

1 **Stem girdling uncouples soybean stomatal conductance from leaf water potential by**  
2 **enhancing leaf xylem ABA concentration**

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11

12 **Abstract**

13 To understand the impact of shoot-to-root ABA transport on water potential of, and xylem  
14 ABA concentration in, different plant tissues during soil drying, soybean (*Glycine max*  
15 cv. Siverka) plants were subjected to drought and girdling in a factorial experiment.  
16 Girdling was achieved by surgically excising the phloem tissue from just above the  
17 cotyledonary node. After girdling and withholding water, ABA concentrations were  
18 determined in xylem saps extracted from individual leaves, detached shoots and de-  
19 topped roots, after measuring stomatal conductance ( $g_s$ ), tissue water potentials, and root  
20 ABA concentrations. Soil drying decreased water potential throughout the plant and  
21 approximately doubled xylem ABA concentrations, coinciding with stomatal closure.  
22 Girdling slightly enhanced water potential, especially in droughted plants. Girdling  
23 diminished the soil-drying induced increase in xylem sap ABA concentration, and  
24 completely prevented root tissue ABA accumulation. Furthermore, girdling decreased  
25 root ABA concentration and increased leaf xylem ABA concentration of well-watered  
26 (WW) plants. Stomatal conductance declined linearly with leaf water potential only in  
27 intact plants, while  $g_s$  declined as leaf xylem ABA concentration increased, independently  
28 of girdling. Thus shoot to root ABA transport not only determines (soil-drying induced)  
29 root ABA accumulation, but also limits ABA accumulation in the shoot to maintain  
30 stomatal opening of WW plants.

31 **Key words:** gas exchange, water status, ABA transport, water stress

32

## 33 **1. Introduction**

34 Soybean is one of the most important crops in the world, but its production is often limited  
35 by drought (Doss *et al.*, 1974; Eck *et al.*, 1987; Liu *et al.*, 2003a; Pardo *et al.*, 2015). Soil  
36 water deficits developing during critical stages of reproductive development can limit  
37 seed set, induce pod abortion and decrease individual seed dry weight, thereby decreasing  
38 soybean yield (Liu *et al.*, 2003a; Pardo *et al.*, 2015). Understanding the physiological and  
39 molecular responses to drought offers opportunities to enhance soybean drought tolerance  
40 by overexpressing key regulatory genes, including those that determine plant hormone  
41 status (Manavalan *et al.*, 2009). Plant hormones control multiple physiological and  
42 developmental processes that determine crop yields (Morgan and King, 1984; Li *et al.*,  
43 2013). Abscisic acid (ABA) is a key phytohormone involved in regulating plant water  
44 status by controlling stomatal aperture (Tardieu *et al.*, 1996; Schurr and Schulze, 1996;  
45 Wilkinson & Davies, 2002) and leaf and root hydraulic conductance (Pantin *et al.*, 2013;  
46 Dodd, 2013).

47

48 During water deficit, ABA concentrations increase throughout the plant, partially closing  
49 the stomata which acts to maintain leaf water status (Liu *et al.*, 2003b; Liu *et al.*, 2005),  
50 but there has been considerable debate as to which organ (roots *versus* shoots) is the first  
51 to perceive soil drying (cf. Kramer, 1988; Passioura, 1988). It was proposed that ABA is  
52 primarily synthesized in the root, then transported in the xylem sap to the shoot where it  
53 accumulates in the leaf apoplast to initiate stomatal closure (Davies and Zhang, 1991),  
54 thus reducing transpiration. Root ABA concentration increases as soil water content and  
55 root water potential decreases (Zhang and Davies, 1989; Puertolas *et al.*, 2013),  
56 suggesting that soil drying increases root ABA biosynthesis. Root ABA concentrations  
57 are linearly related to the concentrations of ABA detected in xylem sap, suggesting that  
58 roots are an important source of xylem ABA (Liang *et al.*, 1997). Moreover, the  
59 concentrations of ABA found in the leaf xylem sap are sufficient to close the stomata of  
60 species such as maize (Zhang and Davies, 1991) and pea (Rothwell *et al.*, 2015), as  
61 determined by experiments that measure the transpiration of detached leaves supplied  
62 with synthetic ABA via the xylem. Nevertheless, in some species, xylem sap ABA  
63 concentrations are insufficient to explain stomatal closure (Munns and King, 1988) and  
64 adding osmotica to the roots caused shoot ABA accumulation prior to any root ABA  
65 accumulation (Christmann *et al.*, 2005). Such observations have challenged the concept

66 of root-to-shoot ABA signalling and prompted the search for other xylem-borne  
67 antitranspirants.

68

69 A further challenge to the concept of root-to-shoot ABA signalling comes from  
70 experiments that have suppressed shoot-to-root ABA transport by girdling (removal of  
71 stem phloem tissue at the root-shoot junction). Using this technique, different studies have  
72 demonstrated the importance of shoot-sourced ABA in explaining root ABA  
73 accumulation in response to water stress induced by chilling (Vernieri *et al.*, 2001) or  
74 drought (Liang *et al.*, 1997; Manzi *et al.*, 2015). In contrast, stem girdling had minimal  
75 effects on root ABA accumulation in both *Xanthium* and tomato, with dehydrated roots  
76 of stem-girdled plants showing 80% of the root ABA accumulation (averaged across both  
77 species) of intact plants (Cornish and Zeevaart, 1985), indicating root-autonomous ABA  
78 biosynthesis. These contrasting results demonstrate the need to further investigate the  
79 origin of the ABA accumulated in roots in response to drought.

80

81 Furthermore, the impact of obstructing the phloem flow on shoot ABA accumulation  
82 remains unclear. Early studies show that petiole girdling can stimulate ABA accumulation  
83 in leaf laminae and trigger stomatal closure (Setter *et al.*, 1980; Setter and Brun, 1981),  
84 while others show that stem girdling has no significant effect on leaf ABA accumulation  
85 (Vernieri *et al.*, 2001; Manzi *et al.*, 2015). In contrast, stem girdling stimulated  
86 pronounced (50% increase) foliar ABA accumulation in young vegetative tissues while  
87 ABA concentrations of mature leaves almost halved (Rivas *et al.*, 2011), indicating that  
88 the effect of girdling on ABA accumulation may intensify with distance from the wound  
89 site. This may be related to basipetal gradients in foliar ABA concentration (Mitchell *et*  
90 *al.*, 2016) and xylem ABA concentration (Soar *et al.*, 2004), which seem important in  
91 regulating stomatal responses. Root xylem ABA concentrations explained more of the  
92 variation in drought-induced stomatal closure than bulk leaf ABA concentration in  
93 soybean (Liu *et al.*, 2003a; b) and other species (Zhang and Davies, 1990). Nevertheless,  
94 the impact of stem girdling on leaf xylem ABA concentration has not yet been  
95 investigated.

