Genetic manipulation of stomatal density influences stomatal size, plant growth and tolerance to restricted water supply across a growth CO_2 gradient

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To investigate the impact of manipulating stomatal density, a collection of Arabidopsis EPIDERMAL PATTERNING FACTOR (EPF) mutants with an approximately 16-fold range of stomatal densities (approximately 20% to 325% of that of control plants), were grown at three atmospheric CO_2 concentrations (200 ppm, 450 ppm and 1000 ppm), and 30% or 70% soil water content. A strong negative correlation between stomatal size (*S*) and stomatal density (*D*) was observed suggesting that factors that control *D* also affect *S*. Under some but not all conditions, mutant plants exhibited abnormal stomatal density responses to CO_2 concentration, suggesting that the EPF signalling pathway may play a role in the environmental adjustment of *D*. In response to reduced water availability, maximal stomatal conductance was adjusted through reductions in *S*, rather than *D*. Plant size negatively correlated with *D*. For example, at 450 ppm CO_2 EPF2-overexpressing plants, with reduced *D*, had larger leaves and increased dry weight in comparison to controls. The growth of these plants was also less adversely affected by reduced water availability than plants with higher *D*, indicating that plants with low *D* may be well suited to growth under predicted future atmospheric CO_2 environments and/or water scarce environments.

Keywords: stomatal density (*D*), stomatal size (*S*); carbon dioxide; signalling peptide; plant growth; EPF

1. INTRODUCTION

Microscopic stomatal pores on the aerial surfaces of higher plants regulate gas exchange between plants and their environment. Two guard cells surround and regulate each stomatal pore in response to a number of environmental cues. To optimise the trade-off between carbon gain and transpirational water loss, stomata sense and integrate a range of environmental signals, including atmospheric CO_2 concentration, and soil moisture [1,2,3]. Considerable progress has been made towards understanding these sensory and signalling mechanisms at the molecular level [3]. However, much less is known about the long-term response of stomata to changing environmental conditions which involves the production of new leaves with altered stomatal size (*S*) and altered stomatal number per unit area, or density (*D*) [4].

When leaves are formed their capacity for exchanging CO_2 and water vapour across the epidermis is set by the maximum diffusive conductance of the stomatal pores to CO_2, $g_{c(max)}$, and water vapour, $g_{w(max)}$,as well as being affected by photosynthetic capacity. These maximum conductances are determined by *S* and *D* [5]. Leaves can regulate stomatal aperture to control diffusive conductance at any point between zero and the maximum, but can only alter $g_{c(max)}$ and $g_{w(max)}$ by altered development. Adaptation of *S* and *D* is therefore central to the long-term response of leaf diffusive conductance to sustained shifts in environmental conditions. Here we examine the interaction of *S* and *D* in response to two globally important and long-term environmental variables, atmospheric CO_2 concentration, c_a, and water availability.

(a) Stomatal size, density and CO_2

Stomatal apertures reduce in response to an increase in c_a, and increase in response to a decrease in c_a. This short-term, reversible response is one of several feedback mechanisms regulating leaf gas exchange [6]. For the majority of species, long-term exposure of plants to elevated c_a results in

leaves with reduced *D* [7,8,9] consistent with a more permanent down-regulation of leaf diffusive conductance. The mature parts of the plant sense c_a and transmit this environmental signal to the developing epidermis to regulate stomatal density [10,11]. We currently know little about how environmental factors, such as CO_2, regulate D but as the transpiration rate of mature leaves correlates with D in developing leaves, a link between the short-term control of stomatal aperture and the long-term regulation of stomatal development has been suggested [12]. The molecular basis of this correlation is unknown. The only gene products known to modulate stomatal development in response to elevated CO_2 are the carbonic anhydrases [13], and the HIGH CARBON DIOXIDE (HIC) protein believed to be involved in biosynthesis of the epicuticular waxes [14,15,16].

Recently, in addition to changes in stomatal aperture and *D*, a third stomatal response to atmospheric CO_2 concentration has been suggested; a change in stomatal size (*S*). Periods of relatively low atmospheric CO_2 concentration in prehistory are characterised by high densities of small stomata, whereas periods of CO_2 concentration much higher than present levels are characterised by low densities of larger stomata [12]. Recent experimental evidence also supports this inverse correlation between CO_2 concentration and *S* [17,18,19]. *S* determines two important physical dimensions contributing to $g_{c(max)}$ and $g_{w(max)}$: the maximum area of the open pore, a_{max}, and the pore depth, or diffusion path length *l*. It is suggested that, because of their shorter aperture diffusion path, high densities of small stomata may represent the best way of achieving maximal gas exchange and preventing CO_2 starvation in periods of low atmospheric CO_2 concentrations. In addition, smaller stomata would be expected to have shorter opening and closing response times allowing for improved control of water loss [12]. On the other hand, a reduced number of, albeit larger, stomata, may perhaps allow a metabolic cost-saving when CO_2 is not scarce [20]. We do not yet know the mechanism by which guard cell size is determined, nor yet whether the signalling components that control guard cell size are shared with, or interact with, those that regulate stomatal

apertures and/or stomatal densities. However, *S* has been shown to strongly correlate with genome size in extant plant specimens [21,22,23].

