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Role of guard-cell ABA in determining steady-state stomatal aperture and prompt vapor-pressure-deficit response

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ABSTRACT

Abscisic acid (ABA) is known to be involved in stomatal closure. However, its role in stomatal response to rapid increases in the vapor pressure deficit (VPD) is unclear. To study this issue, we generated guard cell-specific ABA-insensitive *Arabidopsis* plants (guard-cell specific *abi1-1*; GCabi). Under non-stressed conditions, the stomatal conductance (g_s) and apertures of GCabi plants were greater than those of control plants. This supports guard-cell ABA role as limiting steady-state stomatal aperture under non-stressful conditions. When there was a rapid increase in VPD (0.15 to 1 kPa), the g_s and stomatal apertures of GCabi decreased in a manner similar that observed in the WT control, but different from that observed in WT plants treated with fusicoccin. Low VPD increased the size of the stomatal apertures of the WT, but not of GCabi. We conclude that guard-cell ABA does not play a significant role in the initial, rapid stomatal closure that occurs in response to an increase in VPD, but is important for stomatal adaptation to ambient VPD. We propose a biphasic angiosperm VPD-sensing model that includes an initial ABA-independent phase and a subsequent ABA-dependent steady-state phase in which stomatal behavior is optimized for ambient VPD conditions.

1. Introduction

Stomata are specialized epidermal structures consisting of two guard cells between which there is a pore whose opening is actively regulated, to allow for controlled gas exchange between a plant and the atmosphere. Abscisic acid is a major and evolutionary conserved stomatal regulator [1–3], increasing the flexibility of stomatal control. Nevertheless, the time of transition from passive to ABA dependent control of stomata is still a meter of debate, as ferns exhibit evidence for both passive-hydraulic [4] and ABA dependent stomatal control depending on fern species [1,5] and growing conditions [5]. ABA active-chemical mechanism initiates rapid signal transduction for the depolarization of guard cell membrane potential, decreased osmotic concentration, turgor loss and reduced stomatal aperture [6,7].

Stomatal aperture is known to respond to differences between the vapor concentration within the leaf and the vapor concentration in the air. Mott [8] showed that guard cells do not sense relative humidity (RH) directly, but do respond to changes in the transpiration rate. The difference in vapor pressure between the leaf and the atmosphere (atmospheric vapor pressure deficit; VPD) serves as the driving force for transpiration, determining the rate at which water is lost from the leaf. An increase in the VPD (a greater difference in vapor concentrations) accelerates the loss of water from the leaf and initiates a reduction in stomatal aperture that prevents excessive water loss and protects the leaf from desiccation. Due to its important role in stomatal regulation, ABA has been considered as a possible key player in the mechanism by which the guard-cell respond to changes in the VPD. Nevertheless, there is evidence both in favor of and against ABA involvement in stomatal VPD response, leaving it a matter of debate. In line with evidence supporting the hypothesized role of ABA in stomatal VPD response, McAdam and Brodribb [9] recently showed that a reduction in leaf turgor can trigger ABA biosynthesis and that increased sensitivity of ABA synthesis to leaf turgor corresponds with a higher stomatal sensitivity to VPD. This results suggests that the rapid biosynthesis of ABA in the leaf (10 min) could be responsible for the angiosperms' stomatal VPD response [10,11]. Moreover, an increase in guard-cell ABA was measured 15 min after a drop in humidity (i.e., an increase in VPD) [12]. Indeed, guard-cell were shown to possess the entire ABA biosynthesis pathway [13], which is sufficient for the stomatal response to low RH [14]. These last two findings support the hypothesis that guard-

Abbreviations: ABA, abscisic acid; AXS, artificial xylem sap; RWC, relative water content; g_s, stomatal conductance; VPD, vapor pressure deficit; WUE, water-use efficiency

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cell self-synthesize ABA in response to an increase in VPD. However, other studies have suggested that ABA synthesis is not limited to the guard-cell and that more intense ABA synthesis may take place elsewhere in the leaf [4]. Mutants with impaired ABA metabolism [11,13,16–18; *Pisum sativum, Solanum lycopersicon, Nicotiana plumbaginifolia, Arabidopsis thaliana*] and signaling [15,17,18; *Arabidopsis thaliana*] exhibited in some cases impaired stomatal responses to rapid changes in VPD. It is important to mention that, as described in the next paragraph, some ABA mutants did show a VPD response in these types of experiments. Buckley [19] recently claimed that the increase in ABA content following an increase in the VPD can fill in a gap in the hydroactive feedback hypothesis, demonstrating how an ultimate mechanism (gene regulation) yields an intermediate signal and a proximate effect (stomatal closure).

In line with evidence that ABA does not play a role in stomatal VPD response, a very recent study by Merilo [14] showed that a broad range of Arabidopsis ABA mutants (mostly ABA-deficient) exhibit a reduction in g_s in response to an immediate increase in VPD that is similar or even more intense than that observed for the WT, raising anew the debate regarding the role of ABA in this process. This new evidence corresponds with the work of Assmann et al. [20] which showed that ABA-deficient (*aba1*) and ABA-insensitive (*abi1-1*, *abi2-1*) Arabidopsis mutants have a WT-like stomatal response to VPD, as well as the ABA-independent VPD stomatal closure pathway reported by Yoshida et al. [21] and Merilo et al. [14].

In order to better understand the role of guard-cell ABA in angiosperms' responses to VPD, we generated, for the first time, guard-cell-specific ABA-insensitive plants (GCabi *Arabidopsis thaliana* plants) using the *abi1-1* mutant gene under the control of a guard-cell-specific promoter, resulting in dominant guard-cell ABA insensitivity against a non-manipulated background. This allows, for the first time to the best of our knowledge, the isolation of a guard cell-specific ABA response (as opposed to the whole-plant ABA response). Moreover, by observing stomatal conductance and aperture responses simultaneously, we were able to exclude possible artifacts of gas-exchange measurements. We demonstrate that while guard-cell ABA does play a role in adjusting stomatal aperture to the ambient VPD, it plays no role in sensing rapid changes in ambient VPD, which seems to decrease stomatal conductance and stomatal apertures via a mechanism that is not ABA-dependent.

2. Materials and methods

2.1. Plant material

Arabidopsis (*Arabidopsis thaliana* ecotype Colombia) lines that express GFP or abi1-1 specifically in guard-cell (GCGFP and GCabi lines, respectively) were generated following transformation with GFP or abi1-1 expressed under the KST1 promoter [22] using the floral-dip transformation method [23]. *abi1-1* is a gain-of-function mutation that also has dominant negative features in terms of ABA-sensing [24–26]. Expression of abi1-1 results in ABA insensitivity despite the presence of the WT ABI1 and other redundant PP2Cs, even when the Arabidopsis gene is expressed in poplar (*Populus* x *canescens* [Ait.] Sm.) [27] and tomato (*Lycopersicon esculentum* L.) [28]. Three independent transgenic lines for each construct were identified.

