

1 The Influences of Stomatal Size and Density on Rice

2 Drought, Salinity and VPD Resilience

3 Robert S. Caine¹, Emily L. Harrison¹, Jennifer M. Sloan¹, Paulina M. Flis², Sina
4 Fischer², Nguyen Trong Phuoc³, Nguyen Thi Lang³, Julie E. Gray¹, Holly L. Croft¹

5
6 ¹ Institute for Sustainable Food, School of Biosciences, University of Sheffield,
7 Sheffield, S10 2TN, UK

8 ² Future Food Beacon of Excellence and the School of Biosciences, University of
9 Nottingham, Nottingham, NG7 2RD, UK

10 ³ High Agricultural Technology Research Institute, Can Tho City, Vietnam.

11
12 Authors for correspondence:

13 Dr Robert S. Caine

14 Tel : +44 (0)114 222 0079

15 Email: b.caine@sheffield.ac.uk

16
17 Prof. Julie E. Gray

18 Tel : +44 (0)114 222 4407

19 Email : j.e.gray@sheffield.ac.uk

20
21 Dr. Holly L. Croft

22 Tel : +44 (0)114 222 2000

23 Email: h.croft@sheffield.ac.uk

24

Total word count	6488	No. of Figures	6
Summary:	200	No. of Tables	0
Introduction:	1053	No of Supporting information files	4 (Fig. S1-S3, Table S1)
Materials and Methods:	929		
Results:	2629		
Discussion:	1808		
Acknowledgements:	69		

26 **Summary**

- 27 • A warming climate coupled with reductions in water availability and rising salinity are
28 increasingly affecting rice yields (*Oryza sativa* L.). Elevated temperatures are causing
29 vapour pressure deficit (VPD) rises, leading to stomata closure, further reducing plant
30 productivity and cooling. It is unclear which conformation of stomatal size (SS) and
31 stomatal density (SD) will best suit these future environmental extremes.
- 32 • To understand the influence of stomatal characteristics on rice abiotic stress tolerance,
33 we screened the stomatal characteristics of 72 traditionally-bred varieties. We found
34 significant variation in SS, SD and maximal stomatal conductance (g_{smax}) but did not
35 identify any varieties with SD and g_{smax} as low as the genetically manipulated stomatal
36 development mutant *OsEPF1oe*.
- 37 • Traditionally-bred varieties with high SD and small SS (resulting in high g_{smax}) typically
38 had lower biomasses, and these plants were more resilient to drought than low SD and
39 large SS plants, which were physically larger. None of the varieties tested were as
40 resilient to drought or salinity as low SD *OsEPF1oe* mutants. High SD and small SS
41 rice displayed faster stomatal closure during rising VPD, but photosynthesis and plant
42 cooling were reduced.
- 43 • Compromises will be required when choosing rice SS and SD to tackle multiple future
44 environmental stresses.

45

46 Keywords: stomata, rice, drought, salinity, temperature, VPD, climate change, water

47

48

49

50 Introduction

51 Developing high yielding rice varieties that use less water which can withstand multiple
52 abiotic stresses will be critical for maintaining future global food security (Singh *et al.*,
53 2021). Currently, rice is the most consumed human food crop, providing more than 20%
54 of the total calories consumed worldwide (Fukagawa & Ziska, 2019). It takes around
55 2500 litres of water to produce 1kg of grain, and globally, this equates to around 30% of
56 all the world's fresh water supplies being used for rice production (Bouman, 2009). Half
57 of all cultivation is irrigated, and this boosts crop yields and protects against drought and
58 heat stress, but such practices are highly water-intensive often leading to anaerobic soils
59 and excessive methane production (Singh *et al.*, 2021). The majority of other farmed rice
60 is rain fed, with 34% grown in lowland ecosystems, and 9% in upland environments. As
61 the human population continues to grow, and as climate change intensifies, clean water
62 for irrigation will decrease, and this combined with a need to reduce emissions will
63 prompt a move towards more rain fed rice agriculture (Bouman, 2009; Singh *et al.*, 2021).
64 Such changes in farming practices are forecast to occur at the same time as increased
65 incidences of severe droughts, higher temperatures and rising soil salinity (Livsey *et al.*,
66 2019; IPCC, In press.). Taken together these factors have the potential to massively
67 impact global rice yields exactly when demand for rice (and clean water) is rapidly
68 growing (Panda *et al.*, 2021; Singh *et al.*, 2021).

69

70 Plant water-use is controlled by stomata – microscopic epidermal pores comprised of a
71 pair of guard cells that regulate CO₂ uptake for plant photosynthesis (A), with the
72 concurrent release of water via alterations to stomatal conductance (g_{sw}). As well as
73 regulating gaseous exchanges, stomata facilitate water and nutrient uptake from soils and
74 also aid in plant cooling via increases to the transpiration (E) stream when temperatures
75 rise. Under drought, salinity or increasing vapour-pressure deficit (VPD), stomatal
76 apertures reduce or close, and this restricts photosynthesis, water loss, nutrient uptake,
77 plant cooling and ultimately growth and seed yield (Merilo *et al.*, 2018; Grossiord *et al.*,
78 2020; Ma *et al.*, 2020). Rises in VPD occur when the difference between the maximum
79 amount of water the air can hold, and the actual amount of water in the air increases. This
80 is often the case as temperatures rise (Grossiord *et al.*, 2020), with forecasts predicting
81 VPD will continue to rise until the end of the century (Yuan *et al.*, 2019). Such rises in
82 VPD have thus far offset any CO₂ fertilisation effects associated with rising CO₂
83 concentration.

84 With prolonged changes in environmental stimuli, many plant species are able to
85 modulate their stomatal development by altering stomatal size (SS) and/or stomatal
86 density (SD) – often in opposite directions (Franks & Beerling, 2009; Franks *et al.*, 2012).
87 This developmental adjustment has been widely observed in living plants, herbarium
88 samples and even fossil records, and coincides with CO₂ fluctuations that have occurred
89 during different geological epochs. Typically, high CO₂ environments are associated with
90 an increased SS and reduced SD, and low CO₂ concentrations are associated with the
91 opposite conformation of SS and SD (Franks & Beerling, 2009). Such developmental
92 adjustments have been suggested to alter g_{sw} range by permitting a higher calculated
93 anatomical maximum stomatal conductance (g_{smax}); with plants that have a high SD and
94 small SS being able to potentially achieve high operating g_{sw} and g_{smax} levels (Franks &
95 Beerling, 2009; Bertolino *et al.*, 2019). Alterations to SS and SD have also been suggested
96 to adjust the speed at which stomata respond to environmental cues; with evidence
97 suggesting that plants with smaller SS and higher SD are more rapidly able to respond to
98 environmental conditions, and this can boost water-use efficiency (McAusland *et al.*,
99 2016; Bertolino *et al.*, 2019). For fluctuating light responses, this means faster
100 responsivity of g_{sw} to changes in A , which reduces unnecessary water loss (Lawson &
101 Vialet-Chabrand, 2019). Examples of negative correlations between SS and speed have
102 been observed within a species (or closely related species) but not necessarily between
103 distantly related species (McAusland *et al.*, 2016). However, other studies have shown
104 that stomatal responsiveness is not always related to SS (Eyland *et al.*, 2021), and the
105 improved benefits of small SS on optimising A may be light dependent (Zhang *et al.*,
106 2019). In monocot grasses such as rice, each stomatal guard cell pair is surrounded by a
107 pair of subsidiary cells, which increase the speed of stomatal opening and closure (Franks
108 & Farquhar, 2007; Raissig *et al.*, 2017; Gray *et al.*, 2020).

109

110 It is possible to improve various abiotic stress responses by genetically manipulating
111 stomatal physiology or stomatal development (Huang *et al.*, 2009; Mohammed *et al.*,
112 2019). Previous work manipulating the levels of Epidermal Patterning Factor (EPF)
113 signalling peptides in several species, has shown that SD reductions of up to
114 approximately 50% can lead to significantly lower g_{sw} without significantly impacting A
115 (Hepworth *et al.*, 2015; Caine *et al.*, 2019; Dunn *et al.*, 2019; Mohammed *et al.*, 2019).
116 These larger reductions in g_{sw} compared to A led to improved intrinsic water-use
117 efficiency (iWUE) without negatively impacting seed yield. In fact, moderate reductions

118 in SD, improved rice yields following drought imposition during the flowering stage
119 (Caine *et al.*, 2019). Surprisingly, EPF-driven reductions in SD had inconsistent effects
120 on SS between different rice varieties despite sampling at the same development stage
121 (Caine *et al.*, 2019; Mohammed *et al.*, 2019). Overexpression of the *OsEPF1* gene in
122 transgenic IR-64 plants previously displayed smaller stomata with lower SD (Caine *et al.*,
123 2019), whereas *OsEPF1oe* Nipponbare plants had larger stomata (Mohammed *et al.*,
124 2019).

125

126 It is now clear that reductions in SD can result in reduced crop water loss and increased
127 drought tolerance (Caine *et al.*, 2019; Mohammed *et al.*, 2019). In this study, we
128 investigate whether selecting for specific SD and SS traits could mitigate against not only
129 drought, but also additional climate change associated abiotic stresses including rising
130 salinity and VPD. Specifically, we ask: 1) Is it possible to identify the combination of SS
131 and SD found in IR-64 *OsEPF1oe* (*OsEPF1oe*) in other traditionally-bred high yielding
132 rice varieties? 2) Do *OsEPF1oe* or other plants with fewer (or smaller) stomata perform
133 better under drought or saline stress conditions? And 3) does SD and/or SS affect stomatal
134 responses to high temperature and increased VPD.

135

136 **Material and Methods**

137 Plant Materials

138 A collection of 72 rice varieties previously assayed for salinity tolerance were kindly
139 provided by Jose De Vega, Earlham Institute (Table. S1). Two independently transformed
140 lines of IR-64 variety overexpressing *OsEPF1* have been previously described (Caine *et*
141 *al.*, 2019).

142

143 Plant growth conditions

144 Rice seeds placed in 15-20 ml RO water were sealed in Petri dishes with micropore tape
145 (3M, Saint Paul, Minnesota) and germinated under: 12 h 26°C : 12 h 24°C light:dark
146 cycle at photosynthetically active radiation (PAR) 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Seedlings were sown
147 onto a previously described soil mix (Caine *et al.*, 2019), in 0.8 L pots (IPP, Bytom,
148 Poland). Pots were prepared by first half filling with soil mix, then RO water was mixed
149 through, and then a second equal application of soil was added, and further RO water
150 mixed through to saturate the soil. When drained the soil level was c. 1.5 cm from the pot

151 apex. For preparation of salt-treated pots in Fig. 4, a 20 mM solution of NaCl rather than
152 RO water was applied to saturate the soil during mixing. Seedlings were grown in
153 Conviron growth cabinets (Controlled Environments Ltd, Winnipeg, MB, Canada) to 12
154 h 30°C : 12 h 24°C light : dark cycle, RH 60% with CO₂ concentration between 450-480
155 ppm. For plants in Fig. 4 and Fig. S1, PAR was set at canopy level to 1000 μmol m⁻² s⁻¹.
156 For all other experiments, PAR was 1500 μmol m⁻² s⁻¹. For salt-treated plants in Fig. 5,
157 all samples were initially transplanted into fresh-water-mixed soil, with 50 mM NaCl first
158 applied 8 days after transferring (16 DPG). A constant supply of RO water or salt water
159 was available during experiments.

