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Dynamics of stomatal water relations during the humidity response: implications of two hypothetical mechanisms

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ABSTRACT

The feasibility of two hypothetical mechanisms for the stomatal response to humidity was evaluated by identifying theoretical constraints on these mechanisms and by analysing timecourses of stomatal aperture following a step change in humidity. The two hypothetical mechanisms, which allow guard cell turgor pressure to overcome the epidermal mechanical advantage, are: (1) active regulation of guard cell osmotic pressure, requiring no hydraulic disequilibrium between guard and epidermal cells, and (2) a substantial hydraulic resistance between guard and epidermal cells, resulting in hydraulic disequilibrium between them. Numerical simulations of the system are made possible by recently published empirical relationships between guard cell pressure and volume and between stomatal aperture, guard cell turgor pressure, and epidermal cell turgor pressure; these data allow the hypothetical control variables to be inferred from stomatal aperture and evaporative demand, given physical assumptions that characterize either hypothesis. We show that hypothesis (1) predicts that steady-state π_g is monotonically related to transpiration rate, whereas hypothesis (2) suggests that the relationship between transpiration rate and the steady-state guard to epidermal cell hydraulic resistance may be either positive or negative, and that this resistance must change substantially during the transient phase of the stomatal response to humidity.

Key-words: humidity; hydraulics; model; stomata; water relations.

INTRODUCTION

The mechanism by which stomata open under high atmospheric humidity and close under low atmospheric humidity remains unknown. However, the field of viable hypotheses is limited by a few key empirical facts. First, steady-state stomatal conductance responds to transpiration rate, rather than to any measure of humidity or the humidity gradient *per se* (Mott & Parkhurst 1991). Second, stomatal aperture is controlled by a balance between guard cell turgor, which opens the pore, and epidermal turgor, which closes it (Cowan 1994). Third, epidermal turgor is more effective in controlling stomatal aperture (it has a 'mechanical advantage'), so an equal decrease in guard and epidermal turgors will open the pore (Sharpe, Wu & Spence 1987; Franks, Cowan & Farquhar 1998). Fourth, mesophyll and epidermal turgor pressures decline with increasing transpiration rate (Shackel 1987; Nonami, Schulze & Ziegler 1990). Any valid hypothesis must therefore take an increase in the rate of evaporative water loss from cells in the leaf and turn it into a substantially greater decrease in guard cell turgor than in epidermal cell turgor. Such hypotheses can be divided into two general categories. The first category, hereafter called the 'osmotic regulation model', suggests that the increase in water loss rate triggers a metabolically induced decline in guard cell osmotic pressure (via solute efflux) and therefore in guard cell turgor pressure (e.g. Meidner 1986; Grantz 1990; Buckley & Mott 2001). The second, hereafter called the 'drawdown model', suggests that these responses are the result of a water potential gradient, or drawdown, between epidermal and guard cells (e.g. Raschke & Kuhl 1970; Lange *et al*. 1971; Dewar 1995). It is important to note that in both models, guard cells lose turgor passively, but in the osmotic regulation model, passive water loss is not enough to overcome the epidermal mechanical advantage and cause stomatal closure.

Early studies favoured the drawdown hypothesis (Raschke 1970; Lange 1971), and considerable effort was made to explain stomatal responses to humidity in terms of evaporation from the guard cells and epidermis (e.g. Appleby & Davies 1983; Maier-Maercker 1983; Sheriff 1984). However, experiments with leaves and epidermes bathed in solutions of different osmotic pressure suggest that a metabolic signal from the mesophyll is involved (Grantz & Schwartz 1988), which implies an active regulation of guard cell osmotic pressure. Furthermore, the similarities in kinetics between responses to light and humidity also suggest that active regulation of guard cell osmotic concentration is responsible for humidity responses (Grantz 1990).

Although there is no unequivocal evidence for either of these models, recently published data make it possible to formalize and quantify the mechanistic constraints on each model based on the physics of stomatal movements. The goals of the present study were to provide a mathematical context that permits evaluation of the theoretical plausibility of each hypothesis, and to generate, from this mathematical context, testable criteria for each hypothesis. A

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numerical solution of the mathematical system required to achieve these goals was made possible by recent data characterizing the hydromechanics of stomata in *Vicia faba* L. Franks, Cowan & Farquhar (1995, 1998) characterized the effects of guard cell turgor pressure (P_g) and epidermal cell turgor pressure (P_e) on stomatal aperture (a) in *V. faba* and *Tradescantia virginiana* L., by manipulating P_g with a pressure probe and by altering P_e via the water potential of the bathing medium. These relationships, together with one of the hypothetical mechanisms for the stomatal response to humidity (discussed above) and a set of empirical constraints (discussed in the Appendix, under *Mathematical development*) close the system mathematically. This makes it possible to infer relationships among any arbitrary subset of the variables that control or influence stomatal dynamics. In the present study, this mathematical framework, along with measurements of stomatal aperture in response to changes in humidity, was used to infer either the dynamics of guard cell osmotic pressure or the properties of the epidermal-to-guard cell water potential gradient that must exist for either hypothesis to be consistent with observed stomatal responses.

