Journal of Experimental Botany, Vol. 70, No. 18 pp. 4737–4747, 2019
doi:10.1093/jxb/erz248 Advance Access Publication June 6, 2019
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RESEARCH PAPER

Reduced stomatal density in bread wheat leads to increased water-use efficiency

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Received 17 October 2018; Editorial decision 15 May 2019; Accepted 24 May 2019

Editor: Tracy Lawson, University of Essex, UK

Abstract

Wheat is a staple crop, frequently cultivated in water-restricted environments. Improving crop water-use efficiency would be desirable if grain yield can be maintained. We investigated whether a decrease in wheat stomatal density via the manipulation of epidermal patterning factor (EPF) gene expression could improve water-use efficiency. Our results show that severe reductions in stomatal density in EPF-overexpressing wheat plants have a detrimental outcome on yields. However, wheat plants with a more moderate reduction in stomatal density (i.e. <50% reduction in stomatal density on leaves prior to tillering) had yields indistinguishable from controls, coupled with an increase in intrinsic water-use efficiency. Yields of these moderately reduced stomatal density plants were also comparable with those of control plants under conditions of drought and elevated CO_2. Our data demonstrate that EPF-mediated control of wheat stomatal development follows that observed in other grasses, and we identify the potential of stomatal density as a tool for breeding wheat plants that are better able to withstand water-restricted environments without yield loss.

Keywords: Cereals, drought, photosynthesis, stomata, water-use efficiency, wheat.

Introduction

Agriculture accounts for 80–90% of freshwater usage, and water availability is a key factor limiting crop productivity worldwide. Over the coming decades, as water supplies become scarce and more variable in the face of climate change, unprecedented food production will be required to feed a growing global population (Ray *et al.*, 2013; SOFI, 2018). Ideally, future crop plants will utilize water resources more efficiently to produce 'more crop per drop'; that is, requiring less water for a given amount of photosynthesis and a given amount of grain produced. In addition, increased resilience to variable environmental conditions, including more frequent or more severe periods of drought, is likely to be important for sustainable agriculture. The amount of CO_2 fixed by photosynthesis relative to the amount of water vapour lost to the atmosphere is termed water-use efficiency (WUE). Currently cereals account for 27% of global water consumption (Hoekstra *et al.*, 2012). Wheat (*Triticum aestivum*) is the most widely cultivated crop globally, with 713 Mt produced in 2013 (Long *et al.*, 2015). It is grown across a wide range of environments, many of which face increasing problems of water scarcity and/

or salinity. Measures to improve wheat WUE without cost to yield could have significant agronomic benefit (Chaerle *et al.*, 2005; Bertolino *et al.*, 2019). However, this may not be simple to achieve as decreasing water flux through a plant may have a knock-on effect on reproduction and the process of grain formation and filling (Roche, 2015).

Stomata are key determinants of the trade-off between photosynthetic carbon fixation and water transpired, playing a major role in controlling plant WUE, through their regulation of CO_2 uptake and water loss (Franks *et al.*, 1999). The outcome of rising atmospheric CO_2 levels on crop yield is difficult to predict since, in addition to direct effects as a substrate for photosynthesis and as a regulator of stomatal aperture, CO_2 levels indirectly influence many other aspects of plant physiology. For example, it is believed that many crops may have been inadvertently selected for increased stomatal conductance (Roche, 2015) (either through increased stomatal density or by other means), which is in contrast to the effect that atmospheric CO_2 increases have had on non-crop stomatal densities and conductance (Hetherington *et al.*, 2003). A range of species develop reduced stomatal densities at elevated CO_2 (Woodward *et al.*, 1995), but it is possible that this response has reached saturation in the field and further rises in $[CO_2]$ are unlikely to have a major effect on stomatal development (Serna *et al.*, 2000). Indeed, the effects of increasing atmospheric CO_2 levels on stomatal density are unclear in field conditions. In free air CO_2 enrichment (FACE) experiments, there is limited evidence that plants can reduce stomatal density in response to higher atmospheric CO_2 concentrations, suggesting that transpiration is altered by other factors, such as aperture control or cuticle changes (Tricker *et al.*, 2005; Ainsworth *et al.*, 2007; Yoo *et al.*, 2009). Consequently, several authors have suggested that crop water use could be targeted directly by reducing the density or aperture of stomata (Chaerle *et al.*, 2005; Yoo *et al.*, 2009). In contrast, others have argued that high levels of stomatal conductance are often required in the field to maximize cooling in the midday sun and to enhance photosynthetic yields (Roche, 2015). Crops with genetically manipulated stomatal densities are yet to be tested in field conditions, and the effects on water use and yield therefore remain unclear.

Through work predominantly on the model eudicot *Arabidopsis thaliana*, we have a comprehensive understanding of the process of stomatal formation, with many of the components that regulate the pathway of stomatal differentiation identified. Early in leaf development, undifferentiated epidermal cells progress through a well-defined cell lineage, undergoing a series of divisions and transitions, eventually resulting in functional guard cells (Pillitteri *et al.*, 2007; Zoulias *et al.*, 2018). Progression through this developmental lineage is controlled by the sequential action of the basic helix–loop–helix (bHLH) transcription factors SPEECHLESS, MUTE, and FAMA and their heterodimeric partners SCRM/SCRM2. The activity of these transcription factors is under control of a mitogen-activated protein kinase kinase kinase (MAPKKK) signal transduction pathway which is itself regulated by both exogenous environmental factors (such as light and carbon dioxide levels) and endogenous peptide factors encoded by the *EPIDERMAL PATTERNING FACTOR* (*EPF*) gene family. This includes both negative regulators EPF1 and EPF2 (Hara *et al.*, 2007; Hara *et al.*, 2009; Hunt and Gray, 2009), and positive regulators of stomatal formation, such as EPFL9/STOMAGEN (Hunt *et al.*, 2010; Sugano *et al.*, 2010; Lee *et al.*, 2015). EPFs have been implicated in regulating stomatal density responses to environmental conditions, including light and CO_2 levels (Doheny-Adams *et al.*, 2012; Engineer *et al.*, 2014; Hronková et al., 2015), suggesting a complex interplay between exogenous and endogenous programmes regulating stomatal differentiation.

