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

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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-CO_2 atmospheres.

Key words:

water-use efficiency; $\delta^{13}C$; stomata; Arabidopsis; crops; CO_2, Epidermal Patterning Factor; genetically modified

Introduction

Increased water use efficiency (WUE; the ratio of the rates of CO_2 assimilation to transpiration, A/E) can improve productivity and reduce water stress under drier environmental conditions (Slatyer, 1964; Sinclair et al., 1984; Han et al., 2013). In the short term, plants increase WUE by reducing stomatal apertures and therefore E, but often under prolonged water deficit plants also produce leaves with reduced maximum stomatal conductance ($g_{w(max)}$) via altered stomatal density (D) and/or size (S) (Gindel, 1969; Franks et al., 2009; Doheny-Adams et al., 2012). It is unclear why plants undergo this developmental response to drought in addition to simply reducing stomatal aperture, but it has been suggested that when conditions promote a long-term reduction in the average operating stomatal conductance, production of new leaves with reduced $g_{w(max)}$ maintains more favourable mechanical and energetic conditions for stomatal control (Franks et al., 2009; Franks et al., 2012). This specific adaptation of $g_{w(max)}$ for improved WUE may represent a model for genetic manipulation of WUE. Here, using Arabidopsis epidermal patterning factor (EPF) mutants exhibiting altered stomatal density, we show that WUE can be improved directly by manipulation of the genes controlling the development of stomata to reduce $g_{w(max)}$.

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

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

A subtilisin peptidase STOMATAL DENSITY AND DISTRIBUTION 1 (SDD1) was the first genetic component identified as regulating stomatal development (Berger & Altmann 2000), but it achieves this by a mechanism which remains unknown, and that appears to act independently of the EPF peptides and their receptors (Hunt & Gray, 2009; Hunt et al., 2010). Plants lacking SDD1 expression have increased stomatal density (sdd1-1; approximately 250% of C24 background) and a low level of stomatal pairing. The stomatal conductance of sdd1 plants was consistently higher than that of control plants (following growth at three different light intensities) particularly when measured at higher light intensities (Schlüter et al., 2003). However, photosynthetic assimilation rates, although higher, were not significantly increased in the high D plants except in an experiment following transfer to a higher light intensity before analysis. When sdd1 plants (with inherently high D) were grown at 120 mmol m^{-2} s^{-1} and then shifted for 2 d to high light conditions (500mmol m^{-2} s^{-1}) their maximal photosynthetic capacity was increased by 30% in comparison to wild-type controls, although their conductance was not significantly increased (Schlüter et al., 2003). These pioneering experiments suggested that plants manipulated to have substantially increased D have correspondingly increased levels of stomatal conductance. If allowed to biochemically acclimate to higher light intensity (i.e. typical saturation intensities) these plants may potentially exhibit enhanced CO_2 assimilation without loss of WUE.

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

availability was limited. However, for plants such epf1epf2 exhibiting increased D and $g_{w(max)}$, we did not identify any conditions under which the growth of plants was improved. It is not known why the growth rate of plants with low D was enhanced but it may have resulted from a combination of lower metabolic cost of developing and operating fewer stomata, higher A at the elevated leaf temperature, or improved water status from a reduction in E (Doheny-Adams et al., 2012).

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

Using a range of Arabidopsis stomatal development mutants with no, or low levels of stomatal clustering including epf1 and epf1epf2, (Dow et al., 2014a) reported a strong correlation between $g_{w(max)}$ determined from anatomy (via measurements of S and D) and $g_{w(max)}$ from gas exchange measurements. This proved that the developmental changes in S and D translated to a shift in the operational range of stomatal conductance. If these shifts in $g_{w(max)}$ were uncoupled from photosynthetic biochemistry then WUE should increase with declining $g_{w(max)}$. However, in that study, a significant increase in WUE was only observed with mutants exhibiting high stomatal clustering, an abnormal condition resulting from disruption of stomatal spacing control.

