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RESEARCH PAPER

Up-regulation of *NCED3* and ABA biosynthesis occur within minutes of a decrease in leaf turgor but *AHK1* is not required

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Abstract

A major environmental signal influencing day-time stomatal aperture is the vapour pressure deficit between the leaf and atmosphere (VPD). In angiosperms, increased VPD triggers biosynthesis of abscisic acid (ABA), prompting rapid stomatal closure. Altered cell turgor has been proposed as the trigger for ABA biosynthesis, but the timing and nature of the genetic signals linking these processes have remained uncertain. We investigated this in Arabidopsis by examining changes induced by a decrease in leaf turgor, simulating a natural increase in VPD. We found that the rate-limiting gene within the *de novo* ABA biosynthesis pathway, 9-*cis*-epoxycarotenoid dioxygenase 3 (*NCED3*), was induced and ABA levels increased within just 5 min of decreased leaf turgor. This rapid induction matches the time-frame for initiation of stomatal closure in response to a doubling in VPD. We further examined Arabidopsis histidine kinase1 (AHK1) as the most likely candidate for the turgor-sensing receptor involved, but found no significant difference between wild-type and an *ahk1* null mutant in the induction of ABA-biosynthetic genes, ABA production, or stomatal behaviour. We show that decreased leaf turgor triggers *de novo* ABA biosynthesis within the time-frame of the stomatal response to VPD, but that AHK1 does not fulfil a critical role as a turgor-sensing receptor within this pathway.

Key words: ABA, AHK1, leaf turgor, NCED, stomata, turgor sensor, VPD.

Introduction

A major advancement that allowed plants to successfully colonise dry terrestrial environments was the evolution of stomata as a means of controlling plant water balance during photosynthesis (Raven, 2002). The majority of water loss in land plants occurs by transpiration through open stomata during photosynthesis. Angiosperms have evolved the ability to integrate multiple environmental signals for the precise regulation of stomatal aperture, fine-tuning the balance between maximising photosynthesis and avoiding damaging dehydration (Cowan and Farquhar, 1977; Brodribb and Holbrook, 2003). One of the most important environmental signals to influence stomatal aperture throughout the day is a change in the humidity of the air, or more precisely, the vapour pressure deficit between the leaf and the atmosphere (VPD). The phytohormone abscisic acid (ABA) plays a central role in the closure of angiosperm stomata in response to high VPD (Xie *et al.*, 2006). An increase in ABA level prompts the activation of ion channels in guard cells that

Abbreviations: ABA, abscisic acid; AHK1, Arabidopsis histidine kinase1; NCED, 9-*cis*-epoxycarotenoid dioxygenase; qRT-PCR, quantitative reverse transcription PCR; SLN1, synthetic lethal of N-end rule1; VPD, vapour pressure deficit.

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actively decrease their osmotic potential, causing the guard cells to lose turgor and close the stomatal pore (Keller *et al.*, 1989; Geiger *et al.*, 2009). An increasing body of evidence suggests that ABA biosynthesis at high VPD is essential for normal stomatal closure in angiosperms (Bauerle *et al.*, 2004; Xie *et al.*, 2006; Bauer *et al.*, 2013; McAdam and Brodribb, 2015; McAdam *et al.*, 2016). Studies have shown that (i) a sufficient increase in VPD triggers the biosynthesis of ABA (Bauer *et al.*, 2013; McAdam and Brodribb, 2016), and (ii) by the time stomata have responded to increased VPD (within 20 min), only a single gene encoding the rate-limiting enzyme 9-*cis*-epoxycarotenoid dioxygenase, *NCED*, is significantly up-regulated within the ABA biosynthesis pathway (Qin and Zeevaart, 1999; Thompson *et al.*, 2000; McAdam *et al.*, 2016). However, the stomatal response to VPD can occur over an extremely rapid time-frame, with incipient stomatal closure at increased VPD recorded within a minute following the change in VPD (Fanjul and Jones, 1982). The rapid speed of the stomatal response to VPD has led a number of authors to propose other mechanisms as alternatives to *de novo* ABA biosynthesis, to explain the increase in ABA level driving these stomatal responses (Lee *et al.*, 2006; Georgopoulou and Milborrow, 2012). Precise, time-resolved information about the speed at which *NCED* transcription can be induced and ABA levels can increase, following a change in VPD, would resolve this debate. Even the nature of the signal by which plants sense changes to VPD remains to be explained. Candidates such as leaf turgor and osmotic potential vary in proportion to transpiration rate and have been raised as possibilities, while even direct sensing of atmospheric humidity has been proposed (Buckley, 2016).