Several members of the *EPF* family have been misexpressed in Arabidopsis, producing plants with increased and decreased stomatal density. Physiological analysis of these plants has demonstrated the potential of exploiting *EPF* gene expression to improve WUE and drought tolerance, with, for example, decreased stomatal density resulting from misexpression or overexpression of *EPF2* leading to plants with enhanced WUE and drought tolerance (Doheny-Adams *et al.*, 2012; Franks *et al.*, 2015; Hepworth *et al.*, 2015). More recently, analysis of EPF gene function has been extended to the monocot grasses which comprise the most important crops for human nutrition. Despite the substantial differences in stomatal and leaf structure, it has been shown that major components of Arabidopsis stomatal development are also required in grasses (Hepworth *et al.*, 2018). Similar MAPKKKs and bHLHs regulate grass stomatal formation, and, remarkably, an orthologue of the transcription factor MUTE (BdMUTE) orchestrates subsidiary cell formation in a grass species (Raissig et al., 2016, 2017; Abrash *et al.*, 2018). EPFs also appear to play a similar role in grass stomatal patterning and differentiation, since misexpression or overexpression of *EPF1*- and *EPF2*-like genes in barley and rice leads to the generation of leaves with decreased stomatal density and plants with improved WUE and drought tolerance (Hughes *et al.*, 2017; Yin *et al.*, 2017; Caine *et al.*, 2019). This raises the question of whether similar manipulations of *EPF* expression levels in other crops can also lead to altered stomatal density and water use, and, moreover, what the outcome on crop performance/yield might be.

As in other grasses, wheat stomata are formed in defined rows or files along the adaxial and abaxial epidermis, with each stoma consisting of two dumb-bell shaped guard cells flanked by a pair of subsidiary cells (Liu *et al.*, 2009; Serna, 2011; Facette and Smith, 2012; McKown and Bergmann, 2018). Although it is widely assumed that the genetic regulation of wheat stomatal density is comparable with that uncovered in diploid grasses, functional experimental evidence to confirm this is lacking (Hepworth *et al.*, 2018). Moreover, the relationship of stomatal density to crop WUE is unclear, as both positive and negative correlations between stomatal density and yield under drought have been reported for wheat cultivars (Jäger et al., 2014; Shahinnia *et al.*, 2016).

In this study, we sought to investigate the effect on yield and intrinsic WUE of genetically manipulating stomatal density through altering the level of an EPF in the major crop, wheat. Our objective was to assess the impact of a severe reduction in stomatal gas exchange on WUE and yield.

Materials and methods

Plant material

Putative wheat orthologues of the Arabidopsis negative stomatal density regulators *AtEPF1*/*AtEPF2* were identified by the predicted amino acid sequence similarity of the proteins they encode using wheat genomics resources at TGAC and at Ensembl (http://plants.ensembl.org/Triticum_aestivum/Info/Index). These homeologues are designated *TaEPF1A*, *TaEPF1B*, *TaEPF1D*, *TaEPF2A*, *TaEPF2B*, and *TaEPF2D*, indicating their location on the A, B, or D subgenome (Hughes *et al.*, 2017). *TaEPF1A* was manually annotated using the TGAC Grassroots genomics BLAST server.

Construct assembly

All wheat experiments were performed using *Triticum aestivum* cultivar Fielder, a hexaploid spring bread wheat. *TaEPF1* (TGACv1_scaffold_641036_U: 17 763-18 194) was PCR amplified using KOD DNA polymerase (for primer sequences, see Supplementary Table S1 at *JXB* online), ligated into vector pjJET1.2, excised with *Xho*I and *Xba*I, then ligated into PENTR1A cut with *Sal*I and *Spe*I.

TaEPF2 (located on TGACv1_scaffold_159977_2DL: 31 371-31 905) was similarly amplified from wheat genomic DNA, cut with *Not*I and *Asc*I, and ligated into a similarly cut PENTR/D/TOPO. For Arabidopsis transformation, the entry vectors containing *TaEPF1* and *TaEPF2* were recombined using LR clonase into pCTAPi (Rohila *et al.*, 2004) and transformed into *Agrobacterium* strain C58 by the freeze/thaw method, then transformed into *A. thaliana* ecotype Col-0 by the floral dip method (Clough *et al.*, 1998). Transformants were selected by spraying with BASTA, and stomatal density was measured from T_3 generation plants.

TaEPF1 was recombined using LR clonase into pSc4Act-R1R2-SCV and electrotransformed into *Agrobacterium* strain EHA105 prior to transformation of immature embryos isolated from wheat cultivar Fielder (Perochon *et al.*, 2015; Milner *et al.*, 2018). Forty-seven regenerated wheat plants were confirmed as transformed by PCR for the *TaEPF1* transgene and also by T-DNA copy number assay of *nptII* relative to a single-copy wheat gene amplicon, normalized to a known single-copy wheat line. T-DNA copy number was assessed by iDna genetics (Norwich, UK). Wild-type control Fielder plants were regenerated through tissue culture, but did not undergo transformation, and were grown alongside *TaEPF1* transgenic plants in all experiments. Lines used were homozygous as determined by lack of wild-type phenotype amongst at least 10 individual plants of each line. All plants used in physiology experiments were checked for stomatal density to preclude any wild-type segregants. Experimental data were collected from the T_5 generation, where T_0 indicates the primary transformant.

Expression analysis

Expression data for all putative orthologues were obtained using the published WheatExp RNASeq data sets (Choulet *et al.*, 2014; Pearce *et al.*, 2015 deposited at https://wheat.pw.usda.gov/WheatExp/). Expression levels of *TaEPF1* in transgenic lines were measured using cDNA reverse-transcribed from extracted total RNA extracted from leaf 1 at 3 d after germination, using primers used to amplify the original *TaEPF1B* sequence above. A ubiquitin gene (*UBQ*) was used as a loading control.

Encoded proteins sequences were aligned by Multalin (Corpet, 1988). Phylogenies were constructed using the amino acid sequences using Clustal Omega.

Growth conditions

Arabidopsis plants were grown in square 5 cm pots under a 9 h day (200 μmol m^{-2} s^{-1} light, 22 °C), 15 h night (16 °C) cycle at 60% humidity in a Conviron walk-in growth chamber.

Two experiments were carried out with wheat plants in controlled-environment chambers. The first experiment compared the stomatal gas and photosynthetic assimilation characteristics of *TaEPF1* plant lines. The second compared their seed yields following growth under two different watering regimes and at ambient or elevated atmospheric CO_2 concentrations (shown in Figs 3 and 5). For wheat growth chamber experiments, conditions were as follows: 12 h photoperiod, ~400 μmol m^{-2} s^{-1} light, 22 °C/18 °C day/night temperature, respectively, and 60% relative humidity. CO_2 level was either supplemented to 1000 ppm where specified, or otherwise not modified (measured ambient CO_2 was ~450 ppm). For all chamber experiments, seeds were surface-sterilized with 10% (v/v) bleach for 30 min, then rinsed in deionized water before being germinated in Petri dishes on wet filter paper in the growth chamber. After germination, seeds were transferred to square 13 cm pots with either 6:1 M3 compost:perlite (CO_2 and water restriction experiments, CO_2 response curve experiment) or M3 compost (drought experiment) and were supplemented with slow-release fertilizer (Osmocote Exact Standard 5-6). Before starting the water restriction experiment, 100% field capacity (FC) was determined by fully soaking compost in pots overnight, then draining before weighing. Pots were fully dried, and dry weight was subtracted from weight at FC to calculate the amount of water that the compost could hold. Pots were kept at 80% and 30% FC by adding the corresponding amount of water. Controlled watering started once the first leaf had fully expanded, and was maintained by weighing and adding water every other day throughout the life cycle until ears were no longer green.

