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TECHNICAL ADVANCE

Stomatal action directly feeds back on leaf turgor: new insights into the regulation of the plant water status from non-invasive pressure probe measurements

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SUMMARY

Uptake of CO_2 by the leaf is associated with loss of water. Control of stomatal aperture by volume changes of guard cell pairs optimizes the efficiency of water use. Under water stress, the protein kinase OPEN STOMATA 1 (OST1) activates the guard-cell anion release channel SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1), and thereby triggers stomatal closure. Plants with mutated OST1 and SLAC1 are defective in guard-cell turgor regulation. To study the effect of stomatal movement on leaf turgor using intact leaves of Arabidopsis, we used a new pressure probe to monitor transpiration and turgor pressure simultaneously and non-invasively. This probe permits routine easy access to parameters related to water status and stomatal conductance under physiological conditions using the model plant *Arabidopsis thaliana*. Long-term leaf turgor pressure recordings over several weeks showed a drop in turgor during the day and recovery at night. Thus pressure changes directly correlated with the degree of plant transpiration. Leaf turgor of wild-type plants responded to CO_2, light, humidity, ozone and abscisic acid (ABA) in a guard cell-specific manner. Pressure probe measurements of mutants lacking OST1 and SLAC1 function indicated impairment in stomatal responses to light and humidity. In contrast to wild-type plants, leaves from well-watered *ost1* plants exposed to a dry atmosphere wilted after light-induced stomatal opening. Experiments with open stomata mutants indicated that the hydraulic conductance of leaf stomata is higher than that of the root–shoot continuum. Thus leaf turgor appears to rely to a large extent on the anion channel activity of autonomously regulated stomatal guard cells.

Keywords: pressure probe, leaf hydraulics, stomatal movement, stomatal conductance, abscisic acid.

INTRODUCTION

Plants respond to drought by closing the stomata, and thereby reducing the transpirational water loss from the leaves. Stomatal control of the plant water status is thus pivotal for leaf turgor control. In this context, stomata have been suggested to directly respond to drought-induced changes in the hydraulic properties within the xylem (Meinzer and Grantz, 1990; Salleo *et al.*, 1992; Sperry *et al.*, 1993; Cochard *et al.*, 1996, 2000; Lu *et al.*, 1996). Water transport in the xylem is associated with negative pressures. This metastable system is susceptible to cavitation, i.e. the collapse of water columns under tension (Pickard, 1981). In many plant species, the xylem tension is at the border of

embolism onset, and control of water loss via stomata and xylem pressure is therefore essential for survival under drought conditions (Jones and Sutherland, 1991).

It has been suggested that stomata respond to the overall leaf water status, which is the result of transpiration and the hydraulic properties of the root–shoot continuum (Comstock and Mencuccini, 1998). The xylem pressure within the rachis of the leaf appears to represent a key parameter by which stomata-controlled transpiration prevents massive cavitations when root water availability becomes limiting (Cochard *et al.*, 2002).

Obvious questions therefore are (i) what are the mechanisms by which stomata sense leaf turgor changes, and (ii) how do they re-adjust their aperture accordingly? As initiation of stomatal closure is correlated with the onset of cavitations in the leaf lamina, it has been speculated that this may produce a cavitation-induced closing signal (Salleo *et al.*, 2000; Nardini *et al.*, 2001). However, the chain of events leading to stomatal closure to prevent cavitation is considered the typical physiological phenomenon associated with drought stress. In both cases, the trigger for stomatal closure very likely originates from the mesophyll cells, as this tissue represents a hydraulic and chemical interface between the rachis (xylem) and the stomata (guard cells). The leaf water potential appears to feedback on the sensitivity of guard cells towards ABA (Tardieu and Davies, 1993). ABA is the key factor for induction of stomatal closure under drought stress, a mechanism which is triggered by water stress-induced ABA metabolism in the root and mesophyll, ABA transport to guard cells, and signal transduction within guard cells. However, the precise nature and site at which leaf water status is sensed and the molecular events transducing this signal into a guard cell response remain unknown.

Arabidopsis offers a rich source of available mutants associated with impaired stomatal movement. Mutants that are not able to properly synthesize or sense ABA or that lack the guard-cell anion channel SLAC1 are characterized by a wilting phenotype (Leung *et al.*, 1998; Seo *et al.*, 2000; Mustilli *et al.*, 2002; Negi *et al.*, 2008; Vahisalu *et al.*, 2008). Open-stomata phenotypes are associated with the *aba3*, *abi1*, *abi2* and *ost1* mutations. In *aba3*, ABA synthesis is affected in all cell types in which the gene is expressed, including guard cells. The *ABI1* gene is also expressed in various cell types. *ABI1* encodes a protein phosphatase that is required for seed germination and mesophyll osmoprotection (induction of dehydrin synthesis), amongst others, as well as stomatal closure. ABI1 gain-of-function plants develop an ABA-insensitive phenotype, not just in guard cells (Moes *et al.*, 2008). The SnRK protein kinase OST1 activates the guard cell-specific anion channel SLAC1 in response to ABA (Geiger *et al.*, 2009; Lee *et al.*, 2009) and ozone (Vahisalu *et al.*, 2010), and it has been shown that guard cells of *ost1* mutants do not respond to ABA. However, light- and CO_2-dependent stomatal movement of *ost1-2* appears to be unaffected (Mustilli *et al.*, 2002). As OST1 is predominantly expressed in guard cells and vascular tissues in leaves, lack-of-function mutants were characterized by stomatal malfunction only (Sirichandra *et al.*, 2009a). Thus the OST1 mutant offers the possibility of studying stomata leaf pressure feedback in more detail.

To answer the question of whether and how plant hydraulics affect stomatal movement or vice versa, we performed turgor measurements using a non-invasive leaf pressure probe (Zimmermann *et al.*, 2008, 2010; Westhoff *et al.*, 2009) in combination with IR (infra-red) gas exchange analysis in Arabidopsis plants. These studies showed that the stomatal activity and in turn the leaf turgor of the model plant Arabidopsis and mutants thereof can be recorded non-invasively for a period of weeks. Our experiments show that the new pressure probe monitors the leaf turgor response directly associated with stomatal movement. The hydraulic coupling within the leaf was analyzed using open-stomata mutants. Drought stress causes cavitation-induced wilting in the open-stomata mutant *ost1*. This shows that the stomata at the leaf–atmosphere boundary limit the hydraulic conductivity of the plant. Experiments with excised leaves further showed that active processes in the rachis delay turgor loss and wilting of the entire leaf.