A recent study using a novel method of modifying cell turgor by the application of external pressure indicates that a decline in cell turgor is likely to be the physiological trigger for induction of ABA biosynthesis when leaves are exposed to high VPD in angiosperms (McAdam and Brodribb, 2016). This suggests the existence of a molecular signalling pathway linking the perception of pressure by a turgor-sensing protein to the up-regulation of *NCED* expression and increase in ABA level at high VPD. However, this key molecular component remains as yet unidentified. Results from a series of studies suggest that the transmembrane protein Arabidopsis histidine kinase1 (AHK1) is the most likely candidate for the turgor-sensing receptor within this pathway. AHK1 is a homolog of the yeast protein synthetic lethal of N-end rule1 (SLN1), which acts as a receptor within the osmotic stress signalling pathway and alters expression of downstream stress-response genes via a response regulator and mitogen-activated protein kinase (MAPK) signalling cascade (Urao *et al.*, 1999). SLN1 is usually referred to as an osmosensor; however, the signal actually perceived is thought instead to be changes in either turgor pressure (Reiser *et al.*, 2003), or cell wall structure (Shankarnarayan *et al.*, 2008). In *Arabidopsis thaliana*, AHK1 is a positive regulator of drought and osmotic stress responses, and is involved in ABA signalling (Urao *et al.*, 1999; Tran *et al.*, 2007). *NCED3* and numerous other genes within the ABA-biosynthesis pathway have been found to have reduced expression in an *ahk1* null mutant background and increased expression in a line over-expressing *AHK1* when plants are exposed to high osmolarity over a 10–15 h period (Wohlbach *et al.*, 2008). However, there are conflicting reports over whether this difference in expression results in a significant difference in ABA levels in these plants (Wohlbach *et al.*, 2008; Kumar *et al.*, 2013). While the importance of *AHK1* for sensing changes in water potential over a longer time-frame have been questioned (Kumar *et al.*, 2013), this protein provides the best candidate yet for the hypothesised turgor-sensing receptor that rapidly up-regulates ABA-biosynthesis when plants are exposed to increased VPD.

In this study, we address two key questions: (i) does a controlled reduction in leaf turgor trigger increased expression of *NCED3* over the short time-frame of the stomatal response to VPD, and (ii) is AHK1 the turgor-sensing receptor responsible for up-regulating ABA-biosynthesis during exposure to high VPD?

Materials and methods

Plant materials and growth conditions

Wild-type *Arabidopsis thaliana* Wassilewskija (Ws-2) and null mutant *ahk1-4* (Wohlbach *et al.*, 2008) were grown under the conditions described by McAdam and Brodribb (2016). The turgor of recently excised leaves was reduced by the controlled method of pressurisation in a Scholander pressure chamber and gas exchange measurements of stomatal responses to VPD were undertaken as described by McAdam and Brodribb (2016). In this study, we applied 1.5 MPa of external pressure to excised leaves for 1, 5, 10 or 20 min, after which tissue samples for gene expression analysis were snap-frozen in liquid nitrogen. Leaf samples were taken for the quantification of ABA after 5, 10 and 20 min for wild-type, and after 20 min only for *ahk1-4*. Initial samples for gene expression and ABA quantification were taken prior to application of external pressure for both genotypes, while controls for ABA level comprised leaves that were contained in the pressure chamber without external pressure for 5, 10 and 20 min. The pressure level of 1.5 MPa was chosen as it is known to be beyond the threshold trigger point for ABA biosynthesis in this genotype of Arabidopsis (McAdam and Brodribb 2016).

