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

Pedro Castro [A, B, C], Jaime Puertolas [A] and Ian C. Dodd [A, C]

[A] Lancaster Environment Centre, Lancaster LA1 4YQ, United Kingdom
[B] School of Life Sciences and Centre for Soybean Research of the State Key Laboratory of Agrobiotechnology, The Chinese University of Hong Kong, Sha Tin, Hong Kong Special Administrative Region, China
[C] The Joint Institute for the Environmental Research and Education, Guangzhou, China
Correspondence author email: p.castrovaldecantos@lancaster.ac.uk

Abstract

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

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

1. Introduction

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

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

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

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

To assess these questions, soybean plants were exposed to a factorial combination of soil drying and stem girdling. Stomatal conductance was measured daily and water relations / xylem ABA concentration measured in different parts of the plant (roots, shoots, leaves) to evaluate the dependence of ABA accumulation on tissue water relations. It was hypothesised that shoot to root ABA transport determines ABA distribution in the plant and thus stomatal responses to soil drying.

2. Materials and methods

2.1. Plant materials and experiment design

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

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

2.2. Physiological measurements

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

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

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

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

ABA was determined using a radioimmunoassay using the monoclonal antibody MAC252 (Quarrie *et al.*, 1988). While the sap samples were measured without further purification, the root tissue samples were lyophilized and finely ground. Deionized water was added (1:50 weight ratio), the sample incubated on a shaker at 4°C overnight, then centrifuged to collect the aqueous extract.

2.3. Statistical analysis.

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

3. Results

3.1. Soil water status

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

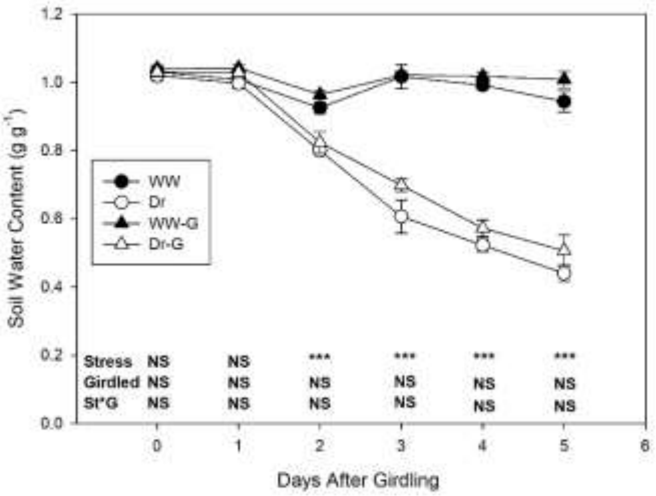

Figure 1. Soil Water Content during the experiment, with water withheld from droughted plants, and girdling on Day 0. Measurements on Day 0 were done before imposing treatments. Filled circles and filled triangles represent well-watered intact and girdled plants (WW and WW-G) respectively, while hollow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

3.2. Effect of girdling and soil drying on plant responses

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

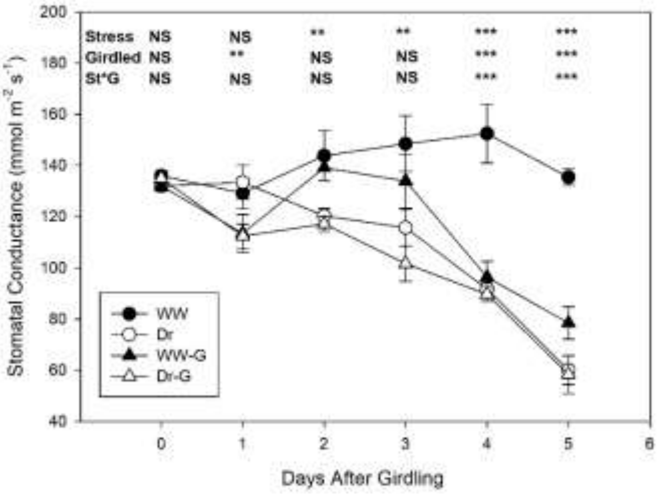

Figure 2. Stomatal conductance during the experiment, with water withheld from droughted plants, and girdling on Day 0. Measurements on Day 0 were done before imposing treatments. Filled circles and filled triangles represent well-watered intact and girdled plants (WW and WW-G) respectively, while hollow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