RESULTS

The stomatal aperture directly feeds back on leaf turgor

Using a non-invasive pressure probe, turgor changes of leaves of well-watered Arabidopsis plants were recorded for several days. This pressure probe measures the attenuated relative pressure response of a leaf. The architecture of the probe causes the following observations: a drop in leaf turgor leads to an increase in the detected leaf patch pressure and vice versa (for details on the method, see Zimmermann *et al.*, 2008, 2010). When exposed to a day/night cycle of 8/16 h, leaf turgor decreased during the day. However, the leaf turgor appeared to recover during the night (Figure 1a). This behaviour is in line with changes in the amplitude of the stomatal conductance. Light-induced stomatal opening leads to loss of water, followed by a leaf turgor decrease. In the dark, when stomata close, the turgor increases again. Stomatal action reflects the current environmental conditions (e.g. changes in CO_2 atmosphere, temperature/humidity) as well as the water status within the plant (Roelfsema and Hedrich, 2005). Therefore, the recorded pressure differences are associated with stomatal movements. Stomatal opening is monitored as an increase in leaf patch pressure (as the result of leaf turgor loss) and closure as a decrease in leaf patch pressure. The leaf turgor started to decrease immediately after the onset of the light period and reached a minimum after approximately 4 h. Peak turgor changes were often accompanied by transient

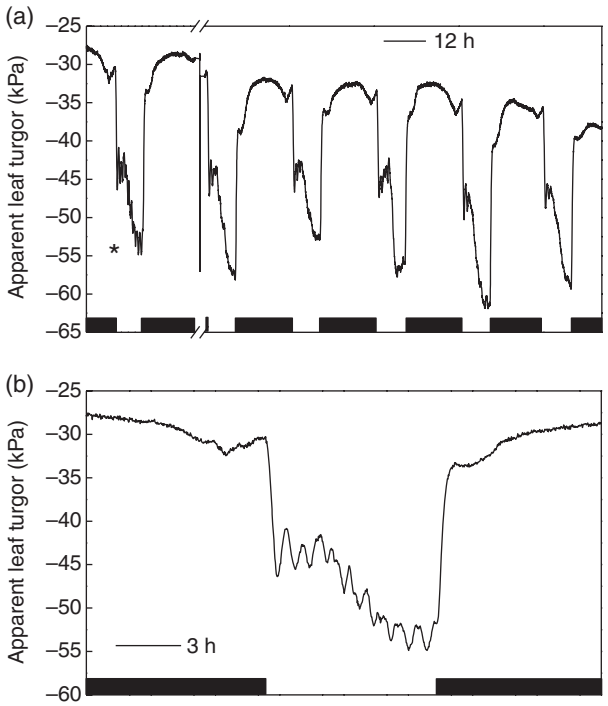

Figure 1. Diurnal leaf turgor changes recorded with *Arabidopsis thaliana* Col-0.
(a) The patch pressure increase reflected a decrease in apparant leaf turgor. Relative turgor changes were inversely related to the stomatal aperture.
(b) Steady-state pressure changes at higher time resolution [indicated by an asterisk in (a)]. Note the presence of turgor oscillations following illumination onset (white bars). The traces shown are representative of three independent experiments. The black bar indicates the dark period.

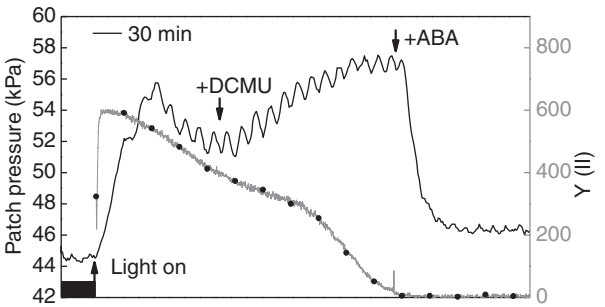

Figure 2. Short-term leaf turgor regulation is photosynthesis-independent.
Simultaneous leaf patch pressure (black line) and PAM measurements (grey line, closed circles). in *Arabidopsis thaliana* Col-0. Upon illumination, the kinetics of the leaf patch pressure increase did not correlate with the onset of photosynthetic electron transport Y(II). Addition of DCMU caused complete loss of Y(II). Note that the photosynthesis inhibitor did not affect leaf patch pressure. ABA-induced stomatal closure was monitored by feedback on leaf turgor. The traces shown are representative of three independent experiments. The black bar indicates the dark period.

complex oscillations around the new steady state (Figure 1b). These oscillations were characterized by periods of 30 min and amplitudes of approximately 20% relative to the major day/night turgor change.

Stomata-dependent leaf turgor changes are photosynthesis-independent

To test whether, in addition to the stomata, the photosynthetic processes in mesophyll cells contribute to the leaf turgor response observed, we used the pulse/amplitude modulation method (PAM) to monitor the effective quantum yield of photosystem II [Y(II)] (Müller *et al.*, 2008) together with the leaf patch pressure (Figure 2). After an initial drop within 4 min after the start of the light period, the quantum yield Y(II) increased to a new steady state, but pressure changes reached a new steady state 30–35 min after stimulus onset. Inhibition of photosystem II by DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) led to total loss of Y(II) (Figure 2). However, the leaf patch pressure remained unaffected under these conditions. After introduction of ABA to the petiole of excised leaves, the stomata closed and the leaf patch pressure increased within 30 min. This behaviour clearly demonstrated that light- and ABA-induced leaf patch pressure changes correlate with stomatal movement rather than with photosynthetic processes. In the short term, the leaf turgor thus appeared to be independent of changes in the photosynthetic status of the mesophyll. However, it may be assumed that metabolic processes within the mesophyll contribute to long-term adaptation to the environment.

