

1 **Measuring stress signalling responses of stomata in isolated**
2 **epidermis of graminaceous species.**

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27 Cereal epidermal peel stomatal assays

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31 **Abstract**

32 Our current understanding of guard cell signalling pathways is derived from studies in a small
33 number of model species. The ability to study stomatal responses in isolated epidermis has
34 been an important factor in elucidating the mechanisms by which the stomata of these species
35 respond to environmental stresses. However, such approaches have rarely been applied to
36 study guard cell signalling in the stomata of graminaceous species (including many of the
37 world's major crops), in which the guard cells have a markedly different morphology to those
38 in other plants. Our understanding of guard cell signalling in these important species is
39 therefore much more limited. Here, we describe a procedure for the isolation of abaxial
40 epidermal peels from barley, wheat and *Brachypodium distachyon*. We show that isolated
41 epidermis from these species contains viable guard cells that exhibit typical responses to
42 abscisic acid (ABA) and CO₂, as determined by measurements of stomatal apertures. We use
43 the epidermal peel assay technique to investigate in more detail interactions between different
44 environmental factors in barley guard cells, and demonstrate that stomatal closure in response
45 to external CO₂ is inhibited at higher temperatures, whilst sensitivity to ABA is enhanced at
46 30°C compared to 20°C and 40°C.

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48

49 **Keywords:**

50 Stomata, guard cells, isolated epidermis, cereal, Graminae, abscisic acid, carbon dioxide,
51 temperature

52

53 **Introduction**

54 The maintenance of global food is one of the greatest challenges currently facing plant
55 scientists. Water availability is a major constraint on crop yield (Sinclair and Rufty 2012) and
56 is the single most important factor limiting food production, with significant yield losses
57 reported under water deficit (Boyer 1982; Mueller *et al.* 2012; van Ittersum *et al.* 2013).
58 Stomata play a key role in determining crop water use efficiency (biomass production or
59 yield per unit of water used), through the regulation of the exchange of water vapour and CO₂
60 between plant tissues and the atmosphere (Mansfield *et al.* 1990; Hetherington & Woodward
61 2003; Yoo *et al.* 2009). This gaseous exchange is controlled by the size of the stomatal pore,
62 which is determined by changes in the turgor of the pair of specialised guard cells that
63 surround the pore and which in turn are driven by fluxes of anions and cations (Pandey *et al.*
64 2007; Kim *et al.* 2010; Hedrich 2012; Kollist *et al.* 2014). Guard cells integrate information
65 from a variety of internal and external environmental signals in order to formulate the optimal
66 pore size for a given set of environmental conditions (Mansfield *et al.* 1990; Hetherington &
67 Woodward 2003; Kim *et al.* 2010). For example, stomata close in response to abscisic acid
68 (ABA), produced under conditions of limited water availability, and to elevated CO₂
69 (Mansfield *et al.* 1990; Hetherington & Woodward 2003; Kim *et al.* 2010). In contrast,
70 stomata open at low CO₂ concentrations (Bunce 2007), in high light (Shimazaki *et al.* 2007)
71 and in response to auxin (Acharya & Assmann 2009). Guard cells also respond to other
72 environmental signals, such as the atmospheric pollutant ozone (Vainonen & Kangasjarvi
73 2014) and pathogenic microbes (Sawinski *et al.* 2013), resulting in stomatal closure and
74 thereby preventing entry to the leaf of damaging chemical and biological agents.

75
76 The diversity of stimuli to which stomata respond, together with the ease with which the
77 response can be quantified (*i.e.* changes in stomatal aperture or conductance), have meant that
78 guard cells have been extensively used as a model system for studying signalling pathways in
79 plant cells (Mansfield *et al.* 1990; Hetherington & Brownlee 2004; Kim *et al.* 2010). This has
80 resulted in the elucidation of a complex signalling network controlling the molecular
81 machinery integrating the different signals to which guard cells are exposed in order to
82 regulate guard cell turgor (Mansfield *et al.* 1990; Hetherington & Woodward 2003; Kim *et al.*
83 2010). The ability to measure changes in stomatal aperture in isolated epidermis, in response
84 to externally-applied signals, and to manipulate these responses both pharmacologically and
85 genetically, has been central to the advances in understanding of guard cell signalling that
86 have been made in the last twenty years.