METHODS AND MATERIALS

Synopsis of the mathematical technique

Using the standard equations of plant cell water relations and gas exchange, a mathematical system was developed to relate stomatal aperture to guard cell osmotic pressure and epidermal-guard cell hydraulic resistance. The complete development of the necessary mathematical system was made possible by recent data, obtained from experiments using a cell pressure probe and a confocal microscope, that provide empirical relationships between (i) stomatal aperture and the turgor pressures of epidermal and guard cells (Franks *et al*. 1998) and (ii) guard cell turgor pressure and guard cell volume (Franks *et al*. 2001). The relationships used in the present study for these fundamental relationships of stomatal hydromechanics are shown in Figs 1 and 2, respectively (equations are given the Appendix). Their main features are: (i) stomatal aperture increases with guard cell turgor in a saturating fashion when epidermal turgor is zero; (ii) aperture increases with guard cell turgor in a sigmoidal fashion when epidermal turgor is high; and (iii) guard cell volume increases in a weakly saturating fashion with guard cell turgor.

This mathematical system, which is developed fully in the Appendix, can be distilled to two equations (A9 and A12b). Two numerical parameters appearing in these equations required determination by experiment. These parameters are: (1) $r_{se} \cdot \gamma$, the product of r_{se} (the hydraulic resistance from the water source, assumed to be at constant water potential, to the epidermal cells) and γ (the scaling factor between stomatal aperture and conductance); and (2) π_e, the epidermal osmotic pressure (also assumed to be constant). Because the terms r_{se} and γ always appear in Eqns A8 and A12b as the product, $r_{se} \cdot \gamma$, it was necessary only to determine a value for this product, rather than for each of these parameters.

To obtain approximate values for $r_{se} \cdot \gamma$ and π_e, we determined the purely hydraulic effects of D (the mole fraction gradient for water vapour from the substomatal cavity to the atmosphere) on epidermal turgor pressure (P_e) and stomatal aperture (a). This was done by equilibrating leaves at a low value of D and then measuring P_e and a as D was increased. These measurements were made rapidly – during the quasi-steady state that is achieved as stomata open in response to an increase in D, before the closing response is initiated.

The procedures for measuring stomatal apertures and epidermal turgor pressures in intact leaves were similar to those described in Mott, Denne & Powell (1997) and Mott & Franks (2001). Briefly, a fully expanded leaf of *Vicia faba* L. was secured to a microscope stage and the ambient humidity was controlled by flowing gas of known composition over the leaf through a 0·5 cm internal diameter latex tube. The leaf was secured to the stage with the adaxial surface facing up. The abaxial surface of the leaf was sealed with clear plastic to prevent gas exchange through that surface. Light was provided by a 500 W xenon bulb and delivered to the adaxial surface by two 0·5 cm fibre-optic bundles. To view stomata on the adaxial surface, light above 700 nm was applied to the abaxial surface through the microscope's condenser. The transmitted light was imaged using a CCD camera (NEC model TI-324 A; NEC Technologies, Woodale, IL, USA) mounted on the microscope. Epidermal turgor was measured with a cell pressure probe mounted to the microscope stage.

It was impossible to monitor the oil–water meniscus of the pressure probe and stomatal apertures simultaneously. Therefore, the effects of D on P_e and a were determined in separate experiments on separate leaves. In all experiments, the leaf was brought to steady state at a temperature of approximately 23 °C, a D of approximately 9 mmol H_2O mol^{-1} air, and a photon flux density of approximately 800 mol photons $m^{-2}\,s^{-1}$. To determine P_e, the pressure probe was inserted in an epidermal cell, and a stable pressure was recorded for about 5 min. D was then increased in steps, and the pressure was recorded continuously over time. For each step in D, the pressure was allowed to stabilize at the new value before D was increased again. The process was continued until D reached 25 mmol mol^{-1} or until the seal around the pressure probe was lost. Five experiments using different leaves were performed to obtain at total of 25 data points, and most experiments were complete within about 15 min. To determine the hydraulic effect of D on a, an image containing one or two stomata was captured at D = 9 mmol mol^{-1} and the D was then increased to 14·5, 18·5, or 21·5 mmol mol^{-1}. Six experiments using different leaves were performed to obtain a total of 12 data points. Images were captured at 1 min intervals for about 20 min, and apertures were measured from these images using image analysis software. A stable aperture was achieved after about 10 min, and this value was used in the analysis below.

