Original Article

Stomatal responses to vapour pressure deficit are regulated by high speed gene expression in angiosperms

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ABSTRACT

Plants dynamically regulate water use by the movement of stomata on the surface of leaves. Stomatal responses to changes in vapour pressure deficit (VPD) are the principal regulator of daytime transpiration and water use efficiency in land plants. In angiosperms, stomatal responses to VPD appear to be regulated by the phytohormone abscisic acid (ABA), yet the origin of this ABA is controversial. After a 20 min exposure of plants, from three diverse angiosperm species, to a doubling in VPD, stomata closed, foliar ABA levels increased and the expression of the gene encoding the key, rate-limiting carotenoid cleavage enzyme (9-cis-epoxycarotenoid dioxygenase, NCED) in the ABA biosynthetic pathway was significantly up-regulated. The NCED gene was the only gene in the ABA biosynthetic pathway to be up-regulated over the short time scale corresponding to the response of stomata. The closure of stomata and rapid increase in foliar ABA levels could not be explained by the release of ABA from internal stores in the leaf or the hydrolysis of the conjugate ABA-glucose ester. These results implicate an extremely rapid de novo biosynthesis of ABA, mediated by a single gene, as the means by which angiosperm stomata respond to natural changes in VPD.

Key-words: abscisic acid (ABA); ABA-GE; 9-cis-epoxycarotenoid dioxygenase; stomata.

INTRODUCTION

The regulation of stomatal movement by changes in the environment has been a field of particular importance to biologists for nearly 200 years (Darwin 1898). One of the most well-recognized and environmentally dynamic signals triggering the movement of guard cells is a change in air humidity, or more precisely, the vapour pressure deficit around the guard cells causing stomata to close (Tardieu & Davies 1993). As an alternative to the flux model, stomatal responses to changes in VPD have been attributed to the rapid release of fettered-ABA from internal stores in the leaf (Georgopoulou & Milborrow 2012), most likely held in the chloroplasts (Loveys 1977).

Recently, however, both of these traditional hypotheses have been challenged by the idea that plants actively regulate gene expression to alter ABA levels, and thereby stomatal responses to changes in VPD. One molecular-based explanation for changes in ABA levels is that plants activate de novo ABA biosynthesis in response to increases in VPD (Bauerle et al. 2004; Bauer et al. 2013a, b; McAdam & Brodribb 2015). There are a number of lines of evidence supporting the idea that increased de novo ABA biosynthesis is the source of the ABA that drives stomatal responses to changes in VPD. These include (i) the stomatal response to VPD is compromised in ABA biosynthetic mutants (Xie et al. 2006; Bauer et al. 2013a); (ii) measurable increases in foliar ABA level in response to high VPD exposure occur over the time scale of minutes (McAdam & Brodribb 2015) to hours (Bauerle et al. 2004); and (iii) at high VPD, there is an increase in the expression of the gene encoding the key protein catalysing the rate-limiting carotenoid cleavage step of ABA biosynthesis by a 9-cis-epoxycarotenoid dioxygenase (NCED) (Bauer et al. 2013a; Pantin et al. 2013). However, changes in the expression of this gene have only been measured in Arabidopsis thaliana plants exposed to increased VPD for an extended period of time (between 1 h and 1 d).

The hypothesis that de novo ABA biosynthesis drives changes in ABA levels during VPD transitions has also been challenged primarily on the basis that the long, multi-step de novo ABA-biosynthetic pathway would be too slow to increase ABA levels in sufficient time to account for the observed rapidity of stomatal responses to VPD (Lee et al. 2006; Xu et al.
2012). Such a rapid increase in ABA levels would require rates of biosynthetic regulation faster than any known plant hormone pathway so far described (Chappell et al. 1984; Tao et al. 2008). In addition, rapid functional increases in ABA levels seen in angiosperms (McAdam & Brodribb 2015) could occur by the single biochemical hydrolysis of the conjugated form of ABA, ABA-glucose ester (ABA-GE), to ABA during an increase in VPD. The conversion of ABA-GE to ABA by a single step through β-glucosidases has long been hypothesized as a means of dynamically increasing ABA levels in both the leaf and xylem sap in response to increased stress (Dietz et al. 2000). A molecular and physiological characterization of the genes encoding β-glucosidases, and their respective mutants, suggests an important role for the hydrolysis of ABA-GE to ABA in plant response to severe water stress (Lee et al. 2006; Xu et al. 2012).