The pressure probe reports the pressure of leaf lamina

In *Arabidopsis thaliana* Landsberg *erecta* (L*er*), the upper (adaxial) surface harbours approximately 30% fewer stomata than the lower (abaxial) side (Razem and Davis, 2002). When the pressure probe was placed with the sensor facing either the upper or lower epidermis, the leaf patch pressure read-outs were identical in shape. The fact that the amplitude was higher for the abaxial leaf side reflects the higher compressibility of the spongy parenchyma of the abaxial side compared to the palisade parenchyma of the adaxial side (Figure S1a) (see also Zimmermann *et al.*, 2008, 2010). Likewise, the position of the pressure probe relative to the leaf lamina (base or tip) did not alter the quality of the signals recorded (Figure S1b). This suggests that leaf patch pressure measurements do not depend on the number and developmental state of the stomata covered by the sensor. In other words, the pressure probe is reporting the gross (apparent) leaf turgor rather than that of the clamped leaf patch.

Leaf turgor responds to guard cell-specific signals

Stomatal movement is triggered by external and internal stimuli. Light and low CO_2 concentrations cause stomatal opening, whereas darkness and high CO_2 levels result in closure of the water gate (Roelfsema and Hedrich, 2005). In addition, the water stress hormone ABA provides a strong closing signal from within the plant (Levchenko *et al.*, 2005).

Light. Stomatal opening in the light depends on two spectral ranges of sunlight. Guard cells sense blue and red light through independent photoreceptors (Roelfsema and Hedrich, 2005). Upon red light illumination, leaf turgor changes to an extent representing 50–60% of the amplitude obtained with white light (Figure 3). In the background of high flux of red light (200 μmol m^{-2} sec^{-1}), addition of low quantum density blue light (15 μmol m^{-2} sec^{-1}) caused a further drop in turgor pressure. Thus red and blue irradiance together triggered changes in amplitude similar to those obtained with white light. The new pressure probe thus appears to be well suited to follow changes in stomatal activity online, e.g. to monitor guard cell responses to diurnal changes in the spectrum of sunlight. Previous studies have shown that the guard cell response to red light very probably represents a CO_2 effect (Roelfsema *et al.*, 2002), i.e. red light activates mesophyll carbon assimilation, causing the CO_2 concentration within the sub-stomatal cavities to drop. This change is recognized by CO_2-sensitive guard cell anion channels (Negi *et al.*, 2008; Vahisalu *et al.*, 2008).

CO_2. To explore the coupling between the stomatal conductance and leaf turgor in response to CO_2, experiments were performed with excised Arabidopsis leaves. The turgor of leaves with their petiole exposed to a water reservoir is not superimposed by root pressure, and thus is not subject to the hydraulic limitations of the root–shoot system. The petioles of excised leaves were allowed to equilibrate with deionized water before being subjected to atmospheres with various CO_2 concentrations. When exposed to 350 ppm CO_2, illumination onset was followed by a progressive loss of leaf turgor, which reached a steady state after 20–30 min. This point reflects the stomatal conductance/transpiration at nearly ambient CO_2 levels in the light. A stepwise increase in CO_2 from ambient to 700 ppm and finally 1000 ppm CO_2 resulted in a gradual increase in leaf turgor (Figure 4a).

ABA. Abscisic acid (ABA) is transported to the guard cells when the plant experiences water stress (Hartung *et al.*, 1998). This plant stress hormone activates guard cell anion channels and thereby induces stomatal closure (Blatt, 2000; Hetherington, 2001; Levchenko *et al.*, 2005; Acharya and Assmann, 2009; Siegel *et al.*, 2009). To compare the kinetics of leaf patch pressure (Figure 4b, black line) and transpiration changes (Figure 4b, blue line), we monitored both processes simultaneously. Arabidopsis plants were placed in a gas exchange chamber with a pressure probe attached (Figure 4b and Figure S1). Three to six minutes after application of ABA via the petiole of excised leaves (the time required for the transpiration stream to reach the stomata), the leaf patch pressure increased. Although the changes in leaf patch pressure (black line) and transpiration (blue line) in response to stomata opening and closing signals exhibited similar qualitative behaviour, their kinetics appeared different. Transpiration decreased to a minimum within 20–30 min, while the leaf patch pressure reached a

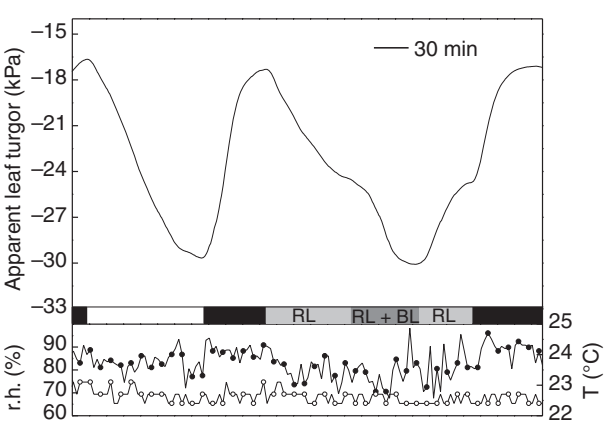

Figure 3. Red- and blue light-specific responses of leaf turgor in *Arabidopsis thaliana* Col-0.
Leaf turgor sensors were attached to leaves of dark-adapted plants. Stomata opened and closed in response to white light/dark cycles. Addition of red light (RL) and blue light (BL) resulted in responses with the same amplitude as those seen for white light. The relative humidity (r.h., closed circles) and temperature (T, open circles) during the experiments are shown in the lower panel. The traces shown are representative of three independent experiments. The black bars indicate the dark period.

