease in plant size (Figure 8; Table 3). At the onset of the RH treatment 14 days after sowing, the plant lines with the *epf2* mutations had slightly smaller projected area, a small enough effect to only appear in the pooled data (Table 3, epf2 main effect; Figure S3). During the differential treatment in weeks 3 and 4, growth under low RH resulted in a smaller relative growth rate in all plant lines, with no significant difference associated with any genetic features (Table S3, Figure S4). Both plant area and dry mass at the end of the experiment were markedly smaller in the lines with the *epf1/2* double mutation; in case of mass, this was due to the main effect of the *epf2* mutation and there was no main effect of *epf1* (Figure 8a; Table 3), while the final area was affected by both *epf1* and *epf2* mutations (Table 3). The *cyp707a1/a3* double mutation also resulted in

a slight decrease in plant dry mass and led to an increased area reduction under low RH compared to other plant lines (Figure 8; Table 3, significant RH*sensitivity interaction).

As a result of the size reduction associated with the *epf1* and *epf2* mutations, particularly that of *epf1/2* double mutants, there was a significant negative correlation between total stomatal density and plant size (Figure 9a, Table S2). The slope of this relationship remained the same under both growth humidities, with the normal RH-grown plants consistently around 3.5 cm^2 larger in their projected area, or 10 mg heavier in their dry mass. Whereas the stomatal count and plant size relationship had a similar downward trend, it was not statistically significant (Figure 9b; Table S2).

Discussion

Both altered stomatal density and sensitivity impact plant water use efficiency and productivity (Doheny-Adams *et al.* 2012; Franks *et al.* 2015; Kimura *et al.* 2020; Sakoda *et al.* 2020), but the potential interaction of signalling pathways governing these traits and their combined effects on plant stomatal conductance, water use efficiency, and growth have not been addressed. Here we combined mutations in *EPF1* and *EPF2* that affect stomatal density and in *HT1* and *CYP707A1/A3* that affect stomatal sensitivity under normal and low RH conditions to address these questions. While the studied mutations that affect stomatal density and sensitivity mostly acted independently (Figures 2, 4, 5, Tables 2, 3), mutations in the *EPF1* gene stood out, as in the high stomatal sensitivity backgrounds *epf1* alone did not increase stomatal density (Figure 5, Table 3). EPF2 can partially replace EPF1 function but not vice versa (Hara *et al.* 2009); it is possible that the high stomatal sensitivity background stimulates *EPF2* expression or activity in the *epf1* single mutants, leading to normal stomatal densities. It has been proposed that a combination of multiple traits could be necessary for engineering more water use efficient and productive crops (Flexas 2016). Our results suggest that combining traits that affect stomatal density and sensitivity could be one approach, as these traits affect stomatal conductance independently. Given the negative relationship between stomatal density and plant growth (Figure 9a), optimisation of stomatal density and sensitivity trait combinations is needed for practical applications.

Our experiments showed an increase in stomatal ratio in response to low air humidity (Figure 7). Allocating more stomata to the adaxial epidermis under dry air conditions may seem counterintuitive, as excessive water loss via adaxial stomata has been proposed as

one of the costs of adaxial stomata in plants (Buckley, John, Scoffoni & Sack 2015; Drake, de Boer, Schymanski & Veneklaas 2019). Yet, a similar increase in stomatal ratio under dry air was found also in several cotton varieties (Devi & Reddy 2018), whereas amphistomatous plant species are common in dry habitats (Wood 1934; Muir 2015; Bucher *et al.* 2017) and tend to have faster stomatal responses than hypostomatous plants (Haworth *et al.* 2018). It is possible that high stomatal ratios allow plants to make better use of favourable conditions when they arise, by increasing CO_2 uptake capacity (Mott, Gibson & O'leary 1982; Muir 2018; Xiong & Flexas 2020). At the same time, densely packed stomata are typically smaller and faster than sparse stomata (Drake, Froend & Franks 2013), ensuring fast and efficient restriction of water loss via speedy stomatal closure upon water stress.

How plants adjust stomatal ratio in response to changes in environmental conditions is not clear. The pronounced increase in stomatal ratio under low RH in the *epf1* single mutant backgrounds suggests that EPF1 as a negative regulator of stomatal development preferentially suppresses stomatal production in the adaxial epidermis in response to a decrease in relative air humidity (Figure 7). The leaf-side specific behaviour of EPFs and other stomatal regulators has not been addressed, but given that silencing of *SPCH* expression or overexpression of a *SPCH* repressor led to preferential loss of abaxial stomata (Dow *et al.* 2014; Qi *et al.* 2019), it is likely that signalling events that govern adaxial and abaxial stomatal formation are regulated independently and at least partly via different mechanisms. Indeed, across-species studies indicate independent evolution of adaxial and abaxial stomatal densities with a greater flexibility of adaxial SD (Muir *et al.* 2023), in line with more prominent increases in adaxial SD in response to low air humidity (Figure 5). Further studies of stomatal ratio and its responses to changes in environment in different mutants of stomatal patterning, environmental and hormonal signalling pathways will help to understand how stomatal ratio adjustments occur in plant development.

In our experiments we found that relative air humidity and high stomatal density independently impair plant growth (Table 3, Figure 8, Figure 9). We anticipated a larger negative effect of low RH in plant lines with increased stomatal conductance that we presumed would suffer from water stress. In our experiment, low RH was uniformly detrimental to all genotypes, despite compensation of high stomatal conductance caused by high stomatal density of the *epf1epf2* mutations by the increased stomatal sensitivity mutations *ht1-2* and *cyp707a1/a3* (Figures 2, 4, 5). It is possible that efficient stomatal

closure under low RH compensated for increased stomatal densities in our experiment (Figure 3b). Indeed, mutants with high stomatal density can efficiently close stomata in response to changes in environmental conditions (Dow *et al.* 2014; Hepworth *et al.* 2015). However, in our recent study, growth under low RH was not more impaired in the *ost1* mutant with large stomatal conductance than in wild-type plants (Tulva *et al.* 2023). As the *ost1* mutant is deficient in stomatal closure in response to low humidity, the lack of stronger growth impairment than in wild-type plants cannot be explained by stomatal closure in response to low RH. Thus, hampered plant growth under low RH seems to occur independently of stomatal traits.