Measurement of ABA levels

Foliar ABA quantification was performed using the method of McAdam (2015). For statistical analysis of ABA levels, one-way ANOVA was conducted with Tukey's HSD *post-hoc* test to compare initial and final (20 min) levels between wild-type and *athk1-4*, and with Dunnett two-sided *t*-tests to compare 5 and 10 min levels with initial levels in the wild-type.

Expression analyses

For expression experiments, RNA was extracted using the Agilent Plant RNA Isolation Mini Kit according to the manufacturer's instructions, and RNA quantification, reverse transcription, and quantitative reverse transcription PCR (qRT-PCR) were conducted as previously described (McAdam *et al.*, 2016). Transcript levels for each gene of interest were evaluated for 2–4 replicates per genotype/time-point against the SAND family gene *MONENSIN SENSITIVITY1* (*MON1*; At2g28390) using the primers of Czechowski *et al.* (2005), as this reference gene was found to be stably expressed in these samples. Primers were as follows: *CYP707A1*, 5′-AACTCAGGAAGCTTGTTCTTCG-3′ and 5′-AGATCGATAGCAACGCAACG-3′; *CYP707A3*, 5′-AAGC

AGGATTAACCGACGAAC-3′ and 5′-ATTGCCATTTGCTCTTC AGTG-3′. Primers for all other genes are as previously described (McAdam *et al.*, 2016).

For calculations of relative gene expression, the comparative C_T method was used to determine the difference in threshold cycle between the gene of interest and *MON1* (ΔC_T) for each sample, and the fold-change between control (initial samples taken prior to treatment) and other sample groups ($2^{-\Delta\Delta CT}$), as previously described (Schmittgen and Livak, 2008). Statistical analysis of expression data was conducted on ΔC_T values for each sample, using one-way ANOVA with either Tukey's HSD *post-hoc* test for comparisons of initial and final levels between genotypes, or Dunnett two-sided *t*-tests for comparisons of multiple time-points with initial values within a genotype, with a significance level of $P<0.05$ unless otherwise stated. All statistical analyses were conducted using IBM SPSS Statistics (version 21).

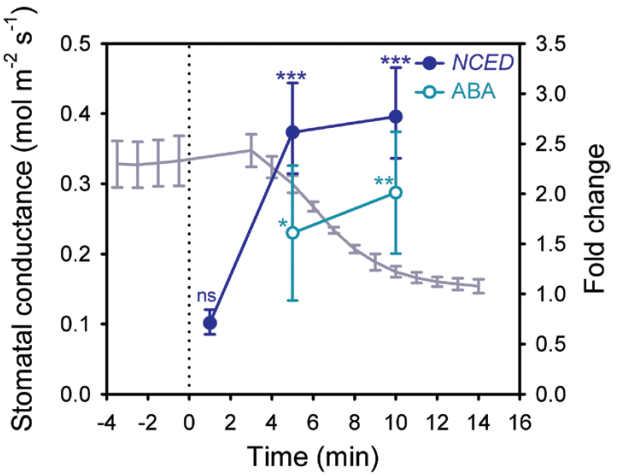

Fig. 1. *NCED3* expression and ABA levels increase after a decrease in leaf turgor and correspond with the timing of stomatal closure after a step-increase in VPD in wild-type Arabidopsis. The mean response of stomatal conductance to a step-change in VPD from 0.75 to 1.5 kPa (line with no symbols; *n*=3, ±SE) and the fold-change in *NCED3* expression (closed circles; *n*=3, ±95% CI) and foliar ABA levels (open circles; *n*=3, ±95% CI) after exposure to external pressure of 1.5 MPa, relative to initial levels. The timing of initiation of the change in external pressure or VPD is denoted by a vertical dotted line. Asterisks denote significant changes compared to initial levels: **P*≤0.05; ***P*≤0.01; ****P*≤0.001; ns, not significant. (This figure is available in colour at *JXB* online.)