The Arabidopsis plants were grown in a growth chamber under short-day conditions (10 h light, light intensity of ˜150 µmol $m^{-2} s^{-1}$) at a controlled temperature of 20–22 °C. Plants were exposed to 50% humidity (VPD = ˜1.4 kPa) or covered with clear plastic lid to maintain 90% humidity (VPD = ˜0.2 kPa). All plants were grown in potting mix containing (w/w) 30% vermiculite, 30% peat, 20% tuff and 20% perlite (Shacham; Beit Haemek, Israel).

Cyrtomium falcatum ferns were obtained from the Givat Brenner Nursery (Israel) and were grown in a tropical greenhouse until they were transferred to the lysimeter system (described below).

2.2. Generation of GCabi and GCGFP plants

The *abi1-1* gene from an *abi1-1* plant (Landsberg ecotype) was cloned into the pDONR™ 221 vector (Invitrogen; Waltham, MA USA) and the KST1 partial promoter; KSTppro (KST) promoter [22,29] was cloned into pDONRP4P1r using Gateway BP reactions, and later recombined into a pK7M24GW two-fragment destination vector [30] using a Gateway LR reaction, according to the manufacturer's instructions. The binary *KST:abi1-1* vector was transformed into Agrobacterium by electroporation. The *KST:GFP* binary vector was constructed using the same method used to construct the *KST:abi1-1* binary vector, except that the *abi1-1* gene was replaced with the GFP (green fluorescent protein) gene. GCabi mutants were identified through the use of high-resolution melt analysis real-time PCR (Corbett Research Rotor-Gene 6000 cycler; Sydney, Australia) using forward (5TGGTCG GTTTGATCCTCAAT3) and reverse (5TAGCTATCTCCTCCGCCAAA3) primers. DNA of plants suspected to be transgenic was sequenced to confirm the presence of the *abi1-1* snip (G to A at position 539) in the plant.

Comparison of three independent GCabi lines to the WT revealed that all of those GCabi lines had significantly higher stomatal conductance and larger stomatal apertures (Suppl. Fig. 1). All experiments were conducted using at least three independent lines of T3 and T4 plants, except for the lysimeter experiment, which included only one transgenic line (GCabi9).

2.3. Confocal microscopy and thermal imaging

Images were acquired using the Olympus IX 81 inverted laser scanning confocal microscope (Fluoview 500; Olympus; Tokyo) equipped with a 488-nm argon ion laser and a 60 × 1.0 NA PlanApo water immersion objective. GFP was excited by 488-nm light and the emission was collected using a BA 505–525 filter. A BA 660 IF emission filter was used to observe chlorophyll autofluorescence. Confocal optical sections were obtained at 0.5-µm increments. The images were color-coded green for GFP and red for chlorophyll autofluorescence. Infrared thermal imaging was taken using FLIR A655sc 50 Hz full frame ResearchIR

2.4. Stomatal measurements

Epidermal peels were soaked in 'closure' enabling solutions, as described in Acharya et al. [31], under a light intensity of ˜150 µmol m − 2 s − 1. After 1.5 h, ABA ((+)-cis, trans abscisic acid; Biosynth; Staad, Switzerland), PEG6000 (Sigma-Aldrich; Rehovot, Isreal) or fusicoccin (Santa Cruz Biotechnology; Heidelberg, Germany) were added to reach desirable concentration. Solvents (ethanol or DMSO) at the same concentration were added to the control. Fusicoccin is a fungal toxin that stimulates stomatal opening by activating the plasma membrane ATPase even in the presence of supplemental ABA. The fusicoccin treatment results in forced open stomata, mimicking wide open, ABA-insensitive stomata (Fig. 5C; [32]) and was, therefore, referred to as a "positive control" for insensitive open stomata.

The stomatal apertures (Figs. 5 and 6), stomatal densities and stomatal indices of the plants were determined using the rapid imprinting technique [33]. This approach allowed us to reliably score hundreds of stomata from each treatment, each of which was sampled at the desired time. In brief, light-bodied vinylpolysiloxane dental resin (Heraeus-Kulzer; Hananu, Germany) was attached to the abaxial leaf side and then removed as soon as it had dried (1 min). The resin epidermal imprints were covered with nail polish, which was removed once it had dried. The nail-polish imprints were mirror images of the resin imprints. The nail-polish imprints were put onto microscope slides.

All stomata were photographed under a bright-field inverted microscope (1M7100; Zeiss; Jena, Germany) on which a Hitachi HV-D30 CCD camera (Hitachi; Tokyo, Japan) was mounted. Stomatal images

were analyzed to determine aperture size using the ImageJ software (http://rsb.info.nih.gov/ij/). A microscopic ruler (Olympus; Tokyo, Japan) was used. Stomatal index was calculated as the number of stomata / (number of stomata + number of epidermal cells).

2.5. Measurements of whole-plant continuous canopy conductance

Relative whole-plant continuous canopy conductance (g_{sc}) was measured using an array of load-cell lysimeters (Plantarray Gravimetric Prototype system, Plant-DiTech Ltd; Rehovot, Israel), as described by Halperin et al. [34]. GCabi and WT Arabidopsis plants were plated on kanamycin (50 mg mL–1) selection medium or antibiotic-free medium, respectively. After 3 weeks, the seedlings were transferred to 3.9-L pots (six plants per pot) in the greenhouse. Pots were covered with plastic wrap and gradually uncovered. *C. falcatum* ferns were planted directly into 3.9-L pots (one plant per pot).

The Arabidopsis plants were grown in a greenhouse under semi-controlled conditions of 26/12 °C (day/night) and natural day length and light conditions in Rehovot, Israel during January and February of 2014. The ferns were grown in the greenhouse under a shade net and semi-controlled conditions of 27/18 °C (day/night) and natural day length in Rehovot, Israel during May and June 2016. Daily measurements were conducted simultaneously for all of the plants in the array, so that all the plants were exposed to similar ambient conditions at each measurement point. Since no differences were observed between the three independent lines of GCabi used in previous experiments, only one line (GCabi9) was used in the lysimeter experiment, which allowed us to increase the number of replicates of GCabi. Each pot was placed on a temperature-compensated load cell. The soil surface surrounding each Arabidopsis plant was covered to prevent evaporation. The pots holding ferns were not sealed, due to the large area from which fronds were initiated and shoot density, which prevent soil evaporation almost completely. The output (weight) of the load cells was monitored every 10 s and 3-min average values were logged in a data-logger for further analysis. Whole-plant transpiration was calculated as a numerical derivative of the load-cell output following a data-smoothing process. The daily water loss rate was normalized to the total plant weight to determine the transpiration rate. Continuous whole-canopy conductance was calculated by dividing the whole-plant transpiration rate by the VPD.

