Rice with reduced stomatal density conserves water and has improved drought tolerance under future climate conditions

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Summary

- Much of humanity relies on rice (Oryza sativa) as a food source, but cultivation is water intensive and the crop is vulnerable to drought and high temperatures. Under climate change, periods of reduced water availability and high temperature are expected to become more frequent, leading to detrimental effects on rice yields. We engineered the high-yielding rice cultivar ‘IR64’ to produce fewer stomata by manipulating the level of a developmental signal. We overexpressed the rice epidermal patterning factor OsEPF1, creating plants with substantially reduced stomatal density and correspondingly low stomatal conductance.

- Low stomatal density rice lines were more able to conserve water, using c. 60% of the normal amount between weeks 4 and 5 post germination. When grown at elevated atmospheric CO2, rice plants with low stomatal density were able to maintain their stomatal conductance and survive drought and high temperature (40°C) for longer than control plants. Low stomatal density rice gave equivalent or even improved yields, despite a reduced rate of photosynthesis in some conditions.

- Rice plants with fewer stomata are drought tolerant and more conservative in their water use, and they should perform better in the future when climate change is expected to threaten food security.

Introduction

The combined impact of rapid human population growth and climate change has been described as a ‘perfect storm’ that threatens our food security (Solomon et al., 2009; Godfray et al., 2010; Porter et al., 2014). Future predicted decreases in water availability and increased frequency of extreme drought and high-temperature events are likely to present particular challenges for farmers, resulting in substantial crop losses (Vikram et al., 2015; Korres et al., 2017). Rice (Oryza sativa) is a major food crop, eaten by billions (Elert, 2014), and to mitigate the threat to global food security there is interest in developing new varieties of rice engineered to be ‘climate ready’.

Rice cultivation is particularly water intensive, using an estimated 2500l of water per 1kg of rice produced (Bouman, 2009). However, almost half of the global rice crop derives from rain-fed agricultural systems where incidences of drought and high temperatures are predicted to become more frequent and damaging under climate change (Vikram et al., 2015; Matsuda et al., 2016; Korres et al., 2017). Like most land plants, rice uses microscopic pores called stomata to regulate CO2 uptake for photosynthesis with the concomitant release of water vapour via transpiration (Zeiger et al., 1987; Murchie et al., 2002). When water is plentiful, stomatal opening also permits regulation of plant temperature by evaporative cooling (Urban et al., 2017). Under water-limiting drought conditions, stomatal closure slows down water loss, with potential trade-offs being reduced carbon assimilation A and increased plant temperature (Hu et al., 2006; Tombesi et al., 2015; Urban et al., 2017). Elevated atmospheric CO2 concentrations also induce stomatal closure and raise plant temperature (Kollist et al., 2014; Engineer et al., 2016), but this response is typically not as important under field conditions as drought-induced stomatal closure (Xu et al., 2016). In predicted future higher CO2 climates, it has been suggested that plants will be more water-use efficient as enhanced photosynthetic A allows stomata to be less open, meaning less water will be lost (Keenan et al., 2016).
et al., 2013). However, despite grain yields increasing in experiments where rice is grown at elevated CO$_2$, a greater volume of water is used than at current CO$_2$ levels, indicating that, in the future, rice cultivation may be even more water intensive than it is today (Kumar et al., 2017).

Rising CO$_2$ levels are expected to result in a warming of 1–4°C in global atmospheric temperatures by the end of the century, and the frequency of heat spikes will also increase (Meyer et al., 2014). Such dramatic rises in temperature are expected to lead to negative impacts on rice yields even in the presence of increased atmospheric CO$_2$ (Ainsworth, 2008; Kumar et al., 2017). Rice is particularly sensitive to heat stress, with the majority of growth stages being affected once temperatures exceed 35°C (Redfern et al., 2012). This is especially the case during the reproductive period, (Redfern et al., 2012; Jagadish et al., 2015), and it is predicted that, by 2050, 27% of rice-growing areas will experience at least 5 d of heat stress temperatures during this stage (Goudji et al., 2013). The impact of heat stress is expected to be exacerbated as water resources diminish and more water-use-efficient practices involving less water are adopted. This may be somewhat mitigated if transpiration-mediated cooling can be maintained, as rice can remain productive in air temperatures of 40°C if humidity remains low (Jagadish et al., 2015).

