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Reducing stomatal density in barley improves drought tolerance without impacting on yield.

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Manipulation of a gene involved in the suppression of stomatal development in barley can reduce stomatal density, leading to improved drought tolerance without deleterious effects on yield.

J.H. and C.H performed barley physiological and statistical analyses, C.H. and J.H. performed the confocal microscopy; C.D. performed qPCR. J.H. carried out Arabidopsis experiments and J.A.D. contributed to the stomatal analysis. L.H., J.S. and R.W. performed barley gene cloning and transformation. J.E.G., L.H. and R.W. conceived and supervised the project. C.H created the figures; C.H., J.H. and J.E.G. analysed the data and wrote the article with input from the other authors.

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Abstract

The epidermal patterning factor (EPF) family of secreted signalling peptides regulate the frequency of stomatal development in model dicot and basal land plant species. Here we identify and manipulate the expression of a barley ortholog and demonstrate that when overexpressed HvEPF1 limits entry to, and progression through, the stomatal development pathway. Despite substantial reductions in leaf gas exchange, barley plants with significantly reduced stomatal density show no reductions in grain yield. In addition, HvEPF1OE barley lines exhibit significantly enhanced water use efficiency, drought tolerance and soil water conservation properties. Our results demonstrate the potential of manipulating stomatal frequency for the protection and optimisation of cereal crop yields under future drier environments.

Introduction

With the global population set to rise to over 9 billion by 2050 and the predicted instability in global climate patterns, fears over global food security continue to grow (Godfray *et al.*, 2010). Prolonged periods of drought and expanded zones of desertification are expected to become increasingly prevalent as this century progresses (IPCC, 2014). The need to expand agriculture into areas of marginal land, where drought is a severe inhibitor of sustainable agriculture (Fita *et al.*, 2015), continues to increase. 70% of global freshwater is already utilised for irrigation and rain-fed agriculture is now the world's largest consumer of water (Foley *et al.*, 2011). A potential way to both futureproof against climate change, and to expand crop production onto water-limited marginal lands would be through improvements to crop drought tolerance and water use efficiency (*WUE*, the ratio of carbon gained to water lost).

The vast majority of water is lost from crops via transpiration and reducing this loss provides a potential route towards improving *WUE* and conserving soil water levels (Hepworth *et al.*, 2015). To this end, much research into the use of anti-transpirants was carried out in 1960's and 70's (Davenport *et al.*, 1972). However, although effective in improving water status and increasing fruit size, these chemical solutions were never economically viable on an agricultural scale.

The majority of water loss from plants occurs via transpiration through epidermal pores known as stomata, making these cellular structures an attractive target in the battle to prevent water loss. Recently several laboratory studies have demonstrated that it is possible to improve drought tolerance and *WUE* by reducing the frequency of stomata on leaves; by using genetic manipulation or mutation to reduce stomatal density (*SD*) improved water use efficiency has been achieved across several model dicot species including Arabidopsis (Yoo *et al.*, 2010; Franks *et al.*, 2015; Hepworth *et al.*, 2015), poplar (Lawson *et al.*, 2014) and tobacco (Yu *et al.*, 2008). In addition, the ectopic expression of a putative transcription factor in maize has led to reduced stomatal density and gas exchange in a monocot (Liu *et al.*, 2015).

The manipulation of *SD* has been facilitated by microscopic studies which characterised the cellular stages of the stomatal lineage, and molecular studies that revealed the developmental mechanisms controlling their progression (Zhao & Sack, 1999; Han & Torii, 2016). The majority of these studies have been carried out using the genetically tractable, model plant species Arabidopsis. During early Arabidopsis leaf development, a subset of epidermal cells known as meristemoid mother cells

(MMCs) become primed to enter the stomatal lineage. Each MMC then undergoes an initial asymmetric entry division to produce a meristemoid in addition to a larger daughter cell known as a stomatal lineage ground cell (SLGC). SLGCs either differentiate directly into epidermal pavement cells or undergo further asymmetric divisions to produce secondary meristemoids. Some meristemoids can themselves undergo further asymmetric divisions, each of which reforms a meristemoid and creates an additional SLGC. Each meristemoid eventually differentiates into a guard mother cell, small and rounded in shape, prior to undergoing a symmetric division to form the guard cell pair of the mature stomatal complex. These cell fate transitions and divisions, which ultimately control the number and proportions of stomata and pavement cells in the mature leaf epidermis, are controlled by a sub-group of related basic helix-loop-helix (bHLH) transcription factors; SPCH, MUTE and FAMA (Ohashi-Ito & Bergmann, 2006; MacAlister *et al.*, 2007; Pillitteri & Torii, 2007). SPCH primarily directs expression of genes controlling meristemoid formation including members of the cysteine-rich EPIDERMAL PATTERNING FACTOR (EPF) family of secreted signalling peptides, which in turn activate a pathway that regulates SPCH stability, thus forming a feedback loop that regulates the number of cells entering the stomatal lineage (Adrian *et al.*, 2015; Simmons & Bergmann, 2016). The best characterised negative regulators of stomatal density in this peptide family are EPF1 and EPF2, which are numbered in order of their discovery (Hara *et al.*, 2007; Hara *et al.*, 2009; Hunt & Gray, 2009). Both peptides act extracellularly within the aerial epidermal cell layer to suppress stomatal development through activation of an intracellular MAP kinase signalling pathway (Bergmann et al., 2004; Wang et al., 2007; Lampard et al., 2008). Although their functions somewhat overlap, EPF2 acts earliest in stomatal development to restrict entry of cells into the stomatal lineage, whilst EPF1 acts later to orient subsequent divisions of meristemoid cells and enforce stomatal spacing through the 'one-cell-spacing' rule via the inhibition of MUTE expression (Hara *et al.*, 2007; Qi *et al.*, 2017). Manipulation of the expression levels of these peptides in Arabidopsis has led to significant improvements in drought tolerance and *WUE* in experiments conducted in controlled-environment plant growth rooms (Doheny-Adams *et al.*, 2012; Hepworth *et al.*, 2015).