2.6. Gas-exchange measurements

Leaves of plants that were 7 to 9 weeks old were excised just before dawn and immediately immersed (petiole-deep) in artificial xylem sap (AXS; 3 mM KNO_3, 1 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 3 mM $CaCl_2$, 0.25 mM NaH_2PO_4, 90 μM EDFS and a micromix of 0.0025 μM $CuSO_4$*$5H_2O$, 0.0025 μM H_2MoO_4, 0.01 μM $MnSO_4$, 0.25 μM KCl, 0.125 μM H_3BO_3*$3H_2O$, 0.01 μM $ZnSO_4$*7 H_2O). Cotton swabs were then used to smear leaves with 10 μM fusicoccin (Santa Cruz Biotechnology) dissolved in ethanol and diluted with AXS, or with AXS containing the same concentration of ethanol. The leaves were then kept in the growth chamber for 1 h to allow the smeared material to dry. Then, the leaves were put into a sealed transparent plastic box, in which they were exposed to elevated humidity, up to 94% (VPD = ˜0. 15 kPa), for 2 h, ensuring identical low VPD steady-state conditions. From the beginning of the experiment, the boxes were kept in the lab under a light intensity of ˜150 μmol m − 2 s − 1. The measurement data described below were collected using leaves from different boxes, in order to ensure a uniform, very humid starting point for all measurements

Gas-exchange measurements were taken using the LI-6400 portable gas-exchange system (LI-COR; Lincoln, NE, USA). In order to simultaneously measure stomatal conductance and aperture, a low-VPD steady state was needed outside of the LI-6400 cuvette, to allow the imprinting of the stomatal aperture. For leaves imprinting before and after the increase in VPD, pairs of leaves were prepared. At the beginning of each measurement, two leaves were taken from a box (VPD = ˜0. 15 kPa), one leaf was immediately imprinted while the second leaf was placed in the LI-COR chamber for 20 min and then immediately imprinted.

Measurements began 3 min after the leaf was placed in chamber, when the conditions in the chamber had stabilized. VPD was adjusted manually by adjusting the desiccant scrub flow during the 20 min (VPD = 0.93–1.07 kPa). The slope of the linear region of leaf response, from 9 to 17 min, was calculated. All measurements were taken between 10:00 and 15:00.

For the fern gas-exchange measurements, fronds were cut under water during the morning hours (8:00–8:30). From each frond, five leaflets (starting from the third leaflet from the top) were cut underwater and inserted into different Eppendorf tubes; the leaflets of each frond constituted a block. All treatments included AXS. Tubes that contained no DMSO were used to assess whether DMSO itself affected the gas exchange of *C. falcatum* (as was found in a previous experiment in which 0.4% DMSO was used). Blocks of the five 1.5-mL tubes were then put into hermetically sealed transparent plastic boxes and left under lights for 1 h. Following that hour, the boxes were opened for 5 min and measurement data was then collected.

2.7. Dark treatments

Two hours after dawn, well-watered whole plants were moved to darkness for 1 h. After that hour, g_s was measured using a leaf porometer (SC-1 Porometer; Decagon Devices, Inc., WA, USA). The plants were then moved back into the light (˜150 μmol m − 2 s − 1) for an additional hour and g_s was then measured once again.

2.8. Stomatal conductance in drying soil

Stomatal conductance of 10- to 13-week-old plants was measured using a leaf porometer (SC-1 Porometer) and volumetric water content was measured with a Prochek probe (Decagon Devices). The equivalent water potential to 80-60, 45-25, 25-0 VWC are 0.0001-0.024, 0.038-0.075, 0.097-0.25Bar respectively. The conversion was don using water retention curve [35] of a similar potting soil. All measurements were carried out between 10:00 and 13:00.

2.9. Petiole-dip perfusion and leaf relative water content

Leaves excised before dawn were immediately immersed (petioles only, as shown in Fig. 3) in AXS and kept at close to zero VPD for 2 h (in a humid, transparent box), followed by 2 h of exposure to ambient VPD (˜ 0.7 kPa) under light (125 μmol s^{-1} m-2). The duration and efficiency of the xylem-loading perfusion were confirmed in separate leaves under the same experimental conditions by following the red dye Safranin O (1% w/v) through the leaf veins (Sigma Cat. No. S2255, 1% w/w in AXS; Fig. 3A).

Relative water content (RWC) was measured as described by Sade et al. [36]. In short, leaf fresh weight (FW) was immediately recorded and leaves were then soaked for 8 h in 5 mM $CaCl_2$ at room temperature in the dark, after which the turgid weight (TW) was recorded. Total dry weight (DW) was recorded after the leaves were dried at 70 °C to a constant weight. RWC was calculated as (FW – DW / TW – DW) × 100.

2.10. Statistical analysis

Student's *t*-test was used for comparisons of two means and the Tukey-Kramer test was used for comparisons of more than two means. Dunnett's test was used for comparisons with the control. The Yuen-Welch test for unequal variances was used when the variance was not homogeneous. All analyses were done using JMP software (SAS; Cary, NC, USA).

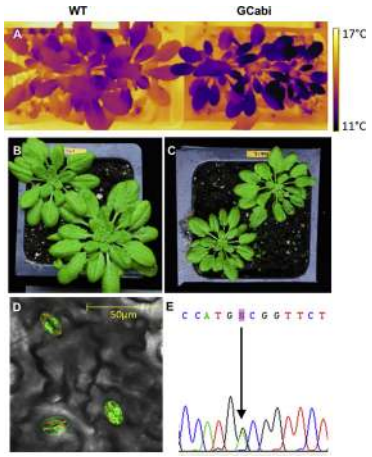

Fig. 1. GCabi plants. (A) Thermal imaging of 9-weeks-old well irrigated WT and GCabi plants. (B) 6-week-old WT Colombia and (C) GCabi plants. (D) A fluorescent image (488-nm excitation; 520-nm emission) of a leaf expressing GFP under the KST promoter. (E) Sequence of KST:abi (GCabi) cDNA, the arrow points to the G→A mutation.

3. Results

3.1. GCabi exhibits no significant stomatal response to external ABA or drought, while responding to osmotic stress and darkness

After confirming the guard-cell-specific expression of GFP under the KST promoter (GCGFP; Fig. 1D), we examined GCabi's stomatal responses to ABA and drought. Initially, we examined the stomatal apertures of WT and GCabi epidermal peels that had been soaked in 10 and 100 μM ABA. ABA caused a significant reduction in the stomatal apertures of the WT, however it had no significant effect on the stomatal aperture of GCabi (Fig. 2A), indicating that the quantity-dependent dominancy of *abi1-1* [37] is maintained under the KST promotor even for high concentration of 100 μM ABA. Moreover, under controlled conditions, the stomatal apertures of GCabi were significantly larger than those of the WT.