The parameters $r_{se} \cdot \gamma$ and π_e were estimated from these

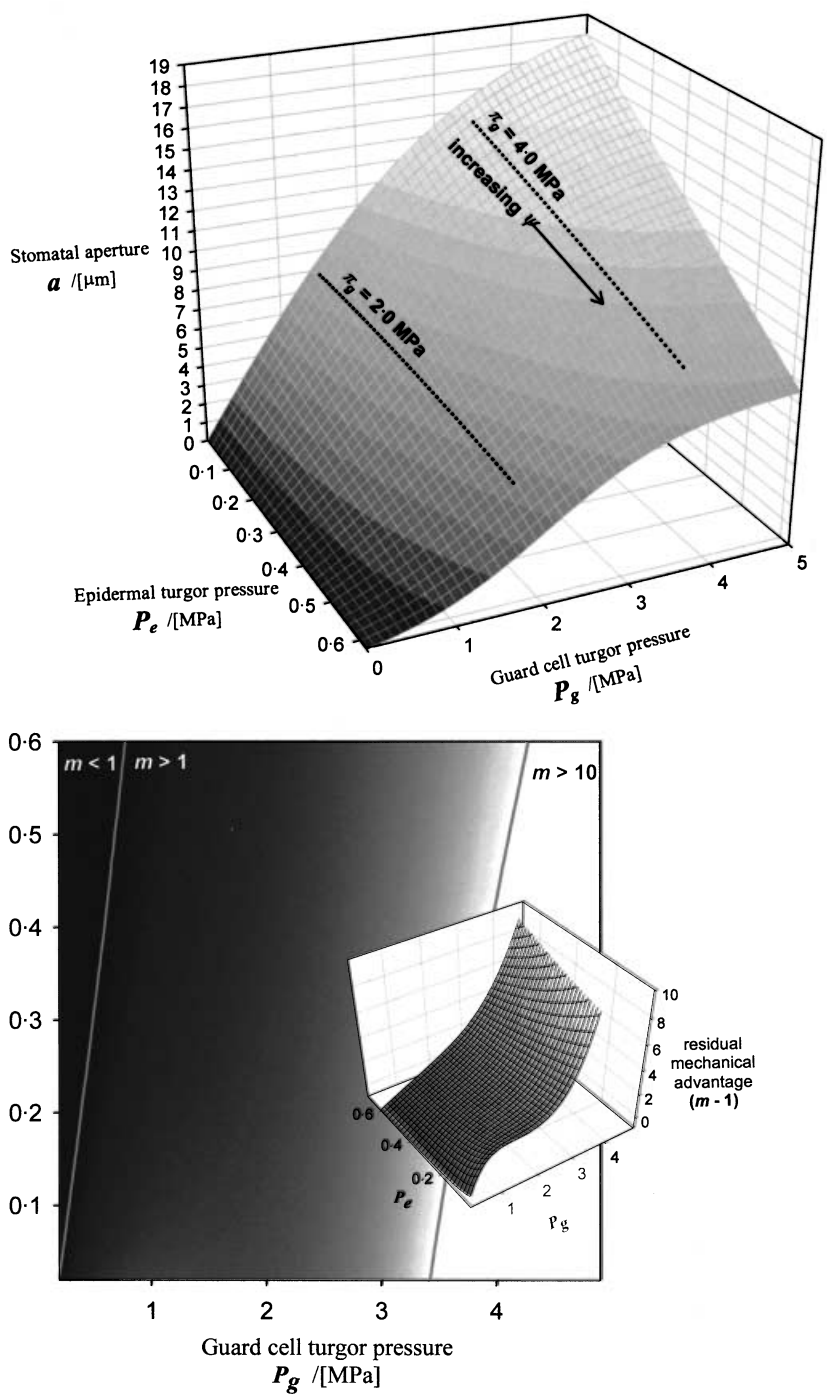

Figure 1. Relationships between stomatal aperture (a, μm) and the turgor pressures of guard and epidermal cells (P_g and P_e, respectively, MPa). Panel (a) shows aperture as a function of P_g and P_e. Superimposed on this surface are two trajectories that would result if water potential were increased from –0·6 to 0 MPa in the absence of decoupling between P_g and P_e, for two values of guard cell osmotic pressure (π_g, 2·0 and 4·0 MPa); both of these trajectories show a continuous decline in aperture with increasing water potential, highlighting the need for a mechanism by which P_g and P_e can be decoupled to produce the observed increase in stomatal aperture with water potential. Panel (b) shows the residual mechanical advantage of the epidermis ($m - 1$, where m is unitless), both as a shaded contour plot (marked with boundaries between regions where $m - 1$ is negative and positive, and where it is less or greater than 10) and as a surface plot.

data in a three-step procedure. First, the data for D versus a were used to create a regression function $a(D)$; second, this function was applied to the data for D versus P_e to infer a from the values of D measured in that experiment; third, the product of the measured values of D and inferred values of a were regressed against values of P_e measured in the same experiment. The slope of the resulting regression (Fig. 3) then provided an estimate of $r_{se} \cdot \gamma$, and the intercept provided an estimate of π_e (assuming that the source water potential was equal to zero).

