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A hydraulic signal in root-to-shoot signalling of water shortage

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Summary

Photosynthesis and biomass production of plants are controlled by the water status of the soil. Upon soil drying, plants can reduce water consumption by minimizing transpiration through stomata, the closable pores of the leaf. The phytohormone abscisic acid (ABA) mediates stomatal closure, and is the assigned signal for communicating water deficit from the root to the shoot. However, our study does not support ABA as the proposed long-distance signal. The shoot response to limited soil water supply is not affected by the capacity to generate ABA in the root; however, the response does require ABA biosynthesis and signalling in the shoot. Soil water stress elicits a hydraulic response in the shoot, which precedes ABA signalling and stomatal closure. Attenuation of the hydraulic response in various plants prevented long-distance signalling of water stress, consistent with root-to-shoot communication by a hydraulic signal.

Keywords: abscisic acid, water stress, drought stress, Arabidopsis, long-distance signal, transpiration.

Introduction

Biomass production by plants is regulated by water availability (Schulze, 1986). Transpiration and the gas exchange of CO_2 and oxygen are controlled by adjusting the stomatal aperture of pores formed by guard cells in the leaf epidermis (Hetherington and Woodward, 2003). Plants can adjust to widely varying water potentials (ψ), ranging from almost zero in well-watered soils down to –8 MPa in arid areas (Odening *et al.*, 1974). A limited water supply in the soil is sensed by roots and communicated to the shoot to allow timely adjustment of the plant's transpiration (Schulze and Hall, 1982). According to current understanding, abscisic acid (ABA) is the long-distance signal that communicates water stress from the root to the shoot (Wilkinson and Davies, 2002).

Abscisic acid plays a pivotal role in adjustment of plants to abiotic stress conditions (Christmann *et al.*, 2006). It mediates stomatal closure, but also regulates plant development in the absence of stress (Cheng *et al.*, 2002). The sesquiterpene ABA is derived from the C15 compound xanthoxin that is generated by cleavage of a carotenoid (Schwartz *et al.*, 2003), and the reaction is stimulated by drought conditions (Qin and Zeevaart, 1999). The enzymes that convert xanthoxin into ABA are expressed in the vascular parenchyma cells of roots and shoots (Cheng *et al.*, 2002; Koiwai *et al.*, 2004). Evidence for root-derived ABA as a long-distance signal has been obtained from split-root experiments with whole plants in which only one part of the root system experienced water deficit (Wilkinson and Davies, 2002). Likewise, maintaining the water status in leaves by pressurization of drought-exposed root systems did not prevent stomatal closure in herbaceous species (Wilkinson and Davies, 2002). However, grafting of tomato shoots onto root stocks with deficiency in ABA biosynthesis indicated that root-generated ABA is not required, or, alternatively, that transported ABA from the shoot may compensate for ABA deficiency in roots (Holbrook *et al.*, 2002). ABA can be stored as physiologically inactive ABA–glucose conjugates from which ABA is released by a specific hydrolase that is activated under water stress (Lee *et al.*, 2006). However, the levels of ABA glucose ester are too small for ABA release from this conjugate to contribute significantly to the overall increase in ABA during water stress (Neill *et al.*, 1983; Priest

et al., 2006). Distinct ABA perception and signal transduction control stomatal aperture, seed germination, growth and flowering time (Finkelstein *et al.*, 2002; Mishra *et al.*, 2006; Razem *et al.*, 2006; Schroeder *et al.*, 2001; Shen *et al.*, 2006).

The function of ABA as a long-distance signal that communicates water shortage from the root to the shoot is still not clear. In this study, we used Arabidopsis and a non-invasive imaging system for ABA action (Christmann *et al.*, 2005) to examine the model. Our data do not support ABA as the long-distance signal controlling water-deficit responses in the shoot; rather we present evidence for a hydraulic signal in root-to-shoot communication of water stress.