The GCabi plants exhibited no significant reduction in their stomatal conductance (g_s) in response to reductions in soil volumetric water content (for equivalent water potential see material and methods 2.8) and their stomatal conductance was significantly higher than that of the WT plants under the soil-drying treatment (25–45 and 0–21 % VWC; Fig. 2B). The unimpaired responses of GCabi stomata to darkness and osmotic stress (7.5% PEG; Fig. 2C, D) indicate that these plants possess a functional stomatal-movement mechanism. When detached leaves immersed (petiole-deep) in artificial xylem sap were exposed to ambient VPD conditions, the higher g_s and water-loss rates of the GCabi and fusicoccin-smeared WT leaves resulted in levels of leaf relative water content (RWC) that were lower than those observed for the WT (Fig. 3). This wilting may indicate that the leaf hydraulic conductance did not sufficiently compensate for the high rate of water loss through stomata. However, when attached leaves, in which bulk flow from the roots was not disturbed, were exposed to ambient VPD conditions, the RWC of GCabi did not differ from that of the WT.

3.2. GCabi plants exhibit a daily whole-plant canopy conductance pattern that is similar to that of the WT

g_s is a dynamic parameter that changes over the course of the day in response to changes in environmental factors such as light and VPD. Since GCabi plants exhibit larger stomatal apertures and higher stomatal conductance, we were interested in monitoring their responses to daily changes in atmospheric conditions in the greenhouse. In order to measure continuously and simultaneously the trends of relative whole-plant canopy conductance (g_{sc}) among the GCabi plants and the control plants we used an array of lysimeters (see Materials and methods). Both WT and GCabi revealed similar patterns of relative g_{sc} in response to the natural changes in the environmental conditions in the greenhouse, which included an increase in relative g_{sc} during the early morning (when VPD is low and light levels are increasing), followed by a decline in relative g_{sc} as VPD increased, down to a steady-state during late

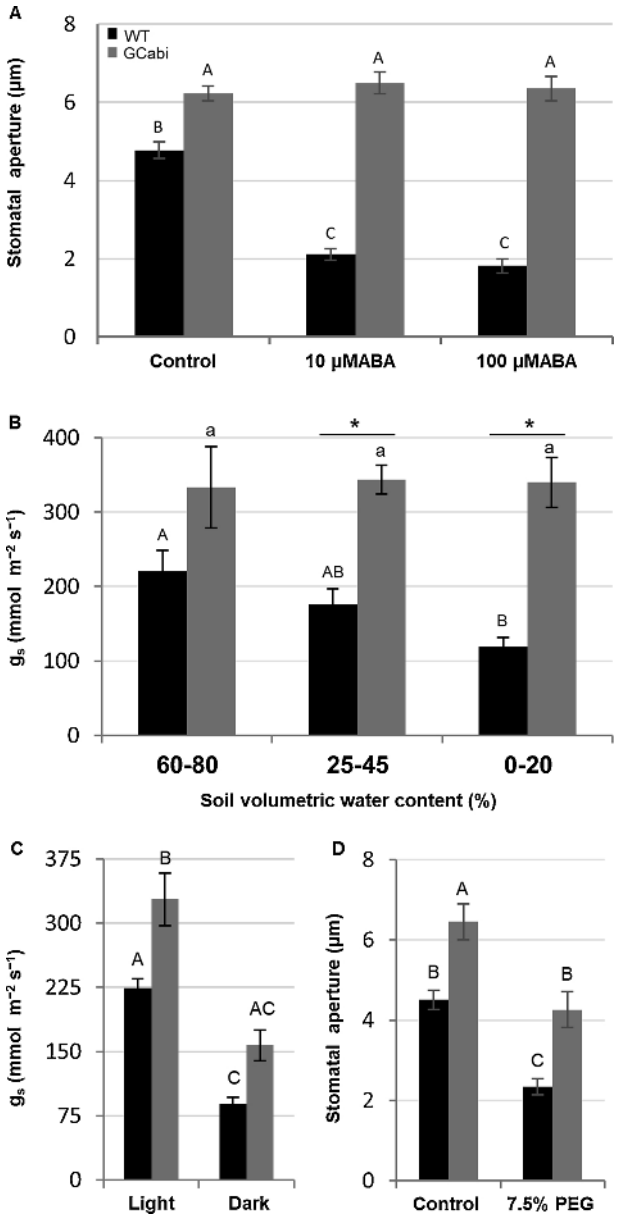

Fig. 2. GCabi exhibits no significant stomatal response to external ABA or drought, while responding to osmotic stress and darkness. (A) Stomatal apertures of 6-9-week-old WT (black bars) and GCabi (gray bars) epidermal peels directly exposed to 10 and 100 μM ABA. (B) Stomatal conductance of 10-13-week-old plants in response to continuous drought and (C) 1 h darkness. (D) Stomatal apertures of epidermal peels directly exposed to 7.5% PEG 6000. Results are means + SE of at least 3 independent experiments and 3 independent lines of GCabi (A–C) or one representing line of GCabi (D). For A, $n > 7$ leaves consisting of 80 > stomata; B, $n > 3$ pots consisting of 25 > leaves; C, $n > 63$ leaves; D, $n > 10$ leaves consisting of 120 > stomata. (A, C, D) Different letters indicate a significant difference according to the Tukey-Kramer test ($P < 0.05$). (B) Different letters indicate a significant difference between treatments within the same line and asterisks indicate a significant difference between lines subjected to the same treatment, according to the Yuen-Welch test for unequal variances ($P < 0.05$).

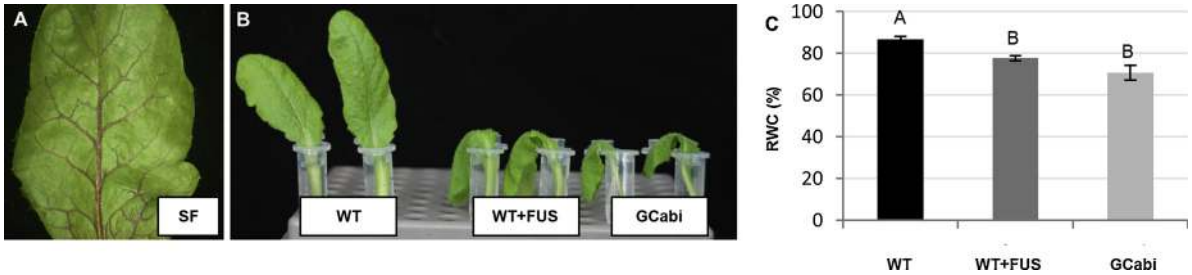

Fig. 3. Perfusion of detached leaves via their petioles (petiole dip). The low-level stomatal regulation of GCabi leads to lower relative water content (RWC) in a manner similar to that observed among WT leaves smeared with fusicoccin (FUS). (A) The efficacy of the petiole-dip perfusion (see Materials and methods) was confirmed by the fact that the xylem-borne dye spread throughout the leaf vasculature. (B) WT leaves, WT leaves smeared with 10 μM fusicoccin and GCabi leaves were petiole-dipped in AXS without any safranin and (C) their relative water contents are shown. Results are means + SE of 3 independent experiments (n = 15). Three independent lines of GCabi plants were used. Different letters indicate a significant difference (Tukey-Kramer test, $P < 0.05$).

