

1 **Title:** Whole plant chamber to examine sensitivity of cereal gas exchange to changes
2 in evaporative demand

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27 **ABSTRACT**

28 Background: Improving plant water use efficiency (WUE) is a major target for
29 improving crop yield resilience to adverse climate change. Identifying genetic variation
30 in WUE usually relies on instantaneous measurements of photosynthesis (A_n) and
31 transpiration (T_r), or integrative measurements of carbon isotope discrimination, at the
32 leaf level. However, leaf gas exchange measurements alone do not adequately
33 represent whole plant responses, especially if evaporative demand around the plant
34 changes.

35 Results: Here we describe a whole plant gas exchange system that can rapidly
36 alter evaporative demand when measuring A_n , T_r and intrinsic WUE (iWUE) and
37 identify genetic variation in this response. A_n was not limited by VPD under steady-
38 state conditions but some wheat cultivars restricted T_r under high evaporative
39 demand, thereby improving iWUE. These changes may be ABA-dependent, since the
40 barley ABA-deficient mutant (*Az34*) failed to restrict T_r under high evaporative
41 demand. Despite higher T_r , *Az34* showed lower A_n than wild-type (WT) barley
42 because of limitations in Rubisco carboxylation activity. T_r and A_n of *Az34* were more
43 sensitive than WT barley to exogenous spraying with ABA, which restricted
44 photosynthesis via substrate limitation and decreasing Rubisco activation.

45 Conclusions: Examining whole plant gas exchange responses to altered VPD
46 can identify genetic variation in whole plant iWUE, and facilitate an understanding of
47 the underlying mechanism(s).

48

BACKGROUND

49 Photosynthesis is a complex process in which light, water and carbon dioxide
50 (CO₂) are used to synthesize carbohydrates. In plants, CO₂ can only diffuse into the
51 leaves via the stomata. When open, the stomata represent the major path of water
52 loss to the atmosphere via transpiration. Approximately 98% of all water taken up
53 through the roots may be transpired through the stomata [1]. Therefore, plants
54 constantly seek to minimise water loss while maintaining CO₂ entry for photosynthesis,
55 by tightly regulating their stomatal responses. Monitoring plant–atmosphere gas
56 exchange is essential for understanding plant responses to a fluctuating environment.

57 Atmospheric vapour pressure deficit (VPD) or evaporative demand is
58 influenced by both air temperature and relative humidity (RH), and is the difference
59 between the saturation vapour pressure and the actual vapour pressure. The driving
60 force for water movement through the plant is caused by the vapour pressure deficit
61 between the substomatal cavity and the surrounding air, known as leaf-to-air vapour
62 pressure deficit (VPD_{leaf}). High VPD_{leaf} increases plant transpiration rates (Tr) [2]. By
63 decreasing their stomatal conductance (g_s), plants can partially limit Tr and the
64 decrease in leaf water status [3]. High ambient VPD and VPD_{leaf} enhances evaporation
65 of water from the leaf, reducing bulk leaf water status and inducing stomatal closure,
66 which is contributed to by a hydropassive response common to all land plants and, in
67 angiosperms, a hydroactive response regulated by abscisic acid (ABA) [4]. Increased
68 VPD rapidly upregulates expression of the *NCED* genes (involved in ABA
69 biosynthesis), thereby increasing leaf [ABA] and decreasing g_s [5]. However, this leaf-
70 based mechanism may not completely explain the spatial and temporal behaviour of
71 whole plant transpiration under increasing evaporative demand: other factors such as
72 patchy stomatal closure [6], changes in leaf [7], root [8,9], or whole plant hydraulic

73 conductivity [10,11] and leaf-age differences in sensitivity to ABA [12], may operate
74 together to limit T_r under increasing VPD.

75 Water use efficiency (WUE) typically refers to the ratio between the biomass
76 produced and cumulative water use. At the physiological level, the ratio of net
77 photosynthesis (A_n) to T_r is known as photosynthetic or intrinsic WUE (iWUE).
78 Maintaining net photosynthesis (A_n) while reducing T_r under high atmospheric
79 evaporative demand may be of adaptive significance under certain conditions, and
80 genetic variability in the sensitivity of g_s to VPD has been described in angiosperms:
81 in some genotypes, T_r increases linearly with increasing VPD, while others restrict T_r
82 at higher VPD. Pioneering work identified the “restricted transpiration” trait [13, 14],
83 and associated low leaf hydraulic conductivity with improved WUE. The trait has been
84 identified in many crops, including cereals [15–16], using gravimetric methods in
85 chambers [17], greenhouses [18], and the field [19]. A potential drawback of
86 decreasing g_s to restrict transpiration under increasing VPD, is that internal CO_2
87 concentration (C_i) may decrease, thereby decreasing A_n via substrate limitation. Field
88 measurements under high VPDs cannot separate effects of VPD on A_n from effects
89 of high temperature *per se*. Consistent with this potential limitation, high evaporative
90 demands and temperatures considerably limit leaf level photosynthesis [20,21].
91 However, similar measurements at the whole plant level have not been made.