Despite these new genetic tools for manipulating D and $g_{w(max)}$, there is no clear evidence of a significant enhancement of WUE via an engineered reduction in $g_{w(max)}$. Our goal in this study was to compare the instantaneous and long-term WUE of Arabidopsis efp1epf2 (high-D) and EPF2OE (low-D) mutants relative to Col-0 control plants. Our hypothesis is that the developmental changes in EPF2OE reduce $g_{w(max)}$ and thereby shift the operating stomatal conductance to a lower state; if the EPF2OE mutation affects $g_{w(max)}$ exclusively then the photosynthetic biochemistry

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

Materials and Methods

Plant Growth

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

Leaf gas exchange measurements

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

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

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

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

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

where constants d and v are, respectively, the diffusivity of H_2O in air and the molar volume of air, D is stomatal density and a_{max} is the average maximum stomatal pore area, which in Arabidopsis approximates a circle with diameter equal to the stomatal pore length p, i.e. $a_{max} = \pi p^2/4$. The quantity $g_{w(max)}$ is the anatomically-determined maximum possible stomatal conductance which sets the theoretical range over which $g_{w(op)}$ can be controlled.

Carbon Isotope Analysis

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

$$\delta^{13}C_{leaf} = \left(\frac{R_{sample}}{R_{standard}} - 1 \right) \times 1000 \qquad [2]$$

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

$$\Delta_{leaf} = \frac{\delta^{13}C_{air} - \delta^{13}C_{leaf}}{1 + \delta^{13}C_{leaf}/1000} \qquad [3]$$

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

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

Statistical analysis

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

Results

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

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

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

observed in natural systems (Franks & Beerling, 2009; Franks et al., 2009) and studies of genetically modified Arabidopsis (Doheny-Adams et al., 2012; Dow et al., 2014b). On both abaxial and adaxial leaf surfaces, EPF2OE showed significantly lower stomatal density and larger stomatal size, while epf1epf2 showed significantly higher stomatal density and smaller stomatal size, compared to the Col-0 control (Fig. 5a,b). These differences translate into substantially lower maximum stomatal conductance ($g_{w(max)}$) in EPF2OE and substantially higher $g_{w(max)}$ in epf1epf2 relative to Col-0 (Fig. 6a), consistent with the pattern observed in stomatal operating point, $g_{w(op)}$ (Fig. 2b). Altered $g_{w(max)}$ appeared to affect the relative stomatal operating point, defined as the ratio $g_{w(op)}/g_{w(max)}$. Lower $g_{w(max)}$ in EPF2OE was associated with significantly higher $g_{w(op)}/g_{w(max)}$, while higher $g_{w(max)}$ in epf1epf2 was associated with lower $g_{w(op)}/g_{w(max)}$ (Fig. 6b). The integrated relationship between S, D and $g_{w(max)}$, as affected by the modification of genes acting directly on stomatal development, is shown in Fig. 7. For both the abaxial and adaxial leaf surfaces, S varies inversely with D (Fig. 7, black lines), with significantly lower $g_{w(max)}$ in EPF2OE achieved through larger S and lower D, and higher $g_{w(max)}$ in epf1ep2 achieved through smaller S and higher D.

Discussion

Direct reduction of $g_{w(max)}$ through genetic modification of EPF gene expression increased WUE without significantly altering photosynthetic capacity, confirming our main hypothesis. Relative to the Col-0 control, both the EPF2OE and epf1epf2 mutants exhibited significantly altered stomatal densities and sizes (Fig 5a,b) that changed $g_{w(max)}$ (Fig. 6a) and consequently shifted the stomatal conductance operating point, $g_{w(op)}$ (Fig. 2b). The accompanying shift in WUE, which is significantly higher for the low-D EPF2OE mutant, and lower for the high-D epf1epf2 mutant, is evident when measured instantaneously for standard conditions (Fig 2d) and also when inferred over the long-term functioning of leaves from leaf $\Delta^{13}C$ (Fig. 1). The targeting of stomatal development through these EPF gene modifications could be a powerful tool for manipulating WUE in Arabidopsis and potentially other plant species.

