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1 Increasing water use efficiency directly through genetic
2 manipulation of stomatal density.

3

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Summary

- Improving crop water-use efficiency (WUE) is a critical priority for regions facing increased drought or diminished groundwater resources. Despite new tools for manipulating stomatal development, engineering plants with high WUE remains a challenge.
- We used *Arabidopsis* epidermal patterning factor (EPF) mutants exhibiting altered stomatal density to test if WUE can be improved directly by manipulation of the genes controlling stomatal density. Specifically, we tested whether constitutive overexpression of *EPF2* reduced stomatal density and maximum stomatal conductance, $g_{w(max)}$, sufficiently to increase WUE.
- We found that reduction of $g_{w(max)}$ via reduced stomatal density in the *EPF2* overexpressing plants (*EPF2OE*) increased both instantaneous and long-term WUE without significantly altering photosynthetic capacity. Conversely, plants lacking both *EPF1* and *EPF2* expression (*epf1epf2*) exhibited higher stomatal density, higher $g_{w(max)}$ and lower instantaneous WUE, as well as lower (but not significantly so) long-term WUE.
- Targeted genetic modification of stomatal conductance such as in *EPF2OE* is a viable approach for engineering higher WUE in crops, particularly in future high-CO₂ atmospheres.

Key words:

water-use efficiency; $\delta^{13}C$; stomata; *Arabidopsis*; crops; CO₂; Epidermal Patterning Factor; genetically modified

51 **Introduction**

52 Increased water use efficiency (WUE; the ratio of the rates of CO₂ assimilation to
53 transpiration, A/E) can improve productivity and reduce water stress under drier
54 environmental conditions (Slatyer, 1964; Sinclair *et al.*, 1984; Han *et al.*, 2013). In the
55 short term, plants increase WUE by reducing stomatal apertures and therefore E , but
56 often under prolonged water deficit plants also produce leaves with reduced
57 maximum stomatal conductance ($g_{w(max)}$) via altered stomatal density (D) and/or size
58 (S) (Gindel, 1969; Franks *et al.*, 2009; Doheny-Adams *et al.*, 2012). It is unclear why
59 plants undergo this developmental response to drought in addition to simply reducing
60 stomatal aperture, but it has been suggested that when conditions promote a long-term
61 reduction in the average operating stomatal conductance, production of new leaves
62 with reduced $g_{w(max)}$ maintains more favourable mechanical and energetic conditions
63 for stomatal control (Franks *et al.*, 2009; Franks *et al.*, 2012). This specific adaptation
64 of $g_{w(max)}$ for improved WUE may represent a model for genetic manipulation of
65 WUE. Here, using *Arabidopsis* epidermal patterning factor (EPF) mutants exhibiting
66 altered stomatal density, we show that WUE can be improved directly by
67 manipulation of the genes controlling the development of stomata to reduce $g_{w(max)}$.

68 The epidermal patterning factors are a family of eleven related small, secreted
69 peptides, several of which regulate the number of stomata formed on *Arabidopsis*
70 leaves. They are characterized by at least six conserved cysteine residues towards
71 their C-terminus and all studied so far are processed at their non-conserved N-
72 terminal end (Ohki *et al.*, 2011; Torii, 2012). Manipulating the expression level of
73 these genes has proved to be a powerful tool to modify stomatal density and
74 patterning. Lack of EPF1, which is normally expressed in guard cells of young
75 stomata and their precursors, results in an increase in stomatal density and clustering
76 on the leaf epidermis (Hara *et al.*, 2007). EPF2 is expressed at slightly earlier stages
77 of stomatal development than EPF1, in stomatal precursors known as meristemoids
78 and guard mother cells. Lack of EPF2 results in higher stomatal and precursor cell
79 densities but clustering of stomata remains rare (Hara *et al.*, 2009; Hunt & Gray,
80 2009). Although constitutively over-expressing either EPF1 or EPF2 results in
81 similar phenotypes with dramatically reduced numbers of stomata, the two gene
82 products appear to act independently; the double mutant *epf1epf2* displays an additive
83 phenotype with approximately twice the density of stomata as Col-0 controls, together

84 with a low level of stomatal pairing and additional precursor cells (Hunt & Gray,
85 2009; Dow *et al.*, 2014b). In plants genetically manipulated to constitutively over-
86 express *EPF1* or *EPF2* (*EPF1OE* and *EPF2OE*), the leaves have very few stomata
87 (Hara *et al.*, 2009).

88 A subtilisin peptidase STOMATAL DENSITY AND DISTRIBUTION 1
89 (*SDD1*) was the first genetic component identified as regulating stomatal
90 development (Berger & Altmann 2000), but it achieves this by a mechanism which
91 remains unknown, and that appears to act independently of the EPF peptides and their
92 receptors (Hunt & Gray, 2009; Hunt *et al.*, 2010). Plants lacking *SDD1* expression
93 have increased stomatal density (*sddl-1*; approximately 250% of C24 background)
94 and a low level of stomatal pairing. The stomatal conductance of *sddl* plants was
95 consistently higher than that of control plants (following growth at three different
96 light intensities) particularly when measured at higher light intensities (Schlüter *et al.*,
97 2003). However, photosynthetic assimilation rates, although higher, were not
98 significantly increased in the high *D* plants except in an experiment following transfer
99 to a higher light intensity before analysis. When *sddl* plants (with inherently high *D*)
100 were grown at 120 mmol m⁻² s⁻¹ and then shifted for 2 d to high light conditions
101 (500mmol m⁻² s⁻¹) their maximal photosynthetic capacity was increased by 30% in
102 comparison to wild-type controls, although their conductance was not significantly
103 increased (Schlüter *et al.*, 2003). These pioneering experiments suggested that plants
104 manipulated to have substantially increased *D* have correspondingly increased levels
105 of stomatal conductance. If allowed to biochemically acclimate to higher light
106 intensity (i.e. typical saturation intensities) these plants may potentially exhibit
107 enhanced CO₂ assimilation without loss of WUE.