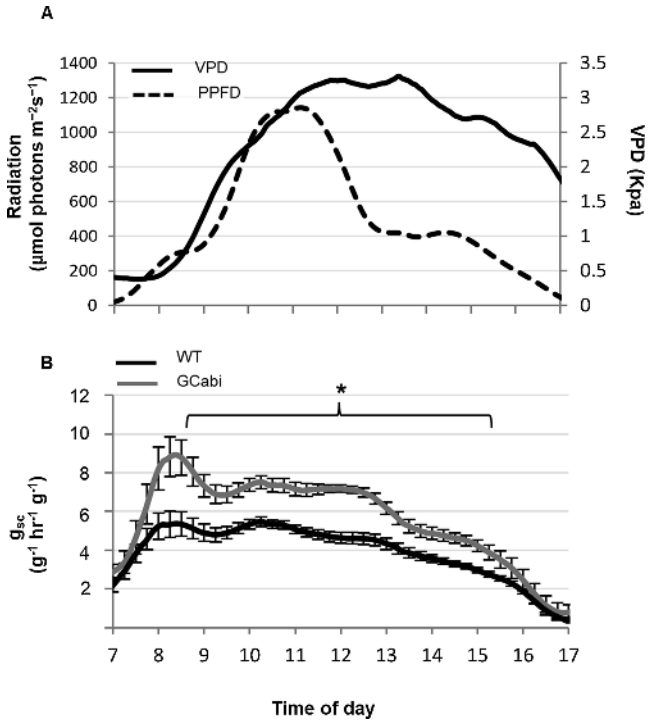

Fig. 4. Daily pattern of relative whole-canopy stomatal conductance. GCabi and WT Arabidopsis plants were grown under well-irrigated greenhouse conditions. (A) Daily VPD (solid line) and light intensity (radiation, dashed line). (B) The relative whole-plant canopy stomatal conductance (g_{sc}; g-1 h-1 unit plant weight-1; plant weight, g) of GCabi (gray) and WT (black) Arabidopsis plants. Curves show the means of 5–9 independent pots. Each pot included 6 plants; WT Arabidopsis plants (black, n = 9), GCabi plants (line GCabi9, gray, n = 5). Data are shown as means ± SE. The asterisk indicates a significant difference between GCabi and WT according Student's t-test ($P < 0.005$).

morning and the middle of the day (Fig. 4A, B). Despite the similar relative g_{sc} patterns of GCabi and the WT, under well-irrigated conditions, GCabi exhibited significantly higher canopy conductance during most of the day (from 08:05 to 15:20; Fig. 4B). In addition, GCabi transpiration values and pattern were similar to the WT despite their smaller size, further reflecting GCabi excessive water loss (Suppl. Fig. 2B). Since we were interested in guard-cells ABA involvement in this g_{sc} daily pattern, we further observed the daily relative g_{sc} pattern of a plant possessing an ABA-insensitive stomata, the fern *C. falcatum*. Ferns insensitivity to ABA has been shown to be species- and growth condition-dependent [5], therefore we confirmed the insensitivity of *C. falcatum* to ABA in our growing conditions (Suppl. Fig. 3A). The ferns' daily relative g_{sc} pattern was different from that observed for Arabidopsis (both WT and GCabi), including an increase in relative g_{sc} during the early morning and a decrease down to the basal level as VPD increased during the early morning (Suppl. Fig. 3D).

3.3. GCabi plants exhibit a WT-like stomatal response to an increase in VPD

The similar responses of the relative g_{sc} pattern and transpiration of GCabi and the WT to changes in ambient conditions point to similar VPD-sensing in both types of plants, or a stronger effect of some other signal such as light. Therefore, we decided to test the VPD-specific response of guard-cell in a tightly controlled gas-exchange experiment.

To study the role of ABA in the regulation of stomatal response to a sharp increase in VPD, we monitored changes in g_s over a period of 20 min, starting 3 min after leaves were transferred from low VPD conditions [0.15 kPa (high humidity)] to higher VPD conditions [1 kPa (lower humidity)], using the LI−COR 6400 chamber. Data were collected as soon as the chamber conditions stabilized (see Materials and methods). We also measured stomatal aperture before and after VPD was increased (Fig. 5). Fusicoccin treatment, which induces irreversible stomatal opening, was used as a "positive control" for non-sensitive open stomata. As expected, the sharp increase in VPD (from 0.15 to 1 kPa) resulted in a reduction in the g_s of the WT (Fig. 5A), which was correlated with a reduction in the stomatal apertures of those leaves (Fig. 5C). Interestingly, WT leaves that had been treated with fusicoccin also exhibited reduced stomatal conductance (Fig. 5A), yet with a significantly more moderate slope than that observed for the untreated WT (Fig. 5B) while not reducing their stomatal aperture (Fig. 5C). As before, the g_s and stomatal apertures of GCabi were significantly greater than those of the WT throughout the experiment (Fig. 5A, C). Nevertheless, the stomatal response patterns of GCabi to the jump in VPD, in terms of both g_s and stomatal aperture, were similar to the response patterns observed for the WT. The g_s graphs of the two sets of plants had the same slope (0.33 + 0.013 mmol m^{-2} s^{-1}/min) and the two sets of plants also exhibited similar reductions in stomatal aperture (48% for the WT and 46% for GCabi; Fig. 5B).

3.4. Responses of GCabi and the WT to different VPD conditions

The higher g_s of the GCabi plants can be explained by their larger stomatal apertures (Figs. 2A, 5 C) and their higher stomatal density. The fact that GCabi plants lose more water through transpiration raises the possibility that lack of leaf turgor may lead to their smaller-leaf phenotype (Fig. 1C, 6 A) and, subsequently, to their higher stomatal density (Fig. 6B). Therefore, we grew the plants under ambient (1.4 kPa) and low VPD (0.2 kPa, with a transparent plastic lid kept over the growth tray to reduce transpiration and increase RWC). Indeed, the low VPD conditions restored the RWC of GCabi to the level observed for the WT leaves. That is, WT plants were able to preserve relatively high RWC under both high- and low-VPD conditions; whereas the RWC of GCabi decreased under ambient VPD conditions (Fig. 6C). Nevertheless, despite the RWC differences, GCabi leaf area did not change and