Plants with high stomatal densities were stunted in growth irrespective of conditions (Figures 8, 9, Table 3), indicating that excess stomatal development impairs plant growth during the first 4 weeks in Arabidopsis. Similar impairments have been found before in mutants with high SD (Doheny-Adams *et al.* 2012; Lawson, Pijut & Michler 2014), although other studies have reported increased photosynthesis, carbon gain or biomass production in high stomatal density mutants (Schlüter *et al.* 2003; Tanaka *et al.* 2013; Sakoda *et al.* 2020). The negative relationship between stomatal density and plant growth may be explained by increased porosity due to higher numbers of substomatal cavities and decreased amounts of photosynthetic tissue in high density mutants. Dow et al. (2017) showed that mesophyll porosity increases together with stomatal density in Arabidopsis lines; similar coupling between stomatal and mesophyll anatomy was shown in wheat (Lundgren *et al.* 2019). Given the negative relationship between stomatal density and plant growth, but not overall leaf stomatal numbers and plant growth (Figure 9), it is possible that the proportion of leaf tissue dedicated to non-stomatal structures that is larger in plant lines with lower stomatal density contributes to their improved growth. Alternatively, the lack of significant correlation between stomatal count and plant size (Figure 9b) may reflect different expansion growth due to differences in energy allocation into cell divisions, leading to a negative relationship between stomatal density and plant size (Figure 9a).

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. It is possible that the energy-consuming process of stomatal formation leads 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, Zhu & Kinoshita 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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Table 1. Plant lines studied, their short names used in this article, and levels of the factors used in ANOVA.

Line	Short name	sensitivity	epf 1	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

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

	gl (60%)	gl (40%)	An (60%)	An (40%)	intrWUE (60%)	intrWUE (40%)	gl (growth RH)	An (growth RH)	intrWUE (growth RH)	SD abaxial	SD adaxial
RH	0.91	**0.00**	**0.00**	**0.00**	0.69	**0.00**	**0.00**	0.10	**0.00**	**0.00**	**0.00**
epf1/2	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
sensitivity	**0.00**	**0.00**	**0.01**	**0.05**	**0.00**	**0.00**	**0.00**	**0.02**	**0.00**	**0.02**	0.51
RH*epf1/2	**0.00**	**0.00**	0.69	0.38	**0.00**	0.10	**0.00**	0.74	0.59	0.28	**0.03**
RH*sensitivity	0.56	0.40	0.99	1.00	**0.03**	0.84	**0.02**	0.97	0.49	0.38	0.40
epf1/2*sensitivity	**0.04**	0.20	0.40	0.78	**0.00**	**0.00**	0.10	0.39	**0.00**	**0.01**	0.83
RH*epf1/2*sensitivity	**0.03**	**0.04**	**0.00**	**0.00**	0.05	0.33	**0.02**	**0.00**	0.26	0.28	0.49

Table 3. P-values of ANOVA on the growth experiment harvest data (collected on days 15-16 after treatment start = 29-30 after sowing), as well as projected plant areas on the day treatment started (day 0) and the last growth day (day 14; 14 and 28 days after sowing resp.). P-values < 0.05 shown in bold. RH, growth air humidity; epf1, state of the *EPF1* gene (wild-type or mutant), epf2 likewise; sensitivity encodes the state of stomatal reaction genes (*ht1-2* mutant, *cyp707a1/a3* double mutant, or wild-type).

Factor	area day 0	area day 14	dry mass	LMA	SD abax	SD adax	SD sum	Stom count abax	Stom count adax	Stom count sum	Stom ratio
RH	0.06	**0.00**	**0.00**	**0.00**	0.06	**0.00**	**0.00**	**0.00**	0.14	**0.04**	**0.00**
epf1	0.96	**0.00**	0.13	**0.01**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.31
epf2	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.69
sensitivity	0.05	**0.00**	**0.00**	0.18	**0.00**	0.16	**0.00**	**0.00**	**0.00**	**0.00**	0.23
RH*epf1	0.59	0.89	0.76	0.89	0.79	**0.02**	0.67	0.28	0.10	0.77	0.11
RH*epf2	0.98	0.97	0.41	0.53	0.38	0.22	0.29	0.63	0.70	0.58	0.87
epf1*epf2	**0.01**	**0.00**	**0.01**	0.30	**0.00**	**0.02**	**0.00**	0.27	0.12	0.82	**0.01**
RH*sensitivity	0.77	**0.03**	0.48	0.34	0.49	0.53	0.97	0.49	0.84	0.81	0.37
epf1*sensitivity	0.26	**0.03**	0.15	0.96	**0.00**	**0.03**	**0.00**	**0.00**	**0.00**	**0.00**	0.29
epf2*sensitivity	0.78	0.06	0.74	0.09	0.20	0.28	0.11	0.34	0.15	0.05	0.36
RH*epf1*epf2	0.39	0.53	0.52	0.53	0.47	0.38	0.50	0.74	0.52	0.71	0.92
RH*epf1*sensitivity	0.69	0.16	0.63	0.98	0.19	0.82	0.41	0.22	0.66	0.47	0.22
RH*epf2*sensitivity	0.09	0.07	0.13	0.73	0.57	0.29	0.93	0.99	0.11	0.72	0.12
epf1*epf2*sensitivity	0.11	0.36	0.15	**0.04**	0.98	0.09	0.66	0.89	**0.01**	0.31	0.45
RH*epf1*epf2*sensitivity	0.72	0.42	0.88	0.61	0.16	0.90	0.40	0.17	0.93	0.35	0.58