In addition to the reversible modification of stomatal apertures, plants in the longer term can adapt their stomatal development to optimize their stomatal conductance $g_s$ to the surrounding environmental conditions, such as light intensity or CO$_2$ concentration (Casson & Gray, 2008). At high temperature, some plant species can produce leaves with altered stomatal density, which can affect transpiration rates and evaporative cooling (Crawford et al., 2012; Jumrani et al., 2017). Currently, however, it is not known whether rice stomatal development is affected by growth temperature. In our study, we have investigated this and the feasibility of creating rice plants that require less water through genetically reducing stomatal density and $g_s$. Our results indicate that in a future world with elevated atmospheric CO$_2$, higher temperature and reduced water availability, stomatal-based water conservation could help to maintain or even improve rice productivity by enhancing water conservation before drought and slowing water loss during drought.

Manipulating the number of stomata that form in plants requires detailed knowledge of the developmental programme. The regulation of stomatal function and development is well studied in the model dicot Arabidopsis thaliana, and recently researchers have begun to translate these findings into monocots, including some cereal crop species (Liu et al., 2009; Hughes et al., 2017; Raissig et al., 2017). During Arabidopsis epidermal development, the extracellular EPIDERMAL PATTERNING FACTOR (EPF) and EPF-LIKE (EPFL) signalling peptides maintain the correct density and spacing of stomatal precursor cells through binding ERECTA-family receptors (Hara et al., 2007, 2009; Hunt & Gray, 2009; Lee et al., 2015). Negative regulators of stomatal development, EPF2 and EPF1, restrict stomatal development. EPF2 primarily regulates asymmetric divisions which facilitate ‘entry’ to the stomatal lineage by forming meristemoids in the early epidermis, and EPF1 acts slightly later, to regulate stomatal spacing and the transition to a guard mother cell (GMC). EPFL9 (also known as STOMAGEN) competes with EPF2 for receptor binding and thus promotes stomatal development (Lee et al., 2015; Zoulas et al., 2018). Recently, it has been shown that epidermal patterning factors also regulate stomatal development in grasses (Hughes et al., 2017; Yin et al., 2017). As in Arabidopsis, there appear to be two $EPF$ gene homologues that may restrict stomatal development in diploid grasses, but unlike Arabidopsis there are also two putative $EPFL9$ genes (rather than one) (Hepworth et al., 2018). The combination of EPF/Ls required, and when they function during stomatal development in grasses, is not yet understood. Given that grass stomata develop in parallel files and have subsidiary cells (Stebbins & Shah, 1960), whereas dicot stomata typically develop in a more random pattern, it is probable that the factors regulating grass stomatal development have evolved additional and or modified functions to their Arabidopsis/dicot counterparts (Facette & Smith, 2012; Raissig et al., 2016). So far, one rice and one barley (Hordeum vulgare) epidermal patterning factor have been shown to affect stomatal development in grasses (Hughes et al., 2017; Yin et al., 2017). In rice, lack of OsEPFL9a expression results in reduced stomatal density (Yin et al., 2017), and overexpression of HvEPF1 in barley leads to reduced stomatal density, with $HvEPF1$ appearing to act both before and after the asymmetric ‘entry’ division; that is, $HvEPF1$ has functional attributes reminiscent of both Arabidopsis EPF1 and EPF2 activities (Hughes et al., 2017). By reducing stomatal density, Hughes et al. improved barley drought tolerance, but did not quantify reductions in water use, nor investigate how fewer stomata impacted on growth at high temperature or elevated atmospheric CO$_2$ concentrations. Here, we investigate how reducing stomatal density in the major food crop, rice, affects water use, drought tolerance and heat stress tolerance, in experiments carried out at atmospheric CO$_2$ levels expected to be prevalent in the field over the next 20–50 years (Solomon et al., 2009; Meyer et al., 2014).

Materials and Methods

Plant growth conditions

Rice cultivar ‘IR64’ (Oryza sativa L. ssp. indica) seeds were germinated and seedlings cultivated for 7–8 d in a Petri dish with 15 ml water in a Sanyo growth cabinet with a 12 h 26°C : 12 h 24°C light : dark cycle, photosynthetically active radiation (PAR) 200 μmol m$^{-2}$ s$^{-1}$. Seedlings were transferred to 13D pots (0.88 l), or for yield experiments to large 19F pots (2.4 l) (East Riding Horticulture, York, UK) containing soil consisting of 71% Kettering Loam (Boughton, UK), 23.5% Vitax John Innes No. 3 (Leicester, UK), 5% silica sand and 0.5% Osmocote Extract Standard 5–6 month slow-release fertilizer (ICL, Ipswich, UK) by volume saturated with water. Plants were grown in a controlled-environment growth cabinets (Controlled Environments Ltd, Winnipeg, MB, Canada) at 12 h 30°C : 12 h 24°C light : dark cycle, PAR 1000 μmol m$^{-2}$ s$^{-1}$ and 60% relative humidity, with a constant supply of water to the pot base and watering from the top once a week unless otherwise stated.