The aim of this study was to resolve these wide-ranging hypotheses behind the origin of functional increases in foliar ABA levels when angiosperms are exposed to increased VPD (McAdam & Brodribb 2015). Using quantitative gene expression analysis, we specifically investigated whether de novo ABA-biosynthetic genes were up-regulated in the leaves of angiosperms over the extremely rapid (less than 20 min) period of time during which the stomata respond to a doubling in VPD. In addition, we used a series of ABA biosynthetic mutants to test whether ABA dynamics and stomatal conductance in these mutants were responsive over short-term VPD transitions. Finally, we concurrently measured changes in both foliar ABA and ABA-GE levels in diverse wild-type angiosperm species, to see if the rapid increase in foliar ABA level after a doubling in VPD could be explained by a parallel decrease in ABA-GE through the activity of β-glucosidases.

**MATERIALS AND METHODS**

**Plant material**

In experiments involving the measurement of gene expression, *Solanum lycopersicon* ‘Rhinelands Rhun’ (Solanaceae), *Pisum sativum* ‘Torsdag’ (Fabaceae) and *A. thaliana* ‘Col-0’ (Brassicaceae) were used; these species were chosen because of their phylogenetic diversity as well as the availability of ABA biosynthetic mutants for these species and knowledge of genes in the ABA biosynthetic pathway, particularly *A. thaliana*, for which genes in the complete biosynthetic pathway for ABA have been characterized. In experiments investigating the possibility of stomatal closure in ABA biosynthetic mutants following an increase in VPD, *S. lycopersicon* ABA biosynthetic mutant lines *flccca* and *sitiens* with wild-type line ‘Rhinelands Rhun’ and *notabilis* with wild-type line ‘Micro-Tom’, and the *P. sativum* ‘wilty’ mutant with wild-type line ‘Torsdag’ were used. These mutants have all been characterized at the molecular level (Burbidge et al. 1999; Sagi et al. 2002; Harrison et al. 2011; McAdam et al. 2015) and together represent a deficiency in every step in the ABA biosynthetic pathway after and including the rate-limiting NCED-mediated carotenoid cleavage step (Supporting Information Fig. S1). For experiments investigating the capacity of plants to utilize ABA-GE as a means of rapidly increasing foliar ABA levels, we selected species that spanned the ecological range of dicot angiosperms including *A. thaliana* ‘Col-0’, *S. lycopersicon* ‘Rhinelands Rhun’ at 30 d of age and at 120 d of age (unlike young plants which have lower levels, older plants have levels of ABA-GE similar to ABA (Weiler 1980)) and *Urena lobata* (Malvaceae), a woody, tropical angiosperm.

All experiments were conducted on plants grown in a controlled environment growth cabinet (PGC-105, Percival Scientific Inc., Perry, IA, USA) between 1100 and 1300 h. Plants of *P. sativum* were grown in 14 cm slim-line pots in a 1:1 mix of vermiculite and dolerite gravel chips topped with 3 cm of potting mix, *S. lycopersicon* and *U. lobata* individuals were grown in 14 cm slim-line pots in potting mix and *A. thaliana* plants were grown in 5 cm pots in potting mix. All plants were watered daily and received weekly applications of liquid fertilizer (Aquasol, Hortico Ltd, Padstow, NSW, Australia). Conditions in the growth cabinet were regulated at 25 °C/16 °C day/night temperature and a 16 h photoperiod, provided by mixed incandescent and fluorescent lights ensuring a minimum 300 μmol quanta m⁻² s⁻¹ at the pot surface. For germination and prior to experiments, a daytime VPD of 1.2 kPa (62% relative humidity) was maintained with temperature and relative humidity monitored every 5 min during this period by a data logger (HOBO Pro Series, Onset, Bourne, MA, USA).