92 Leaf gas exchange measurements fail to capture whole plant responses since:
93 ¹⁾ transpiration inside the leaf cuvette of an infra-red gas analysis system reflects the
94 controls imposed on that environment (i.e. mixing of air to control boundary layer
95 conductance, chosen temperature, choice of light source, leaf area used for
96 measurement, flow rate); ²⁾ leaf measurements cannot adequately describe whole
97 plant A_n due to spatial variation in the light environment of different leaves [22,23]; ³⁾

98 naturally occurring microclimates across the plant affect its interaction with the
99 environment. Thus, several chambers have been built to characterize whole plant gas
100 exchange of plants such as *Arabidopsis* [24–26], shrubs [27–29], or even trees [30],
101 but with limited regulation of environmental conditions inside the chamber. As a
102 consequence, such measurements may be bedeviled by leaks, flow rate fluctuations,
103 overheating of the larger chambers [31], and high humidity/condensation that can
104 cause severe failures of IRGAs [32,33]. These technical difficulties probably explain
105 why relatively few researchers have built whole plant systems to study transpiration
106 responses to increasing evaporative demand [7,18,34,35].

107 In the present manuscript, we describe a whole plant gas exchange system to
108 measure A_n , T_r and $iWUE$ under increasing VPD. We tested whether different cereal
109 genotypes, previously demonstrated to show variation in transpiration response to
110 VPD [16] and variation in leaf-level photosynthesis [36], showed variation in whole
111 plant $iWUE$ as evaporative demand changed. Because higher photosynthetic rates
112 correlate with high yield [36] and stomatal responses to VPD governs diurnal plant
113 transpiration [39], identifying useful genetic variation in $iWUE$ at high VPD will be of
114 interest to plant physiologists and breeders. Our whole plant gas exchange system is
115 relevant to achieving this goal.

116 Since the role of ABA in regulating stomatal responses to VPD is not completely
117 clear (cf. [35,37]), we used the whole plant gas exchange system to investigate the
118 responses of an ABA-deficient barley mutant and its wild-type under contrasting VPD,
119 and in response to foliar ABA spraying. Previous observations indicate that exogenous
120 ABA application limits photosynthesis of ABA-deficient plants (*flacca* tomato mutant
121 and *Arabidopsis* lines) [35, 38], even if the mechanistic interpretation is not clear. Our
122 working hypothesis is that stomatal hypersensitivity of the ABA deficient mutant (*Az34*)

123 to exogenous ABA spraying constrains photosynthesis via substrate limitation,
124 decreasing Rubisco activation state, and limiting net photosynthesis.

125

126 **MATERIA METHODS**

127 **Growth conditions and plant material**

128 Wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) were pre-germinated
129 on moistened filter paper (Whatman #1) in petri dishes. The dishes were covered with
130 foil and placed in dark conditions at room temperature ($24^{\circ}\text{C} \pm 5\%$) for 48 h. Once
131 germinated, two seeds were placed at about 2.5 cm depth in rectangular 2 l pots (10.5
132 x 10.5 x 20 cm height) containing a commercial growing substrate (Petersfield
133 Products, UK) with a slow-release fertilizer (Osmocote, Scotts UK Professional, UK).
134 After the first true leaf emerged, one of the plants was removed from each pot to
135 maintain one plant per pot. Twelve days after transplanting, the plants were supported
136 in a sealing sleeve (Fig. 1, Supplemental Fig. 1). The plants grew for six weeks until
137 reaching the phenological stage Zadoks 39-45. Plants were watered every 2-3 days
138 to reach drip point, the maximum water content of the substrate, and were randomly
139 allocated in the greenhouse and rotated weekly to assure homogeneity.

140 Plants were placed in a naturally lit greenhouse at Lancaster University
141 (54.0104°N , 2.7877°W) with supplementary lighting (14 h per day), and controlled
142 temperature (lights turn off if air temperature exceeds 30°C). To maintain atmospheric
143 VPD lower than 2.5 kPa throughout a diurnal cycle, a ten heads humidifier (Growell,
144 UK) was placed in the greenhouse, to avoid developmental VPD priming of plants
145 growing in different periods in the greenhouse.