Results

ABA pools and ABA signalling in response to root water stress

We studied the dynamics of the action of ABA during water stress throughout the plant by non-invasively visualizing ABA signalling via luciferase expression in Arabidopsis. Seedlings water-stressed by exposure of roots to a medium of high osmotic concentration showed prominent ABA signalling in shoot tissue, namely guard cells (Christmann *et al.*, 2005). Under such conditions of low water potential (−1.0 MPa), a weak activation of ABA signalling was observed in root cells. Responses were localized to the proximity of the vascular bundles (Figure 1a), and were absent in control seedlings exposed to high water potential (−0.2 MPa) as well as in the Arabidopsis mutants *aba2-1* (Figure 1a) and *abi1-1* (not shown) that are deficient in ABA biosynthesis and defective in ABA signalling, respectively (Gonzalez-Guzman *et al.*, 2002; Koornneef *et al.*, 1984; Léon-Kloosterziel *et al.*, 1996; Meyer *et al.*, 1994).

In addition to the plant's capacity to synthesize and transduce the ABA signal, steady-state stomatal apertures depended on the perirhizal water potential. Complete stomatal closure required a water potential (ψ) <−0.8 MPa in the substratum. Adjustment of the water potential using either mannitol (Figure 1b) or polyethylene glycol (not shown) yielded identical responses, with detectable closing of stomata 1.5 h after onset (Figure 1c). Feeding 30 μM ABA to roots in the absence of water stress activated ABA signalling (Figure 1a) and induced stomatal closure with kinetics comparable to those of water-stressed plants (Figure 1c). ABA feeding also rescued stomatal closure in *aba2-1* at low water potential of the substratum (Ψ = −1.0 MPa, data not shown).

Water deficit at the roots resulted in an approximately tenfold increase in ABA levels (Figure 2a) and a comparable ABA-responsive reporter gene expression in the shoot (Figure 2b). As seen by *in vivo* imaging, ABA signalling was confined to the stele of water-stressed roots. However, the levels were too low to detect significant changes in cell-free extracts (Figure 2a,b). It is possible that ABA may have

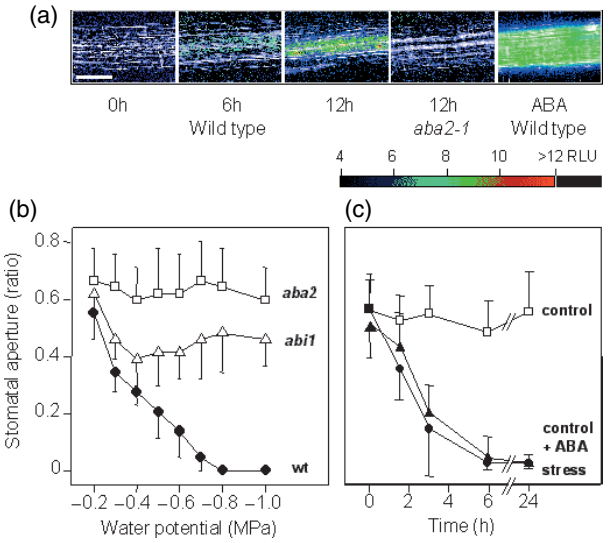

Figure 1. Abscisic acid (ABA) signalling and stomatal responses in water-stressed Arabidopsis.
(a) Activation of ABA signalling in water-stressed roots. ABA-indicative light emission from roots (Ψ = −1.0 MPa) of a wild type ABA-responsive reporter line (*pAtHB6::LUC*) is compared to the response in the *aba2-1* mutant carrying the reporter and the response seen in the wild-type after up to 12 h of exposure to 30 μM ABA. Luminescence activity is depicted in false colors, and the color scale for the relative light units (RLU) measured is provided. The signal sensitivity for the image of an ABA-exposed root was 20-fold reduced.
(b) Stomatal responses to decreasing water potential. Arabidopsis seedlings were water-stressed (24 h) by exposing roots to solidified medium supplemented with various concentrations of mannitol to yield the indicated water potential. Filled circles, wt; open triangles, ABA-insensitive *abi1-1*; open squares, ABA-deficient *aba2-1*. Values are means ± SD ($n \geq 45$).
(c) Stomatal closure in response to root-inflicted water stress (filled circles, Ψ = −1.0 MPa) and to exogenous ABA (30 μM) in the absence of water stress (closed triangles). Open squares indicate the stomatal aperture in non-treated plants. Values are means ± SD ($n \geq 45$).

leaked into the substratum. During 24 h of stress treatment, each seedling delivered on average 30 fmol of ABA to the medium (Table 1). Deuterated ABA was added to the substratum as an internal standard to correct for ABA recovery. During the stress exposure, shoot ABA levels increased by approximately 290 fmol, indicating that phytohormone leakage is significant but accounts for less than 10% of the total ABA in water-stressed seedlings (Table 1). Thus, our failure to observe increased ABA pools in the root may be attributable to some ABA leakage under the experimental conditions. The much higher ABA content in the shoot, however, made us question whether root-to-shoot signalling of water stress does indeed rely on a supply of ABA from the root.