**Physiological and molecular measurements over the VPD transition**

For all experiments, plants were initially grown for a week at 0.7 kPa (±0.05 kPa) maintained by the presence of containers of water and a 1 m² surface of wet hessian and 22 °C. After this acclimation period to low VPD, the simultaneous monitoring of leaf gas exchange, foliar ABA (and ABA-GE) levels, leaf water potential and harvesting of leaf tissue for gene expression analysis was undertaken on each of the four individuals at midday. Methods for each of these are described in the succeeding discussions. After this initial simultaneous measurement, VPD was increased to 1.5 kPa (42% relative humidity) using a condensing dehumidifier (SeccoUltra 00563; Olimpia-Splendid, Gualtieri, Italy) in the growth cabinet. Temperature and relative humidity were monitored every 30 s during the experimental period by a humidity probe (HMP45AC; Vaisala, Vantala, Finland) and thermocouple connected to a data logger (CR10X; Campbell Scientific, Logan, UT, USA). A VPD of 1.5 kPa was maintained for 20 min after which leaf gas exchange, foliar ABA (and ABA-GE) level, leaf water potential and harvesting of leaf tissue for gene expression analysis were again undertaken. VPD was then returned to 0.7 kPa, and measurements were again conducted after a further 20 min. The small volume of air in the growth cabinet (3 m³) resulted in a fast half time for the VPD transition of 150 s, with no hysteresis.

**Leaf gas exchange and water potential measurements**

Leaf gas exchange was measured in fully irradiated leaves using an infrared gas analyser (LI-6400, Li-Cor Biosciences).
Conditions in the leaf cuvette were maintained as close as possible to the conditions in the growth cabinet, with VPD regulated by a portable dew point generator (LI-610; Li-Cor Biosciences, Lincoln, NE, USA) and light intensity set at 1000 μmol quanta m⁻² s⁻¹. Leaves were enclosed in the cuvette, and instantaneous gas exchange was logged following stability in cuvette conditions (after approximately 30 s). Following gas exchange measurements, the same leaf was then excised and sampled for foliar ABA (and ABA-GE) quantification.

A neighbouring leaf was excised and immediately wrapped in damp paper towel and double bagged for leaf water potential assessment using a Scholander pressure chamber and microscope to accurately determine the balance pressure. A further neighbouring leaf or leaflet was harvested for expression analysis (see below), by immediate freezing in liquid nitrogen.

**Foliar ABA and ABA-GE extraction, purification and quantification**

Samples harvested for foliar ABA and ABA-GE quantification were immediately weighed (±0.0001 g; MS204S; Mettler-Toledo, Greifensee, Switzerland) into 50 mL tubes, covered in approximately 15 mL of cold (0°C) 80% methanol in water (v/v) with 250 mg L⁻¹ (m/v) of added butylated hydroxytoluene and transferred to –20°C. Foliar ABA was extracted, purified and quantified by physicochemical methods using an added internal standard and ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS) according to the a previously reported method of McAdam & Brodribb (2014). Extraction and purification of foliar ABA-GE was the same as for foliar ABA. ABA-GE was also analysed by UPLC-MS, having an approximate retention time of 6.6 min. The mass spectrometer was operated in negative ion electrospray mode with a needle voltage of 2.8 kV and multiple reaction monitoring (MRM) for detection. For ABA-GE, the MRM transition, precursor (m/z) 425.2 [M-H]⁻ to product (m/z) 263.2 [M-H]⁻ was used for quantitation with a dwell time of 22 ms. Cone voltage was 25 V, and collision energy was 14 V for this transition. The ion source temperature was 130°C, the desolvation gas was N₂ at 950 L h⁻¹, the cone gas flow was 100 L h⁻¹ and the desolvation temperature was 450°C. Data were processed using Waters MassLynx software (Milford, MA, USA). For all samples, the ratio of endogenous ABA-GE ion intensity to the [2H₆]-ABA internal standard ion intensity was calculated. The product of this ratio and the amount of internal standard added were divided by the fresh weight of tissue sampled to determine the level of ABA-GE per gramme of fresh weight.