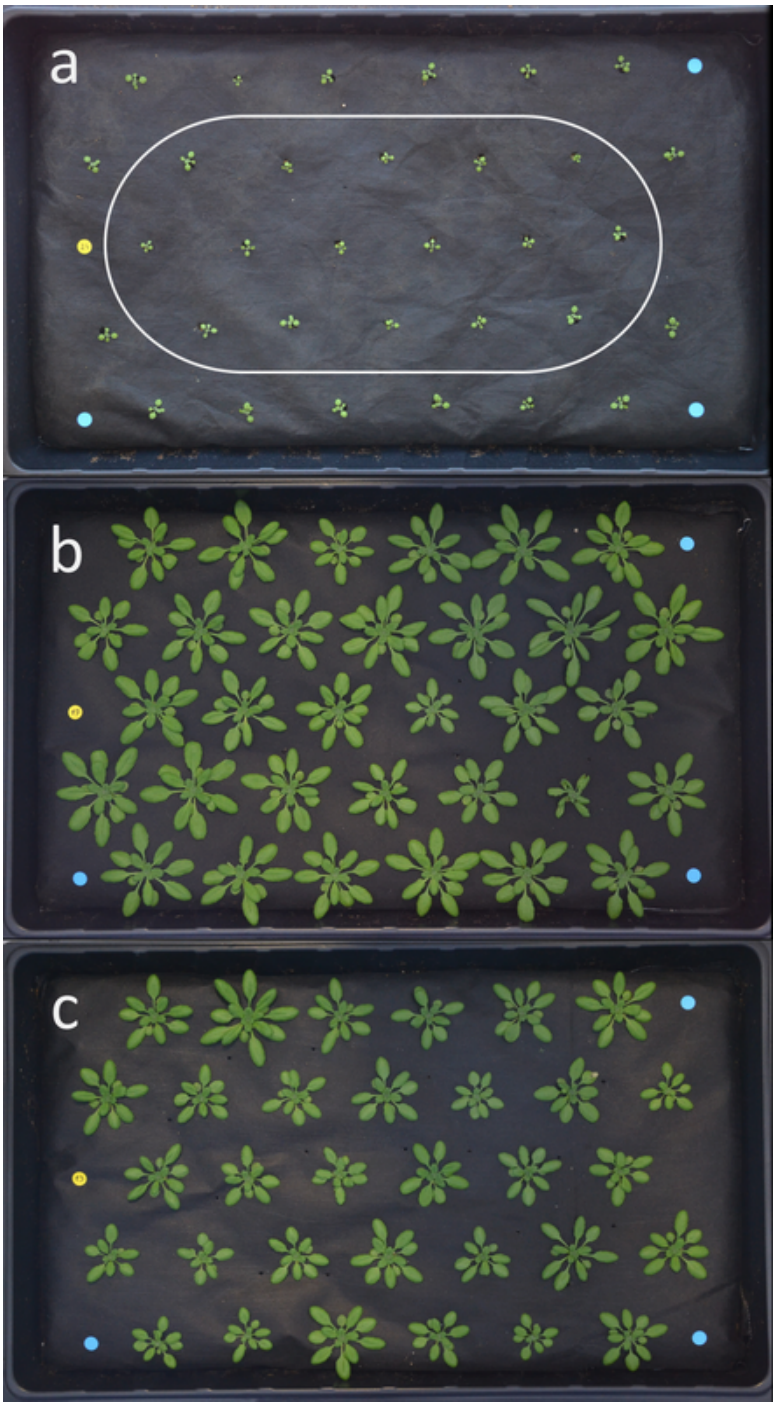

Figure 1. Layout of the trays in the growth experiment. (a), an example tray on Day 0 (14th day after sowing). The region surrounded by the oval included the studied plants in randomised positions, others outside were Col-0. (b), an example control tray on Day 14; (c), an example low-RH tray on Day 14.