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

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

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

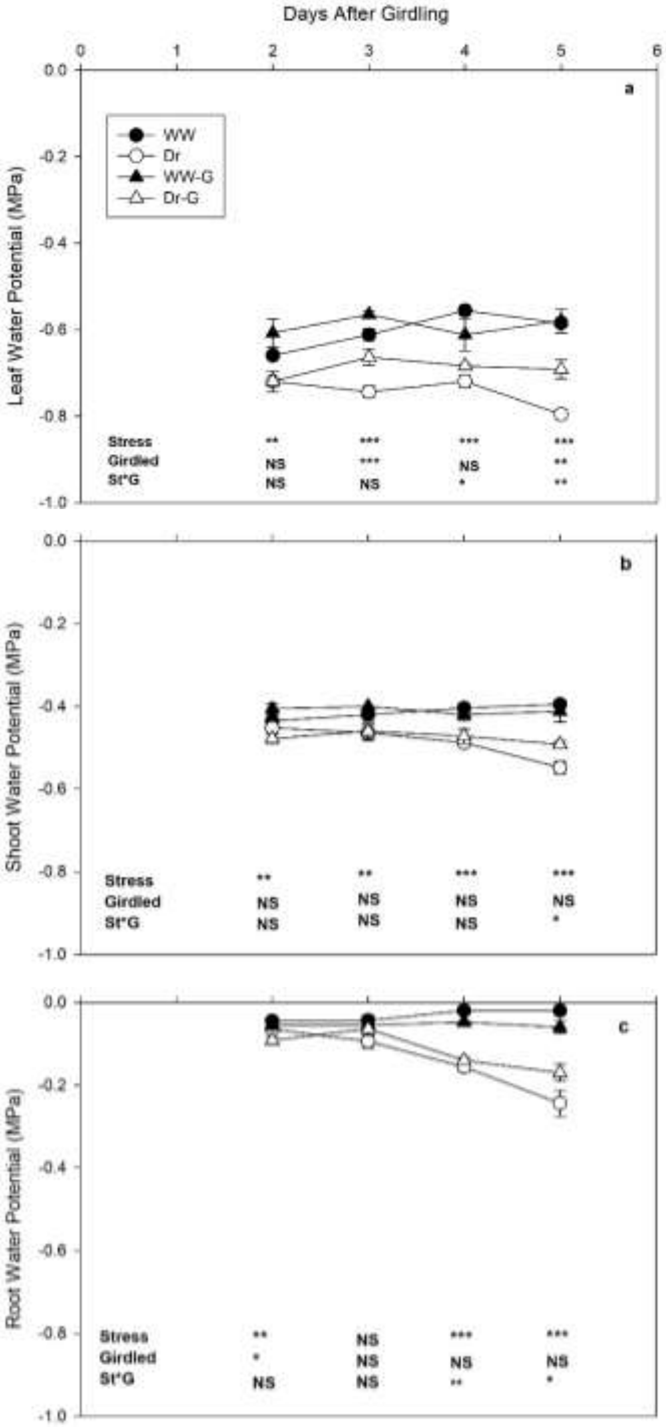

Figure 3. Leaf (a), Shoot (b) and Root (c) Water Potential during the experiment, with water withheld from droughted plants, and girdling on Day 0. Filled circles and filled triangles represent well-watered intact and girdled plants (WW and WW-G) respectively, while hollow circles and hollow triangles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