**Expression analysis**

Quantitative reverse transcription PCR analysis was used to determine expression of all of the characterized ABA biosynthetic genes of *A. thaliana*, *S. lycopersicon* and *P. sativum*, as well as *PsNCED2* which, while not characterized by a mutant, is the only *P. sativum* homolog in the well-characterized clade of eudicot, foliar ABA-biosynthetic specific NCED genes (Supporting Information Fig. S2). This clade includes *AtNCED3*, which is the gene responsible for ABA biosynthesis in leaves of *A. thaliana* (Iuchi et al. 2001), *SINCEI*, the gene affected in the classic *notabilis* mutant (Burbidge et al. 1999) and the characterized NCED genes in the legumes *Vigna unguiculata* and *Phaseolus vulgaris*, which are specifically responsible for increases in foliar ABA levels during water stress (Qin & Zeevaart 1999; Iuchi et al. 2000). RNA was extracted using the SV total RNA isolation system (Promega, Madison, WI, USA), and RNA concentrations were determined using a NanoDrop 8000 (Thermo Scientific, Waltham, MA, USA). Reverse transcription was conducted in 20 μL with 1 μg of total RNA using the Tetro cDNA synthesis kit (Bioline, London, UK) according to the manufacturer’s instructions. RT-negative (no enzyme) controls were performed to monitor for contamination with genomic DNA. First-strand cDNA was diluted five times, and 2 μL was used in each PCR. Reactions using SYBR green chemistry (SensiFAST, Bioline) were set up with a CAS-1200N robotic liquid handling system (Corbett Research, Mortlake NSW, Australia) and run for 50 cycles in a Rotor-Gene Q (Qiagen, Valencia, CA, USA). Two technical replicates were performed for each sample. Transcript levels for each gene of interest were evaluated against housekeeping genes that were stably expressed in leaves through the VPD transition. Primer details are given in Supporting Information Table S4.

**RESULTS**

When a leaf is exposed to a mild, step-increase in VPD for 20 min (from 0.7 to 1.5 kPa), stomata close rapidly (Fig. 1). The three species examined all exhibited stomatal closure over 20 min when whole plants were exposed to a doubling in VPD (Fig. 2). Increases in foliar ABA levels over this relatively short period of time ranged between twofold and ninefold, depending on the species (Fig. 2; Supporting Information Tables S1 and S2). In each of these species, the expression of the specific NCED gene with a characterized or predicted role in foliar ABA biosynthesis (Supporting Information Fig. S2) was significantly up-regulated (between threefold and tenfold relative to background) over this transition in VPD (Fig. 2). This increase in the gene expression was particularly high in *A. thaliana* and *P. sativum*, which also had the greatest fold increases in foliar ABA level (Fig. 2). No other genes examined within the ABA biosynthetic pathway showed a significant change in expression over this period (Fig. 2).

In all species, there was very little change in the expression of ABA biosynthetic genes after returning to 0.7 kPa for 20 min (Supporting Information Table S3). In *A. thaliana* and *S. lycopersicon*, the expression of NCED genes remained high after a 20 min transition back to 0.7 kPa, which was consistent with foliar ABA levels, which also remained high in these species (Supporting Information Table S3). As a result, there was pronounced hysteresis in the recovery of stomatal conductance on returning to 0.7 kPa for 20 min in all species examined (Supporting Information Table S2). The lack of recovery in stomatal conductance resulted in leaves being more hydrated upon returning to 0.7 kPa for 20 min than at the start of the experiment (Supporting Information Table S5).
Characterized ABA biosynthetic mutants, with mutations in genes spanning the ABA biosynthetic pathway after and including the carotenoid cleavage step catalysed by NCED enzymes, had no significant change in stomatal conductance in response to an increase in VPD (Supporting Information Table S2). In addition, no ABA biosynthetic mutant plants showed a significant increase in foliar ABA level 20 min after a transition in VPD from 0.7 to 1.5 kPa, unlike wild-type plants (Fig. 3). The absence of a rapid stomatal response to the increase in VPD in these ABA biosynthetic mutants occurred despite a number of these mutant plants having the same foliar ABA levels as wild-type plants prior to the VPD transition (Fig. 3; Supporting Information Table S1). On returning to 0.7 kPa for 20 min, leaf hydration in the ABA biosynthetic mutant plants was the same as at the start of the experiment (Supporting Information Table S5).