(b) Molecular basis of stomatal development

Our understanding of the molecular control of stomatal development has benefitted from studies of *Arabidopsis thaliana* mutants [24]. A family of secreted peptide signals known as the EPIDERMAL PATTERNING FACTORs (or EPFs) are proposed to compete for a putative cell surface receptor, believed to comprise the receptor-like protein TOO MANY MOUTHS (TMM) and a putative leucine-rich repeat receptor-like protein kinase [25,26]. Evidence suggests that receptor binding activates an intracellular mitogen-activated protein kinase cascade which phosphorylates and destabilises a bHLH transcription factor required, early in leaf development, for cells to enter the stomatal lineage [27].

The role of three EPF signals has been described. Two act as inhibitors of stomatal development, and the third as an activator of stomatal development. The relative expression levels of these activator and inhibitor peptides during leaf development appear to determine *D* in mature leaves. EPF1 and EPF2 act to inhibit the formation of stomatal precursors by performing distinct but overlapping functions [28,29]. EPF1 is principally involved in orienting cell divisions and prevents stomata from forming in clusters or pairs. Thus, mature leaves of plants lacking EPF1 (*epf1* knockout mutants) have increased numbers of stomata, and frequent stomatal pairs. EPF2 principally inhibits the formation of meristemoids and also promotes the formation of pavement cells. Thus, leaves of *epf2* knockout plants have increased stomatal densities but also form small arrested stomatal lineage cells. The double mutant lacking both EPF1 and EPF2 has an additive phenotype. *epf1epf2* plants exhibit greatly increased stomatal densities, and also have stomatal pairing and additional arrested cells. Plants manipulated to constitutively over-express EPF1 or EPF2 (*EPF1*OE and *EPF2OE*) have very few stomata [30, 28,29].

The third peptide, EPFL9/STOMAGEN has essentially the opposite function to EPF1 and EPF2, the promotion of stomatal development [31,32,33]. This activator peptide is secreted from mesophyll cells and is proposed to prevent inhibitory peptides like EPF1 or EPF2 from binding their receptor and inhibiting stomatal development [26]. Plants manipulated to have reduced levels of EPFL9/STOMAGEN (*EPFL9*RNAi) have decreased *D*, whereas plants manipulated to over-express EPFL9/STOMAGEN (*EPFL9*OE) have increased *D* [32,33,34]. In line with this hypothesis, plants lacking EPF1 and EPF2, and over-expressing EPFL9/STOMAGEN would be expected to have exceptionally high *D*. We have created plants with this triple mutant *epf1epf2EPFL9*OE genotype for use in this study.

(c) Stomatal mutants as tools for studying *S* versus *D*

The availability of plants with, for the first time, a 16-fold range of *D* in the same genetic background has allowed us to investigate a number of outstanding questions: (i) Are changes in *D* accompanied by correlated changes in *S* (ii) Are increases or decreases in D beneficial at relatively low or high atmospheric CO_2 concentrations, respectively, and particularly under water-stress? Here, we compare the phenotypes of Arabidopsis mutants ranging from *EPF2*OE which has an approximately 80% reduction in *D* to *epf1epf2EPFL9*OE which has approximately 225% increase in *D*, following their growth in controlled environments at sub-ambient and elevated atmospheric CO_2 concentrations. In a further experiment, we compare plants grown in well watered and water-deficit conditions (70% and 30% soil water content) under these CO_2 concentrations.