96

97 To assess these questions, soybean plants were exposed to a factorial combination of soil  
98 drying and stem girdling. Stomatal conductance was measured daily and water relations  
99 / xylem ABA concentration measured in different parts of the plant (roots, shoots, leaves)

100 to evaluate the dependence of ABA accumulation on tissue water relations. It was  
101 hypothesised that shoot to root ABA transport determines ABA distribution in the plant  
102 and thus stomatal responses to soil drying.

103

## 104 **2. Materials and methods**

### 105 **2.1. Plant materials and experiment design**

106 Soybean (*Glycine max* L. Merr. cv. Siverka) seeds were germinated in the dark on  
107 moistened filter paper for 3 days, then sown in pots which fitted perfectly inside a  
108 Scholander-type pressure chamber (Soil Moisture Equipment Crop., Santa Barbara, CA,  
109 USA). Pots were 6.5 cm in diameter and 23 cm in length (762 cm<sup>3</sup> in volume), with a  
110 steel mesh (0.7 mm aperture) base to allow drainage. Pots were filled with an organic  
111 loam (John Innes No. 2, J. Arthur Bowers, UK), watered to the drip point and then  
112 seedlings of uniform development (radical length 30-50 mm) transplanted.

113

114 Plants were grown in a naturally lit greenhouse with an average daytime temperature of  
115  $27 \pm 2^\circ\text{C}$ , with a relative humidity of 30-40% and supplementary lighting providing a  
116 PPFD at bench height of 250-400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for a 13 h photoperiod (0700-2000h). A  
117 commercial liquid fertilizer Miracle-Gro (24:8:16 N:P:K) was applied once to the plants  
118 at the appearance of the first trifoliolate leaf. All the plants were irrigated to drained  
119 capacity at 1600h daily (by replacing evapotranspirational losses, determined  
120 gravimetrically). During expansion of the third trifoliolate leaf, the plants were randomized  
121 into 4 groups, comprising the treatments applied: soil drying (WW: well-watered; DR:  
122 droughted) and girdling (NG: intact plants; G: Girdled plants) respectively. Five plants  
123 from each treatment were harvested each day. Girdling was achieved surgically (at 1400h  
124 on Day 0), when the third trifoliolate leaf was completely expanded, by excising 10 mm of  
125 phloem tissue from the stem (at 100-110 mm above the soil surface) with a sharp razor  
126 blade. Plants were girdled between the cotyledonary node and the second node, where the  
127 unifoliolate leaf was located. At this time, the cotyledons had either naturally abscised or  
128 were excised, to prevent them influencing root hormone concentrations (Waadt *et al.*,  
129 2014). Water was withheld from half of the girdled and non-girdled plants after the  
130 girdling was complete on Day 0. Thus 20 hours elapsed between girdling and stomatal  
131 conductance measurements on the following day (Day 1).

132

133 **2.2. Physiological measurements**

134 Measurements were made on the third trifoliolate leaf (when it was completely expanded)  
135 throughout the experiment. Stomatal conductance ( $g_s$ ) was measured daily at 1000h  
136 (except on Day 0 that was at 1200h) on the central leaflet of the third trifoliolate leaf with  
137 a porometer (Model AP4, Delta-T Devices, Burwell, UK). Two measurements were  
138 sequentially made on each plant and averaged.

139

140 Leaf, shoot and root water potential were measured with a Scholander-style pressure  
141 chamber (Soil Moisture Equipment Crop., Santa Barbara, CA, USA). After measuring  
142 stomatal conductance, the leaf was excised at the petiole junction with the stem, then leaf  
143 water potential measured. Then the shoot was de-topped 6-7 cm from the stem base (in  
144 the middle of the girdled tissue to avoid phloem contamination of xylem sap samples)  
145 and placed in the pressure chamber to measure shoot water potential. Finally the entire  
146 pot was sealed in the chamber with sufficient stem protruding to measure root water  
147 potential. For all water potential measurements, the chamber was gradually pressurized  
148 at  $0.03 \text{ MPa s}^{-1}$  until the meniscus of the sap appeared, at which time the pressure was  
149 recorded.

150

151 Once the water potential of each organ was measured, xylem sap was collected at 0.3  
152 MPa overpressure (Dodd, 2007) above the balancing pressure. Xylem sap was collected  
153 in Eppendorf vials and immediately frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$  for  
154 further analysis. On the last day of harvest, when the soil volume was extracted from the  
155 pot, 15-20 mg (dry weight – determined retrospectively) of the root system was removed  
156 from the middle of the pot, briefly washed (to remove adhering soil debris), then frozen  
157 in liquid nitrogen. After measuring root water potential (and collecting root samples on  
158 the last day of the experiment), the entire soil volume was removed from the pot, weighed  
159 and then placed in a drying oven until constant weight, to calculate gravimetric soil water  
160 content ( $\theta$ ) with the following relationship:

161

162 
$$\text{Soil Water Content } (\theta) = (\text{Fresh soil weight} - \text{Dry soil weight}) / \text{Dry soil weight}$$

163

164 ABA was determined using a radioimmunoassay using the monoclonal antibody  
165 MAC252 (Quarrie *et al.*, 1988). While the sap samples were measured without further

166 purification, the root tissue samples were lyophilized and finely ground. Deionized water  
167 was added (1:50 weight ratio), the sample incubated on a shaker at 4°C overnight, then  
168 centrifuged to collect the aqueous extract.

169

### 170 **2.3. Statistical analysis.**

171 The experiment was repeated twice with qualitatively similar results, thus data from a  
172 single experiment are presented. Two-way analysis of variance (ANOVA) determined the  
173 effects of water treatment, girdling and their interaction. Heterogeneous groups were  
174 separated by Tukey's Honestly Significant Difference (HSD) test ( $P < 0.05$ ) to  
175 discriminate differences between treatment x girdling combinations. Analysis of  
176 covariance (ANCOVA) and regression analyses determined whether girdling affected  
177 relationships between plant and soil variables (eg. Fig. 6; 7 and Table 1; 2 respectively).

