Stomatal Response to Humidity: Blurring the Boundary between Active and Passive Movement

Land plants often experience conflicting demands for carbon assimilation and water conservation. The epidermal surface of plant leaves includes pores, or stomata, that allow atmospheric CO_2 to enter the air space within leaf for photosynthetic carbon fixation by the mesophyll cells. Stomata also facilitate transpiration, that is, the diffusion of water vapor from the leaf air spaces to the outer atmosphere. The water vapor content within the leaf is usually close to saturation. So, a dry atmosphere gives rise to a large gradient in water vapor across the stomatal pore, that is, a leaf-to-air vapor pressure deficit (VPD), which increases as the relative humidity outside falls. Increasing VPD leads to a proportional increase in transpiration rate through the stomatal pore, driven by diffusion. Stomata therefore generally close at high VPD to prevent excessive water loss.

Pairs of guard cells surround each stoma to regulate the stomatal aperture and, hence, the stomatal conductance (g_s, defined as the rate of transpiration divided by the VPD, the driving force). Guard cells open and close the pore, driven by solute uptake and loss—notably of K^+ and Cl^-—and by the synthesis and metabolism of organic solutes, especially malate. Solute flux and metabolism generate osmotic gradients for water flux across the guard cell plasma membrane, which in turn leads to changes in guard cell volume and turgor. Increasing volume and turgor promotes stomatal opening as the guard cells press apart from one another, and decreasing volume and turgor reverses this effect. Much research has focused on the water stress hormone abscisic acid (ABA) and its connection to stomatal closing. These studies have highlighted roles for Ca^{2+}-dependent signaling, cytosolic pH, reactive oxygen and nitrogen species, and protein phosphorylation in regulating a number of critical ion transport processes of the guard cell needed to generate the osmotic gradients for stomatal movement (Jezek and Blatt, 2017).

By contrast, how guard cells perceive and respond to changes in VPD has been a matter of debate, much of it centered on whether stomata respond passively or actively to VPD. This debate is intimately connected with discussions about the evolution of stomatal physiology (Brodribb and McAdam, 2017; Cai et al., 2017; Hõrak et al., 2017). Arguments for passive (so-called hydropassive) stomatal movements are based around leaf hydration and its effect in adding water to, or removing water from the guard cells. Hydropassive models consider stomata to operate entirely on the basis of passive water flux driven by evaporation and osmotic equilibration, without reference to guard cell solute transport or its consequences for guard cell turgor, and stomatal aperture (Brodribb and McAdam, 2011).

Arguments for active stomatal movements are supported by findings that changes in VPD associate with some elements of the signal cascades regulating ion transport, including those engaged by ABA. Initially, a role for ABA was discarded following publication of a seminal paper (Assmann et al., 2000) indicating that stomata respond to VPD changes in the Arabidopsis (*Arabidopsis thaliana*) *aba1* mutant, which is deficient in ABA biosynthesis, and in the ABA-insensitive mutants *abi1-1* and *abi2-1*, which affect two key protein phosphatases essential for ABA signal transmission. Arguments around a role for ABA reopened when a screen for genes associated with altered responses to VPD yielded mutations in *ABA2* and *OST1* (Xie et al., 2006). The *ABA2* gene encodes a dehydrogenase that contributes to the synthesis of ABA; the *OST1* gene encodes a protein kinase that affects the elevation of reactive oxygen species and cytosolic-free $[Ca^{2+}]$ and activates the guard cell SLAC1 Cl^- channel in response to ABA (Jezek and Blatt, 2017). In fact, both Assmann et al. (2000) and Xie et al. (2006) report very similar results: ABA-associated mutants still show a decrease in g_s with a step up in VPD, but from elevated background g_s which opens the findings to multiple interpretations.

In this issue of *Plant Physiology*, Merilo et al. (2018) present results that reconcile many of the perceived differences between these datasets. Their findings must raise a question also about the conventional wisdom of separating hydropassive and active responses of stomata, and we return to this question later. One clear-cut finding of Merilo et al. (2018) is that mutants affecting ABA biosynthesis responded to a step up in VPD by reducing g_s. These mutants generally showed a higher g_s than wild-type plants, both before and after a VPD step. Most important, a genotypic ranking of steady-state g_s was inversely related to the ABA levels found in the plant, with g_s highest in the mutants with the lowest leaf ABA content. In effect, the background g_s of the leaf appears to be restricted by ABA, even when VPD is low (high relative humidity). This observation is not new, but the comprehensive dataset that Merilo et al. present further supports the conclusion that native ABA content and sensitivity are important in determining the background of stomatal transpiration. Restricting g_s, even in well-watered conditions, allows the plant to retain water for leaf expansion and thus facilitate growth when the evaporative demand is significant (Pantin et al., 2011).

