Viewpoints

Molecular mechanisms of stomatal closure in response to rising vapour pressure deficit

Summary

Vapour pressure deficit (VPD), the difference between the saturation and actual air vapour pressures, indicates the level of atmospheric drought and evaporative pressure on plants. VPD increases during climate change due to changes in air temperature and relative humidity. Rising VPD induces stomatal closure to counteract the VPD-mediated evaporative water loss from plants. There are important gaps in our understanding of the molecular VPD-sensing and signalling mechanisms in stomatal guard cells. Here, we discuss recent advances, research directions and open questions with respect to the three components that participate in VPD-induced stomatal closure in *Arabidopsis*, including: (1) abscisic acid (ABA)-dependent and (2) ABA-independent regulation of the protein kinase OPEN STOMATA 1 (OST1), and (3) the passive hydraulic stomatal response. In the ABA-dependent component, two models are proposed: ABA may be rapidly synthesised or its basal levels may be involved in the stomatal VPD response. Further studies on stomatal VPD signalling should clarify: (1) whether OST1 activation above basal activity is needed for VPD responses, (2) which components are involved in ABA-independent regulation of OST1, (3) the role of other potential OST1 targets in VPD signalling, and (4) to which extent OST1 contributes to stomatal VPD sensitivity in other plant species.

Introduction

Stomata are small pores formed by two guard cells on the leaf epidermis that control photosynthetic carbon assimilation, water transpiration and leaf cooling. During climate change, atmospheric vapour pressure deficit (VPD), the difference between the saturation and actual air vapour pressures, increases due to rising temperatures and reduction in relative air humidity (Ficklin & Novick, 2017; Vicente-Serrano *et al.*, 2018). VPD is the measure of evaporative potential and its increase causes higher transpiration from plants, followed by stomatal closure to reduce excessive water loss. Globally, over 50% of vegetated areas have experienced higher VPD since the late 1990s and different Earth system models have predicted that VPD will continue to increase during the 21st century (Yuan *et al.*, 2019). Despite the large impact of VPD on plants, for example on vegetation index and terrestrial gross primary production, crop yields and tree/forest mortality (Williams *et al.*, 2013; Lobell *et al.*, 2014; Hsiao *et al.*, 2019; Yuan *et al.*, 2019), stomatal VPD sensing and signalling are incompletely understood. Here, we present stomatal VPD responses of *Arabidopsis* abscisic acid (ABA) synthesis and signalling mutants based on recent studies (Merilo *et al.*, 2018; Jalakas *et al.*, 2021) to dissect the possible proportions of passive and active ABA-dependent and ABA-independent stomatal closure. We also discuss relevant questions and research directions to uncover the mechanisms of VPD-induced stomatal regulation. Modelling approaches to improve the predictions of VPD responses from plant to ecosystem levels have been discussed in a recent review (Grossiord *et al.*, 2020).

Mechanisms contributing to high VPD-induced stomatal closure

VPD-induced stomatal closure was reported to be a passive hydraulic ABA-independent process in ferns and conifers (Brodribb & McAdam, 2011; McAdam & Brodribb, 2015). In these basal vascular plants, reductions in leaf water potential and leaf and guard cell turgor pressure have been reported to directly promote stomatal closure in response to high VPD (Brodribb & McAdam, 2011; Buckley, 2019). This conclusion was supported by findings that fern stomata did not respond to ABA (Brodribb & McAdam, 2011). By contrast, ABA caused stomatal closure in the moss *Physcomitrella patens* and the lycophyte *Selaginella uncinata* (Chater *et al.*, 2011; Ruszala *et al.*, 2011). Significant, although relatively weak, ABA responses have been also observed in some fern species (ABA-induced stomatal closure was *c.* 20% in ferns compared with at least 40% in *Arabidopsis*) (Hõrak *et al.*, 2017). More evidence supporting active CO_2-mediated and ABA-mediated stomatal regulation of ferns has been provided by Franks & Britton-Harper (2016) and Cai *et al.* (2017). Therefore, basal vascular plants may combine passive and active stomatal regulation depending on species and environmental conditions (Cai *et al.*, 2017; Hõrak *et al.*, 2017). In angiosperms, epidermal turgor contributes to the regulation of stomatal apertures. Following an increase in VPD, a rapid rise in transpiration results in reduced epidermal turgor and in less backpressure on the guard cells, promoting a transient passive 'wrong-way' stomatal opening (Ivanoff, 1928; Mott & Franks, 2001; Buckley, 2019). As a result, an active response is required to counteract the opening and ensure stomatal closure in angiosperms (Franks, 2013; Buckley, 2019). For the active process, ABA was proposed to be involved for two reasons: (1) a rapid (within 10 min) VPD-induced or turgor-induced increase in foliar ABA concentration preceded stomatal closure; and (2) VPD-induced stomatal closure was absent in ABA-deficient mutants (McAdam *et al.*, 2016;

Sussmilch *et al.*, 2017; Cardoso *et al.*, 2020). While rapid (15 min), although relatively weak, low air humidity-induced guard cell ABA concentration increases were also observed using the ABA biosensor ABAleon2.1, this slight concentration increase could result from a reduction in guard cell volume after 15 min and stomatal closure rather than from rapid ABA synthesis (Waadt *et al.*, 2014). The half-response time of high VPD-induced stomatal closure was short: 6.1 ± 0.32 min in *Arabidopsis* Col-0 wild-type (Merilo *et al.*, 2018). Therefore, time-resolved investigations are required to determine which process occurs first, stomatal closure or guard cell ABA biosynthesis.

As discussed above, in angiosperms a passive transient 'wrong-way' stomatal opening is detected in response to high VPD (Buckley, 2019). Currently it is unclear whether angiosperms can display passive hydraulic stomatal closure or whether they rely only on active ion channel-mediated ion efflux processes to close stomata in response to elevated VPD. McAdam & Brodribb (2015) proposed that, in response to rising VPD, the ancestral passive regulation of stomatal closure is an important contributing component in some angiosperm species, for example *Quercus robur* in their dataset. We return later to this model of passive stomatal closure in angiosperms.