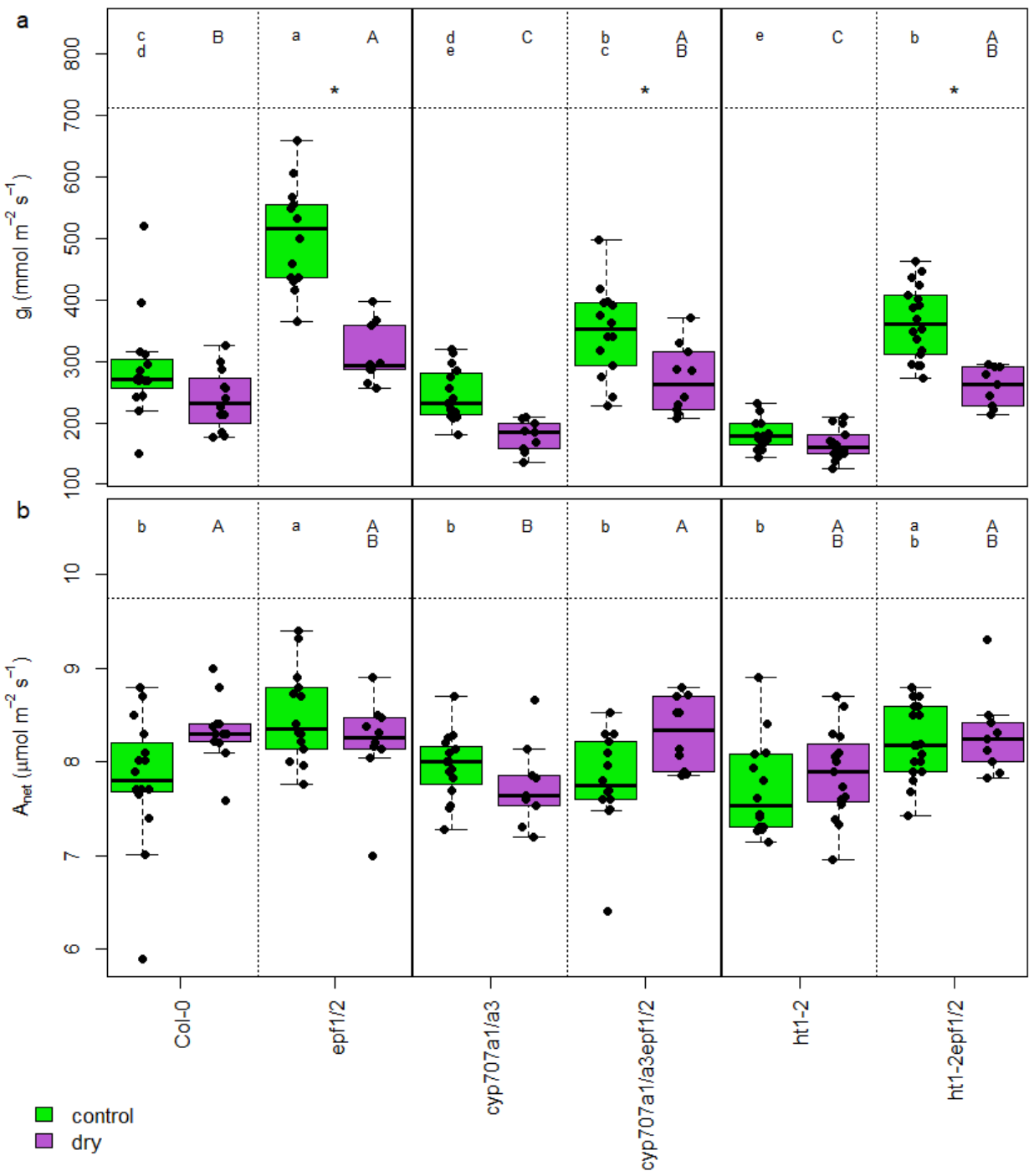

Figure 2. Leaf conductance to water vapour (a) and net photosynthesis (b), measured with RH in the cuvette equal to the growth RH for each plant. Green boxes, normal growth RH; magenta boxes, low RH. The boxes span from the first to the third quartile, with median indicated with the horizontal line; the whiskers span the non-outlier range. Individual data points 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 low RH treatment.

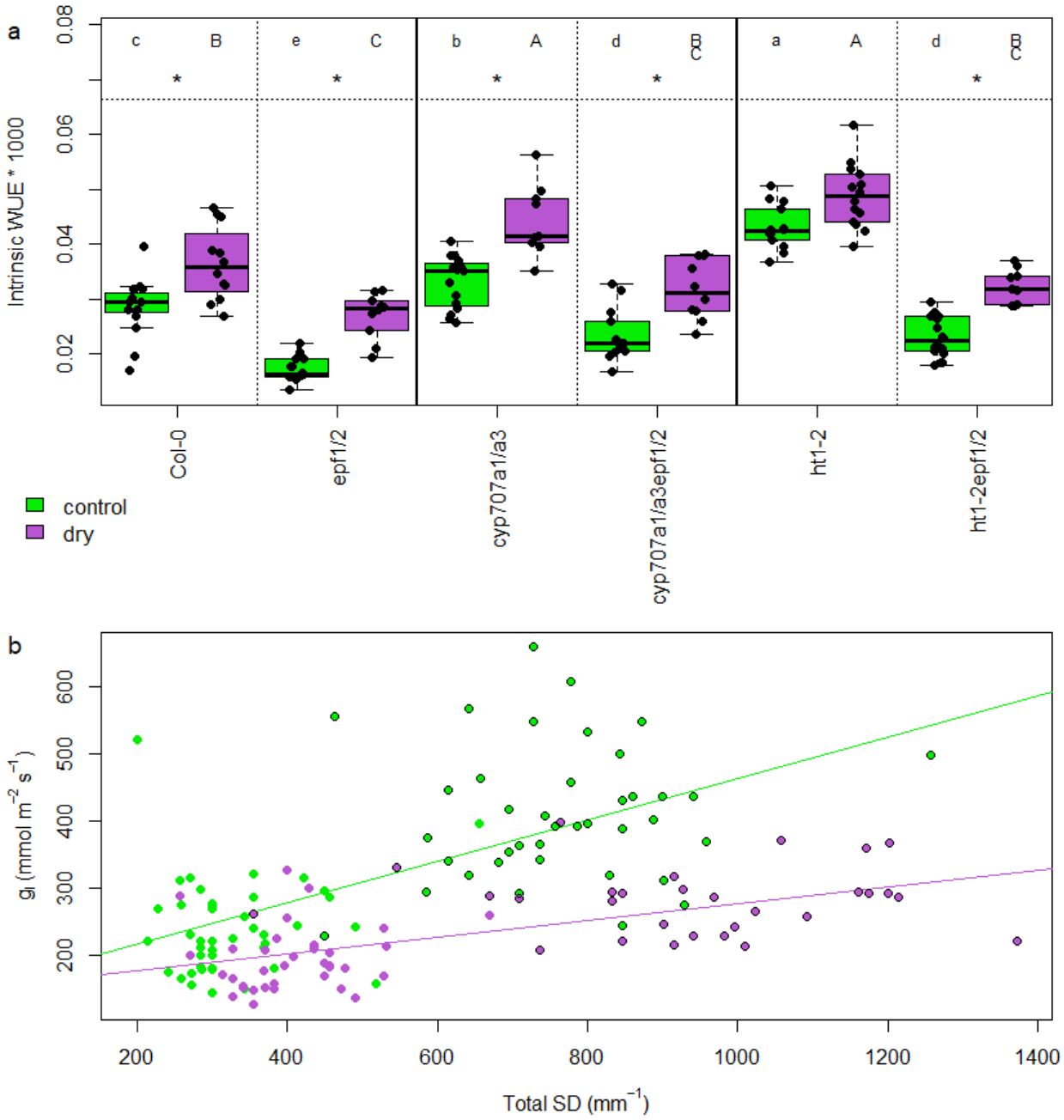

Figure 3. (A) Intrinsic water use efficiency in the gas exchange experiment, measured with RH in the cuvette equal to the growth RH for each plant. Green boxes, normal growth RH; magenta boxes, low RH. The boxes span from the first to the third quartile, with median indicated with the horizontal line; the whiskers span the non-outlier range. Individual data points 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. (b) Relationship between stomatal density and leaf conductance at growth conditions. Each dot is one plant; the plants with the *epf1/2* double mutation are circled. Slopes of the linear regressions differ significantly from zero ($p<0.05$) and from each other ($p<0.001$).