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

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

The shift in the relative stomatal conductance operating point, $g_{w(op)}/g_{w(max)}$, in both the EPF2OE and epf1epf2 mutants (Fig. 6b) indicates a partial physiological counteraction of genetically altered $g_{w(max)}$. Thus, EPF2OE, with inherently lower $g_{w(max)}$ compared to Col-0, operates at close to 50% of $g_{w(max)}$, while epf1epf2, with inherently higher $g_{w(max)}$ operates at just below 20% of $g_{w(max)}$. A similar pattern of higher $g_{w(op)}/g_{w(max)}$ in low-D Arabidopsis mutants is evident in the results of (Dow et al., 2014a). Previous work has shown that in typical environmental conditions plants tend to operate at around 20% of $g_{w(max)}$ (Franks et al., 2011; Dow et al., 2014a), in the region of greatest stomatal sensitivity and mechanical efficiency per unit guard cell pressure (Franks et al., 2012). However, under atypical conditions which promote wider stomatal apertures, such as the need to counteract CO_2 starvation at low atmospheric CO_2, $g_{w(op)}/g_{w(max)}$ can be much higher (Dow et al., 2014a). Similarly, the higher $g_{w(op)}/g_{w(max)}$ in EPF2OE plants helps to maintain photosynthesis closer to optimum, but in doing so stomata must operate sub-optimally from a mechanical perspective. When exposed to high atmospheric CO_2 concentration, low-D (and therefore low-$g_{w(max)}$) Arabidopsis mutants operate closer to 20% of $g_{w(max)}$ (Dow et al., 2014a).

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

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

The significant reduction in $g_{w(op)}$ and $g_{w(max)}$ with conservation of J_{max} in EPF2OE plants as a result of their genetic modification resembles the generally observed natural adaptation of plants to growth under elevated atmospheric CO_2 concentration (Ainsworth & Rogers, 2007; Franks et al., 2013). This translates to reduced rates of transpiration per unit CO_2 assimilation and therefore increased WUE. EPF2OE plants may therefore be regarded as genetically pre-adapted to future high-CO_2 atmospheres. There is potential for application of this technology to improve WUE in crops under future high-CO_2 scenarios, particularly those that may exhibit limited natural capacity for adaptation to high atmospheric CO_2 concentration.

Improving WUE in EPF2OE by genetically altering stomatal density requires the natural physiological coupling between stomatal conductance and photosynthetic capacity to be broken. Within and across plant species, and under a variety of conditions, there is a strong correlation between $g_{w(op)}$ and A_{op} which tends to conserve the relative gradient for CO_2 diffusion into the leaf (Wong et al., 1979; Field & Mooney, 1986; Hetherington & Woodward, 2003). This suggests that stomatal development and function is normally closely coordinated with the biochemical capacity for photosynthesis across developmental and evolutionary timescales. The strength of this coupling is further evident in crop breeding where selection for higher productivity is usually accompanied by higher stomatal conductance and lower WUE (French & Schulz, 1984; Fischer et al., 1998; Condon et al., 2004). Disruption of this correlation has been shown in transgenic plants which maintain normal stomatal conductance despite an impaired photosynthetic mechanism (Quick et al., 1991; von Caemmerer et al., 2004). In these cases, impaired photosynthesis results in reduced WUE, but breaking the stomatal/photosynthesis connection in this way provided early indications that stomata could be similarly targeted for manipulation, independently of photosynthetic capacity, to change WUE. Our results with EPF2OE are consistent with this and suggest that under a future climate of high atmospheric CO_2

concentrations and in regions of diminishing water supply, targeted genetic modification of stomatal conductance such as in EPF2OE is a viable approach for improving WUE in crops.

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474
475

Figure legends

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

Figure 2. Higher instantaneous WUE in EPF2OE under typical high-light conditions. (a) operating CO_2 assimilation rate, $A_{(op)}$, (b) operating stomatal conductance to water vapour, $g_{w(op)}$, (c) ratio of leaf intercellular to ambient CO_2 concentration, c_i/c_a, and (d) water-use efficiency, WUE, as $A_{(op)}/E$, for the EPF2OE, Col-0 and epf1epf2 Arabidopsis thaliana genotypes. Different letters above standard error bars indicate the means are significantly different.

Figure 3. Photosynthetic biochemistry not significantly affected in either of the EPF mutants. Shown is CO_2 assimilation rate versus leaf intercellular CO_2 concentration for the EPF2OE, Col-0 and epf1epf2 Arabidopsis thaliana genotypes. Individual data points represent mean and standard error for three–five plants. Blue, black and red arrows indicate the operating point, as determined by stomatal conductance, for epf1epf2, Col-0 and EPF2OE, respectively, at 400 ppm CO_2 and saturating light. Similar photosynthetic biochemistry but lower operating stomatal conductance in EPF2OE mutants (see Fig. 2b) results in higher WUE.