108 We have previously reported that the manipulation of stomatal density through
109 alteration of *EPF* gene expression leads to altered *E* (Doheny-Adams *et al.*, 2012).
110 Across a range of *EPF* mutants with stomatal densities ranging from approximately
111 20% to 325% of Col-0 controls, there was a negative correlation between the
112 predicted maximum stomatal conductance to water vapour ($g_{w(max)}$) and leaf
113 temperature, suggesting plants with reduced $g_{w(max)}$ operated with lower stomatal
114 conductance and *E*. These changes in *D* and *E* translated to differences in growth, but
115 in this case only when *D* and *E* were reduced relative to Col-0. For *EPF2OE* plants,
116 with substantially reduced *D* and *E*, leaf rosettes were larger, particularly when water

117 availability was limited. However, for plants such *epfl epf2* exhibiting increased D
118 and $g_{w(\max)}$, we did not identify any conditions under which the growth of plants was
119 improved. It is not known why the growth rate of plants with low D was enhanced but
120 it may have resulted from a combination of lower metabolic cost of developing and
121 operating fewer stomata, higher A at the elevated leaf temperature, or improved water
122 status from a reduction in E (Doheny-Adams *et al.*, 2012).

123 Recently several other reports have explored the effects of altering D on
124 stomatal conductance. Using plants with manipulated levels of STOMAGEN, a
125 secretory peptide promoting stomatal development, (Tanaka *et al.*, 2013) showed that
126 plants with increased D (ST-OX; 372% of Col-0 wild-type) have increased E and
127 stomatal conductance, and at high light intensities also have increased A . The same
128 study reported no significant differences in E or A in plants with reduced D (ST-
129 RNAi; 32% of Col-0). No significant differences were reported in WUE from
130 gravimetric analyses, but importantly, the trend suggested a negative correlation with
131 D .

132 Using a range of Arabidopsis stomatal development mutants with no, or low
133 levels of stomatal clustering including *epfl* and *epfl epf2*, (Dow *et al.*, 2014a) reported
134 a strong correlation between $g_{w(\max)}$ determined from anatomy (via measurements of S
135 and D) and $g_{w(\max)}$ from gas exchange measurements. This proved that the
136 developmental changes in S and D translated to a shift in the operational range of
137 stomatal conductance. If these shifts in $g_{w(\max)}$ were uncoupled from photosynthetic
138 biochemistry then WUE should increase with declining $g_{w(\max)}$. However, in that
139 study, a significant increase in WUE was only observed with mutants exhibiting high
140 stomatal clustering, an abnormal condition resulting from disruption of stomatal
141 spacing control.

142 Despite these new genetic tools for manipulating D and $g_{w(\max)}$, there is no
143 clear evidence of a significant enhancement of WUE via an engineered reduction in
144 $g_{w(\max)}$. Our goal in this study was to compare the instantaneous and long-term WUE
145 of Arabidopsis *epfl epf2* (high- D) and *EPF2OE* (low- D) mutants relative to Col-0
146 control plants. Our hypothesis is that the developmental changes in *EPF2OE* reduce
147 $g_{w(\max)}$ and thereby shift the operating stomatal conductance to a lower state; if the
148 *EPF2OE* mutation affects $g_{w(\max)}$ exclusively then the photosynthetic biochemistry

149 will remain unchanged when leaves are grown under typical saturating light
150 conditions, resulting in higher WUE.

151 **Materials and Methods**

152 Plant Growth

153 Plants were *Arabidopsis thaliana* Col-0 background and have been described
154 previously (Hunt & Gray, 2009; Hunt *et al.*, 2010). Two separate experiments were
155 performed under different growth environments: (1) plants grown in a greenhouse
156 (University of Sydney) in full natural sunlight (high-light) and (2) plants grown in a
157 controlled environment chamber (Convicon model BDR16; University of Sheffield)
158 with photosynthetically active radiation (PAR) set at $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ (low light).
159 Other conditions were similar across the two experiments: plants well-watered at all
160 times; commercial compost soil in 100 ml pots; ambient CO_2 450ppm in growth
161 chamber and 390 in greenhouse; 9 hours day length; $22^\circ\text{C}/16^\circ\text{C}$ day/night
162 temperature. Seeds were stratified at 4°C in distilled water for 72 hours. Plant
163 positioning was altered weekly. Measurements were performed on the largest mature
164 rosette leaves at the initiation of the floral bolt. All measurements were performed on
165 the greenhouse-grown plants (carbon isotope analysis, stomatal anatomy and leaf gas
166 exchange), whereas only carbon isotope analysis was performed on the chamber-
167 grown plants. The main goal was to compare the physiological attributes of the
168 genotypes under high, natural light (greenhouse conditions). The additional
169 measurements on plants grown under low-light in a growth chamber were to test if the
170 pattern of carbon isotope discrimination, indicative of WUE, was consistent across
171 greenhouse and growth-chamber environments.