146 Table 1 describes the different experiments done. The wheat cultivars (cv.)
147 Krichauff and Drysdale were chosen because they showed contrasting T_r under
148 increasing VPD [16]. The wheat cultivars cv. Cadenza, Gatsby, Mercato, Gladiator,
149 Zebedee were chosen because they showed contrasting leaf photosynthesis (A) in a
150 field experiment [36]. The barley ABA-deficient mutant *Az34* mutant (and its
151 corresponding wild-type, WT) was chosen since it shows reduced capacity to produce
152 ABA in response to water deficit, caused by a pleiotropic deficiency in the molybdenum
153 cofactor that decreases aldehyde oxidase activity, which catalyses the ultimate step
154 in the ABA biosynthesis pathway [40]. This mutant has higher T_r than the wild-type
155 (WT) Steptoe in an early stage, both under control VPD and after increasing air
156 temperature and, therefore, VPD [41].

157

158 **Whole plant gas exchange system**

159 We re-designed the whole plant gas exchange system previously described
160 [18]. With the new configuration and upgrades, the equipment can measure A_n and
161 $iWUE$, in addition to T_r , under increasing VPD (Fig. 1). Hereafter, transpiration
162 determined with this chamber is termed T_{IRGA} to avoid confusion with T_r obtained by
163 gravimetric methods. The new system incorporates: ¹⁾ a powerful
164 humidifier/dehumidifier system (Supplemental Fig. 5) that can more rapidly change
165 chamber relative humidity (5 min compared to ~30 min required previously [18])
166 allowing higher VPDs (> 4 kPa) while maintaining temperatures below 30°C; ²⁾ a mass
167 flow controller to tightly control the flow in the system by allowing a certain amount of
168 pressure from the compressor while the previous version [18] pulled in air via a fan ³⁾
169 multiple probes within the chamber to monitor environmental conditions including

170 temperature, relative humidity and light, which were absent in [18]; ⁴) a LI- 6400XT (LI-
171 COR, Lincoln, NE, USA) to simultaneously measure the gas exchange by logging the
172 data measured using the various probes. The diagrams of the different parts are
173 supplied in Supplemental Fig.s 1-4.

174

175 **IRGA and external probes**

176 A LI-6400XT equipped with a 9964-053 Sample Cell Outlet Manifold Kit (LI-
177 COR, Lincoln, NE, USA) to reduce the gas analyzer sample volume, was used to
178 determine CO₂ and H₂O vapour concentrations. Using a LI-6400XT allowed external
179 probes to be connected to the console to calculate A_n and T_{IRGA}, using a protocol
180 provided by LI-COR (LI-6400 Portable Photosynthesis System, Application Note 2) to
181 communicate with the external probes as well as the IRGA. A temperature-humidity
182 probe (Vaisala Humitter 50Y, Helsinki, Finland), a flow rate transducer (TSI 8450,
183 Aliflow Instruments, USA) and a temperature probe (LI-COR, Lincoln, Nebraska, USA)
184 were added.

185 The LI-6400XT head was connected to the chamber using vinyl flexible tubing
186 (Swagelok, UK) and aluminium tube fittings and adapters (Swagelok, UK). Gas was
187 driven through the LI-6400XT head using an external pump (model TD-4X2NA,
188 Brailsford & CO, USA), which tightly controlled the flow of air. The flow rate achieved
189 was checked every week. All tubing was covered with thermal insulation to stabilise
190 dew point temperatures.

191

192 **The chamber**

193 A chamber of total volume of 30 l (25 x 20 x 60 cm) was built from Perspex,
194 with a nominal thickness of 3.5 mm. Light was supplied by two Son-T high-pressure
195 sodium lamps (Philips, Netherlands) providing $450 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD at the top of
196 the plants. The light action spectrum that it is transmitted into the chamber was
197 measured between 200 nm and 1100 nm by placing the spectroradiometer (SR9910-
198 V7, Macam Photometrics, Livingston, UK) inside the closed chamber, at 25 nm
199 intervals (Supplemental Fig. 6).

200 To insert the plant into the chamber, one side consisted of a removable door
201 (see Supplemental Fig. 3a) sealed with 1 cm wide neoprene sponge rubber and
202 closed using eight metal clips. A sealable slot at the base of the chamber (see sealing
203 sleeve description) isolated the root and shoot of the plant.

204 The chamber is hermetically sealed and works under a slight overpressure. It
205 is noteworthy that no leaks were detected in our system (Fig. 2A-B). Four fans
206 (Ebmpapas 512Ft, Hungary) were placed inside the chamber (two fans on the top
207 quarter and two fans on the bottom quarter of the chamber) to lower boundary layer
208 resistance, with a combined capacity of $310 \text{ m}^3 \text{ h}^{-1}$. Fan placement ensured
209 homogenous airflow which was checked using smoke (data not shown). The
210 equipment is operated in the laboratory, allowing the temperature to remain stable at
211 $27.5^\circ\text{C} \pm 5\%$ when the fans are on (Fig. 2C). Temperature and relative humidity inside
212 the chamber remains comparable (Supplemental Table 1).