Figure 4. No significant differences in photosynthetic potential. (a), maximum velocity of Rubisco for carboxylation (V_{cmax}) and (b) the potential rate of electron transport under saturating light, J_{max}, do not differ significantly (0.05 level) across the three genotypes EPF2OE, Col-0 and epf1epf2.

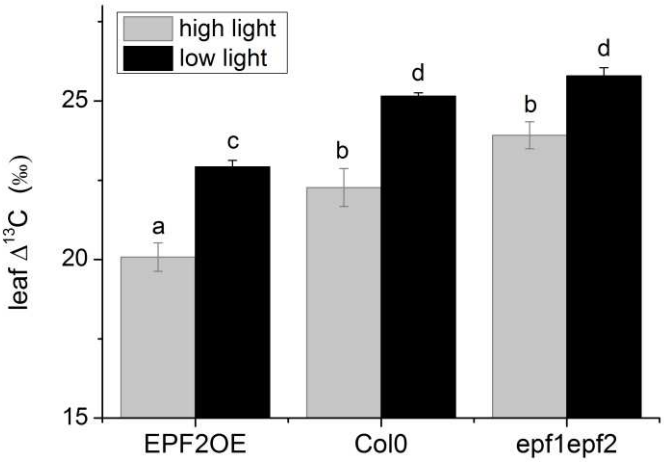
Figure 5. Altered stomatal density (a) and size (b) in EPF2OE and epf1epf2 Arabidopsis thaliana mutants. Significantly lower density and larger stomatal size in EPF2OE contributes to lower maximum stomatal conductance (see Fig. 6a).

Different letters above standard error bars indicate the means are significantly different.

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

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

523 FIGURES



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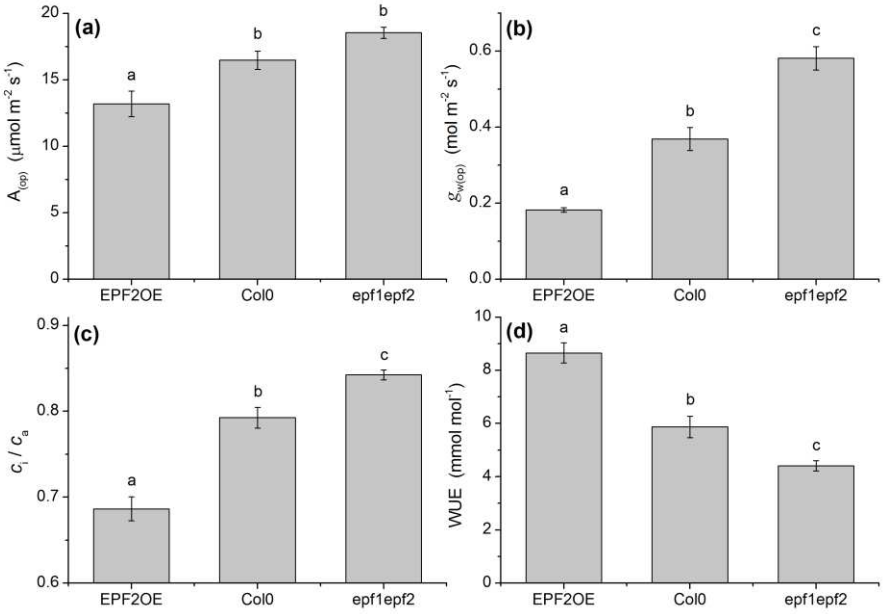