Shoot-derived ABA is necessary and sufficient for stomatal closure

In order to test the hypothesis that ABA acts as a long-distance signal, we generated reciprocal grafts between wild-

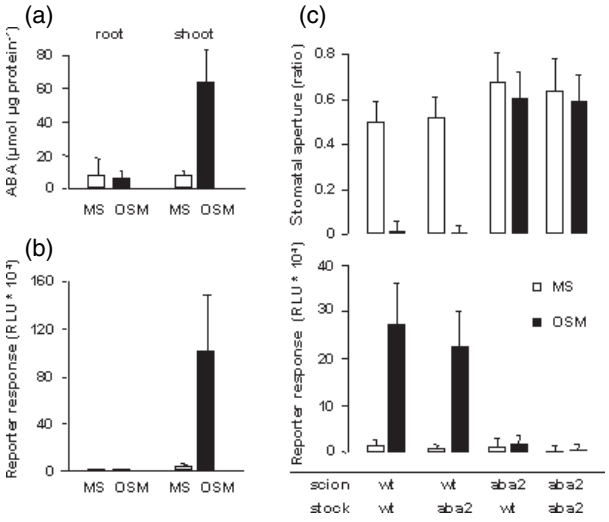

Figure 2. Root-sensed water shortage requires shoot-derived abscisic acid (ABA) for stomatal closure.

(a, b) Induction of ABA levels (a) correlates with ABA-specific reporter activation (b) in response to root water stress (Ψ = −1.0 MPa). Eight-week-old *pAtHB6::LUC* transgenic plants cultivated under short-day conditions (10 h light, 14 h dark) were water-stressed for 24 h on solidified medium supplemented with mannitol (OSM) or transferred to non-stress medium (MS). ABA levels were subsequently determined in root and shoot extracts of non-stressed and water-stressed plants, and ABA-responsive light emission was determined in parallel.

(c) Stomatal aperture and ABA-indicative light emission in shoots of grafts between *pAtHB6::LUC* wild-type and *aba2-1* mutants carrying the reporter. Grafts (8 weeks old) were water-stressed by exposure of the roots to agar medium supplemented with mannitol to yield a water potential of −1.0 MPa for 24 h. As a control, plants were transferred to fresh MS medium. Stomatal apertures ($n = 3 \times 45$) were then determined in three grafts per scion/stock combination (wt/wt, wt/*aba2-1*, *aba2-1*/wt and *aba2-1*/*aba2-1*). ABA-responsive light emission was recorded in roots and shoots of non-stressed and water-stressed grafts. Values are means ± SD [$n = 4$ plants in (a) and (b); $n = 3$ grafts in (c)], and error bars indicate SD.

Table 1 Abscisic acid (ABA) levels in substratum, root and shoot in response to low water potential (Ψ = −1.0 MPa/24 h) in the substratum. Seedlings of Arabidopsis were stressed by exposure of roots to mannitol-containing medium (Ψ = −1.0 MPa) for 24 h (water stress) or to non-stress medium (control) prior ABA analysis in extracts of medium, roots and shoots

	ABA level (fmol)		
	Control	Water stress	Induction (fold increase)
Agar medium[a]	<10	30.3 ± 4.3	> 3
Root	15 ± 4.1	14.1 ± 9.0	1
Shoot	5.4 ± 3.5	295.2 ± 17.7	55

[a]Calculated per seedling.

type and *aba2-1* (ABA-deficient) mutant reporter lines. Grafted plants containing a wild-type-derived scion on root stock of wild-type or ABA-deficient plants closed their stomata in response to perirhizal water shortage ($P < 0.001$), resulting in mean stomatal width-to-length aperture ratios of 0.02 (Figure 2c). Irrespective of the root's capacity to generate ABA, stomata of grafts containing an ABA-deficient scion were unable to respond to water deficit ($P < 0.001$). Stomata remained open (mean aperture ratio of 0.59), and no ABA-dependent reporter induction was detectable (Figure 2d). From these results, we concluded that (i) root-inflicted water stress elicits shoot ABA responses in the absence of root-generated ABA, and (ii) shoot-derived ABA is necessary and sufficient for stomatal closure. The data do not support the model of root-derived ABA translocated from water-stressed roots to the shoot as the long-distance signal.