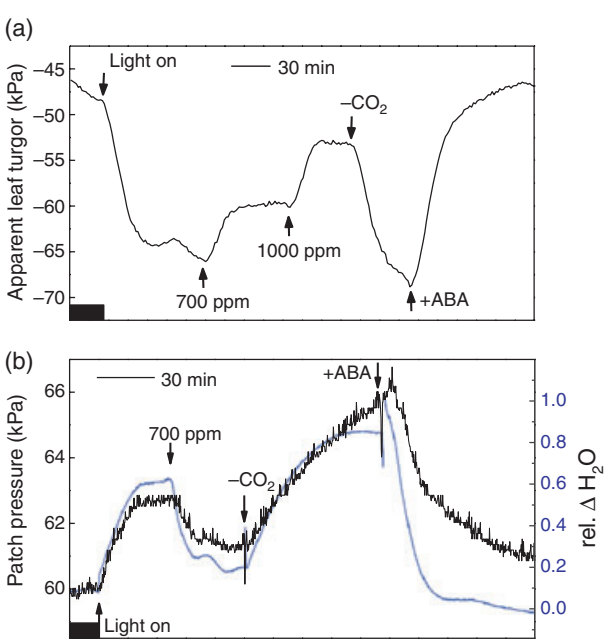

Figure 4. Leaf turgor responses of excised leaves to CO_2 and ABA.
(a) Leaf turgor decreased upon illumination whereas high CO_2 caused an increase in leaf turgor. In CO_2-free air, the turgor decreased again. Feeding of ABA to the leaf petiole returned the turgor to values observed in darkness.
(b) Simultaneous measurements of water loss (blue line) and patch pressure (black line) for the same leaf. Note that, in response to guard cell-specific signals, relative leaf turgor amplitudes and stomatal conductance are inversely related. The traces shown are representative of three independent experiments. The black bar indicates the dark period.

maximum 10–30 min later (Figure 4b). When compared to water loss via the stomata (transpiration), leaf patch pressure changes are slower to reach a new steady state. This difference in kinetics reflects the apparent time constants of the hydraulic parameters that transduce the major change in transpiration into the turgor response of the whole leaf.

SLAC1 is the key player in regulation of stomatal action

In loss-of-function mutants of the guard cell plasma membrane anion channel SLAC1, stomatal opening and closure have been reported to be impaired (Negi *et al.*, 2008; Vahisalu *et al.*, 2008). During the day (illumination), leaf patch pressure measurements with the *slac1-5* mutant revealed behaviour similar to that of wild-type plants (Figure 5). However, following onset of the night period, the leaf turgor recovery of *slac1-5* leaves was delayed.

The *slac1-1* mutation was found in a screen for O_3 sensitivity. To test whether occurrence of the O_3-triggered rapid transient decrease in stomatal conductance present in *Arabidopsis thaliana* Col-0 and absent in *slac1-1* (Vahisalu *et al.*, 2008) also correlates with leaf turgor changes, we monitored the leaf patch pressure and whole-plant gas exchange simultaneously before and after ozone treatment (Figure 6). Follwing a 3 min O_3 challenge, a transient decrease in stomatal conductance and leaf turgor was observed in Col-0 wild-type plants (Figure 6a). Note that the turgor change was delayed by 5–10 min relative to the stomatal conductance. In contrast, in *slac1-1* mutant plants, ozone treatment did not affect either the stomatal conductance or the leaf turgor of mutant plants (Figure 6b). *ost1-2* exhibited similar delayed stomatal closure behaviour in darkness (Figure 7) to that seen in *slac1-5* (Figure 5). The leaf turgor responses with mutants *ost1-2* and *slac1-5* indicate that the activity of the guard cell anion release channel is pivotal not only for control of the stomatal aperture but also for the maintenance of leaf turgor under water stress.

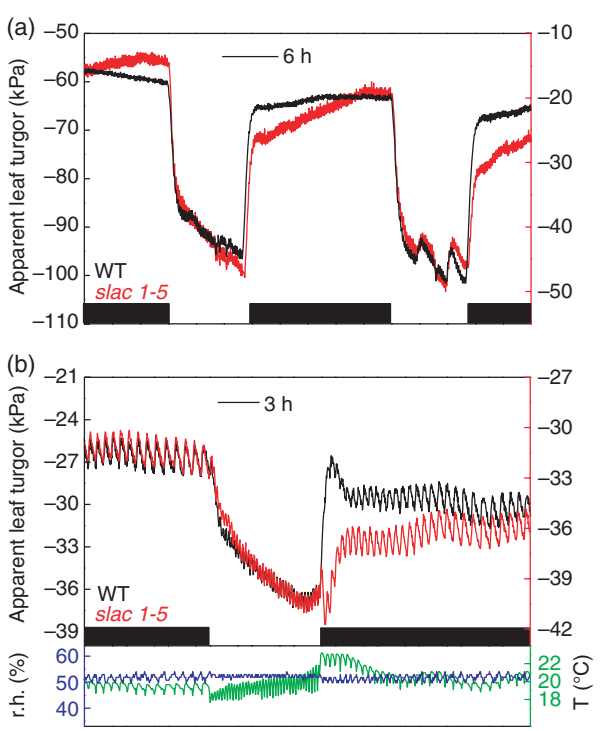

Figure 5. Impaired stomatal closure and leaf turgor control with the guard-cell anion channel mutant *slac1-5*.
(a) Diurnal leaf turgor measurements with intact wild-type Arabidopsis plants and the *slac1-5* mutant. Day/night temperatures were 24/18°C. The two major peaks during the illumination phase of the second day are the result of watering, which required opening of the climate chamber.
(b) Constant humidity (r.h. 50%, blue line) and temperature (20°C, green line) accentuate the phenotype. The *slac1-5* mutant, which shows impaired stomatal closure, also showed delayed dark-induced leaf turgor regeneration. The traces shown are representative of four independent experiments. The black bars indicate the dark period.