160

161 Drought and salinity experiments

162 For droughted plants (n = 10 or 11), water was withheld for 5 days from 30-35 days post
163 germination (DPG). New leaves were classified as visible new growth emerging from
164 sheaves of pre-existing tillers at 42 DPG. For salinity experiments, salt water was applied
165 when required, and trays changed weekly for 20 mM NaCl experiments (Fig. 4) (n = 6),
166 or two-weekly for the 50 mM NaCl experiment (n = 7 to 9, Fig.5). For both experiments,
167 salt water or fresh water (for controls), was applied from above every time salt trays were
168 changed. Leaf blade and tiller base (2 cm from soil) Φ PSII measurements were collected
169 using a FluorPen FP 110 (PSI, Drasov, Czech Republic). Leaf discs were collected from
170 35 day-old fresh water and salt grown plants and dried in a 40°C oven for 3 days prior to
171 analysis.

172

173 Gas exchange and thermal imaging measurements

174 Measurements of g_{sw} shown in Fig.1 and 2 were collected using a LI-600 porometer (LI-
175 COR, Lincoln, USA) set to a flow rate of 150 μmol s⁻¹ (n = 7). Thermal images in Fig.1
176 were captured using a FLIR T650sc (Wilsonville, USA). Steady state and dynamic gas
177 exchange experiments were performed using LI-6800 Portable Photosynthesis Systems
178 (LI-COR, Lincoln, USA) and attached MultiphaseFlash Fluorometer (6800-01A). Fully
179 expanded leaves of 19-25 day old plants (leaf 5 or 6) were used to collect steady-state
180 measurements in Fig. 4. Leaf chamber conditions were set to light intensity 2000 μmol
181 m⁻² s⁻¹ PAR, relative humidity (RH) 60%, Tair 30°C, flow 300 μmol s⁻¹ and [CO₂]_{ref} 480
182 ppm. Over a 5-minute period, 10 readings were taken then averaged (n = 6 plants). For
183 VPD gas exchange experiments in Fig. 6, measurements were conducted on fully
184 expanded leaf 5 of 19-23 DPG plants, with the leaf chamber set to 2200 μmol m⁻² s⁻¹

185 PAR, 55% RH, Tair 32°C, flow 400 $\mu\text{mol s}^{-1}$ and 450 ppm $[\text{CO}_2]_{\text{ref}}$. For fluctuations in
186 RH during this experiment see Fig. S3. Once steady-state was reached, two readings were
187 collected at the end of 6-minute intervals, then the temperature was increased by 2.5°C in
188 4x 6 minute intervals, with readings taken immediately prior to each subsequent
189 temperature increase. Four further readings were recorded at 42 °C ($n = 7$ or 8 plants).
190 Leaf chamber matching was conducted prior to each reading. For steady-state VPD
191 experiments in Fig. S3, chamber settings were the same as in Fig. 6, except Tair was 39°C
192 and RH maintained at 55% throughout ($n = 5$ or 6).

193

194 Analysis and quantification of stomatal traits

195 Epidermal imaging and quantification was conducted on IMAGEJ using nail varnish
196 impressions of dental resin imprints taken from leaf 5 (19-23 DPG), (Fig. 4, 6 and Fig.
197 S1 and 3) or leaf 8 (28-33 DPG), (Fig1). Two 0.44 mm^2 fields of view (FOV) per replicate
198 were used to calculate SD with 5 stomata per biological replicate used to assess guard cell
199 length. Calculations for g_{max} were performed as in Caine *et al.* (2019) based on double
200 end-corrected version of the Franks & Farquhar (2001) equation from Dow *et al.* (2014).
201 Graphs and statistical analysis were produced using R software.

202

203 Inductively Coupled Plasma-Mass Spectrometry

204 Sample preparation was as described previously (Danku *et al.*, 2013). In brief, dried plant
205 material was digested with 1ml concentrated nitric acid (trace metal grade, Fisher
206 Chemicals) spiked with Indium (internal standard) in dry block heaters (SCP Science;
207 QMX Laboratories) at 115°C for 4h. The samples were then diluted to 10ml with Milli-
208 Q Direct water (18.2 $\text{M}\Omega\text{cm}$, Merck Millipore) and analysed using ICP-MS (PerkinElmer
209 NexION 2000) in the collision mode (He). Reference material (pooled samples) was run
210 to correct for variation within ICP-MS analysis run. Calibration standards were prepared
211 from single element standards solutions (Inorganic Ventures, Essex Scientific Laboratory
212 Supplies Ltd, Essex, UK). The final element concentrations were obtained by normalizing
213 concentrations to sample dry weight.

214

215

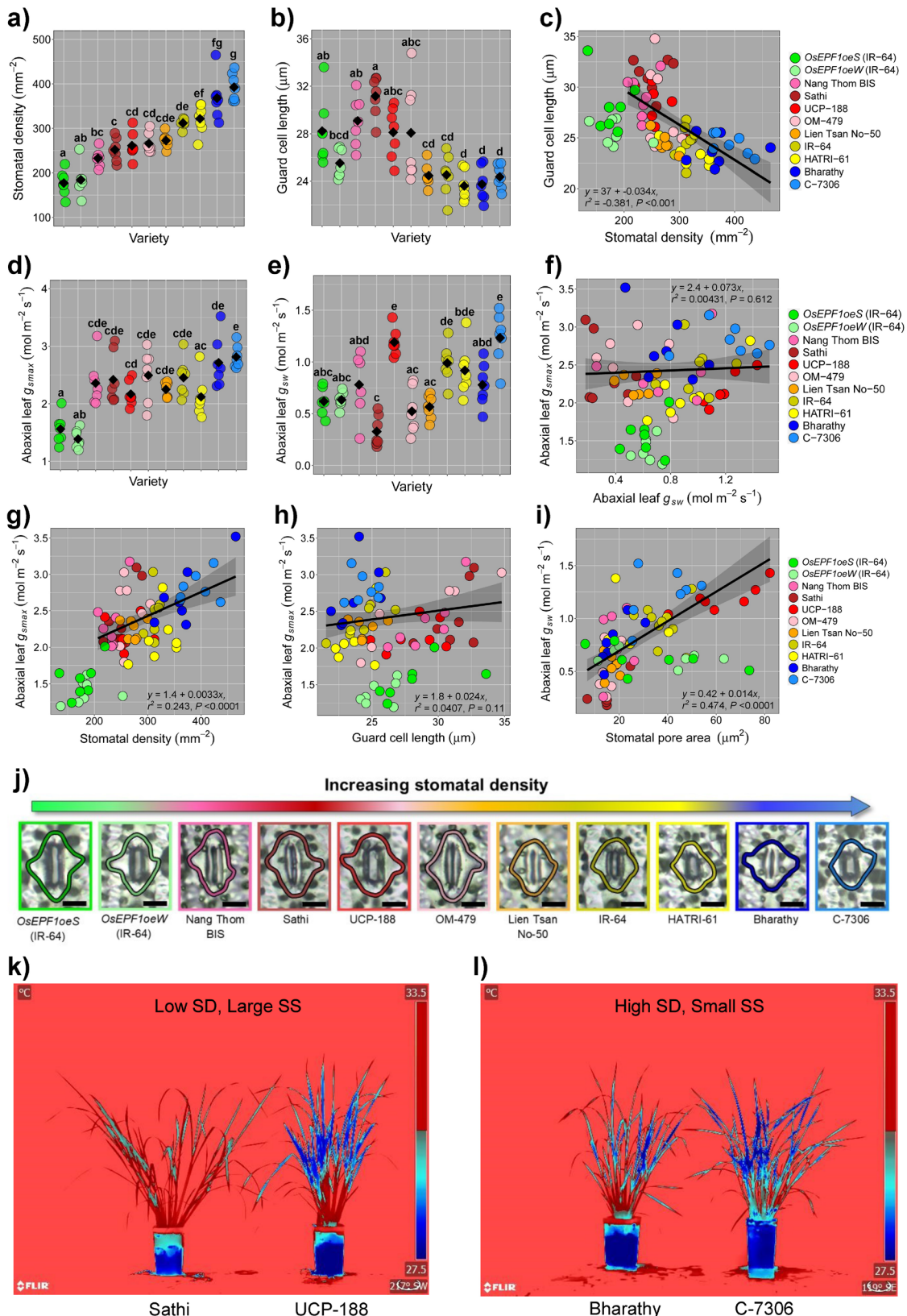
216

217 **Results**

218 **Rice stomatal size and density are negatively correlated and contribute to operating**
219 **and maximum potential stomatal conductance**

220 By overexpressing an Epidermal Patterning Factor, we previously showed that reductions
221 in SD reduces rice water requirements and thus improves iWUE and drought tolerance
222 (Caine *et al.*, 2019; Mohammed *et al.*, 2019). Here we carried out a screen of 72
223 traditionally-bred rice varieties alongside two independently transformed *OsEPF1oe*
224 lines to survey and compare stomatal traits between genetically engineered plants and a
225 non-transgenic population (**Fig. S1, Table. S1**). Although SD differed widely across the
226 collection, we were unable identify a non-transgenic variety with a mean SD as low as
227 either of the IR-64 *OsEPF1oe* rice lines. We did, however, observe a negative correlation
228 between SS and SD within the full collection of traditionally-bred varieties ($r^2 = -0.17$, P
229 < 0.0001 , **Fig. S1**), with lower SD varieties typically having larger SS (with the exception
230 of *OsEPF1oe* lines), and higher SD varieties typically having smaller SS. To study how
231 these differences in stomatal traits might affect abiotic stress responses to increased
232 drought, salinity and VPD, we selected nine varieties spanning the range of SS and SD,
233 and conducted a series of experiments alongside *OsEPF1oe* plants. We began by
234 measuring and comparing stomatal morphology, gas exchange and pore size on the leaves
235 of tillering rice plants (**Fig. 1**).

236



237

238 **Fig. 1.** Stomatal size, density and pore aperture contributions to rice gaseous exchange
 239 on plants grown in high light conditions (1500 PAR). (a) Abaxial stomatal density (SD)
 240 plotted low to high of nine selected rice varieties and two transgenic *OsEPF1oe* plants.
 241 (b) Corresponding stomatal size (SS) (guard cell length) measurements of plants in (a).