2. MATERIAL AND METHODS

(a) Plant Material and Growth Conditions

*Arabidopsis thaliana epf1-1, epf1-1epf2-1, EPF2*OE (*p35S:EPF2-CTAPi*), *p35S:EPFL9RNAi* mutant seeds were as previously described [29,34]. *epf1epf2EPFL9OE* was generated by

transforming *epf1epf2* with *p35S:EPFL9-CTAPi* as previously described [34]. Gene accession numbers are *EPF1 At2g20875*, *EPF2 At1g34245*, *EPFL9 At4g12970*. T3 generation plants were used in these experiments following genotype confirmation by PCR. Mutant plants and their background ecotype Col-0, were stratified on wet M3 compost/perlite (4:1) at 4ºC in dark for 72 h to synchronise germination. Plants were grown in three matched controlled environment growth chambers (Conviron model BDR16) at 200, 450 and 1000 ppm CO_2 at 22ºC/16 ºC 9h light/15h dark cycle. CO_2 levels were constantly monitored by VAISALA CO_2 sensors. In one cabinet CO_2 was scrubbed from the air with a mixture of soda lime and charcoal, and CO_2 free air mixed with ambient air to achieve 200 ppm CO_2. In the other two cabinets ambient air was supplemented with CO_2 from a gas cylinder, derived from fossil carbon sources (BOC).

After two weeks of growth, plants were transferred to 100 ml volume plant pots and covered with cling-film for the first 5 days. For the first experiment (presented in figure 1) plants were grown to maturity in these pots. When the plants were just beginning to initiate a floral bolt (stage 3.9 on Boyes scale [35]; at approximately 82 days, 74 days, and 68 days post germination for 200 ppm CO_2, 450 ppm CO_2 and 1000 ppm CO_2 respectively), fully expanded leaves were removed for stomatal analyses. Plants grown for the second experiment (presented in figures 2 and 3) were grown in 100 ml pots and well-watered for 4-6 weeks until the plants had developed 12-15 true leaves (41 dpg, 32 dpg and 28 dpg for 200 ppm CO_2, 450 ppm CO_2 and 1000 ppm CO_2 respectively), at which point they were transplanted to larger 1000 ml volume pots filled with M5 compost/perlite (4:1). Pots with established plants and water-saturated soil were weighed and water was withheld from a subset of 'water-restricted' plants until the compost reached 30% water saturation, whereupon the soil was watered to 30% water saturation every 2-3 days. 'Well-watered' plants were grown in the same manner except compost was watered to 70% water saturation every 2-3 days. The plants were maintained under well-watered and water restricted conditions for a further 49, 49 or 42 days for 200 ppm CO_2, 450 ppm CO_2 and 1000 ppm CO_2 respectively.

(b) Stomatal density, index, size and maximum stomatal conductance measurements

Dental resin (Coltene Whaledent, Switzerland) was applied to both surfaces of fully expanded leaves and nail varnish peels were taken from set resin. Cell counts were taken from the widest area of 2 leaves each from at least 4 plants of each genotype at each growth condition. *S* was calculated from measurements of guard cell length and width following incubation of leaf samples with the fungal toxin fusicoccin to open pores. Abaxial leaf epidermal peels of mature leaves were removed 2-3 hours into the photoperiod and floated on opening buffer (10mM KCl, 10mM MES , pH 6.2 , supplemented with 500nM fusicoccin (Sigma-Aldrich)) and incubated at 22ºC for 2 h [36, 37]. Stomatal measurements and maximum apertures were determined by light microscopy (Olympus BX51), using a fitted camera (Olympus DP70), and measured using ImageJ software version 1.43u. Maximum stomatal aperture was estimated from:

$$a_{max} = \frac{\pi * Wa * La}{4} \quad (1),$$

where W_a is aperture width and L_a is aperture length. Stomatal size (*S*) was estimated from:

$$S = \frac{\pi * Ws * Ls}{4} - a_{max} \quad (2),$$

where W_s is the width of the fully inflated guard cell pair and L_s is stomatal length.

Maximum leaf conductance to water vapour was calculated from:

$$g_{wmax} = \frac{d}{v} * D * amax / \left(1 + \frac{\pi}{2} \sqrt{\frac{amax}{\pi}}\right) \quad (3),$$

d is the diffusivity of water vapour in air at 22ºC ($m^2 \cdot s^{-1}$), *v* is the molar volume of air at 22ºC and 1 atm ($m^3 \cdot mol^{-1}$), *D* is stomatal density (m^{-1}) and l is defined as guard cell width (m) [17].

Leaf size was estimated from digital photos using ImageJ. Mean leaf size was used to calculate the number of stomata per leaf. Rosette leaf area was calculated as the sum of the areas of all mature leaves on a plant.

(c) Infrared thermal imaging

Infrared images were taken using a FLIR SC660 camera (FLIR systems) vertically positioned approximately one metre above the leaf rosette. Infrared images of plants in the first experiment (presented in figure 1) were taken immediately after their removal from the growth cabinet. Sixty infrared images of all plants involved in the second experiment (presented in figures 2 and 3) were taken within the growth cabinets over a period of 1 hour, beginning 2 hours after the start of the photoperiod. 3-4 plants of each genotype were imaged and average temperature data was recorded from the widest uncovered regions of 3 leaves per plants. Images were analysed using ThermaCAM Researcher 2.9 Professional (FLIR systems) and corrected for small variations in growth cabinet temperatures in comparison to background temperatures recorded using the same camera system (<0.5ºC).