Generally, ABA plays a central role in regulating active stomatal closure (Hetherington, 2001). The ABA signalling pathway consists of cytosolic PYR1/PYL/RCAR ABA receptors, type 2C protein phosphatase co-receptors (PP2Cs) and SnRK2-type protein kinases (including OST1). The molecular action of ABA involves its perception by the receptors, the formation of receptor–ABA–PP2C complexes, resulting in inactivation of PP2Cs, and the activation of SnRK2 protein kinases (Fig. 1a; Ma *et al.*, 2009; Park *et al.*, 2009). Further, OST1 activates downstream transcription factors and ion channels, for example the S-type anion channel SLAC1 and the R-type anion channel ALMT12/QUAC1, triggering stomatal closure (Geiger *et al.*, 2009; Imes *et al.*, 2013). OST1 has two regulatory domains in the C-terminus, domain II (ABA-box) for ABA-dependent activation and domain I (SnRK2-box) for ABA-independent osmotic stress and low humidity activation (Belin *et al.*, 2006; Yoshida *et al.*, 2006). Interestingly, an OST1 mutant isoform that lacked the ABA-box was impaired in ABA activation, but remained responsive to osmotic stress and low humidity in *Arabidopsis* T87 cells and transgenic whole seedlings, providing evidence for an ABA-independent activation of OST1 (Yoshida *et al.*, 2006).

The involvement of ABA in VPD-induced stomatal regulation is controversial. Stomata of ABA-deficient mutants were independently reported to close in response to high VPD as wild-type (Assmann *et al.*, 2000; Merilo *et al.*, 2013; Merilo *et al.*, 2018) or in a disturbed, but still functional manner (Xie *et al.*, 2006). Xie *et al.* (2006) discussed that either basal ABA levels are present in ABA-deficient mutants or the VPD response is partially ABA-independent, explaining the response of ABA-deficient mutants. Viable ABA biosynthesis mutants are known to have reduced, but measurable, levels of ABA, while their stress-induced ABA accumulation is strongly reduced (Gonzalez-Guzman *et al.*, 2002; Frey *et al.*, 2012). Guard cell-specific complementation of ABA biosynthesis was sufficient to prevent the high VPD-induced leaf wilting of ABA-deficient *aba3-1* mutant plants, pointing to the role of guard cell synthesised ABA. However, under high VPD, parental *aba3-1* mutants also displayed a marked decrease in the guard cell K^+ content, similar in the percentage of K^+ content decrease to that of wild-type controls, indicating VPD-induced stomatal closure in

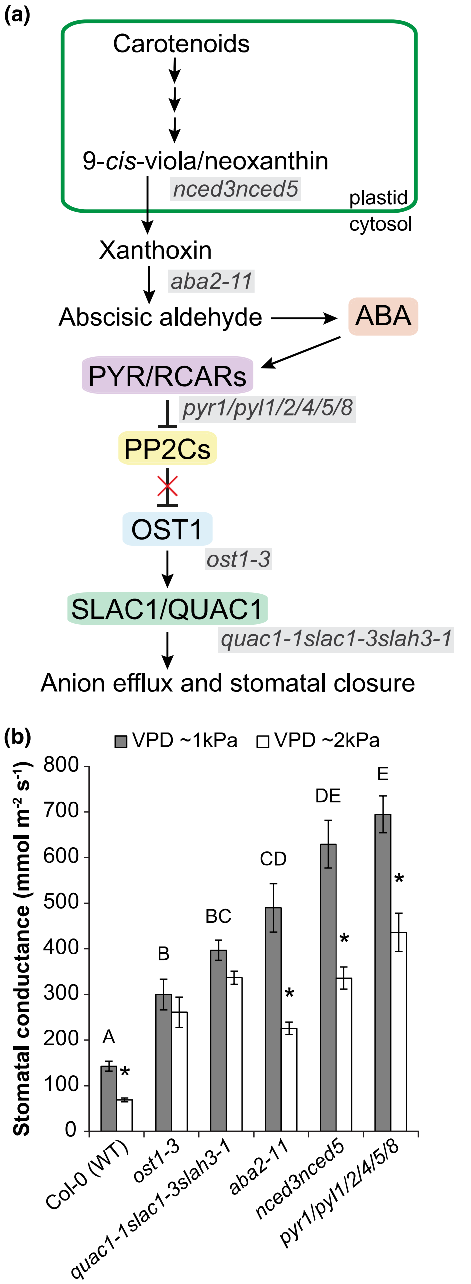

Fig. 1 (a) Schematic overview of abscisic acid (ABA) biosynthesis and signalling in *Arabidopsis*. ABA biosynthesis is condensed to present only the defective steps for which mutants are discussed (highlighted in grey). ABA signalling is explained in the main text. (b) Values of steady-state stomatal conductance at low and high vapour pressure deficits (VPD) (1.2 and 2.2 kPa, respectively) for the studied *Arabidopsis* lines. Capital letters denote statistically significant differences between the genotypes at low VPD (GLM Linear Models and Tukey post hoc test). Stars point at significant differences of stomatal conductance's between low and high VPD conditions for that genotype (GLM Linear Models and Repeated Measures ANOVA; n = 7–12, average and standard errors).

aba3-1 plants (Bauer *et al.*, 2013a). Overall, these results contradicted a model in which ABA would be the sole regulator of the stomatal VPD response in higher plants. Conversely, OST1, a positive transducer of stomatal closing in response to several stimuli including ABA, is crucially involved in stomatal VPD sensitivity (Xie *et al.*, 2006; Merilo *et al.*, 2013, 2018; Jalakas *et al.*, 2017). Interestingly, a linkage between VPD and ABA signalling was detected also during leaf development; the stomatal ABA sensitivity of young leaves was triggered by exposure to dry air (Pantin *et al.*, 2013). Note that mutants defective in ABA biosynthesis and ABA signalling exhibited a strongly increased steady-state stomatal conductance, which in turn accelerated VPD-induced water loss from leaves (Fig. 1b; Merilo *et al.*, 2018). Independent studies are needed to confirm the rapid VPD-induced or turgor-induced increase in leaf ABA concentration (McAdam *et al.*, 2016; Sussmilch *et al.*, 2017). Of importance, over a longer timescale, growth at different relative air humidities led to differences in foliar ABA concentrations in some species (Arve *et al.*, 2013). However, no long-term effect of growth air humidity on foliar ABA concentrations was observed in other species (Innes *et al.*, 2021), indicating that short-term versus long-term (i.e. acclimatised) VPD sensitivity of leaf ABA levels may vary among different species.