Glasshouse experiments to determine wheat yield were performed with plants sown in early January and harvested in June. Light intensity varied with sunlight, which peaked at ~2000 μmol m^{-2} s^{-1} light. Supplemental lighting was provided by 315 W metal halide bulbs (MASTERColour CDM-T Elite MW) when required to maintain a minimum daytime light intensity of 200 μmol m^{-2} s^{-1}. Day/night temperatures were maintained at 20 °C/16 °C, respectively, (ambient) relative humidity was ~60%, and mean daily ambient CO_2 was measured at 400 ppm. Soil-germinated plants were transferred to square 13 cm plant pots at 3 weeks old, and regularly watered with an automated watering system so that each plant under each treatment was given an equal daily amount of water. This was adjusted to maintain one set of control plants at ~80% soil relative water content (RWC), and another set at ~30% soil RWC. Soil moisture meter readings were taken using a Delta Devices HH2 (Cambridge, UK) on all plant pots every 2–3 d.

Leaf anatomy measurements

Dental resin (Coltene Whaledent) was used to make a negative impression of the leaf surface from the abaxial surface at the widest part of a fully expanded leaf. For Arabidopsis, three regions from a leaf of a 6-week-old plant were sampled from five plants per line. For wheat, since light conditions are known to influence stomatal density (Hronková et al., 2015), five 1 mm^2 areas of either the first true leaf (leaf 1 for controlled-environment room in Fig. 1) or the flag leaf (from glasshouse-grown plants in Supplementary Fig. S4) were sampled from five plants. Resin impressions were coated in clear nail varnish, and the nail varnish impressions were mounted on slides and visualized using a light microscope. Images were recorded on Olympus BX52 microscope (Tokyo, Japan) using a Nikon AS-F DX (Tokyo, Japan). The numbers of stomata and epidermal pavement cells were counted and the mean calculated to give the stomatal density for each plant. The stomatal index was calculated as the ratio of stomata/epidermal cells plus stomata.

Physiology measurements

Photosynthesis and stomatal conductance measurements were made using infrared gas analysis during the mid-photoperiod (between 2 h after lights were turned on and 2 h before lights were turned off). Measurements were performed using a LI-COR LI-6400XT portable photosynthesis system (Lincoln, NE, USA) attached to a leaf chamber fluorimeter with a 2 cm^2 leaf area (LI-COR 6400-40) on five plants per line. In all experiments, gas exchange measurements were taken from the middle of the blade of leaf number 5 when fully expanded. Throughout experiments, humidity was maintained at 60–70% using self-indicating desiccant (Drierite, Xenia, OH, USA), and CO_2 was scrubbed from

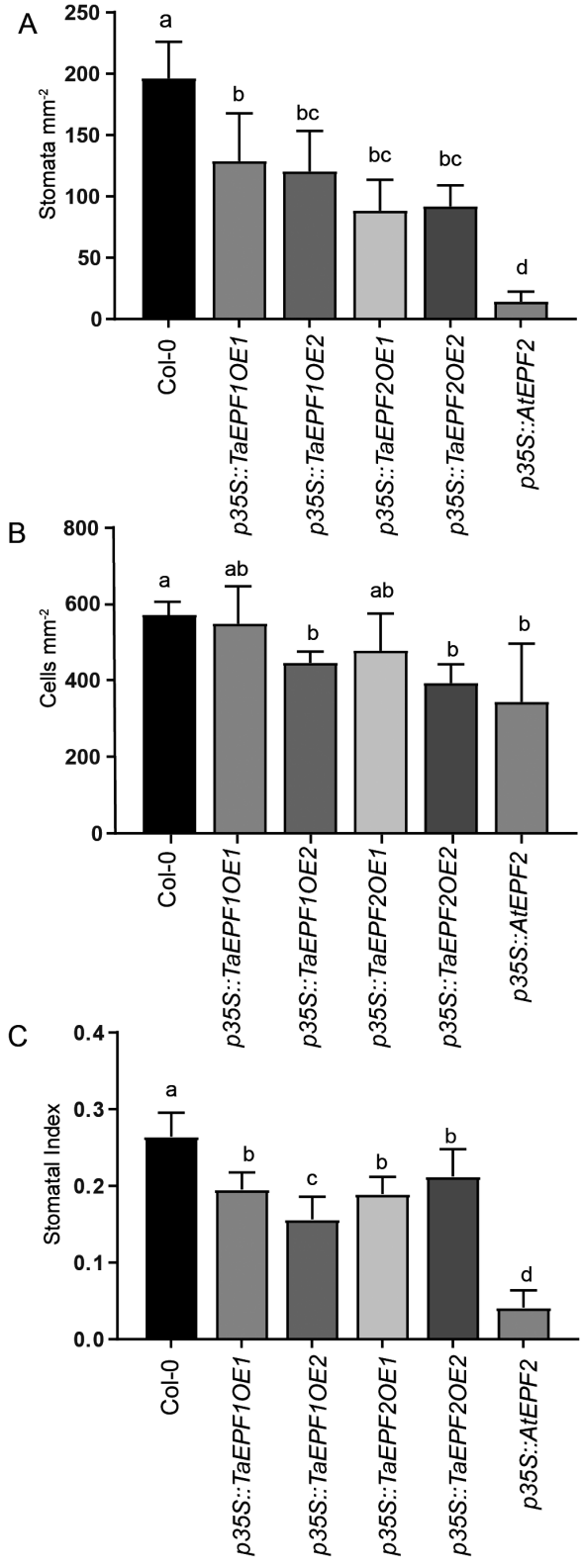

Fig. 1. Overexpression of wheat EPFs in Arabidopsis leads to a decreased stomatal density. (A) Stomatal density, (B) pavement cell density, and (C) stomatal index were measured in fully expanded leaves from a series of 6-week-old transgenic Arabidopsis plants (Col-0) transformed with either *TaEPF1* or *TaEPF2* under control of the 35S promoter. Samples were analysed using one-way ANOVA with Fisher's LSD test. Col-0, five plants; *p35STaEPF1-1*, *p35STaEPF1-2*, and *p35STaEPF2-2*, four plants; *p35STaEPF2-2* and *p35SAtEPF2*, three plants. (A–C) show means, bars=SE.

incoming air using soda lime and resupplied from a 12 g liquid CO_2 cartridge (Repcelak, Hungary). Steady-state gas exchange measurements of the carbon assimilation rate (A) and stomatal conductance (g_s) were made in the LI-COR leaf chamber with conditions matched to the growth conditions. This comprised 400 µmol m^{-2} s^{-1} light, 22 °C block temperature, and 60–70% relative humidity, 450 ppm sample [CO_2]. Once a steady state of both assimilation and conductance had been reached (not increasing or decreasing; ~20 min), the reference and sample chambers were matched, and then gas exchange measurements were logged every 30 s for 10 min. An average of these data was taken for each leaf. Intrinsic WUE was calculated as A/g_s from these averages.