ABA-insensitive mutants lack turgor control

Mustilli *et al.* (2002) showed that *ost1-2* stomata behaved like those of wild-type after 2.5 h darkness/light triggering or upon 3 h incubation with CO_2-free air. In addition to these observations, our long-term measurements with the *ost1-2* mutant revealed differences from wild-type with respect to the light-dependent stomatal movement (Figure 7a,b). Upon illumination, the wild-type exhibited a transient overshoot in the loss of leaf turgor induced by stomatal opening. This

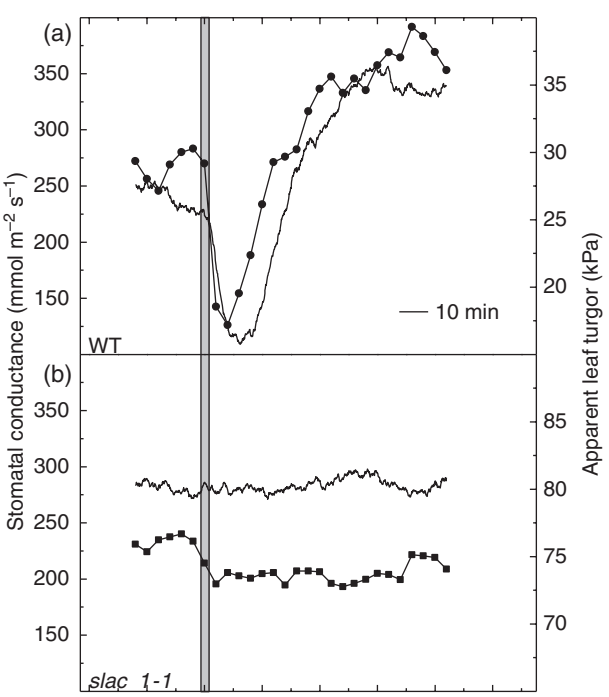

Figure 6. The guard cell anion channel mutant *slac1-1* showed an impaired O_3-triggered stomatal response.
Patterns of stomatal conductance (line with squares/circles) and leaf turgor (line) of Arabidopsis wild-type (wt) and *slac1-1* mutant plants exposed to 350 ppb of O_3 for 3 min. Application of O_3 is indicated by the grey bar. The experiment was repeated twice with similar results.

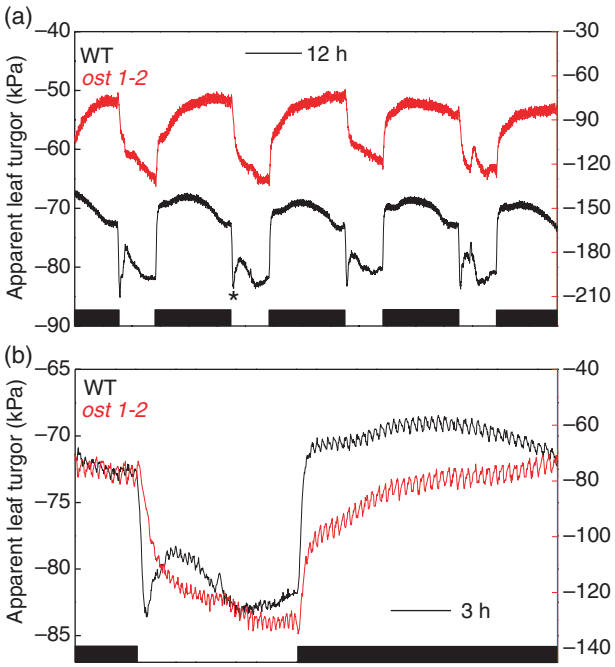

Figure 7. Impaired stomatal closure and leaf turgor control of the open-stomata mutant *ost1-2*.
Diurnal leaf turgor measurements with intact wild-type Arabidopsis plants and the guard cell protein kinase mutant *ost1-2*.
(a) The *ost1-2* mutant, which shows impaired SLAC1 regulation and stomatal closure, showed delayed dark-induced leaf turgor regeneration.
(b) Magnification of the section in (a) marked by an asterisk. Day/night temperatures were 23/16°C. The traces shown are representative of four independent experiments. The black bars indicate the dark period.

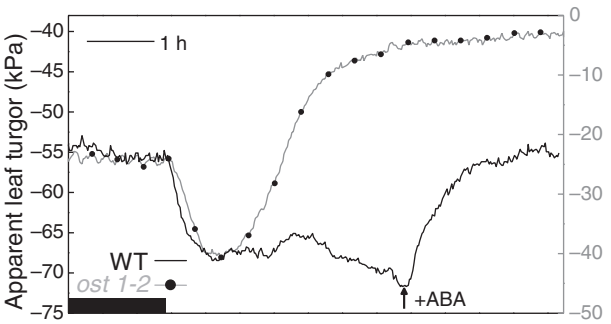

Figure 8. Collapse of leaf turgor in the light in excised *ost1-2* leaves.
The initial response of turgor to light was similar for wild-type leaves and the ABA-insensitive *ost1* mutant. However, mutant leaves wilted and finally collapsed within 2 h of light stimulation (grey line, closed circles). Upon ABA application (25 μM fed via the petiole), the turgor of wild-type leaves increased indicating stomatal closure but showed no effect on the *ost1* mutant. The black bar indicates the dark period.

behaviour probably represents feedback regulation of the guard cells in response to CO_2 changes in the sub-stomatal cavities while stomatal pores begin to open. With ongoing Calvin cycle activity in the mesophyll cells, intercellular CO_2 levels decrease again, and the stomatal aperture reaches its final size. *ost1-2* plants lacked this stomatal response, which indicates a phenotype less sensitive to CO_2. Upon onset of darkness, the stomata of wild-type plants closed. However, in the *ost1-2* mutant, stomatal closure was not completed before the end of night (Figure 7b). The phenotype of *ost1-2* was even more pronounced than that of *slac1-5* in this respect.

To study the response to low humidity and ABA, we excised leaves from wild-type plants and the *ost1-2* mutant at night and attached the pressure probe. Upon illumination, wild-type leaves opened their stomata and adjusted their aperture to the environmental settings. After the leaf patch pressure had reached steady state, ABA was applied via the petiole (Figure 8). As expected from previous experiments, ABA induced stomatal closure and leaf turgor increased 3–6 min after hormone application. In contrast, *ost1-2* leaves, which are unable to properly control guard cell action, kept their stomata wide open. Thus sensing of drought stress in the mutant appeared not to be followed by stomatal closure. As a result, mutant plants lost water excessively and suffered from irreversible wilting. The latter phase was characterized by a decrease in leaf patch pressure below the set point of the clamp initially adjusted for a turgescent leaf, and this very likely represents the point of no return. To quantify the kinetics of turgor loss in the entire leaf, we followed the movement of the leaf tip relative to its position at light onset (t_0) using a time-lapse camera. To prevent sudden wilting of *ost1-2*, low light intensities were used in this experiment. Under these conditions, we followed the leaf tip movement and leaf patch pressure changes simultaneously. Figure 9 shows the onset of wilting under illumination. In contrast to *ost1-2*, wild-type leaves did not show any wilting symptoms (data not shown). Upon termination of light treatment before the permanent wilting point was reached, *ost1-2* mutants regained leaf turgor. Note