87
88 To date, studies of guard cell signalling have focused on a small number of model species,
89 notably *Vicia faba*, *Commelina communis* and latterly, *Arabidopsis thaliana* (for reviews, see
90 Hetherington & Brownlee 2004; Kim *et al.* 2010). The stomata of all of these species possess
91 kidney bean-shaped guard cells, which are typical of the large majority of plant families,
92 including the mosses, ferns, gymnosperms and most angiosperms (Wilmer & Fricker 1996).
93 However, the stomata of the monocotyledonous family, Graminae (Poaceae; the true grasses),
94 which includes the world's major cereal crops, have a different morphology, possessing
95 characteristic dumb-bell shape guard cells and a pair of specialised subsidiary cells. The
96 different morphology of graminaceous stomata provide them with different mechanical
97 properties, which likely allow them to open and close more rapidly in response to
98 environmental signals (Franks & Farquhar 2007). It is therefore critical to understand fully
99 the molecular mechanisms by which the stomata of the graminaceous species respond to
100 environmental stresses, particularly in relation to the protection of global food security and
101 the challenge of producing “more crop per drop” (Kijne *et al.* 2003) posed by future
102 environmental changes in global temperature, CO₂ levels and water availability.

103

104 Although assays of stomatal responses have been performed using isolated epidermis from
105 maize and wild grasses (Pallaghy 1971; Incoll & Whitelam 1977; Jewer & Incoll 1980;
106 Rodriguez and Davies 1982), in general, graminaceous species are commonly regarded as
107 poorly tractable systems for epidermal peel isolation. Other authors have isolated epidermal
108 tissue for other purposes, such as microscopy (Zou *et al.* 2011) or metabolite analysis (Falter
109 *et al.* 2015), but in these cases, tissues were not demonstrated to be suitable for stomatal
110 assays. Here, we demonstrate that the epidermal peel assay used so extensively in other
111 model systems is also applicable to model grass species, and we use it to identify interactions
112 between temperature and signals stimulating stomatal closure in barley.

113

114

115 **Materials and Methods**

116

117 **Plant material**

118 Seeds of *Brachypodium distachyon* (line Bd21; Vogel *et al.* 2006) were sown in a 1:1 mix of
119 Sinclair multipurpose compost and silver sand (Sinclair Horticultural, UK) and grown under
120 a 16-hour photoperiod at 22°C ±2°C, 70% relative humidity and 120 μmol m⁻² s⁻¹
121 photosynthetic photon flux density (PPFD) in a Microclima growth cabinet (CEC, Glasgow,
122 UK). Wheat (cultivar Cadenza) and barley (cultivars Golden Promise and Optic) were grown
123 in Levington M3 peat-based compost in a heated, passively ventilated glasshouse (minimum
124 temperature 15°C, mean day-time temperature 25°C) with 14 h of supplementary lighting
125 supplied by 125 W 50/60 Hz High output, Correct Spectrum Class 11 energy saving bulbs
126 (wheat) or Osram Greenpower 600 W high pressure sodium lamps (barley). Two days prior
127 to peeling, plants were moved to controlled environment chambers set at 22°C, with a
128 photoperiod of 14 h (wheat) or 16 h (barley) with Osram fluora lamps delivering 70-100
129 μmol m⁻² s⁻¹ light.

130

131 **Preparation of isolated epidermis**

132 Isolated epidermis was obtained from the abaxial surface of the first true leaf of 8-14 day old
133 wheat and barley plants, when the first true leaf had stopped expanding (5-8 cm long). For
134 *Brachypodium distachyon*, the youngest fully expanded leaves of 3 to 4 week old plants were
135 used. Leaves were cut from the plant and bent over the forefinger with the adaxial surface
136 facing upwards. A shallow cut was made with a sharp razor blade horizontally across the leaf
137 and a flap of leaf tissue lifted with a razor, leaving the lower epidermis intact (Fig. 1). The
138 leaf tissue was removed from the epidermis with forceps. Once a section of epidermis
139 approximately 1 cm long was exposed, it was cut from the leaf and floated cuticle-side-up in
140 10 mM MES/KOH (pH 6.2), 50 mM KCl.