Stomatal VPD responses of plants defective in ABA synthesis, signalling and guard cell anion transport

High VPD caused an increase in the steady-state rosette transpiration rate (plants were kept at the respective VPDs for at least 60 min) in wild-type Col-0, ABA synthesis (*aba2-11*) and ABA signalling (*ost1-3*) mutants (Fig. 2a). In Col-0 and *aba2-11*, but not in the *ost1-3* mutant, this was accompanied by stomatal closure (Figs 2b, 3a). A significantly higher slope of the linear regression between VPD and transpiration for *ost1-3* compared with Col-0 and *aba2-11* (Fig. 2a) indicated that, under strong evaporative demand, a rise in the transpiration in *ost1-3* leaves was considerably higher than in Col-0 and *aba2-11*. Pantin & Blatt (2018) discussed that stomatal closure in response to a stepwise increase of VPD from low to high values in plants with high stomatal conductance could be a methodological artefact. Namely, in the calculations of stomatal conductance, it is assumed that the water vapour pressure within the leaf reaches saturation in the substomatal chamber, an assumption that may not hold in mutants with very open stomata (Pantin & Blatt, 2018). However, the calculations of transpiration do not include such assumptions (see Supporting Information Methods S1). Therefore, Fig. 2(a) clearly indicates that the high VPD-induced rise in *ost1-3* transpiration was different from wild-type and *aba2-11*, and that the latter two genotypes were able to limit their transpiration under high VPD due to stomatal closure.

ABA-insensitive ABA receptor sextuple mutant plants (*pyr1/pyl1/2/4/5/8*) displayed delayed, but still functional, high VPD responses, showing that ABA is involved but not critical for stomatal VPD sensitivity (Fig. 3a,b; Merilo *et al.*, 2013, 2018). A triple mutant defective in S-type and R-type anion channels (*quac1-1slac1-3slah3-1*) exhibited nearly as strongly impaired high VPD responses as *ost1-3* (Fig. 3a,b; Jalakas *et al.*, 2021). Conversely, VPD-induced stomatal closure was detected in all studied ABA biosynthesis mutants (of *Arabidopsis*, pea and tomato; Fig. 3a,b; Merilo *et al.*, 2018). Returning to high VPD-induced passive hydraulic stomatal closure in angiosperms, Fig. S1 presents relative VPD responses of *ost1-3* plants that were kept under low CO_2 (40 ppm) conditions or given additional blue light, to boost stomatal conductance (Merilo *et al.*, 2018). Interestingly, in those plants, clear VPD-induced stomatal closure was evident. A passive stomatal closure appears to be a plausible explanation for this response, provided that the initial stomatal conductance was high and assuming that active stomatal closure is absent in the *ost1-3* mutant. Therefore, passive hydraulic stomatal closure may contribute a conditional limited response with clearly different closure kinetics compared with the active response of Col-0 wild-type plants (Fig. S1) in VPD-induced stomatal regulation of angiosperms.

A redundant, transpiration-linked perspective to the passive stomatal VPD response was proposed by Outlaw (2003), connecting a high VPD-induced increase in the transpiration rate with solute accumulation in the guard cell apoplast. In apoplastic phloem loaders (*Arabidopsis* included), this solute may be sucrose, which is able to reduce stomatal aperture, when accumulating in the apoplast (Zhang & Outlaw, 2001; Outlaw, 2003; Kang *et al.*, 2007).

Hypothetical model to dissect components involved in the stomatal VPD response of angiosperms

To differentiate and discuss processes involved in VPD-induced stomatal closure, we propose a hypothetical model outlining VPD responses in a diverse range of *Arabidopsis* stomatal mutants. From dynamic absolute stomatal conductance responses to rising VPD (Fig. 3a, from Merilo *et al.*, 2018; Jalakas *et al.*, 2021), we first calculated relative responses (Fig. 3b). Then to visualise the relative contributions of different components, we used the Col-0 wild-type relative stomatal response as a reference and subtracted the relative responses of plants defective in ABA synthesis (average of *aba2-11* and *nced3nced5*), ABA signalling upstream of OST1 (ABA receptor hextuple *pyr1/pyl1/2/4/5/8*), OST1 (*ost1-3*) and guard cell S-type and R-type anion channels (*quac1-1slac1-3slah3-1*) (Fig. 3c). Positive and negative scales in this figure point to larger or weaker VPD responses relative to Col-0.

The blue area between *ost1-3* and *quac1-1slac1-3slah3-1* indicates the proportion of an OST1-dependent response that is independent of these three anion channels. Several other substrates of OST1 are known that could potentially contribute to the VPD response (Wang *et al.*, 2020). For example, OST1 is involved in the ABA-induced inhibition of guard cell H^+-ATPases (Hayashi *et al.*, 2011). However, considering the overlap between the relative responses of the *ost1-3* and the anion channel triple mutant (Fig. 3b), the proportion of the OST1-dependent and anion channel-independent response can be viewed as negligible.

The yellow area between the responses of the *quac1-1slac1-3slah3-1* triple mutant and the ABA-insensitive genotype (*pyr1/pyl1/2/4/5/8)* represents an active ABA-independent stomatal VPD response that is mediated by OST1 and anion channels and that was