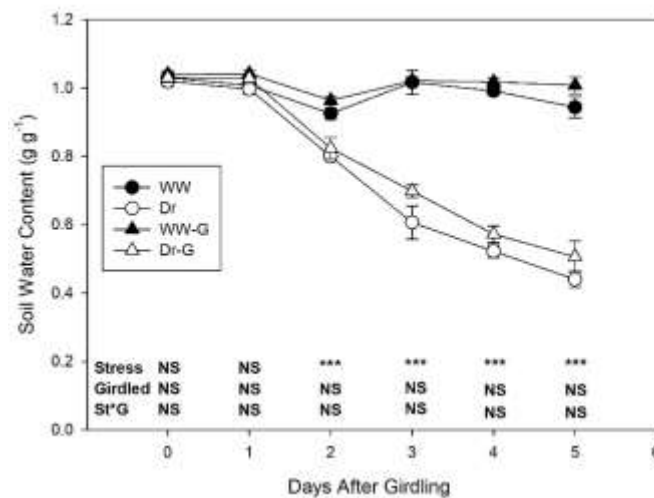
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## 179 **3. Results**

### 180 **3.1. Soil water status**

181 Soil water content of both well-watered treatments remained around 1 g g<sup>-1</sup> during the  
182 experiment (Fig. 1). Withholding water for 5 days decreased soil water content similarly,  
183 by *circa* 60% compared to well-watered plants, in both droughted treatments. Girdling  
184 had no significant effect on soil water dynamics during the experiment, even if  
185 droughted–girdled plants dried the soil slightly slower.

186



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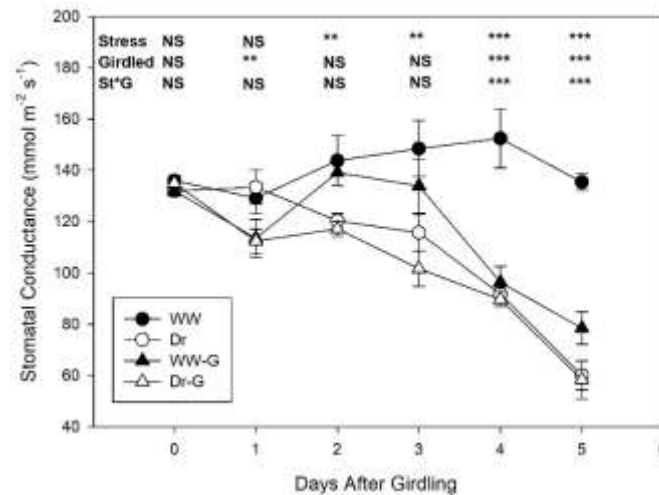
188 **Figure 1.** Soil Water Content during the experiment, with water withheld from droughted  
 189 plants, and girdling on Day 0. Measurements on Day 0 were done before imposing  
 190 treatments. Filled circles and filled triangles represent well-watered intact and girdled  
 191 plants (WW and WW-G) respectively, while hollow circles and hollow triangles represent  
 192 droughted intact and girdled plants (Dr and Dr-G) respectively. Symbols indicate mean  $\pm$   
 193 s.e. (n=5). Effects of watering treatment (Stress or St), girdling (Girdled or G) and their  
 194 interaction are indicated thus: NS, non-significant; \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

195

### 196 3.2. Effect of girdling and soil drying on plant responses

197 Stomatal conductance ( $g_s$ ) of well-watered, intact plants remained between 130 and 150  
 198  $\text{mmol m}^{-2} \text{s}^{-1}$  during the experiment, unlike the other treatments (Fig. 2). One day after  
 199 girdling,  $g_s$  decreased by 15% (averaged across both water treatments). Girdling  
 200 significantly decreased  $g_s$  of well-watered plants 4 days after girdling, and was almost  
 201 half that of well-watered intact plants at the end of the experiment. Soil drying decreased  
 202  $g_s$  within 2 days of withholding water, and  $g_s$  steadily decreased during the experiment in  
 203 both girdled and intact plants. Towards the end of the experiment, the effects of girdling  
 204 on stomatal conductance depended on soil water status (significant girdling x treatment  
 205 interaction), since girdling substantially decreased  $g_s$  of well-watered plants but had no  
 206 significant effect on plants in drying soil.

207



208

209 **Figure 2.** Stomatal conductance during the experiment, with water withheld from  
 210 droughted plants, and girdling on Day 0. Measurements on Day 0 were done before  
 211 imposing treatments. Filled circles and filled triangles represent well-watered intact and  
 212 girdled plants (WW and WW-G) respectively, while hollow circles and hollow triangles  
 213 represent droughted intact and girdled plants (Dr and Dr-G) respectively. Vertical bars  
 214 indicate mean  $\pm$  s.e. (n=5). Effects of watering treatment (Stress or St), girdling (Girdled  
 215 or G) and their interaction are indicated thus: NS, non-significant; \*  $P < 0.05$ ; \*\*  $P < 0.01$ ;  
 216 \*\*\*  $P < 0.001$ .

217

218 Soil drying decreased water potential of all tissues (Fig. 3). Soil drying decreased leaf  
 219 water potential ( $\Psi_{\text{leaf}}$ ) throughout the experiment, such that  $\Psi_{\text{leaf}}$  was 0.1 MPa and 0.2  
 220 MPa lower than well-watered plants for girdled and intact plants respectively (Fig. 3a).  
 221 Girdling increased  $\Psi_{\text{leaf}}$  by 0.12 MPa (averaged across both water treatments) on Day 3  
 222 and increased  $\Psi_{\text{leaf}}$  of plants grown in drying soil on Day 5. On Day 5, the effects of  
 223 girdling on  $\Psi_{\text{leaf}}$  depended on soil water status (significant girdling x treatment  
 224 interaction) since girdling had no effect on  $\Psi_{\text{leaf}}$  of well-watered plants but significantly  
 225 increased  $\Psi_{\text{leaf}}$  of plants in drying soil.