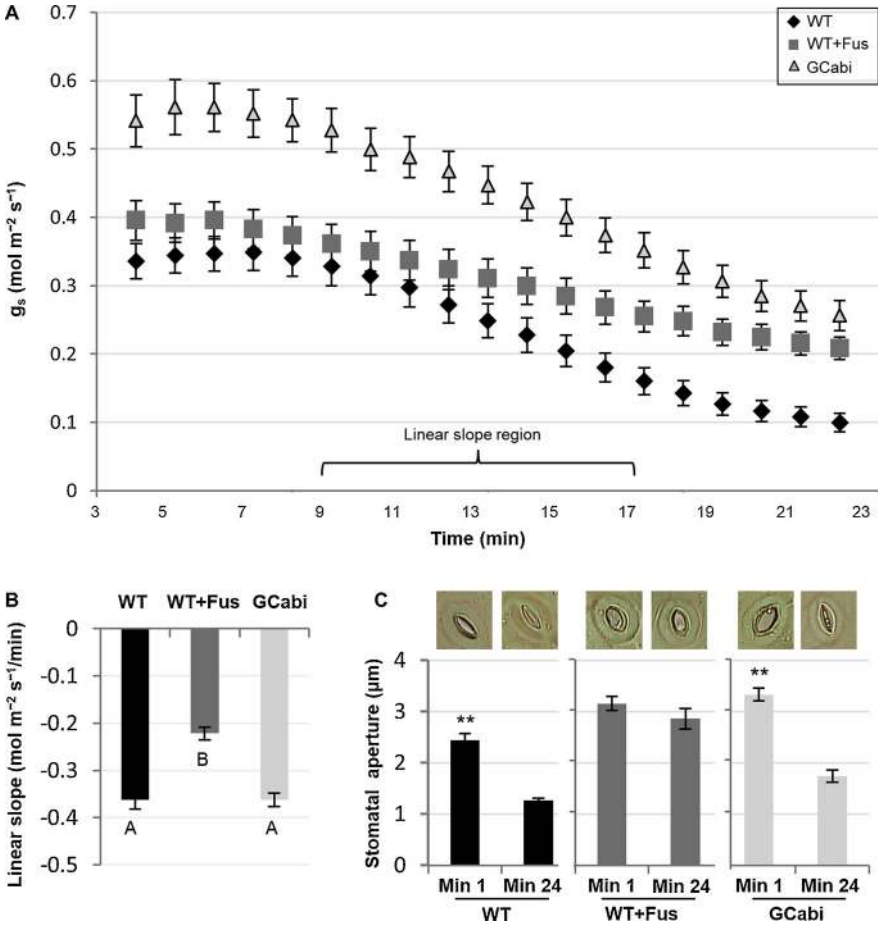

Fig. 5. GCabi plants exhibit a WT-like stomatal response to a rapid increase in VPD. (A) Changes in stomatal conductance over time in response to an increase in VPD from 0.15 kPa to 1 kPa; WT (diamond), WT smeared with 10 µM fusicoccin (square) and GCabi (triangle). Measurements began 3 min after a leaf was placed in the gas-exchange chamber (see Material and methods). (B) The linear slope of g_s (9 to 17 min). (C) Bright-field microscopy images of stomatal imprints, measured on duplicate leaves exposed to a change in VPD at Minute 1 and Minute 24. The measurement data are also presented in bar graphs. Data are shown as means ± SE (three independent lines for GCabi. For A, B $n = 20$, for C $n = 20$ leaves consisting of $380 >$ stomata). Significant differences are indicated by letters (Tukey-Kramer test, $P < 0.05$) or by asterisks (*t*-test, $P < 0.01$).

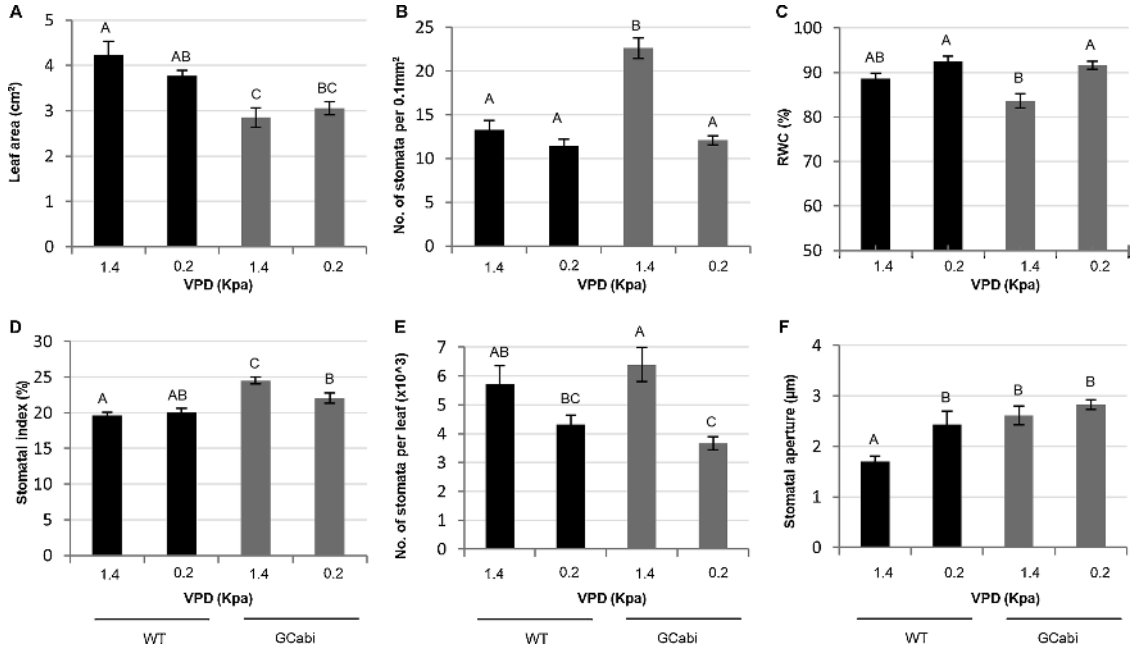

Fig. 6. Stomatal characteristics of GCabi and WT Arabidopsis plants grown under ambient and low VPD conditions. Eight-week-old GCabi and WT plants were grown under ambient (1.4 kPa) or low (0.2 kPa) VPD conditions. (A) Leaf area; (B) stomatal density per 0.1 mm^2 of leaf area; (C) leaf RWC; (D) stomatal index; (E) number of stomata per leaf and (F) stomatal aperture. Stomatal density, aperture and index were examined in 3 regions of 5 leaves from each treatment. For leaf area and RWC, $n = 15$. Results are means ± SE; different letters indicate a significant difference (Tukey-Kramer test, $P < 0.05$).

remained smaller than that of the WT under both high and low VPD conditions (Fig. 6A). The small size of the GCabi leaves cannot fully explain GCabi's higher stomatal density under higher VPD conditions. (WT stomata were 1.48 times larger; whereas GCabi stomata were 1.7 times denser). Therefore, stomatal index (number of stomata per epidermal cell) was quantified as well. The GCabi stomatal index was higher than that of the WT under ambient conditions and decreased to match the unchanged stomatal index of the WT under low-VPD conditions (Fig. 6D). The total number of stomata per leaf for GCabi and WT was similar under both VPD growing conditions (Fig. 6E). In addition, the long-term, low-VPD conditions increased WT stomatal apertures to the level seen for GCabi. In contrast, GCabi stomatal aperture remained constant under the two VPD conditions (Fig. 6F).