The carbon assimilation rate was measured at a range of leaf intercellular CO_2 concentrations (C_i) under saturating light conditions (2000 µmol m^{-2} s^{-1}) using the LI-6400XT. A/C_i curves were plotted for five well-watered plants per line grown at ambient CO_2 in the growth chamber. Flow was maintained at 500 µmol s^{-1} and temperature was maintained at 21 °C. Leaves were acclimated under ambient CO_2 and saturating light for 45–60 min before sample [CO_2] conditions were altered stepwise through 500, 400, 300, 200, 100, 80, 60, 40, and 500 ppm. After a reacclimation at 500 ppm until assimilation and conductance had reached a steady state (~15–30 min), [CO_2] was increased stepwise through 600, 800, 1000, 1200, 1400, 1600, 1800, and 2000 ppm. R and S chambers were matched at each CO_2 concentration prior to measurements. At each CO_2 concentration, leaves were allowed to acclimate for 90–180 s (subambient region of the curve) or 3–5 min (above ambient region of the curve). A/C_i response curve measurements were fitted to the Farquhar–von Caemmerer–Berry model of photosynthesis using the plantecophys package in R (Duursma, 2015), to yield estimates of V_{cmax} (the maximum rate of Rubisco carboxylation) and J_{max} (the maximum rate of electron transport), both measures of photosynthetic capacity (Farquhar *et al.*, 1980).

Yield was measured on plants that had been allowed to fully dry down in the growth chambers or glasshouse (until the tiller weight had not changed for at least a week). Total number and weight of seeds from each tiller were recorded, as well as the total number of tillers and aboveground dry biomass produced.

Statistical analyses

Statistical analyses were performed using one- or two-way ANOVAs. Uncorrected Fisher's least significant difference (LSD) tests were applied for multiple comparisons in GraphPadPrism 7. ANOVA tables for Figs 1A–C, 3C–E, 4A, B, and 5A and C are shown in Supplementary Table S2.

Results and discussion

Wheat expresses EPIDERMAL PATTERNING FACTOR *genes suggesting evolutionary conservation of function*

A comparison of the Chinese Spring bread wheat genome (https://www.wheatgenome.org/Tools-and-Resources/Sequences) with the Arabidopsis AtEPF1 and AtEPF2 peptide sequences revealed the presence of three *EPF1*-like and three *EPF2*-like genes annotated across all three chromosomes (*TaEPF1A*, *TaEPF1B*, *TaEPF1D*, *TaEPF2A*, *TaEPF2B*, and *TaEPF2D*) (Supplementary Fig. S1A). Notably, all six sequences possess the conserved pair of cysteine residues that are present in Arabidopsis EPF1 and EPF2 sequences but absent from EPFL9/STOMAGEN sequences, reflecting a high degree of homology in the C-terminal regions of these potential negative regulators of stomatal development (Hepworth *et al.*, 2018). Analysis of available RNASeq data (Pearce *et al.*, 2015) provided evidence that all three *TaEPF1* homeologues and two of the *TaEPF2* homeologues (*TaEPF2B* and *TaEPF2D*)

are expressed in wheat plants. While the timing of expression differed between the genes, at least one of the five genes is expressed at each of the developmental stages and tissue types sampled (Supplementary Fig. S1B), with relatively higher levels of expression during leaf and stem development where differentiating stomata are found.

To investigate the conservation of function of the wheat EPFs, *TaEPF1B* and *TaEPF2D* were constitutively misexpressed in *A. thaliana* Col-0 plants. The results showed that ectopic expression of both wheat *EPF1*-like and *EPF2*-like genes led to a significant decrease in stomatal density (Fig. 1A), demonstrating a degree of functional conservation of the wheat *EPF* genes in the Arabidopsis system. However, in this experiment, the wheat EPFs did not inhibit stomatal density as effectively as the endogenous Arabidopsis EPF2; that is, overexpression of *TaEPF1B* and *TaEPF2D* reduced stomatal density by 28–41% whereas Arabidopsis *EPF2* overexpression reduced it by 86%. Analysis of the transgenic Arabidopsis plants indicated a concomitant decrease in pavement cell density in the lines overexpressing *TaEPF1* genes, although this was only significant in two of the four overexpression lines (Fig. 1B). Finally, an analysis of the stomatal index (the proportion of epidermal cells that are stomata) indicated a decreased value relative to controls in all of the transgenic lines (including those overexpressing *TaEPF1* and *TaEPF2*) (Fig. 1C), thus the number of stomata formed relative to pavement cells decreased after constitutive overexpression of wheat *TaEPF1B* and *TaEPF2D* in the Arabidopsis background. Again, the decreases in stomatal index brought about by the overexpression of the wheat *EPF* genes were not as severe as that brought about by overexpression of endogenous At*EPF2*.

These observations are consistent with previous work on barley, rice, and the evolutionarily more distant moss *Physcomitrella patens*, where the relevant *EPF* gene orthologues have been expressed in Arabidopsis (Caine et al., 2016, 2019; Hughes *et al.*, 2017). Furthermore, overexpression of barley *EPF1* decreased stomatal density, but similar to the overexpression of Arabidopsis *EPF1*, also resulted in the excessive formation of stomatal precursor cells (Hughes *et al.*, 2017). Together, these results indicate that the function of all grass EPF1- and EPF2-related peptides studied so far is more reminiscent of that of Arabidopsis EPF1 than EPF2—they can restrict stomatal development once initiated but, distinct from Arabidopsis EPF2, they are unable to restrict the asymmetric division that leads to the formation of stomatal precursor cells.

TaEPF1 *misexpression decreases stomatal density and arrests stomatal development in wheat*

Next, we investigated the outcome of misexpression of endogenous *EPF* genes in wheat. To do this we created a series of transgenic lines in which the coding region for *TaEPF1B* (hereafter referred to as *TaEPF1*) was constitutively overexpressed from a rice actin promoter in the bread wheat cultivar Fielder. Genotyping indicated that the lines contained one or more copies of the *TaEPF1* construct (Supplementary Fig. S2A), and reverse transcription–PCR (RT–PCR) analysis confirmed that *TaEPF1* mRNA accumulated in these lines to a higher level than in control, non-transformed cv Fielder plants, with a larger increase observed in those lines showing the largest reduction in stomatal density (Supplementary Fig. S2B).