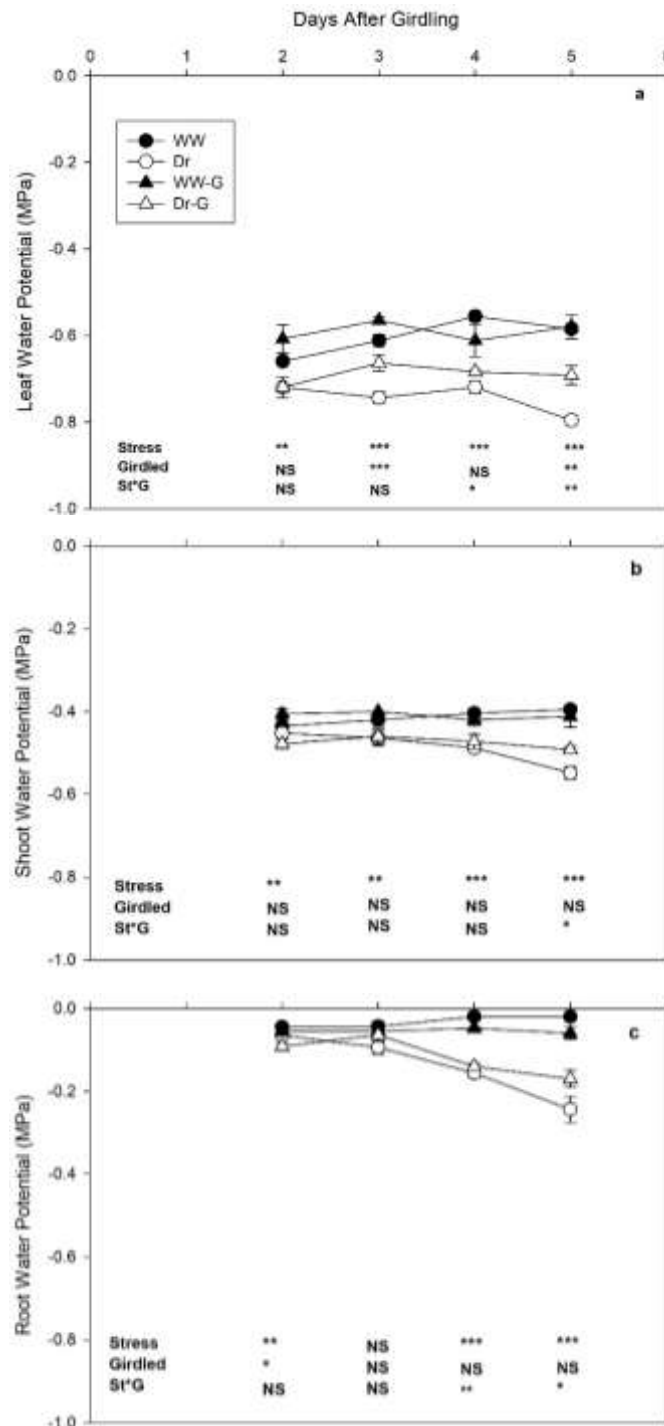
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227 Similarly, the effects of girdling on shoot water potential ( $\Psi_{\text{shoot}}$ ) on Day 5 depended on  
 228 soil water status, even though girdling had no significant effect throughout the  
 229 experiment. Soil drying decreased  $\Psi_{\text{shoot}}$  by 0.15 MPa (intact plants) and 0.08 MPa  
 230 (girdled plants) during the experiment.

231



232 Root water potential ( $\Psi_{\text{root}}$ ) did not differ between the two groups of well-watered plants  
233 throughout the experiment. Soil drying significantly decreased  $\Psi_{\text{root}}$  on Days 2, 4 and 5  
234 after withholding water. At the end of the experiment, soil drying decreased  $\Psi_{\text{root}}$  to -0.22  
235 and -0.11 MPa in intact and girdled plants respectively (Fig. 3c). On the last two days of  
236 the experiment, the effect of soil drying on  $\Psi_{\text{root}}$  depended on girdling (significant girdling  
237 x treatment interaction) such that girdling decreased the  $\Psi_{\text{root}}$  of well-watered plants (by  
238 0.04 MPa) but increased the  $\Psi_{\text{root}}$  of plants in drying soil (by 0.07 MPa). Taken together,  
239 soil drying decreased  $\Psi$  throughout the plant, but girdling mitigated this effect in all  
240 tissues, especially on the last day of measurements.  
241



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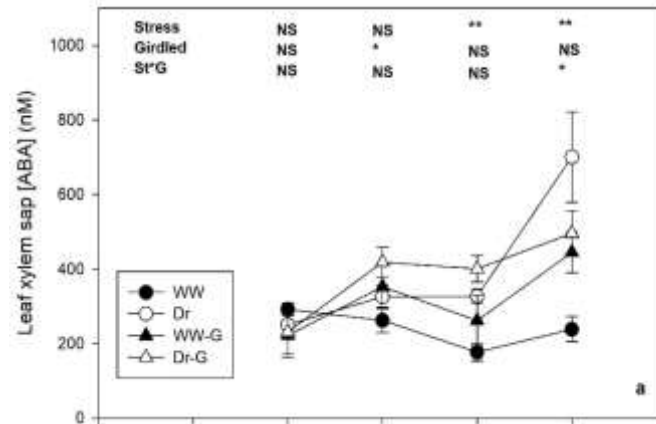
245 **Figure 3.** Leaf (a), Shoot (b) and Root (c) Water Potential during the experiment, with  
 246 water withheld from droughted plants, and girdling on Day 0. Filled circles and filled  
 247 triangles represent well-watered intact and girdled plants (WW and WW-G) respectively,  
 248 while hollow circles and hollow triangles represent droughted intact and girdled plants  
 249 (Dr and Dr-G) respectively. Vertical bars indicate mean  $\pm$  s.e. (n=5). Effects of watering  
 250 treatment (Stress or St), girdling (Girdled or G) and their interaction are indicated thus:  
 251 NS, non-significant; \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

252

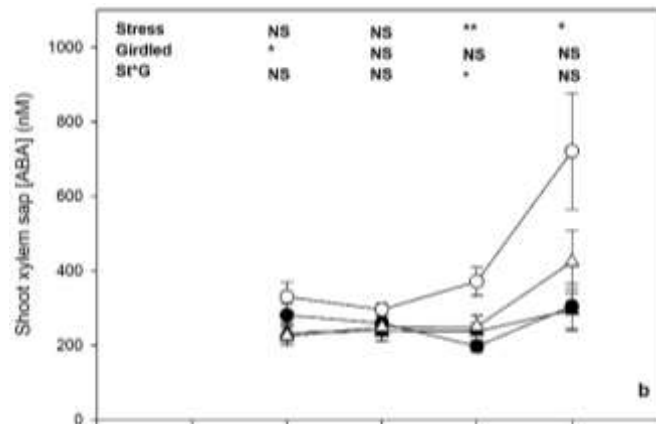
253 In well-watered intact plants, xylem sap ABA concentrations were stable throughout the  
254 experiment, averaging 126, 260 and 242 nM in samples collected from the roots, shoots  
255 and leaves respectively (Fig. 4). In well-watered plants, girdling increased leaf xylem sap  
256 ABA concentration (by 60% averaged over Days 3-5 of the experiment) (Fig. 4a), had no  
257 effect on shoot xylem ABA concentration (Fig. 4b) and decreased root xylem sap ABA  
258 concentration (by 66% averaged over the entire experiment) (Fig. 4c) compared with  
259 well-watered intact plants. Girdling decreased root xylem ABA concentration within two  
260 days.