172 Leaf gas exchange measurements

173 Steady state leaf gas exchange parameters (CO_2 assimilation rate A , transpiration rate
174 E and stomatal conductance to water vapour, g_w) were measured with a portable,
175 open-flow photosynthesis monitor incorporating an infrared gas analyser (IRGA)
176 (model 6400, Li-COR, Lincoln, NE). CO_2 was removed from external air using soda
177 lime and mixed with pure CO_2 to control leaf cuvette air CO_2 concentration (c_a).
178 Conditions in the cuvette were maintained at $c_a = 390$ ppm, corresponding to ambient
179 c_a for glasshouse grown plants; 20°C leaf temperature, $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, 1 kPa

180 leaf-to-air water vapour pressure difference. One mature leaf (remaining attached to
 181 the rosette) was clamped inside the leaf cuvette and A and g_w were allowed to
 182 stabilize under the controlled conditions (minimum 45 minutes). At this stage the
 183 operating CO_2 assimilation rate and stomatal conductance, $A_{(\text{op})}$ and $g_{w(\text{op})}$, were
 184 recorded, together with E and the ratio of leaf internal to ambient CO_2 concentration,
 185 c_i/c_a . Instantaneous WUE was calculated as $A_{(\text{op})}/E$. The relationship between A and
 186 c_i was then determined by adjusting the IRGA reference CO_2 concentration
 187 incrementally in the following order: 200, 100, 50, 600, 1000, 1200, 1600, 1000 ppm.
 188 Using the tool developed KP Tu (www.landflux.org) and following (Ethier &
 189 Livingston, 2004) the C_3 photosynthesis model of (Farquhar *et al.*, 1980) was fitted to
 190 the A vs c_i relationship to obtain the maximum velocity of Rubisco for carboxylation
 191 (V_{cmax}) and the potential rate of electron transport under saturating light, J_{max} . To
 192 ensure full hydration during gas exchange experiments the base of the pot was placed
 193 in 5–10 mm deionised water for the duration of the measurements. At least three
 194 plants of each genotype were analysed for each experiment.

195 Stomatal size, density and $g_{w(\text{max})}$

196 For each leaf in which gas exchange was measured, approximately 0.5 cm^2 of
 197 epidermis was dissected from the abaxial and adaxial surfaces of the leaf, mounted in
 198 water on a glass microscope slide and examined at 400 times magnification using a
 199 light microscope. Stomatal size ($S, \mu\text{m}^2$) was calculated as guard cell length \times guard
 200 cell pair width; stomatal density (D, mm^{-2}) was calculated as number of stomata per
 201 0.140 mm^2 field of view. For each epidermal peel, 20 stomata were sampled for size
 202 and ten 0.140 mm^2 fields were sampled for density. Maximum stomatal conductance
 203 to water vapour, $g_{w(\text{max})}$, was calculated using the basic diffusion equation (Franks and
 204 Beerling, 2009):

$$205 \quad g_{w(\text{max})} = \frac{d}{v} \cdot D \cdot a_{\text{max}} \left/ \left(l + \frac{\pi}{2} \sqrt{a_{\text{max}}/\pi} \right) \right. , \quad [1]$$

206 where constants d and v are, respectively, the diffusivity of H₂O in air and the molar
207 volume of air, D is stomatal density and a_{\max} is the average maximum stomatal pore
208 area, which in *Arabidopsis* approximates a circle with diameter equal to the stomatal
209 pore length p , i.e. $a_{\max} = \pi p^2/4$. The quantity $g_{w(\max)}$ is the anatomically-determined
210 maximum possible stomatal conductance which sets the theoretical range over which
211 $g_{w(\text{op})}$ can be controlled.

212 Carbon Isotope Analysis

213 Four mature leaves from each plant were dried overnight at 75 °C and ground to
214 powder. The ratio of ¹³C to ¹²C was measured with a continuous flow mass
215 spectrometer (ANCA GSL 20-20, Sercon PDZ Europa, Sercon Ltd., Cheshire, UK for
216 Experiment 1 and Thermo Finnigan Delta V IRMS, Finnigan MAT GmbH,
217 Barkhausenstr, Germany for Experiment 2). The carbon isotope composition of leaf
218 tissue ($\delta^{13}\text{C}_{\text{leaf}}$, in per mil, ‰) was calculated as (Farquhar *et al.*, 1989):

219

$$220 \quad \delta^{13}\text{C}_{\text{leaf}} = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000 \quad [2]$$

221

222 where R_{sample} and R_{standard} are the ¹³C/¹²C ratios of the leaf tissue and the V-PDB
223 standard, respectively. $\delta^{13}\text{C}_{\text{leaf}}$ was then converted to leaf carbon isotope
224 discrimination Δ_{leaf} (‰) using (Farquhar & Richards, 1984):

225

$$226 \quad \Delta_{\text{leaf}} = \frac{\delta^{13}\text{C}_{\text{air}} - \delta^{13}\text{C}_{\text{leaf}}}{1 + \delta^{13}\text{C}_{\text{leaf}}/1000} \quad [3]$$

227

228 with $\delta^{13}\text{C}_{\text{air}}$ and $\delta^{13}\text{C}_{\text{leaf}}$ in units of ‰. Because air in the greenhouse was well-mixed
229 with outside ambient air the $\delta^{13}\text{C}_{\text{air}}$ for greenhouse-grown plants was taken as -8.2 ‰
230 (Carbon Dioxide Information Analysis Centre (CDIAC), Oak Ridge National
231 Laboratory, Oak Ridge, USA; <ftp://ftp.cmdl.noaa.gov/ccg/co2c13>). For the growth-

232 chamber plants, $\delta^{13}\text{C}_{\text{air}}$ was measured as -10.4 ‰ using the method in Fletcher *et al.*
233 (2006).