213

214 **Sealing sleeve**

215 A sealing sleeve, made of PVC (12 x 8 x 0.2 cm) (Supplemental Fig. 1) isolated
216 the above and below-ground parts of the plant. In most cases, tiller development inside

217 the sealing sleeve isolated the roots from the shoots, but to ensure gas tightness
218 Sylgard Silicone elastomer (Dow Corning, UK) was applied inside the sealing sleeve
219 two days prior to measurements. A neoprene sponge rubber ensured a tight fit of the
220 plant into the chamber.

221

222 **The circuit**

223 Air from outside the building was supplied to the chamber to assure a stable
224 [CO₂]. The [CO₂] in this source changed less than 10 ppm during a typical day. To
225 provide air under positive pressure, we used a compressor (OF1202-40MQ3, Junk Air,
226 USA) with an extensive cooling system for temperature control. The compressed air
227 was circulated through a 2 m pipe (1 cm internal \varnothing) filled with silica gel to dehydrate it
228 to approx. 5% RH. The silica gel was replenished after every 4-6 h of use. Thus
229 conditioned, the air was supplied to the chamber at a stable rate of 30 l min⁻¹ (with an
230 error of 0.1%) via a mass flow controller (Alicat CMR 500 SLPM, Alicat Scientific,
231 USA). This flow rate allows ¹⁾ an acceptable air renewal of one chamber volume per
232 minute; ²⁾ a reasonable [CO₂] differential across the chamber (between -18 to -25 μmol
233 CO₂ m⁻² s⁻¹); ³⁾ avoidance of high system pressure.

234 If necessary (at high VPDs or when plant leaf area exceeded 400 cm²), the flow
235 rate was increased. A water bath (Fig. 1, Supplemental Fig. 2) containing an Ultrasonic
236 humidifier with ten heads (Growell, UK) was developed to re-humidify the air (if
237 needed). Manually operated low-pressure valves (Swagelok, UK) were used to control
238 the amount of air passing through the water bath. The system established RH values
239 in the range 5-75% by passing air through the water bath, and when higher RH was
240 desired, the ultrasonic humidifier was connected. Although most of the tubing in the

241 system has 0.4 cm internal \varnothing , the tube that connects the humidifier system with the
242 pre-mixer chamber has 1 cm internal \varnothing to avoid condensation inside it.

243 To homogenize the air, it passes through a pre-mixer box (30 x 30 x 30 cm)
244 (Fig. 1, Supplemental Fig. 4). Next, prior to entering the chamber, the air transits a
245 PVC pipe (3 cm internal \varnothing , 40 cm length) where flow rate, temperature and humidity
246 probes and the reference line for LI-6400XT are assembled. The flow rate used for the
247 gas exchange calculations is computed here. Typically, the conductance of this pipe
248 averages $175 \mu\text{mol air s}^{-1}$ with an error of 5%. The pipe ends in the base of the
249 chamber, circulating air upwards. Air exits the chamber via another PVC pipe where
250 the sample line for the LI-6400XT is connected. A thermocouple (connected to the LI-
251 6400XT) measures the temperature of a selected leaf from the top of the canopy.

252

253 **Data collection**

254 At the beginning of each measurement sequence, the plants were acclimated
255 for ~ 20 min to a VPD of 2.5 kPa, the maximum VPD experienced by plants in the
256 greenhouse. Differences in $[\text{CO}_2]$ and $[\text{H}_2\text{O}]$ between air entering and exiting the
257 chamber were measured and recorded using the LI-6400XT. Once the exchange of
258 CO_2 and H_2O had been steady for more than 5 min (steady-state, Fig. 2D), values
259 were logged every 20 seconds for 3-5 min, and a median value was established. Then,
260 the relative humidity in the system was adjusted to inside the system were changed to
261 achieve the next desired VPD level, usually requiring 15-30 min to reach a new steady-
262 state. For VPD curves, VPD was gradually decreased to the minimum achievable in
263 decrements. After that, VPD was increased in 0.5 kPa (or 0.75 kPa) increments to a
264 maximum of 3.75 kPa during winter and above 4 kPa during summer experiments.

265 Each plant was exposed to a minimum of 7 different VPDs. After measuring whole
266 shoot gas exchange response to changing VPD, each plant was removed from the
267 chamber to determine leaf area (LI-3100C Area Meter, Lincoln, NE, USA). T_{IRGA} did
268 not significantly differ from gravimetrically determined T_r (see Supplemental Fig. 7)

269 To examine the effects of the *Az34* mutation in barley plants, leaf [ABA] was
270 measured as previously described [12]. Frozen leaf tissues were freeze-dried and
271 then powdered in a mortar. The ABA was extracted in distilled water (1:50, w/w) at 4
272 °C overnight in a shaker. ABA concentration was determined in aqueous extracts by
273 a radioimmunoassay with the monoclonal antibody MAC252 as previously described
274 [42]. The assay was conducted with two technical replicates per biological sample
275 (Supplemental table 2).