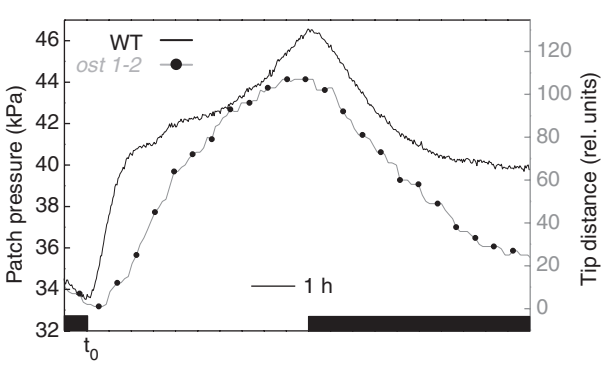

Figure 9. Wilting-induced leaf tip movement in the Arabidopsis *ost1-2* mutant.
Movement of the leaf tip in *ost1-2* plants (relative to its base) was monitored by time-lapse video. Leaf turgor was monitored using the pressure probe. The traces shown are representative of three independent experiments. The black bars indicate the dark period. The tip distance at t_0 = 0; the maximum tip distance = 100.

that leaf tip movements were seen approximately 15–20 min after the onset of the leaf turgor response. However, the kinetics of both processes correlated during the recovery phase.

The fact that wild-type plants closed their stomata but the open stomata mutant *ost1-2* wilted indicates that hydraulic conductance of the leaf vasculature is a limiting factor.

Vascular bundle sheath architecture might control leaf water flow

To identify the bottleneck in hydraulic conductance, we studied the ultrastructure of the cells in the vascular bundle and adjacent mesophyll cells. We found that the bundle sheath cells interconnected with their mesophyll cell neighbours via numerous plasmodesmata (Figure 10a–c). In contrast, no symplastic continuum appeared to exist between the bundle sheath and the phloem and xylem (data not shown). Given this symplastic uncoupling, it is tempting to speculate that water movement from the xylem to the bundle sheath cells represents the limiting step in leaf turgor adjustment (Figure 10d).

DISCUSSION

Here we showed that a new leaf turgor pressure probe (Zimmermann *et al.*, 2008, 2010) is a suitable tool to non-invasively and routinely study guard cell-dependent regulation of the leaf turgor in wild-type Arabidopsis plants and various mutants. In contrast to cell and xylem pressure probes, the leaf patch pressure probe enables long-term measurements with this model plant for up to a month. Application of the sensor at the upper or lower epidermis, leaf tip or base did not affect the quality of the read-out. This indicates that the sensor is monitoring the relative apparent turgor of the entire leaf lamina rather than the stomatal conductance (stomatal aperture and density) of the surface to which the pressure clamp is attached. By changing environmental factors such as light quality and quantity, CO_2, humidity and ozone, we demonstrated that the leaf turgor responds in a guard cell-specific manner, i.e. stomata change their aperture according to external and internal signals. Stomatal opening for physical/chemical reasons causes an increase in the hydraulic/stomatal conductivity, loss of water and finally a decrease in leaf turgor. As a result of the time constants and transfer functions that are dependent on the elastic modulus of the leaf and the absolute turgor, pressure changes appear delayed in time and amplitude (for details, see Zimmermann *et al.*, 2008, 2010). The leaf turgor changes via back pressure of the common epidermal cells, for example, feedback on fine regulation of the stomatal aperture. As a result, oscillations of both stomatal aperture and leaf turgor are visible until a new environment-dependent steady state is reached. Due to the high sensitivity of the sensor, this effect is resolved in time and amplitude, but this is not achieved using the less sensitive method of infra-red gas analysis. The sensor can be used with leaves of intact plants as well as excised leaves. After feeding of ABA to the petiole of excised leaves (simulating water stress signaling), stomata closed and leaf turgor increased.