234

235 Statistical analysis

236 All statistical analyses were carried using OriginPro software (OriginLab Corp.,
237 Northampton, MA, USA). Significant difference between means (0.05 level) was
238 determined using one- and two-way analysis of variance (ANOVA) and post-hoc
239 mean comparison tests (Tukey and Scheffe multiple comparisons).

240 **Results**

241 Constitutive overexpression of *EPF2* led to higher time-integrated WUE irrespective
242 of growth irradiance, as indicated by significantly lower leaf $\Delta^{13}\text{C}$ in the *EPF2OE*
243 genotype grown under both high and low light intensity (Fig. 1). The double mutant
244 *epf1epf2*, with characteristically higher stomatal density, showed lower WUE (higher
245 leaf $\Delta^{13}\text{C}$) than the Col-0 control, but the means were not significantly different (Fig.
246 1). Within each genotype, plants grown under low light had significantly lower WUE
247 (higher leaf $\Delta^{13}\text{C}$) than those grown under high light.

248 Instantaneous leaf gas exchange data reflect the pattern observed in leaf $\Delta^{13}\text{C}$.
249 The operating CO_2 assimilation rate (A_{op}) was significantly lower in *EPF2OE*
250 compared to Col-0 (Fig. 2a), but the greater relative reduction in operating stomatal
251 conductance ($g_{\text{w(op)}}$) (Fig. 2b) and lower operating c_i/c_a (Fig. 2c) explain the
252 significantly higher instantaneous WUE in *EPF2OE* (Fig. 2d). Remarkably, potential
253 rate of photosynthesis at any given leaf intercellular CO_2 concentration is virtually the
254 same in each genotype (Fig. 3) despite different operating points (see arrows in Fig.
255 3) on account of differences in stomatal conductance. Further evidence of unaltered
256 photosynthetic potential in the genetically modified plants is confirmed by the lack of
257 any significant differences in V_{cmax} and J_{max} (Fig 4). These results indicate that the
258 genetically altered stomatal density in *EPF2OE* and *epf1epf2* is decoupled from
259 photosynthetic biochemistry.

260 The genetic modification of stomatal density appears to be inextricably
261 accompanied by a qualitatively similar but opposite change in stomatal size, as

262 observed in natural systems (Franks & Beerling, 2009; Franks *et al.*, 2009) and
263 studies of genetically modified *Arabidopsis* (Doheny-Adams *et al.*, 2012; Dow *et al.*,
264 2014b). On both abaxial and adaxial leaf surfaces, *EPF2OE* showed significantly
265 lower stomatal density and larger stomatal size, while *epflepf2* showed significantly
266 higher stomatal density and smaller stomatal size, compared to the Col-0 control (Fig.
267 5a,b). These differences translate into substantially lower maximum stomatal
268 conductance ($g_{w(max)}$) in *EPF2OE* and substantially higher $g_{w(max)}$ in *epflepf2* relative
269 to Col-0 (Fig. 6a), consistent with the pattern observed in stomatal operating point,
270 $g_{w(op)}$ (Fig. 2b). Altered $g_{w(max)}$ appeared to affect the *relative* stomatal operating
271 point, defined as the ratio $g_{w(op)}/g_{w(max)}$. Lower $g_{w(max)}$ in *EPF2OE* was associated
272 with significantly higher $g_{w(op)}/g_{w(max)}$, while higher $g_{w(max)}$ in *epflepf2* was associated
273 with lower $g_{w(op)}/g_{w(max)}$ (Fig. 6b). The integrated relationship between *S*, *D* and
274 $g_{w(max)}$, as affected by the modification of genes acting directly on stomatal
275 development, is shown in Fig. 7. For both the abaxial and adaxial leaf surfaces, *S*
276 varies inversely with *D* (Fig. 7, black lines), with significantly lower $g_{w(max)}$ in
277 *EPF2OE* achieved through larger *S* and lower *D*, and higher $g_{w(max)}$ in *epflepf2*
278 achieved through smaller *S* and higher *D*.

279 Discussion

280 Direct reduction of $g_{w(max)}$ through genetic modification of *EPF* gene expression
281 increased WUE without significantly altering photosynthetic capacity, confirming our
282 main hypothesis. Relative to the Col-0 control, both the *EPF2OE* and *epflepf2*
283 mutants exhibited significantly altered stomatal densities and sizes (Fig 5a,b) that
284 changed $g_{w(max)}$ (Fig. 6a) and consequently shifted the stomatal conductance operating
285 point, $g_{w(op)}$ (Fig. 2b). The accompanying shift in WUE, which is significantly higher
286 for the low-*D* *EPF2OE* mutant, and lower for the high-*D* *epflepf2* mutant, is evident
287 when measured instantaneously for standard conditions (Fig 2d) and also when
288 inferred over the long-term functioning of leaves from leaf $\Delta^{13}C$ (Fig. 1). The
289 targeting of stomatal development through these *EPF* gene modifications could be a
290 powerful tool for manipulating WUE in *Arabidopsis* and potentially other plant
291 species.