Results and Discussion

The speed of induction of ABA biosynthesis via NCED3

We first investigated wild-type expression of *NCED3*, the gene encoding the key, rate-limiting enzyme for ABA-biosynthesis (Qin and Zeevaart, 1999; Thompson *et al.*, 2000). We examined *NCED3* induction over a short time-course of 1–10 min after exposure of leaves to an external pressure of 1.5 MPa, a method that specifically reduces leaf turgor similar to the effect of a natural increase in VPD (McAdam and Brodribb, 2016). Relative to initial levels, expression of *NCED3* was not significantly different after 1 min of exposure to external pressure (P=0.185), but a significant doubling of *NCED3* levels occurred within 5 min of exposure (P=0.001; Fig. 1). We also detected a functionally significant increase in foliar ABA level within 5 min of altered leaf turgor pressure (P=0.047), which was more pronounced after 10 min of exposure (P=0.005; Fig. 1). The time-frame for detected increases in *NCED3* and ABA levels coincided with the initiation of stomatal closure in response to a doubling in VPD (Fig. 1) (Xie *et al.*, 2006), providing evidence that *de novo* ABA biosynthesis, triggered by reduced leaf turgor, can occur within this narrow time-frame.

We also examined the genes that encode the first enzyme in the major ABA catabolism pathway, ABA 8′-hydroxylase, in guard cells and vascular tissue, *CYP707A1* and *CYP707A3*, respectively (Kushiro *et al.*, 2004; Okamoto *et al.*, 2009). We found *CYP707A3* to be significantly upregulated within 5 min of altered leaf turgor pressure (P=0.0003), while *CYP707A1* was significantly up-regulated after 10 min of exposure (P=0.0004), relative to initial levels (Supplementary Fig. S1 at *JXB* online). These results are consistent with previous findings that show these genes to be up-regulated by drought stress, a response that is at least partially ABA-dependent (Kushiro *et al.*, 2004; Saito *et al.*, 2004; Umezawa *et al.*, 2006). Our results confirm there is no down-regulation in catabolism contributing to the rapid increase in ABA levels observed in response to altered leaf turgor, and instead support the conclusion that this rapid increase in ABA levels is due to an increase in ABA biosynthesis, via rapid *NCED3* up-regulation.

For further confirmation that the application of external pressure was the trigger for *NCED3* induction in our short time-course experiment, we conducted additional replicate experiments. Firstly, we showed that no significant change in *NCED3* expression was induced in wild-type leaves in the pressure chamber when no pressure was applied, even after up to 20 min (P=0.688; Supplementary Fig. S2), nor were ABA levels changed in wild-type leaves at any time point when no pressure was applied (P=0.911; Supplementary Fig. S3). Secondly, as our methods of external pressure application in air could potentially affect carbon dioxide (CO_2) availability, and increased CO_2 levels can trigger stomatal closure (Mott, 1988), we ruled out the possibility that the induction of *NCED3* we detected may have been due to altered CO_2 levels, by repeating this experiment under compressed nitrogen gas. We found that *NCED3* was similarly induced by application of external pressure for 20 min when performed under nitrogen as when performed under air (Supplementary Fig. S4). The results from these combined experiments provide evidence that *de novo* biosynthesis of ABA is up-regulated in response to decreased leaf turgor via the rapid induction of *NCED3*, which can occur within a time-frame that matches the speed of the stomatal response to VPD in Arabidopsis.