(d) Statistical analysis

Two and three way analysis of variance was calculated using the R programming language available from CRAN family of internet sites. Variance was considered significant for $p<0.05$. Individual student T tests were conducted with Microsoft Excel.

3. RESULTS

(a) *D* versus *S* across EPF mutants

Mean stomatal densities of mature leaves on plants grown at 450 ppm CO_2 ranged from approximately 40 mm^{-2} for *EPF2*OE, up to 650 mm^{-2} for *epf1epf2 EPFL9*OE (figures 1a, c-h) and, with the exception of the *epf1epf2EPFL9*OE plants which were created for this study, were consistent with their previously published values of *D* [29-34]. There was a significant effect of genotype and CO_2 conditions, as well as a significant interaction between genotype and CO_2 conditions on *D* (see Supplementary Table 1 for analysis of variance results). Four out of six

genotypes exhibited a significant reduction in *D* following growth at elevated CO_2 (1000 ppm CO_2) as would be expected from previous studies of Arabidopsis [10]. However, none of the EPF mutant genotypes showed increases in *D* at the low CO_2 concentration (200 ppm CO_2), perhaps indicating that without an intact EPF signalling pathway plants are unable to respond to sub-ambient CO_2 concentrations. Non-stomatal cell densities were also recorded and stomatal index (the number of stomata divided by the total number of stomata and other epidermal cells) was calculated (supplementary figure 1 and supplementary table 1). Stomatal indices were in line with the EPF mutant phenotypes described previously [29-34] but in contrast to *D*, stomatal indices were generally not affected by growth at either the higher or lower CO_2 environments (200 ppm CO_2 or 1000 ppm CO_2).

There was significant effect of genotype on *S*. The mean *S* of *EPF2*OE and *EPFL9*RNAi open stomata were significantly larger, and *epf1epf2* and *epf1epf2EPFL9*OE were significantly smaller than the Col-0 control *S* (figure 1b and supplementary table 1). Thus, a strong inverse correlation between *S* and *D* was apparent in this experiment (figure 1o) and for individual plants (supplementary figure 2).

(b) Effect of growth CO_2 concentration on leaf temperature

We used infrared thermal imaging to record the temperature of plant leaf rosette surfaces. This technique measures the level of leaf evaporative cooling due to transpiration, and hence provides a proxy measurement for transpiration [38,39,40]. There were significant effects of genotype and CO_2 concentration on the mean rosette leaf temperatures. Mean temperatures for each genotype grown at 1000 ppm CO_2 were higher with respect to those grown at 450 ppm CO_2 which were in turn higher than those grown at 200 ppm CO_2 (figure 1p) indicating that stomata of the EPF family mutants are functional in transpiration, and that their stomatal aperture opening/closing response to CO_2 is intact. Plants with high *D* (*epf1epf2* and *epf1epf2*EPFL9OE) transpired more than those with lower

D (*EPFL9*RNAi, Col-0 and *epf2*) under all CO_2 conditions (figures 1i-n and p). The difference between mean leaf temperatures of genotypes with the most extreme *D* (*EPFL9*RNAi and *epf1epf2 EPFL9*OE) was 0.64ºC, 0.56ºC and 0.67ºC for plants grown at 200 ppm CO_2, 450 ppm CO_2 and 1000 ppm CO_2 respectively. Thus a clear correlation between *D* and the rate of leaf transpiration was observed which was remarkably consistent between the different CO_2 conditions. Furthermore, plants with high *D*, such as *epf1epf2*, had significantly smaller stomata (figure 1o and p) and the highest transpiration rates, confirming expections from physical diffusion theory [5].

(c) Interaction of growth, CO_2 and water restriction

In our second experiment, we compared the growth and stomatal characteristics of plants with differing *D* grown under well-watered or water-restricted conditions (70% or 30% relative soil water content) in matched chambers at the same three CO_2 levels as above; 200 ppm, 450 ppm and 1000 ppm CO_2. Three genotypes were used for this experiment; *EPF2*OE with low *D*, *epf1epf2* with high *D* and Col-0 control plants. Plants with reduced *D* (*EPF2*OE) were larger, and those with increased *D* (*epf1epf2*) smaller, than Col-0 control plants at each CO_2 level (figure 2a-c) and in comparison to plants grown at 70% soil water content (figure 2a-c), plants of all genotypes were smaller when grown at 30% (figure 2d-f). We investigated this further by measuring mean dry weight (figure 2g) and mean rosette area (figure 2h). There were significant effects of genotype, CO_2 concentration and water availability on rosette area and dry weight, as well as significant effects of the interaction between genotype and CO_2 concentration and between water availability and CO_2 concentration on both rosette area and dry weight (supplementary table 1). Biomass was most severely restricted in the plants with high *D* (*epf1epf2*) at 200 ppm CO_2 and 450 ppm CO_2 when transpiration would be expected to be highest. In addition, *epf1epf2* plants showed the largest decrease in dry weight and rosette diameter following growth under water restriction at both 200