292 Apart from reported differences in plant size (Doheny-Adams *et al.*, 2012), the
293 retention of photosynthetic capacity and normal reproductive function in the *EPF*

294 mutants stands in contrast to other potential methods of genetically modifying WUE
295 via altered stomatal density. Loss-of-function mutations in the mitogen-activated
296 protein kinase (MAPK) kinase gene *YODA* result in plants with high stomatal density
297 but a dwarfed stature with defective and sterile inflorescences (Bergmann *et al.*,
298 2004). Mutations in the *ERECTA* gene influence WUE (Masle *et al.*, 2005) but also
299 confer multiple phenotypic changes in inflorescences, fruits and leaves (Torii *et al.*,
300 1996; van Zanten *et al.*, 2009). Loss-of-function *ERECTA* mutants exhibit lower
301 WUE due to both increased stomatal conductance (through increased *D*) and reduced
302 biochemical capacity for photosynthesis (Masle *et al.*, 2005). Our results show that
303 mutations in the *EPF* gene can achieve both increases and decreases in WUE, via
304 altered stomatal properties, without altering photosynthetic biochemistry (Fig. 3, 4).

305 The shift in the relative stomatal conductance operating point, $g_{w(op)}/g_{w(max)}$, in
306 both the *EPF2OE* and *epf1epf2* mutants (Fig. 6b) indicates a partial physiological
307 counteraction of genetically altered $g_{w(max)}$. Thus, *EPF2OE*, with inherently lower
308 $g_{w(max)}$ compared to Col-0, operates at close to 50% of $g_{w(max)}$, while *epf1epf2*, with
309 inherently higher $g_{w(max)}$ operates at just below 20% of $g_{w(max)}$. A similar pattern of
310 higher $g_{w(op)}/g_{w(max)}$ in low-*D Arabidopsis* mutants is evident in the results of (Dow *et*
311 *al.*, 2014a). Previous work has shown that in typical environmental conditions plants
312 tend to operate at around 20% of $g_{w(max)}$ (Franks *et al.*, 2011; Dow *et al.*, 2014a), in
313 the region of greatest stomatal sensitivity and mechanical efficiency per unit guard
314 cell pressure (Franks *et al.*, 2012). However, under atypical conditions which
315 promote wider stomatal apertures, such as the need to counteract CO₂ starvation at
316 low atmospheric CO₂, $g_{w(op)}/g_{w(max)}$ can be much higher (Dow *et al.*, 2014a).
317 Similarly, the higher $g_{w(op)}/g_{w(max)}$ in *EPF2OE* plants helps to maintain photosynthesis
318 closer to optimum, but in doing so stomata must operate sub-optimally from a
319 mechanical perspective. When exposed to high atmospheric CO₂ concentration, low-
320 *D* (and therefore low- $g_{w(max)}$) *Arabidopsis* mutants operate closer to 20% of $g_{w(max)}$
321 (Dow *et al.*, 2014a).

322 The two primary morphological characteristics determining $g_{w(max)}$ are
323 stomatal size (*S*, which strongly determines maximum stomatal aperture, a_{max}) and
324 density (*D*) (Franks and Beerling, 2009; Franks *et al.* 2009). For any given $g_{w(max)}$
325 there is in theory an infinite number of *S/D* combinations. The four lines in Fig 7 are
326 loci of constant $g_{w(max)}$ for different combinations of *S* and *D*, calculated using Eqn 1,

327 as described in Franks and Beerling, 2009. These lines are overlaid on the actual
328 $g_{w(max)}$ data points to show how genetic modification of stomatal density (and
329 associated changes in stomatal size) moves $g_{w(max)}$ on the surface represented by
330 stomatal size versus density. Thus, in *EPF2OE* mutants, lower $g_{w(max)}$ on both the
331 abaxial and adaxial leaf surfaces is due to fewer and larger stomata, while in *epf1epf2*
332 mutants the opposite trend is seen.

333 The significant reduction in $g_{w(op)}$ and $g_{w(max)}$ with conservation of J_{max} in
334 *EPF2OE* plants as a result of their genetic modification resembles the generally
335 observed natural adaptation of plants to growth under elevated atmospheric CO₂
336 concentration (Ainsworth & Rogers, 2007; Franks *et al.*, 2013). This translates to
337 reduced rates of transpiration per unit CO₂ assimilation and therefore increased WUE.
338 *EPF2OE* plants may therefore be regarded as genetically pre-adapted to future high-
339 CO₂ atmospheres. There is potential for application of this technology to improve
340 WUE in crops under future high-CO₂ scenarios, particularly those that may exhibit
341 limited natural capacity for adaptation to high atmospheric CO₂ concentration.

342 Improving WUE in *EPF2OE* by genetically altering stomatal density requires
343 the natural physiological coupling between stomatal conductance and photosynthetic
344 capacity to be broken. Within and across plant species, and under a variety of
345 conditions, there is a strong correlation between $g_{w(op)}$ and A_{op} which tends to
346 conserve the relative gradient for CO₂ diffusion into the leaf (Wong *et al.*, 1979; Field
347 & Mooney, 1986; Hetherington & Woodward, 2003). This suggests that stomatal
348 development and function is normally closely coordinated with the biochemical
349 capacity for photosynthesis across developmental and evolutionary timescales. The
350 strength of this coupling is further evident in crop breeding where selection for higher
351 productivity is usually accompanied by higher stomatal conductance and lower WUE
352 (French & Schulz, 1984; Fischer *et al.*, 1998; Condon *et al.*, 2004). Disruption of this
353 correlation has been shown in transgenic plants which maintain normal stomatal
354 conductance despite an impaired photosynthetic mechanism (Quick *et al.*, 1991; von
355 Caemmerer *et al.*, 2004). In these cases, impaired photosynthesis results in reduced
356 WUE, but breaking the stomatal/photosynthesis connection in this way provided early
357 indications that stomata could be similarly targeted for manipulation, independently
358 of photosynthetic capacity, to change WUE. Our results with *EPF2OE* are consistent
359 with this and suggest that under a future climate of high atmospheric CO₂

360 concentrations and in regions of diminishing water supply, targeted genetic
361 modification of stomatal conductance such as in *EPF2*OE is a viable approach for
362 improving WUE in crops.