276 In some experiments, ABA was sprayed on the leaves to inhibit T_r . ABA was
277 dissolved in ethanol to make a stock solution at 0.05 M, which was diluted to 10 μ M in
278 H₂O prior to use. ABA 10 μ M was applied with a wetting agent Silwet (L-77, De
279 Sangosse Ltd, Cambridge, UK) at 0.025 %. We applied 10-15 ml per plant, depending
280 on leaf area, using an atomizer (Perfume Pod, Amazon, UK). ABA-sprayed plants
281 were used to measure whole plant gas exchange after 1 h (Supplemental Fig. 7).

282 Flag leaf gas exchange measurements were also made as part of these
283 experiments spraying ABA over whole plants. The conditions in the LI-6400XT
284 chamber were 1.5 kPa air VPD (to avoid stomatal limitations at high VPD), 500 μ mol
285 s⁻¹ air flow, 400 ppm CO₂, 25°C leaf temperature (same as the *in vitro* Rubisco assay)
286 and 460 μ mol m⁻² s⁻¹ PPFD.

287 Flag leaf Rubisco *in vitro* activity was measured with a non-radioactive
288 spectrophotometric assay with the modifications described by [43, 44]. The assay uses

289 five enzymatic reactions to couple ribulose 1,5-bisphosphate (RuBP) carboxylation
290 and 3-PGA formation to NADH oxidation. Rubisco activity is calculated based on
291 RuBP consumption by monitoring the decrease in NADH concentration in the well,
292 tracking the absorbance at 340 nm using UV-transparent 96-well plates in a microplate
293 reader (SpectroStars, BMG Labtech, Germany) at 25°C. Firstly, leaves were extracted
294 as described by [36]. The Rubisco total activity (V_t) was assayed after incubating the
295 extract for 5 min in the presence of CO₂ and MgCl₂, while the initial activity (V_i) was
296 measured directly after extraction. The Rubisco activation state is the ratio V_i / V_t .

297

298 **Statistical analysis**

299 One- or two-way ANOVA [45] was used to test statistical significance of
300 differences in means of each trait between genotypes or between genotypes and ABA
301 treatments, respectively. Where significance of effects was observed ($P < 0.05$),
302 multiple pairwise comparisons between treatments used the Tukey-b test.

303 To detect the T_{IRGA} breakpoint, the R package “segmented” [46] was used.
304 When the results lacked biological meaning (resulting from statistical artefacts
305 associated with exceeding the VPD operating boundaries of the chamber), or when
306 the slope after the breakpoint was higher than the one before, a linear regression was
307 used. Breakpoint calculations were made for each plant individually (Supplemental
308 tables 3-5). Regression results were confirmed using the software Prism 7 (GraphPad
309 Software Inc., San Diego, USA; Supplemental table 6).

310

311 **RESULTS**

312 **Reliability of the whole plant system**

313 Without a plant inside the chamber, ΔH_2O and ΔCO_2 were stable over
314 time at a steady flow rate (Fig. 2A), and at different flow rates (Fig. 2B), indicating
315 that leaks were absent or minimal. Rapid and large VPD changes (0.5-4 kPa) were
316 possible in just a few min (Supplemental Fig. 5) while maintaining a temperature of
317 $27.5^\circ C \pm 5\%$, which is faster than in previously reported chambers [7,18,34,35].

318 The whole plant system shows similar stability with a plant inside the chamber.
319 Temperature and relative humidity were stable over time (Fig. 2C) because ¹⁾ the
320 system was mounted in a temperature-controlled laboratory, and ²⁾ the pipes were
321 thermally insulated. Moreover, the water bath design (Supplemental Fig. 2) assured
322 stability of VPD (Fig. 2C). With a wheat plant in the chamber, whole plant A_n and
323 T_{IRGA} remained stable over time (Fig. 2D). T_{IRGA} measurements did not produce
324 different results from paired gravimetric measurements (Supplemental Figure 7).