ppm and 450 ppm CO_2 conditions (figure 2g). However, the restriction of water availability had no significant effect on the dry weight of any genotype at 1000 ppm CO_2 when transpiration would be expected to be low (figure 2g and 2h). Interestingly, *EPF2*OE plants which have low stomatal densities, grown at 450 ppm CO_2 acquired a significantly larger biomass and rosette area than Col-0 plants under both watering regimes (figure 2g and 2h). Thus at 450 ppm CO_2, plants with the least stomata appeared to be the most successful; growing larger even under restricted water availability. In contrast, plants with increased *D* (*epf1epf2*) did not appear to show any growth advantage even under sub-ambient CO_2 conditions and *epf1epf2* dry weights were significantly lower than Col-0 at all CO_2 conditions when water was restricted.

The mean abaxial stomatal densities of plants grown with reduced water availability were not significantly different from those of well-watered plants, except for a reduction in *epf1epf2* stomatal density at 450 ppm CO_2 and a small increase in *EPF2*OE stomatal densities at 200 ppm and 1000 ppm CO_2 (supplementary figure 3 and supplementary table 1). The mean adaxial values of *D* were similar to the abaxial *D* although the *D* of Col-0 grown at 1000 ppm CO_2 was significantly lower than the *D* of Col-0 grown at 450 ppm and 200 ppm CO_2 (supplementary figure 4 and supplementary table 1). *epf1epf2* plants did not respond to increasing CO_2 by reducing *D* on either the abaxial or adaxial side of the leaf in this experiment. It is possible that the breakdown of the stomatal density response to CO_2 in these mutants is due to the lack of EPF1 and EPF2 inhibitory peptides; however this is not consistent with the results of our first experiment (figure 1a). All three genotypes showed a significant reduction in mean stomatal size following growth under drought conditions at 200 ppm CO_2 and 450 ppm CO_2 but not at 1000 ppm CO_2 (supplementary figure 5 and supplementary table1). Thus, the inverse correlation between stomatal density and stomatal size that we observed in our first experiment appeared to be modulated by water availability but remained consistent across the genotypes within those conditions (figure 3a). When plants were

grown under water restriction, total stomatal aperture area appeared to be reduced by changes in *S* rather than reductions in *D* (supplementary figures 4 and 5 and supplementary table 1).

Although values of *D* were largely unaffected by water-restriction, plants of all genotypes were significantly smaller following growth at 30% soil water content, at 450 ppm CO_2 and 200 ppm CO_2 as described above (figure 2d-f). In light of the dramatic differences in leaf size, we calculated the mean total number of abaxial stomata per leaf (supplementary figure 6). The number of stomata per leaf remained stable for *EPF2*OE and Col-0 at all three CO_2 conditions and also under water restriction, except for Col-0 following growth under water-restriction at 450 ppm CO_2. The *epf1epf2* plants however, which have high *D*, had reduced numbers of stomata per leaf following growth under water-restriction at 200 ppm CO_2 and 450 ppm CO_2. Our results indicate that Arabidopsis plants respond to restricted water availability primarily by producing smaller leaves with smaller stomata. Plants with the highest *D* (*epf1epf2*) responded by a more extreme restriction in leaf size whilst *D* remained relatively consistent resulting in fewer stomata per leaf under water restriction (supplementary figures 3, 4 and 6).

To assess transpiration, mean rosette leaf temperatures of Arabidopsis plants were taken by infrared thermography. Leaf temperatures ranged from 22.27ºC for well-watered plants with high *D* grown at sub-ambient CO_2 (*epf1epf2*, 70% soil water content, 200 ppm CO_2), to 24.02ºC for plants with reduced *D*, grown with restricted water availability at elevated CO_2 (*EPF2*OE, 30% soil water content, 1000 ppm CO_2) (figures 3b, c, supplementary figure 7 and supplementary table 1). There were significant effects of genotype, CO_2 concentration, and water availability on leaf temperature as well as a significant interaction between genotype and water availability on mean leaf temperature (supplementary table 1). At 70% soil water content, plants with low *D* (*EPF2*OE) had significantly warmer leaves than either Col-0 or *epf1epf2* across all CO_2 conditions, and plants with high *D* (*epf1epf2)* were significantly cooler than Col-0 at 200 ppm CO_2 (figure 5b, supplementary figure 7 and supplementary table 1). Thus a negative correlation between maximum