Analysis of the abaxial epidermis in three independent lines (*TaEPF1-OE1*, *TaEPF1-OE2*, and *TaEPF1-OE3*) revealed a reduced stomatal density in all three transgenic lines, with mean values ranging from 12 to 4 stomata mm^{-2}, compared with a mean density of >25 stomata mm^{-2} in the wild-type cv Fielder control (Fig. 2A). The most severe phenotype (observed in *TaEPF1-OE3*) involved a decrease in mean stomatal density of 87%, whereas the most moderate phenotype (*TaEPF1-OE1*) led to a 46% decrease in stomatal density. Further analysis of the epidermis of transgenic plants overexpressing *TaEPF1* indicated that although many of the stomata formed appeared normal (consisting of a pair of guard cells surrounded by two subsidiary cells), they were interspersed with cells in which the stereotypical pattern of division which leads to normal stomatal differentiation in grasses had apparently been abrogated before a mature, functional stomatal complex could form (compare Fig. 2B and C). These abnormal or truncated division patterns were generally located within a leaf epidermal cell file (which form in parallel lines from the leaf tip to base) that also contained apparently normal stomata, consistent with the idea that they represent stomatal precursors whose differentiation has been terminated. Examples of these abrogated pre-stomatal cells are shown in Fig. 2D–G. They ranged from epidermal cells which, by their position relative to normal stomata, could be expected to initiate stomatal differentiation yet these epidermal cells underwent only very limited axial expansion (Fig. 2D, E), to cells which had undergone both axial expansion and a single longitudinal division (Fig. 2F), as well as cells which had undergone a transverse division without any major axial expansion (Fig. 2G). This latter phenotype is reminiscent of that observed after ectopic expression of *EPF1* in Arabidopsis which leads to an increase in arrested stomatal precursors (Hara *et al.*, 2007) and can also be compared with the altered patterns seen in other grasses where *EPF1* genes have been misregulated (Hughes *et al.*, 2017; Caine *et al.*, 2019). Thus, the function of the wheat EPF orthologue reported here is more similar to that of Arabidopsis EPF1 than EPF2, in common with the previously reported grass EPFs.

Overexpression of EPF1 or EPF2 has been shown to reduce stomatal density in Arabidopsis and other species (Hara *et al.*, 2007, 2009; Hunt *et al.*, 2009; Caine et al., 2016, 2019; Wang *et al.*, 2016; Hughes *et al.*, 2017). A range of stomatal densities were found in the different *TaEPF1-OE* lines reported here, with a maximum decrease of ~90% being found in the most extreme phenotype. This can be compared with the reductions reported in rice (up to 75%) and barley (up to 99%), so the phenotypes are comparable in the different grass systems, with the differences possibly relating to the different gene promoters used [a rice actin promoter being used here and a ubiquitin or *Cauliflower mosaic virus* (CaMV) 35S promoter being used in other systems].

Overall, the data reported here are consistent with the hypothesis that the EPF patterning system is highly conserved within the grasses and that overexpression of an EPF1 orthologue is sufficient to lead to a decrease in stomatal density (Hepworth *et al.*, 2018).

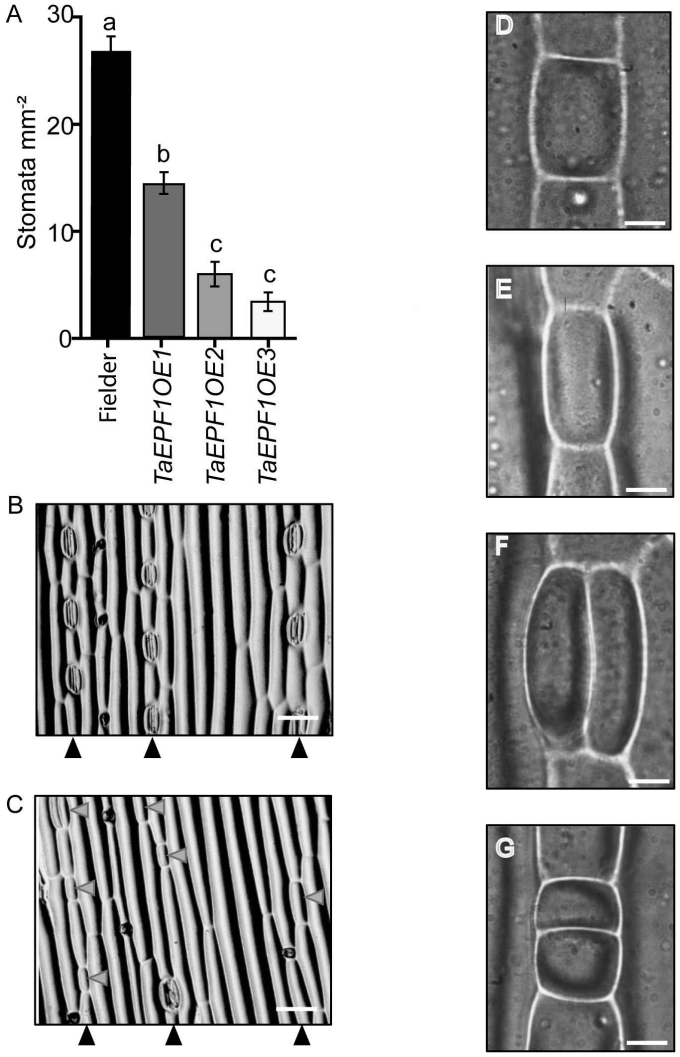

Fig. 2. Stomatal density is reduced in wheat (*Triticum aestivum*) lines overexpressing *TaEPF1*. (A) Stomatal densities of wild-type cv Fielder and three independently transformed *TaEPF1-OE* lines were measured in fully expanded leaf 1. Comparison of stomatal densities was performed using a one-way ANOVA and Fisher's LSD test, Means that are not significantly different from each other (P<0.05) are indicated with the same letter (n=5–8 leaves). Bars=SE. (B) Image of wild-type wheat epidermis of the abaxial side of leaf 1, with stomatal files labelled (purple arrows). (C) as in (B) but a *TaEPF1* transgenic plant (*TaEPF-OE3*) with stomatal files (purple arrows) and arrested precursor cells (green arrows). (D–G) Example images of arrested or altered cell division patterns in *TaEPF-OE3* in positions predicted to form stomata normally. Scale bars (B, C)=20 µm, (D–G)=5 µm.

TaEPF1 *misexpression leads to improved WUE*

Despite the observed decrease in stomatal density, when raised in controlled-environment chambers and kept well watered, the *TaEPF1-OE* plants grew and developed apparently normally and were visibly indistinguishable from wild-type cv Fielder controls (Fig. 3A). As shown in Fig. 3B, analysis of A/Ci curves under saturating light indicated very similar assimilation rates at all CO_2 levels, with a maximal mean rate of >30 µmol CO_2 m^{-2} s^{-1} being measured in all lines. In addition, estimates of V_{cmax} (the maximum rate of Rubisco carboxylation) and J_{max} (the maximum rate of electron transport) revealed no differences in these key measures of photosynthetic capacity (Farquhar *et al.*, 1980) (Supplementary Fig. S3).