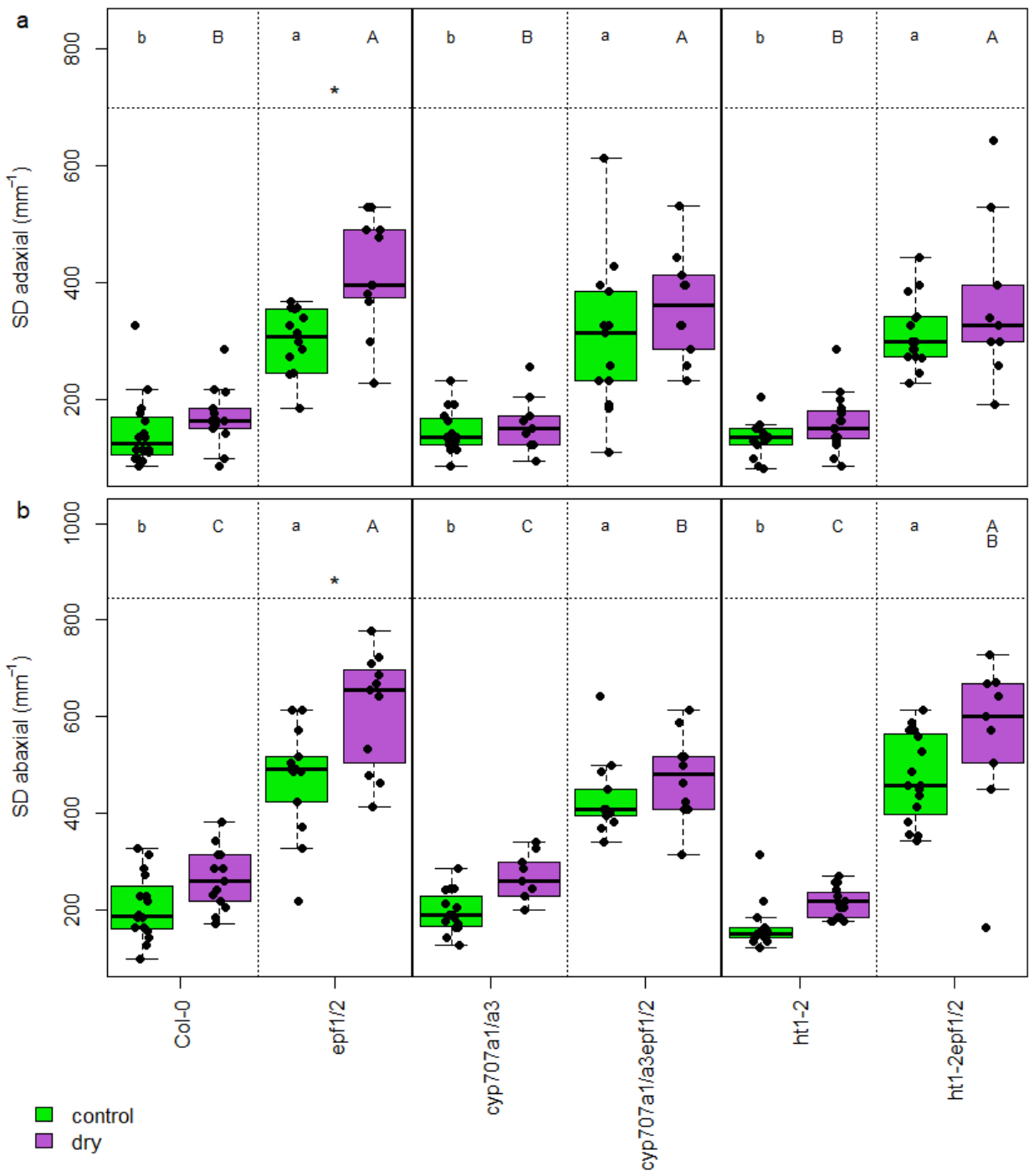

Figure 4. Adaxial (a) and abaxial (b) stomatal densities in the gas exchange experiment. Green boxes, normal growth RH; magenta boxes, low RH. The boxes span from the first to the third quartile, with median indicated with the horizontal line; the whiskers span the non-outlier range. Individual data points 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.