In contrast to the Arabidopsis model system, our knowledge of stomatal development in crops is relatively limited (Raissig *et al.*, 2016). Although the grasses include many of our major global crops, our molecular understanding of their transpirational control mechanisms remains extremely limited. It is known from microscopic observations that grass stomata are formed by a single asymmetric cell division that forms a stomatal precursor cell (a guard mother cell) and an epidermal pavement cell (Stebbins & Jain, 1960). There are no further asymmetric divisions of the stomatal lineage cells analogous to the repeated possible divisions that meristemoids undergo in Arabidopsis (Serna,

2011). The mature grass stomatal complex is formed by division of two neighbouring cells that give rise to flanking subsidiary cells, and a symmetric division of the guard mother which produces two dumbbell-shaped guard cells - rather than the characteristically kidney-shaped guard cells of most dicots (Hetherington & Woodward, 2003; Serna, 2011). In contrast to dicots, all grass stomatal development initiates at the leaf base. The patterning of stomata within the leaf epidermis also differs in grasses, with stomata forming in straight files parallel to the leaf vein as opposed to the 'scattered' distribution seen in Arabidopsis (Stebbins & Khush, 1961; Geisler & Sack, 2002; Serna, 2011)

Despite these differences in stomatal shape and patterning it appears that the molecular control of stomatal development has similarities across a wide range of plant species. Functional orthologs of genes encoding for bHLH transcription factors involved in Arabidopsis stomatal development have been identified in grasses including; rice, maize (Liu *et al.*, 2009) and brachypodium (Raissig *et al.*, 2016) and recently in the early diverging non-vascular mosses (Chater *et al.*, 2016). EPF orthologs are encoded across a range of plant genomes and have recently been shown to effectively regulate moss stomatal patterning (Caine et al., 2016). However, currently it is still not known whether EPFs function in controlling stomatal development in grasses. With the sequencing of the barley genome in 2012 we were able to identify a putative EPF ortholog (*HvEPF*1, MLOC_67484) that is expressed at low levels during development of aerial tissues (IBSC, 2012). Here we characterise the function of an epidermal patterning factor in grasses. We report the ectopic overexpression of *HvEPF1* and the production of transgenic barley lines exhibiting altered stomatal development. Furthermore, our generation of barley lines with reduced *SD* has provided us with the necessary tools to determine the effect of reduced *SD* on transpiration, drought tolerance, water use efficiency and yield in a cereal crop.

Results

11 genes encoding putative EPF-like secreted peptides were identified in the barley genome sequence (IBSC, 2012) (Fig.S1) . MLOC67484 which we refer to here as *HvEPF1* encodes a peptide with extensive similarity to Arabidopsis epidermal patterning factors, and contains the 6 conserved cysteine residues (Fig. 1a) that are characteristic of Arabidopsis epidermal patterning factors (Ohki *et al.*, 2011; Lau & Bergmann, 2012). Phylogenetic analysis of the encoded mature peptide sequence indicated that within the Arabidopsis EPF family, HvEPF1 is most closely related to the known inhibitors of stomatal development EPF1 and EPF2 which each contain two additional cysteine residues (Fig. S1). To confirm that this barley peptide gene could function in stomatal regulation, *HvEPF1* was ectopically overexpressed in Arabidopsis under the control of the *CaMV35S* promoter.

Analysis of cellular patterning on the epidermis of Arabidopsis plants overexpressing *HvEPF1* confirmed that stomatal development had been disrupted; a phenotype similar to that observed on overexpression of Arabidopsis *EPF1,* namely a significant decrease in leaf stomatal density (Fig. 1b) and an increased number of arrested meristemoids (Fig. 1c) (Hara *et al.*, 2007; Hara *et al.*, 2009; Hunt & Gray, 2009).

Next, barley plants ectopically over-expressing the epidermal patterning factor HvEPF1 under the control of a ubiquitin gene promoter were produced. Stomatal density (*SD*) was assessed from 13 transgenic lines of *HvEPF1OE* in the T1 generation under growth room conditions. The first leaves of seedling plants had *SD* ranging from approximately 70% down to < 1% of that of control plants (transformed with the empty-vector) (Fig. 2a). Two lines were selected for further phenotyping: *HvEPF1OE*-(47%) and *HvEPF1OE*-(0.6%), which displayed approximately 47% and 0.6% of the *SD* of controls respectively. Significantly reduced leaf *SD* was observed in abaxial epidermal impressions (Fig. 2b) and unusually large patches of epidermis with an absence of stomates were seen in the leaves of HvEPF1OE (0.6%). Furthermore, arrested stomatal precursor cells were frequently observed in the mature, fully expanded, epidermis which were extremely rare in controls (black arrow in Fig. 2c.