Under standard growth conditions (non-saturating light) in controlled-environment chambers, assimilation rates of *TaEPF1-OE1* and *TaEPF1-OE2* plants were indistinguishable between control cv Fielder and transgenic plants (Fig. 3C), although the assimilation rate in *TaEPF1-OE3*, the line showing the most severe decrease in stomatal density, was significantly decreased (P<0.05). Analysis of steady-state stomatal conductance (g_s) revealed a significant decrease in all three *TaEPF1-OE* lines compared with the wild-type cv Fielder control (Fig. 3D), with *TaEPF1-OE3* having the maximal decrease in stomatal conductance. The maintenance of the assimilation rate, or a moderate decrease in the most severe line *TaEPF1-OE3*, and reduction in stomatal conductance in all lines meant that the calculated intrinsic WUE (iWUE) was significantly higher for all of the *TaEPF1-OE* lines relative to the wild-type cv Fielder control (Fig. 3E).

Previous experiments in which stomatal density has been engineered to be at least 50% lower in crops such as barley and rice have also demonstrated substantial improvements in WUE (Hughes *et al.*, 2017; Caine *et al.*, 2019). In both cases, improved WUE occurred through a large reduction in g_s and a smaller or insignificant reduction in photosynthetic rate. Despite the large reductions in stomatal density and gas exchange capacity in these experiments, small reductions in photosynthesis could only be measured under conditions where stomatal gas exchange would be expected to be high, for example in barley experiments when water was plentiful, or at above normal growth light intensities for rice experiments.

Overall, it seems that generating leaves with a lower stomatal density is a potential approach to increasing WUE in cereals, with the main advantage being a decreased water loss with little penalty in terms of loss of assimilation rate. This is especially the case in those transgenic lines in which the decrease in stomatal density is moderate, namely up to 50% reduction in stomatal density on leaves prior to tillering (Caine *et al.*, 2019).

A moderate decrease in stomatal density does not reduce yield under a normal watering regime

To explore the relationship of stomatal density and yield, particularly under drought conditions where lower stomatal densities might be advantageous, we grew plants to reproductive maturity and analysed total seed yield under restricted or non-restricted watering conditions in a glasshouse. The transgenic lines *TaEPF1-OE2* and *TaEPF1-OE4* continued to show a similar stomatal density reduction relative to the control in glasshouse conditions; moreover, the water restriction regime imposed did not significantly alter this phenotype (both lines had ~20% reduction in stomatal density of the flag leaf; Supplementary Fig. S4A). Similarly, transgenic lines which showed a more severe decrease in stomatal density in controlled-environment chambers (*TaEPF1-OE3* and *TaEPF1-OE5* with ~30% reduction) continued to display this phenotype under glasshouse conditions, irrespective of watering condition (Supplementary Fig. S4B). Earlier work on a different wheat cultivar showed small alterations in stomatal density following water stress or abscisic acid (ABA) treatment

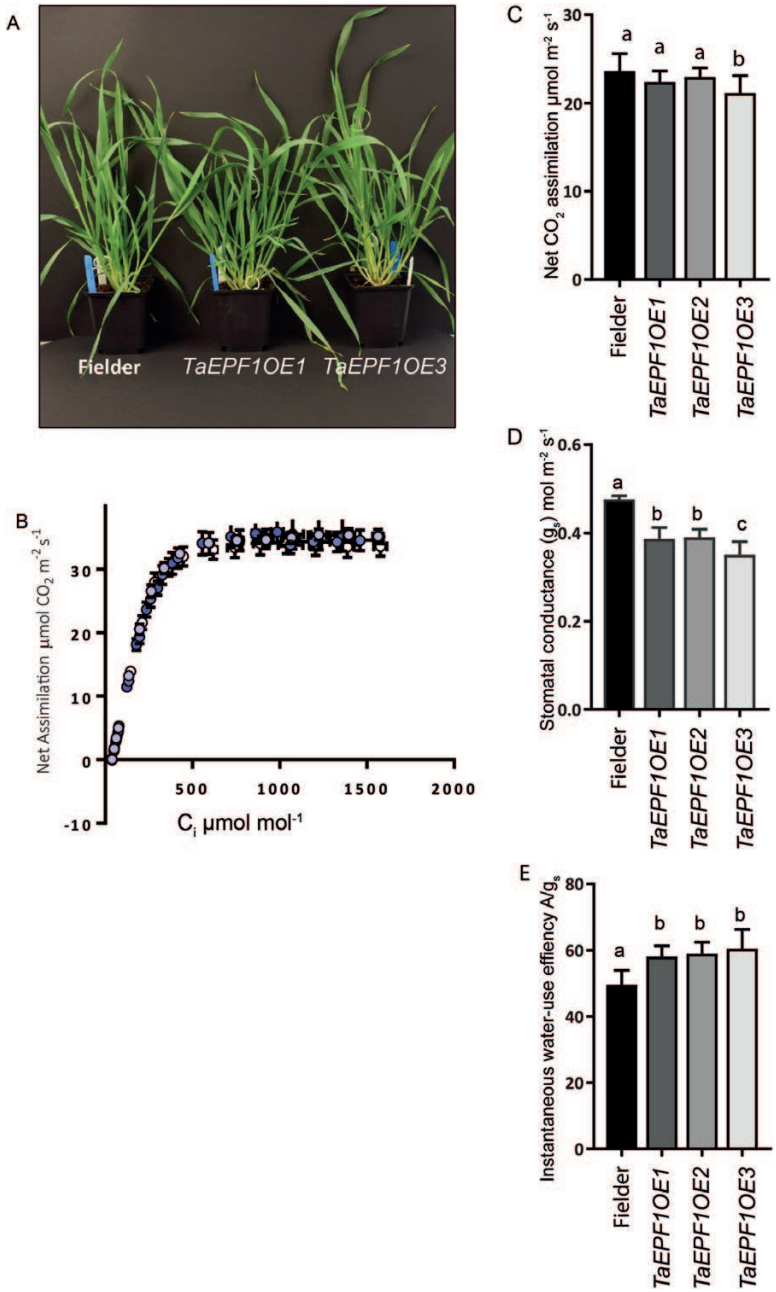

Fig. 3. Wheat plants overexpressing *TaEPF1* grow normally and are more water-use efficient. (A) Wheat transgenic lines *TaEPF1-OE1* (moderate decrease in stomatal density) and *TaEPF1-OE3* (more severe decrease in stomatal density) are not visibly distinguishable from wild-type cv Fielder plants. (B) A/C_i curves under saturating light for the wild type (white circles) and *TaEPF1* overexpressors *TaEPF1-OE1* and *TaEPF1-OE3* (shaded circles) cannot be distinguished, indicating that biochemical limitations on photosynthesis do not differ between the lines. (C) Assimilation rates under standard growth conditions do not differ significantly between wild-type cv Fielder and *TaEPF1* overexpressors *TaEPF1-OE* and *TaEPF1-OE2* (which show a moderate and intermediate decrease in stomatal density, respectively), while the more severe line *TaEPF1-OE3* shows a decrease in assimilation rate under these conditions. (D) Stomatal conductance, g_s, of the TaEPF1 overexpression lines is decreased compared with wild-type cv Fielder. (E) iWUE is higher in the TaEPF1 overexpression lines compared with wild-type cv Fielder. For (C, D, E), a one-way ANOVA was performed followed by uncorrected Fisher's LSD test. Lines that cannot be distinguished from each other (P<0.05) are indicated with the same letter (n=5 plants per line). Bars=SE.