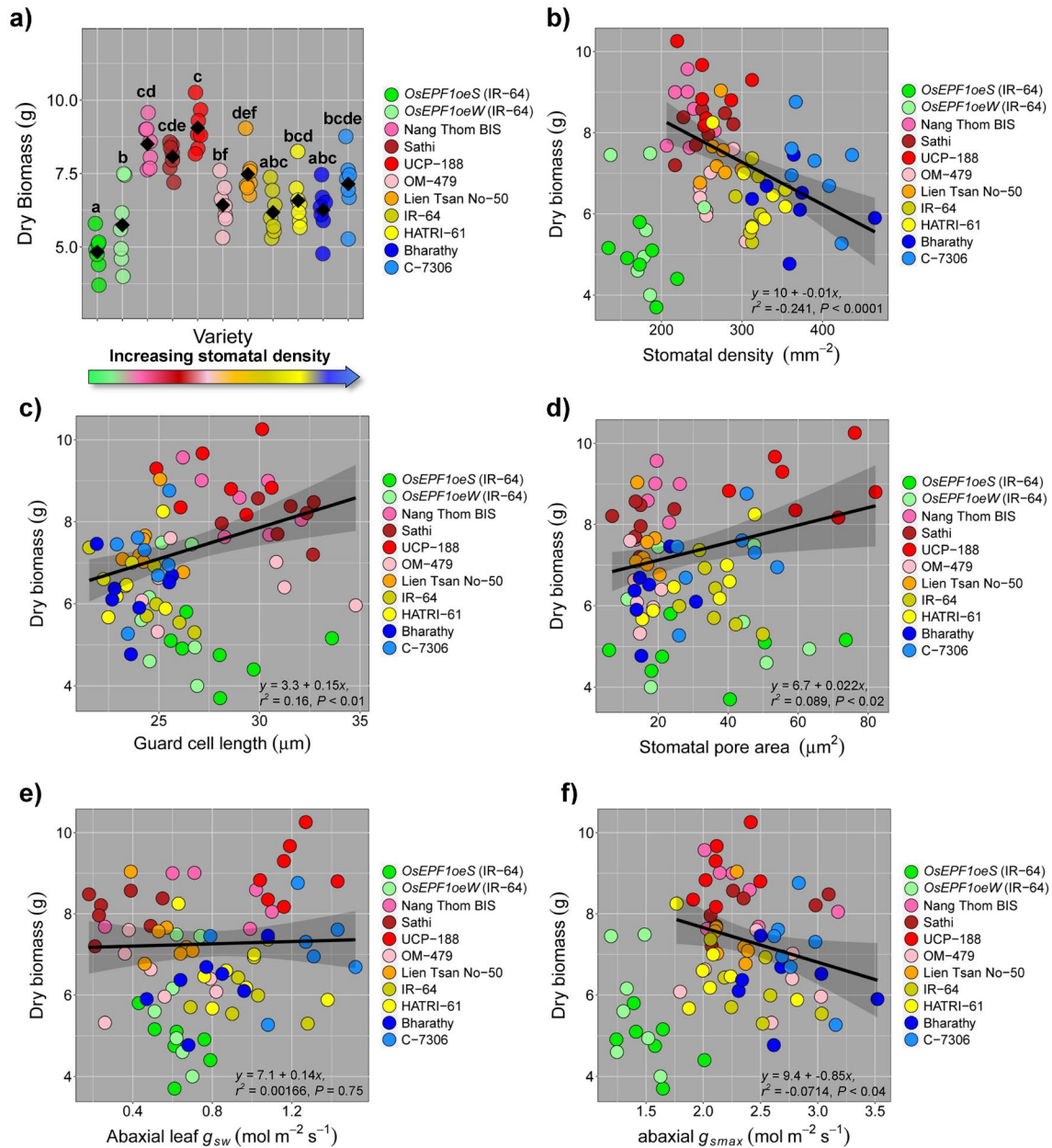
242 (c) Regression analysis highlighting relationship between SS and SD. (d) Calculated
243 maximal stomatal conductance (g_{smax}), and (e) corresponding operating stomatal
244 conductance (g_{sw}) of abaxial leaf surface. (f-i) Regression analysis of (f) abaxial g_{smax} and
245 operating g_{sw} , (g) abaxial g_{smax} and SD, (h) abaxial g_{smax} and SS and (i) abaxial g_{sw} and
246 stomatal pore area. (j) Examples of stomatal openness (bars, 10 μ m). (k and l) Thermal
247 images illustrating temperature differences in plant temperature driven by changes in pore
248 apertures between (k) plants with low SD and large SS and (l) high SD and small SS.
249 Different letters on graphs indicate a significant difference between the means (One-way
250 ANOVA, Tukey HSD test, $P < 0.05$). Black diamonds represent means. Regression
251 analysis and trend lines are based on linear models. *OsEPF1oe* plants are excluded from
252 regression analyses. (a-i) $n = 7$ plants.

253 Assessment of SD on leaf 8 of tillering rice revealed traditionally-bred varieties had mean
254 SD values ranging from 233-393 stomata per mm² whereas the two independent
255 *OsEPF1oe* lines had c. 180 stomata per mm² (**Fig. 1a**). As expected, a negative
256 correlation between SS and SD was observed between varieties, with those with higher
257 SD typically having smaller guard cells ($r^2 = -0.38$; $P < 0.0001$, **Fig. 1b** and **c**). *OsEPF1oe*
258 lines, which had the lowest SD, did not follow the same trend line, but surprisingly, by
259 the leaf 8 stage (28-33 DPG), stomata were either equal to (*OsEPF1oeW*) or significantly
260 larger (*OsEPF1oeIS*) than IR-64 control plants. In the case of *OsEPF1oeS*, this result was
261 opposite to what we had previously found in IR-64 plants at the leaf 5 stage (Caine *et al.*,
262 2019). Despite having larger than expected stomata, very low SD led to *OsEPF1oe* plants
263 having the lowest g_{smax} (**Fig. 1d**). Within the nine varieties selected, there were limited
264 differences between calculated g_{smax} , but notably, the varieties with the two highest SD
265 also had the highest mean g_{smax} values.

266 To investigate if operating g_{sw} followed a similar trend to g_{smax} , we measured leaf g_{sw}
267 using a porometer (**Fig. 1e**). Whilst we found no overall correlation between operating
268 g_{sw} and calculated g_{smax} across varieties (**Fig. 1f**), we did detect positive relationships
269 between SD and g_{smax} ($r^2 = 0.24$; $P < 0.0001$, **Fig. 1g**) and between SD and operating g_{sw}
270 ($r^2 = 0.13$; $P < 0.01$, **Fig. S2a**). There was no significant correlation between SS and g_{smax}
271 ($r^2 = 0.04$; $P = 0.11$, **Fig. 1h**), but we did identify a weak negative relationship between
272 SS and g_{sw} ($r^2 = 0.09$; $P < 0.02$, **Fig. S2b**). Despite very low calculated g_{smax} , both
273 *OsEPF1oe* lines were able to maintain a similar operating g_{sw} comparatively to some of
274 the other selected varieties even though SD was around 30% lower (**Fig. 1a, d, e**), and

275 whilst low SD typically meant low operating g_{sw} , UCP-188 bucked this trend having the
276 equal highest g_{sw} (**Fig. 1a, e**). Our results suggested that factors other than SS and SD
277 drove the observed differences in operating g_{sw} , so we next assessed stomatal pore area.
278 Overall we observed a robust correlation ($r^2 = 0.47$; $P < 0.0001$) between gas exchange
279 and the extent of stomatal opening across the nine selected varieties (**Fig. 1i, j**). Rates of
280 stomatal water loss were further explored by assessing whole-plant surface temperatures
281 as a proxy for evaporative transportation – with lower temperatures indicative of higher
282 water loss. Thermal imaging confirmed firstly that the open-pored UCP-188 variety with
283 large stomata was cooler than the Sathi variety with equivalent SS and SD, and secondly,
284 that the open-pored C-7306 variety, with high SD was cooler than the Bharathy variety
285 which again had similar conformations of SS and SD. These observed similarities
286 between surface temperatures and leaf g_{sw} values, indicate that in addition to SS and SD,
287 stomatal pore aperture (and perhaps other root and vascular features) can differ
288 substantially between rice varieties and this also has the potential to greatly influence
289 water loss (**Fig. 1i-l**).

290 To investigate if the differences in stomatal morphology and physiology (**Fig. 1**) were
291 associated with overall plant growth, we next measured vegetative-stage above ground
292 biomass at 35 DPG (**Fig. 2**). Within the nine selected varieties, we found that low SD
293 varieties typically had greater biomass than plants with higher SD (**Fig. 2a**). The
294 exceptions to this were the *OsEPF1oe* transgenic plants. With *OsEPF1oe* transgenic lines
295 excluded from regression analyses, a moderate negative correlation was observed
296 between SD and plant biomass ($r^2 = -0.24$; $P < 0.001$), and a weaker positive relationship
297 was detected between SS and biomass ($r^2 = 0.16$; $P < 0.01$) (**Fig. 2b-c**). We also found
298 that pore area was weakly associated with greater plant biomass ($r^2 = 0.09$; $P < 0.02$) (**Fig.**
299 **2d**), but no such relationship was detected for operating g_{sw} (**Fig. 2e**). Assessment of g_{smax}
300 values revealed a weak negative correlation with plant biomass ($r^2 = -0.07$; $P < 0.04$) (**Fig.**
301 **2f**), with smaller plants typically having marginally higher g_{smax} values, although this was
302 not the case for *OsEPF1oe* plants.