261

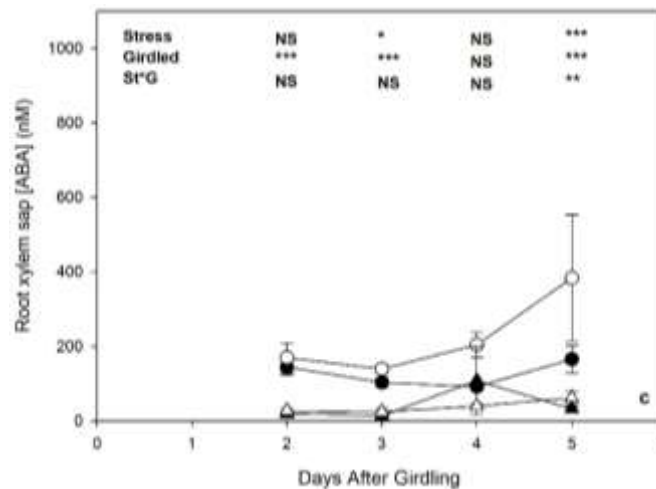
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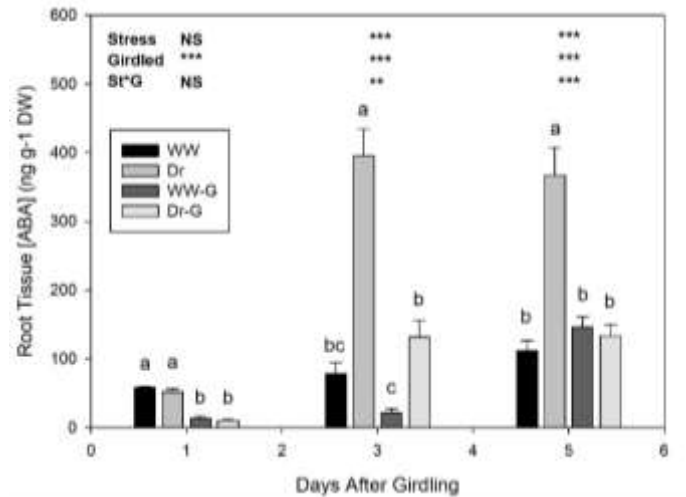
265 **Figure 4.** Leaf (a), Shoot (b) and Root (c) xylem sap ABA concentration during the  
 266 experiment, with water withheld from droughted plants, and girdling on Day 0. Filled  
 267 circles and filled triangles represent well-watered intact and girdled plants (WW and  
 268 WW-G) respectively, while hollow circles and hollow triangles represent droughted  
 269 intact and girdled plants (Dr and Dr-G) respectively. Vertical bars indicate mean  $\pm$  s.e.  
 270 (n=5). Effects of watering treatment (Stress or St), girdling (Girdled or G) and their  
 271 interaction are indicated thus: NS, non-significant; \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .  
 272

273 In intact plants, soil drying increased root, shoot and leaf xylem ABA concentrations  
274 within 3-4 days of withholding water, with significant differences from well-watered  
275 plants first detected in root xylem ABA concentration. By the end of the experiment, soil  
276 drying increased root and shoot xylem ABA concentrations by 2.3-fold and in the leaf by  
277 3-fold compared to well-watered intact plants. Girdling attenuated this soil-drying  
278 induced increase throughout the plant, such that at the end of the experiment, root, shoot  
279 and leaf xylem ABA concentrations were 84, 42 and 30% lower than in intact plants  
280 exposed to soil drying. Indeed, on Day 5, girdling resulted in well-watered plants and  
281 those exposed to drying soil having statistically similar xylem ABA concentrations  
282 throughout the plant.

283

284 Girdling decreased root ABA concentration by nearly 80% (compared to intact plants)  
285 within 20 hours (Day 1), a disparity that was maintained in well-watered plants on Day 3  
286 (Fig. 5). In intact plants, 3 days of soil drying increased root ABA concentration by 4-  
287 fold compared to well-watered plants, but the magnitude of this increase was attenuated  
288 in girdled plants (3-fold increase). Thus well-watered intact plants and girdled plants  
289 exposed to drying soil had statistically similar root ABA concentrations on Day 3.  
290 Significant drought-induced root ABA accumulation occurred in intact plants also on Day  
291 5, while in girdled plants an increase in root ABA concentration of well-watered plants  
292 resulted in no statistical differences from those exposed to soil drying. By Day 5, only  
293 intact plants exposed to soil drying had higher root ABA concentrations than the other  
294 treatments. Thus girdling decreased root ABA concentration of well-watered plants  
295 shortly after treatment (Days 1, 3), and attenuated drought-induced root ABA  
296 accumulation.

297



298

299 **Figure 5.** Root tissue ABA concentration of well-watered intact plants (WW), well-  
 300 watered girdled plants (WW-G), droughted intact plants (Dr) and droughted girdled plants  
 301 (Dr-G) during the experiment. Vertical bars indicate mean  $\pm$  s.e. (n=5). Different letters  
 302 indicate significant differences ( $P < 0.05$ ) according to the Tukey's test on each day.  
 303 Effects of watering treatment (Stress or St), girdling (Girdled or G) and their interaction  
 304 are indicated thus: NS, non-significant; \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

305

### 306 3.3. Relationship of stomatal conductance to different variables

307 Stomatal conductance decreased as leaf water potential decreased in intact plants (Table  
 308 1), although girdling attenuated stomatal sensitivity to leaf water potential (significant  
 309 girdling  $\times$   $\Psi_{leaf}$  interaction - Fig. 6a). In contrast, girdling did not affect the relationships  
 310 between stomatal conductance and either leaf xylem ABA concentration (Fig. 6b) or soil  
 311 water content (Fig. 6c). Stomatal conductance of individual well-watered plants varied 3-  
 312 fold (with the lowest values in girdled plants), but was not related to soil water content,  
 313 while soil drying below  $0.6 \text{ g g}^{-1}$  significantly decreased  $g_s$ . Thus girdling altered stomatal  
 314 response to leaf water potential (Table 1), but not other putative regulatory variables.