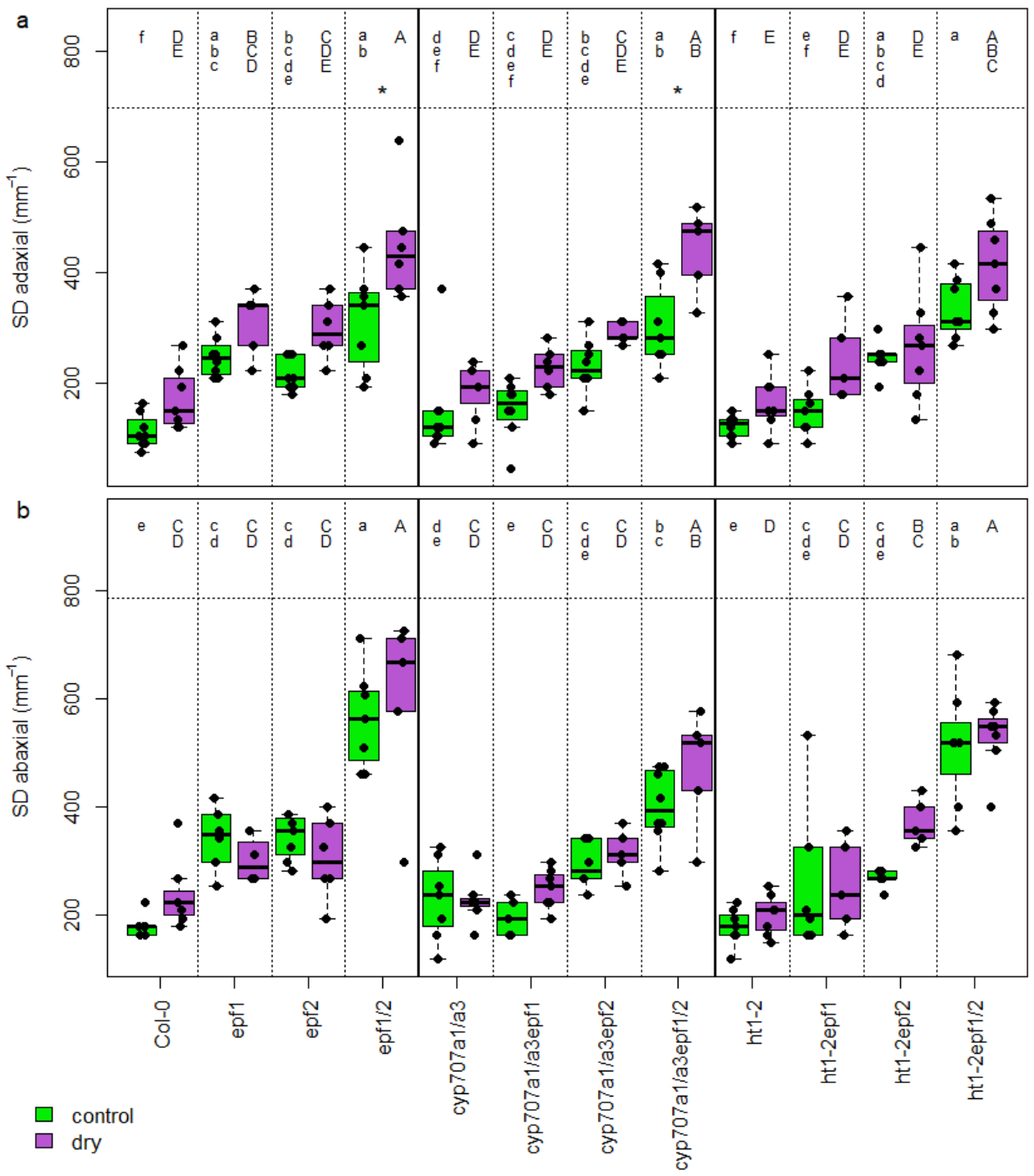

Figure 5. Stomatal density at the adaxial (a) and abaxial (b) leaf surface in the growth experiment. Green boxes, normal growth RH; magenta boxes, low RH. The boxes span from the first to the third quartile, with median indicated with the horizontal line; the whiskers span the non-outlier range. Individual data points 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.