RESULTS AND DISCUSSION

Empirical relationships of stomatal hydromechanics

Figure 1a shows the empirical relationship (Eqn A1) between stomatal aperture and the turgor pressures of epidermal and guard cells, which is based on the measurements reported by Franks *et al.* (1998) for *Vicia faba* L. The most evident feature of the relationship between aperture and the turgor pressures of epidermal and guard cells is its

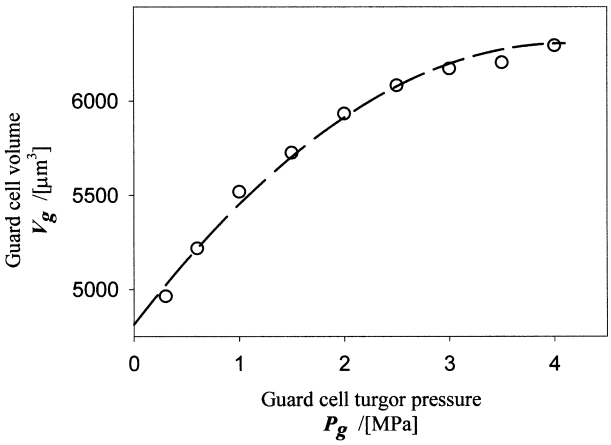

Figure 2. Relationship between guard cell turgor pressure (P_g, MPa) and volume (V_g, μm^3), redrawn from Franks *et al.* (2001).

shift from a sigmoidal relationship between a and P_g at high P_e to a simpler, saturating curve at low P_e; note this is essentially a three-dimensional expansion of Fig. 7 in Franks *et al.* (1998), with different parameter values for *V. faba*. Although it is possible for the physical state of a stomatal pore to occupy any position on this surface, this would require guard-cell and epidermal-cell turgor pressures to be independent. In a normally functioning leaf, these two pressures are hydraulically coupled through the effect of transpiration on epidermal water potential (Eqn A5). To demonstrate this, two dotted lines superimposed on the surface in Fig. 1a show the trajectories of stomatal aperture that would result as water potential increased from –0·6 MPa to 0·0 MPa with no decoupling of epidermal and guard cell turgors (i.e. constant guard cell osmotic pressure and equal epidermal and guard cell water potentials), for two different values of guard cell osmotic pressure (2 and 4 MPa).

Figure 1a shows that aperture declines with increasing water potential if guard cell and epidermal cell water potentials are perfectly coupled. This is a result of the epidermal mechanical advantage, m (Eqn A14). The value of m determines the amount by which the pore will open in response to an equal drop in turgor pressure of both guard and epidermal cells, so the amount by which m exceeds unity – the *residual mechanical advantage* (m – 1, plotted in Fig. 1b) – determines how much decoupling must occur between guard and epidermal cell water potentials (either by guard cell osmoregulation or a water potential gradient) to make aperture decline with decreasing water potential, and thus with increasing transpiration rate. Note that if the residual mechanical advantage is negative, passive equilibration of guard- and epidermal-cell water potentials should produce the correct steady-state humidity response, but Fig. 1b shows that, empirically, m – 1 is negative only at very low values of P_g.

The relationship between guard-cell volume and turgor pressure used in the present study was obtained by confocal microscopy and pressure probe measurements (Franks *et al.* 2001), and is described by a second-order polynomial function (Eqn A4; Fig. 2). Guard cell volume increased in a saturating fashion with turgor pressure for the three cells measured in that study.