141

142 **Viability staining**

143 Isolated epidermis was floated cuticle-side-up in 10 mM MES/KOH (pH 6.2), 50 mM KCl
144 and incubated in a water bath at 22°C at a PPFD of 50-100 μmol m⁻² s⁻¹ provided by an array
145 of 5 fluorescent tube lights (Sylvania White F13W) underneath the tank. Pieces of epidermis
146 were transferred at intervals to a 0.001% (w/v) solution of fluorescein diacetate (FDA) in 10
147 mM MES/KOH (pH 6.2), 50 mM KCl and incubated for 30 minutes prior to observation
148 under the fluorescence microscope (McAinsh *et al.* 1996). The percentage of viable guard
149 cells was determined by comparing the fluorescent images to bright-field images.

150

151 **Promotion of stomatal closure assays**

152 Isolated epidermis was floated cuticle-side-up in Petri dishes containing 10 ml of 10 mM
153 MES/KOH (pH 6.2), 50 mM KCl (a standard buffer to promote stomatal opening; McAinsh
154 *et al.* 1991) under the conditions described above. Microlances, inserted through small holes
155 in the lids, were used to deliver CO₂-free air to the Petri dishes. Pieces of epidermis were
156 incubated for 2 hours to promote stomatal opening, following which, they were transferred to
157 fresh buffer containing the appropriate concentrations of ABA and incubated for a further one
158 (barley) or two (wheat, *Brachypodium*) hours. ABA was diluted from a 10 mM stock of (±)-
159 cis,trans-ABA dissolved in ethanol. Control solutions lacking ABA always contained ethanol
160 equivalent to the concentration of the highest ABA concentration used. For experiments
161 where CO₂ concentration was varied, gas from a balanced CO₂-air cylinder (BOC Industrial
162 Gases, UK) at the appropriate concentration was bubbled through the incubation medium.

163

164 **Measurement of stomatal apertures**

165 Stomatal apertures were measured at the end of the incubation period by mounting pieces of
166 isolated epidermis onto a microscope slide in a drop of assay buffer with a coverslip.

167 Measurements of stomatal apertures were made using an inverted microscope connected to a
168 sideport-mounted video monitor. A calibrated scale was used to make measurements of the
169 width of the stomatal pore directly from the screen. Stomata containing non-viable guard
170 cells (identifiable by FDA staining) were typically fully closed. Fully closed stomata were
171 therefore not used for measurements. Where comparisons of multiple variables measured in
172 experiments conducted at different times were required, stomatal apertures were expressed as
173 relative values. Relative stomatal aperture was defined as the ratio of stomatal aperture
174 measured under a treatment variable to that measured from the relevant control group.

175

176

177 **Results**

178

179 **Guard cells from wheat, barley and *Brachypodium* remain viable in epidermal peels.**

180 Although grasses are less amenable than current model species used for stomatal research, we
181 developed an approach that could be used routinely to generate intact abaxial epidermis
182 isolated from leaves of wheat, barley and *Brachypodium* seedlings (see ‘Materials and
183 Methods’). Peels were free of mesophyll cells and contained viable guard cells, subsidiary
184 cells and pavement cells, as determined by FDA staining. Stomata were significantly larger in
185 barley and wheat (typically around 25-50 µm in length) compared to those in the smaller
186 *Brachypodium* plants (guard cells typically 6 – 9 µm in length. In order to be useful for
187 measuring guard cell-mediated stomatal responses via an *in vitro* assay, it is essential that
188 guard cell viability is maintained in isolated epidermis. We monitored viability over the
189 period when assays are typically performed by performing FDA staining at regular intervals
190 for up to four hours following isolation. Fig. 2 illustrates that guard cell viability was around
191 80% 30 minutes after isolation for all species. This level was maintained throughout the test
192 period for both barley and *Brachypodium*, whilst viability gradually declined for wheat.