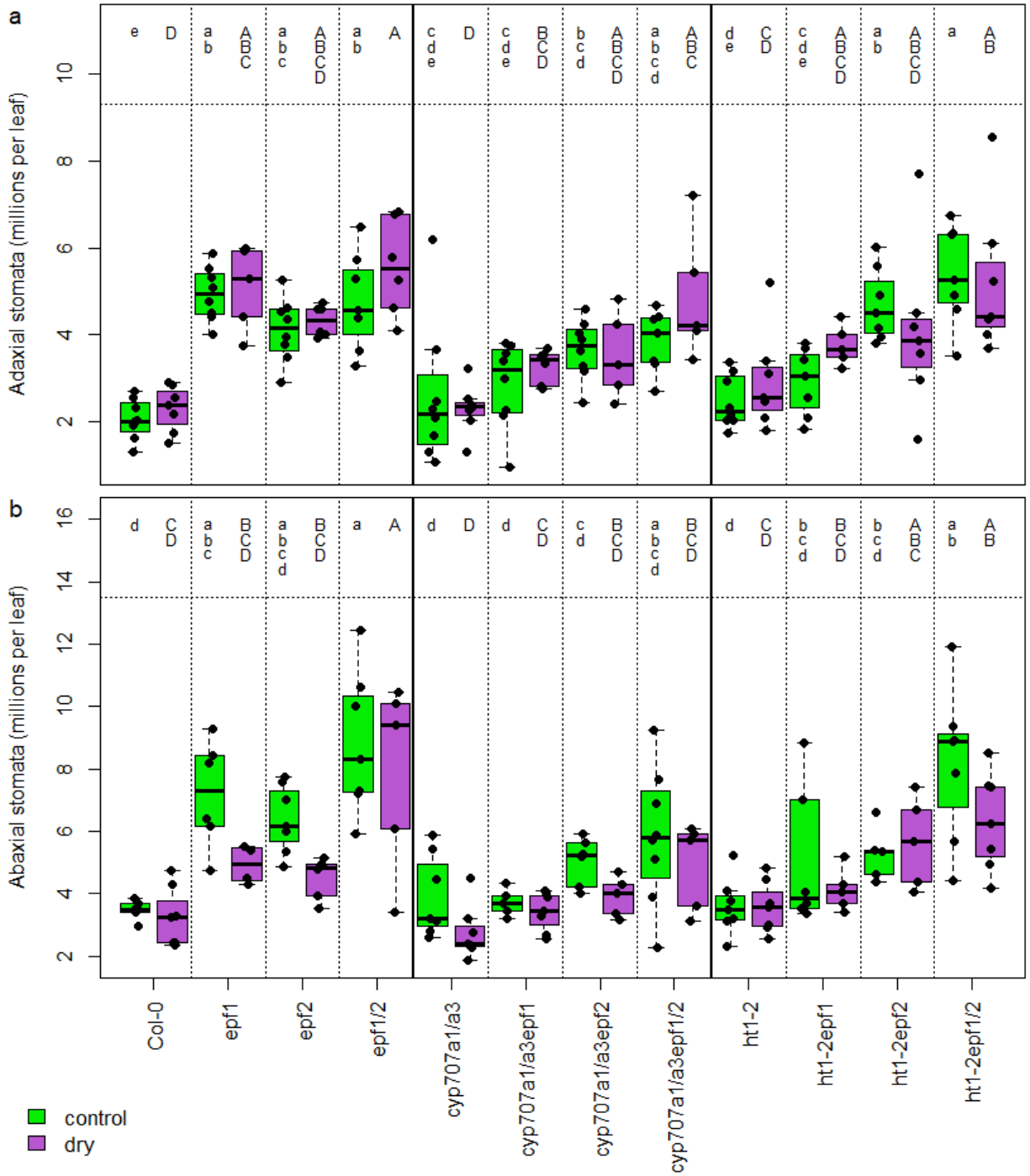

Figure 6. Stomatal count on the adaxial (a) and abaxial (b) leaf surface in the growth experiment. Green boxes, normal growth RH; magenta boxes, low RH. The boxes span from the first to the third quartile, with median indicated with the horizontal line; the whiskers span the non-outlier range. Individual data points 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.

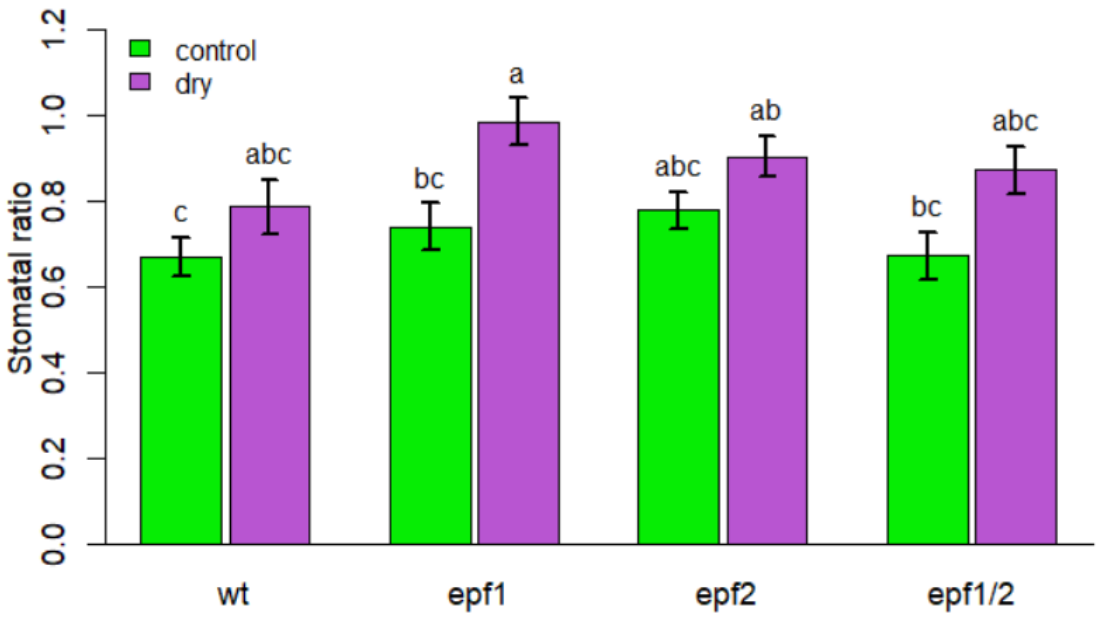

Figure 7. Comparison of stomatal ratio (adaxial/abaxial) in the growth experiment depending on the state of *EPF1* or *EPF2*, with other genes' data pooled. Green bars, normal growth RH; magenta bars, low RH. Shared letter(s) above the bars denote no significant difference according to Tukey HSD test (α=0.05).