To address the role for ABA in stomatal response to VPD in more depth, Merilo et al. (2018) revisit the *ost1*

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mutant (Xie et al., 2006) to show that stomata of this mutant are virtually insensitive to VPD changes. One difficulty in interpreting this finding, like that of Xie et al. (2006), is that the OST1 kinase can be activated independently of ABA (Yoshida et al., 2006). Furthermore, analysis of the target mutant alone cannot distinguish between a direct action of the wild-type gene product in transmitting a signal and an indirect (permissive) role, in this case for the kinase in what we might describe as "gating" the sensitivity of the guard cells to VPD. To explore the question of ABA mediation, Merilo et al. challenged the sextuple ABA-receptor mutant *pyr1pyl1pyl2pyl4pyl5pyl8* as before with steps in VPD. Remarkably, like the ABA biosynthesis mutants, the sextuple receptor mutant showed an elevated background g_s—and enhanced changes in g_s with VPD—that greatly exceeded the wild-type response. It might be argued that the effect was still associated with ABA because several ABA receptors remain functional in the mutant, but Merilo et al. rule out this possibility by demonstrating that g_s of the sextuple mutant did not respond to exogenously applied ABA. A reasonable conclusion, then, is that ABA sets the background g_s of the leaf but does not contribute directly in the short-term response to changing VPD.

Is the stomatal response to VPD hydropassive, then? Brodribb and McAdam (2011) have argued from a thermodynamic stance that hydropassive stomatal movements should be entirely reversible and symmetric, and have used these criteria to differentiate between hydropassive and active responses dependent on ABA. They point out that osmotic water flux is expected to show first-order kinetics, and they conclude that any kinetic hysteresis between closing and opening must arise from metabolic, or active, effects. One difficulty with this argument is that thermodynamics alone cannot speak to the kinetics of a process or its underlying mechanism. Furthermore, even if osmotic water flux can be described as a reversible, first-order process, it does not rule out the possibility of similar kinetics in the case that stomatal responses are active. Nonetheless, based on these criteria, Merilo et al. (2018) suggest that the largely symmetrical response to VPD in the sextuple receptor mutant, by contrast with stomata of wild-type plants, indicates hydropassive regulation.

How, then, might this interpretation be reconciled with active regulation, as implied by the loss of a g_s response in the *ost1* mutation? Merilo et al. recovered a partial and near-symmetrical response to VPD in the *ost1* mutant when the background g_s was first elevated using blue light or low CO_2. They reconcile these data and the VPD sensitivity of the sextuple receptor mutant by arguing that a minimum threshold g_s must be exceeded before a symmetric hydropassive response is evident. Such ad hoc reasoning does not sit comfortably as an explanation, however, notably because the background g_s was higher in several ABA synthesis mutants yet VPD evoked asymmetrical responses in every case.

One trivial explanation, at least for the response to VPD steps when g_s was very high, is that of a methodological artifact. To estimate g_s, it is commonly assumed that the water vapor pressure within the leaf reaches saturation adjacent to the guard cells. While this assumption is a reasonable approximation in many circumstances (Farquhar and Raschke, 1978), reducing the external relative humidity when g_s is very high is likely to