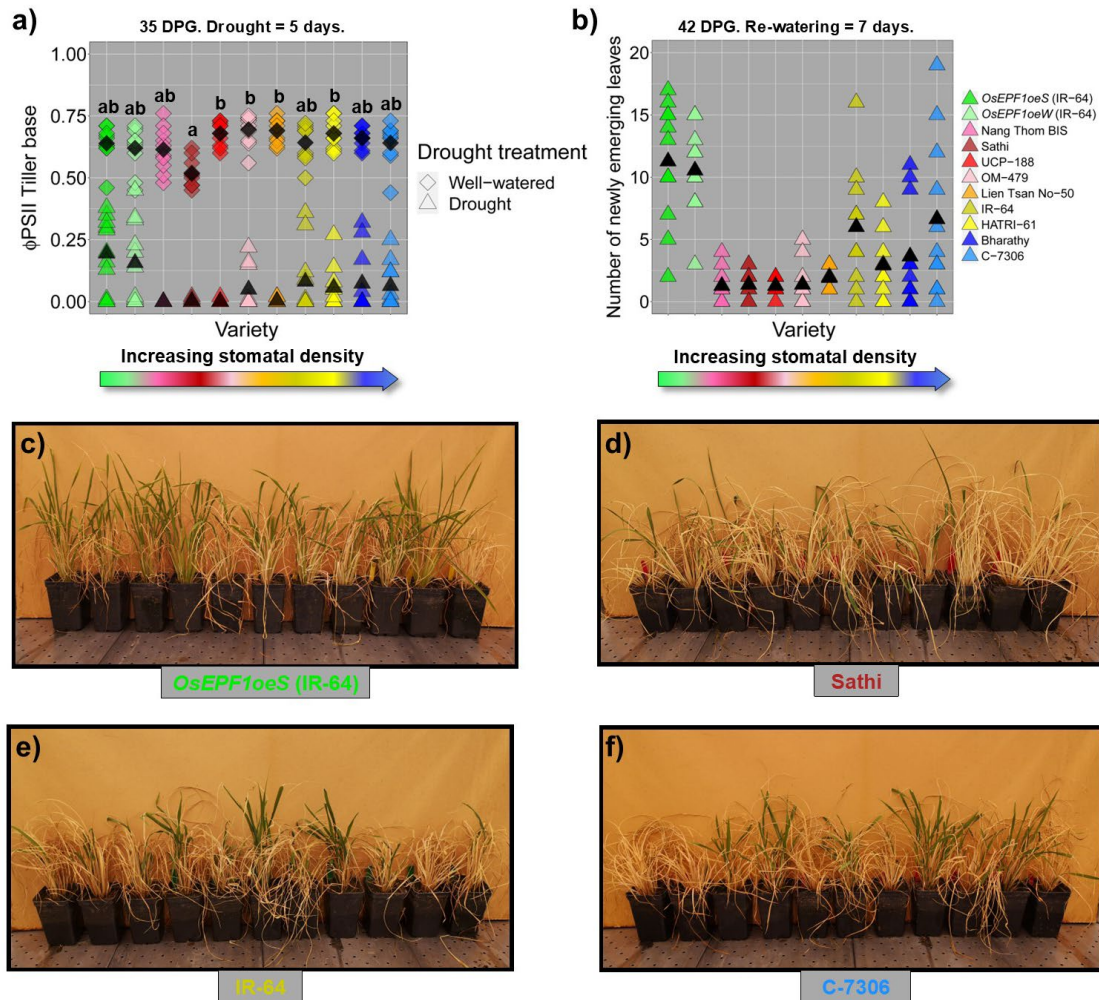
303

304 **Fig. 2.** Stomatal size, density, pore area and anatomical g_{smax} correlate with whole plant
 305 biomass during vegetative tillering. (a) Dry plant biomass. (b-f) Regression analysis
 306 conducted between biomass and (b) stomatal density, (c) stomatal size (guard cell length),
 307 (d) stomatal pore area, (e) leaf stomatal conductance (g_{sw}), and (f) g_{smax} . Different letters
 308 indicate a significant difference between the means (One-way ANOVA, Tukey HSD test,
 309 $P < 0.05$). Black diamonds represent means. Regression analysis and trend lines are based
 310 on linear models. *OsEPF1oe* plants are excluded from regression analyses. (a-f) $n = 7$
 311 plants.

312

313 The association of stomatal traits on rice resilience to drought stress

314 To understand how our nine selected varieties might compare to the *OsEPF1oe* plants
315 when exposed to abiotic stress, we first imposed a drought for five days from 30 DPG
316 (Fig. 3). Chlorophyll fluorescence measurements were taken to assess the efficiency of
317 Photosystem II (Φ PSII) using a drop in the Φ PSII value to indicate plant stress (Caine *et*
318 *al.*, 2019).



319
320 **Fig. 3.** Varieties with higher stomatal density (SD) and smaller stomatal size (SS) respond
321 better to drought than those with lower SD and large SS. (a) Drought responses assessed
322 at the tiller base using Φ PSII to measure plant health. (b) Number of new regenerative
323 leaves one week after re-watering. (c-f) Examples of varieties with (c) very low SD
324 (*OsEPF1oeS*), (d) low SD and large SS (Sathi), (e) medium SD and small SS (IR-64) and,
325 (f) high SD and small SS (C-7306) recovering from drought at 42 days, one week after
326 re-watering. Different letters indicate a significant difference between the means (One-

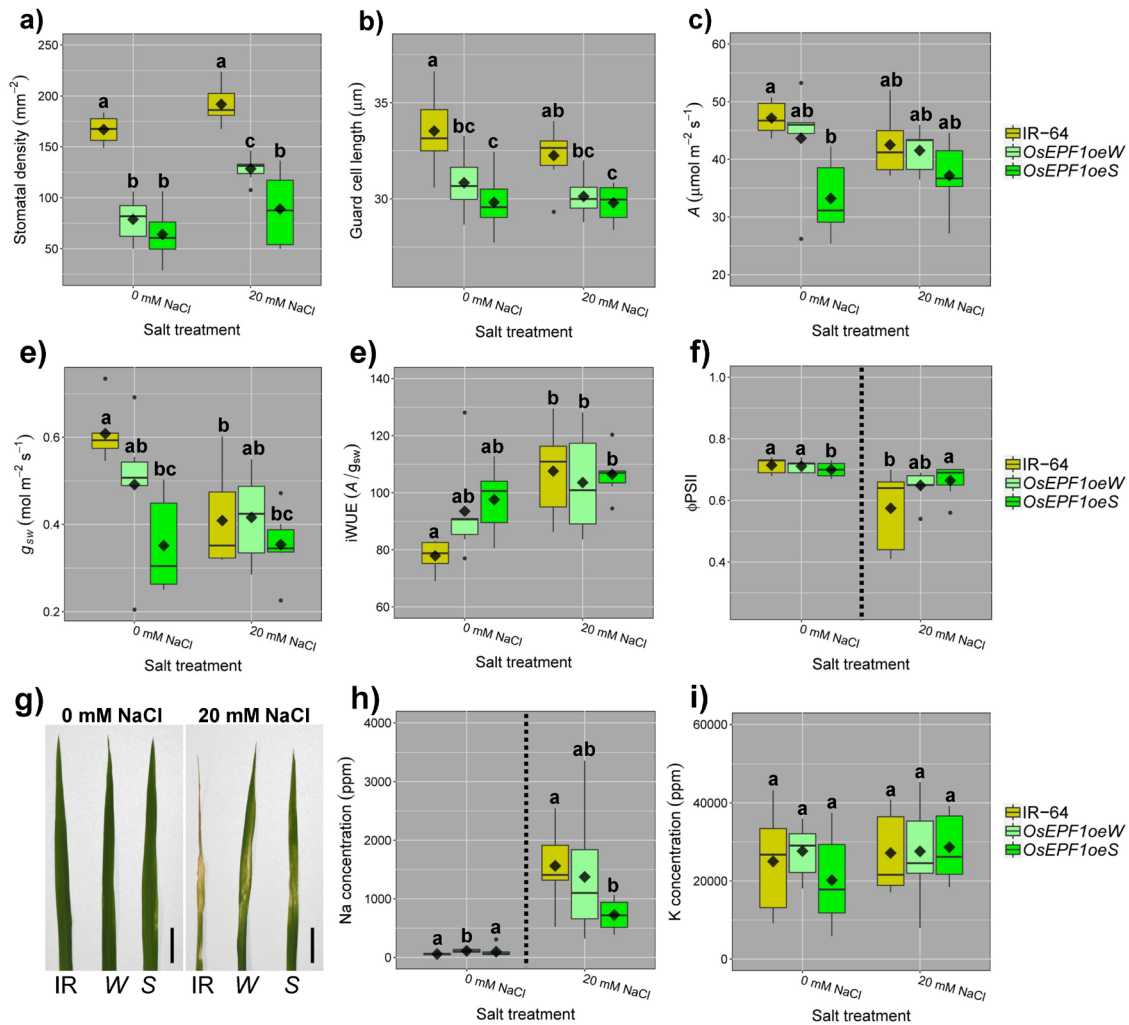
327 way ANOVA, Tukey HSD test, $P < 0.05$). Black diamonds represent means for well-
328 watered plants, and triangles represent means for droughted plants. (a-b) $n = 10-11$ plants.

329 Under well-watered conditions, the *OsEPF1oe* lines and most of the selected varieties
330 shared similar Φ PSII values at the tiller base, with only Sathi displaying slightly (but
331 significantly) lower values than 4 of the other varieties. Following five days of severe
332 drought treatment the Φ PSII values of all traditionally-bred varieties and *OsEPF1oe* lines
333 were dramatically lower than in well-watered controls. Varieties previously identified as
334 having low SD (mostly with large stomata: Nang Thom BIS, Sathi, UCP-188 and OM-
335 479) did not perform well, with most exhibiting Φ PSII values of 0 at the end of the
336 drought (36 out of 44 plants), suggesting that the tillers of these plants were severely
337 stressed (**Fig. 3a**). Plants previously identified to have medium to high SDs (typically
338 with smaller SS: IR-64, HATRI-61, Bharathy and C-7306) performed better, with just
339 over half of plants recording Φ PSII values of 0 (25 out of 44). Although there was
340 variability between individual plants, the *OsEPF1oe* lines fared best and maintained the
341 highest mean Φ PSII values (only 4 out of 22 plants displayed a Φ PSII value of 0).

342 All plants were then resupplied with water and new leaf growth was assessed after 7 days
343 (**Fig. 3b-f**). This revealed that the 35 DAG Φ PSII measurements were a good indicator
344 of plants drought resilience. Plants with low densities of large stomata recovered most
345 slowly and had the fewest new leaves (averaging 1-2 new leaves). Medium and high SD
346 varieties recovered better (averaging 3-7 new leaves) and the transgenic *OsEPF1oe* plants
347 had the highest drought tolerance (averaging 10-11 new leaves per plant).

348 ***OsEPF1oe* lines have enhanced salt tolerance**

349 To understand if stomatal traits can influence how plants perform under saline conditions,
350 we compared the performance of the *OsEPF1oe* to IR-64 control plants grown in 20 mM
351 NaCl.