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367

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476 **Figure legends**

477

478 **Figure 1.** Higher WUE from constitutive overexpression of *EPF2*. Lower leaf tissue
479 $\Delta^{13}\text{C}$ in *EPF2OE* mutants relative to Col-0 wild type plants confirms that they
480 typically operate with lower c_i/c_a and higher WUE. Different letters between
481 genotypes of the same light treatment, and between light treatments of the same
482 genotype, indicate the means are significantly different (0.05 level). Plants grown
483 under photosynthesis-saturating sunlight ('high light') showed significantly lower leaf
484 $\Delta^{13}\text{C}$ and hence higher WUE than plants growing in controlled environment chambers
485 under fluorescent light ('low light').

486 **Figure 2.** Higher instantaneous WUE in *EPF2OE* under typical high-light
487 conditions. (a) operating CO_2 assimilation rate, $A_{(\text{op})}$, (b) operating stomatal
488 conductance to water vapour, $g_{w(\text{op})}$, (c) ratio of leaf intercellular to ambient CO_2
489 concentration, c_i/c_a , and (d) water-use efficiency, WUE, as $A_{(\text{op})}/E$, for the *EPF2OE*,
490 Col-0 and *epfl epf2 Arabidopsis thaliana* genotypes. Different letters above standard
491 error bars indicate the means are significantly different.

492 **Figure 3.** Photosynthetic biochemistry not significantly affected in either of the EPF
493 mutants. Shown is CO_2 assimilation rate versus leaf intercellular CO_2 concentration
494 for the *EPF2OE*, Col-0 and *epfl epf2 Arabidopsis thaliana* genotypes. Individual data
495 points represent mean and standard error for three–five plants. Blue, black and red
496 arrows indicate the operating point, as determined by stomatal conductance, for
497 *epfl epf2*, Col-0 and *EPF2OE*, respectively, at 400 ppm CO_2 and saturating light.
498 Similar photosynthetic biochemistry but lower operating stomatal conductance in
499 *EPF2OE* mutants (see Fig. 2b) results in higher WUE.

500 **Figure 4.** No significant differences in photosynthetic potential. (a), maximum
501 velocity of Rubisco for carboxylation (V_{cmax}) and (b) the potential rate of electron
502 transport under saturating light, J_{max} , do not differ significantly (0.05 level) across the
503 three genotypes *EPF2OE*, Col-0 and *epfl epf2*.

504 **Figure 5.** Altered stomatal density (a) and size (b) in *EPF2OE* and *epfl epf2*
505 *Arabidopsis thaliana* mutants. Significantly lower density and larger stomatal size in
506 *EPF2OE* contributes to lower maximum stomatal conductance (see Fig. 6a).

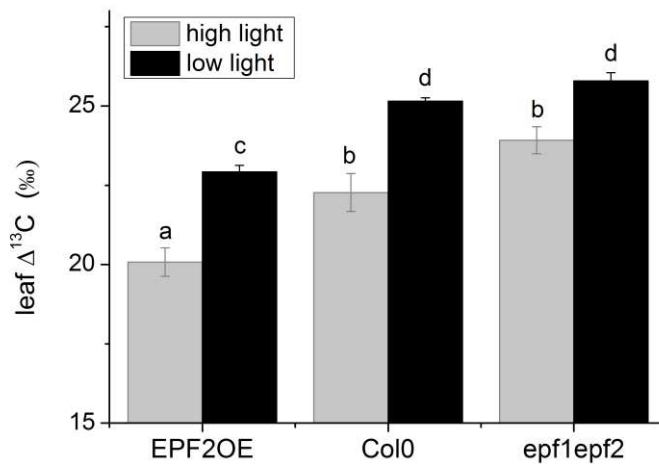
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508 different.

509 **Figure 6.** Lower maximum stomatal conductance in *EPF2OE*. (a) Maximum
510 stomatal conductance to water vapour, $g_{w(max)}$, calculated from stomatal size and
511 density, and (b) the ratio of the operating stomatal conductance to maximum stomatal
512 conductance, $g_{w(op)}/g_{w(max)}$, for the *EPF2OE*, Col-0 and *epf1epf2 Arabidopsis thaliana*
513 genotypes. Different letters above standard error bars indicate the means are
514 significantly different.

515 **Figure 7.** Shift in maximum stomatal conductance via coordinated changes in
516 stomatal size and density. Black lines connect genotypes from lowest to highest
517 stomatal density (*EPF2OE* is genetically modified for low stomatal density; *epf1epf2*
518 is genetically modified for high stomatal density relative to Col-0 control). Red lines
519 indicate combinations of *S* and *D* giving constant maximum stomatal conductance to
520 water vapour, $g_{w(max)}$. Increasing $g_{w(max)}$ on both abaxial and adaxial leaf surfaces
521 follows the classical negative log-log relationship between *S* and *D*.