For more detailed physiological analysis, homozygous barley lines harbouring a single copy of the transgene (Supplementary table 1) were isolated (referred to as *HvEPF1OE*-1 and *HvEPF1OE*-2 and indicated by the left and right red asterisks in Fig.2a respectively). T2 generation plants were grown under controlled chamber conditions and the abaxial *SD* of the second true leaf was significantly reduced by approximately 52% and 56% of controls for *HvEPFOE*-1 and *HvEPFOE*-2 respectively (Fig. 3a). In addition, the stomates that formed were smaller; guard cell length was significantly reduced in both *HvEPF1OE* lines (Fig. 3b). However, we observed no significant increase in epidermal pavement cell density (Fig. 3c). These differences in cell densities combined to produce large reductions in stomatal index (*SI*; stomatal density as a percentage of all cells on the epidermis). *SI* of *HvEPF1OE* plants was reduced to approximately 50% of control values (Fig. 3d). Again we observed a significant increase in the number of arrested stomatal precursor cells in *HvEPF1OE* barley leaves (as shown in Figure 2). To calculate whether the number of arrested stomatal precursor cells could entirely account for the observed reductions in *SD* we calculated the 'stomatal lineage cell index' (the percentage of stomata and arrested stomatal lineage cells compared to all cells on the epidermis). This indicated that if all arrested stomatal precursor cells were to have progressed normally to produce stomata, there would still be a significant reduction in stomatal index, suggesting that both the priming of cells to enter the stomatal lineage, and the progression of cells through the stomatal lineage are compromised by HvEPF1 overexpression (Fig. 3e).

Having shown that HvEPF1 can effectively regulate the frequency of stomatal development, we next explored whether any other aspects of *HvEPF1OE* leaves were affected. In particular, we investigated the internal structure of leaves. Stacked confocal images were produced to visualise *HvEPF1OE* substomatal cavities. This revealed similar internal cellular structures, and mature *HvEPF1OE* stomatal complexes had guard cells positioned normally above substomatal cavities as in controls (yellow asterisks, fig. 4a). However, on the same images, a lack of cavity formation was observed under the arrested stomatal precursor cells in both *HvEPF1OE*-1 and HvEPF1OE-2 lines (white asterisks, fig. 4b).

To more fully investigate the effect of reduced *SD* on drought tolerance, T2 generation plants were grown in a greenhouse with natural and supplemental lighting and temperature control. 5-week-old *HvEPF1OE*-1, *HvEPF1OE*-2 and control plants were subjected to a terminal drought experiment alongside a parallel set of plants that were kept well-watered (maintained at 60% maximum soil water content). Pots were weighed at the same time each day and this was used to calculate soil water loss. The results of this experiment revealed that both transformed barley lines lost water much more slowly and exhibited significantly greater soil water conservation in their pots from day 2 until day 14 under water-withheld conditions (Fig. 5a). Chlorophyll fluorescence measurements were used to measure any reductions in photosystem II efficiency, an indicator of plant stress. The light adapted quantum yield of photosystem II (Φ_{PSII}) was measured daily for both well-watered and water-withheld plants throughout the terminal drought experiment. There were no differences between the Φ_{PSII} of *HvEPF1OE* and control plants at the start of the experiment or between genotypes under well-watered conditions indicating that the reduced stomatal density of the *HvEPF1OE* leaves was not restricting photosystem II efficiency. Remarkably however, the *HvEPF1OE* plants that had water withheld, displayed significantly enhanced rates of Φ_{PSII} versus water-withheld controls from day 10 until day 14; both *HvEPF1OE*-1 and *HvEPF1OE*-2 plants maintained their photosystem II efficiency for approximately 4 days longer than controls under severe drought conditions. On day 6 of terminal drought, leaf samples were taken for leaf relative water content (*RWC)* estimation. This result indicated no significant difference in leaf *RWC* between controls and *HvEPF1OE* plants under well-watered conditions. However, under water-withheld conditions, both *HvEPF1OE* lines displayed significantly higher levels of leaf *RWC* versus controls (Fig. 5c), indicating an enhanced ability to retain water in their leaves under drought conditions. In addition, the *HvEPF1OE* plants were less susceptible to wilting and appeared visibly more ‘drought tolerant’ on day 6 of water-withheld conditions (Fig. 5d).