325

326 **Whole plant gas exchange at a single VPD**

327 At a single VPD ($2.5 \text{ kPa} \pm 0.15$) and constant temperature ($27.5^\circ C \pm 1\%$),
328 different wheat cultivars showed significant differences in whole plant gas exchange
329 (Fig. 3). Transpiration varied ca. 17%, with Cadenza having higher T_{IRGA} than
330 Mercato, Zebedee and Gladiator, while Gatsby, Drysdale and Krichauff, had
331 intermediate values (Fig. 3A). Photosynthesis varied ca. 30%, with Gatsby having
332 higher A_n than Gladiator, Krichauff and Cadenza, while Mercato, Zebedee and
333 Drysdale had intermediate values (Fig. 3B). $iWUE$ was more influenced by whole plant
334 A_n than T_{IRGA} (Fig. 3C). Gatsby and Zebedee had higher $iWUE$ than Cadenza,
335 Gladiator and Krichauff, with Mercato and Drysdale having intermediate values. Since

336 whole plant iWUE of Drysdale and Krichauff was similar, their gas exchange was
337 studied under contrasting VPD levels.

338

339 **Effects of changing VPD on whole plant gas exchange**

340 A representative example of the data required to examine the presence of the
341 T_{IRGA} breakpoint (BP) is shown in Fig. 4. Measurements commenced at 2.5 kPa, the
342 VPD experienced by plants in the greenhouse; then VPD was decreased to the
343 minimum achievable in 0.5 kPa steps (Fig. 4A). After that, VPD was increased in 0.5-
344 0.75 kPa steps to a maximum of 3.75 kPa. Air temperature inside the chamber
345 remained stable during data collection (Fig. 4A). Following this protocol, plant gas
346 exchange usually equilibrates within about 15-30 min because of the small (usually
347 0.5 kPa) VPD changes over time, and because, as grasses, *Triticum spp.* show
348 relatively rapid stomatal movement due to their stomatal conformation [47]. Each VPD
349 response curve took 3-4 h, and no pronounced hysteresis was detected when plants
350 were exposed to ascending and descending series of VPDs (Supplemental Fig. 9).
351 To avoid hydraulic limitations of transpiration that occur if the upper layers of the
352 substrate dry out [48], water was added to the pot every hour during measurement
353 until leaching was observed (since the pot could be irrigated without opening the
354 chamber).

355

356 **Restriction of whole plant gas exchange under high vapour pressure deficit**

357 In the wheat cv. Drysdale, T_{IRGA} increased with increasing VPD and showed a
358 BP at 2 ± 0.3 kPa ($R^2 = 0.96$), while in cv. Krichauff, T_{IRGA} increased linearly with VPD
359 ($R^2 = 0.91$) (Fig. 5A,D; Supplemental Table 3), as previously described [16]. Across

360 the entire range of VPDs, T_{IRGA} was significantly ($P = 0.002$) higher in cv. Krichauff
361 than cv. Drysdale. When comparing T_{IRGA} below the Drysdale BP, both cultivars
362 showed similar sensitivity of T_{IRGA} to VPD (same slope, $P = 0.21$). Beyond this BP,
363 the slopes significantly differ ($P = 0.003$) with T_{IRGA} less sensitive to VPD in cv.
364 Drysdale. Drysdale plants had a significantly ($P = 0.003$) higher An. Taken together,
365 cv. Drysdale had a significantly ($P = 0.005$) higher iWUE than cv. Krichauff over the
366 entire VPD range.

367 In wild-type (WT) barley, transpiration increased linearly with VPD up to $1.9 \pm$
368 0.3 kPa ($R^2 = 0.96$), but VPDs above this threshold restricted transpiration (Fig. 5G,
369 Supplemental Table 3). In contrast, transpiration of the ABA-deficient *Az34* barley
370 mutant increased linearly and continuously with increasing VPD ($R^2 = 0.91$) (Fig. 5J).
371 Absolute T_{IRGA} of the *Az34* mutant was similar to WT over the entire VPD range.
372 Before the BP at 1.9 kPa, the slope of the T_{IRGA} versus VPD response was similar (P
373 $= 0.55$) between genotypes; beyond this BP, T_{IRGA} was more sensitive to VPD in the
374 WT ($P = 0.0037$). An was significantly lower in *Az34* than WT plants ($P < 0.001$), and
375 decreased as VPD increased for the mutant only (Fig. 5 H,K; Supplemental Table 4).
376 *Az34* plants had a significantly ($P = 0.002$) lower An than WT both before ($P < 0.001$)
377 and after ($P < 0.001$) the BP (1.9 kPa). In *Az34* plants, iWUE decreased exponentially
378 as VPD increased, while in WT plants, iWUE decreased as VPD increased in the low
379 range, but remained stable once VPDs exceeded the BP (Fig. 5 I,L). Across the entire
380 range of VPDs, iWUE did not significantly differ between genotypes. However, *Az34*
381 had a significantly ($P < 0.001$) lower iWUE after the BP (1.9 kPa). Thus, the importance
382 of ABA in determining iWUE of these genotypes varied according to the VPD.