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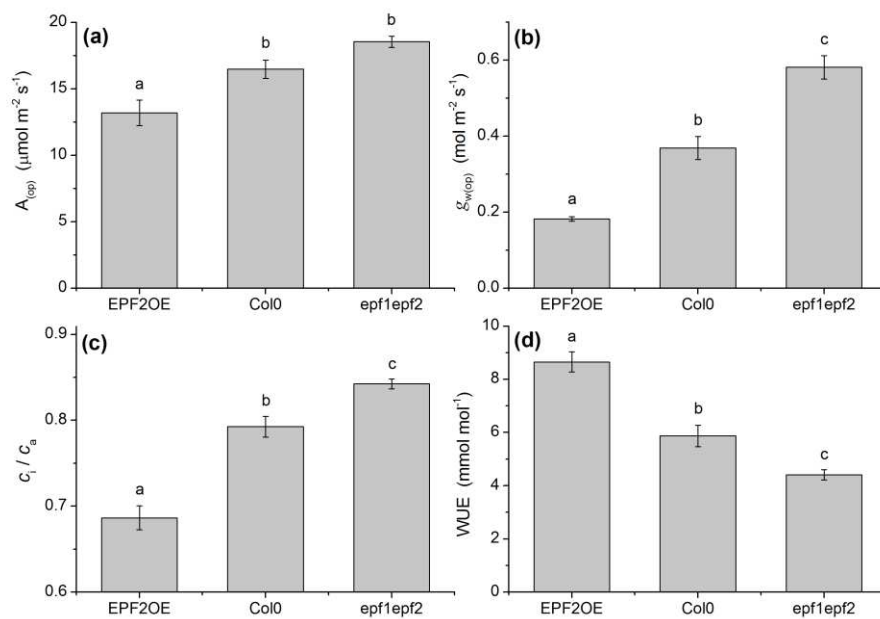
523 FIGURES



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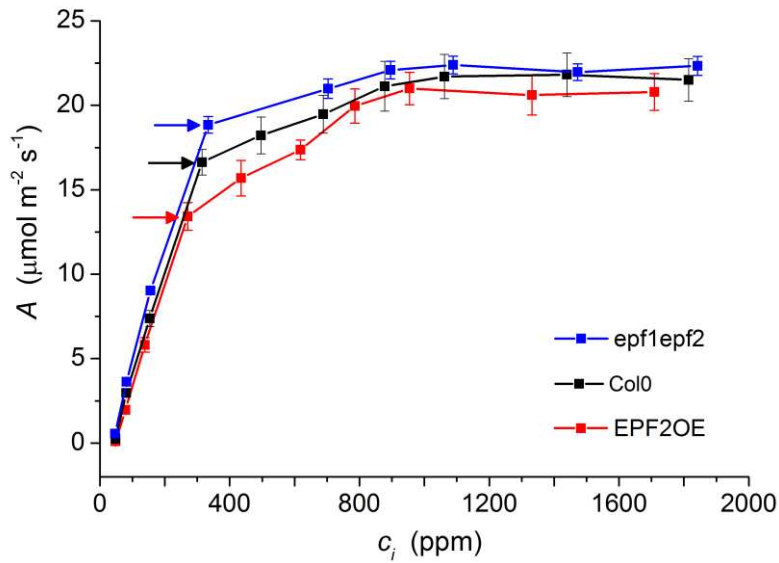


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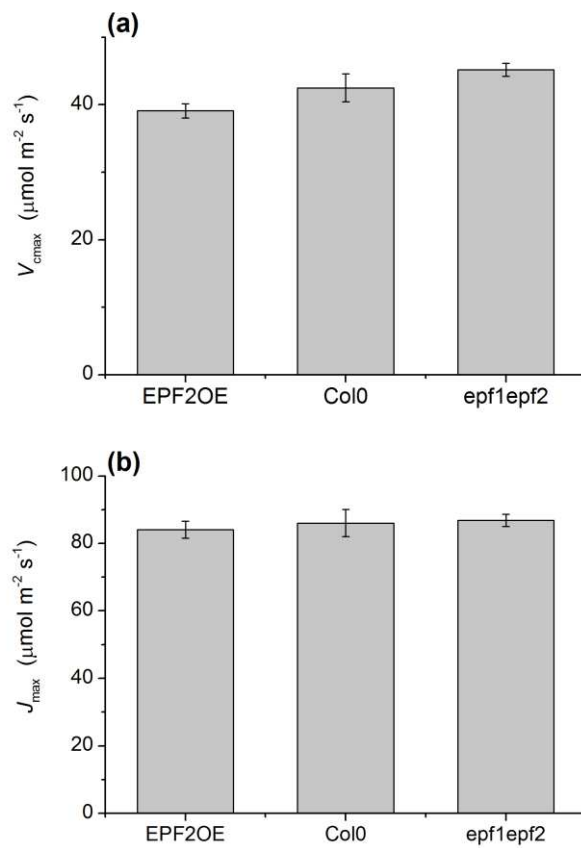