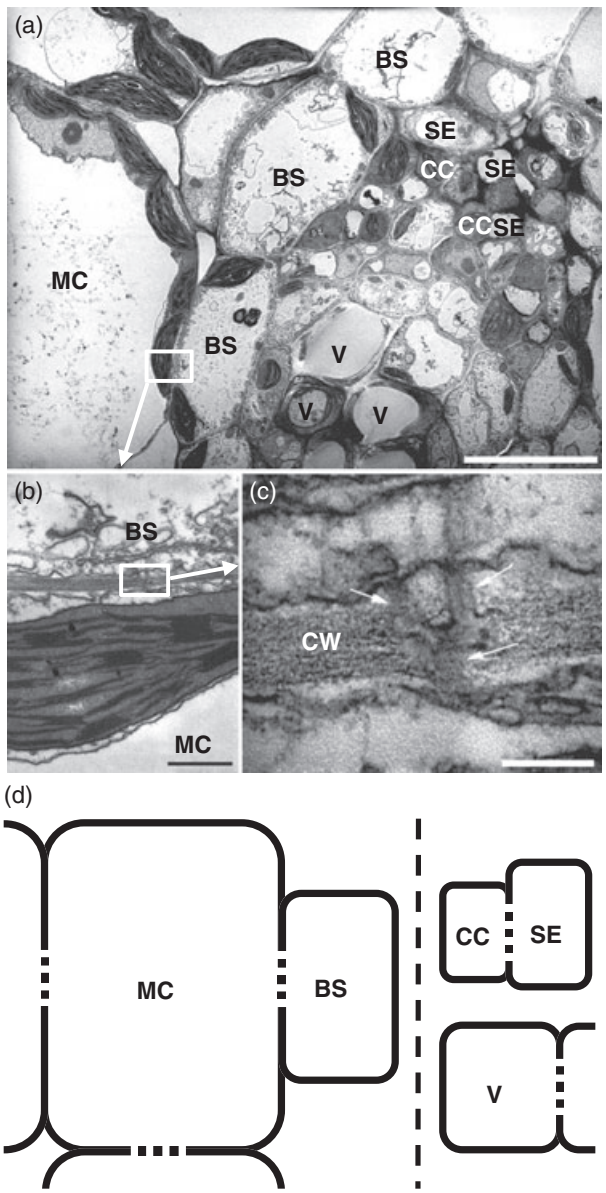

Figure 10. Arabidopsis leaf water flow is restricted at the xylem–bundle sheath transition.
(a) Overview of an Arabidopsis vascular bundle. MC, mesophyll cells; BC, bundle sheath cells; V, vessels; CC, companion cells; SE, sieve elements. Scale bar = 28 μm.
(b) Magnification of the BC/MC cell wall Scale bar = 2.3 μm.
(c) Plasmodesma between a mesophyll cell and a bundle sheath cells. cw, cell wall. Scale bar = 0.5 μm.
(d) Model of the mesophyll/bundle sheath to vascular tissue interface. Numerous plasmodesmata facilitate water flow between mesophyll cells and bundle sheath cells. An apoplastic barrier exists between bundle sheath cells and vascular tissues.

This turgor sensor could be easily and reproducibly applied to Arabidopsis plants to obtain robust results on the influence of stomatal movement on leaf turgor. To study the role of guard cell-specific key regulatory elements, we expanded our studies on the Arabidopsis mutants of OST1 and SLAC1 characterized by de-regulated stomatal closure. In response to darkness and low humidity, both mutants exhibited delayed stomatal closure and thus pronounced leaf turgor loss compared to the wild-type. However, the stomata and leaf turgor phenotype of the OST1 loss-of-function mutant was more pronounced than that of the SLAC1 mutant. This could be explained by the fact that the protein kinase OST1, which activates SLAC1 in guard cells, also addresses targets other than the anion channel. One of these is the NADPH oxidase AtrbohF, which is responsible for ABA-induced ROS production, also leading to stomatal closure (Sirichandra *et al.*, 2009b). OST1 also appears to be involved in ROS-coupled ABA signal transduction from ABA-producing vascular parenchyma to bundle sheath cells, which also very likely involves NADPH oxidase phosphorylation by OST1 (Galvez-Valdivieso *et al.*, 2009).

In addition to turgor measurements on the leaf lamina, we developed a turgor loss-dependent leaf bending (leaf tip movement) assay. The leaf bending is associated with the turgor-dependent tension of the leaf rachis. Experiments with the OST1 mutant suggested that the leaf rachis is a kind of water reservoir that may act as a 'turgor buffer'. It was previously shown that, during water loss and finally wilting, solutes appear to be redistributed from the leaf lamina to the leaf rachis (Levitt, 1986, Weisz *et al.*, 1989). In line with this finding in wilting *ost1-2* leaves, we observed oscillations in leaf bending that very probably reflect transient changes in rachis turgor (Figure 9). These changes might originate from xylem/mesophyll solute transport and water channel activity of the xylem parenchyma (Kirch *et al.*, 2000). As in the root, the vasculature of leaves from C4- and C3-type plants is enclosed by bundle sheath cells (Leegood, 2008). Bundle sheath cells control the import and export of solutes and water in the parenchyma, controlling long-distance xylem and phloem transport. Bundle sheath cells, but not vascular parenchyma cells, are connected to ordinary mesophyll cells via numerous plasmodesmata. The presence of two compartments separated by an apoplastic barrier may allow build up of concentration and pressure gradients between the leaf lamina and the rachis.

The potassium release channel GORK is expressed in guard cells and the leaf vasculature (Ache *et al.*, 2000). GORK expression is under the control of ABA and requires the presence of ABI1 (Becker *et al.*, 2003). Under water stress, a turgor decrease below the plant-specific threshold value in the leaf lamina leads to ABA production (Liu *et al.*, 1978; Pierce and Raschke, 1981; Lee *et al.*, 2006). The phytohormone induces the expression of GORK and dehydrines (Li *et al.*, 2002). It is tempting to speculate that, under such conditions, K^+ and other solutes are released from the xylem parenchyma and taken up by the bundle sheath cells. The latter process is active (requires metabolic energy), and this would explain why leaf tip movement, which to a large extent depends on rachis turgor, appears delayed with respect to the decrease in turgor pressure in the leaf lamina (Figure 9). A mechanism independent of metabolic energy may explain why the upward movement of the leaf tip (recovery of rachis turgor) and increase in leaf lamina pressure show similar kinetics.

A mechanism related to one hypothesized for steady-state xylem pressure control is known for guard cell turgor and volume control. Turgor changes in guard cells are mediated by transport of solute (K^+ salts) (MacRobbie, 2006). Guard cells of open stomata accumulate K^+ salts in large quantities. Under hypertonic conditions, stomata in epidermal peels close due to osmotic water movement from the guard cells into the external osmoticum. Following replacement of the osmoticum by one with a water potential higher than that of the guard cells, the stomata regain turgor hydro-passively. Excised leaves exposed to water stress thus appear to exhibit an osmotic behaviour similar to that of open stomata removed from leaf lamina challenged with hypertonic medium.

CONCLUSION

Excised leaves with the petiole in equilibrium with pure water transpire excessively and even wilt when the stomatal aperture is de-regulated in mutants such as *slac1* and *ost1*. Thus the water conductivity of the leaf mesophyll–bundle sheath–xylem interface appears to represent the bottleneck in the hydraulic xylem–stomata continuum. It is thus tempting to speculate that the pressure of the leaf lamina (mesophyll–bundle sheath) and rachis (xylem–phloem) is not only directly dependent on stomatal movement but may use similar mechanisms of turgor control as guard cells.

EXPERIMENTAL PROCEDURES

Leaf patch pressure probe

Pressure probes with a spring clamp (Zimmermann *et al.*, 2008) or magnetic pressure adjustment (Westhoff *et al.*, 2009; Zimmermann *et al.*, 2010) were used as described previously. Sensors were obtained from Raumedic (http://www.raumedic.com/) or Keller (http://www.keller-druck.com/english/homee/hme.html). Leaf patch pressure probes were attached to the leaves 12–16 h prior to the experiment to allow relaxation of the clamped patches. The starting pressure varied from leaf to leaf depending on the individual compressibility of the clamped leaf area and the force applied via the probe.