stomatal conductance and leaf temperature was observed (figure 3b). At 1000 ppm CO_2, the temperatures of all plants were significantly higher than those grown at 200 ppm CO_2 and 450 ppm CO_2 probably due to reductions in stomatal aperture. However, no significant difference in temperature between well-watered plants grown at 200 ppm CO_2 and 450 ppm CO_2 was observed, suggesting that transpiration rate does not differ greatly between leaves acclimatised to these CO_2 levels. Following growth under water restriction all plants had significantly higher leaf temperatures (supplementary figure 7 and supplementary table 1) and a negative correlation between stomatal density and leaf temperature was observed in plants grown at 1000 ppm CO_2 (figure 3c). Plants with reduced stomatal density (*EPF2*OE) had higher leaf temperature (implying reduced transpiration rate) under water restriction at 200 ppm CO_2 in comparison to Col-0. However, mean leaf temperatures of *epf1epf2* plants grown under water restriction at 200 ppm or 450 ppm CO_2, were not significantly different from Col-0 controls, perhaps because these plants with high *D* had begun to wilt under these conditions. Despite this the plants with increased stomatal density (*epf1epf2*) had a larger difference in temperature between growth at 70% SWC and 30% SWC and appeared more able to increase transpiration when well-watered, than plants with normal or reduced stomatal density (Col-0 and *EPF2*OE).

(d) Effect of manipulating EPF signalling on non-stomatal leaf cells

Stomatal density is influenced by direct effects on stomatal development but also via indirect effects on the size and number of non-stomatal cells. These indirect effects are also likely to be influenced by changes in atmospheric CO_2 concentration and water availability. Our analysis of stomatal indices, leaf size, and calculation of the number of stomata per leaf showed that plants lacking EPF2 and EPFL9 had a higher proportion of non-stomatal epidermal cells (figure S1 and supplementary table 1), and that plants manipulated to have low or high *D* (*EPFL9*OE, *epf1epf2*) developed larger or smaller leaves respectively (figure S8). Although manipulation of EPF levels also altered leaf size, *EPFL9*OE still had significantly decreased, and *epf1epf2* still had significantly increased,

numbers of stomata per leaf (figure S5 and supplementary table 1). All genotypes showed a dramatic reduction in leaf size following growth under restricted water availability under all three CO_2 conditions, with the exception of *epf1epf2* at 1000 ppm (figure S8).

4. DISCUSSION

It has recently been observed from the study of fossilised plants that, over the past 400 million years, changes in stomatal density have been accompanied by large changes in stomatal size [17]. Our results show that this inverse relationship between stomatal size and stomatal density holds true across the *Arabidopsis* genotypes characterised in this study. Altering EPF family expression levels to increase or decrease *D* caused an opposite effect in *S*. Thus the pathways controlling *D* and *S* appear to be linked but whether the EPF signalling pathway impacts directly, or indirectly, on *S* remains to be explored.

Current atmospheric CO_2 concentration is approximately 390 ppm (2010 average at Mauna Loa Observatory was 389.78) and if it continues to rise at the current rate it is expected to reach 450 ppm in the next 25-30 years [41]. Our results suggest that plants with reduced *D* and increased *S* may have advantageous growth characteristics in such future CO_2 environments. At 450 ppm CO_2, *Arabidopsis* plants manipulated to have lower *D* and large *S* had reduced transpiration, a larger biomass, and increased growth tolerance to limited water availability. The improved growth rate of plants with substantially decreased stomatal density perhaps reflects a combination of improved tissue water status from lower transpiration rate, improved CO_2 assimilation rate from favourably higher leaf temperature, and lower metabolic cost from developing and operating less guard cells [21]. However, why this trait, which can be brought about by changing the expression level of just one gene (*EPF2*), has not evolved in the natural population is not clear. It is possible that, under the current rapidly rising atmospheric CO_2 concentration, plants have not yet fully adapted their

stomatal densities to be optimal for growth. Alternatively there may be unknown adverse effects associated with developing lower *D* when plants are growing in a competitive natural environment. Despite the apparent benefits of reduced *D* on Arabidopsis leaves in the highly controlled growth conditions that we used, it is not known whether the same beneficial effects would be observed by reducing *D* of economically useful plants in harsher or fluctuating environments. Indeed, decreased transpirational cooling resulting from lower levels of conductance has been associated with lower agronomic yields of irrigated pima cotton (*Gossypium barbadense*), as well as bread wheat (*Triticum aestivum*), in hot environments [42]. Nevertheless, in marked contrast to the enhanced growth characteristics of plants with reduced *D*, we did not identify any growth conditions in which plants manipulated to have increased *D* (e.g. *epf1epf2*) grew larger. However, we did not attempt to assess seed yield in our experiments and it is possible that such plants may partition biomass differently into their reproductive structures.