Attenuation of root-to-shoot water stress signalling

We then explored the nature of the long-distance signal, which could be electrical, chemical or hydraulic in nature. According to the cohesion/tension theory for the ascent of sap in plants, the capillary water of the xylem is part of a continuous hydraulic system that links absorbing surfaces of the roots to evaporating surfaces in the leaves, and hydraulic forces are fairly rapidly transmitted from roots to shoots (Steudle, 2001; Wei *et al.*, 1999). Hence, the hydraulic system has the potential to provide an effective long-distance relay of the plant's water status (Malone, 1993). To test the potential role of hydraulic signals, we attempted to attenuate signalling of water stress by providing water directly to leaves of water-stressed plants. There was no closure of stomata in response to root-experienced water stress when water was placed directly on leaf surfaces of Arabidopsis seedlings, and ABA-enhanced reporter expression was attenuated (Figure 3a,b). In the root, a marginal activation of ABA-dependent reporter expression was observed upon attenuation of water stress in the shoot. The water feeding performed may intercept the relay of chemical or electric signals or compensate the hydraulic signal. Exogenous application of 30 µM ABA fully rescued stomatal response of water-fed leaves. Six hours after the onset of water stress in roots, the stomata were closed and aperture ratios were indistinguishable from those of seedlings that were subjected to water stress and not water-fed (aperture 0.02 ± 0.07, $n = 45$ stomata). The data show that root-to-shoot transport of a chemical signal was not blocked and that leaf stomata responded to transported ABA. In order to rule out the possibility that water feeding interfered with root-to-shoot signalling by a water stress-induced increase in the pH of the apoplast (Wilkinson and Davies, 2002), microdroplets (0.4 µl) of pure water were repeatedly administered to the air-exposed leaf surface of root-stressed plantlets to avoid dilution of a pH signal. In addition, microdroplets of solutions adjusted to various pH values (pH = 4.8 to 8.0 with increments of 0.4 units), including those observed in the xylem sap from droughted plants (pH around 7.0), were applied. Such droplets were sufficient to attenuate