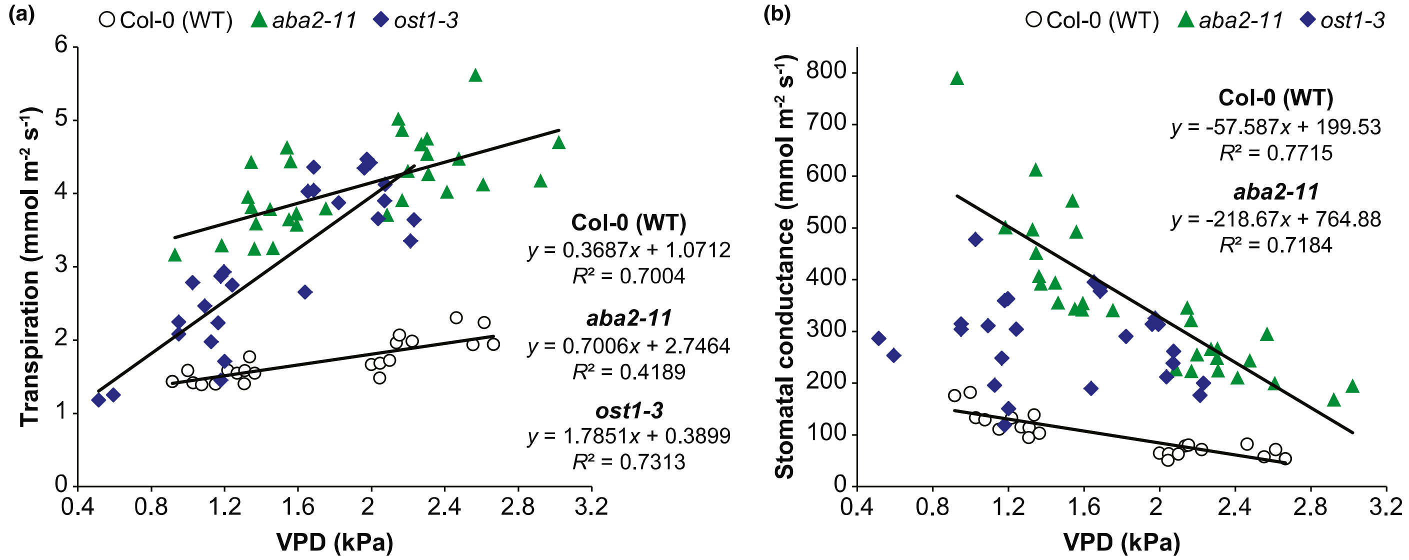

Fig. 2 Relationships between (a) vapour pressure deficit (VPD) and rosette transpiration and (b) VPD and stomatal conductance in *Arabidopsis* wild-type Col-0, ABA-deficient *aba2-11* and ABA-insensitive *ost1-3*. The values represent steady-states at the respective VPDs. (a) The slope of the linear regression of *ost1-3* was significantly higher ($P = 0.0001$; *t*-test) compared with those of Col-0 and *aba2-11*. The slopes of Col-0 and *aba2-11* differed from each other at $P = 0.0705$. (b) Stomatal conductance of *ost1-3* did not correlate with the imposed VPD. Equations for *aba2-11* and Col-0 (wild-type) are shown.

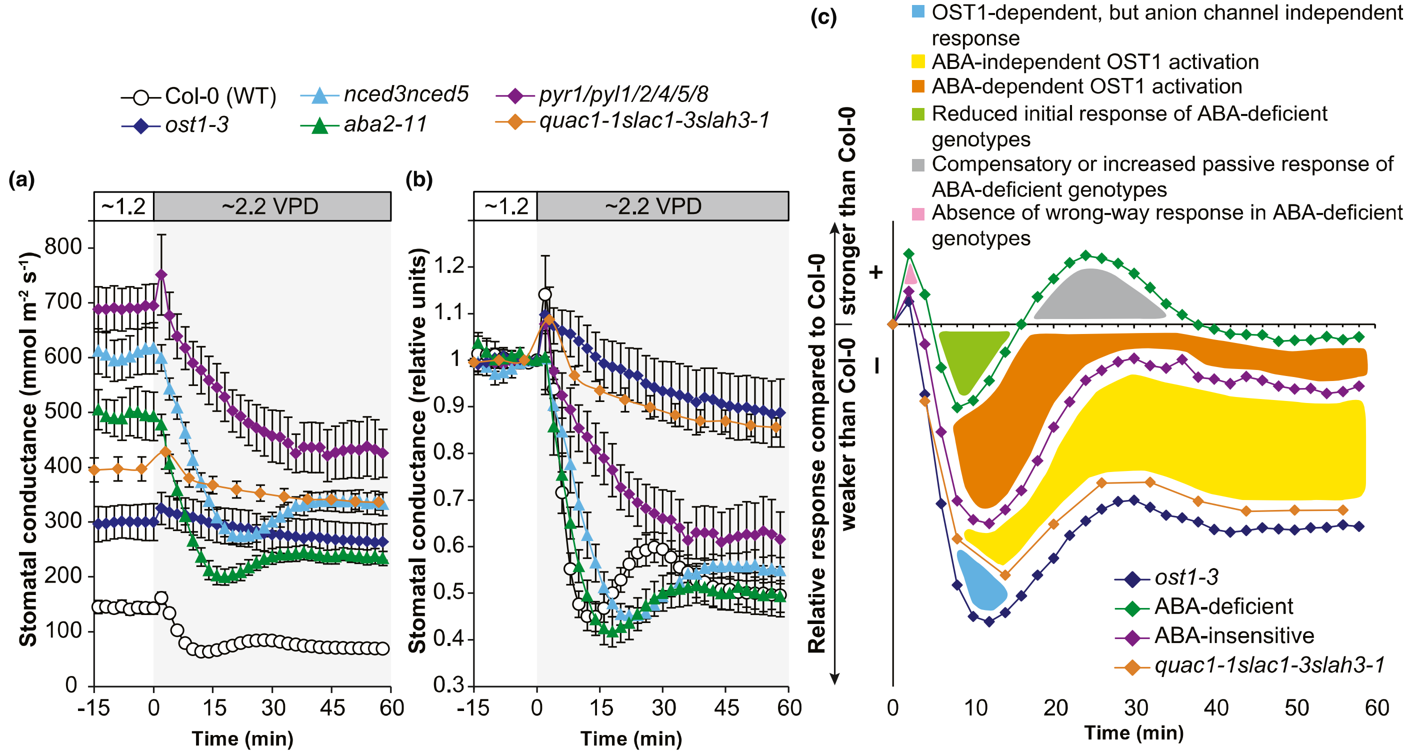

Fig. 3 (a) Stomatal vapour pressure deficit (VPD) responses of *Arabidopsis* plants defective in abscisic acid (ABA) synthesis, signalling and guard cell anion transport in absolute units (from Merilo *et al.*, 2018; Jalakas *et al.*, 2021; $n = 7$–12, average and standard errors). (b) Responses normalised to the average values of the last two time points before the change in VPD. (c) Visualisation of differential stomatal VPD responses of ABA-deficient, ABA-insensitive, *ost1-3* and triple anion channel mutant lines compared with Col-0 wild-type. Col-0 here corresponds to $y = 0$ throughout the time course. Negative *y*-axis values indicate weaker than wild-type responses and positive values indicate stronger than wild-type responses presented on the time axis after a step increase in VPD from 1.2 to 2.2 kPa at $t = 0$ min. The values were calculated by subtracting the relative responses of the mutants as shown in (b) from the relative response of Col-0 wild-type at the same time point. Yellow, OST1-dependent, but ABA-independent response; orange, ABA-dependent OST1 activation; green, early reduced response of ABA-deficient genotypes due to lower ABA levels; grey, stronger than wild-type later response of ABA-deficient genotypes; blue, difference between OST1-dependent and anion channel-dependent responses; pink, absence of wrong-way response in ABA-deficient lines. See text for explanatory details (in the section on Hypothetical model to dissect components involved in the stomatal VPD response of angiosperms).

observed even when ABA sensitivity is seriously impaired, as in the sextuple ABA receptor mutant (Gonzalez-Guzman *et al.*, 2012; Merilo *et al.*, 2018). Without the PYR1/PYL/RCAR receptors, OST1 is not activated through ABA, but can be activated by osmotic stress and low air humidity by the SnRK2-box (Belin *et al.*, 2006; Yoshida *et al.*, 2006). Recent reports have shown ABA-induced and osmotic stress-induced activation of SnRK2s by Raf-like M3Ks, which might be involved in active VPD-induced stomatal regulation (Lin *et al.*, 2020; Soma *et al.*, 2020; Takahashi *et al.*, 2020). As shown in Fig. 3(c), the contribution of ABA-independent OST1 signalling to the VPD response increases with time. The orange area indicates the proportion of an ABA-dependent activation of OST1. We propose that this area may represent both OST1 activation by basal ABA levels present in ABA-deficient lines and/or *de novo* synthesised ABA. Note that foliar ABA concentrations in *aba2-11* and *nced3nced5* were *c.* 30% of wild-type values in no-stress conditions (Hsu *et al.*, 2018; Merilo *et al.*, 2018).