193

194 **Cereal leaf epidermis demonstrates stomatal closure in response to ABA and CO₂.**

195 To test the validity of the stomatal assay in isolated epidermis of graminaceous plants, we
196 examined the well-known response of guard cells to ABA in a promotion of closure assay. As
197 expected, we observed that wheat, barley and *Brachypodium* all exhibit a characteristic dose-
198 dependent response to ABA in the epidermal peel assay (Fig. 3). We also measured responses
199 to external CO₂. In comparison with CO₂-free air, stomatal closure was promoted by ambient
200 CO₂ (360 ppm) in barley, but we observed no further response at elevated CO₂ (800 ppm)
201 (Fig. 4A). *Brachypodium* stomatal apertures were also reduced by CO₂ (Fig. 4A). We next

202 examined the interaction between ABA and CO₂ signalling in barley guard cells. We
203 observed a clear additive effect of ABA and CO₂, with lower stomatal apertures at all
204 concentrations of ABA in the presence of either ambient or elevated CO₂ relative to CO₂-free
205 controls (Fig. 4B).

206

207 **Barley responses to ABA and CO₂ are modified by elevated temperature.**

208 Stomatal responses to ABA and CO₂ have been shown to be temperature-dependent (*e.g.*
209 Raschke 1970; Rodriguez and Davies 1982; Spence *et al.* 1984; Honour *et al.* 1995). We
210 therefore used the epidermal peel assay to examine the temperature-dependence of ABA- and
211 CO₂-induced stomatal closure in isolated epidermis of barley. First, we generated dose-
212 response curves for ABA in isolated epidermis incubated at 20, 30 or 40°C. The results
213 presented in Fig. 5A, show that in comparison with the response at 20°C, incubation at 30°C
214 significantly increased the sensitivity of guard cells to ABA. Increasing the temperature to
215 30°C had no effect on apertures in the absence of ABA, but apertures were reduced for all
216 concentrations of ABA tested. By contrast, incubation at 40°C caused a significant increase
217 in apertures in the absence of ABA and with 10⁻⁹ M ABA. At higher ABA concentrations,
218 apertures were similar to, or slightly larger than, those observed at 20°C, suggesting a degree
219 of inhibition of ABA sensitivity at 40°C. We also examined the response of barley guard
220 cells to CO₂ at these three temperatures. Guard cell responses were again temperature-
221 dependent. However, unlike the response to ABA, we observed maximum CO₂-induced
222 stomatal closure at 20°C, with increasing temperatures causing an increasing degree of
223 inhibition of the CO₂ response (Fig. 5B).

224

225

226 **Discussion**

227 With few exceptions, all of the components of the guard cell signalling network have to date
228 been identified in model species with kidney bean shaped guard cells, and similar responses
229 have been assumed for graminaceous species containing dumb-bell shaped guard cells.
230 However, there is increasing evidence for species-specific responses to common regulatory
231 cues, driven by different environmental conditions (Prokic *et al.* 2006; Mori & Murata 2011;
232 Merilo *et al.* 2014). It is therefore important to consider signalling in key crop species as well
233 as laboratory models. Given the current concerns over our ability to increase food production
234 in the face of environmental change and to maintain global food security (van Ittersum *et al.*
235 2013), it is desirable to establish a robust experimental system for investigating guard cell
236 signalling responses to the multiple environmental stresses currently faced by cereal crops.