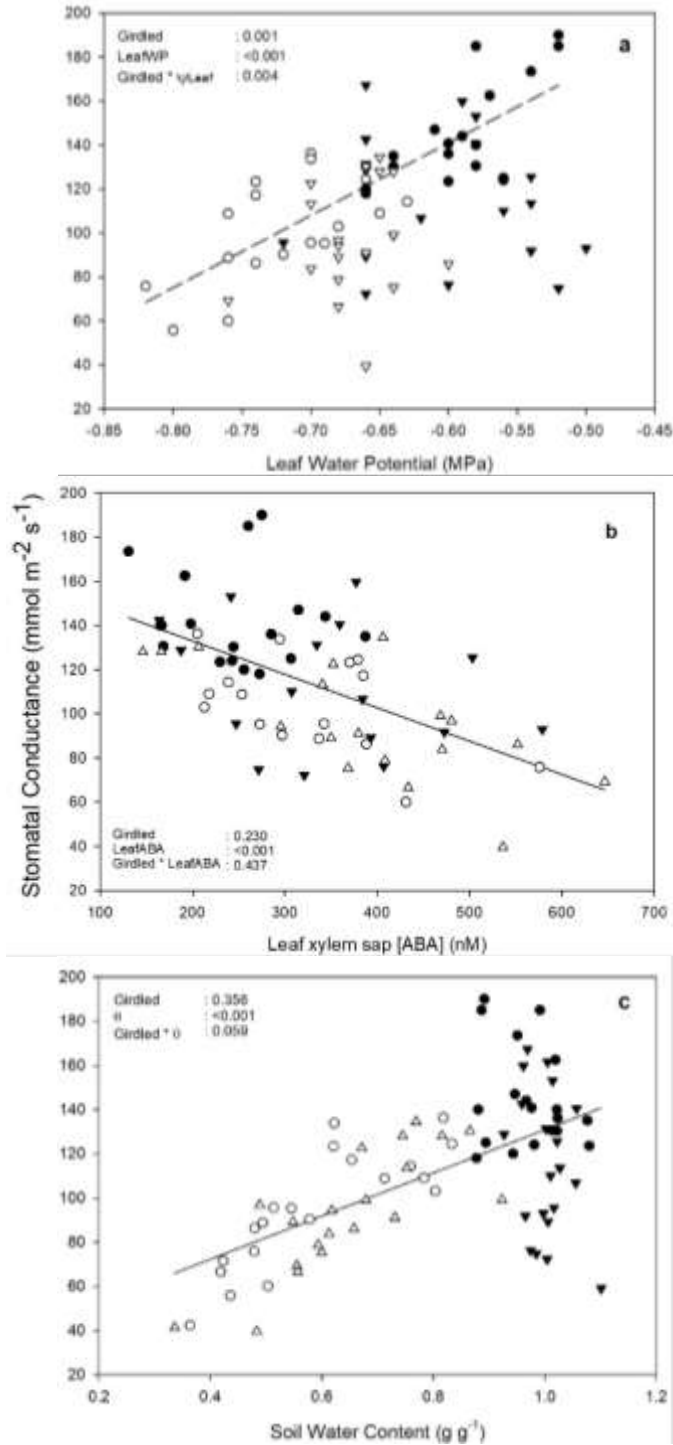
	Girdled plants		Intact plants		All plants	
	$p$ -value	$r^2$	$p$ -value	$r^2$	$p$ -value	$r^2$
$g_s$ vs $\Psi_{leaf}$	0.348	0.03	<0.001	0.70		
$g_s$ vs Leaf xylem [ABA]	0.001	0.28	0.001	0.28	<0.001	0.34
$g_s$ vs $\theta$	0.001	0.24	<0.001	0.64	<0.001	0.38

315 **Table 1.** Linear regression values ( $p$ -value and  $r^2$ ) for the relationships between stomatal  
 316 conductance ( $g_s$ ) and leaf water potential ( $\Psi_{leaf}$ ), leaf xylem sap [ABA] and soil water

317 content ( $\theta$ ) in girdled plants, intact plants and all plants. Each column represents all values  
 318 from girdled plants, intact plants and all plants. Where a significant girdling x x-variable  
 319 interaction exists (indicating that girdling affects the relationship), it is inappropriate to  
 320 pool data for “all plants”.

321

322



323

324

325 **Figure 6.** Relationships between stomatal conductance and leaf water potential (a), leaf  
 326 xylem sap [ABA] (b) and soil water content (c). Filled circles and filled triangles represent

327 well-watered intact and girdled plants (WW and WW-G) respectively, while hollow  
 328 circles and hollow triangles represent droughted intact and girdled plants (Dr and Dr-G),  
 329 respectively. Each symbol is an individual plant and regression lines were fitted to intact  
 330 plants (a) and all data (b, c) where  $P < 0.05$ .  $p$ -values determined by ANCOVA for each  
 331 main effect ( $x$ -variable and girdling) and their interaction are reported.

332

### 333 3.4. Effect of soil drying on xylem ABA concentration

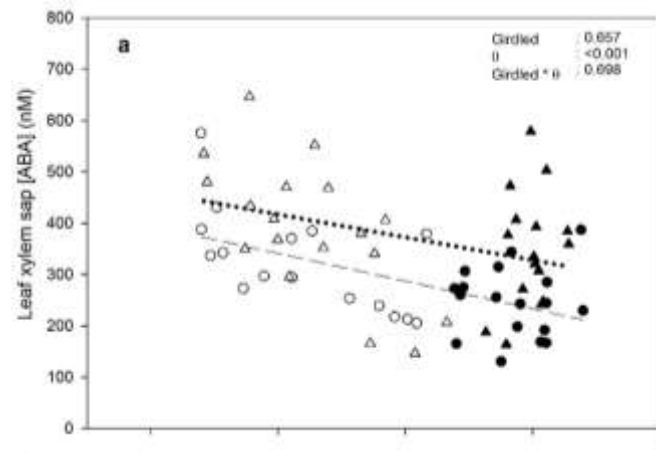
334 Girdling resulted in no significant relationships between tissue water status and xylem  
 335 ABA concentrations of those tissues (Table 2). Although leaf xylem ABA concentration  
 336 was not significantly related to leaf water potential in intact plants, shoot and root xylem  
 337 ABA concentrations significantly increased as shoot and root water potentials decreased  
 338 (Table 2). In all tissues, xylem ABA concentration increased as the soil water content  
 339 decreased in intact plants (Table 2; Fig. 7). Although girdling did not significantly affect  
 340 the relationships between leaf and shoot xylem ABA concentrations and soil water  
 341 content (Fig. 7a, b), it attenuated the sensitivity of root xylem ABA concentration to the  
 342 soil water content (significant girdling x soil water content interaction). Thus soil drying  
 343 increased root xylem sap [ABA] to a greater extent (4.6-fold) in intact plants than girdled  
 344 plants (Fig. 7c).