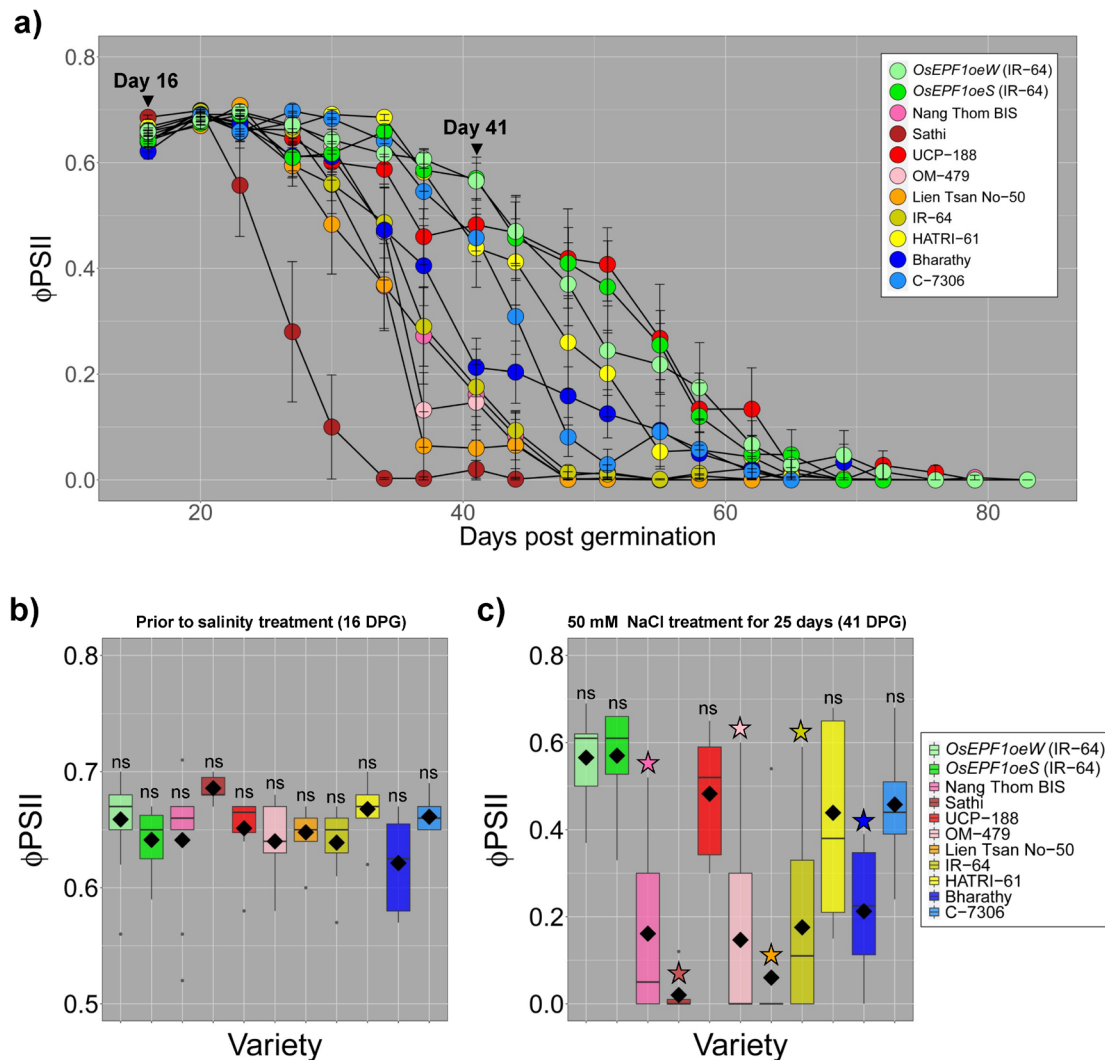
352

353 **Fig. 4.** *OsEPF1oe* plants with reduced stomatal density (SD) display increased salinity
 354 tolerance during seedling and tillering stages. (a) 19-21 DPG Leaf 5 SD and (b) Stomatal
 355 size (guard cell length) of fresh water and salt grown IR-64 and *OsEPF1oe* plants. (c-f)
 356 Fresh water and salt grown rice plant gas exchange measurements of (c) Assimilation (A),
 357 (d) stomatal conductance (g_{sw}) and (e) intrinsic water-use efficiency (iWUE). (f) Φ PSII
 358 leaf measurements of apical leaves at 28 DPG with (g) representative leaf images from
 359 fresh water and salinity treated plants (bars, 2 cm). (h) Sodium (Na), and (i) Potassium
 360 (K) concentrations in auxiliary leaves of 35 DPG tillering plants. Whiskers indicate the
 361 ranges of the minimum and maximum values and different letters indicate a significant
 362 difference between the means (Two-way ANOVA, Tukey HSD test, $P < 0.05$). For (f)
 363 and (h), 2 separate Kruskal-Wallis one-way ANOVAs were performed due to unequal
 364 variances ($P < 0.05$). Black diamonds represent means and black dots are outliers. $n = 6$ -
 365 7 plants.

366 Growth under saline conditions resulted in an increase in mean SD (19-21 DPG), with
367 salt-treated *OsEPF1oeW* seedlings having significantly more stomata mm^{-2} than
368 equivalent fresh-water grown plants when leaf 5 seedlings were assessed (**Fig. 4a**). SS
369 was unaffected by salt treatment, although *OsEPF1oeS* plants had the smallest SS when
370 grown in fresh water or 20 mM NaCl (**Fig. 4b**) (this is more similar to SS trends
371 previously reported for seedling leaves by Caine et al (2019)., than that reported for leaf
372 8 of tillering plants in **Fig. 1b**). Gas exchange analysis showed that *OsEPF1oeS* (but not
373 *OsEPF1oeW*) had significantly reduced A and g_{sw} relative to IR-64 when grown in fresh-
374 water (**Fig. 4c** and **d**). Under saline conditions, IR-64 g_{sw} was greatly reduced, and the A
375 and g_{sw} rates were no longer significantly higher than either *OsEPF1oe* line (**Fig. 4c** and
376 **d**). However, the (already low) g_{sw} of *OsEPF1oe* plants remained relatively similar
377 between fresh water controls and salt-treated equivalents. The reduced A and g_{sw} of salt-
378 treated IR-64 plants resulted in an increased intrinsic water-use efficiency (iWUE),
379 whereas for *OsEPF1oe* plants, iWUE did not significantly increase under saline
380 conditions (**Fig. 4e**).

381 Plants were left to develop further and at 28 DPG the continuing impact of salt uptake
382 was investigated using ΦPSII values as a proxy for plant health (**Fig. 4f**). Under normal
383 fresh water conditions the ΦPSII of *OsEPF1oeS* (but not *OsEPF1oeW*) leaves were was
384 significantly lower than IR-64. Salinity treatment had a more severe impact on IR-64 than
385 the *OsEPF1oe* lines and salt-treated *OsEPF1oeS* had higher ΦPSII values than IR-64,
386 with visibly healthier leaves (**Fig. 4f-g**). At 35 DPG the concentration of accumulated salt
387 in auxiliary leaves was measured. The plants with the lowest SD accumulated
388 significantly less salt in their leaves, with *OsEPF1oeS* salt-grown plants having c. 50%
389 lower Na than IR-64 equivalents (**Fig. 4h**). In comparison, we did not detect any
390 differences in K levels across genotypes or treatments (**Fig. 4i**).

391 Next, we investigated how the nine selected traditionally-bred varieties and two
392 transgenic lines performed under exposure to 50 mM NaCl from 16 DPG onward. We
393 measured ΦPSII every 3 to 4 days for the next 67 days (**Fig. 5**).



394

395 **Fig. 5.** *OsEPF1oe* plants maintain higher leaf Φ PSII values for equal or longer than all
 396 traditionally-bred varieties. (a) Apical leaf Φ PSII measurements of the nine selected
 397 varieties and two *OsEPF1oe* lines grown in 50 mM NaCl solution for 67 days. Vertical
 398 lines above and below individual points show one standard error (b) There was no
 399 statistically significant differences between *OsEPF1oeW* Φ PSII and the other
 400 traditionally-bred varieties or the *OsEPF1oeS* line prior to plants beginning the salt
 401 treatment at day 16 (denoted ns). (c) Φ PSII values on the apical leaf at 41 DPG, 25 days
 402 after the commencement of the salt treatment. For (b) and (c), whiskers indicate the ranges
 403 of the minimum and maximum values and stars indicate a significant difference from salt
 404 tolerant *OsEPF1oeW* (One-way ANOVA, Tukey HSD test, $P < 0.05$). Black diamonds
 405 represent means. $n = 7-9$ plants.

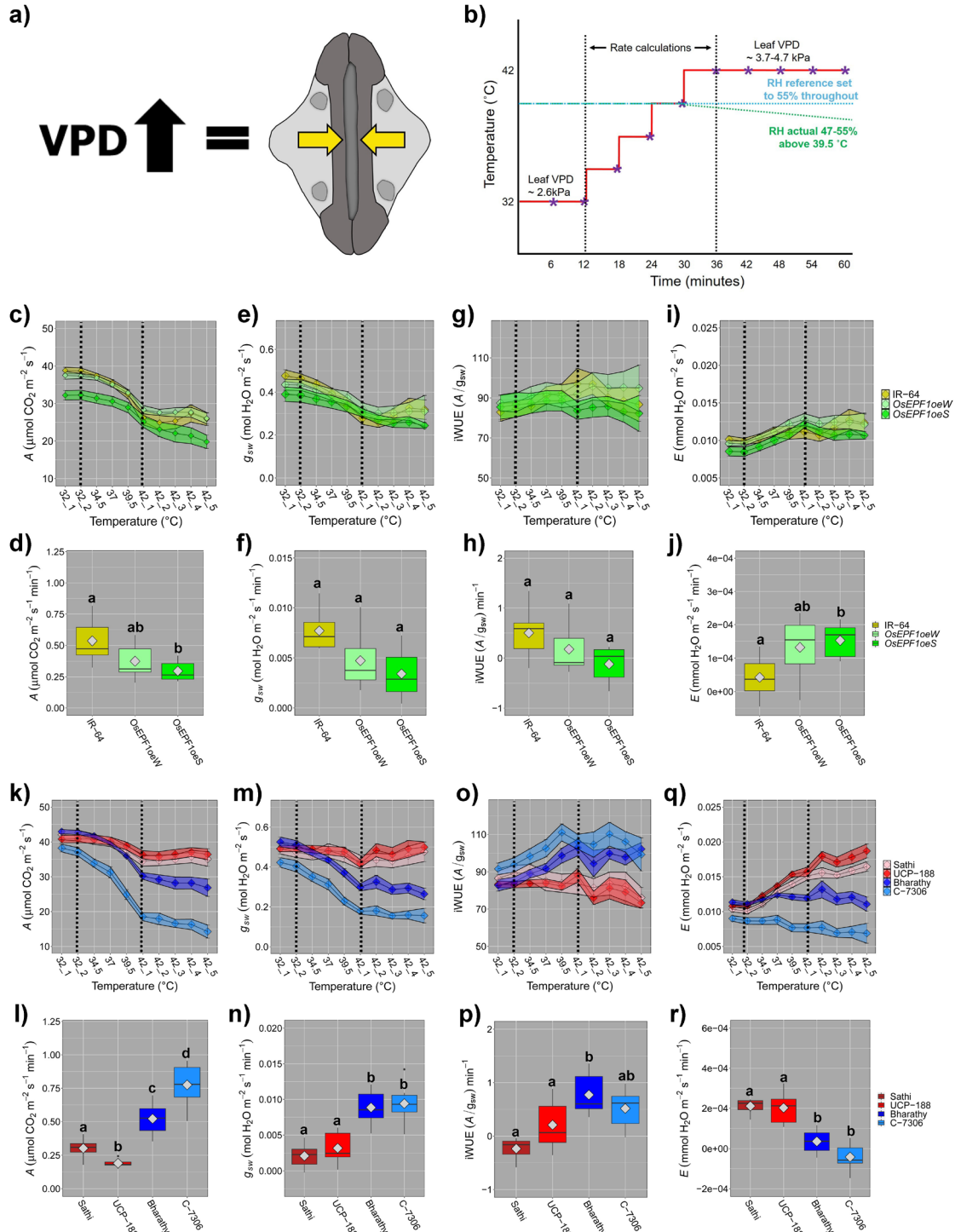
406 Prior to the start of salt treatment (16 DPG), no significant differences between control
 407 *OsEPF1oeW* leaf Φ PSII values and any of the other varieties tested (**Fig. 5a, b**). After 25

408 days of salt treatment (41 DPG), six out of nine varieties (including IR-64) showed
409 significant reductions in Φ PSII (**Fig. 5a, c**). Post 41 DPG, *OsEPF1oe* plant leaves
410 continued to show a slower decline in health than the majority of varieties, with the
411 exceptions being UCP-188. Within the selected varieties, neither SS nor SD appeared to
412 be associated with the degree of salinity tolerance.