Investigating AHK1 as a candidate turgor-sensing receptor

We next examined AHK1 as a possible candidate for the turgor-sensing receptor acting upstream of *NCED* in this VPD-response pathway, using the null *ahk1-4* mutant (Wohlbach *et al.*, 2008). In wild-type Arabidopsis, consistent with previous results following exposure to increased VPD (McAdam *et al.*, 2016), we found that *NCED3* was the only gene within the ABA biosynthetic or conjugation pathway to show a

significant change in expression within 20 min of exposure of excised leaves to an external pressure of 1.5 MPa (Fig. 2A). However, we detected no significant difference between the wild-type and *ahk1-4* mutant in either initial (P=0.058) or final (P=0.387) levels of *NCED3* expression, and both genotypes showed a similar pressure-induced increase in *NCED3* expression (Fig. 2A). Accordingly, we found no significant difference in ABA levels between wild-type and *ahk1-4* before (P=0.421) or after 20 min of external pressure (P=0.994), and both genotypes also had a similar and significant pressure-induced increase in ABA level (Fig. 2B). In addition, we found that the stomatal response to a step-increase in VPD was not altered in the absence of AHK1 function (Fig. 2C). These results show that AHK1 does not function as an essential turgor-sensing receptor in the pathway for ABA-mediated stomatal closure in response to increased VPD.

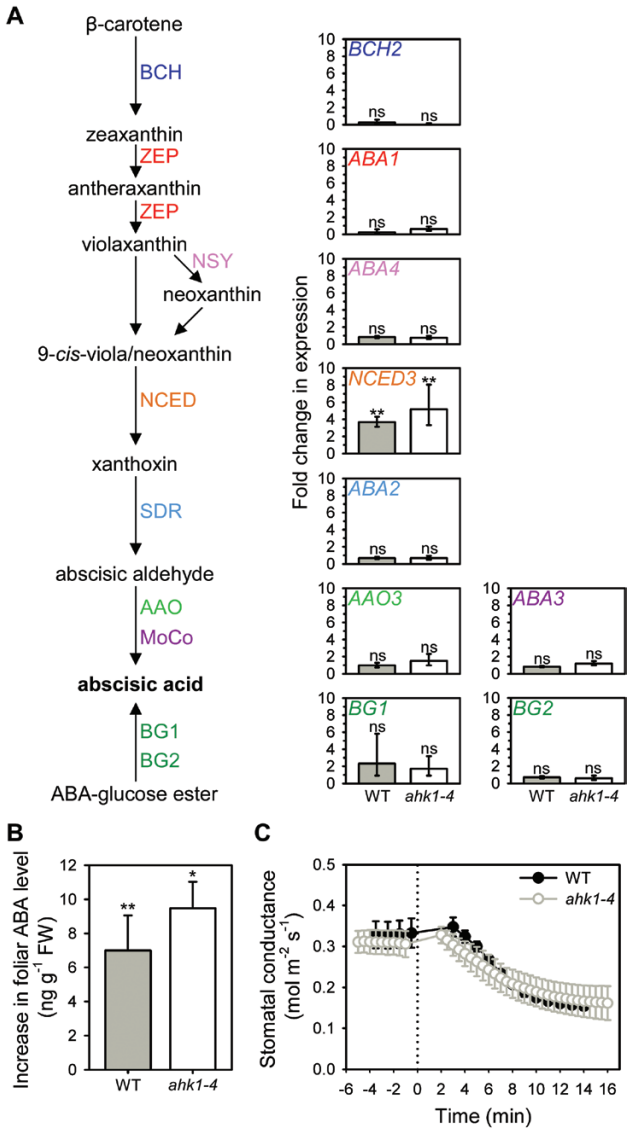

Fig. 2. Loss of *AHK1* function does not affect the expression of ABA biosynthesis genes or ABA level after a decrease in leaf cell turgor, or the stomatal response to increasing VPD. (A) The ABA biosynthesis and ABA-glucose ester hydrolysis pathways showing the fold-change in relative expression of the key genes involved (±95% CI, n=2–3), and (B) the mean increase in foliar ABA level (±95% CI, n=3), after 20 min exposure to external pressure of 1.5 MPa, relative to initial values. Asterisks denote significant changes compared to initial levels within each genotype: *P≤0.05; **P≤0.01; ns, not significant. (C) Mean response of stomatal conductance to a step-change in VPD from 0.75 to 1.5 kPa [n=3, ±SE; dotted line denotes time of VPD change; wild-type (WT) data is also shown in Fig. 1]. (This figure is available in colour at *JXB* online.)