The pink area in Fig. 3(c) depicts an initially stronger response of ABA-deficient mutants compared with wild-type, which is explained by a missing wrong-way response (Fig. 3a,b). The initial wrong-way response was absent in all studied *Arabidopsis* ABA-deficient mutants, such as *aba1-1*, *aba3-1*, *aba4-3* and *aao3-1* in addition to *aba2-11* and *nced3nced5* (Merilo *et al.*, 2018), but was detected in *pyr1/pyl1/2/4/5/8*, the genotype with the highest stomatal conductance (Figs 1b, 3a). Buckley (2019) proposed that a flaccid and limp epidermis could explain why the epidermal backpressure on the guard cells and wrong-way response were missing in ABA-deficient mutants. When epidermal pressure on guard cells is missing, passive hydraulic stomatal closure might contribute to the VPD response similarly to that found in basal vascular plants in the process of equilibration between apoplastic and guard cell water potentials (Peak & Mott, 2011). The green area in Fig. 3(c) indicates a reduced, but still clearly detectable, stomatal closure in ABA-deficient genotypes during the first 15 min under high VPD. These data are consistent with findings that ABA-deficient genotypes contain sufficient ABA levels in guard cells for basal ABA signalling (Gonzalez-Guzman *et al.*, 2002; Frey *et al.*, 2012; Hsu *et al.*, 2018; Merilo *et al.*, 2018), contributing to the VPD-induced stomatal closure, albeit perhaps in a reduced manner. In addition to *de novo* ABA synthesis, β-glucosidase AtBG1-mediated ABA release from conjugated ABA-glucose ester might also participate in the production of ABA under low relative humidity (Lee *et al.*, 2006). β-Glucosidase was reported to be a key enzyme regulating the levels of active ABA in rose plants as well (Arve *et al.*, 2013). However, we note that gas-exchange analyses using the *Arabidopsis Atbg1Atbg2* double mutant knock out plants showed wild-type-like high VPD-induced stomatal closure (Merilo *et al.*, 2015). The grey area, which indicates a stronger response of ABA-deficient genotypes compared with wild-type controls, might point at compensatory mechanisms to counteract ABA deficiency. The ABA signalling pathway may become less or more sensitive to ABA, depending on the expression levels of mRNAs of signalling components (Szostkiewicz *et al.*, 2010; Dittrich *et al.*, 2019). Due to the higher steady-state stomatal conductance and no wrong-way response of ABA-deficient mutants, a larger contribution of passive hydraulic stomatal closure may also be feasible as part of the grey area (Fig. 1b; Merilo *et al.*, 2018). We also point at different dynamics of VPD-induced stomatal closure between Col-0 and ABA-deficient mutants, with a clear bi-phasic response detected only in wild-type (Fig. 3b).

Conclusions and important questions

Mainly three components contribute to the VPD-induced stomatal closure of angiosperms:

(**1**) ABA-dependent activation of OST1. In this process, ABA is either rapidly synthesised or basal ABA levels mediate the response without the need for a rapid ABA concentration increase. Further research is needed to determine the contributions of basal and *de novo* synthesised ABA.

(**2**) ABA-independent activation of OST1, potentially by the domain I SnRK2-box.

(**3**) A putative contribution of passive hydraulic closure, for example in genotypes with conditionally very high stomatal conductance and no wrong-way response.

Answering the following open questions below would help to elucidate the mechanism of VPD-induced stomatal regulation:

(**1**) Recent studies have shown that in contrast to a rise in guard cell ABA concentrations, basal ABA concentrations and basal OST1/SnRK2 kinase activities enhance stomatal responses to other stimuli, including elevated carbon dioxide (Hsu *et al.*, 2018; Zhang *et al.*, 2020). Analyses of bulk leaf ABA concentrations are not a good proxy for guard cell ABA levels due to high basal ABA concentrations in guard cells (Lahr & Raschke, 1988; Waadt *et al.*, 2014; Hsu *et al.*, 2018) and guard cell-specific *de novo* ABA synthesis, transport and catabolism (Yoshida *et al.*, 2019). Measurements of ABA concentrations with cellular and real-time resolution would serve to clarify whether guard cell ABA levels consistently increase upon a rise in VPD during the first minutes. Genetically encoded biosensors could be used to analyse the dynamics of ABA in guard cells (Jones *et al.*, 2014; Waadt *et al.*, 2014; Hsu *et al.*, 2018). Whether in high VPD stress OST1 is activated above basal levels prevailing in guard cells could be measured using genetically encoded real-time reporters for OST1 activity (SNACS; Zhang *et al.*, 2020).

(**2**) To fully understand the proportion of active and passive components of stomatal VPD sensitivity in angiosperms, CRISPR/Cas9 could be used to disrupt OST1 orthologues in plant species other than *Arabidopsis* and then VPD responses could be compared with wild-type responses, as it has been done in *Arabidopsis* (Merilo *et al.*, 2013, 2018). Note however, that the OST1-related *Arabidopsis* proteins SnRK2.2 and SnRK2.3 also participate in stomatal ABA signalling (e.g. Zhang *et al.*, 2020) and cell-type specific CRISPR deletion may be needed for orthologues of the three ABA-activated SnRK2 protein kinases.

(**3**) The early steps of VPD signalling and the details of VPD sensing have not been clarified to date. It has been hypothesised that guard cell sugar metabolism and transport may be involved, either because sugars act as osmotica, or cell wall sugar-loading might serve as a VPD-sensing mechanism (Bauer *et al.*, 2013b). Our data indicate that the ABC-transporter ABCG22, which may be phosphorylated by OST1 according to Wang *et al.* (2020) contributes to initial steps of stomatal VPD-regulation (Merilo *et al.*, 2015). Therefore, it is important to identify the compound that is transported by ABCG22. Interestingly, missing steps in sensing and early signalling events apply to osmotic stress sensing in general (i.e. soil drought and high salinity), not only to atmospheric water stress (for review see Fàbregas *et al.*, 2020).