383

384 **Differences in whole plant gas exchange in response to ABA in an ABA-**
385 **deficient mutant**

386 Before applying ABA and while at 2.5 kPa VPD, T_{IRGA} and A_n were 13% higher
387 in WT than *Az34* plants, while $iWUE$ did not significantly differ between genotypes
388 (Table 2). Foliar ABA application reduced T_{IRGA} within 5 min in both the ABA-deficient
389 mutant *Az34* and WT barley, with T_{IRGA} stabilising after 1 h (Supplemental Fig. 7).
390 Whole plant T_{IRGA} decreased by 40% and 23% in *Az34* and WT plants respectively
391 (Table 2); with the response almost significantly greater in *Az34* ($P = 0.053$ for
392 genotype x ABA interaction). Interestingly, ABA application did not significantly affect
393 A_n of WT plants, but decreased A_n by 30% in *Az34*. ABA treatment increased $iWUE$
394 similarly in both genotypes (no significant genotype x ABA interaction). Taken
395 together, whole plant gas exchange of the ABA-deficient mutant *Az34* was more
396 responsive than WT plants to foliar ABA application.

397

398 **Leaf-level measurements**

399 To further investigate the mechanisms by which ABA limits photosynthesis, the
400 flag leaves of *Az34* and WT plants were sprayed with ABA (Table 3). Stomatal
401 conductance and leaf internal CO_2 concentration (C_i) were 50% and 15% higher,
402 respectively, in *Az34* than WT plants in the greenhouse prior to applying ABA.
403 Following ABA application, both g_s and C_i decreased, more severely in *Az34* plants
404 as indicated by significant ($P < 0.001$ and $P < 0.009$) genotype x ABA interactions
405 (Table 3). *Az34* had ca. 50% less total soluble protein (TSP) and Rubisco V_t than WT
406 plants. *Az34* showed higher Rubisco activation state than WT prior to ABA application,
407 with the opposite observed after ABA application as indicated by the significant ($P <$

408 0.001) genotype x ABA interaction (Table 3). ABA had no significant effect ($P > 0.05$)
409 on TSP or Rubisco V_t for either genotype. However, while activation states were not
410 affected by the ABA treatment in WT plants, *Az34* significantly reduced the activation
411 state by ca. 25% (Table 3). Flag leaf ABA concentration of *Az34* was approximately
412 half of the value in WT plants before spraying, and ABA application increased leaf
413 [ABA] of both genotypes by 6-7 fold (Supplemental Table 5). Taken together, stomatal
414 and photosynthetic responses of *Az34* were more responsive to exogenous ABA
415 spraying, despite similar proportional changes in foliar ABA accumulation.

416

417 **DISCUSSION**

418 **The whole plant chamber can identify genetic diversity in gas exchange**

419 A whole plant gas exchange chamber was adapted to study plant T_{IRGA} , An and iWUE
420 responses to changing VPD. The findings with wheat and barley genotypes support
421 the idea that the chamber enables a robust assessment of these responses. A
422 previous study demonstrated that some wheat genotypes restrict Tr at high VPD, such
423 as cv. Drysdale [16], here we also show that this response significantly improves iWUE
424 since photosynthesis is not limited above the BP. This reinforces the idea that iWUE
425 can be improved by including the restricted transpiration trait at high VPD in those
426 wheat genotypes that do not show it because An is not limited by VPD, making this an
427 effective strategy to implement in breeding programs for drought-prone environments
428 in elite plants [49-50].

429 The whole plant gas exchange system was developed for phenotyping whole
430 plant iWUE at different VPDs, and identified genetic differences. At a single VPD,
431 genotypic differences in An correlated with single-leaf measurements done in field

432 conditions in a previous experiment [36]. Nevertheless, at that specific VPD, a similar
433 T_{IRGA} was found between Drysdale and Krichauff, in contrast with the results when
434 comparing such genotypes under different VPD, reinforcing the importance of the VPD
435 response curves in ranking T_{IRGA} . It is important to note that plants were exposed to
436 high VPD by maintaining air temperatures lower than 30°C, which does not limit wheat
437 photosynthesis [51,52]. However, under natural conditions, high VPD and
438 temperatures occur together, with inhibition of A_n by high VPD attributed to
439 excessively high temperatures [53]. Moreover, under non-steady state conditions, high
440 VPD can constrain photosynthetic induction: the time required to reach the maximum
441 A_n after the transition from low to high light [54]. Taken together, our results show that
442 restricting T_{IRGA} at high VPDs at an optimal temperature range and under steady-state
443 conditions does not affect carbon assimilation in commercial wheat and barley
444 cultivars. It is essential to understand the physiological mechanisms regulating these
445 responses.