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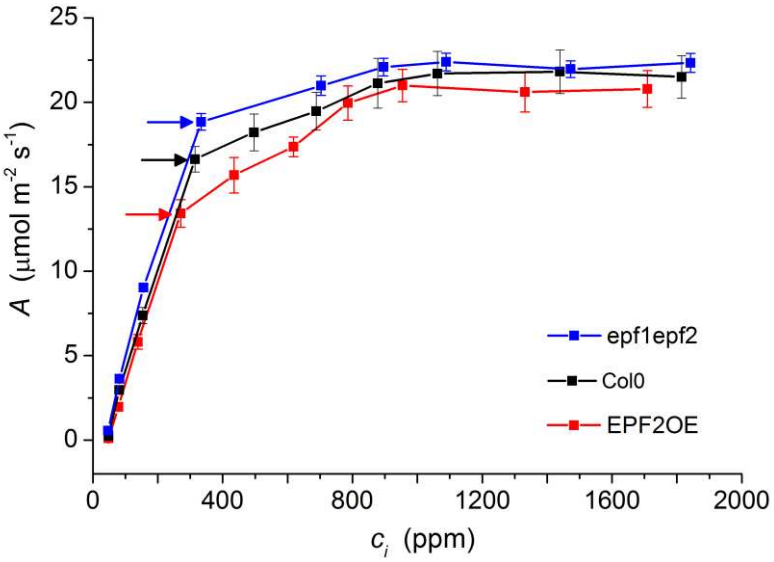

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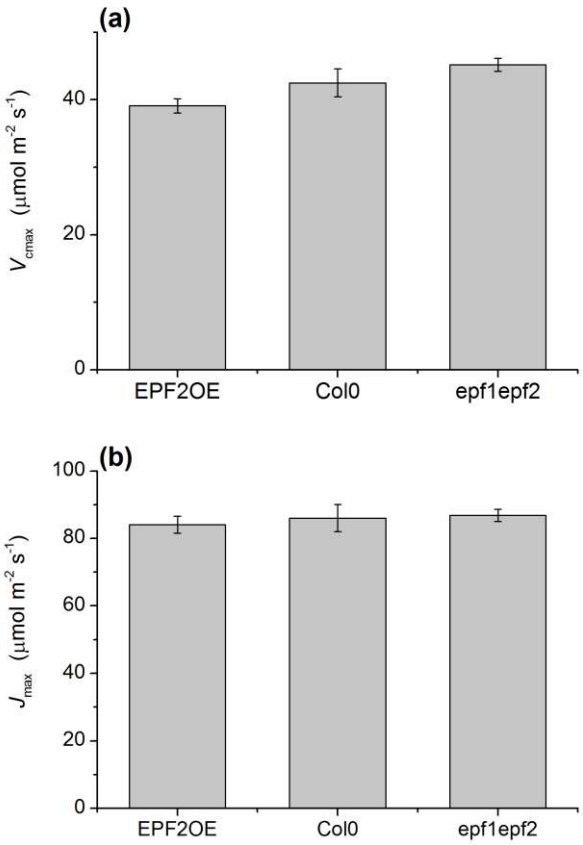

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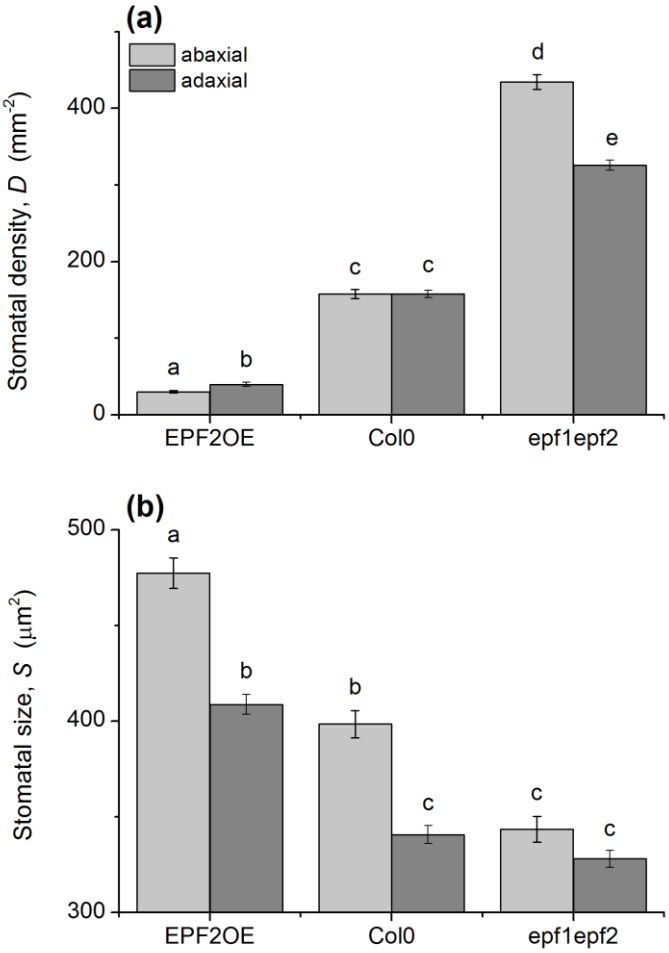