413 **Stomatal responses to raising temperature and vapour pressure deficit**

414 At higher temperatures the amount of water the air can hold increases and this often leads
415 to rising VPD bringing about stomatal closure. We next investigated how increases in
416 VPD impacted stomatal dynamics by undertaking gas exchange experiments that either
417 rapidly increased VPD by increasing temperature (**Fig. 6** and **Fig. S3**), or by exposing a
418 leaf to a constantly high VPD (**Fig. S3**). High temperature and VPD conditions were
419 imposed inside an infrared gas analyser (IRGA) leaf chamber and gas exchange
420 simultaneously measured. For our rapid VPD experiments, assays were conducted over a
421 1 hour duration, with a 10 °C increase in temperature inside the chamber (from 32 °C to
422 42 °C), beginning 12 minutes into the experiment. The leaf VPD inside the chamber
423 increased from c. 2.6 kPa to 3.7 - 4.7 kPa (**Fig. 6b**, **Fig. S3**). Variation in leaf VPD and
424 chamber relative humidity (RH) at higher temperatures (≥ 39.5 °C) was due to reductions
425 in water flow caused by increased stomatal closure and by inability of the IRGA to
426 maintain RH.

427



428

429 **Fig. 6. Dynamic stomatal responses to rising Vapour Pressure Deficit (VPD) lead to**
 430 **large alterations in plant photosynthesis and water-use efficiency (a)** When VPD rises,
 431 usually driven by increasing temperature, stomata close. (b) Program performed in LI-
 432 COR 6800 gas analysers used to study stomatal responses to temperature and leaf VPD.
 433 Around 39.5 °C and above, many varieties were unable to maintain relative humidity at
 434 55% due to additive RH reaching its maximum possible set point (see also Fig. S3). Purple

435 stars indicate points data was recorded. Parallel vertical dotted lines throughout indicate
436 period used to calculate rate changes. (c-j) Comparison of IR-64 plants and *OsEPF1oeW*
437 and *S* plants using leaf 5 to investigate responses to raising temperature and VPD. (c)
438 Assimilation (*A*) responses over a one hour duration and (d) rate of change per minute
439 during the 24-minute incline period. (e-f) Equivalent stomatal conductance (g_{sw})
440 responses and rates of change over 24-minute period. (g-h) Corresponding intrinsic water-
441 use efficiency (iWUE) responses and rates of change and (i-j) transpiration (*E*) responses
442 and rates of change. (k-r) Comparisons between low SD, large SS Sathi and UCP-188
443 varieties and high SD, small SS Bharathy and C-7306. (k) *A* responses over one hour
444 assay and (l) rate of change per minute during the 24-minute incline period. (m-n)
445 Equivalent stomatal conductance (g_{sw}) responses and rates of change. (o-p)
446 Corresponding intrinsic water-use efficiency (iWUE) responses and rates of change and
447 (q-r) transpiration (*E*) responses and rates of change. Ribbons highlight standard error of
448 the mean. Boxplot whiskers indicate the ranges of the minimum and maximum values
449 and different letters indicate a significant difference between the means (One-way
450 ANOVA, Tukey HSD test, $P < 0.05$). Grey diamonds represent means. $n = 7-8$ plants.

451 We first assayed IR-64 and *OsEPF1oe* plants and found that all plants showed a
452 significant decrease in *A* as temperature and VPD increased (ANOVA, $P < 0.0001$, **Fig.**
453 **6c**), but the reduction in *A* occurred significantly more slowly for *OsEPF1oeS* than in IR-
454 64, but not for *OsEPF1oeW* (**Fig. 6d**). Like *A*, g_{sw} also reduced with increasing
455 temperature (ANOVA, $P < 0.001$, **Fig. 6e**), however there were no significant differences
456 in the rate of change between genotypes, despite a clear trend for a slower rate in
457 *OsEPF1oeS* (Kruskal-Wallis one-way ANOVA, overall $P = 0.056$) (**Fig. 6f**). The slower
458 decreases in *A* and (to some extent g_{sw}) resulted in a trend toward lower iWUE for
459 *OsEPF1oeS* during the temperature incline relative to IR-64 (**Fig. 6g and h**) (Kruskal-
460 Wallis one-way ANOVA, overall $P = 0.1052$). Conversely, transpiration (*E*) responses
461 generally increased across all genotypes with *OsEPF1oeS* plants having increased rates
462 of *E* relative to IR-64 during the temperature incline, peaking at the end of the increase.
463 The *E* of IR-64 peaked later and by the end of the 1 hour experiment reached a similar
464 level to *OsEPF1oe* plants (**Fig. 6i-j**).

465 To investigate if a combination of SS and SD contributed to rice VPD responses, we
466 compared two selected varieties which had low SD and large SS (Sathi and UCP-188),
467 with two varieties which had high SD and low SS (Bharathy and C-7306) (**Fig. 6k-r** and

468 **Fig. S3**). There were no significant differences in A or g_{sw} at 32 °C between the two low
469 SD, large SS varieties and the two high SD, small SS varieties at the beginning of the
470 experiments, but large differences were detectable in response to rising temperature and
471 VPD (**Fig. 6k-n**). Specifically, varieties with high SD with small SS showed significantly
472 faster rates of reduction for both A and g_{sw} in response to rising temperature and VPD in
473 comparison to varieties with low SD and large SS (**Fig. 6l-n**). This faster response
474 resulted in a large increase in iWUE (**Fig. 6g, o-p**). We also detected striking differences
475 in E in response to rising VPD, with varieties with low SD and large SS displaying a rapid
476 increase in E with increasing temperature which was in contrast to plants with high SD
477 and small SS that showed little change (or a slight drop) in E (**Fig. 6q-r**). Thus, the two
478 varieties with higher SD and smaller SS reduced A and g_{sw} relatively quickly in response
479 to an increase in VPD whereas for low SD varieties with large SS, A and g_{sw} remained
480 higher and this led to increasing E .

481 The experiments in Fig. 6 highlight that high SD, small SS rice varieties were unable to
482 maintain high A , g_{sw} and E when exposed to increasingly high temperature and VPD. It
483 is possible that that this may have, at least in-part, been due to a transient drop in RH
484 within the chamber during the rapid closure of small stomatal that contributed to leaves
485 having higher VPD (represented by green dotted line **Fig. 6b**, see also **Fig. S3**). To
486 address this, we conducted a subsequent experiment where we held plants under steady-
487 state conditions with temperature fixed at 39 °C where chamber RH was maintained at
488 stable at 55%, and this led to more similar leaf VPD values between varieties (**Fig. S3**).
489 These conditions captured the maximum point where all 4 different rice varieties were
490 able to maintain steady state conditions. This removed any additional stress on the plant
491 leaves caused by insufficient supply of RH as observed in our Fig. 6 experiments. Like
492 with our dynamic response Fig. 6 experiments, plants with low SD and large SS
493 maintained a higher A and g_{sw} than those with high SD and small SS. Reductions in A
494 and g_{sw} again had the opposite effect on iWUE, with high SD, small SS plants typically
495 having higher iWUE at the expense of A and g_{sw} (**Fig. S3**). And also like with our dynamic
496 response experiments in Fig. 6, these changes appeared to be entwined with an increased
497 capability of plants with low SD and large SS plants to increase E , whereas for plants
498 with high SD and small SS, E stayed low. These combined responses led to plants with
499 lower SD and larger SS having lower leaf temperatures based on calculated energy
500 balance (**Fig. S3**).

501 **Discussion**

502 **Stomatal size and density impact on plant gas exchange**

503 A low SD (often associated with larger SS) and/or small SS (often associated with high
504 SD) have frequently been correlated with improvements in water-use and/or drought
505 tolerance (Hepworth *et al.*, 2015; McAusland *et al.*, 2016; Dittberner *et al.*, 2018;
506 Kardiman & Ræbild, 2018; Caine *et al.*, 2019; Mohammed *et al.*, 2019). In this study, we
507 took a stomatal-focused approach to investigate how differences in SS and SD can affect
508 rice performance under several separate abiotic stresses. Anatomical screening of a rice
509 population identified significant variation in both traits across the population. We then
510 compared the abiotic stress responses of selected varieties with a range of differences in
511 SS and SD and *OsEPF1oe* plants which have an unusual combination of low SD and
512 small SS (on their seedling leaves). None of the varieties examined had an SD as low as
513 *OsEPF1oe* lines, suggesting that finding traditionally-bred rice varieties with SDs and
514 g_{smax} values equivalent to *OsEPF1oe* is perhaps unlikely.

515 Previous research has shown that high SD (often accompanied by small SS) can lead to a
516 higher maximum anatomical g_{smax} (Franks & Beerling, 2009), and this leads to a higher
517 operating g_{sw} and greater responsivity of stomatal apertures to environmental changes
518 (Franks *et al.*, 2012; Bertolino *et al.*, 2019). Our results only partially support these
519 findings. We observed the expected correlations between SD and both g_{smax} and g_{sw} ; with
520 the two varieties with highest SD and small SS (Bharathy and C-7306) having the highest
521 average g_{smax} . We did not, however, find a correlation between operating g_{sw} and g_{smax}
522 under well-watered conditions underlining that factors other than SD and SS, including
523 stomatal openness, also impact on operating g_{sw} . For example, the UCP-188 variety with
524 low SD and large SS, despite having a low calculated g_{smax} , had the equal highest
525 operating g_{sw} , and *OsEPF1oe* lines with only 50-72% of the g_{smax} of the traditionally-bred
526 varieties, had an operating g_{sw} similar to other low SD varieties (except UCP-188). Indeed,
527 plotting stomatal pore area measurements against g_{sw} indicated that *OsEPF1oe* plants can
528 counteract their reduced SD by increasing stomatal pore areas (**Fig. 1i**).