In a separate greenhouse experiment, we investigated whether the reduced *SD* of *HvEPF1OE* barley plants could confer any advantage to growth under conditions of limited water availability (rather than on complete withholding of water as above). *HvEPF1OE*-1, *HvEPF1OE*-2 and controls plants were grown under well-watered (60% soil water content) and water-restricted (25% soil water content) in parallel under controlled greenhouse conditions. This water-restricted regime was severe enough to attenuate the growth rate of the barley plants but not severe enough to cause visible signs of wilting (Fig. S2). Stomatal density and steady state gas exchange measurements were taken from the sixth fully expanded leaf of the primary tiller of mature plants. This revealed that *SD* and photosynthetic assimilation (*A*) were significantly reduced in comparison to controls in both *HvEPF1OE* lines under well-watered conditions. On these leaves the *SD* of HvEPF1 OE-1/2 were 24% and 12% of control values respectively. There was a significant decrease in *A* in both lines under well watered conditions but no significant differences in *A* between *HvEPF1OE* or control plants that had been grown under water-restriction (Fig. 6a). In addition, there was a significant reduction in stomatal conductance (*gs*) between *HvEPF1OE* and control plants within the well-watered treatment group and a reduction in the *gs* of all plants within the water-restricted treatment (Fig. 6b). As a result of the large reductions in *gs* and relatively small reductions in *A*, intrinsic WUE *(iWUE,* the value of A divided by gs*)* was calculated to be significantly increased in the *HvEPF1OE*-2 line under well-watered conditions. There was no increase in *iWUE* observed in either *HvEPF1OE* line under water-restricted conditions (fig. 6c). After 11 weeks of drought, *WUE* across the photosynthetic lifetime of the barley flag leaves was then assessed by delta-carbon isotope analysis. This revealed that, under water-restriction, both *HvEPF1OE* lines displayed lower levels of ^{13}C discrimination and thus a greater level of *WUE*. In agreement with the gas exchange results, only *HvEPF1OE*-2 plants (which had more severely reduced *SD*) displayed increased *WUE* under well-watered conditions (Fig. 6d).

Further gas exchange measurements were carried out on the flag leaf to investigate whether photosynthetic biochemistry could have been altered by overexpression of *HvEPFL1*. In line with our previopus Arabidopsis based studies (Franks et al., 2015), we observed no differences in the maximum velocity of Rubisco for carboxylation (Vcmax) or the potential rate of electron transport under saturating light (Jmax). Our calculations indicate that any improvements in WUE are due to increased limitation to stomatal gas exchange, rather than altered photosynthetic biochemistry.

Finally, to assess the impact of reduced *SD* on barley yield and biomass, plants were left to grow under the well-watered and water-restricted regimes described above until plant peduncles had lost colour. At this point plants were allowed to dry and were then harvested. Analysis of the grain yield suggested that a reduction in *SD* did not have a deleterious effect on seed number, seed weight, the

228 average weight of seed, nor the harvest index (the ratio of above ground biomass to seed weight)
229 under either watering condition (Fig. 7 a-d). In addition, no differences in plant height nor above
230 ground biomass were found between any of the barley lines under either watering regime (Figs. S3,
231 S4).

232

233 **Discussion**

234 Grasses are an economically important plant group, with the cereal grasses being of critical
235 importance for both food and energy production. Considering future predicted climate scenarios,
236 the creation of drought tolerant cereals is a priority area for both crop improvement and scientific
237 research.

238 The bHLH transcription factors and epidermal patterning factors which were first discovered to be
239 regulators of stomatal development in Arabidopsis have been conserved from basal land plants
240 through to angiosperms including the grasses, and have been suggested as potential targets for crop
241 improvement (Peterson *et al.*, 2010; Ran *et al.*, 2013; Caine *et al.*, 2016; Raissig *et al.*, 2016). Here we
242 report the characterisation of a functional barley EPF ortholog, named HvEPF1, which acts in a
243 similar way to the Arabidopsis EPF1 and EPF2 signalling peptides to limit entry to and progression
244 through the stomatal cell lineage. Our overexpression of the barley *HvEPF1* transcript in Arabidopsis
245 led to a significant reduction in *SD* indicating a level of conservation in peptide function between
246 monocots and dicots. The overexpression of HvEPF1 in barley led to severe reductions in both
247 stomatal formation, and in the entry of epidermal cells into the stomatal lineage, adding weight to
248 this conclusion.

249 The frequent presence of arrested stomatal precursor cells on the epidermis of both Arabidopsis and
250 barley *HvEPF1OE* plants (Fig. 1c and 2b) suggests that the mode of action of HvEPF1 is most similar
251 to that of Arabidopsis EPF1, which generates a similar epidermal phenotype when overexpressed
252 (Hara *et al.*, 2007; Hara *et al.*, 2009). That is, stomatal precursors enter the developmental lineage
253 but become arrested before the final symmetric cell division and maturation of the stomatal
254 complex. These *HvEPF1OE* oval-shaped arrested cells appear to halt their development at a
255 meristemoid-like or early guard mother cell stage, prior to transition into mature guard mother cells.
256 Thus, in addition to entry to the stomatal lineage, the transition to a mature guard mother cell that
257 is competent to divide and form a pair of guard cells appears to be regulated by HvEPF1. In
258 Arabidopsis this cellular transition step is under the control of the transcription factor MUTE (Fig. 8)
259 whose activity promotes expression of the receptor-like kinase ERECTA-LIKE1, which in turn

mediates EPF1 signalling and the subsequent autocrine inhibition of MUTE (Qi *et al.*, 2017). Barley MUTE may be regulated by HvEPF1 by a similar autocrine pathway and/or by phosphorylation as grass *MUTE* genes (unlike Arabidopsis *MUTE*) encode potential MAP kinase phosphorylation sites (Liu *et al.*, 2009). Recent work in the monocot *Brachypodium,* has revealed MUTE to also be involved in the formation of subsidiary cells (Raissig *et al.*, 2017). In *HvEPF1OE* plants, stomatal precursors arrest prior to the establishment of subsidiary cells suggesting the overexpression of HvEPF1 may act to inhibit the expression of MUTE.