(Quarrie and Jones, 1997). Our observation that watering regime had no effect on Fielder stomatal density is interesting as it indicates that some bread wheat cultivars are unable to adjust stomatal development to suit water availability.

Under the 80% control soil RWC regime, seed mass from the *TaEPF1-OE2* and *TaEPF1-OE4* plants was indistinguishable from that of controls (Fig. 4A). Under restricted watering, both control and transgenic lines showed a significant decrease in seed yield, with the decrease in yield being similar in all cases (Fig. 4A). For the transgenic lines displaying a more severe decrease in stomatal density, the decrease in seed yield after watering restriction was relatively less severe, but this mainly reflected the fact that even under a normal watering regime these plants showed a low seed yield; that is, a lower plateau of yield had already been reached in these lines (Fig. 4B). Although the absolute yield of control plants under normal

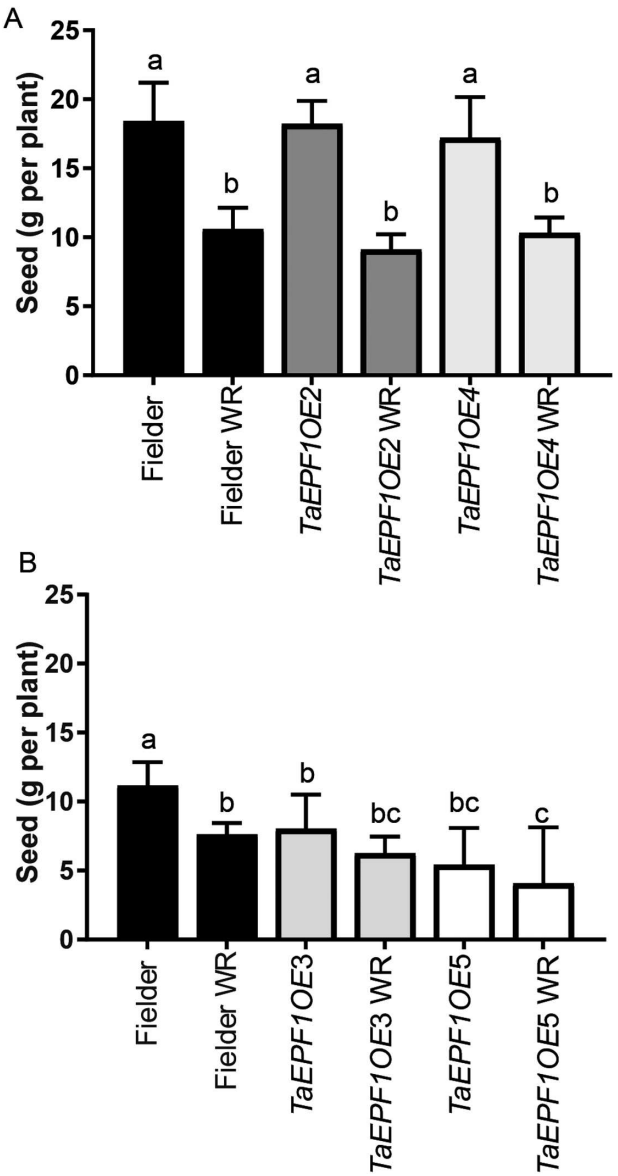

Fig. 4. Seed yield is maintained in wheat plants with moderately decreased stomatal density. Plants were grown to seed in the glasshouse in two separate experiments. In (A), transgenic lines with moderate or intermediate decreases in stomatal density were analysed (*TaEPF1-OE2* and *TaEPF1-OE4*) and in (B) transgenic lines with more extreme decreases in stomatal density (*TaEPF1-OE3* and *TaEPF1-OE5*), with independent control lines of cv Fielder grown in both series of experiments. In (A) and (B), plants were either provided a normal watering regime (80% soil RWC) or were grown with a restricted water regime, WR (30% soil RWC). Seed yield at maturity (g of seed per plant) was measured and the yields compared (one-way ANOVA and Fisher's LSD, n=5 plants per line). Samples designated with the same letter cannot be distinguished from each other at the 95% confidence limit. Bars are SE.

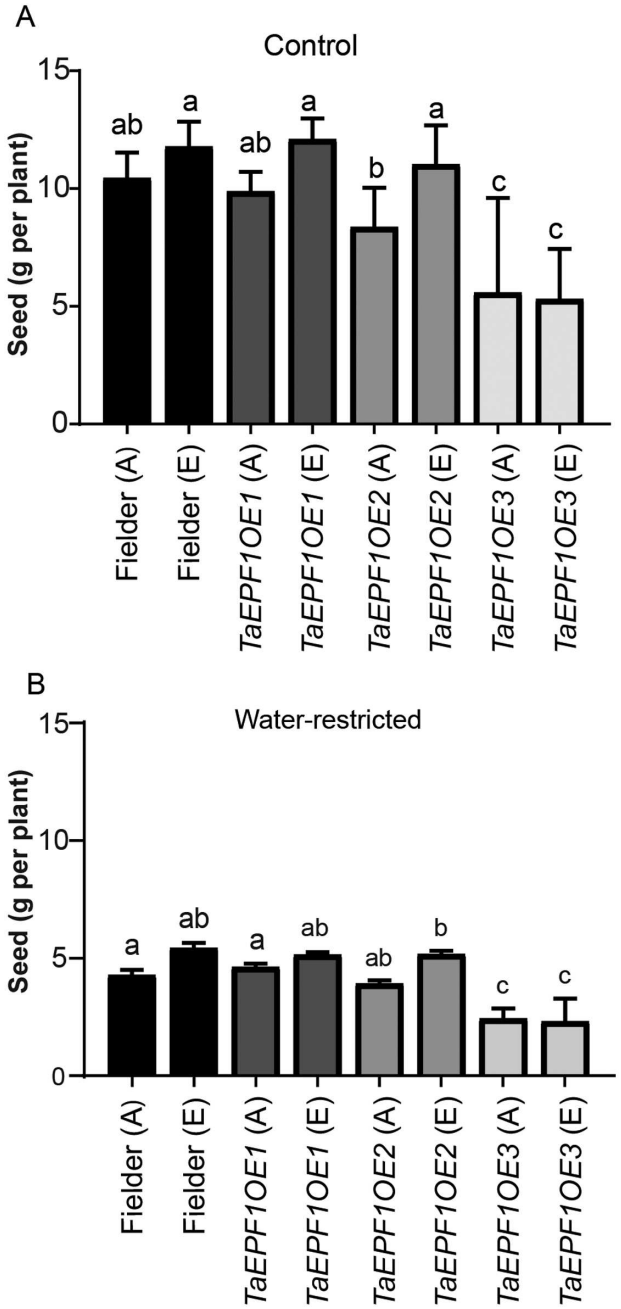

Fig. 5. Water restriction negates any potential yield gain in elevated CO_2 conditions irrespective of stomatal density in wheat. Plants with a spectrum of decreased stomatal density (moderate, *TaEPF1-OE1*; intermediate, *TaEPF1-OE2*; extreme, *TaEPF1-OE3*) as well as the control, non-transgenic cv Fielder were grown to seed under (A) a control watering regime (80% FC) or (B) a restricted watering (30% FC). In addition, plants were grown under either an ambient CO_2 level (indicated by A) or elevated 1000 ppm (indicated by E). Seed yield at maturity (g of seed per plant) was measured and the yields compared (one-way ANOVA and Fisher's LSD, n=5 plants per line). Samples designated with the same letter cannot be distinguished from each other at the 95% confidence limit. Bars are SE.

watering was low in this experiment, there was still a significant decrease in yield observed after watering restriction in the control plants (Fig. 4B).