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

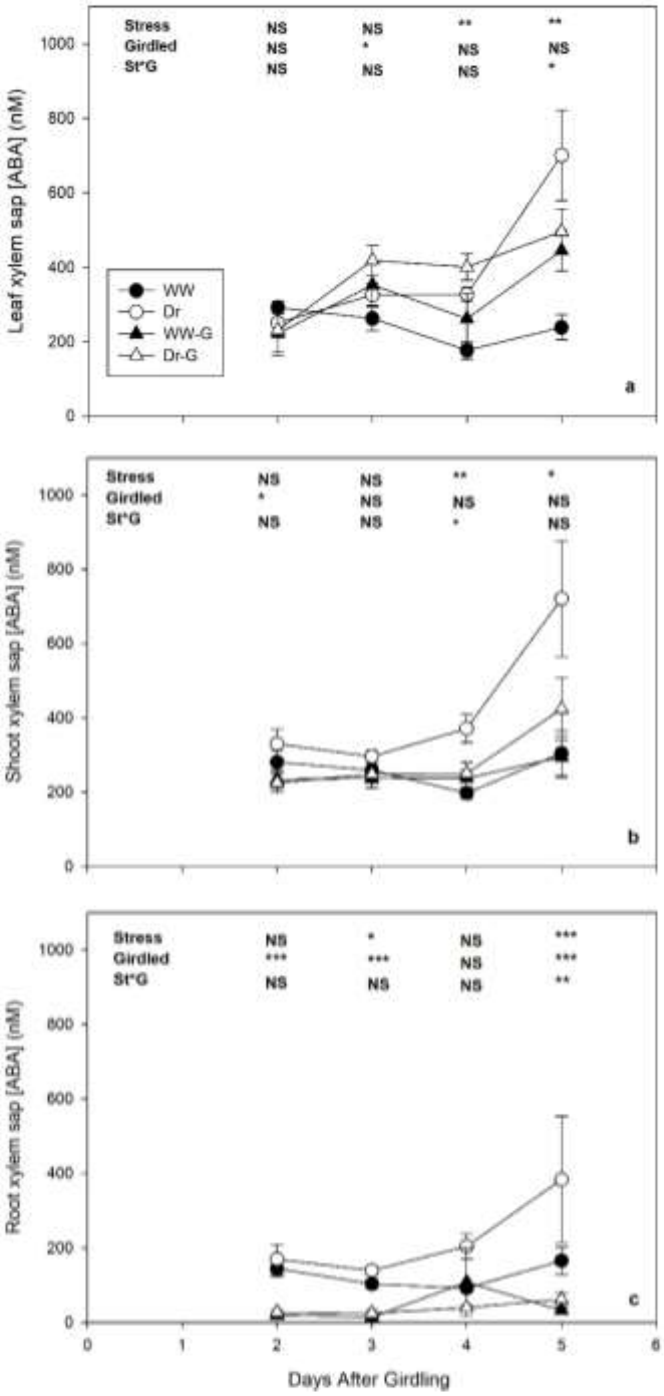

Figure 4. Leaf (a), Shoot (b) and Root (c) xylem sap ABA concentration during the experiment, with water withheld from droughted plants, and girdling on Day 0. Filled circles and filled triangles represent well-watered intact and girdled plants (WW and WW-G) respectively, while hollow circles and hollow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