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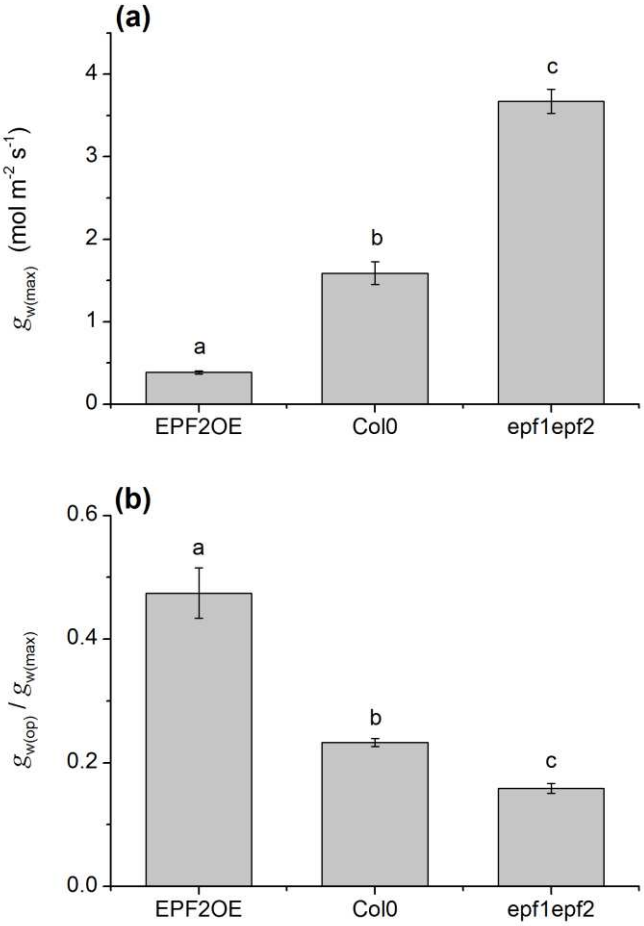

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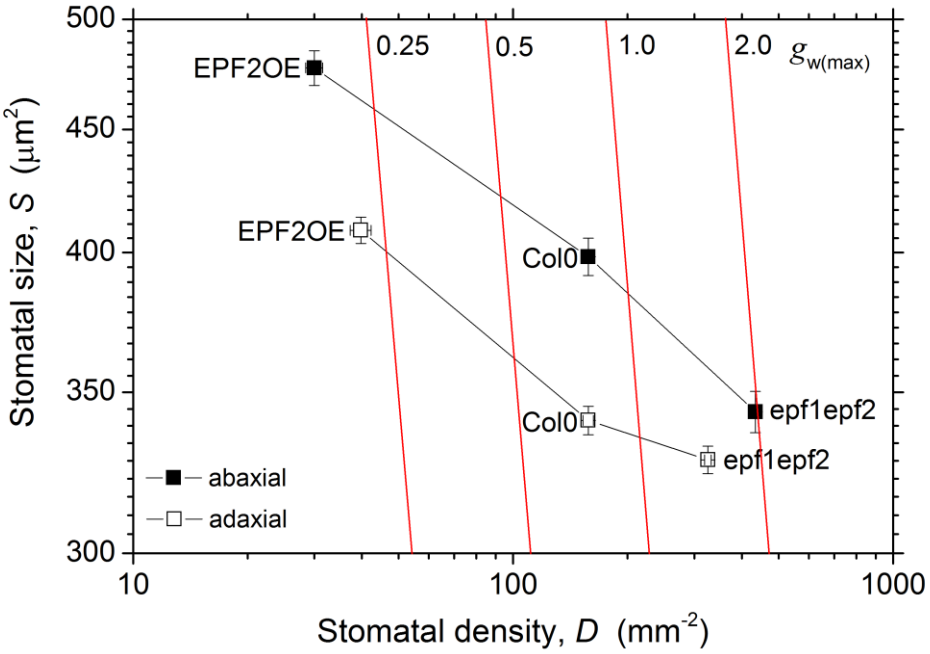

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