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Plants were propagated at an atmospheric CO₂ concentration of 450–480 ppm maintained by a pressurized CO₂ tank (BOC) to the ambient conditions of the growth chamber when required. For higher temperature experiments, the daytime temperature was raised to 35 or 40°C and humidity adjusted to maintain 60% relative humidity.

For yield experiments, plants were fertilized every 14 d from 42 d with 0.5 g l⁻¹ Chempak High Nitrogen Feed No. 2, except when water was withheld (Thompson & Morgan, Ipswich, UK). Treatment 1 plants, which were well watered throughout the experiment, were harvested after 105 d. Treatment 2, which were droughted twice during vegetative growth at 28 d (for 9 d) and at 56 d (for 7 d), were harvested after 120 d. Treatment 3 plants, which were droughted during flowering at 88 d (for 3 d), were harvested after 126 d. Plant tissue was dried at room temperature for 1 month for yield analysis. Cumulative water loss was assessed by weighing and returning to different positions every day. The were placed in randomized positions within the growth chamber, replenished to 750 ml. Soil-filled pots, with or without plants, were converted to cDNA using M-MLV Reverse Transcriptase Removal Kit (Thermo Fisher Scientific, Waltham, MA, USA) and then a PCR-amplified sequence (F: TCTAAACGCCACCAACGGAC; R: GAGGTTGAGGAAA GAGGTTG) as described (Yin et al., 2017).

Analysis of OsEPF transcript levels

RNA from whole 8-d-old rice seedlings was extracted using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Gillingham, UK), adjusted to 100 ng µl⁻¹ per sample, treated with DNA-free™ DNA Removal Kit (Thermo Fisher Scientific, Waltham, MA, USA) and converted to cDNA using M-MLV Reverse Transcriptase (200 U µl⁻¹) (Thermo Fisher Scientific). Real-time quantitative PCR analysis was performed using the Rotor-Gene SYBR Green PCR Kit (400) and a Corbett Rotor Gene 6000 (Qagen) using primers (F: CCCCTTTTTCACATGATGATGTA; R: GCTGTGGGCTGTGTGAGA). Relative expression values were calculated by normalizing the take-off value and amplification efficiency of the genes analysed relative to the Profilin (LOC_Os06g05880) housekeeping gene (van Campen et al., 2016).

Epidermal imaging, quantification and calculation of gₛ max

Cell densities and tracings of Arabidopsis epidermis were produced in PAINT.NET (https://www.getpaint.net/) from nail varnish peels of dental resin impressions of fully expanded leaves from 63-d-old plants. For Fig. 1 (8-d-old rice seedlings, leaf 1) and Fig. 5 (21-d-old rice plants, leaf 5), epidermal cell densities were also recorded from nail varnish peels of dental resin impressions, calculating averages at six veins in from the leaf edge, from four 0.147 mm² confocal stacks per replicate, taken on a Nikon A1.

Supporting Information Fig. S1 were measured using the cell counter plugin of IMAGEJ (Fiji v.1.51u). Complex size (Fig. S2) was manually measured in IMAGEJ from a total of 30 stomata from each genotype, taken from six biological replicates (five
complexes per plant). The images in Figs 2 and S2 were taken as previously described (Hughes et al., 2017). For calculating pore aperture, guard cell area and \( g_{\text{sm} \alpha \text{max}} \) (Figs 5, S3), 20 stomata per plant (five per field of view) from six (40°C) or seven (30°C) biological replicates were imaged. Pore area was calculated as an ellipse from the major axis of measured aperture length, and the minor axis measured aperture width at the centre of the pore. Guard cell area was calculated as an ellipse from the axes of measured guard cell length and the doubled guard cell width at the centre of the stoma. Maximum pore aperture \( a_{\text{max}} \) (\( \mu \text{m}^2 \)) was calculated as an ellipse from axes equal to the measured aperture length and half of the aperture length. Pore depth \( l \) (\( \mu \text{m} \)) was taken as equal to guard cell width at the centre of the stoma. Abaxial anatomical \( g_{\text{sm} \alpha \text{max}} \) was calculated using the double end-corrected version of the Franks & Farquhar (2001) equation, from Dow et al. (2014):