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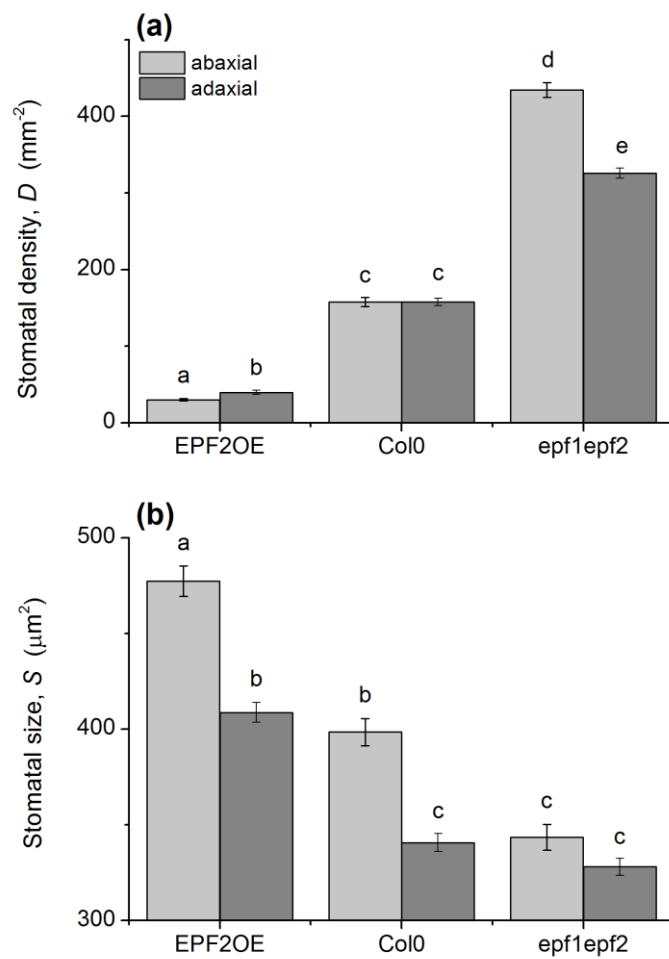


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559 velocity of Rubisco for carboxylation (V_{cmax}) and (b) the potential rate of electron
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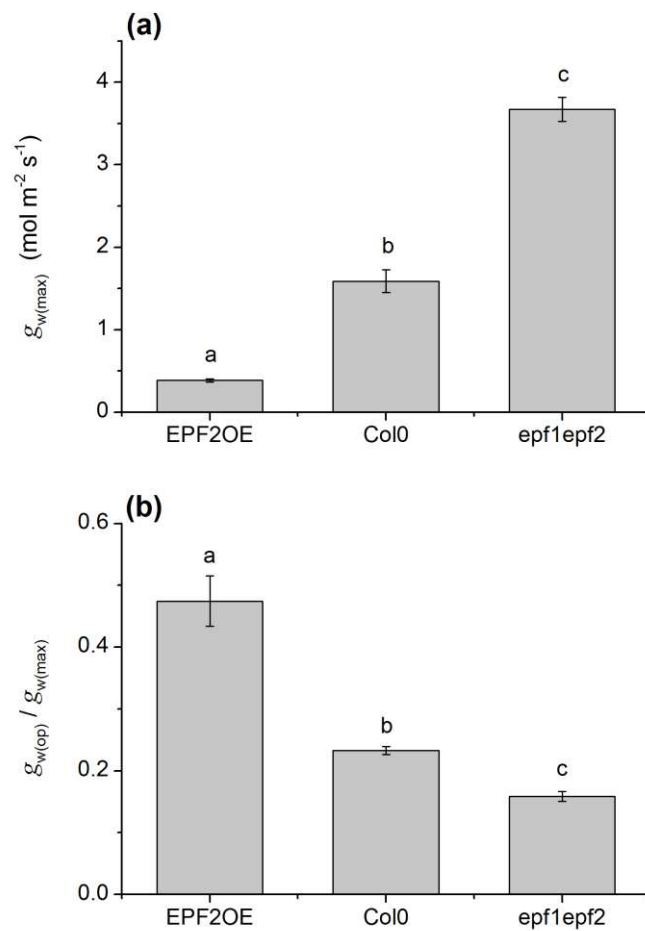
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 568 Different letters above standard error bars within a genotype, and within
 569 corresponding leaf surfaces (abaxial or adaxial) between genotypes, indicate the
 570 means are significantly different.

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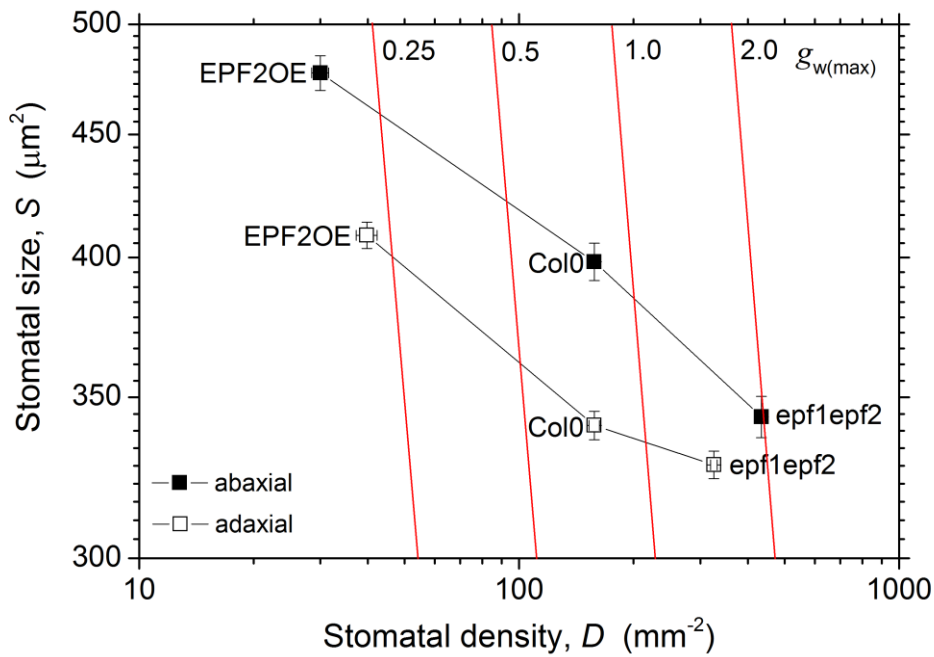
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