529 Our observed correlations between plant biomass and SD, and plant biomass and SS
530 suggest that stomatal characteristics at the tillering stage are reasonable predictors of plant
531 size (**Fig. 2**). Plants with low SDs and large SS typically had higher biomasses (with the
532 exception of *OsEPF1oe*), and plants with a high SD and small SS typically had lower

533 biomasses. Importantly, differences in biomass have the potential to exacerbate
534 differences in water-use, as plants with a larger surface area often require more water
535 (Feldman *et al.*, 2018) and may also close their stomata more slowly than smaller plants
536 (Drake *et al.*, 2013; McAusland *et al.*, 2016; Lawson & Vialet-Chabrand, 2019).

537 **Stomatal associations with drought tolerance**

538 Our drought stress experiments were designed to investigate if larger plants with large SS
539 displayed slower stomatal responsiveness to water stress (**Fig. 3**). We found that
540 traditionally-bred varieties, with high or medium SD with small SS, maintained higher
541 Φ PSII values for longer during drought than varieties with low SD and large SS,
542 indicative of better plant health. However, this was not the case for the *OsEPF1oe* plants.
543 Both lines of these very low SD plants had lower biomass, and these combinations of
544 traits resulted in *OsEPF1oe* plants maintaining the highest Φ PSII at the end of the drought.
545 This was despite *OsEPF1oe* plants showing similar operating g_{sw} levels as other varieties
546 with low SD, large SS when assessed under well-watered conditions (**Fig. 1e**). These
547 results suggest that the ideal combination of traits for growth of rice under drought
548 conditions, would be small plant size together with either a very low SD (leading to a
549 very low g_{smax}), or a higher SD combined with a small SS (leading to a high g_{smax} and
550 greater stomatal responsiveness).

551 **Stomatal contributions to salinity tolerance**

552 Modelling of root water and solute uptake has suggested that passive transport and uptake
553 of water and solutes into plants may be negligible (Foster & Miklavcic, 2017). However,
554 recent research in rice has suggested that reduced SD and g_{sw} , caused by increased activity
555 of a histone deacetylase, improved both drought and salinity tolerance (Zhao *et al.*, 2021).
556 In our study, we tested this potential relationship between SD and salt tolerance and found
557 that *OsEPF1oe* plants with reduced SD showed improved salinity treatment (**Fig. 4**). We
558 found that these plants with maintained higher leaf Φ PSII values when grown in salt water,
559 and also accumulated less than half the amount of Na^+ in leaves after approximately 5
560 weeks of growth (**Fig. 4**). Salt toxicity often leads to deficiencies in other elements such
561 as K^+ (Wang *et al.*, 2013) but we did not detect this in *OsEPF1oe* plants or controls. We
562 compared the performance of traditionally-bred varieties against *OsEPF1oe* and found
563 that, as in the drought experiments, *OsEPF1oe* plants performed well, maintaining leaf

564 Φ PSII the equal longest of all varieties surveyed. In contrast, other varieties with low SD
565 and large SS performed the least well (with the exception of UCP-188) (**Fig. 5a, c**).

566 **Stomatal responsiveness to rising temperature and VPD**

567 Rising temperatures leading to increased VPD has the potential to shut stomata at a time
568 when plants might otherwise utilise transpiration-driven evaporative cooling to maintain
569 a high photosynthetic output (Urban *et al.*, 2017; Yuan *et al.*, 2019; Grossiord *et al.*, 2020).
570 Comparisons between IR-64 and transgenic plants revealed that *OsEPF1oeS* seedlings
571 had slower rates of photosynthetic decline at higher temperature and VPD, and this was
572 linked with a trend towards a decreased rate of g_{sw} change per minute (**Fig. 6**). These
573 slower reductions in A and operating g_{sw} were coupled with increased rates of E ,
574 suggesting that *OsEPF1oeS* leaves with very low SD lost more water in comparison to
575 IR-64 as VPD stress increased. This slower response suggests that having vastly reduced
576 stomata may be detrimental when temperature and VPD are constantly fluctuating, but
577 this requires further study to confirm.

578 Small SS in nature is often associated with faster stomatal responsiveness, which in-turn
579 can result in higher water-use efficiency under fluctuating conditions (McAusland *et al.*,
580 2016; Lawson & Vialet-Chabrand, 2018; Inoue *et al.*, 2021). However, if plants with
581 small SS were to close stomata rapidly on sensing high VPD, this could lead to
582 detrimental reductions in A and evaporative cooling. We therefore tested whether
583 varieties with small SS had faster or slower stomatal VPD responses by comparing two
584 varieties with the highest SD and small SS with two varieties which had lowest SD and
585 large SS (**Fig. 6** and **Fig. S3**). We found that, unlike *OsEPF1oe* lines, the varieties with
586 small SS (and higher SD) reduced their g_{sw} much more rapidly than those with large SS.
587 Indeed, the extremely efficient closure of small SS (and high SD) reduced g_{sw} so
588 effectively that the IRGA equipment measuring the plants was unable to maintain RH
589 levels within the leaf chamber, whereas this was not apparent for plants with larger SS
590 (and low SD) (**Fig. S3c**). This rapid stomatal closure in plants with small SS resulted in
591 improved iWUE at higher VPD levels, but this was at the expense of A . In contrast, the
592 varieties with larger SS maintained higher rates of A and g_{sw} fairly steadily with increasing
593 temperature and VPD, but their level of E increased markedly. These results illustrate
594 how differing stomatal configurations are associated with differing responses; small SS
595 with high SD can react more quickly to reduce g_{sw} whereas plants with larger SS and low

596 SD are usually less responsive in controlling their water loss via stomata at high
597 temperature and VPD. These differing stomatal strategies most probably allow plants to
598 inhabit and thrive under different environments. For example, the fast responses of small
599 stomata (potentially with high SD) would be expected to be beneficial in water restricted
600 conditions, but higher levels of E associated with plants with larger SS (potentially with
601 lower SD) could have a greater effect on the microenvironment. This may be a positive
602 trait when growing in high temperature or VPD environments if sufficient water is
603 available to power an enhanced transpiration stream.

604 **Effects on Stomatal Development**

605 Our results support previous observations (Zhang et al., 2019) showing that rice SD and
606 SS are, in general, negatively correlated (**Fig. 1 and Fig. S1**). During leaf development,
607 epidermal cell division is usually synchronised with cell expansion to achieve a specific
608 SS and SD on a given leaf. This relationship is perturbed in *OsEPF1oe* seedlings, where
609 plants with extremely low SD, typically have smaller SS at the seedling stage (Caine *et*
610 *al.*, 2019). However, as we show here, by leaf 8 stage when plants are rapidly tillering,
611 this is no longer the case, and *OsEPF1oe* plants had comparatively larger SS (than IR-64)
612 in their more mature leaves. SD is also under developmental control, and in our
613 experiments the leaves of mature plants had considerably more stomata than seedling
614 leaves. Thus, the potency or nature of factors driving SS and/or SD regulation must
615 change as a plant develops. Recently GWAS analysis has identified genetic traits
616 associated with both SS and SD which could be useful in breeding for these traits (Chen
617 *et al.*, 2020).

618 **Conclusion**

619 Previously we have shown that *OsEPF1oe* plants with extremely low SD, have improved
620 drought tolerance and survivability, even at high temperatures. Here, we show that these
621 plants are also less susceptible to salinity toxicity, most probably because they accumulate
622 salt at much lower levels. By screening a range of traditionally-bred rice varieties, we
623 show that varieties with low SD and large SS typically have lower operating g_{sw} when
624 sufficient water is available, but this is often associated with increased biomass that could
625 promote increased drought susceptibility. *OsEPF1oe* plants were physically smaller and
626 yet had the lowest SD and g_{smax} of all varieties studied, traits which together appeared to
627 positively impact on both drought and salinity tolerance. Based on our screen, it seems

628 unlikely that similar conformations of stomata with correspondingly small plant size will
629 be found in traditionally-bred rice varieties. This means that stomatal-based stress
630 tolerance may best be obtained via genetic manipulation of stomatal development rather
631 than via conventional breeding practices.

632 Assessment of VPD responses suggest that strong over-expression of *EPFL* diminishes
633 stomatal closure responses relative to IR-64 leading to reduced short-term iWUE. This
634 slower stomatal responsiveness leads to increased *E*. Conversely, traditionally-bred plants
635 with high SD and small SS greatly reduce g_{sw} at high VPD, preventing *E* increasing, and
636 this may be beneficial for short-term iWUE, but detrimental to long-term plant
637 productivity and cooling. Whilst *OsEPFLoe* plants showed enhanced tolerance to several
638 abiotic stresses, taken together our results highlight that there is unlikely to be an ideal
639 SS and SD for all future climatic conditions, and thought must be given as to which
640 conformation(s) of stomata will best suit a given growth environment.

641 **Acknowledgments**

642 We thank Dr Jose De Vega, Earlham Institute, for providing the rice germplasm. We are
643 grateful for funding from BBSRC Newton Fund (BB/N013646/1) and a Leverhulme
644 Trust Senior Research Fellowship (SRF\R1\21000149) to JEG, a University of Sheffield
645 QR GCRF Fellowship (Research England institutional allocation) to RSC, and a BBSRC
646 DTP studentship to ELH (BB/M011151/1). HLC was supported by the UK Research and
647 Innovation (UKRI) Future Leaders Fellowship scheme [MR/T01993X/1]

648 **Author contributions**

649 R.S.C., N.T.L., J.E.G. designed the study. R.S.C., E.L.H., J.M.S., P.M.F., and S.F.
650 undertook the experiments. N.T.L., N.T.P., H.L.C. and J.E.G. contributed materials and
651 advice. R.S.C., H.L.C. and J.E.G. wrote the paper with comments from E.L.H, J.M.S.,
652 P.M.F., S.F., N.T.L. and N.T.P. All authors read, commented on and approved the final
653 version of the manuscript.