\[
Abaxial \text{ anatomical } g_{\text{sm} \alpha \text{max}} = \left( \frac{d \cdot D \cdot a_{\text{max}}}{v \cdot (l + (\pi/2) \cdot \sqrt{a_{\text{max}}/\pi})} \right)
\]

where \( d \) (\( \text{m}^2 \text{s}^{-1} \)) is the diffusivity of water in air and \( v \) (\( \text{m}^3 \text{mol}^{-1} \)) is the molar volume of air. Assuming equal stomatal densities on both sides of the leaf, this value was doubled to give total anatomical \( g_{\text{sm} \alpha \text{max}} \). Values used in calculations are shown in Table S1.

**Physiological measurements**

Gas exchange measurements were performed on 21-d-old plants on fully expanded true leaf 5. Measurements for Figs 3(a,b) and S4(a) were taken on a LiCOR 6400 infrared gas analyser.
The leaf chamber conditions were: light intensity 1000 μmol m⁻² s⁻¹ PAR, humidity 60%, leaf temperature 30°C, flow 300 μmol s⁻¹ and CO₂ concentration 480 ppm. For Figs 3(c,d), 5(d–g) and S4(b,c), a Li-Cor 6800 infrared gas analyser (Lincoln, NE, USA) was used. Light curve analysis was conducted using the same chamber conditions with 3–5 min stabilization between each light level. The light levels were 2000, 1500, 1200, 1000, 800, 600, 480, 340, 200, 100 and 50 μmol m⁻² s⁻¹ PAR. CO₂ response curves were taken under saturating light (2000 μmol m⁻² s⁻¹ PAR) starting at 480 ppm CO₂ concentration and then lowered to 340, 200, 150, 125, 100, 75, 50, 25 and finally 0 ppm CO₂. Plants were re-acclimatized at 480 ppm CO₂ and then CO₂ was raised to 600, 800, 1000, 1250 and 1500 ppm. For all steps, plants were allowed 2.5–5 min stabilization time. Values of maximum rate of Rubisco carboxylase activity Vcmax and potential rate of electron transport Jmax were calculated using the EXCEL tool from Sharkey et al. (2007). For plants grown at different temperatures (30, 35 or 40°C), leaf chamber temperature was set equivalent to growth temperature with light set to 2000 μmol m⁻² s⁻¹ PAR and other conditions were as already noted. Fv/Fm values were measured 1 h before onset of photoperiod, with a FluorPen FP 100 (PSI, Drasov, Czech Republic). Thermal images were captured using an FLIR T650sc, and quantification of temperature was performed using FLIR TOOLS (www.flir.co.uk). Data were collected from equivalent areas of mature leaves across treatments.

Leaf area analysis

Total leaf area was measured from five 28-d-old plants per genotype, by excising every leaf where it emerged from the sheath, flattening and imaging. Areas were calculated in IMAGEJ (Fiji v.1.51u) using thresholding and the magic wand tool.

Amino acid sequence alignments

The Arabidopsis EPF2 peptide sequence was used in BLAST searches for EPF peptides sequences in ‘IR64’ rice via the
Rice SNP-Seek database (http://snp-seek.irri.org/_locus.zul;sessionid=096476AC6709F1EED57798F6D6756EE0) (Alexandrovet al., 2015). Arabidopsis and barley sequences were obtained from PHYTOZOME v.12.1.6 (Goodstein et al., 2012) and aligned using MUSCLE using defaults setting on JALVIEW v.2 (Edgar, 2004; Waterhouse et al., 2009).

Graphs and statistical analysis
Graphs were produced and statistical analysis conducted using SIGMAPLOT v.13 (Systat Software, Inc., San Jose, CA, USA) and one-way ANOVA, except in Figs 5(b) and S3, where two-way ANOVAs were performed. If unequal variances were detected in ANOVAs, a Kruskal–Wallis one-way ANOVA on ranks was performed.