446

447 **Determining the role of ABA in VPD responses**

448 Previous measurements at whole plant level using gravimetric methods [8, 41]
449 have implicated ABA in regulating cereal transpiration under varying evaporative
450 demands. Similarly, transpiration of the ABA-deficient barley mutant *Az34* was
451 unrestricted at high VPDs, but unexpectedly, A_n was limited (Fig. 4K; Supplemental
452 Table 3). Single-leaf measurements were required to confirm the mechanistic
453 response to the reduction of photosynthesis in ABA-deficient plants. Despite higher
454 intercellular CO_2 concentrations due to greater stomatal opening, *Az34* had a lower
455 Rubisco activity (ca. 70% reduction compared to WT plants). Since *Az34* is nitrate-

456 reductase deficient [40], plants are expected to be N limited with approximately half
457 the total soluble protein content compared to WT plants (Table 3). Thus, the limited
458 biomass of *Az34* not only results from its inability to control water loss under moderate-
459 high VPD [41, 55], which induces leaf water deficit, but also from reduced Rubisco
460 carboxylation that lowers photosynthesis.

461 To further demonstrate that dynamic whole plant responses can be detected
462 with our system, ABA was sprayed on the leaves [12, 38]. Exogenous ABA application
463 decreased Tr by ca. 25% in WT plants but even more so in *Az34* (by 40%), indicating
464 greater stomatal sensitivity of the ABA-deficient mutant. These differences in whole
465 plant transpiration sensitivity to ABA were confirmed in flag leaves (Table 3). Several
466 ABA-deficient mutants in *Arabidopsis* (*aba2-11*, *nced3 nced 5*, *aba1-1*, *aba4-3*, *aao3-*
467 *2*, *aba3-1*) and other species (*wilty* pea and *flacca* tomato) were described as
468 hypersensitive to exogenous ABA application [35], attributed to a higher pre-treatment
469 gs. Further work is required to investigate possible feedback regulation of genes for
470 ABA sensitivity by ABA status in ABA-deficient mutants.

471 The mechanisms by which exogenous ABA limits photosynthesis remain under
472 debate. While stomatal closure after ABA application decreases C_i even in ABA-
473 deficient mutants ([38]; Table 3 here), the ABA molecule has been suggested to bind
474 to the Rubisco active site blocking Rubisco activity [57]. While foliar ABA spraying did
475 not affect photosynthesis of WT plants (Table 2), A_n was decreased by 30% in *Az34*,
476 as in a previous comparison of WT and ABA-deficient tomatoes (*flacca* mutant) grown
477 under non-saturating light (Bradford et al. 1983). ABA application decreased Rubisco
478 activation state of *Az34* flag leaves but not WT leaves (Table 2). Similar to *in vitro* ABA
479 experiments [57], activation of such plants might be disrupted by the ABA molecule.
480 Alternatively, *Az34* may have higher CO_2 availability under standard conditions in the

481 greenhouse (before ABA application). The larger decreases in g_s and C_i observed in
482 *Az34* after ABA application may deactivate Rubisco because of the limited CO_2
483 availability, thereby decreasing photosynthesis. Whether such limitations occur
484 because stomatal and mesophyll conductance are co-ordinated [58], or due to a
485 mechanistic constraint of ABA on Rubisco activity, is still unknown. In either case, the
486 lower Rubisco activity of *Az34* makes its photosynthesis more vulnerable to
487 environmental constraints, such as high VPD, than WT plants.

488

489 **CONCLUSIONS**

490 Our chamber was designed, built and operated to evaluate whole plant A_n ,
491 T_{IRGA} and $iWUE$ under increasing evaporative demand in small-grain cereals. This
492 instrumentation is sufficiently precise to detect genetic differences in plant responses.
493 In wild-type genotypes, photosynthesis was not restricted by VPD “*per se*”, even
494 though some genotypes restricted T_{IRGA} under high VPD, which is of direct interest to
495 plant breeders seeking to increase $iWUE$. Furthermore, ABA-deficient barley
496 responded more sensitively to exogenous ABA application, with greater transpirational
497 restriction and decreased Rubisco activation state. Photosynthesis of ABA-deficient
498 barley plants was also limited at high VPD, likely due to reduced Rubisco activity.

499

500 **DECLARATIONS**

501 **Author’s contribution**

502 ICD designed the research with input from all authors; IJ developed the system and
503 conducted the experiments with initial input from SAR; IJ analyzed the results; all

504 authors contributed to interpret the results; IJ and ICD wrote the manuscript with
505 contributions from SAR, SHT, MAJP, ECS. All authors read and approved the final
506 manuscript.

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512 **Competing interests**

513 The authors declare that they have no competing interests.

514 **Availability of data and materials**

515 All data generated or analyzed during this study are included in this published article.
516 The datasets used and analyzed during the current study are available from the
517 corresponding author on reasonable request.

518 **Consent for publication**

519 Not applicable.

520 **Ethics approval and consent to participate**

521 Not applicable.

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526

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