Mathematical criteria for the two hypothetical mechanisms

Stomatal aperture and conductance are observed to decline as the leaf-to-air evaporative gradient increases (see reviews by Grantz 1990; Monteith 1995; Buckley & Mott 2001; direct observations by Kappen & Haeger 1991; Kappen, Schultz & Vanselow 1994; Mott *et al.* 1997). In the absence of 'apparent feedforward' (wherein transpiration rate declines at very low humidity; Franks *et al.* 1997; Farquhar 1978) stomatal aperture and conductance also decline with increasing transpiration rate. Since this 'apparent feedforward' response occurs only at very high values of D and may be hysteretic (Franks, Cowan & Farquhar 1997), it is excluded from the discussion below. As noted above, in most plants for which data are available, stomatal aperture is more sensitive to epidermal turgor than to guard cell turgor (i.e. the 'mechanical advantage' is greater than unity, as is true for *V. faba* under most conditions; Franks *et al.* 1998). For aperture to decline with increasing transpiration rate in such a leaf, an increase in transpiration rate must lead to a greater reduction in turgor in guard cells than in epidermal cells. In other words, guard cell turgor must be actively decoupled from epidermal turgor to overcome the mechanical advantage. This decoupling may be achieved either by decoupling the water potentials of guard and epidermal cells, or by decoupling guard cell turgor pressure from water potential. The first possibility demands that the hydraulic resistance between epidermal and guard cells is substantial. The second possibility demands that

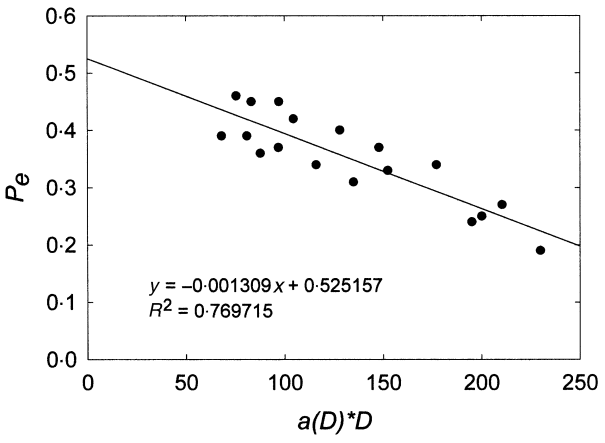

Figure 3. Data relating epidermal turgor pressure (P_e, MPa) with rate of water loss (expressed as the product of stomatal aperture [a, μm] and evaporative demand [D, mmol H_2O mol air^{-1}]), collected for the purpose of estimating the source-epidermis hydraulic resistance factor, $r_{se} \cdot \gamma$. See text for discussion.

guard cell osmotic pressure is actively regulated in response to changes in humidity. Mathematical criteria for each of these hypotheses are presented below; detailed derivations are presented in the Appendix. The general criterion for aperture to decrease with increasing transpiration rate is given by Eqn 1 (Eqn A20 in the Appendix):

$$\left[-\frac{\mathrm{d}\pi_g}{\mathrm{d}[aD]}+\left(r_{eg}^{*}+\frac{\mathrm{d}r_{eg}^{*}}{\mathrm{d}[\ln aD]}\right)\right]\Big/\left(r_{se}\gamma+\frac{\mathrm{d}[r_{se}\gamma]}{\mathrm{d}[\ln aD]}\right)>(m-1) \quad (1)$$

In Eqn 1, π_g is guard cell osmotic pressure, aD is the transpiration rate, r_{eg}^{*} is the hydraulic resistance between epidermal and guard cells (multiplied by two factors assumed constant: the ratio, K, of evaporation rates from guard and epidermal cells, and the scaling factor, γ, between aperture and conductance), r_{se} is the resistance from a hydraulic 'source' to the epidermal cells, and m is the epidermal mechanical advantage. If hydraulic supply is assumed to be insensitive to transpiration rate (that is, if increasing transpiration does not affect either the source water potential or the resistance of the hydraulic supply pathway to the epidermis; this is not strictly correct, but for low and moderate evaporative demands, r_{se} is fairly insensitive to leaf water potential, and thus to E; see Sperry 2000): the criterion can be simplified to Eqn 2 (A20b):

$$\frac{\mathrm{d}\pi_g}{\mathrm{d}P_e}+\left(r_{eg}^{*}+\frac{\mathrm{d}r_{eg}^{*}}{\mathrm{d}[\ln aD]}\right)\Big/[r_{se}\gamma]>(m-1) \quad (2)$$

The two alternative hypotheses (guard cell osmoregulation or water potential drawdown between epidermal and guard cells) are represented by degenerate versions (Eqns 3 and 4, respectively; A20c and A20d) of Eqn 2:

$$\frac{\mathrm{d}\pi_g}{\mathrm{d}P_e}>(m-1) \quad (3)$$

$$\left(r_{eg}^{*}+\frac{\mathrm{d}r_{eg}^{*}}{\mathrm{d}[\ln aD]}\right)\Big/[r_{se}\gamma]>(m-1) \quad (4)$$

Equation 3 says that, for the osmoregulation hypothesis to be correct, steady-state changes in guard cell osmotic pressure (π_g) must outpace changes in epidermal turgor (P_e) by a factor equal to the residual mechanical advantage ($m-1$).