Plant material and growth conditions

Arabidopsis thaliana plants were grown in soil and cultivated in a greenhouse under short-day conditions (8 h light/16 h darkness, 22°C/16°C) with illumination by 100 μmol m^{-2} sec^{-1} white light

(25 W, 230 V, Osram TL70 F32T8/TL 741, Philips, http://www.philips.com). Unless otherwise stated, experiments were performed using *Arabidopsis thaliana* Col-0 wild-type. Mutant plants *ost1-2* (Merlot *et al.*, 2002) and *slac1-5* (also known as *osz1*; Saji *et al.*, 2008) and *slac1-1* (Vahisalu *et al.*, 2008) were compared with their wild-types L*er*, *gl1-1* and Col-0, respectively.

Long-term leaf turgor measurements

Six to eight-week-old plants were acclimatized for at least 1 week in growth chambers (8 h light/16 h darkness, 24°C/18°C). The relative humidity varied between 60% (day) and 95% (night). For measurements under constant conditions, fluctuations were limited to 1–2°C and 20% relative humidity, and were monitored by a data logger (Tinytag RS Components GmbH, http://de.rs-online.com).

Excised leaves

During the dark period, *Arabidopsis thaliana* leaves were cut with a razor blade at the lower part of the petiole and re-cut immediately under water to prevent xylem embolism. Leaves were then placed into deionized water until the beginning of the experiment with the pressure probe attached.

Gas exchange

A single leaf was enclosed in a self-made cuvette (Figure S2) with a light-permeable (>300 nm) lid, and illuminated with white light (350 μmol m^{-2} sec^{-1}). The petiole of the leaf was kept in water. ABA feeding to the petiole was accomplished by applying 25 μM of the hormone via a resealable hole in the cuvette lid. Unless indicated otherwise, the flow rate of gas, usually at 350 ppm CO_2, through the cuvette was 0.4 l min^{-1} at 23–25°C and 40–45% relative humidity. CO_2 and transpiration were measured by an infra-red gas analysis technique using a Binos instrument (Heraeus, http://www.heraeus.de).

Chlorophyll fluorescence measurements using PAM fluorometry

The PAM method (Schreiber, 2004; Lichtenthaler *et al.*, 2005, and references therein) was used for chlorophyll fluorescence measurements. The Junior-PAM (Walz, http://www.walz.com/) was used according to the manufacturer's instructions with the following parameters: darkness – saturation pulse intensity 6, pulse frequency 1–2 min; light – saturation pulse intensity 6, pulse frequency 20 sec, actinic light intensity 8. The effective photochemical quantum yield of photosystem II [Y(II)] was calculated as described by Genty *et al.* (1989).

Red/blue light illumination

Six to eight-week-old plants were illuminated in a climate chamber with white light ('cold light' source KL 1500, Schott, http://www.schott.com) at 200 μmol m^{-2} sec^{-1}. For red light illumination (200 μmol m^{-2} sec^{-1}0, the light was passed through a long-pass glass filter ($\lambda_{½}$ 610–780 nm, RD610, Schott). Blue light (18 μmol m^{-2} sec^{-1}) was obtained by using a broad-bandpass filter transmitting 340–480 nm light (DT-Blue, Linos Photonics, http://www.linos.com). The radiation was measured using a quantum sensor (LI-190, Li-Cor, http://www.licor.com). Graphs were smoothed over 30 data points with an adjacent-averaging filter using Origin 8 software (http://www.originlab.com).

Leaf-tip assay

Excised leaves of similar size (approximately 3.5 cm from tip to base) from 6–8-week-old plants were illuminated at 80 μmol m^{-2} sec^{-1} by neon tubes (Fluora 18W/Lumilux 18W, Osram, http://www.osram.com) at 22 ± 2°C and 40 ± 15% relative humidity. Leaf movement and wilting were monitored by time-lapse video using a digital photo camera (A580; Canon, http://www.canon.de). The position of the leaf tip relative to its position at light onset (t_0) was determined (Figure S3) and plotted against time.

Transmission electron microscopy (TEM)

Small sections of leaf tissue were cut with a razor blade and immediately immersed for 4 h in fixation medium containing 1% w/v formaldehyde, 1 mM EGTA, 50 mM cacodylat buffer and 5% glutaraldehyde. Subsequently the tissue was post-fixed with 2% w/v osmium tetroxide overnight at room temperature, and then stained with 3% w/v uranyl acetate in 20% ethanol for 1 h, dehydrated using a graded series of ethanol, and embedded in Spurr's epoxy resin (Spurr, 1969). Ultra-thin sections were cut using a diamond knife on an ultramicrotome (Ultratome Nova, LKB, formally Bromma, Sweden), transferred onto copper grids coated with Formvar (polyvinyl formal, http://www.plano-em.de) and stained with lead citrate. Sections were examined using a Zeiss EM 10c transmission electron microscope (http://www.zeiss.com/) at 80 kV.

Ozone treatments

Arabidopsis thaliana plants were grown singly in 10 × 10 × 6 cm plastic pots on a well-watered 3:4 vermiculite:peat mixture in a growth chamber (AR-66LX, Percival Scientific, http://www.percival-scientific.com) under a light/dark cycle of 12/12 h, with a photon flux density 120 μmol m^{-2} sec^{-1}, temperature 23/18°C, air relative humidity 65/80%, for 4 weeks. For gas exchange and pressure probe recordings, whole rosettes were enclosed in the cuvettes of a rapid-response O_3 exposure/gas exchange measurement device (Kollist *et al.*, 2007), with conditions close to those used during growth. When stable values of water vapour exchange were reached, the rosettes were exposed to approximately 350 ppb O_3 for 3 min. The time patterns of stomatal conductance, averaged over all leaves in the rosette, were calculated from water vapour exchange recordings as described previously (Kollist *et al.*, 2007).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. The pressure probe signal is independent of the mounting position on the leaf.

Figure S2. Gas-exchange cuvette for simultaneous pressure probe and PAM recordings.

Figure S3. Leaf tip assay set-up.

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