	Girdled plants		Intact plants		All plants	
	$p$ -value	$r^2$	$p$ -value	$r^2$	$p$ -value	$r^2$
<b>Leaf xylem [ABA] vs <math>\theta</math></b>	0.043	0.12	<0.001	0.37	<0.001	0.17
<b>Shoot xylem [ABA] vs <math>\theta</math></b>	0.045	0.11	<0.001	0.42	<0.001	0.25
<b>Root xylem [ABA] vs <math>\theta</math></b>	<0.001	0.28	<0.001	0.41		
<b>Leaf xylem [ABA] vs <math>\Psi_{\text{leaf}}</math></b>	0.952	0.00	0.155	0.06	0.408	0.01
<b>Shoot xylem [ABA] vs <math>\Psi_{\text{shoot}}</math></b>	0.854	0.00	0.045	0.12	0.106	0.04
<b>Root xylem [ABA] vs <math>\Psi_{\text{root}}</math></b>	0.309	0.03	<0.001	0.34	0.004	0.12

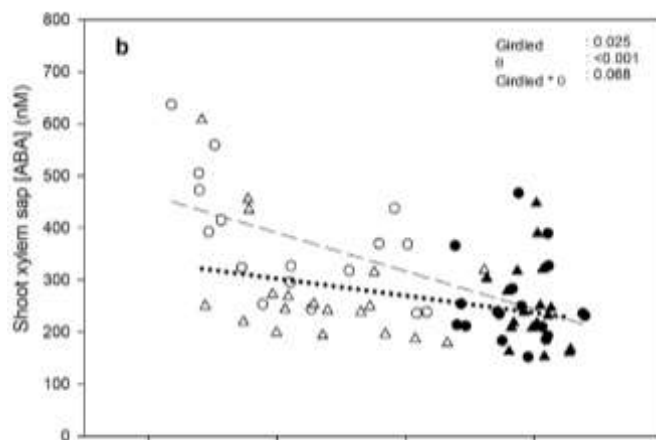
345 **Table 2.** Linear regression values ( $p$ -value and  $r^2$ ) for the relationships between leaf  
 346 xylem sap [ABA], shoot xylem sap [ABA], root xylem sap [ABA] and soil water content  
 347 ( $\theta$ ) and leaf / shoot / root water potential ( $\Psi_{\text{leaf}} / \Psi_{\text{shoot}} / \Psi_{\text{root}}$ ). Each column represents all  
 348 values from girdled plants, intact plants and all plants. Where a significant girdling x  $x$ -  
 349 variable interaction exists (indicating that girdling affects the relationship), it is  
 350 inappropriate to pool data for “all plants”.



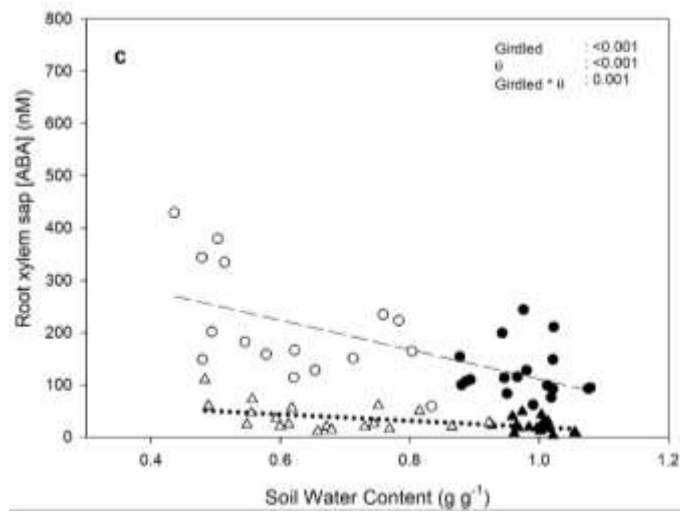
351



352



353



354

355 **Figure 7.** Relationships between leaf xylem sap [ABA] (a), shoot xylem sap [ABA] (b),  
356 root xylem sap [ABA] (c) and soil water content. Filled circles and filled triangles  
357 represent well-watered intact and girdled plants (WW and WW-G) respectively, while  
358 hollow circles and hollow triangles represent droughted intact and girdled plants (Dr and  
359 Dr-G), respectively. Each symbol is an individual plant and regression lines (dashed lines  
360 = intact plants; dotted lines = girdled plants) were fitted where  $P < 0.05$ .  $p$ -values

361 determined by ANCOVA for each main effect ( $x$ -variable and girdling) and their  
362 interaction are reported.

363

#### 364 **4. Discussion**

365 Recent studies emphasise the importance of foliar [ABA] in regulating stomatal  
366 conductance (Bauer *et al.*, 2013; McAdam and Brodribb, 2018). Increased foliar ABA  
367 levels have been correlated with decreased leaf water status (Sack *et al.*, 2018, Pierce and  
368 Raschke, 1980, McAdam and Brodribb, 2016). Our results show a unifying relationship  
369 between  $g_s$  and leaf xylem [ABA] irrespective of whether the plants were girdled (Fig.  
370 6b), whereas  $g_s$  was only correlated with  $\Psi_{\text{leaf}}$  in intact plants (Fig. 6a), suggesting that  
371 foliar [ABA] regulates stomatal aperture regardless leaf water status when shoot to root  
372 ABA transport is interrupted. Similarly, frequent measurements of both variables as the  
373 soil dries demonstrated that leaf xylem ABA concentration increases prior to any change  
374 in  $\Psi_{\text{leaf}}$  (Liu *et al.*, 2005) and better explained early stomatal closure (than leaf ABA  
375 levels) during the initial stages of soil drying (Liu *et al.*, 2003a, b). Moreover, girdling  
376 increased  $\Psi_{\text{leaf}}$  in both drying soil (Fig. 3a) and under well-watered conditions (Setter *et al.*,  
377 1980; Mitchell *et al.*, 2016), while promoting ABA accumulation and stomatal  
378 closure, suggesting that ABA-mediated stomatal closure acted to maintain  $\Psi_{\text{leaf}}$ . Indeed,  
379 in other species, soil drying induced stomatal closure can be associated with increased  
380  $\Psi_{\text{leaf}}$  (Kudoyarova *et al.*, 2007; Visentin *et al.*, 2016) suggesting that  $\Psi_{\text{leaf}}$  can be  
381 regulated by stomatal response. Taken together, these studies suggest that leaf ABA  
382 accumulation is not always associated with decreased leaf water status but in some  
383 situations can also be determined by ABA transport to and from the leaf.