654 **ORCID identifiers**

655 Robert S. Caine : <http://orcid.org/0000-0002-6480-218X>

656 Emily L. Harrison : <https://orcid.org/0000-0002-4109-8919>

657 Jennifer M. Sloan: <http://orcid.org/0000-0003-0334-3722>

658 Paulina M. Flis: <https://orcid.org/0000-0002-5529-7599>

659 Sina Fischer: <https://orcid.org/0000-0001-8368-3262>
660 Julie E. Gray: <http://orcid.org/0000-0001-9972-5156>
661 Holly L. Croft: <https://orcid.org/0000-0002-1653-1071>

662

663 References

- 664 **Bertolino LT, Caine RS, Gray JE. 2019.** Impact of Stomatal Density and Morphology on Water-
665 Use Efficiency in a Changing World. *Frontiers in Plant Science* **10**: 225.
- 666 **Bouman B 2009.** How much water does rice use? *Rice Today*. Manilla, Philippines: IRRI. 29.
- 667 **Caine RS, Yin X, Sloan J, Harrison EL, Mohammed U, Fulton T, Biswal AK, Dionora J, Chater**
668 **CC, Coe RA, et al. 2019.** Rice with reduced stomatal density conserves water and has
669 improved drought tolerance under future climate conditions. *New Phytologist* **221**(1):
670 371-384.
- 671 **Chen H, Zhao X, Zhai L, Shao K, Jiang K, Shen C, Chen K, Wang S, Wang Y, Xu J. 2020.** Genetic
672 Bases of the Stomata-Related Traits Revealed by a Genome-Wide Association Analysis
673 in Rice (*Oryza sativa* L.). *Frontiers in Genetics* **11**.
- 674 **Danku JMC, Lahner B, Yakubova E, Salt DE 2013.** Large-Scale Plant Ionomics. In: Maathuis FJM
675 ed. *Plant Mineral Nutrients: Methods and Protocols*. Totowa, NJ: Humana Press, 255-
676 276.
- 677 **Dittberner H, Korte A, Mettler-Altmann T, Weber APM, Monroe G, de Meaux J. 2018.** Natural
678 variation in stomata size contributes to the local adaptation of water-use efficiency in
679 *Arabidopsis thaliana*. *Molecular Ecology* **27**(20): 4052-4065.
- 680 **Dow GJ, Bergmann DC, Berry JA. 2014.** An integrated model of stomatal development and leaf
681 physiology. *New Phytologist* **201**(4): 1218-1226.
- 682 **Drake PL, Froend RH, Franks PJ. 2013.** Smaller, faster stomata: scaling of stomatal size, rate of
683 response, and stomatal conductance. *Journal of Experimental Botany* **64**(2): 495-505.
- 684 **Dunn J, Hunt L, Afsharinafar M, Meselmani MA, Mitchell A, Howells R, Wallington E, Fleming**
685 **AJ, Gray JE. 2019.** Reduced stomatal density in bread wheat leads to increased water-
686 use efficiency. *Journal of Experimental Botany* **70**(18): 4737-4748.
- 687 **Eyland D, van Wesemael J, Lawson T, Carpentier S. 2021.** The impact of slow stomatal kinetics
688 on photosynthesis and water use efficiency under fluctuating light. *Plant Physiology*
689 **186**(2): 998-1012.
- 690 **Feldman MJ, Ellsworth PZ, Fahlgren N, Gehan MA, Cousins AB, Baxter I. 2018.** Components of
691 Water Use Efficiency Have Unique Genetic Signatures in the Model C(4) Grass *Setaria*.
692 *Plant physiology* **178**(2): 699-715.
- 693 **Foster KJ, Miklavcic SJ. 2017.** A Comprehensive Biophysical Model of Ion and Water Transport
694 in Plant Roots. I. Clarifying the Roles of Endodermal Barriers in the Salt Stress
695 Response. *Frontiers in Plant Science* **8**: 1326.
- 696 **Franks PJ, Beerling DJ. 2009.** Maximum leaf conductance driven by CO₂ effects on stomatal
697 size and density over geologic time. *Proceedings of the National Academy of Sciences*
698 *of the United States of America* **106**(25): 10343-10347.
- 699 **Franks PJ, Farquhar GD. 2001.** The effect of exogenous abscisic acid on stomatal development,
700 stomatal mechanics, and leaf gas exchange in *Tradescantia virginiana*. *Plant Physiology*
701 **125**(2): 935-942.
- 702 **Franks PJ, Farquhar GD. 2007.** The mechanical diversity of stomata and its significance in gas-
703 exchange control. *Plant Physiol* **143**(1): 78-87.
- 704 **Franks PJ, Leitch IJ, Ruszala EM, Hetherington AM, Beerling DJ. 2012.** Physiological framework
705 for adaptation of stomata to CO₂ from glacial to future concentrations. *Philosophical*
706 *Transactions of the Royal Society B-Biological Sciences* **367**(1588): 537-546.

- 707 **Fukagawa NK, Ziska LH. 2019.** Rice: Importance for Global Nutrition. *Journal of Nutritional*
708 *Science and Vitaminology* **65**(Supplement): S2-S3.
- 709 **Gray A, Liu L, Facette M. 2020.** Flanking Support: How Subsidiary Cells Contribute to Stomatal
710 Form and Function. *Frontiers in Plant Science* **11**: 881.
- 711 **Grossiord C, Buckley TN, Cernusak LA, Novick KA, Poulter B, Siegwolf RTW, Sperry JS,**
712 **McDowell NG. 2020.** Plant responses to rising vapor pressure deficit. *New Phytologist*
713 **226**(6): 1550-1566.
- 714 **Hepworth C, Doheny-Adams T, Hunt L, Cameron DD, Gray JE. 2015.** Manipulating stomatal
715 density enhances drought tolerance without deleterious effect on nutrient uptake. *The*
716 *New phytologist* **208**(2): 336-341.
- 717 **Huang XY, Chao DY, Gao JP, Zhu MZ, Shi M, Lin HX. 2009.** A previously unknown zinc finger
718 protein, DST, regulates drought and salt tolerance in rice via stomatal aperture control.
719 *Genes Dev* **23**(15): 1805-1817.
- 720 **Inoue T, Sunaga M, Ito M, Yuchen Q, Matsushima Y, Sakoda K, Yamori W. 2021.** Minimizing
721 VPD Fluctuations Maintains Higher Stomatal Conductance and Photosynthesis,
722 Resulting in Improvement of Plant Growth in Lettuce. *Frontiers in Plant Science* **12**.
- 723 **IPCC. In press.** Masson-Delmotte V, P. Zhai, A. Pirani, S.L. Connors, C. Péan, S. Berger, N. Caud,
724 Y. Chen, L. Goldfarb, M.I. Gomis, M. Huang, K. Leitzell, E. Lonnoy, J.B.R. Matthews, T.K.
725 Maycock, T. Waterfield, O. Yelekçi, R. Yu, and B. Zhou., ed. AR6 Climate Change
726 2021:The Physical Science Basis: IPCC.
- 727 **Kardiman R, Ræbild A. 2018.** Relationship between stomatal density, size and speed of
728 opening in Sumatran rainforest species. *Tree Physiology* **38**(5): 696-705.
- 729 **Lawson T, Vialet-Chabrand S. 2018.** Speedy stomata, photosynthesis and plant water use
730 efficiency. *New Phytologist* **0**(0).
- 731 **Lawson T, Vialet-Chabrand S. 2019.** Speedy stomata, photosynthesis and plant water use
732 efficiency. *New Phytologist* **221**(1): 93-98.
- 733 **Livsey J, Kätterer T, Vico G, Lyon SW, Lindborg R, Scaini A, Da CT, Manzoni S. 2019.** Do
734 alternative irrigation strategies for rice cultivation decrease water footprints at the
735 cost of long-term soil health? *Environmental Research Letters* **14**(7): 074011.
- 736 **Ma Y, Dias MC, Freitas H. 2020.** Drought and Salinity Stress Responses and Microbe-Induced
737 Tolerance in Plants. *Frontiers in Plant Science* **11**: 1750.
- 738 **McAusland L, Vialet-Chabrand S, Davey P, Baker Neil R, Brendel O, Lawson T. 2016.** Effects of
739 kinetics of light-induced stomatal responses on photosynthesis and water-use
740 efficiency. *New Phytologist* **211**(4): 1209-1220.
- 741 **Merilo E, Yarmolinsky D, Jalakas P, Parik H, Tulva I, Rasulov B, Kilk K, Kollist H. 2018.** Stomatal
742 VPD Response: There Is More to the Story Than ABA. *Plant Physiology* **176**(1): 851-864.
- 743 **Mohammed U, Caine RS, Atkinson JA, Harrison EL, Wells D, Chater CC, Gray JE, Swarup R,**
744 **Murchie EH. 2019.** Rice plants overexpressing OsEPF1 show reduced stomatal density
745 and increased root cortical aerenchyma formation. *Scientific Reports* **9**(1): 5584.
- 746 **Panda D, Mishra SS, Behera PK. 2021.** Drought Tolerance in Rice: Focus on Recent
747 Mechanisms and Approaches. *Rice Science* **28**(2): 119-132.
- 748 **Raissig MT, Matos JL, Gil MXA, Kornfeld A, Bettadapur A, Abrash E, Allison HR, Badgley G,**
749 **Vogel JP, Berry JA, et al. 2017.** Mobile MUTE specifies subsidiary cells to build
750 physiologically improved grass stomata. *Science* **355**(6330): 1215.
- 751 **Singh B, Mishra S, Bisht DS, Joshi R 2021.** Growing Rice with Less Water: Improving
752 Productivity by Decreasing Water Demand. In: Ali J, Wani SH eds. *Rice Improvement:*
753 *Physiological, Molecular Breeding and Genetic Perspectives*. Cham: Springer
754 International Publishing, 147-170.
- 755 **Urban J, Ingwers MW, McGuire MA, Teskey RO. 2017.** Increase in leaf temperature opens
756 stomata and decouples net photosynthesis from stomatal conductance in *Pinus taeda*
757 and *Populus deltoides* x *nigra*. *J Exp Bot* **68**(7): 1757-1767.

- 758 **Wang M, Zheng Q, Shen Q, Guo S. 2013.** The critical role of potassium in plant stress response.
759 *International journal of molecular sciences* **14**(4): 7370-7390.
- 760 **Yuan W, Zheng Y, Piao S, Ciais P, Lombardozzi D, Wang Y, Ryu Y, Chen G, Dong W, Hu Z, et al.**
761 **2019.** Increased atmospheric vapor pressure deficit reduces global vegetation growth.
762 *Science Advances* **5**(8): eaax1396.
- 763 **Zhang Q, Peng S, Li Y. 2019.** Increase rate of light-induced stomatal conductance is related to
764 stomatal size in the genus *Oryza*. *Journal of Experimental Botany* **70**(19): 5259-5269.
- 765 **Zhao J, Zhang W, da Silva JAT, Liu X, Duan J. 2021.** Rice histone deacetylase HDA704 positively
766 regulates drought and salt tolerance by controlling stomatal aperture and density.
767 *Planta* **254**(4): 79.
- 768 **Fig. S1.** Rice leaf 5 stomatal size and density screen of 72 traditionally-bred rice varieties and 2
769 transgenic varieties.
- 770 **Table. S1.** List of 72 rice varieties and corresponding stomatal size and densities.
- 771 **Fig. S2.** Stomatal size and density relationships to stomatal conductance.
- 772 **Fig. S3.** Rapid and steady-state VPD responses of rice with differing stomatal size and density.