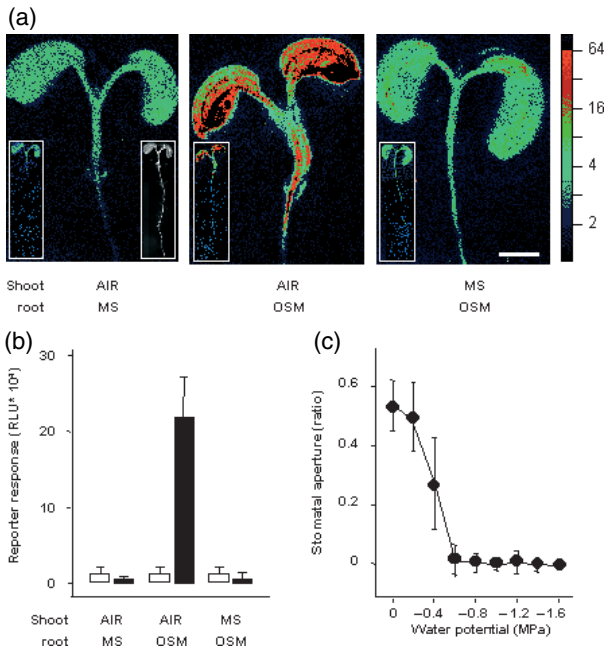

Figure 3. Attenuation of root-to-shoot signalling.
(a) Abscisic acid (ABA) signalling in shoots in response to water stress. Seedlings of the ABA-responsive reporter line were water-stressed by exposing roots to medium of low water potential (OSM; Ψ = −1.0 MPa,). Shoots were either exposed to air, generating the shoot/root combination AIR/OSM, or were in contact with mannitol-free MS medium (Ψ = −0.2 MPa; MS/OSM). The combination AIR/MS (shoot/root) is shown as a control. Light emission was recorded 8 h after treatment and is shown for single seedlings in false color. The color scale for the relative light units (RLU) is provided. The inset reveals reporter activity of the entire seedling. The black and white image is shown to visualise the root of the control.
(b) ABA-dependent light emission of seedlings treated as in (a) at the start of the experiment (open bars) and after 8 h of treatment (closed bars). Values are means ± SD (n = 45 seedlings).
(c) Dependence of the stomatal response on the water potential of leaf-fed water. Roots of seedlings were water-stressed (Ψ = −1.0 MPa) while water or mannitol solutions of various water potentials were simultaneously applied selectively to the shoots. Stomatal aperture was determined after 8 h of exposure. Values are means ± SD (n = 50 stomata).

the ABA responses in leaves irrespective of their pH. Consistent with a hydraulic signal, the stomatal response depended on the water potential of the shoot-fed solution (Figure 3c). Water potentials of the feeding solution close to or more negative than the leaf water potential (–0.6 ± 0.05 MPa) did not attenuate the response, while more positive values partially or fully suppressed it (Figure 3c). Hence, conditions that are predicted to allow net water influx into leaf tissue, which presumably proceeds via the hydathodes, reduced or abolished the closing of stomata. The data therefore favour a hydraulic over an electric or chemical signal.

Detection of a hydraulic signal in leaves in response to root water stress

Transpiration under conditions of water shortage can result in high tensions of almost 10 MPa in the xylem, affecting the turgor pressure of living cells in the vicinity of the xylem (Steudle, 2001), and extensive water deficits lower cell turgor throughout the plant (Hsiao, 1973). Mesophyll cells of well-watered plants revealed a positive cell pressure (turgor) of approximately 0.38 ± 0.04 MPa. Severe water stress reduced the turgor pressure of the leaf cell to zero within 200 sec (Figure 4a). Water deficit was imposed on whole plants by lowering the water potential of the soil using a sorbitol solution (–0.8 MPa). Drainage of sorbitol from the soil by excessive watering reversed the effect and almost recovered the initial turgor values (0.31 ± 0.10 MPa). Similar changes were observed in the *aba2* and *abi1* mutants (data not shown), and therefore are independent of ABA biosynthesis and signalling. Compared to changes in turgor, stomatal responses were delayed, and full closure resulted after approximately 1 h (Figure 4b).

When leaves were fed with water, a decrease in soil water potential sufficient to elicit a strong response as described above, now only generated a minor change of turgor (Figure 4c). Under these conditions, mesophyll cells remained turgid (turgor approximately 0.3 MPa), and

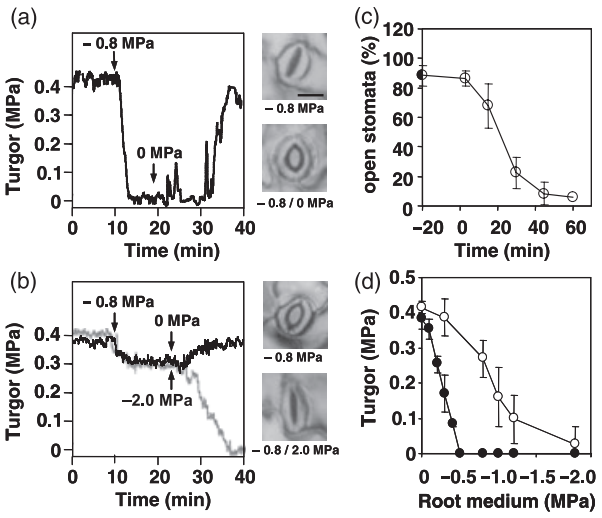

Figure 4. Root-to-shoot signalling of water deficit induces rapid turgor changes in mesophyll cells.
(a, b) Changes in turgor pressure of mesophyll cells after water stress was imposed on intact Arabidopsis plants by shifting the water potential of the soil. The arrows mark the administration of sorbitol solution (–0.8 or –2.0 MPa) or pure water (0 MPa) to the pot. The images on the right show typical stomata of the abaxial leaf epidermis 2 h after changing the water potential of the soil. Representative traces of single cell turgor measurements with a cell-pressure probe are shown for leaves that were not fed with water (a) or leaves fed with water on their adaxial sides (b). Bar = 10 μm.
(c) Kinetics of stomatal closure in response to water stress in intact plants imposed by administration of sorbitol solution (–0.8 MPa). The percentage of open stomata (width > 0.2 μm) is given out of a total of more than 300 stomata from nine leaves per data point. Values are means ± SD.
(d) Steady-state levels of turgor pressure in mesophyll cells after application of water to the soil or of sorbitol solutions adjusted to different water potentials. Rosette leaves of 4-week-old plants were fed with water on their adaxial sides (open symbols) or not (filled symbols). Values are means ± SD (n = 3 plants).