(**4**) Components contributing to the ABA-independent activation of OST1 in response to high VPD remain to be identified, and also the aspects of crosstalk between ABA-dependent and ABA-independent signalling. In this respect, future research should investigate the potential involvement of Raf-like M3Ks (Lin *et al.*, 2020; Takahashi *et al.*, 2020).

(**5**) In the calculations of stomatal conductance, the water vapour pressure within the leaf is assumed to reach saturation at a given leaf temperature. However, a considerable deviation from saturation was found in grey poplar lines with impaired stomatal regulation (Cernusak *et al.*, 2019). The possibility that leaf intercellular vapour pressure is not saturated, and its extent and effect on stomatal conductance measurements need further attention.

In crops, variation in the response of transpiration to VPD is high. Some genotypes showed linear increases of transpiration with VPD, as do *Arabidopsis* lines in Fig. 2(a). In others, transpiration increased only up to a certain threshold value of VPD, above which reduced or plateaued transpiration was detected (Jauregui *et al.*, 2018; Medina *et al.*, 2019). Limitations on maximum transpiration rates at high VPD values were modelled to increase transpiration efficiency and conserve soil water, resulting in yield increases in unfavourable low-yielding years (Sinclair *et al.*, 2005; Messina *et al.*, 2015). By contrast, in water-limited Mediterranean environments, wheat lines exhibiting a linear transpiration versus VPD relationship had greater grain yields and greenness indexes associated with better uptake, transport and metabolism of nitrogen, carbon and water compared with the lines showing limited transpiration increases under rising VPD (Medina *et al.*, 2019). The transpirational response to VPD is therefore an important trait to select crop varieties better adapted to specific local conditions in terms of timing and extent of water stress.

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Author contributions

EM designed the research, PJ and EM performed experiments, PJ, RW, YT, JIS and EM analysed the data, EM wrote the manuscript with input from all co-authors.

ORCID

Pirko Jalakas https://orcid.org/0000-0001-9981-8088
Ebe Merilo https://orcid.org/0000-0003-1767-6413
Julian I. Schroeder https://orcid.org/0000-0002-3283-5972
Yohei Takahashi https://orcid.org/0000-0002-9406-4093
Rainer Waadt https://orcid.org/0000-0002-6772-3006

Pirko Jalakas[1], Yohei Takahashi[2], Rainer Waadt[1], Julian I. Schroeder[2] and Ebe Merilo[1]*

[1]Institute of Technology, University of Tartu, Tartu 50411, Estonia;
[2]Cell and Developmental Biology Section, Division of Biological Sciences, University of California, San Diego, La Jolla, CA 92093-0116, USA
(*Author for correspondence: email ebe.merilo@ut.ee)

References

Arve LE, Terfa MT, Gislerød HR, Olsen JE, Torre S. 2013. High relative air humidity and continuous light reduce stomata functionality by affecting the ABA regulation in rose leaves. *Plant, Cell & Environment* **36**: 382–392.

Assmann SM, Snyder JA, Lee Y-RJ. 2000. ABA-deficient (*aba1*) and ABA-insensitive (*abi1-1, abi2-1*) mutants of *Arabidopsis* have a wild-type stomatal response to humidity. *Plant, Cell & Environment* **23**: 387–395.

Bauer H, Ache P, Lautner S, Fromm J, Hartung W, Al-Rasheid K, Sonnewald S, Sonnewald U, Kneitz S, Lachmann N *et al.* 2013a. The stomatal response to reduced relative humidity requires guard cell-autonomous ABA synthesis. *Current Biology* **23**: 53–57.

Bauer H, Ache P, Wohlfart F, Al-Rasheid KAS, Sonnewald S, Sonnewald U, Kneitz S, Hetherington AM, Hedrich R. 2013b. How do stomata sense reductions in atmospheric relative humidity? *Molecular Plant* **6**: 1703–1706.

Belin C, de Franco P-O, Bourbousse C, Chaignepain S, Schmitter J-M, Vavasseur A, Giraudat J, Barbier-Brygoo H, Thomine S. 2006. Identification of features regulating OST1 kinase activity and OST1 function in guard cells. *Plant Physiology* **141**: 1316–1327.

Brodribb TJ, McAdam SAM. 2011. Passive origins of stomatal control in vascular plants. *Science* **331**: 582–585.

Buckley TN. 2019. How do stomata respond to water status? *New Phytologist* **224**: 21–36.

Cai S, Chen G, Wang Y, Huang Y, Marchant DB, Wang Y, Yang Q, Dai F, Hills A, Franks PJ *et al.* 2017. Evolutionary conservation of ABA signaling for stomatal closure. *Plant Physiology* **174**: 732–747.

Cardoso AA, Brodribb TJ, Kane CN, DaMatta FM, McAdam SAM. 2020. Osmotic adjustment and hormonal regulation of stomatal responses to vapour pressure deficit in sunflower. *AoB Plants* **12**: plaa025.

Cernusak LA, Goldsmith GR, Arend M, Siegwolf RTW. 2019. Effect of vapor pressure deficit on gas exchange in wild-type and abscisic acid-insensitive plants. *Plant Physiology* **181**: 1573.

Chater C, Kamisugi Y, Movahedi M, Fleming A, Cuming AC, Gray JE, Beerling DJ. 2011. Regulatory mechanism controlling stomatal behavior conserved across 400 million years of land plant evolution. *Current Biology* **21**: 1025–1029.

Dittrich M, Mueller HM, Bauer H, Peirats-Llobet M, Rodriguez PL, Geilfus C-M, Carpentier SC, Al Rasheid KAS, Kollist H, Merilo E *et al.* 2019. The role of *Arabidopsis* ABA receptors from the PYR/PYL/RCAR family in stomatal acclimation and closure signal integration. *Nature Plants* **5**: 1002–1011.

Fàbregas N, Yoshida T, Fernie AR. 2020. Role of Raf-like kinases in SnRK2 activation and osmotic stress response in plants. *Nature Communications* **11**: 6184.

Ficklin DL, Novick KA. 2017. Historic and projected changes in vapor pressure deficit suggest a continental-scale drying of the United States atmosphere. *Journal of Geophysical Research: Atmospheres* **122**: 2061–2079.

Franks PJ. 2013. Passive and active stomatal control: either or both? *New Phytologist* **198**: 325–327.

Franks PJ, Britton-Harper ZJ. 2016. No evidence of general CO_2 insensitivity in ferns: one stomatal control mechanism for all land plants? *New Phytologist* **211**: 819–827.

Frey A, Effroy D, Lefebvre V, Seo M, Perreau F, Berger A, Sechet J, To A, North HM, Marion-Poll A. 2012. Epoxycarotenoid cleavage by NCED5 fine-tunes ABA accumulation and affects seed dormancy and drought tolerance with other NCED family members. *The Plant Journal* **70**: 501–512.