Despite their importance, we know remarkably little about the sequence of events leading to the production of the air-filled spaces that underlie stomata. In conjunction with the stomatal pores, these substomatal cavities facilitate high levels of gas exchange into plant photosynthetic mesophyll cells, and mediate leaf water loss via transpiration. Using confocal microscopy, we could see no evidence for the separation of mesophyll cells below arrested stomatal precursor cells in HvEPF1OE leaves. Our observations begin to throw light on the developmental sequence leading to cavity formation. The arrested stomatal precursor cells in *HvEPF1OE* do not form substomatal cavities, suggesting that these cavities form following either GMC maturation, like the subsidiary cells of the stomatal complex, or after guard cell pair formation. Alternatively, the formation of a substomatal cavity may be required for guard mother cell maturation.

There is much evidence to support a negative correlation between stomatal density and stomatal size across a range of species and Arabidopsis stomatal mutants i.e. those plants with relatively low *SD* tend to produce larger stomates (Miskin & Rasmusson, 1970; Franks & Beerling, 2009; Doheny-Adams *et al.*, 2012). Interestingly, the overexpression of HvEPF1 did not conform to this trend, and led to barley plants with smaller, shorter guard cells. Thus if the EPF signalling pathway directly regulates stomatal size in dicot species (and this remains to be demonstrated), it appears to act in the opposite manner in grass stomatal size determination.

Through the ectopic over-expression of HvEPF1 we have created barley transformants with a range of reductions in *SD*. Although barley plants with substantially reduced numbers of stomata showed some attenuation of photosynthetic rates when well-watered, they exhibited strong drought avoidance and drought tolerance traits when water was withheld. They had lower levels of water loss via transpiration, and they were able to maintain higher levels of soil water content, and delayed the onset of photosynthetic stress responses for several days longer than controls. Remarkably when grown under water-limiting conditions (25% soil pot water content) two barley lines with reductions in *SD* demonstrated significant improvements in *WUE* without any deleterious effects on either plant growth or seed yield (biomass, seed weight or seed number). Indeed, it would

be interesting to determine whether both *WUE* and yield may be further optimised in reduced stomatal density lines under less severe watering regimes or through less drastic reductions in *SD*

HvEPF1OE-2 plants (which had the lowest *SD* in this experiment) also displayed significantly enhanced levels of drought tolerance and *WUE* under well-watered conditions, without accompanying decreases in either grain yield or plant biomass. The increased *iWUE* observed in these experiments was a result of a relatively moderate drop in *A* compared to a larger decrease in *gs*, suggesting that *A* was not limited by internal CO_2 concentration under the growth conditions of our experiment (Yoo *et al.*, 2009). This may also be a factor in explaining why reductions in *SD* did not impact on the yield of *HvEPF1OE* plants. Further explanations include significantly reduced rates of *gs* and thus water loss in *HvEPF1OE* plants allowing for more resources to be allocated to the generation of seed and above ground biomass, at the potential cost to root development, as described previously in Arabidopsis EPF over-expressing plants (Hepworth *et al.*, 2016), or increased soil water content leading to improved nutrient uptake and *gs* under water limitation (Van Vuuren *et al.*, 1997; Hepworth *et al.*, 2015). Thus, although not tested in this study, reducing *SD* may also enhance resource allocation or nutrient uptake capacity under water-restriction.

To conclude, this study describes the function and physiological effect of overexpressing a native epidermal patterning factor in a grass species. The manipulation of *HvEPF1* expression levels has improved our understanding of stomatal developmental mechanisms in grasses, and has generated a range of barley plants displaying significantly reduced *SD*. These barley plants exhibit substantially improved drought tolerance and *WUE* without reductions in grain yield. This novel discovery adds strength to the proposition that stomatal development represents an attractive target for breeders when attempting to future-proof crops.

Materials and Methods

Vector Construction

HvEPF1 genomic gene was PCR amplified from *Hordeum vulgare* cultivar Golden Promise DNA using primers in Table S1. The HVEPF1 gene is annotated as MLOC67484 at Ensembl Plants but is incorrectly translated in this prediction. We used FGENESH to generate an alternative translation which includes a putative signal sequence at the N-terminus. The PCR product was recombined pENTR/D/TOP0 then by LR recombination into pCTAPi (Rohila *et al.*, 2004) transformation vector under the control of the CaMV35S promoter, and introduced into *Arabidopsis thaliana* Col-0 background by floral dip (Clough & Bent, 1998). Transformation and expression of the transgene were confirmed by PCR and RT-PCR using the primers in Supplementary Table S2.