Results
In Arabidopsis and barley, AtEPF2 or HvEPF1 overexpression reduces stomatal density, leading to improved drought tolerance (Hara et al., 2009; Hunt & Gray, 2009; Franks et al., 2015; Hepworth et al., 2015; Hughes et al., 2017). Two closely related rice gene products have been identified as orthologues of Arabidopsis EPF1 and EPF2, both potentially involved in regulating stomatal development (Hepworth et al., 2018) (Fig. 1a). In the ‘IR64’ rice cultivar genome, OSIR64_00232g011350.1 encodes the most similar gene product to AtEPF2 and HvEPF1. We studied the function of this rice gene product by fusing the OSIR64_00232g011350.1 coding sequence to the native AtEPF2 promoter and expressing the gene construct in the Arabidopsis epf2 knockout background (Figs 1, S1). Expression of this
‘OsEPF rescue’ gene construct restored the Arabidopsis epf2 stomatal density from c. 250 mm⁻² back to normal levels (c. 160 mm⁻²) (Fig. 1b–e). However, high numbers of aborted stomatal lineage cells, characteristic of epf2, persisted in the epidermis, suggesting that in Arabidopsis plants the expression of the ‘OsEPF rescue’ gene could not adequately restrict the number of asymmetric ‘entry’ divisions at the start of the stomatal development pathway (Figs 1b–d, S1). Excessive stomatal lineage cells formed but were unable to progress to stomata; this phenotype was previously observed in Arabidopsis EPF1 overexpression experiments (Hara et al., 2009). Owing to the large number of aborted stomatal lineage cells, the stomatal indices (ratio of stomata to stomata plus other epidermal cells) of the OsEPF rescue plants remained similar to epf2 plants (Fig. 1f). Ectopic overexpression of OSIR64_00232g011350.1 in Arabidopsis, directed by the CaMV35S promoter, led to a marked reduction in both OSIR64_00232g011350.1 as was previously observed in Arabidopsis formed but were unable to progress to stomata; this phenotype of asymmetric ‘entry’ divisions at the start of the stomatal development affected evaporative cooling. In treatment 1 plants were cooler than ‘IR64’ controls (Fig. S4). Conversely, when watering ceased during a single drought period (for 3 d) when plants were 98 d old, they were 0.3°C cooler towards the end of the drought period (for 3 d) when plants were 88 d old and flowers had emerged from panicle sheaths.

We measured gas exchange across a range of light intensities and in this experiment found no significant differences in A between genotypes at and below the growth light intensity (1000 µmol m⁻² s⁻¹ PAR; Fig. S4). However, above this light intensity, A was reduced relative to the ‘IR64’ controls in both OsEPF1oe lines. To assess whether the maximum rate of Rubisco carboxylase activity Vₘₐₓ or the potential rate of electron transport Jₘₐₓ was altered in plants with reduced stomatal density, we measured A and intercellular CO₂ at a range of CO₂ concentrations (Figs 3c,d, S4). We did not detect any significant differences in the rates of either Vₘₐₓ or Jₘₐₓ, suggesting that the photosynthetic apparatus in OsEPF1oe plants can perform at equivalent rates to controls. To see whether changes in stomatal density and gas exchange properties reduced whole-plant water use, we directly measured water loss over between weeks 4 and 5 (Fig. 3e). Over this 1 wk period, both OsEPF1oe lines used significantly less water than ‘IR64’ controls did, with OsEPF1oeW using 42% less water and OsEPF1oeS using 38% less water. To determine whether the observed reduction in water loss could be affected by plant size, we measured whole plant leaf area on a subset of 4-wk-old plants and found that OsEPF1oeW plants had no reduction in size (P = 0.33), but OsEPF1oeS had a 14% reduction in leaf area (P = 0.04; Fig. 3f).

To test whether the substantial reductions in OsEPF1oe stomatal density could lead to improvements in drought tolerance, plants were grown in 2.4 l pots and subjected to one of three different watering regimes (Figs 4, S6, S7). Treatment 1 plants were watered normally; treatment 2 plants were subjected to two periods without water during vegetative growth at 28 d (for 9 d) and at 56 d (for 7 d); and treatment 3 plants were subjected to a single drought period (for 3 d) when plants were 88 d old and flowers had emerged from panicle sheaths.

We used infrared thermal imaging to assess how altering stomatal development affected evaporative cooling. In treatment 1 conditions, low stomatal density OsEPF1oe lines were c. 0.3°C warmer than ‘IR64’ controls at the maximum tillering stage (49 d old), suggesting a small but significant reduction in water loss and cooling (Fig. 4a,b). Conversely, when watering ceased during treatments 2 and 3, OsEPF1oe plants were cooler than ‘IR64’ controls (OsEPF1oeW were 0.3°C cooler towards the end of drought period during treatment 2; OsEPF1oeW and OsEPF1oeS were 0.7 and 0.6°C cooler during treatment 3; Figs 4c–f, S6). Thus, OsEPF1oe plants were able to maintain evaporative cooling.
at higher levels than controls during drought, suggesting that initial improved water conservation in the reduced stomatal density lines allowed plants to keep their stomata open for longer under drought conditions.