stomata did not close. Removal of sorbitol from the root system recovered the initial turgor of mesophyll cells. However, when the osmotic potential of the sorbitol solution was lowered to −2 MPa and leaves were fed with water, there was a complete loss of turgor, and a subsequent closure of stomata occurred. Attenuation of the hydraulic signal in mesophyll cells shifted the soil water potential required for half-maximal turgor loss from –0.28 ± 0.02 MPa to potentials that were three times more negative (ψ = −0.93 ± 0.12 MPa; Figure 4d).

In order to extend our findings to other plants, seedlings of maple (*Acer pseudoplatanus*) and beech (*Fagus sylvatica*) were analyzed for the importance of hydraulic signals in the stomatal response to water stress. Exposing roots to water shortage resulted in stomatal closure in the shoot, unless the shoots were provided with water via the cotelydons or the leaves (Table 2). However, replacing water with solutions of low water potential in the feeding experiment did not prevent stomatal closure. Taken together, the data show that water stress applied to the root generates a hydraulic signature in the shoot. The response was independent of ABA and preceded stomatal closure. The data imply that a hydraulic signal functions in the long-distance communication of water status.

Discussion

Water stress in the presence of ongoing photosynthesis puts a major strain on plants. The dilemma is whether to keep stomata closed and combat the lack of the electron acceptor CO_2, or to open stomata and tolerate water loss by transpiration. Hence, control of stomatal aperture is based on a regulatory network in which several input signals for opening and closing such as CO_2 deficiency and ABA are integrated (Hetherington, 2001; Schroeder *et al.*, 2001; Young *et al.*, 2006). Optimal regulation of leaf transpiration requires the integration of the soil water status. Plants are able to respond rapidly to transient water shortage at midday by closing the stomata and causing midday suppression of photosynthesis (Schulze and Hall, 1982). Such a short-term adjustment is difficult to reconcile with the long-distance transport of a chemical signal in trees.

Transport of a chemical signal from roots to the canopy of tall trees may take more than a day based on the transpirational streaming in conifer xylem with transport velocities of up to 2 m/h (Zimmermann and Brown, 1971). During night or water shortage, transpiration is reduced, and, simultaneously, basi-acropetal transport in the xylem is also reduced (Jackson, 1997). The grafting analyses with ABA-deficient Arabidopsis clearly showed a requirement for ABA in the response to soil-borne water stress, but not as the long-distance signal. ABA biosynthesis in the shoot was necessary and sufficient to mediate stomatal closure of plants water-stressed at the roots, confirming similar grafting experiments with tomato (Holbrook *et al.*, 2002). The detailed analysis of ABA action during the stress response disclosed some ABA signalling in the root stele. A measurable increase in root-localized ABA was not found, and ABA leakage into the substratum may have contributed to this finding. In the shoot, a 10-fold up to more than 20-fold induction of ABA levels was observed during water stress, paralleled by a strong activation of ABA signalling. Neither the constraints on transport velocity in the transpiration stream or our experimental data favour ABA as the root-to-shoot signal. The identification of a change in turgor pressure of mesophyll cells within minutes of root-evoked water stress provided evidence for the involvement of a hydraulic signal.

Stomatal closure in response to water stress required both ABA and a hydraulic signal in the shoot. Attenuation of the hydraulic response prevented ABA signalling and subsequent physiological responses. The attenuation was overcome by feeding ABA to the root system, compatible with a single response pathway of ABA and the hydraulic signal. Generation of the hydraulic signal neither depended on ABA biosynthesis nor on ABA signalling. In response to water stress, the mutants *aba2* and *abi1* revealed a hydraulic response comparable to that of wild-type; however, the stomata did not close in the mutants. Taken together, the data reveal a signal pathway in which ABA acts downstream of the hydraulic signal in communicating water stress between root and shoot.