Although in previous studies, Arabidopsis leaves of another stomatal density mutant were apparently able to compensate for altered *D* by adjusting stomatal aperture size [43], our results suggest that the genotypes in the current study were unable to completely compensate for altered *D*, by adjusting either *S* or aperture. Under two different watering regimes (70% and 30% soil water content) and across three atmospheric CO_2 conditions (200 ppm, 450 ppm and 1000 ppm), *EPF2*OE leaves with reduced *D* transpired less than controls, as evidenced by increased leaf temperature. In addition, *epf1epf2* and *epf1epf2EPFL9*OE plants, both with increased *D*, generally had higher transpiration rates than their counterpart controls at all three CO_2 conditions. Thus, our results indicate a strong correlation between *D*, and transpiration rate. Given the widespread conservation of the EPF family across different plant species [44], we consider genetic manipulation of this peptide family a potential route for altering leaf conductance without disrupting stomatal aperture control.

In our experiments, plants lacking inhibitors of stomatal development EPF1 and EPF2 were unable to increase *D* at sub-ambient atmospheric CO_2 concentrations, or to decrease *D* at elevated CO_2 concentrations when grown in larger pots (figure 1a and figures S3 and S4). Thus, it could be concluded that an intact EPF signalling pathway is required for the adjustment of *D* in response to atmospheric CO_2 concentration. However, as *D* is responsive to many environmental signals and some results were inconsistent between our two experiments this possibility requires further study before firm conclusions can be drawn.

In summary, genetic manipulation of the EPF signalling pathway not only produced plants with dramatically altered stomatal densities but it also affected stomatal size, plant transpiration, rosette growth, and tolerance to restricted water availability across a range of atmospheric CO_2 environments.

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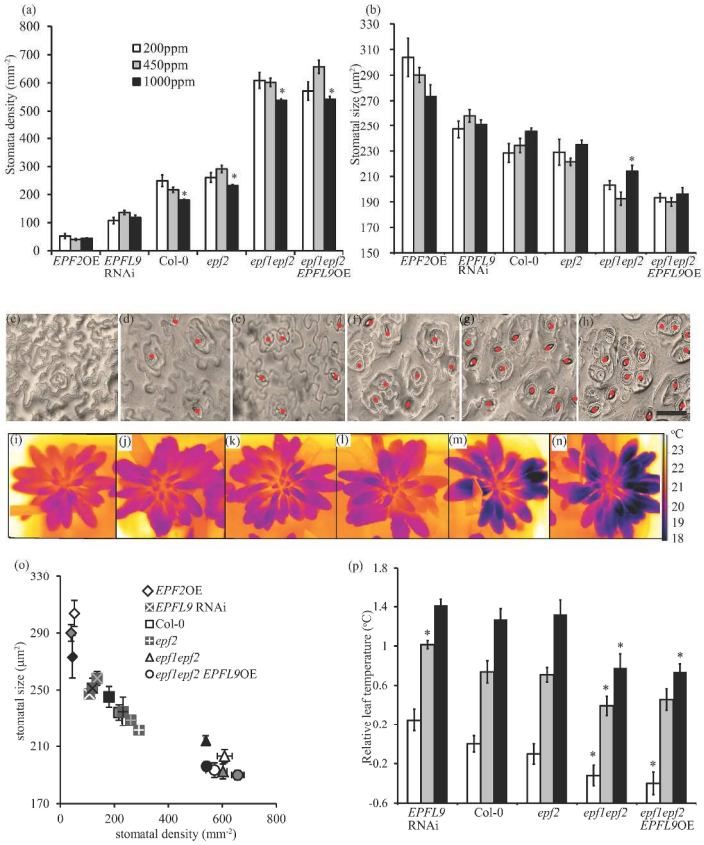
Figure Captions

Figure 1. Stomatal properties of Arabidopsis EPF family mutants grown at 200 ppm (white), 450 ppm (grey) or 1000 ppm (black) atmospheric CO_2 concentration. (a) Mean stomatal densities (n=3-8). (b) Mean stomatal sizes (n=3-8). (c-h) Micrographs of epidermal peels of leaves from *EPF2*OE, *EPFL9*RNAi, Col-0, *epf2*, *epf1epf2* and *epf1epf2EPFL9*OE respectively with red dots added to highlight stomatal pores. Scale bar = 25 µm. (i-n) False colour IR images of *EPF2*OE, *EPFL9*RNAi, Col-0, *epf2*, *epf1epf2* and *epf1epf2 EFL9*OE respectively grown at 1000 ppm CO_2.