Equation 4 is more difficult to interpret. The central element in this equation is r_{eg}^{*}, which we call the 'epidermal-guard cell hydraulic drawdown factor.' This quantity, which is the product of r_{eg} (the resistance between epidermal and guard cells) with K (the ratio of evaporation rates from guard and epidermal cells) and γ (the proportionality between aperture and conductance), must be large enough to draw down guard cell water potential relative to epidermal water potential, in order to overcome the epidermal mechanical advantage. Note that this could be accomplished either by large K (a large proportion of evaporation occurring from guard cells) or by large r_{eg} (a large resistance in water supply to guard cells). The formal mathematical requirement is not simply $r_{eg}^{*} > (m-1)$, however. There are two other factors. First, r_{eg} is normalized to r_{se}, which is the supply resistance for epidermal cells; this shows that it is the *balance* of water supplies to guard and epidermal cells that is critical, and this makes sense intuitively because the goal is to decouple the water potentials of these two cells. Second, either K or r_{eg} may, in principle, change with transpiration rate (either passively or actively), which provides an additional way for drawdown to overcome the mechanical advantage. Even if the balance of water supplies to guard and epidermal cells is small to begin with (i.e. even if $K \cdot r_{eg}/r_{se}$ is small), a large *change* in that balance would also decouple epidermal and guard-cell water potentials by making them depend differently on E. Therefore, the sensitivity of r_{eg}^{*} to changes in transpiration rate [strictly, to normalized changes, hence the natural logarithm: $\mathrm{d}r_{eg}^{*}/\mathrm{d}(\ln aD)$] also contributes to the effort of overcoming the mechanical advantage.

What is required for either decoupling factor to produce the steady-state response?

We have taken two approaches to evaluating the feasibility of these hypotheses. First, the mathematical system described in the Appendix was constrained by implementing each hypothesis separately. This allowed us to generate the three-dimensional relationships (Figs 4 and 5) between aperture, evaporative gradient, and each decoupling factor (π_g or r_{eg}^{*}) implied by each hypothesis. These relationships, in turn, permit simple visual evaluation of the feasibility of each hypothesis for producing the observed *steady-state* response of stomata to humidity, as discussed below. Second, the mathematical system was constrained with measured timecourses of stomatal aperture following a step change in humidity. This allows inference of the timecourses (Fig. 6) of the hypothetical decoupling factor implied by each hypothesis, which in turn reveal the ability of each decoupling factor to produce the observed *transient dynamics and kinetics* of the stomatal response to humidity.

Figure 4 shows how stomatal aperture is affected by guard cell osmotic pressure (π_g) and evaporative gradient (D) in the limiting case of hydraulic equilibrium between epidermal and guard cells, and Fig. 5 shows how aperture is controlled by the epidermis-guard cell drawdown factor (r_{eg}^{*}) and D in the limiting case where guard cell osmotic pressure is constant. In Fig. 4, aperture always increases with both π_g and D except at extremely high values of π_g and D, where aperture decreases with D. This small planar section at the far upper corner of the surface corresponds to zero epidermal turgor; that is, these values of π_g and D produce a transpiration rate that draws down ψ_e to be equal to $-\pi_e$, below which further water loss causes plasmolysis. The requirement that aperture must decline as D increases has a very simple topological interpretation on each of these surfaces: any empirically plausible response of the decoupling factor to D must produce a trajectory of steady states that move progressively *downhill*. Examples of implausible and plausible trajectories are overlaid on the surfaces in Figs 4 and 5.

Figure 4 shows that π_g must decline monotonically with D to produce the correct steady-state response of aperture