4. Discussion

4.1. The role of ABA in regulating stomatal aperture under non-stressful conditions

The fact that the guard cell-specific ABA-insensitive plants (GCabi) had significantly larger stomatal apertures and greater stomatal and relative-canopy conductance than the WT under well-irrigated conditions (Figs. 2, 4B, 5 AC, 6 F) supports the findings of previous studies, which have suggested that ABA plays a housekeeping role in limiting the size of stomatal apertures under non-stressful conditions [14,38,39]. Moreover, exposure to steady and higher VPD growing conditions altered the WT stomatal aperture; whereas the stomatal apertures of GCabi were unaffected by those conditions (Fig. 6F). Our results emphasize the fact that the role of ABA in limiting steady-state stomatal aperture is related specifically to the guard-cell, as opposed to being an indirect outcome of ABA's reduction of hydraulic conductivity [40,41], which, in turn, may cause stomatal closure [40]. Such an indirect effect of ABA on stomata could have been involved in previously reported observations of whole-plant ABA-mutant lines. The steady-state aperture mediated by ABA has been hypothesized to play a role in the following optimization processes: 1) the improvement of plant water-use efficiency (WUE) [42]; 2) the coordination of transpiration with photosynthesis [38]; and 3) the coordination of transpiration with vascular and non-vascular hydraulic limitations that may make the plant incapable of supporting excessive transpiration [43], resulting in reduced leaf water potential [40,41]. Lack of sufficient hydraulic conductivity can also explain the low RWC of the GCabi and WT + Fus detached leaves, as compared to the RWC of the untreated WT (Fig. 3C), despite the fact that all of the leaves were submerged in solution and xylem-borne dye moved freely through each leaf (Fig. 3A; [40,44]).

4.2. Stomata–VPD relations and the role of ABA in the g_s response

Typically, the daily pattern of g_s is strongly correlated with daily changes in VPD. This daily g_s–VPD pattern is characterized by high g_s in the early morning when VPD is low and a decrease in g_s as VPD increases over the course of the morning, as the temperature rises and the relative humidity drops (Fig. 4, Suppl. Fig. 3BD [34,38,45–47]). Obviously, this daily g_s–VPD pattern is influenced by seasonal conditions, for example, higher steady-state aperture and a slower decrease in g_s are observed during rainy seasons, when VPD is relatively low [47]. The daily relative g_{sc}–VPD pattern observed for GCabi was similar to that observed for the WT, but with greater g_{sc} throughout the day (Fig. 4). This further supports the hypothesized role of ABA in limiting steady-state stomatal apertures under non-stressful conditions. However, it also seems to suggest that ABA plays no substantial role in the daily response pattern of g_{sc} to VPD. Since the apparent daily g_s reduction seems to be ABA-independent, we hypothesized that it may be a hydro-passive response of the stomata, previously associated with the response to VPD by some fern stomata [4,48,49]. Therefore, we monitored the daily relative g_{sc}–VPD pattern of the fern *C. falcatum* (insensitive to ABA; Suppl. Fig. 3). The sharp decline in the fern's relative g_{sc} peak, back to basal level by late morning, emphasizes that the apparent daily g_{sc} reduction occurs and is even more pronounced in ABA-insensitive stomata. It may also involve a hydro-passive mechanism, as has been suggested for some ferns.

4.3. ABA-independent responses to a prompt increase in VPD

The Arabidopsis ABA-independent response to an increase in VPD (daily VPD pattern) was challenged in a tightly controlled gas-exchange chamber (prompt VPD increase), in which the patterns of stomatal aperture and g_s responses of GCabi leaves were similar to those observed for the WT (Fig. 5). In previous studies conducted using whole-plant ABA-mutant lines or lines in which ABA production was limited to the guard cells [13,14] or phloem [14], the effect of ABA on internal tissues [40,41] could be disrupted, altering any indirect effect of ABA on stomata, as described above (Section 4.2). For example, Merilo et al. [14] showed that an ABA-insensitive mutant (whole-plant mutant; mutant 112458) exhibited a different response to a prompt increase in VPD than that observed for the WT. That finding contrasts with our results (similar response pattern of GCabi and WT). The main difference between the two ABA-insensitive plants is the sensing tissue (only guard cells vs. whole plant in the 112458 mutant). This suggests that the altered stomatal response of the 112458 mutant may have been due to disruption of the indirect effect of ABA on stomata, as opposed to disruption of guard-cell ABA signaling. This further emphasizes the importance of internal-tissue feedback for stomatal activity.

The ABA-independent response of guard cells to VPD may be due to the passive-hydraulic response mechanism, which could be due to ancestral regulation that has remained significant in some angiosperm species, including Arabidopsis [4] or to a mechanism that regulates the guard-cell response to a new steady-state in bulk leaf turgor (i.e., the new balance between the pressures of the guard cell and the epidermal cells [50,51]). The second possibility could explain GCabi's larger apertures under ambient conditions (operating close to turgor loss point; Fig. 3), so that guard-cell turgor dominates epidermal pressure, as in a "continuous wrong-way response". However, GCabi stomatal aperture was unchanged when RWC increased (i.e., higher turgor, low VPD; Fig. 6CF), weakening that argument. In addition, differences in the balance of pressure between the epidermis and the guard cell are expected to be reflected in stomatal-closure dynamics that differ from those observed for the WT control, such as those seen for the fusicoccin-treated WT leaves, but not for GCabi (Fig. 5).

Alternatively, the ABA-independent response of guard cells to VPD could be due to a physical, as yet unknown parameter that varies with transpiration, so that the guard cell senses changes in the flux of water through the stomate [8,20]. It has been suggested that solutes (mainly sucrose) carried toward the stomata by the transpiration stream might act as such an osmotic and/or molecular signal, linking photosynthesis, transpiration and stomatal regulation [52–55]. It has also been suggested that this sugar-induced stomatal closure might be mediated by ABA [39], yet the GCabi guard-cell osmotic response (Fig. 2D) seems to conflict with that suggestion and, in fact, suggests that osmotic signaling converges with ABA signal transduction downstream of PP2C. OST1 (a protein kinase active downstream of ABA) might be involved in such an ABA-independent response [14,21,56], but its relation to sucrose has not yet been determined.