237

238 The epidermal peel assay has been used to measure stomatal guard cell responses to external
239 stimuli for several decades (Mansfield *et al.* 1990; Kim *et al.* 2010; Kollist *et al.* 2014).
240 Although it has been suggested that the removal of the stomatal complex from the
241 biochemical and physical influences of the mesophyll tissues means that the epidermal peel
242 assay cannot always accurately reflect stomatal responses in intact leaves (Lee & Bowling
243 1992; Roelfsema & Hedrich 2002), it has nevertheless served as an important tool in the
244 elucidation of the complex signalling network within guard cells (Mansfield *et al.* 1990;
245 Hetherington & Brownlee 2004; Kim *et al.* 2010). Despite this fact, few studies have
246 examined the molecular mechanisms by which the dumb-bell shaped guard cells of the
247 Graminae respond to environmental stimuli and whether these reflect our current
248 understanding of the signalling network in the kidney bean-shaped guard cells of the model
249 species studied to date. This has been due largely to graminaceous species being considered
250 intractable to the necessary cell physiological techniques. We have demonstrated here that the
251 isolation of epidermis containing viable guard cells, whilst technically more demanding than

252 in other model species, can be established as a routine technique to permit such
253 investigations.

254
255 Epidermis was most easily obtained from young plants and guard cell viability was capable
256 of being maintained at levels suitable for collection of aperture data from large numbers of
257 stomata. While in all three species tested there was significant variability between individual
258 peels, overall guard cell viability was maintained at around 70-80% for 4 hours in barley and
259 *Brachypodium*, although it declined to around 40% in wheat. This compares to between 85%
260 (*Pisum sativum*) and 100% (*C. communis* and *V. faba*), respectively (Weyers & Travis 1981).
261 All three species exhibited stomatal closure in response to ABA and CO₂, consistent with
262 previous reports of ABA- and CO₂-induced stomatal closure in isolated epidermis of model
263 species with kidney bean-shaped guard cells, (for reviews, see Hetherington & Brownlee
264 2004; Kim *et al.* 2010; Mori & Murata 2011). These responses highlight the epidermal peel
265 assay as a useful tool for dissecting guard cell signalling pathways in grasses.

266
267 We used the epidermal peel assay to measure barley guard cell responses to ABA, CO₂ and
268 temperature alone and in combination. In experiments where we simultaneously applied two
269 closing signals, ABA and CO₂, we observed a simple additive response at 20°C, whereby
270 apertures were smaller in the presence of both ABA and CO₂ than for the individual stimuli.
271 Interactions between either ABA or CO₂ and temperature, however, were more complex.
272 Incubation of epidermal strips under opening conditions (light, CO₂-free air, no ABA) at
273 different temperatures resulted in similar apertures, but with a small but statistically
274 significant increase in aperture at 40°C. Upon addition of ABA, apertures were reduced much
275 more markedly at 30°C than at 20°C or at 40°C. The enhanced closure at 30°C could result
276 either from altered biophysical properties of the stomatal complexes, or from increased
277 sensitivity to ABA. Since the effect was only apparent at 30°C, and not at 40°C, a simple
278 biophysical effect of temperature seems less likely, and we therefore suggest that the
279 temperature-dependency of ABA-induced stomatal closure in barley reflects interactions
280 between temperature and ABA signalling pathways. Similar increases in sensitivity to ABA
281 at elevated temperatures have previously been observed in some dicotyledonous species
282 (Cousson 2003; Honour *et al.* 1995). Interestingly, the response of the Arabidopsis
283 *RESPONSIVE TO DESSICATION 29A (RD29A)* promoter to exogenous ABA was also
284 enhanced at elevated temperature (Xiong *et al.* 1999), suggesting the possibility of a more
285 general increase in ABA sensitivity at elevated temperature.

286
287 Temperature had a different and very pronounced effect on the response of barley guard cells
288 to external CO₂. At 20°C, both ambient and elevated CO₂ treatments resulted in a substantial
289 reduction in stomatal aperture. At 30°C, the effect of ambient CO₂ was strongly diminished,
290 and whilst guard cells still responded to elevated CO₂ at 30°C, responses to both ambient and
291 elevated CO₂ concentrations were lost at 40°C. These observations are consistent with
292 previous work in maize (Raschke 1970) and bean (Spence *et al.* 1984), where a loss of
293 stomatal responses to CO₂ at higher temperatures was found for both species. Since barley
294 guard cells are able to close in response to ABA at 40°C (Fig. 4A), an interaction between
295 temperature and CO₂ signalling pathways again provides the simplest explanation of our data.