The question of which turgor receptor may be fulfilling this critical role in angiosperms still remains. Based on knowledge of the molecular components involved in osmosensing in the simple eukaryote yeast, a number of other possible candidates have been suggested, including other histidine kinases closely related to AHK1, receptor-like kinases (RLKs; ~600 in Arabidopsis), and integrin-like proteins, which remain to be investigated for a role in regulating ABA biosynthesis (Christmann *et al.*, 2013; Osakabe *et al.*, 2014). Also, although it is likely that a single turgor-sensing protein first evolved to regulate ABA biosynthesis, as the simplest evolutionary model, it remains a possibility that a number of proteins may have since evolved some level of redundancy in this role.

Here, we have shown that specifically altering leaf turgor through the application of external pressure is sufficient to trigger ABA biosynthesis through the up-regulation of *NCED3* within the rapid time-frame of the stomatal response to VPD. This adds further support to the argument that altered turgor constitutes the signal by which angiosperms detect day-time variation in VPD, in order to adjust stomatal aperture accordingly. We have summarised these findings in an updated mechanistic model for ABA-mediated stomatal closure in response to increased VPD in angiosperms (Fig. 3). While our results do not focus on the specific location of ABA-biosynthesis within the leaf, the speed of the response strongly suggests that the site of synthesis, and therefore the location of the turgor sensor that triggers *NCED3* up-regulation, is relatively close to the stomata, in the transpiration stream. All genes involved in ABA biosynthesis are expressed within guard cells, indicating that ABA biosynthesis can occur within guard cells (Bauer *et al.*, 2013). However, a number of lines of evidence suggest that ABA derived from either leaf vascular or mesophyll tissue that is transported into guard cells plays the predominant role in driving stomatal closure at high VPD (Fig. 3). Firstly, *NCED3* and genes that control the subsequent steps in the ABA biosynthetic pathway, *ABA2* and *AAO3*, are expressed predominantly in leaf vascular tissues (Cheng *et al.*, 2002; Koiwai *et al.*, 2004; Endo *et al.*, 2008). Secondly, mutants that lack the function of ABA uptake transporters in the guard cell membrane wilt faster than wild-type plants in response to drought stress (Kang *et al.*, 2010). Lastly, as ABA synthesis must precede loss of guard cell turgor for ABA-driven stomatal closure, in order for the VPD response to be controlled by guard-cell autonomous synthesis of ABA at high VPD there would need to be an as yet unidentified, turgor-independent trigger for ABA biosynthesis that directly senses humidity changes, an arguably less likely scenario (Grantz, 1990). It is worth noting that a recent study has shown that extreme mechanical changes occur in response to transpiration driven decreases in leaf water potential, with considerable yet reversible collapse of minor veins occurring within 20 s following a decrease in water potential in angiosperm leaves (Zhang *et al.*, 2016).