4.4. ABA-independent and ABA-dependent responses to VPD

In light of the evidence presented above, it seems that (at least in Arabidopsis) the stomatal response to a sharp increase in VPD involves three elements: leaf hydraulic status, an ABA-independent mechanism and an ABA-dependent mechanism. A possible explanation that includes all three of these elements could be that ABA is not the initial cause of stomata closure, but rather a consequence of that closure. The

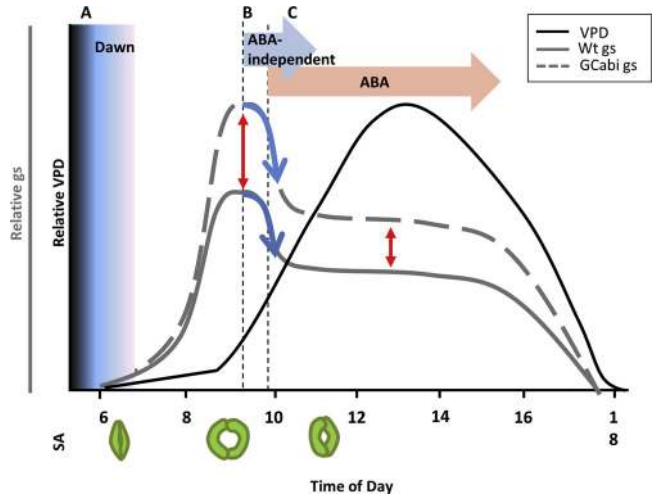

Fig. 7. Our hypothetical biphasic stomatal VPD-sensing model. This model suggests that under well-irrigated conditions (A) the stomatal conductance (g_s) of the WT (solid gray line) and GCabi (dashed gray line) generally increases rapidly, beginning at the first light at dawn, when VPD is low (black line), and maximal g_s (and maximal stomatal aperture, SA, vertical red arrow) is reached during the morning, in coordination with the sum of signals perceived by the guard-cell, including the basal ABA level. (B) The increasing VPD induces a higher rate of transpiration, which triggers a reduction in SA and g_s via an ABA-independent mechanism (blue arrows). (C) The ABA-independent signal induces the synthesis of ABA (horizontal red arrow), triggering the start of an ABA-dependent phase, which regulates the steady-state g_s and SA throughout the middle of the day and the afternoon (vertical red arrow). This VPD–ABA synthesis feedback may serve as a regulatory mechanism that enables the plant to optimize its SA under the prevailing VPD conditions.
ern Stomatal responses to ABA and CO 2 depend on species and growth conditio

(symplastic) isolation of guard cells from epidermal cells in the leaves of angiosperms [57,58] limits such a hydraulic response, as compared to the hydraulic response observed in ABA-insensitive ferns. This "hydraulic independency" allows for larger stomatal apertures, but also means that active turgor loss is required to reduce stomatal aperture beyond the reduction caused by the initial hydraulic passive response. We hypothesized that the potential advantage of this combined strategy is the flexibility associated with having two modes of action: 1) high WUE with high stomatal conductance (i.e., enabling high CO_2 intake) during low VPD periods and 2) the ability to keep stomata slightly open during periods of higher VPD, even at the price of lower WUE (risk-taking anisohydric behavior [59,60). In this hypothetical biphasic model, an ABA-independent passive response triggers an active ABA-dependent response and ABA enables the optimal steady-state g_s for each phase, corresponding to ambient conditions (i.e., soil water content and VPD).

The main role of ABA in evolution may have been in the adjustment of stomatal opening, as opposed to the common understanding of ABA as the stomatal-closing phytohormone. This would also explain angiosperms' relatively long phase of steady-state g_s (guard cells of both WT and GCabi maintained some turgor) during late morning and midday (Fig. 4B; [34,38,45–47]) and the lag in the rate at which stomata open following a sharp increase in VPD, as compared to the rate at which they close [4,9,14].

4.5. ABA's role in adaptation to ambient VPD

The goal of growing plants under low VPD was to reduce their water loss and increase their RWC. This experiment also uncovered some interesting long-term effects of VPD on stomatal development and the stomatal response to ABA. Low-VPD conditions increased the size of WT apertures, but did not affect the larger stomatal apertures of the GCabi plants (Fig. 6F). This is in agreement with previous studies that have shown that VPD conditions (1–4 days of exposure) affect stomatal aperture [61–63], the quantity of leaf ABA [64–66] and stomatal sensitivity to ABA [39,64,66–68]. These effects are reversed by the application of ABA [61,62] or air movement [63] during the low-VPD period. Growing GCabi plants under constant low-VPD conditions did not increase their leaf area, despite the observed increase in their RWC (Fig. 6A, C). This suggests that a lack of turgor (Figs. 3, 6C) is not the main cause of the small size of GCabi leaves and that the higher stomatal density of GCabi (Fig. 6B) is at least partially due to developmental modification, as seen in their higher stomatal index (Fig. 6D). Expression of *abi1-1* under a guard cell-specific promoter was sufficient to increase stomatal density, as observed for the *abi1-1* mutants [69]. This explains why the g_s of the GCabi leaves was higher than that of the WT leaves that were treated with fusicoccin, despite their similar stomatal apertures (Fig. 5A, C). Moreover, the fact that the promoter used to construct the GCabi plants is guard cell-specific [22,29,70]; Fig. 1D) and likely activated late in or even after differentiation suggests that ABA may have an indirect effect on stomatal proliferation through stomatal aperture and the transpiration rate [71], in addition to the direct effect suggested by Tanaka et al. [69]. The greater stomatal density of GCabi was VPD-dependent and was reduced when those plants were grown under low-VPD conditions (Fig. 6B). This contrasts with the findings of previous studies, which suggested that low RH (high VPD) [63,72,73] and/or ABA [69] suppress stomatal proliferation. In our experiment, reducing the VPD increased the size of stomatal apertures (Fig. 6F) and, subsequently, WT g_s [61,62,65]. In contrast, the stomatal apertures of GCabi, which were large to begin with, had lower g_s under lower-VPD conditions, which could prevent the transduction of stress signals, reducing GCabi's stomatal density and stomatal index to WT levels. The long-term outcome of these conditions was the developmental changes observed, possibly balancing the loss of water through stomata with stomatal proliferation [72].

5. Conclusion

In this study, we describe a biphasic guard-cell response to ABA. We demonstrate the importance of guard-cell ABA for restricting stomatal apertures under well-irrigated conditions, in contrast to its insignificance in the immediate guard-cell response to increases in VPD. In addition, we demonstrate that guard cell-specific ABA plays an indirect role in stomatal proliferation.

We summarize this study with a daily VPD–g_s response-curve hypothesis (Fig. 7). This dynamic response-curve hypothesis is based on the notion that stomatal aperture always reflects the sum of signals sensed by the guard cell (e.g., light, CO_2 and ABA). The VPD signal has a special dual effect as it is the physical force that drives transpiration and also serves as a (direct or indirect) closing signal. Hence, under the natural dynamic pattern of daily signals, a typical g_s curve of a well-irrigated plant is expected to show the following pattern (as illustrated in Fig. 7): The first daylight initiates stomatal opening. At that point, a continuum between the leaf substomatal cavity and the atmosphere (i.e., VPD) is established (i.e., the opening of stomata initiates the soil-plant-atmosphere continuum, which was blocked while the stomata were closed). At this early hour, VPD is at its lowest level and stomatal apertures are at their largest. As the temperature rises, VPD increases gradually, which leads to proportional water flux through the stomata that causes a passive, ABA-independent reduction in g_s. That passive response also triggers corresponding ABA synthesis within minutes [10,11]. The amount of ABA produced and the sensitivity of the guard cells to ABA restricts the size of the stomatal apertures, to keep g_s at a steady-state level that is appropriate for the prevailing ambient conditions.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2018.12.027.

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