In wild-type plants from diverse angiosperm species, increases in foliar ABA level after a 20 min exposure to a doubling in VPD was not accompanied by a corresponding decrease in the foliar level of ABA-GE, as would be expected if the source of the rapid rise in ABA was conversion from ABA-GE (Fig. 4). Foliar ABA-GE levels in young plants were several orders of magnitude less than levels of ABA in the same leaves (Supporting Information Table S6). In A. thaliana, while ABA was easily detected in leaf samples, no evidence of ABA-GE could be detected by UPLC-MS analysis in the same leaf samples of these plants. In young plants of S. lycopersicon and U. lobata, the change in foliar ABA-GE levels was not significant, but in mature S. lycopersicon plants, which had foliar ABA-GE levels that were approximately 25% foliar ABA levels (Supplementary Table 6), there was a significant increase
in both foliar ABA and ABA-GE levels after 20 min at high VPD, contrary to the expectation if a pool of ABA-GE was the source for a rapid rise in foliar ABA (Fig. 4).

**DISCUSSION**

Rapid increases in functionally relevant levels of foliar ABA occur in angiosperms following even mild increases in VPD (McAdam & Brodribb 2015), and we show here that this increase in foliar ABA level has its origin in *de novo* biosynthesis. We found that the NCED genes, which mediate the carotenoid cleavage step in ABA biosynthesis were significantly up-regulated after only 20 min following a doubling in VPD, and that these were the only genes in the ABA biosynthetic pathway observed to be up-regulated. It has long been known, from studies investigating the origin of increased ABA levels in severely desiccated plants, that the NCED-mediated step in ABA biosynthesis is rate limiting (Qin & Zeevaart 1999; Thompson *et al.* 2000), and one of the first to be up-regulated in plants exposed to soil water stress (Seo & Koshiba 2002) or VPD (Bauer *et al.* 2013a; Pantin *et al.* 2013), but these previous experiments did not demonstrate that increased NCED expression was fast enough to account for the stomatal responses to VPD that occur over minutes. We show here that changes in the expression of NCED genes can also occur dynamically in response to typical changes in atmospheric water status and over the very fast timescale relevant to stomatal control. With changes in VPD being one of the greatest determinants of

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**Figure 3.** Mean foliar abscisic acid (ABA) levels (left panels) and stomatal conductance (right panels) before and after a 20 min step change in vapour pressure deficit (VPD) from 0.7 to 1.5 kPa in wild type (white) and ABA biosynthetic mutant plants (grey; *n* = 4 and ±SE) of *Pisum sativum* (wild-type Torsdag) and *Solanum lycopersicon* (wild type for mutants sitiens and flacca was Rhinelands Rhun and for notabilis was Micro-Tom). Different letters denote significant differences between means.
daytime canopy transpiration and water use efficiency, our data suggest that the dynamic expression of the NCED gene may have very large-scale consequences for plant canopy–atmosphere interactions.

Documented changes in hormone levels over very short time frames are extremely rare, especially those that involve the up-regulation of biosynthesis. The levels of other dynamic hormones such as auxin have been shown to increase over an hour in response to increased light levels (Tao et al. 2008) and ethylene levels over an hour in response to a fungal elicitor (Chappell et al. 1984). Thus, the evolution of ABA-mediated stomatal responses to VPD in the earliest angiosperms (McAdam & Brodribb 2015) required the evolution of an extraordinarily rapid means of activating hormone biosynthesis. The rapid mechanism through which NCED expression is up-regulated by a reduction in turgor during a VPD transition remains unknown, although a number of candidate, turgor-activated transcription factors that might regulate ABA biosynthesis have been suggested (Christmann et al. 2007).