In earlier studies on root-to-shoot communication of water shortage, stress was imposed gradually and no changes in leaf water relations were detected (Wilkinson and Davies, 2002). In our study, the stress was evoked suddenly and enabled us to monitor rapid root-to-shoot transmission of a hydraulic signal in the intact plant. The

Table 2 Attenuation of stomatal responses to perirhizal water deficit, measured in terms of the stomatal aperture (width vs. length)

	Ambient air (AIR)/Murashige and Skoog (MS)	AIR/osmotic stress medium (OSM)	MS/OSM	OSM/OSM
Arabidopsis	0.54 ± 0.14	0.01 ± 0.02	0.53 ± 0.10	0.01 ± 0.05
Maple	0.51 ± 0.16	0.01 ± 0.06	0.59 ± 0.14	0.03 ± 0.07
Beech	0.49 ± 0.15	0.03 ± 0.07	0.49 ± 0.15	0.02 ± 0.06

Seedlings of Arabidopsis, beech and maple were in contact at the root with solidified MS medium or osmotic stress medium (Ψ = −1.0 MPa, OSM). The shoot was either exposed to air (AIR/MS or AIR/OSM) or was in contact with solidified medium (MS/OSM or OSM/OSM). Values are for stomatal apertures (n = 90) after one day of exposure.

experimental set-up would have allowed generation and transmission of a stress-triggered chemical signal to act on leaf stomata, and exogenous ABA was indeed transmitted from roots to the shoot. However, there was no indication of such a stomata-controlling signal when we attenuated the hydraulic signal. Instead, stomata remained open in dependence of the water potential of the feeding solution.

The hydraulic signal preceding ABA must be decoded in the shoot, leading to ABA action. Complete turgor loss in leaf tissues has been suggested to stimulate ABA biosynthesis in leaves (Pierce and Raschke, 1980), but it is not known how the mechanical signal is sensed to activate ABA for stomatal closure. Hydraulic forces generated in the capillary system of plants might stimulate stretch-activated sensors (Kung, 2005; MacRobbie, 2006; Sukharev and Corey, 2004) that subsequently elicit ABA action, e.g. by enhancing ABA biosynthesis. Within the hydraulic continuum of the root system, water availability in various sectors is integrated, and the resulting hydraulic information is transmitted to the above-ground plant parts. The hydraulic mode of signal transfer allows rapid relay of information over long distances. In non-compressible pipes, the hydraulic signal can propagate at the speed of sound (Malone, 1993; Wildon *et al.*, 1992), providing an efficient tool for communicating the water status between root and shoot to optimize plant performance and photosynthesis in a environment of limiting and changing water availability.

Experimental procedures

Plant materials and growth conditions

Arabidopsis thaliana seeds were cultivated in Petri dishes containing Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 10 g/l sucrose and 0.9% agar as described previously (Christmann *et al.*, 2005). Plates were kept for 2 days at 4°C, and thereafter at 22°C under continuous illumination (60 μE m^{-2} sec^{-1}). Seedlings of maple (*Acer pseudoplatanus*) and beech (*Fagus sylvatica*) were grown on MS plates without sucrose under long-day conditions with 16 h light (160 μE m^{-2} sec^{-1}), at 22°C and 16°C during day and night, respectively.

Arabidopsis grafts

Grafts of *pAtHB6::LUC* reporter lines (Christmann *et al.*, 2005), wild-type (Columbia) and *aba2* mutant background were generated using 5-day-old seedlings by a micrografting technique (Turnbull *et al.*, 2003). Grafts were kept under short-day conditions with 10 h light (50 μE m^{-2} sec^{-1}), 22°C, and 14 h dark, 17°C, on solidified mineral MS medium. Petri dishes with grafts were sealed with both Nescofilm (Bando Chemical; http://www.bando.co.jp; lower half) and a porous tape (Soehngen Pore; Soehngen; http://www.soehngen.de; upper half), to allow gas exchange with the chamber environment while reducing evaporation. Grafts were transferred to fresh Petri dishes every 2 weeks, and were used for experiments after development of at least eight rosette leaves (approximately 5 weeks after grafting).