Geiger D, Scherzer S, Mumm P, Stange A, Marten I, Bauer H, Ache P, Matschi S, Liese A, Al-Rasheid KAS *et al.* 2009. Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling kinase-phosphatase pair. *Proceedings of the National Academy of Sciences, USA* **106**: 21425–21430.

Gonzalez-Guzman M, Apostolova N, Belles JM, Barrero JM, Piqueras P, Ponce MR, Micol JL, Serrano R, Rodriguez PL. 2002. The short-chain alcohol dehydrogenase ABA2 catalyzes the conversion of xanthoxin to abscisic aldehyde. *Plant Cell* **14**: 1833–1846.

Gonzalez-Guzman M, Pizzio GA, Antoni R, Vera-Sirera F, Merilo E, Bassel GW, Fernández MA, Holdsworth MJ, Perez-Amador MA, Kollist H *et al.* 2012. Arabidopsis PYR/PYL/RCAR receptors play a major role in quantitative regulation of stomatal aperture and transcriptional response to abscisic acid. *Plant Cell* **24**: 2483–2496.

Grossiord C, Buckley TN, Cernusak LA, Novick KA, Poulter B, Siegwolf RTW, Sperry JS, McDowell NG. 2020. Plant responses to rising vapor pressure deficit. *New Phytologist* **226**: 1550–1566.

Hayashi M, Inoue S, Takahashi K, Kinoshita T. 2011. Immunohistochemical detection of blue light-induced phosphorylation of the plasma membrane H^+-ATPase in stomatal guard cells. *Plant and Cell Physiology* **52**: 1238–1248.

Hetherington AM. 2001. Guard cell signaling. *Cell* **107**: 711–714.

Hõrak H, Kollist H, Merilo E. 2017. Fern stomatal responses to ABA and CO_2 depend on species and growth conditions. *Plant Physiology* **174**: 672–679.

Hsiao J, Swann ALS, Kim S-H. 2019. Maize yield under a changing climate: the hidden role of vapor pressure deficit. *Agricultural and Forest Meteorology* **279**: 1027692.

Hsu P-K, Takahashi Y, Munemasa S, Merilo E, Laanemets K, Waadt R, Pater D, Kollist H, Schroeder JI. 2018. Abscisic acid-independent stomatal CO_2 signal transduction pathway and convergence of CO_2 and ABA signaling downstream of OST1 kinase. *Proceedings of the National Academy of Sciences, USA* **115**: E9971.

Imes D, Mumm P, Böhm J, Al-Rasheid KAS, Marten I, Geiger D, Hedrich R. 2013. Open stomata 1 (OST1) kinase controls R–type anion channel QUAC1 in *Arabidopsis* guard cells. *The Plant Journal* **74**: 372–382.

Innes SN, Solhaug KA, Torre S, Dodd IC. 2021. Different abscisic acid-deficient mutants show unique morphological and hydraulic responses to high air humidity. *Physiologia Plantarum* **172**: 1795–1807.

Ivanoff L. 1928. Zur Methodik der Transpirationsbestimmung am Standort. *Berichte der Deutschen Botanischen Gesellschaft* **46**: 306–310.

Jalakas P, Huang Y-C, Yeh Y-H, Zimmerli L, Merilo E, Kollist H, Brosché M. 2017. The role of ENHANCED RESPONSES TO ABA1 (ERA1) in Arabidopsis stomatal responses is beyond ABA signaling. *Plant Physiology* **174**: 665–671.

Jalakas P, Nuhkat M, Vahisalu T, Merilo E, Brosché M, Kollist H. 2021. Combined action of guard cell plasma membrane rapid- and slow-type anion channels in stomatal regulation. *Plant Physiology.* doi: 10.1093/plphys/kiab202

Jauregui I, Rothwell SA, Taylor SH, Parry MAJ, Carmo-Silva E, Dodd IC. 2018. Whole plant chamber to examine sensitivity of cereal gas exchange to changes in evaporative demand. *Plant Methods* **14**: 97.

Jones AM, Danielson JA, Manojkumar SN, Lanquar V, Grossmann G, Frommer WB. 2014. Abscisic acid dynamics in roots detected with genetically encoded FRET sensors. *eLife* **3**: e01741.

Kang Y, Outlaw WH Jr, Andersen PC, Fiore GB. 2007. Guard-cell apoplastic sucrose concentration – a link between leaf photosynthesis and stomatal aperture size in the apoplastic phloem loader *Vicia faba* L. *Plant, Cell & Environment* **30**: 551–558.

Lahr W, Raschke K. 1988. Abscisic-acid contents and concentrations in protoplasts from guard cells and mesophyll cells of *Vicia faba* L. *Planta* **173**: 528–531.

Lee KH, Piao HL, Kim H-Y, Choi SM, Jiang F, Hartung W, Hwang I, Kwak JM, Lee I-J, Hwang I. 2006. Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. *Cell* **126**: 1109–1120.

Lin Z, Li Y, Zhang Z, Liu X, Hsu C-C, Du Y, Sang T, Zhu C, Wang Y, Satheesh V *et al.* 2020. A RAF-SnRK2 kinase cascade mediates early osmotic stress signaling in higher plants. *Nature Communications* **11**: 613.

Lobell DB, Roberts MJ, Schlenker W, Braun N, Little BB, Rejesus RM, Hammer GL. 2014. Greater sensitivity to drought accompanies maize yield increase in the U.S. Midwest. *Science* **344**: 516.

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E. 2009. Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* **324**: 1064–1068.

McAdam SAM, Brodribb TJ. 2015. The evolution of mechanisms driving the stomatal response to vapor pressure deficit. *Plant Physiology* **167**: 833–843.

McAdam SAM, Sussmilch FC, Brodribb TJ. 2016. Stomatal responses to vapour pressure deficit are regulated by high speed gene expression in angiosperms. *Plant, Cell & Environment* **39**: 485–491.

Medina S, Vicente R, Nieto-Taladriz MT, Aparicio N, Chairi F, Vergara-Diaz O, Araus JL. 2019. The plant-transpiration response to vapor pressure deficit (VPD) in Durum wheat is associated with differential yield performance and specific expression of genes involved in primary metabolism and water transport. *Frontiers in Plant Science* **9**: 1994.

Merilo E, Jalakas P, Kollist H, Brosché M. 2015. The role of ABA recycling and transporter proteins in rapid stomatal responses to reduced air humidity, elevated CO_2, and exogenous ABA. *Molecular Plant* **8**: 657–659.