296
297 Together, our results clearly demonstrate the suitability of the epidermal peel assay for
298 studying guard cell signalling networks in the dumbbell-shaped guard cells of the Graminae
299 and that these studies can provide important insights into the mechanisms by which the
300 stomata of the world's major cereal crops respond to the multiple stresses resulting from
301 predicted future changes in global temperature, CO₂ levels and water availability (Stocker *et*

302 *al.*, 2013). Such studies will help to inform future strategies for improving the water use
303 efficiency of cereal crops and for mitigating the adverse effects of climate change on cereal
304 crop production.

305

306 **Conflict of interest statement**

307 The authors declare that the research was conducted in the absence of any commercial or
308 financial relationships that could be construed as a potential conflict of interest.

309

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Figure Legends

Figure 1. Technique for removing abaxial epidermis. (A) The first true leaf is removed from the plant, bent over the finger adaxial side up, and a cut made across the lamina using a scalpel blade. The tip of the leaf blade is bent back and forth to detach a small section of the mesophyll from the lower epidermis. (B) The upper layer was then peeled back using forceps to leave the lower epidermis attached to the leaf tip. The size of the epidermal peel obtained typically varies from around 1 to 3 cm. (C) Bright field micrograph illustrating open stomata in a typical abaxial epidermal peel from wheat (20 μm scale bar).

Figure 2. Guard cell viability in epidermal peels. Peels were incubated in CO_2 -free MES-KCl buffer at 22°C and then stained for 30 min in FDA. The x-axis indicates the time from the start of the initial incubation period until microscopic observation of FDA staining. Data represent counts from three areas of approximately 1 mm^2 from each piece of epidermis and three independent biological replicates.

Figure 3. Promotion of closure of cereal stomata by ABA. Following incubation under opening conditions, epidermal peels were exposed to zero (white bars), 10^{-7} M (grey bars), or 10^{-6} M (black bars) ABA at 20°C . Values shown are mean stomatal apertures \pm SE from $n = 240$ (barley), $n = 90$ (wheat), and $n = 120$ (*Brachypodium*) measurements. Letters indicate statistically different means within species, determined using one-way ANOVA and a Tukey post-test.

Figure 4. Responses of cereal stomata to external CO_2 . (A) Following incubation under opening conditions, epidermal peels were exposed to CO_2 -free air, (white bars), or air with ambient CO_2 (grey bar; 360 ppm, barley), or elevated CO_2 (black bars; 800 ppm, barley; 700 ppm, *Brachypodium*) at 20°C . Values shown are mean stomatal apertures \pm SE ($n = 120$). Letters indicate statistically different means within species, determined using one-way ANOVA with Tukey post-test and a Student's *t*-test for barley and *Brachypodium* data respectively. (B) Interaction between CO_2 and ABA. Following incubation under opening conditions, barley epidermal peels were exposed to ABA at the concentrations shown on the x-axis under either CO_2 -free air, (filled circles), or air with ambient (open circles) or elevated CO_2 (filled triangles) at 20°C . Values shown are mean stomatal apertures \pm SE ($n = 240$).

Figure 5. Temperature-dependence of the responses of barley stomata to ABA and CO_2 . (A) Interaction between temperature and ABA. Promotion of closure assays were performed at a range of concentrations of ABA in CO_2 -free air at either 20°C , (filled circles), 30°C (open circles), or 40°C (filled triangles). To normalise for variation between different experiments, stomatal apertures are expressed relative to that of the control (stomatal aperture at 20°C , no ABA). Data from at least 3 sets of independent experiments were pooled and values are means of at least 120 measurements \pm SE. (B) Interaction between temperature and CO_2 . Promotion of closure assays were performed in CO_2 -free air or at ambient or elevated CO_2 at either 20°C , (filled circles), 30°C (open circles), or 40°C (filled triangles). To normalise for variation between different experiments, stomatal apertures are expressed

482 relative to that of the control (stomatal aperture at 20°C, no CO₂). Data from at least 3 sets of
483 independent experiments were pooled and values are means of at least 120 measurements ±
484 SE.