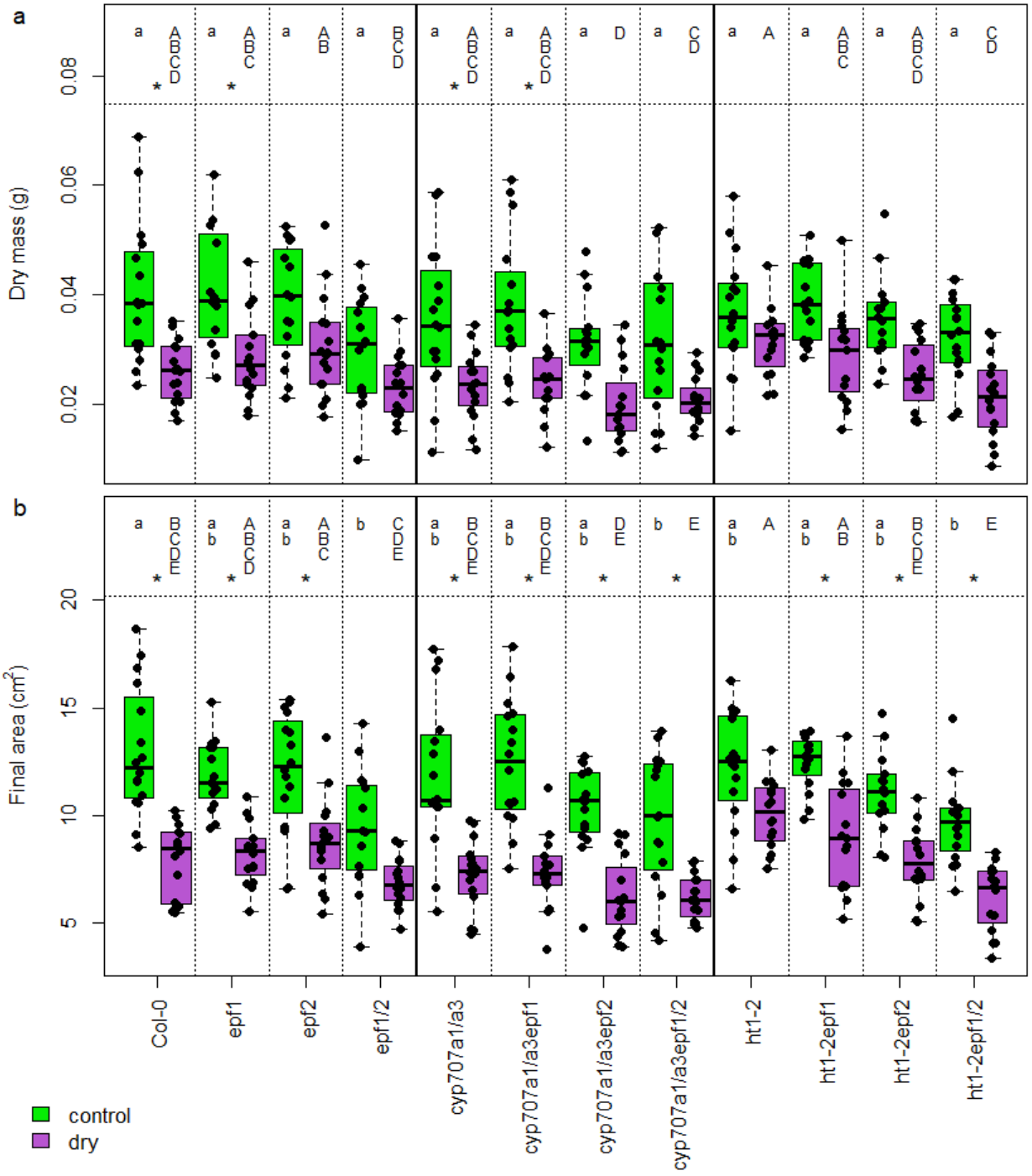

Figure 8. Plant above-ground dry mass (29-30 days old; a) and projected area (28 days old; b). Green boxes, normal growth RH; magenta boxes, low RH. The boxes span from the first to the third quartile, with median indicated with the horizontal line; the whiskers span the non-outlier range. Individual data points 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.

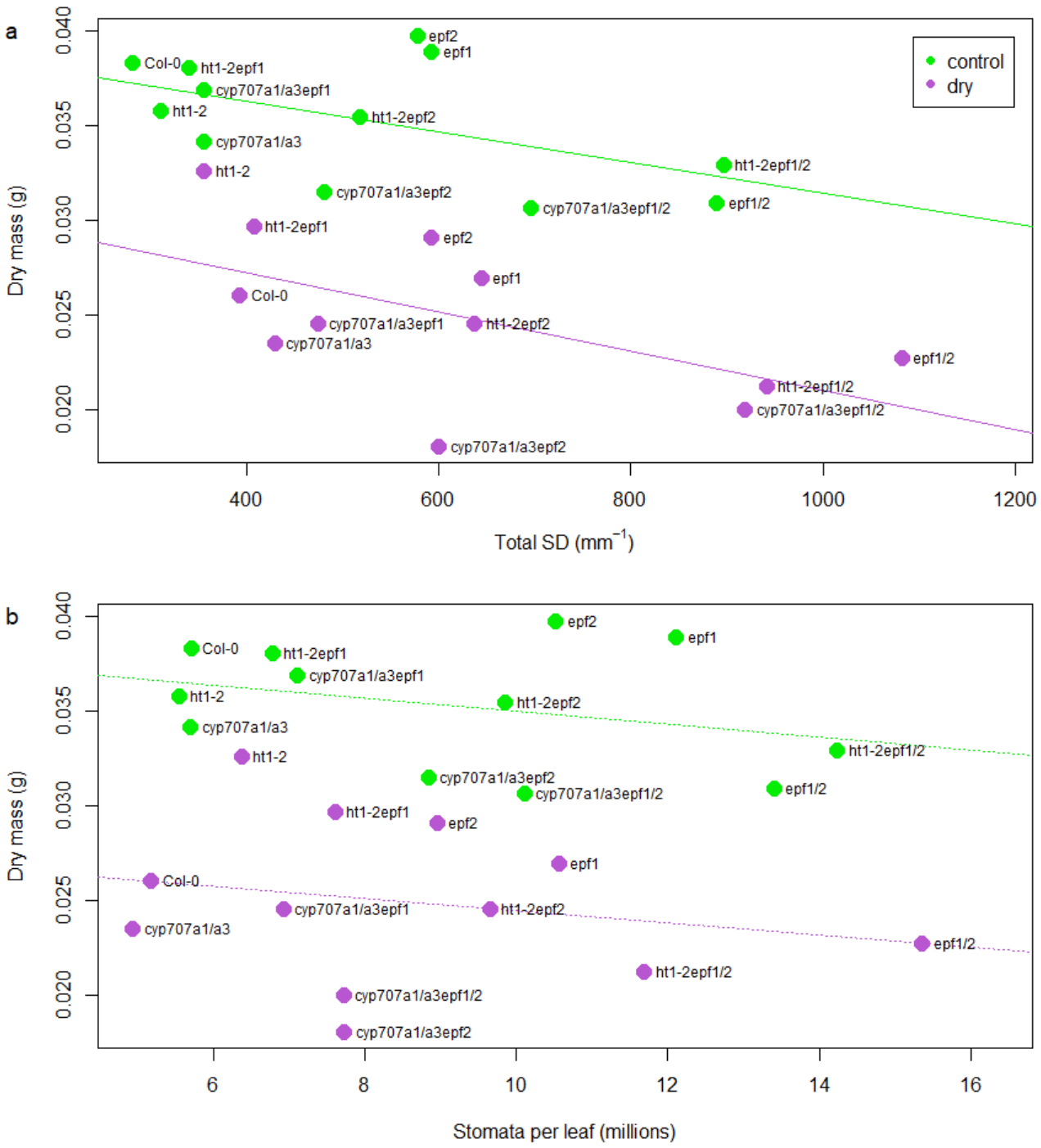

Figure 9. Dependence of above-ground dry mass (29-30 days old) on total stomatal density of leaf 6 (sum of both leaf sides; a) or estimated total number of stomata on leaf 6 (b). Green markers, normal growth RH; magenta markers, low RH. Each dot indicates the median values (in both dimensions) of the 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.