In field experiments with the wheat cultivar *Ytipi*, elevated CO_2 (550 ppm) has been shown to increase both yield and WUE, and reduce total water consumption (O'Leary *et al.*, 2015). As a first approach to investigating the potential outcome of decreased stomatal density on wheat yields in a future climate of elevated CO_2, we grew the *TaEPF1*-overexpressing plants in chambers with ambient or elevated CO_2 levels. We used a [CO_2] of 1000 ppm which is higher than that expected to be reached in the field by 2050 (IPCC, 2018). In addition, we investigated the outcome of water restriction on seed yield under this altered CO_2 regime, to see how the plants might respond to or cope with multiple environmental challenges. In this experiment, transgenic

lines and Fielder control plants were maintained at soil water levels of 80% FC (non-limiting) or 30% FC (limiting). The results indicated that in the controlled-environment chambers under a non-limiting watering regime and ambient CO_2 conditions, one out of the three transgenic lines tested gave a decreased yield in comparison with non-transgenic controls. This decrease was only observed in plants with the most severe decrease in stomatal density (*TaEPF1-OE3*). Transgenic lines *TaEPF1-OE2*, which showed an intermediate decrease in stomatal density, and *TaEPF1-OE1* which showed a moderate decrease in stomatal density, both had no significant difference in yield in comparison with Fielder controls (Fig. 5A). These results from controlled-environment ambient CO_2 conditions are comparable with the results observed under glasshouse conditions. Furthermore, when grown under elevated CO_2 and well watered, the yield of the intermediate *TaEPF1-OE2* line was higher relative to growth at ambient CO_2, and this yield was not significantly different from the non-transgenic control under the same conditions. Perhaps surprisingly, for the other lines (*TaEPF1-OE1* and *TaEPF1-OE3*) and the Fielder controls, yields were similar under both ambient and elevated CO_2 (Fig. 5A).

When the plants grown under elevated CO_2 were also subjected to a restricted watering regime, the yield of all transgenic and control lines decreased. No significant differences in yield were observed when each line grown under elevated CO_2 with restricted water was compared with the same line grown under ambient CO_2 with restricted water. There were also no differences in yield between *TaEPF1-OE1* and *TaEPF1-OE2* and control Fielder plants grown under the same conditions (Fig. 5B). Under the restricted watering regime, only the transgenic line with the most severe decrease in stomatal density (*TaEPF1-OE3*) showed yield losses relative to the Fielder line under elevated CO_2, which is in line with the results observed under non-limited watering (Fig. 5A).

These observations can be compared with those made on other crops engineered to have more severely decreased stomatal density where no loss of seed yield was reported (Hughes *et al.*, 2017; Caine *et al.*, 2019). None of the *TaEPF1-OE* lines reported here showed any relative increase in yield compared with controls under conditions of restricted watering, whereas yield enhancements were observed in some water stress conditions when similar manipulations were performed in rice (Caine *et al.*, 2019).

The reasons for this difference are unclear. The most obvious explanation for a lack of enhanced seed set in the wheat transgenic lines might be the decreased photosynthetic assimilation leading to reduced yield. However, this appears to be unlikely as only the more severe *TaEPF1-OE3* line showed a reduction in assimilation (Fig. 3C) and yields could not be restored by growth at high CO_2 levels under either well-watered or water-restricted conditions (Fig. 5). Alternatively, yield differences could be due to alterations to root architecture (Mohammed *et al.*, 2019), or to lack of nutrients or metabolites reaching the reproductive organs as a consequence of a reduced transpiration stream (Hepworth *et al.*, 2015). Other plausible explanations could include effects of EPF overexpression that are unrelated to alterations in gas exchange. For example, an excess of EPF ligand may interfere with the activity of ERECTA family receptors which are involved in ovule and anther development (Pillitteri *et al.*, 2007; Hord *et al.*, 2008) or a reduction in stomatal number may retard pollen dehiscence, as has been proposed for spores from capsules of moss (Chater *et al.*, 2015). Further work is required to explore whether wheat yields are particularly sensitive to these factors in comparison with other cereals.

Despite no increase in yield being observed in any of the *EPF1-OE* lines under any conditions, in those lines showing a moderate decrease in stomatal density, yield was comparable with that obtained in control plants grown under similar conditions, both with ample and with restricted watering. If the improved iWUE measured in these plants with decreased stomatal density is translated to the whole-plant level over the duration of crop growth, one would expect less water to have been used to obtain a similar final seed yield. Future work at the field level to investigate this possibility will reveal whether manipulation of TaEPF1 levels to engineer decreased stomatal density in wheat is a plausible strategy to achieve the goal of 'more crop per drop'. Bearing in mind that wheat is often grown on land with limited water availability, such an advance may be extremely advantageous for this crop.

In summary, we have demonstrated that wheat has orthologues of the Arabidopsis EPFs and that overexpression of one of these *EPF*-like genes leads to arrest in stomatal development and, consequently, transgenic lines with a range of decreased stomatal densities. We show that in controlled-environment and glasshouse conditions, lines with moderate suppression of stomatal differentiation have decreased water use while maintaining photosynthesis, leading to an improved WUE. We also demonstrate that under controlled conditions lines with moderately decreased stomatal density can maintain yields equivalent to wild-type control plants.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Homology and expression of wheat EPF1/2 homologues.

Fig. S2. Analysis of transgenic wheat lines.

Fig. S3. Photosynthetic capacity of transgenic wheat lines.

Fig. S4. Stomatal density of glasshouse-grown transgenic wheat lines.

Table S1. Oligonucleotide primer sequences.

Table S2. Statistical analyses to accompany the graphs in the figures.

Acknowledgements

We acknowledge Japan Tobacco Inc. as the patent holder for the technology included in the PureWheat® Wheat Package licensed from Japan Tobacco. This work was funded by a White Rose BBSRC DTP studentship to JD; MA and AM were funded by BBSRC SARIC grant BB/N004167/1 to JEG and LH; and RMH was funded by BBSRC grant BB/J019356/ to EW. We would like to thank Melanie Craze and Sarah Bowden for assisting with wheat transformation.

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