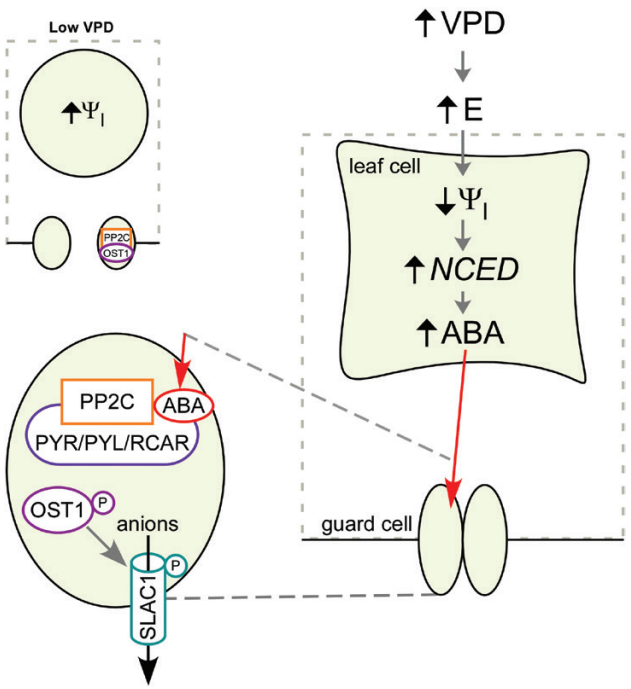

Fig. 3. A proposed mechanistic model for ABA-mediated stomatal closure in response to increased VPD in angiosperms. At low VPD (left), leaf water potential (ψ_l) and turgor pressure are high, and PROTEIN PHOSPHATASE TYPE 2C (PP2C) proteins inhibit the function of the key ABA-signalling pathway, the protein kinase OPEN STOMATA1 (OST1) (Ng *et al.*, 2011). This leaves OST1 incapable of activating the anion channel SLOW ANION CHANNEL 1 (SLAC1) in the guard cell membrane (Geiger *et al.*, 2009). When VPD increases (right), transpiration (E) increases, decreasing leaf water potential (ψ_l) and leaf turgor pressure. Our results indicate that altered turgor is the likely trigger for rapid up-regulation of *NCED3*, the rate-limiting step in the ABA-biosynthesis pathway, resulting in an increase in ABA level within 5 min. The speed of this response indicates that the turgor sensor involved is located close to the stomata, and we hypothesise that it is in the mesophyll cells. ABA is transported into the guard cells (enhanced, centre), from the main site of synthesis in the leaf, where it binds with ABA receptors within the PYRABACTIN RESISTANCE 1 (PYR)/PYR1-LIKE (PYL)/REGULATORY COMPONENT OF ABA RECEPTOR (RCAR) family, which alleviate inhibition of OST1 by binding the catalytic site of PP2C proteins (Park *et al.*, 2009; Soon *et al.*, 2012). OST1 phosphorylates and thereby activates SLAC1, resulting in a flow of ions and loss of osmotic potential from the guard cells, which deflate, closing the stomatal pore (Chen *et al.*, 2010). (This figure is available in colour at *JXB* online.)

This mechanical deformation of the xylem conduits would place mechanical stress on the surrounding cells and may contribute as a mechanical trigger in addition to altered turgor for up-regulation of ABA; a possibility that remains to be investigated.

Conclusions

In conclusion, our results show that *de novo* biosynthesis of ABA, via up-regulation of the rate-limiting *NCED3* gene, can be triggered by changes in leaf turgor over the extremely fast timeframe required to initiate a stomatal response to VPD. These stomatal responses to VPD, governed by *NCED3*, are some of the most important determinants of diurnal gas exchange in land plants (Zhao and Running, 2010). As a result, the regulation of this gene has arguably one of the greatest influences on terrestrial productivity and transpiration of any signalling pathway in land plants. We find that a loss of function of the transmembrane protein AHK1 does not affect either the induction of ABA biosynthesis in response to altered leaf turgor, or stomatal closure in response to increased VPD. These results indicate that AHK1 does not function as the critical turgor-sensing receptor in the pathway for ABA-mediated stomatal closure in response to increased VPD. Instead, we suggest that the continued search for the main turgor receptor in this pathway should focus on the vascular tissue, which could be the predominant site of ABA synthesis (Endo *et al.*, 2008). Future characterisation of this important turgor-sensing receptor will be essential for shedding much needed light on this central means for regulating day-time transpiration in angiosperms.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Expression of ABA catabolism genes.

Fig. S2. Confirmation that *NCED3* up-regulation is triggered by application of external pressure.

Fig. S3. Confirmation that foliar ABA levels do not increase without the application of external pressure.

Fig. S4. Confirmation that *NCED3* up-regulation is triggered by changes to turgor pressure and not altered CO_2 levels when external pressure is applied.

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Author contributions

SM conceived and designed the research and TB provided expertise. FS and SM conducted the experiments and analysed the data. FS wrote the manuscript. All authors read and approved the manuscript.

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