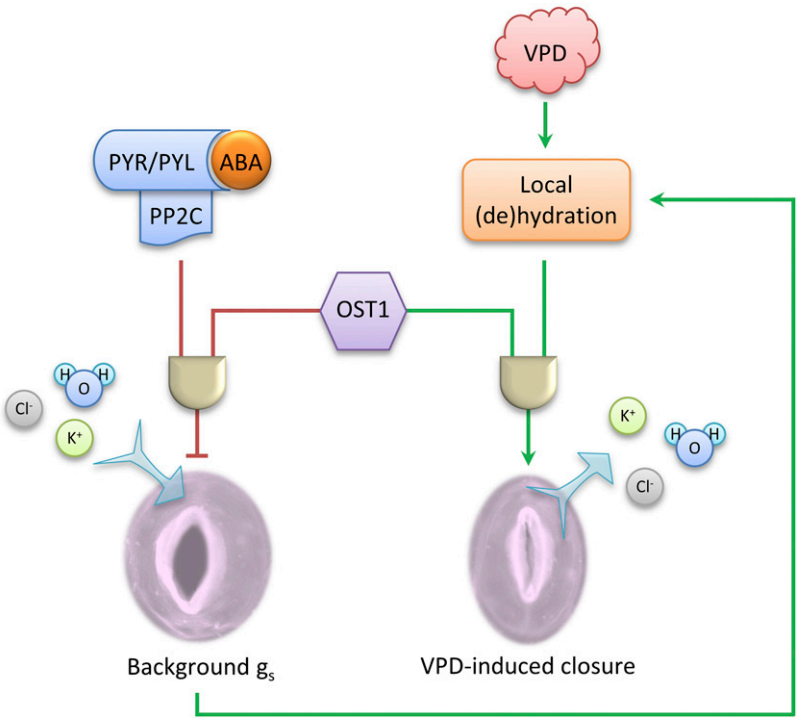

Figure 1. Gating model for stomatal response to low humidity. The background g_s in control conditions is restricted by ABA-mediated regulation of ion and water transport, which is gated by the OST1 kinase. Stomatal closure with a step increase in VPD is initiated by a drop in water potential at the vicinity of the guard cells, especially at high g_s. This local dehydration triggers ion and water efflux, a response which is gated by OST1. The green arrows denote a stimulatory process, and the red bars denote an inhibitory process. Note this simplified model assumes that the VPD responses observed by Merilo et al. (2018) at extremely high g_s result from genuine stomatal movements and not from artifacts due to subsaturation of water vapor close to the stomatal pore.

depress the water vapor pressure below saturation within the substomatal cavity. In these circumstances, the assumption leads to an overestimation of the VPD across the stomatal pore and, hence, an underestimation of the true g_s (Buckley et al., 2017), potentially accounting for the apparent drop in g_s with VPD steps observed by Merilo et al. (2018). To eliminate this explanation, alternative porometry techniques (Farquhar and Raschke, 1978; Pantin et al., 2013) are needed to assess g_s independent of evapotranspiration. Such techniques would clarify whether the response to VPD resulted from a change in stomatal aperture or from changes in water status within the leaf.

If stomata of the ABA synthesis and sextuple receptor mutants do close with VPD, but stomata of the *ost1* mutant normally do not, then the arguments differentiating hydropassive and active movements become self-contradictory. Perhaps the logic is simply misguided, and it is time to reframe the questions around solute and water flux. In a recent paper, Wang et al. (2017) take this approach and, in the process, unify the macroscopic water relations and evapotranspiration of the whole plant with the microscopic events of subcellular transport in the guard cells. They extend the quantitative OnGuard systems platform by incorporating the concept of a VPD gradient that extends through the stomatal pore and into the substomatal cavity adjacent to the guard cells (Peak and Mott, 2011). OnGuard is a proven computational platform for modeling stomatal physiology that encompasses guard cell transport, signaling, and homeostasis, and has predicted stomatal behavior across species (Chen et al., 2012; Wang et al., 2012). In OnGuard2, Wang et al. (2017) connect these processes, largely ignored in past models of g_s (Dewar, 2002; Peak and Mott, 2011), with water in the guard cell wall. They allow the wall to equilibrate with the water vapor pressure in the substomatal cavity adjacent to the guard cells. In turn, the water potential of the cell wall affects the osmotic balance of the guard cell, and hence both water and ion fluxes across the guard cell membranes, stomatal aperture, and g_s. To use the parlance of the hydropassive versus active debate, in OnGuard2 the two processes are combined within a single, overarching framework that describes stomatal movements in explicit mechanistic terms.

Using OnGuard2, Wang et al. (2017) accurately predict changes in transport, stomatal aperture, and g_s dynamics in response to VPD in wild-type Arabidopsis and in the *slac1* and *ost2* mutants that they subsequently validated experimentally. Both mutations affect solute flux rather than passive water flux per se. The *slac1* mutation eliminates the major Cl^- channel that facilitates Cl^- efflux during stomatal closure, while the *ost2* mutation confers constitutive activation of the H^+-ATPase at the guard cell plasma membrane. Yet the *slac1* mutation enhanced the asymmetry in g_s dynamics between closing and opening, while the *ost2* mutation had the opposite effect. These findings, and the associated model predictions, clearly illustrate the failure of symmetry as a criterion to differentiate kinetic mechanisms. Equally important, a "hardwired" ABA signal was not needed to recapitulate the VPD responses within OnGuard2. The findings do not speak to a role for ABA in determining the background g_s. However, the effects of VPD steps, predicted by OnGuard2 and demonstrated experimentally, indicate only a partial overlap with the effects on ion transport that are known to underpin ABA-mediated stomatal closure. Thus, ABA is unlikely to contribute in any short-term response to VPD. In summary, the studies of Merilo et al. (2018) and Wang et al. (2017) are consistent with an alternative scheme (Fig. 1) in which neither OST1 nor ABA couple the VPD stimulus to stomatal response. Instead, OST1 "gates" the sensitivity of one or more transporters essential for the response; ABA sets the poise of the transduction and response network independent of VPD; and hydropassive and active stomatal movements are interlocked within a single, mechanistic framework.

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