To investigate whether either the reduced $g_s$ that we observed when plants were well watered or the enhanced evaporative cooling observed during vegetative and reproductive drought could affect plant growth or productivity, we grew the OsEPF1oe and

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Fig. 4 OsEPF1 overexpression affects leaf water loss and temperature, and enhances yield following flowering drought in ‘IR64’ rice (Oryza sativa ssp. indica). (a, b, g) Treatment 1: well-watered plants. (c, d, h) Treatment 2: water withheld during vegetative growth at 28 d for 9 d and at 56 d for 7 d. (e, f, i) Treatment 3: water withheld during reproductive stage at 88 d for 3 d. Surface temperatures of (a) treatment 1 plants, well-watered at 49 d old, (c) treatment 2 plants, 62 d old at the end of 7 d drought period, and (e) treatment 3 plants 90 d old at the end of 3 d drought period. Infrared thermal images in (b), (d) and (f) are from representative plants used to compile data in (a), (c) and (e). Dark blue denotes coolest areas, as indicated on scale on right. (g–i) Total grain yields of (g) well-watered, (h) vegetative drought and (i) flowering drought plants. For all box plots graphs, horizontal lines within boxes indicate the median with boxes covering the upper (75%) and lower (25%) quartiles. Whiskers indicate the ranges of the minimum and maximum values, and letters indicate significantly different mean values ($P < 0.05$, one-way ANOVA). Owing to unequal variances, in (g) a Kruskal–Wallis one-way ANOVA on ranks was performed: (a, b, g) $n = 8$; (c, d, h) $n = 5–7$; (e, f, j) $n = 6–7$. 

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control plants to maturity. After treatments 1 and 2, OsEPF1oe plant biomass and grain yield were equivalent to the ‘IR64’ control plants (Figs 4g,h, S7). Interestingly, following treatment 3, drought during the flowering period, the OsEPF1oeW line produced significantly more aboveground biomass (26% increase) and grain yield (27% increase) than ‘IR64’ controls did ($P<0.01$ and $P<0.05$), whilst OsEPF1oeS yields remained comparable to ‘IR64’ (Figs 4i, S7). The 1000 grain weight of both OsEPF1oe lines was also significantly higher than that of ‘IR64’ controls in treatment 3 ($P<0.01$), suggesting that having fewer stomata has a positive effect on grain filling when plants experience drought during flowering (Fig. S7).

To examine whether OsEPF1oe plants have altered heat stress tolerance, a series of experiments was performed at elevated atmospheric CO₂ and elevated daytime temperatures (35 or 40°C compared with a normal growth condition of 30°C) (Fig. 5). We noted that growth at higher temperatures affected stomatal development in rice controls: ‘IR64’ produced leaves with a 31% increase in stomatal density at 35°C and a 40% increase at 40°C. OsEPF1oe plants, however, were unable to adjust stomatal density across temperature treatments, suggesting that this developmental response may require modulation of EPF levels (Fig. 5a). To see whether changes in stomatal density were accompanied by anatomical changes to stomata at high temperature, we also measured guard cell size and stomatal pore area of plants grown at 30 and 40°C (Figs 5b,c, S3). At 30 and 40°C, OsEPF1oeW had similar-sized guard cells to controls, whereas OsEPF1oeS guard cells were significantly smaller (30°C, $P<0.001$ and 40°C $P<0.01$, Fig. S3). For all plants, stomatal pore area was significantly increased at 40°C ($P<0.05$), with OsEPF1oeS plants having significantly larger pore areas than controls at 30 and 40°C (Fig. 5b,c).

![Fig. 5](image-url)
data suggest that OsEPF1oe plants increased stomatal aperture (but not guard cell size) to compensate for reduced stomatal density, with this response being particularly noticeable at 40°C.