Temperature scale bar on right. (o) Relationship between mean stomatal size and mean stomatal density. (p) Mean leaf temperatures of leaves relative to Col-0 grown at 200 ppm (n=4-8). Error bars represent SE. Asterisks indicate values that are significantly different from same genotype grown at the same 450 ppm ($p<0.05$; (a) and (b) or values that are significantly different from Col-0 grown at the same CO_2 concentration ($p<0.05$; (p)).

Figure 2. Size and dry weights of EPF family mutants following growth at 200 ppm (white), 450 ppm (grey) or 1000 ppm (black) atmospheric CO_2 concentration with either 30% or 70% relative soil water content. (a-f) Images of plants grown at 450 ppm CO_2 at 70% (a-c) or 30% (d-f) soil water content at 70 dpg. Scale bar = 10 cm. (g) Mean dry weight and (h) mean leaf rosette area (n=3-4). Error bars represent SE. Letters indicate values that are significantly different from a, the same genotype grown at 450 ppm CO_2, b, the same genotype grown at 70% soil water content and c, Col-0 grown in the same conditions ($p<0.05$).

Figure 3. Stomatal characteristics of EPF family mutants following growth at 200 ppm (white), 450 ppm (grey) or 1000 ppm (black) atmospheric CO_2 concentration with either 30% or 70% relative soil water content. (a) Relationship between mean stomatal size and density across the CO_2 conditions (n=3-4). (b-c) Relationship between mean leaf temperature and mean maximum stomatal conductance to water vapour at (a) 70% and (b) 30% soil water conditions (n=3-4). Error bars represent SE.



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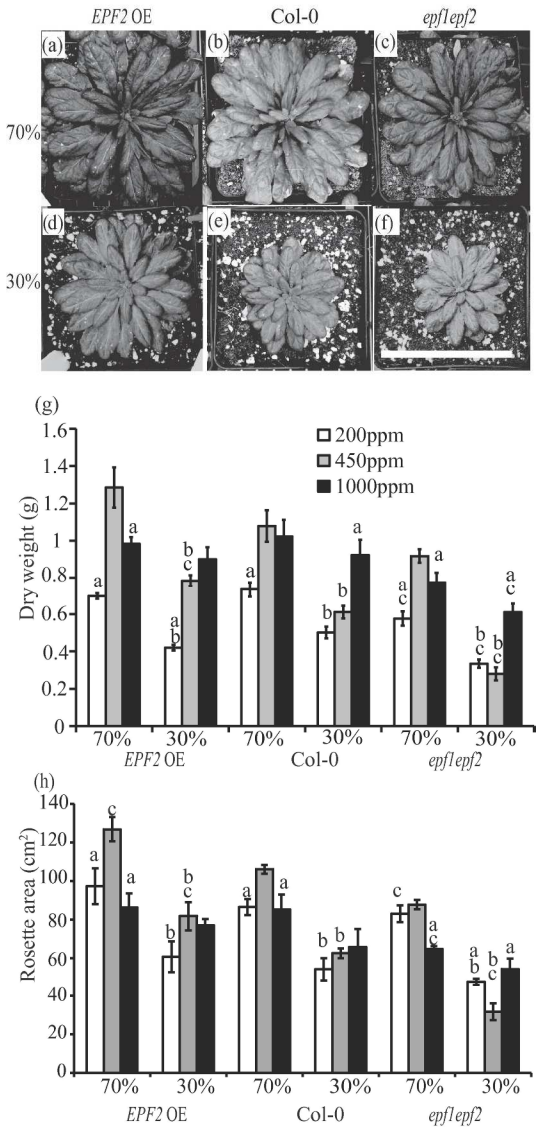

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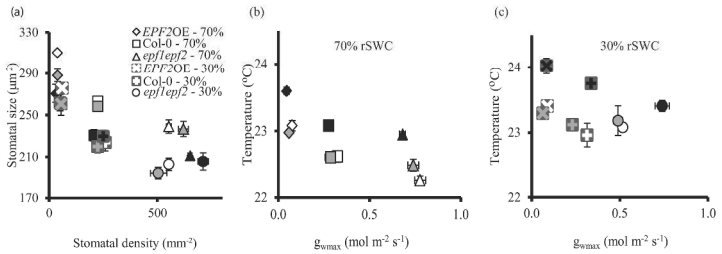

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