For barley transformation the *HvEPF1* genomic gene was introduced by LR recombination into pBRACT214 gateway vector under the control of the maize ubiquitin promoter, adjacent to a hygromycin resistance gene under the control of a CaMV35S promoter (Fig. S4). Barley transformations were carried out in background Golden Promise using the method described by (Harwood *et al.*, 2009). Plants harbouring just the hygromycin resistance cassette were regenerated alongside to produce 'empty-vector control' plants. Potentially transformed plants were regenerated on selective medium and T0 individuals genotyped to confirm gene insertion by PCR. Gene copy number was estimated byIDna Genetics Ltd (www.idnagenetics.com) using a PCR based method HvEPF1 overexpression was confirmed by RT-qPCR of T2 generation plants (Fig. S6). Total RNA was extracted from 10 day old seedlings using Spectrum plant total RNA kit (Sigma, UK) and reverse transcribed using Maxima H Minus Reverse Transcriptase cDNA synthesis kit (Thermo Scientific). RT-qPCR was performed using a Rotor-Gene SYBR® Green PCR kit (Qiagen) with tubulin and GADPH used as housekeeping reference genes, and primers outlined in the supplementary supporting information (Supplementary table 2). Three plants of each transformed line were amplified to confirm overexpression of the *HvEPF1* gene. Fold induction values of gene expression were normalised to average $2^{\Delta Ct}$ values relative to empty-vector control samples.

Plant Growth Conditions

For plant growth, seeds were surfaced sterilised in 50% vol/vol ethanol/bleach before being placed onto water saturated filter paper and placed into sealed Petri dishes in the appropriate growth chamber. Arabidopsis plants were grown in a controlled growth chamber (Conviron model MTPS120) at 22°C/16°C, 9 hours light, 150-200 µmol m^{-2} s^{-1}, 15 hours dark, ambient [CO_2] and 60% humidity. Arabidopsis plants were kept well-watered throughout. Barley plants were grown in a MTPS120 growth chamber at 21°C/15°C, 11 hours light at 300µmol.m^{-2}.s^{1}, 13 hours dark, ambient [CO_2] and 60% humidity. For plants grown under greenhouse conditions (Fig. 5, Fig. 6), temperature was set at 20°C/16°C, 12 hours light, ambient humidity, and supplementary lighting ensured a minimum of 200 µmol m^{-2} s^{-1} at bench level.

At 5 days post-germination individual barley seedlings were placed into 13cm diameter pots containing homogenised M3 compost/perlite (4:1) with the addition of Osmocote. For initial phenotyping and leaf developmental characterisation (Fig. 2, Fig. 3, Fig. 4) plants were kept well-watered. For the water-restricted experiment, (Fig. 6, Fig. 7) plants were maintained at either 60% (well-watered) or 25% (water-restricted) of soil saturation by the daily weighing of pots.

Microscopy and cell counts

For both Arabidopsis and barley, stomatal and epidermal cell counts were taken from the abaxial surface of mature, fully expanded leaves or cotyledons. Cell counts were taken from the widest section of the first true leaf avoiding the mid vein. Dental resin (Coltene Whaledent, Switzerland) was applied in the region of maximum leaf width and left to set before removing the leaf and applying clear nail varnish to the resin. Stomatal counts were determined from nail varnish impressions by light microscopy (Olympus BX51). 5 areas per leaf were sampled from 4-8 plants of each genotype and treatment. For epidermal imaging (Fig. 2b-d), mature leaves were excised and the central vein of the leaf cut away. Leaf tissue was then serially dehydrated in ethanol. Samples were then placed into modified Clarke's solution (4:1 ethanol to glacial acetic acid solution) then cleared in 50% bleach overnight.

For epidermal phenotyping, the second fully expanded mature leaf of seedings were excised and a 3-5cm strip midway along the proximodistal axis of these leaves were cut out. These leaf samples were then submerged in Clarke's solution (3:1 ethanol to glacial acetic acid solution). Following 1 hour of vacuum infiltration the samples were left in Clarke's solution for 24 hours for fixation. Once fixed the samples were transferred into 100% ethanol. Prior to imaging the leaf samples were cleared in 50% bleach solution overnight. The midrib of each sample was then excised and the remaining leaf sections mounted in deionised water on microscope slides for imaging. Samples were viewed by light microscopy (Olympus BX51) using differential interference contrast functionality. For confocal microscopy (Fig. 4a, Fig4b), barley samples were prepared as described (Wuyts *et al.*, 2010) and viewed on a Olympus FV1000 using 20X UPlan S-Apo N.A. 0.75 objective, 543nm laser, 555-655nm emission and Fluorview software .

Physiological measurements

Throughout the terminal drought experiment the light adapted quantum yield of photosystem II (ΦPSII) was measured daily for both well-watered and water-withheld plants. The most recent fully expanded leaf of the primary tiller was selected for the measurement at day 1 and the same leaf was then monitored throughout the experiment. Readings were taken using a FluorPen FP100 (Photon Systems Instruments) with a saturating pulse of 3000 µmol m^{-2} s^{-1}. Following the onset of the drought treatment the pots were weighed every day and used to calculate the percentage of initial soil water content remaining. Well-watered controls were maintained at 60% soil water content.

Leaf relative water content was determined from excised leaves from well-watered or droughted and their fresh weight measured immediately and leaves were floated on water overnight and weighed to record the hydrated weight. They were oven-dried overnight and weighed to obtain their

dry weight; the RWC was calculated using the following formula *RWC (%)* = (fresh weight−dry weight)/ (hydrated weight−dry weight)*100.