To see how OsEPF1oe plants might perform at high temperature with light levels similar to a bright sunny day in the field (Murchie et al., 2002), we conducted steady-state infrared gas exchange analysis on plants grown at the 30, 35 and 40°C with leaf chamber light levels set to 2000 μmol m⁻² s⁻¹ PAR (Fig. 5d–f). At 30°C all OsEPF1oe plants had significantly lower A and g, than ‘IR64’ controls did (P < 0.05; Fig. 5d). However, when OsEPF1oeW plants were grown at 35°C, A and g were both comparable to controls; and when grown at 40°C, neither of the OsEPF1oe lines differed significantly from ‘IR64’ plants in these parameters (Fig. 5d,e). Calculation of intrinsic water use efficiency A/g (iwUE) showed that at 30°C OsEPF1oe plants performed significantly better than ‘IR64’ controls (P < 0.05); at 35 or 40°C this was not the case, and OsEPF1oe iwUE levels were similar to controls (Fig. 5f). The failure of OsEPF1oe plants to maintain improved iwUE at higher temperatures may be explained by the increase in the g, of OsEPF1oe to a level similar to that of control plants (Fig. 5d). Taken together with our finding that OsEPF1 plants had larger apertures when grown at 40°C (Fig. 5b,c), our data suggest that plants with reduced stomatal density can compensate for having fewer stomata by increasing stomatal aperture when assayed under high temperature and high light intensity.

To estimate the physical limitations associated with having a reduced stomatal density we calculated maximum stomatal conductance g,,, using the formula set out in Dow et al. (2014) (Fig. 5g; Table S1). By comparing the calculated potential g,,, with the actual g, values measured at 2000 μmol m⁻² s⁻¹ PAR, it can be seen that at 30 and 40°C the OsEPF1oeS plants are operating at over a third of their maximum capacity whereas controls operate at below 20% capacity. These data suggest that there are clearly opportunities to reduce stomatal density whilst maintaining g, in the hot conditions expected to become more prevalent in the coming decades.

Having measured the performance of the OsEPF1oe reduced stomatal density plants under different drought or heat treatments, we investigated the combined effects of both these abiotic stresses (Fig. 6). Before imposing the drought treatment, we measured temperatures of plants grown at 40°C and observed no differences between OsEPF1oe plants and controls, indicating that reducing stomatal density did not cause overheating under these conditions (Fig. S8). From 28 d post germination we imposed severe drought at either 30°C (for 8 d) or 40°C (for 7 d) during the vegetative growth period. During the water withdrawal period, OsEPF1oeW plants lost water (indicated by a reduction in pot weight) at a similar rate to control plants, whereas OsEPF1oeS plants showed significantly increased water conservation at both temperatures (for 3 d at 30°C, P < 0.05, and 2 d at 40°C, P < 0.001; Fig. 6a,e). As when grown at 30°C, we noticed that OsEPF1oeS plants appeared smaller when grown at 40°C, so we assessed tiller development at 5 wk post germination and found that, although not significantly different (P = 0.057), OsEPF1oeS showed a trend towards reduced tiller number (Fig. S8). Analysis of dark-adapted F₀/Fm chlorophyll fluorescence values (an indicator of abiotic stress, with low values representing reduced photosystem II function) highlighted that both OsEPF1oe lines maintained F₀/Fm levels for at least a day longer than ‘IR64’ controls under drought conditions at 30°C, and the OsEPF1oeS line also at 40°C (Fig. 6b,f). When the plants were rewatered, 100% of OsEPF1oe plants grown at 30°C survived the drought period compared with only 50% of ‘IR64’ control plants (Fig. 6c,d). At 40°C, 50% of OsEPF1oeS plants survived the drought treatment, whereas all other plants died (Fig. 6g,h).

Thus, reducing stomatal density leads to increased survival under severe drought, although at 40°C this was only apparent in OsEPF1oeS plants.

**Discussion**

It is probable that 50% of rice crops already experience drought-associated yield losses (Matsuda et al., 2016). Confronted with human population increases, climate change and water scarcity, there is an urgent need to reduce crop water use whilst maintaining photosynthesis, yield and heat tolerance at higher atmospheric CO₂ concentrations (Ainsworth, 2008; Gago et al., 2014; Jagadish et al., 2015). To simulate future conditions, we conducted experiments at an elevated 450–480 ppm CO₂ concentration. As reported previously in barley (Hughes et al., 2017), overexpression of OsEPF1 in rice led to arrested stomatal development, resulting in reductions in stomatal density, stomatal index and, in some cases, stomatal size. Both in rice and barley, these phenotypic changes at the leaf surface led to increased drought tolerance by restricting water loss, both when water was plentiful and under drought conditions. As rice is typically grown in warm, bright tropical climates, we have further explored how plants with fewer stomata respond to high temperature (including under drought conditions) and at high light intensity to determine whether crops with reduced stomatal density could perform well in warmer, drier climates.