We observed here that the high levels of foliar ABA in plants exposed to an increase in VPD tend to remain high for an extended period after plants are returned to lower VPDs. This delay in the reduction of ABA levels results in hysteresis in both stomatal conductance and leaf water potential. Hysteresis in the stomatal response to VPD appears to be due not only to a delay in the activation of ABA catabolism or conjugation but also because transcript levels of NCED genes remain high on returning to low VPD (Supporting Information Table S3). The maintenance of relatively high NCED transcript levels after the rehydration of droughted plants has been shown previously in A. thaliana (Iuchi et al. 2000). Whether hysteresis in the stomatal response to VPD in angiosperms has a selective advantage, or is simply an artefact of plants having no means of rapidly deactivating NCED transcription, remains to be tested.

Alternative explanations, including the release of ABA from internal stores (Georgopoulou & Milborrow 2012) or the hydrolysis of the conjugate ABA-GE (Dietz et al. 2000), cannot explain our observations of rapid increases in foliar ABA levels in angiosperms in response to increased VPD. While not relevant for stomatal responses to VPD transitions, the release of fetttered-ABA from the chloroplasts may play a major role in triggering the substantial biosynthesis of foliar ABA levels when plants are drought stressed beyond turgor loss point (Loveys 1977; Pierce & Raschke 1981; Georgopoulou & Milborrow 2012).

Our data on ABA-GE levels, though, indicate that the hydrolysis of ABA-GE to ABA is only ever likely to contribute to a very small portion of increased ABA levels during water stress.

The large increase in foliar ABA level and up-regulation of expression of NCED in the leaf adds to a growing body of evidence suggesting that the leaf provides the vast majority of ABA responsible for closing stomata (Holbrook et al. 2002; Christmann et al. 2007; McAdam & Brodribb 2015). Alternative explanations for the main site of ABA biosynthesis in the plant include the roots (Zhang et al. 1987) and the guard cells (Bauer et al. 2013a). Elegant grafting studies however suggest that an inability of roots to synthesize ABA has no effect on stomatal control (Holbrook et al. 2002; Christmann et al. 2007). Whether guard cells provide sufficient ABA to elicit a functional stomatal response to VPD is uncertain. Recently, it has been shown that ABA levels in the epidermis (unlike the leaf) do not increase until after major increases in foliar ABA levels and stomatal closure during a VPD transition (McAdam & Brodribb 2015). While a recent study that sequenced RNA profiles of A. thaliana during a transition in VPD found that NCED3 expression in the guard cells was up-regulated twofold after a quadrupling in VPD for at least an hour (Bauer et al. 2013a), this limited increase contrasts with the 14-fold increase in NCED3 expression observed in A. thaliana leaf tissue following a doubling in VPD for only 20 min in this study. These findings, as well as others, which highlight the importance of ABA transporters for functional stomatal behaviour (Kuromori et al. 2011; Kanno et al. 2012; Kuromori et al. 2014; Merilo et al. 2015), support the idea that ABA synthesized in the leaf vasculature is by far the main contributor to ABA-mediated stomatal closure.

In conclusion, we show that extremely rapid de novo biosynthesis of foliar ABA, through the up-regulation of a single gene in the ABA-biosynthetic pathway, drives ABA-mediated stomatal responses to VPD in angiosperms. The single step that is up-regulated in response to increased VPD is the rate-limiting cleavage of carotenoid precursors by the NCED enzyme. We show that the expression of NCED has enormous implications for diurnal fluxes of water in angiosperms. Extending our understanding of the regulation of this single gene has important mechanistic implications for the modelling of global gas exchange.

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REFERENCES


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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Figure S1.** The biosynthetic pathway for ABA with known enzymes and characterised mutants of these enzymes.

**Figure S2.** Phylogram of angiosperm NCED proteins. *Table S1. Mean foliar ABA levels. Table S2. Mean stomatal conductance. Table S3. Mean relative expression of ABA biosynthetic genes. Table S4. qRT-PCR primer details. Table S5. Mean leaf water potentials. Table S6. Foliar ABA-GE levels. Table S7. Details of sequences. *Data Set S1. Alignment used for Supplemental Figure 2.*