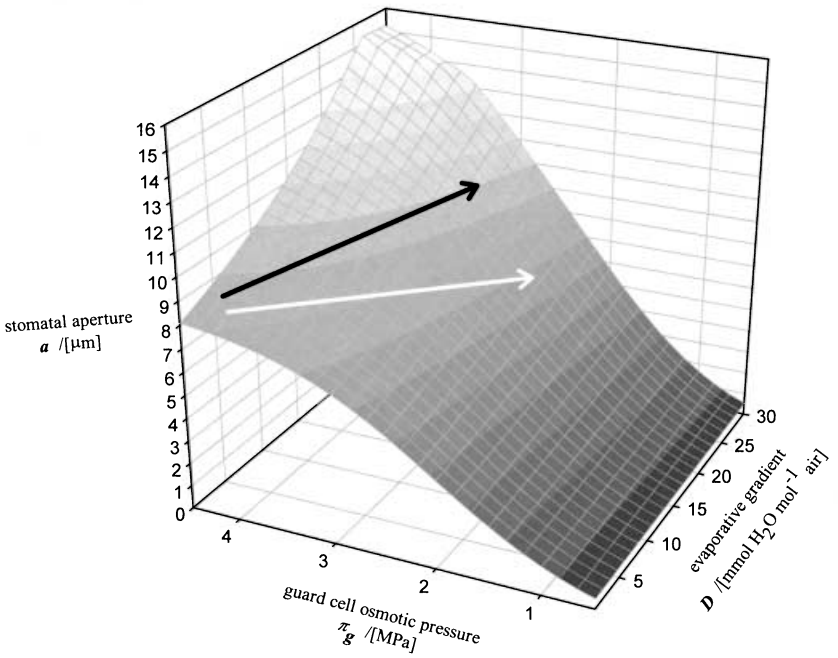

Figure 4. Model output showing the effect of guard cell osmotic pressure (π_g, MPa) and evaporative demand (D, mmol H_2O mol^{-1} air) on stomatal aperture (a, μm). Arrows superimposed on the surface show two hypothetical trajectories, one of which is consistent with observation (the white arrow, which has a declining with increasing D) and one of which is inconsistent with observation (the black arrow, which has a increasing with increasing D).

to humidity; in particular, π_g must decline with D more than the planar contours that transect the surface in Fig. 4, as shown by the 'plausible' trajectory that is drawn on the surface. Another interesting property of Fig. 4 is that at high evaporative demands, stomatal aperture is more sensitive to guard cell osmotic pressure (excluding the zero–P_e region). As D rises, smaller increments in π_g are required to cause a given change in a. This means that stomata can respond more rapidly and more efficiently to changes in other environmental variables under conditions of high evaporative demand (as reported by Mott, Shope & Buckley 1999 for stomatal responses to light) – consistent with the idea that stomata are adapted to optimize the carbon–water tradeoff when water is limiting (Cowan & Farquhar 1977). Another property of the system defined by the osmoregulation hypothesis is the implicit maximum steady-state aperture that occurs at the high-π_g–low-D corner of the surface (using the hydromechanical parameters reported by Franks *et al.* (1998, 2001), this value is about 8·1 μm). Because steady-state aperture declines monotonically with increasing D, but increases monotonically with increasing π_g, steady-state aperture will never occupy the region of the surface that rises higher than this corner (i.e. all points with a >8·1 μm in this example), although this region can be occupied during transients.

Figure 5 contains three different surfaces, each for a different constant value of π_g (2·5, 3·0, and 3·5 MPa), showing how aperture would vary with r_{eg}* and D if the drawdown hypothesis were correct. All three surfaces have the same basic shape, in that aperture increases with D when r_{eg}* = 0 (as one would expect), but decreases with D for sufficiently large r_{eg}*. However, the surfaces differ in two ways. First, the predicted aperture increases with the imposed value of π_g for any given set of values for r_{eg}* and D. Second, the critical value of r_{eg}* (above which the drawdown hypothesis produces the correct response, that is, aperture declines with increasing D for all values of D) increases with π_g, and is 0·22, 0·30, and 0·50 (MPa mol air mmol H_2O^{-1} μm^{-1}) when π_g is 2·5, 3·0 and 3·5 MPa, respectively.

Can either decoupling factor produce the observed transients and response kinetics?

Figures 6(a) and (d) show timecourses of stomatal aperture measured before and after a step change in D from 9 to 18·5 mmol mol^{-1}. These timecourses are consistent with previously published data and show the typical rapid transient opening followed by a slower closing response. The inferred timecourses of π_g (Fig. 6b, e) and r_{eg}* (Fig. 6c, f) are presented below the aperture measurements. Figures 6(b) and (e) show that, according to the osmoregulation hypothesis (i.e. if r_{eg}* is zero, meaning the $\psi_g = \psi_e$), π_g must decrease continuously and monotonically over time following a step increase in D to produce the observed dynamics of stomatal aperture. Because it is already well established that guard cell osmotic pressure can be actively and continuously regulated across a broad range, there is little reason to question the plausibility of the inferred π_g timecourses in Fig. 6(b, e). Furthermore, a continuous and monotonic decline of π_g during the approach to a new steady-state is easily explained by the kinetics of metabolically controlled solute uptake by guard cells, if the 'target' steady-state value of π_g is hypothesized to be controlled by direct feedback to either epidermal or mesophyll water potential (Grantz 1990; Haefner, Buckley & Mott 1997).