Infrared gas exchange analysis performed on plants with fewer than half the normal density of stomata showed no reductions in A at light intensities below 1000 μmol m⁻² s⁻¹ PAR. Despite increased plant temperatures and decreases in A under some growth conditions (e.g. when well watered at 30°C and 2000 μmol m⁻² s⁻¹ PAR), plants with reduced stomatal density consistently produced grain yields equivalent to, or greater than, ‘IR64’ controls when grown in growth chambers set to 1000 μmol m⁻² s⁻¹ light intensity. Furthermore, OsEPF1oe plants showed lower levels of water use at 30°C, only requiring c. 60% of the water used by controls when consumption was measured between weeks 4 and 5. Owing to their enhanced water conservation, OsEPF1oe plants could maintain transpiration for longer under drought, leading to an extended period of A and cooling relative to controls. Following drought during flowering (treatment 3), the OsEPF1oeW plants produced increased yield relative to both control and OsEPF1oeS plants. This suggests that a moderate reduction in stomatal density (OsEPF1oeW) rather
than a severe reduction (OsEPF1oeS) was more beneficial under these conditions, perhaps because the recovery of large flowering plants after drought was hindered in the plants with the fewest stomata.

Reduced levels of transpiration and associated cooling, as seen in the well-watered OsEPF1oe plants, might be expected to increase plant susceptibility to heat stress, but this is not what we observed. Our experiments growing plants at high temperature
and elevated CO₂ during the vegetative stage gave important insights into how crops with different stomatal density might perform in the future. We discovered that rice plants naturally increase the number of stomata that develop on leaves when grown at higher temperatures. Whilst OsEPF1oe lines did not do this, they were able to adapt effectively by increasing stomatal pore area. When assayed at high temperature and high light conditions this response enabled OsEPF1oe plants to increase gₗ (and A) up to a level equivalent to ‘IR64’ controls.

Somewhat counterintuitively, when combined high growth temperature (40°C) and severe drought stress treatments were applied, half of the OsEPF1oeS plants were able to survive the harsh conditions when all other control and OsEPF1oeW plants died. We propose that the reduced stomatal density of OsEPF1oeS permitted improved water conservation before and during the drought, leading to an extended period of gₗ and enhanced plant survival. Taken together with the results discussed earlier from drought experiments with more mature plants during flowering, this indicates that the optimum stomatal density required to perform well during and after episodes of drought is not always the same. Clearly, the optimization of stomatal characteristics to particular drought and temperature scenarios will require further investigation.

All our findings support the idea that cultivated rice may currently have higher gₗ capacity than is required to maintain yields (Hu et al., 2006). In a future, warmer high-CO₂ world where water availability will decrease, altering stomatal density, size and or pore aperture could provide a solution that maintains yields and conserves water. Our data provide promise for future water-use-efficient rice that is more drought and heat tolerant. However, the effect of reducing stomatal density (and altering stomatal size and pore aperture) on field-grown rice, experiencing other environmental fluctuations, remains untested. Based on our results, we suggest that reducing stomatal density may conserve water and protect, and in some cases even improve, rice yields under future climate conditions. Finally, by combining stomata-related water use efficiency and drought tolerance with other stress-responsive traits, we foresee further advances that could lead to the development of rice increasingly fine-tuned for future warmer, drier, high-CO₂ climates.

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References


Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article:

**Fig. S1** Peptide sequence alignment and functional studies of the rice *OsEPF1 (OSIRI64_00232g011350)* gene.

**Fig. S2** Confocal microscopy imaging of stomata and underlying sub-stomatal cavity formation and vein development in leaf 5 of 21-d-old rice plants.

**Fig. S3** Total guard cell area of IR64 control and *OsEPF1oe* plants grown at 30 and 40°C.
**Fig. S4** Leaf 5 analysis of gas exchange and photochemistry in *OsEPF1oe* plants.

**Fig. S5** Number of insertions and expression profiling in *OsEPF1* overexpressing lines.

**Fig. S6** *OsEPF1oe* plants droughted from 4 wk after germination.

**Fig. S7** *OsEPF1oe* biomass and grain yield.

**Fig. S8** Temperature and growth properties of IR64 control and *OsEPF1oe* plants grown at 40°C.

**Table S1** Values used for the calculation of anatomical $g_{\text{m,ax}}$.

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