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

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

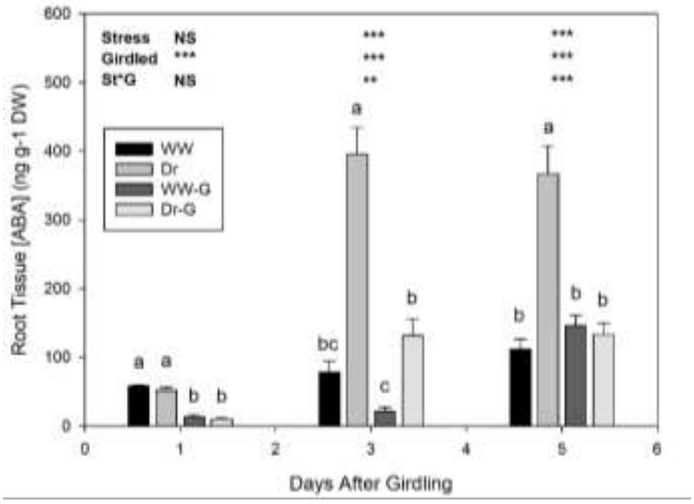

Figure 5. Root tissue ABA concentration of well-watered intact plants (WW), well-watered girdled plants (WW-G), droughted intact plants (Dr) and droughted girdled plants (Dr-G) during the experiment. Vertical bars indicate mean ± s.e. (n=5). Different letters indicate significant differences ($P < 0.05$)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

3.3. Relationship of stomatal conductance to different variables

Stomatal conductance decreased as leaf water potential decreased in intact plants (Table 1), although girdling attenuated stomatal sensitivity to leaf water potential (significant girdling x Ψ_{leaf} interaction - Fig. 6a). In contrast, girdling did not affect the relationships between stomatal conductance and either leaf xylem ABA concentration (Fig. 6b) or soil water content (Fig. 6c). Stomatal conductance of individual well-watered plants varied 3-fold (with the lowest values in girdled plants), but was not related to soil water content, while soil drying below 0.6 g g^{-1} significantly decreased g_s. Thus girdling altered stomatal 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 Ψ_{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 θ	0.001	0.24	<0.001	0.64	<0.001	0.38

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

content (θ) in girdled plants, intact plants and all plants. Each column represents all values from girdled plants, intact plants and all plants. Where a significant girdling x *x*-variable interaction exists (indicating that girdling affects the relationship), it is inappropriate to pool data for "all plants".