Merilo E, Laanemets K, Hu H, Xue S, Jakobson L, Tulva I, Gonzalez-Guzman M, Rodriguez PL, Schroeder JI, Broschè M *et al.* 2013. PYR/RCAR receptors contribute to ozone-, reduced air humidity-, darkness-, and CO_2-induced stomatal regulation. *Plant Physiology* **162**: 1652–1668.

Merilo E, Yarmolinsky D, Jalakas P, Parik H, Tulva I, Rasulov B, Kilk K, Kollist H. 2018. Stomatal VPD response: there is more to the story than ABA. *Plant Physiology* **176**: 851–864.

Messina CD, Sinclair TR, Hammer GL, Curan D, Thompson J, Oler Z, Gho C, Cooper M. 2015. Limited-transpiration trait may increase maize drought tolerance in the US Corn Belt. *Agronomy Journal* **107**: 1978–1986.

Mott KA, Franks PJ. 2001. The role of epidermal turgor in stomatal interactions following a local perturbation in humidity. *Plant, Cell & Environment* **24**: 657–662.

Outlaw WH Jr. 2003. Integration of cellular and physiological functions of guard cells. *Critical Reviews in Plant Sciences* **24**: 2483–2496.

Pantin F, Blatt MR. 2018. Stomatal response to humidity: blurring the boundary between active and passive movement. *Plant Physiology* **176**: 485.

Pantin F, Renaud J, Barbier F, Vavasseur A, Le Thiec D, Rose C, Bariac T, Casson S, McLachlan DH, Hetherington AM *et al.* 2013. Developmental priming of stomatal sensitivity to abscisic acid by leaf microclimate. *Current Biology* **23**: 1805–1811.

Park S-Y, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow T-FF *et al.* 2009. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* **324**: 1068–1071.

Park Williams A, Allen CD, Macalady AK, Griffin D, Woodhouse CA, Meko DM, Swetnam TW, Rauscher SA, Seager R, Grissino-Mayer HD *et al.* 2013. Temperature as a potent driver of regional forest drought stress and tree mortality. *Nature Climate Change* **3**: 292–297.

Peak D, Mott KA. 2011. A new, vapour-phase mechanism for stomatal responses to humidity and temperature. *Plant, Cell & Environment* **34**: 162–178.

Ruszala EM, Beerling DJ, Franks PJ, Chater C, Casson SA, Gray JE, Hetherington AM. 2011. Land plants acquired active stomatal control early in their evolutionary history. *Current Biology* **21**: 1030–1035.

Sinclair TR, Hammer GL, van Oosterom EJ. 2005. Potential yield and water-use efficiency benefits in sorghum from limited maximum transpiration rate. *Functional Plant Biology* **32**: 945–952.

Soma F, Takahashi F, Suzuki T, Shinozaki K, Yamaguchi-Shinozaki K. 2020. Plant Raf-like kinases regulate the mRNA population upstream of ABA-unresponsive SnRK2 kinases under drought stress. *Nature Communications* **11**: 1373.

Sussmilch FC, Brodribb TJ, McAdam SAM. 2017. Up-regulation of *NCED3* and ABA biosynthesis occur within minutes of a decrease in leaf turgor but *AHK1* is not required. *Journal of Experimental Botany* **68**: 2913–2918.

Szostkiewicz I, Richter K, Kepka M, Demmel S, Ma Y, Korte A, Assaad FF, Christmann A, Grill E. 2010. Closely related receptor complexes differ in their ABA selectivity and sensitivity. *The Plant Journal* **61**: 25–35.

Takahashi Y, Zhang J, Hsu P-K, Ceciliato PHO, Zhang Li, Dubeaux G, Munemasa S, Ge C, Zhao Y, Hauser F *et al.* 2020. MAP3Kinase-dependent

SnRK2-kinase activation is required for abscisic acid signal transduction and rapid osmotic stress response. *Nature Communications* 11: 12.

Vicente-Serrano SM, Nieto R, Gimeno L, Azorin-Molina C, Drumond A, El Kenawy A, Dominguez-Castro F, Tomas-Burguera M, Peña-Gallardo M. 2018. Recent changes of relative humidity: regional connections with land and ocean processes. *Earth System Dynamics* 9: 915–937.

Waadt R, Hitomi K, Nishimura N, Hitomi C, Adams SR, Getzoff ED, Schroeder JI. 2014. FRET-based reporters for the direct visualization of abscisic acid concentration changes and distribution in Arabidopsis. *eLife* 3: e01739.

Wang P, Hsu C-C, Du Y, Zhu P, Zhao C, Fu X, Zhang C, Paez JS, Macho AP, Tao WA *et al.* 2020. Mapping proteome-wide targets of protein kinases in plant stress responses. *Proceedings of the National Academy of Sciences, USA* 117: 3270.

Xie X, Wang Y, Williamson L, Holroyd GH, Tagliavia C, Murchie E, Theobald J, Knight MR, Davies WJ, Leyser HMO *et al.* 2006. The identification of genes involved in the stomatal response to reduced atmospheric relative humidity. *Current Biology* 16: 882–887.

Yoshida R, Umezawa T, Mizoguchi T, Takahashi S, Takahashi F, Shinozaki K. 2006. The regulatory domain of SRK2E/OST1/SnRK2.6 interacts with ABI1 and integrates abscisic acid (ABA) and osmotic stress signals controlling stomatal closure in *Arabidopsis*. *The Journal of Biological Chemistry* 281: 5310–5318.

Yoshida T, Christmann A, Yamaguchi-Shinozaki K, Grill E, Fernie AR. 2019. Revisiting the basal role of ABA – roles outside of stress. *Trends in Plant Science* 24: 625–635.

Yuan W, Zheng Yi, Piao S, Ciais P, Lombardozzi D, Wang Y, Ryu Y, Chen G, Dong W, Hu Z *et al.* 2019. Increased atmospheric vapor pressure deficit reduces global vegetation growth. *Science Advances* 5: eaax1396.

Zhang L, Takahashi Y, Hsu P-K, Kollist H, Merilo E, Krysan PJ, Schroeder JI. 2020. FRET kinase sensor development reveals SnRK2/OST1 activation by ABA but not by MeJA and high CO$_2$ during stomatal closure. *eLife* 9: e56351.

Zhang ZQ, Outlaw WH Jr. 2001. The guard-cell apoplast as a site of abscisic acid accumulation in *Vicia faba* L. *Plant, Cell & Environment* 24: 347–355.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Relative stomatal VPD response of *ost1-3* plants with boosted stomatal conductance.

Methods S1 Supporting methods.

Key words: abscisic acid (ABA), *Arabidopsis*, passive hydraulic stomatal closure, stomata, transpiration, vapour pressure deficit.

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