A LI-6400 portable photosynthesis system (Licor, Lincoln, NE) was used to carry out infrared gas analysis (IRGA) on the sixth, fully expanded, leaf from the primary tiller whilst still attached to the plant. Relative humidity inside the IRGA chamber was kept at 60%-65% using self-indicating desiccant, flow rate was set at 300 µmol.s^{-1} and leaf temperature at 20°C. Reference [CO_2] was maintained at 500ppm and light intensity at 200µmol.m^{-2}.s^{1}. Plants were allowed to equilibrate for 40-45 minutes the IRGA chamber being matched at least every 15 minutes. Once readings were stable measurements were taken every 20 seconds for 5 minutes. For soil water content calculations, the weight of pots containing water saturated (100% water content) or oven dried (0%) compost mix was first determined. Pots were then maintained at either 60% or 25% soil water content by weighing and addition of the appropriate amount of water every two days.

For carbon isotope discrimination (Fig. 6d), δ13C was assessed from the flag leaf of 5 plants from each of the two watering regimes (well-watered and restricted-watered), as described previously (Hepworth *et al.*, 2015).

Once plants had matured and dried down the plants were harvested, with the total number and weight of seeds per plant being recorded and the average seed weight being calculated. All above-ground vegetative tissue was dried in an oven at 80oC for two days and then weighed to provide the dry weight. Harvest index (ratio of yield to above-ground biomass) was then calculated.

Statistical analysis

All comparisons were performed on Graph Pad Prism software. The appropriate post-hoc tests were conducted once significance was confirmed using an ANOVA test and an alpha level of 0.05 or below as significant.

Figure legends

Figure 1. HvEPF1 shares sequence similarity with Arabidopsis EPF1 and EPF2, and can restrict Arabidopsis stomatal development. (a) Alignment of the putative HvEPF1 mature signalling peptide with members of the Arabidopsis EPF family of signalling peptides. Conserved cysteine residues are highlighted. Amino acid sequences for the mature peptide region were aligned using Multalin and displayed using Boxshade. (b) Overexpression of *HvEPF1* under the control of the *CaMV35S* promoter in Arabidopsis leads to a significant decrease in stomatal density. (c) Epidermal tracings

from Arabidopsis cotyledons overexpressing *EPF1*, *EPF2*, and *HvEPF1* alongside the background control Col-0. Red dots mark location of stomata whilst green dots mark location of arrested meristemoids. N=5 plants, asterisks indicate P<0.05, (Dunnett's test after one-way ANOVA). Error bars represent SE.

Figure 2. Over-expression of HvEPF1 in barley arrests stomatal development and reduces stomatal density. (a) The abaxial stomatal density *(SD)* of barley plants transformed to ectopically over-express *HvEPF1* (grey bars) compared to control lines transformed with the empty-vector (black bars). All T1 generation *HvEPF1* over-expressing lines demonstrated a significant reduction in *SD* in comparison to both control lines. Lines chosen for further phenotyping in T2 generations are indicated (red asterisks). (b) Traced abaxial epidermal impressions of T1 generation control, *HvEPF1OE*-(47%) and *HvEPF1OE*-(0.6%) lines illustrating the reduction in *SD*. Red dots denote positions of stomatal complexes. (c) Abaxial epidermal micrographs of *HvEPF1OE* plants. Black arrow indicates arrested stomatal precursor cell. N=4-8 plants. Asterisks indicated significance to at least P<0.05 versus control lines (Dunnett's test after one-way ANOVA. (Error bars represent SE.

Figure 3. Stomatal characteristics of barley plants overexpressing *HvEPF1.* (a) Abaxial stomatal densities of *HvEPF1* overexpressing T2 barley lines harbouring a single copy of the transgene are significantly decreased. *HvEPF1OE*-1 (white bars) and *HvEPF1OE*-2 (grey bars) compared to control lines (black bars). (b) Guard cell length is significantly decreased in both *HvEPF1OE* lines. (c) Pavement cell density is similar to that of the control in both *HvEPF1OE* lines. (d) Stomatal index is significantly decreased in both *HvEPF1OE* lines. (e) Stomatal lineage index (the ratio of stomata and arrested stomatal precursor cells to the total number of epidermal cells) is significantly decreased in both *HvEPF1OE* lines. N=5 plants, asterisks indicate P<0.05, (Dunnett's test after one-way ANOVA). Error bars represent SE.

Figure 4. Cellular structure of *HvEPF1OE* stomatal complexes. (a) Representative propidium iodide stained confocal image of a Z-plane below the *HvEPF1OE*-1 abaxial epidermal surface. Yellow asterisks mark the location of the substomatal cavity under mature guard cells. (b) Higher Z-plane image of the same field of view as (a) to reveal position of stomata. White asterisks mark the location of arrested stomatal precursors and the lack of underlying substomatal cavities in (a).