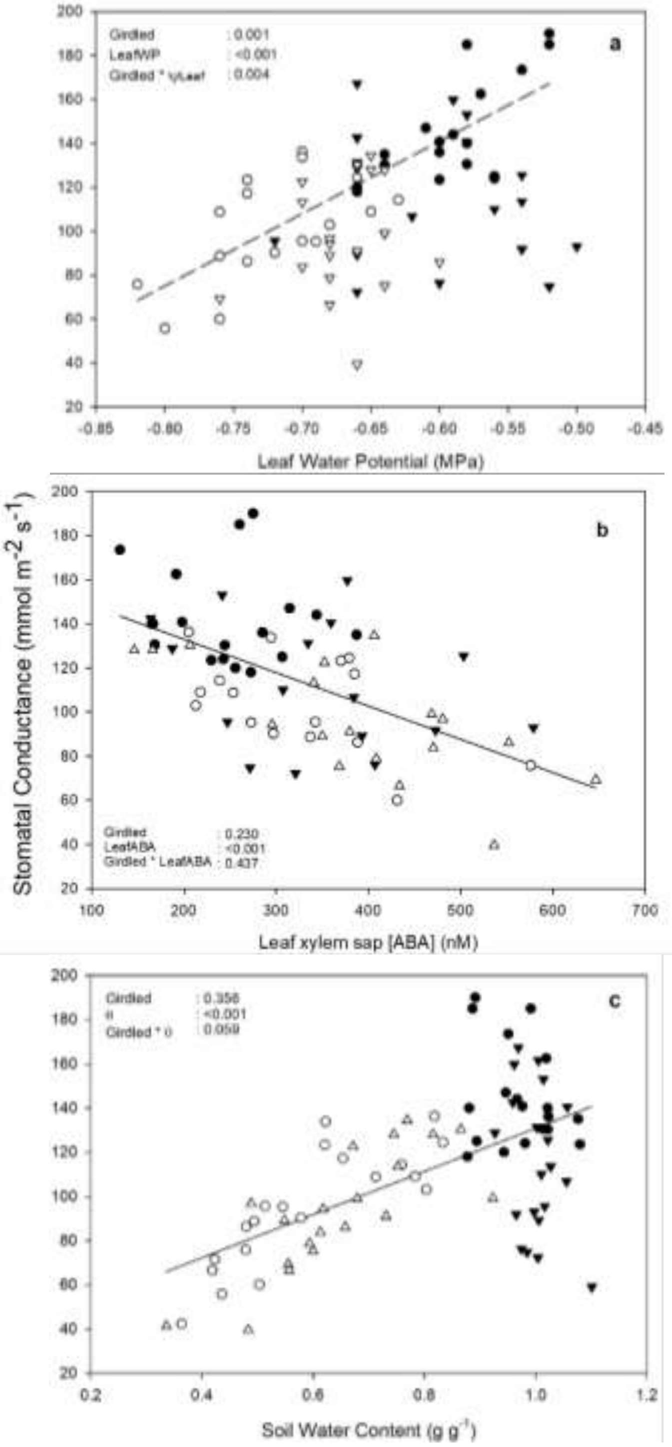

Figure 6. Relationships between stomatal conductance and leaf water potential (a), leaf xylem sap [ABA] (b) and soil water content (c). Filled circles and filled triangles represent

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

3.4. Effect of soil drying on xylem ABA concentration

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

	Girdled plants		**Intact plants**		**All plants**	
	p-value	r^2	p-value	r^2	p-value	r^2
Leaf xylem [ABA] vs θ	0.043	0.12	<0.001	0.37	<0.001	0.17
Shoot xylem [ABA] vs θ	0.045	0.11	<0.001	0.42	<0.001	0.25
Root xylem [ABA] vs θ	<0.001	0. 28	<0.001	0.41		
Leaf xylem [ABA] vs Ψ_{leaf}	0.952	0.00	0.155	0.06	0.408	0.01
Shoot xylem [ABA] vs Ψ_{shoot}	0.854	0.00	0.045	0.12	0.106	0.04
Root xylem [ABA] vs Ψ_{root}	0.309	0.03	<0.001	0.34	0.004	0.12