ABA and stress treatments

During stress treatments, the roots of plants were selectively exposed to solidified medium such that the shoot was not in contact with the root medium. The water potential of the original root medium (MS: ψ = −0.2 MPa) was adjusted by adding mannitol. The osmotic stress medium (OSM) contained 0.32 M mannitol in MS (OSM: ψ = −1.0 MPa). Seedlings (4 days old) and grafts (5–6 weeks old) of Arabidopsis, seedlings of maple (*A. pseudo-platanus,* 3 weeks old), and beech (*F. sylvatica,* 3 weeks old) were water-stressed for 24 h on OSM or incubated on MS as a control unless otherwise indicated. To attenuate the hydraulic signal, either water (supplemented with mannitol or not) or a buffered solution (5 mM MES in water, pH 4.8–7.2, or 5 mM Tris–Cl, pH 7.6 and 8) was applied as a droplet (3–5 μl) or repeatedly for 8 h as microdroplets (0.4 μl) to the adaxial side of leaves or cotyledons. Alternatively, the shoot was brought into contact with a solidified nutrient solution, which was physically separated by a small air gap (1 mm) from the root medium. Subsequently, stomatal apertures were determined or luciferase activity was recorded. A solution of 5 mM (+)-ABA (Lomon Bio Technology; http://www.lomonbio.com) in buffer (10 mM MES in water at pH 7) was used as a stock for feeding experiments.

Measurement of stomatal aperture

Stomatal apertures were determined in cotyledons or epidermal peels from Arabidopsis leaves, as described previously (Himmelbach *et al.*, 2002). The lengths and widths of the stomatal pores were measured using digital photographs and IMAGEJ 1.32J software (http://rsb.info.nih.gov/ij/). The stomatal aperture is given as a ratio of the pore width vs. length (Roelfsema and Prins, 1995). Stomata with a pore width of 0.2 μm or less were considered as 'closed', while those with a pore width >0.2 μm were defined as 'open'.

ABA analysis

ABA was extracted from seedlings using methanol containing 10 pmol of [^{2}H]-ABA per sample, and was quantified by GC-MS/MS according to the method described by Müller *et al.* (2002).

Solidified MS medium that had been in contact with roots of 60 seedlings for 24 h was freeze-dried after addition of [^{2}H]-ABA standard, and extracted with methanol. Acetic acid (0.1 N) was added to the methanolic extracts, which were then reduced to the aqueous phase and loaded onto C18 solid-phase cartridges (Chromafix C18ec, Macherey-Nagel; http://www.macherey-nagel.com). The cartridges were washed with acetic acid (0.1 N) and acetic acid (0.1 N):methanol (85:15, v/v). ABA was eluted from the cartridges with acetic acid (0.1 N):methanol (50:50, v/v) and quantified by GC-MS/MS as described above.

Imaging of luciferase activity

Imaging of LUC activity in plants was carried out as described previously (Christmann *et al.*, 2005) using an intensified CCD camera (ORCAII ERG, Hamamatsu Photonics; http://www.hamamatsu.com) and an inverted microscope (Axiovert 200, Zeiss; http://www.zeiss.com/).

Cell turgor measurements

Turgor measurements were carried out on intact Arabidopsis plants grown under well-watered conditions (–0.01 MPa soil water potential) in 0.04 m × 0.04 m pots using the cell-pressure probe technique (Steudle, 1993). We employed the instrumentation described by Henzler *et al.* (1999). Intact leaves attached to the plant were mounted with the help of magnetic bars on a metal plate covered by a layer of cotton wool. The cotton wool layer was either dry ambient air (AIR)–OSM treatments) or soaked with deionized water using a circulating system (AIR+H_2O–OSM treatments). Leaves were incubated for 2 h prior to the start of the measurements. A mesophyll cell in the midrib area of a rosette leaf was then punctured with the cell-pressure probe and turgor pressure was recorded. Deionized water or sorbitol solutions with a water potential between –0.1 and –2.0 MPa were applied to the soil (twice the maximum water holding volume of the soil), and changes in turgor pressure were monitored until the turgor remained constant again for at least 5 min. Treatment was continued with further application of appropriate solutions of sorbitol or deionized water.

Statistical analysis

Data were analysed using the Mann–Whitney *U* test and WINSTAT® software (R. Fitch Software; http://www.winstat.de). All experiments were repeated at least three times with similar results.

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