Figure 5. Reducing barley stomatal density enhances drought tolerance though conserving soil and plant water content. (a) 5 week old *Hv*EPF1OE-1 and *Hv*EPF1OE-2 barley plants maintain significantly higher soil water content in comparison to control plants when water is withheld from days 2-14. (b) Both *Hv*EPF1OE-1 and *Hv*EPF1OE-2 lines show significantly higher light adapted quantum yields (ΦPSII) from 10 to 14 days after water was withheld (square symbols; plants from

same experiment as (a)). There were no significant differences between ΦPSII of well-watered plants (circular symbols). (c) Relative water content (*RWC*) of barley leaves from *Hv*EPF1OE lines was significantly higher than controls after 6 days without watering. There were no differences in *RWC* between well-watered plants. (d) Photograph of representative plants to illustrate enhanced turgor maintenance in *Hv*EPF1OE-1 and *Hv*EPF1OE-2 on day 6 of water-withheld conditions. N=5 plants, asterisk indicates significance to at least P<0.05 (Dunnett's tests after one-way ANOVA for each watering group). Error bars represent SE.

Figure 6. Reducing barley stomatal density lowers stomatal conductance and enhances water use efficiency. (a) Under well-watered conditions a significant decrease in rate of carbon assimilation was observed in both *HvEPF1OE* lines. Under water-restricted conditions there was no difference in assimilation. (b) Stomatal conductance (*gs*) was significant decreased in *HvEPF1OE* lines grown under well-watered conditions in comparison to controls. Under water-restricted conditions there was no difference in *gs*. (c) Under well-watered conditions, a significant improvement in intrinsic water use efficiency (i*WUE*) was observed in the *HvEPF1OE*-2 line when compared to control plants. Under water-restricted conditions there was no difference in i*WUE*. (d) Carbon isotope discrimination revealed a significant improvement in water use efficiency of the *HvEPF1OE*-2 barley line under well-watered conditions. Under water-restricted conditions, both *HvEPF1OE* lines displayed significantly improved water use efficiency in comparison to controls. N=5 plants, asterisk indicates significance to at least P<0.05 (Dunnett's tests after one-way ANOVA for each watering group). Error bars represent SE.

Figure 7. Reducing stomatal density in barley has no deleterious effect on yield. No significant differences in (a) seed number, (b) total weight of seed per plant, (c) average weight of individual seeds, (d) harvest index (the ratio of yield to total shoot biomass) were observed between *HvEPF1OE*-1, *HvEPF1OE*-2 and control plants under either watering condition. N=5 plants. Error bars represent SE.

Figure 8. HvEPF1 acts to prevent cells entering the stomatal lineage, guard mother cell maturation and substomatal cavity and subsidiary cell formation. Schematic to illustrate the putative mode of action of HvEPF1 in barley stomatal development. Left to right: Undifferentiated epidermal cells at the base of leaves are formed in cellular files. Cells in some files gain the capacity to divide asymmetrically to create small stomatal precursor cells shown here as immature guard mother cells (GMC, green). A developmental step, potentially under the control of the transcription factor MUTE, stimulates guard mother cell maturation (dark green) and division of adjacent epidermal cells to form subsidiary cells (SC, orange). Mature GMCs then divide symmetrically to form pairs of dumbbell

shaped guard cells (red). In the underlying mesophyll layer (M, green shaded regions) a substomatal cavity forms during either the mature GMC or guard cell stage, although the exact developmental staging of this is process is unknown. In the *HvEPF1* overexpressing plants, HvEPF1 prevents GMC maturation perhaps through the suppression of MUTE activity, resulting in arrested GMCs which are unable to differentiate into mature stomatal complexes complete with subsidiary cells, guard cells and substomatal cavities. Drawn with reference to Brachypodium development in Raissig *et al.* 2016.

Supplemental Data

Supplemental Figure 1. Phylogenetic tree of predicted Arabidopsis and barley epidermal patterning factor peptide sequences constructed using Multalin. Barley annotations taken from Ensembl Plants apart from HvSto7, which is a putative unannotated EPFL9/Stomagen on Chromosome 7. HvEPF1 highlighted in red.

Supplemental Figure 2. Growth of barley plants is inhibited by the water-restricted conditions used in this study (25% soil water content) in comparison to growth in well-watered conditions (60% soil water). From left to right: Control plant well-watered, control water-restricted, *HvEPF1OE*-1 well watered, *HvEPF1OE*-1 water-restricted, *HvEPF1OE*-2 well-watered and *HvEPF1OE*-2 water-restricted.

Supplemental Figure 3. Plant heights of controls and *HvEPF1OE*-1 or *HvEPF1OE*-2 were not significantly different within either well-watered or water-restricted conditions. Error bars represent SE.

Supplemental Figure 4. Above ground biomass of control and *HvEPF1OE*-1 or *HvEPF1OE*-2 plant lines were not significantly different under either well-watered or water-restricted conditions. N=5 plants. Error bars represent SE.

Supplemental Figure 5. Schematic of the gene expression construct inserted into the barley genome to overexpress the *HvEPF1* gene

Supplemental Figure 6. qPCR results the confirming significant overexpression of *HvEPF1* the barley lines detailed in the manuscript. N=5 plants, asterisk indicates significance to at least $P<0.05$ (Dunnett's tests after one-way ANOVA). Error bars represent SE.

Supplemental Table 1. Copy number data for transformed plant lines used in this study.

Supplemental Table 2. Primer sequences used for PCR and RT-qPCR detailed in the methods section of the manuscript.

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