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

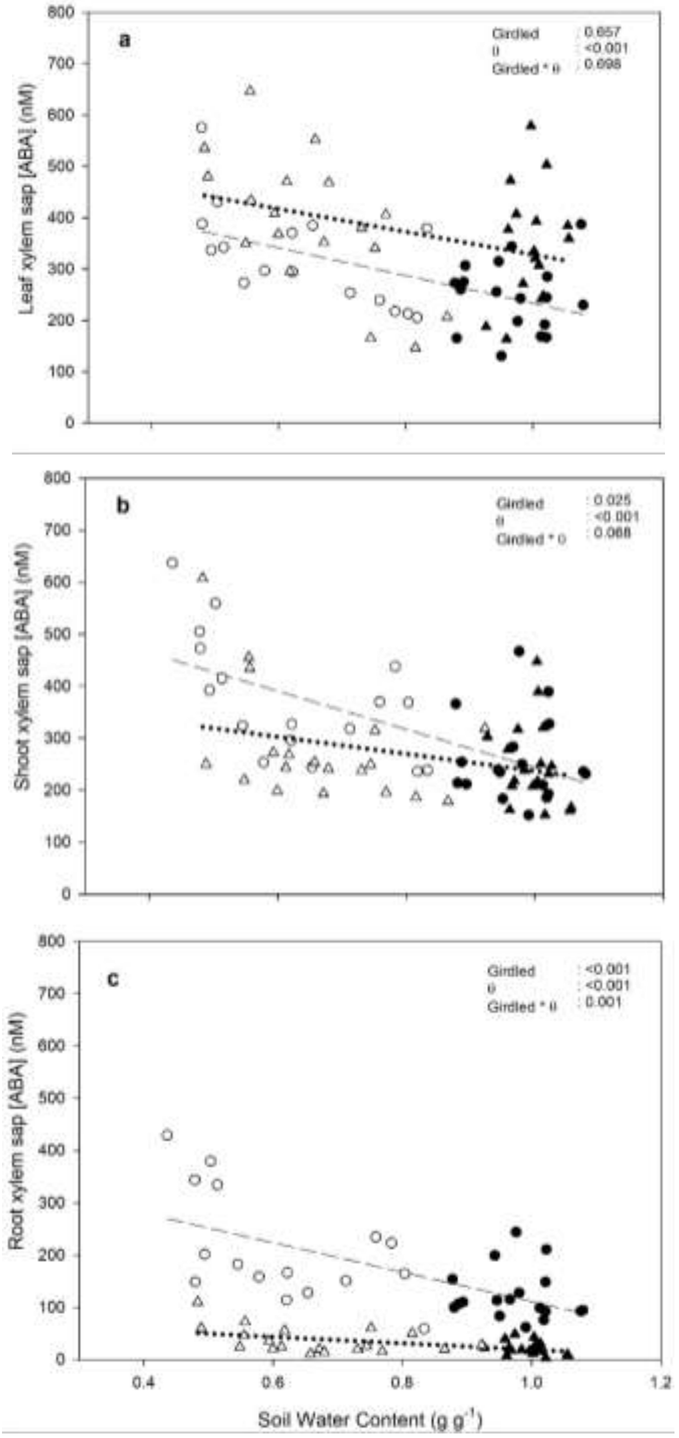

Figure 7. Relationships between leaf xylem sap [ABA] (a), shoot xylem sap [ABA] (b), root xylem sap [ABA] (c) and soil water content. Filled circles and filled triangles represent well-watered intact and girdled plants (WW and WW-G) respectively, while hollow circles and hollow triangles represent droughted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

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

4. Discussion

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

Since ABA is an important stomatal regulator, it is necessary to understand where in the plant it is produced. By compromising communication between the aerial part of the plant and the roots via the phloem, stem girdling attenuated (Day 3) or eliminated (Day 5) root ABA accumulation in response to drying soil (Fig. 5). Similarly, girdled citrus plants showed attenuated root ABA accumulation following an initial (3 day) soil drying cycle, but following a 3 day recovery (re-watered soil) period, no drought-induced ABA accumulation during a subsequent drying cycle (Manzi *et al.*, 2015). Furthermore, stem girdling attenuated root hormone export to the shoot via the xylem as the soil dried (Fig. 7c). Drying soil increased xylem sap ABA concentrations irrespective of sampling position in intact plants, but girdling attenuated the increase in xylem ABA concentration as the soil dried (Fig. 4). This suggests that root ABA export partially depends on shoot-to-root ABA transport in the phloem (Slovik *et al.*, 1995). Recycling of ABA between phloem and xylem in the roots made a variable contribution to the root-to-shoot ABA signal depending on soil water status, comprising 45 and 72% of root ABA export under salinized and non-salinized conditions respectively (Wolf *et al.*, 1990). The remaining contribution originated from *de novo* root ABA biosynthesis, which was accentuated when roots were exposed to salinity. Taken together, *de novo* ABA synthesis in the roots makes a variable contribution to root ABA accumulation and xylem export, with clear impacts during the early stages of soil drying seemingly being abolished following more intense (Fig. 5) or repeated (Manzi *et al.*, 2015) soil drying, as time since girdling increased.

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

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

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

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

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

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Highlights

-Girdling eliminates shoot-to-root ABA transport, altering plant physiology.

-Girdling decreased stomatal conductance by increasing leaf xylem ABA concentration.

-Girdling decreased root ABA concentration of well-watered plants.

-Soil-drying induced root ABA accumulation requires shoot-to-root ABA transport.

-Soil water content better explained variation in [ABA] than tissue water status.

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