384

385 Since ABA is an important stomatal regulator, it is necessary to understand where in the  
386 plant it is produced. By compromising communication between the aerial part of the plant  
387 and the roots via the phloem, stem girdling attenuated (Day 3) or eliminated (Day 5) root  
388 ABA accumulation in response to drying soil (Fig. 5). Similarly, girdled citrus plants  
389 showed attenuated root ABA accumulation following an initial (3 day) soil drying cycle,  
390 but following a 3 day recovery (re-watered soil) period, no drought-induced ABA  
391 accumulation during a subsequent drying cycle (Manzi *et al.*, 2015). Furthermore, stem  
392 girdling attenuated root hormone export to the shoot via the xylem as the soil dried (Fig.  
393 7c). Drying soil increased xylem sap ABA concentrations irrespective of sampling

394 position in intact plants, but girdling attenuated the increase in xylem ABA concentration  
395 as the soil dried (Fig. 4). This suggests that root ABA export partially depends on shoot-  
396 to-root ABA transport in the phloem (Slovik *et al.*, 1995). Recycling of ABA between  
397 phloem and xylem in the roots made a variable contribution to the root-to-shoot ABA  
398 signal depending on soil water status, comprising 45 and 72% of root ABA export under  
399 salinized and non-salinized conditions respectively (Wolf *et al.*, 1990). The remaining  
400 contribution originated from *de novo* root ABA biosynthesis, which was accentuated  
401 when roots were exposed to salinity. Taken together, *de novo* ABA synthesis in the roots  
402 makes a variable contribution to root ABA accumulation and xylem export, with clear  
403 impacts during the early stages of soil drying seemingly being abolished following more  
404 intense (Fig. 5) or repeated (Manzi *et al.*, 2015) soil drying, as time since girdling  
405 increased.

406

407 Similarly, girdling eliminated root ABA accumulation in bean plants exposed to chilling  
408 temperatures (Vernieri *et al.*, 2001) and when citrus plants were repeatedly exposed to  
409 soil drying (Manzi *et al.*, 2015), with girdling attenuating root ABA accumulation during  
410 an initial drying cycle. This temporal response was initially interpreted as being due to a  
411 limited supply of (unspecified) ABA precursors from the shoot (Ren *et al.*, 2007; Manzi  
412 *et al.*, 2015), but further studies in citrus did not find a direct relationship between  
413 carotenoid abundance and root ABA biosynthesis (Manzi *et al.*, 2016). The physiological  
414 significance of species differences in the ability of roots for *de novo* ABA synthesis in  
415 response to soil drying requires additional experiments to determine its local (eg. root  
416 hydraulic conductance) and long-distance (eg. stomatal conductance) physiological  
417 effects.

418

419 Leaf xylem ABA concentration increased even in well-watered, girdled plants (Fig. 4a)  
420 despite no significant root ABA export (Fig. 4c). It is therefore important to distinguish  
421 whether elevated leaf xylem ABA concentrations reflect *in situ* leaf ABA synthesis.  
422 Xylem sap collected by pressurising detached leaves (as conducted here) comes from  
423 both apoplastic and symplastic sources (Hartung *et al.*, 1988; Borel and Simonneau,  
424 2002). Collecting large sap volumes (relative to apoplastic volume) from small leaves  
425 (which is often necessary to ensure sufficient sap volume for ABA analysis) increases the  
426 contribution of symplastic (membrane-filtered) sap, ensuring that leaf xylem sap ABA  
427 concentrations are closely related to leaf tissue ABA concentrations (Borel and

428 Simonneau, 2002). Thus the higher shoot and leaf xylem ABA concentrations (Fig. 4)  
429 likely reflect relative tissue ABA concentrations, since leaves have much higher ABA  
430 concentrations than roots (Liang *et al.*, 1997; Liu *et al.*, 2005; Manzi *et al.*, 2015). Thus  
431 phloem transport of ABA to the roots not only determines root ABA accumulation (Manzi  
432 *et al.*, 2015; McAdam *et al.*, 2016) but also leaf xylem ABA concentration, suggesting  
433 that much of the ABA in the xylem sap is actually shoot-sourced.

434

435 Alternatively, increased shoot ABA levels in well-watered girdled plants may represent  
436 a wound response (Hildmann *et al.*, 1992), even though wounding more commonly elicits  
437 the synthesis of other signalling hormones as jasmonic acid (JA) and its precursor the  
438 oxylipin 12-OPDA (Savchenko *et al.*, 2014). Since both xylem-borne ABA and JA act as  
439 antitranspirants (De Ollas *et al.*, 2018), synthesis of jasmonates in response to girdling  
440 may explain the lower stomatal conductance occurring one day after girdling (Fig. 2),  
441 likely prior to any xylem ABA accumulation (Fig. 3). Nevertheless, the sustained  
442 decrease in  $g_s$  of well-watered girdled plants after Day 3 coincides with increased leaf  
443 xylem ABA concentration (cf. Fig. 2, 4a). Moreover, the consistent relationship between  
444 leaf xylem ABA concentration and stomatal conductance independent of girdling (Fig.  
445 6b) suggests that hormonal synthesis induced by girdling had no long-term influence on  
446 the regulation of stomatal conductance.

447

448 In conclusion, shoot-sourced ABA was necessary to allow root accumulation in response  
449 to soil drying (Fig. 5), and maintain root-to-shoot ABA signalling in response to soil  
450 drying (Fig. 7c) in soybean. Shoot to root ABA translocation also maintained high  
451 stomatal conductance by preventing increases in foliar ABA concentration under well-  
452 watered conditions.

453

## 454 **5. Acknowledgements**

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457

458 **Highlights**

- 459 -Girdling eliminates shoot-to-root ABA transport, altering plant physiology.  
460 -Girdling decreased stomatal conductance by increasing leaf xylem ABA concentration.  
461 -Girdling decreased root ABA concentration of well-watered plants.  
462 -Soil-drying induced root ABA accumulation requires shoot-to-root ABA transport.  
463 -Soil water content better explained variation in [ABA] than tissue water status.

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