Figures 6(c) and (f) reveal an important and novel implication of this analysis: the 'pure' drawdown hypothesis, which requires that π_g play no role in the response to

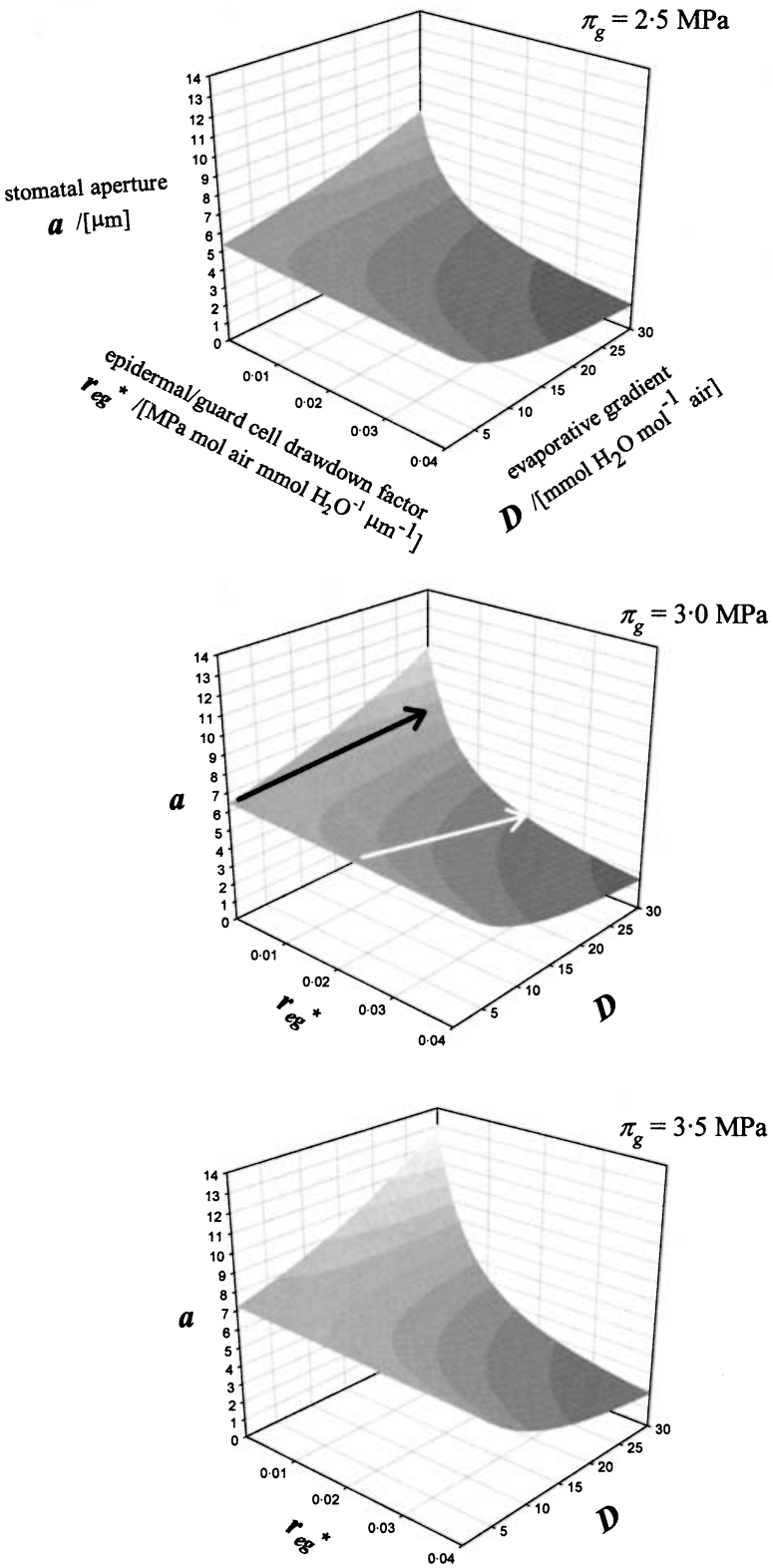

Figure 5. Model output showing the effect of the epidermal/guard cell drawdown factor (r_{eg}*, MPa μm^{-1}) and evaporative demand (D, mmol H_2O mol^{-1} air) on stomatal aperture (a, μm), for each of three values of guard cell osmotic pressure (π_g, MPa). Arrows superimposed on the surface for π_g = 3·0 MPa represent trajectories that are consistent (white) and inconsistent (black) with the observation that aperture declines with increasing evaporative demand.

humidity and therefore must remain constant, requires that r_{eg}* change substantially during the response if this hypothesis is to explain the observed transient dynamics of aperture. The nature and magnitude of the variation in r_{eg}* depend on the assumed value of π_g. (Since π_g was unknown in the plants for which timecourse data are presented, we imposed three values of π_g to infer numerical values of r_{eg}*.) Although it has never been proposed to our knowledge,

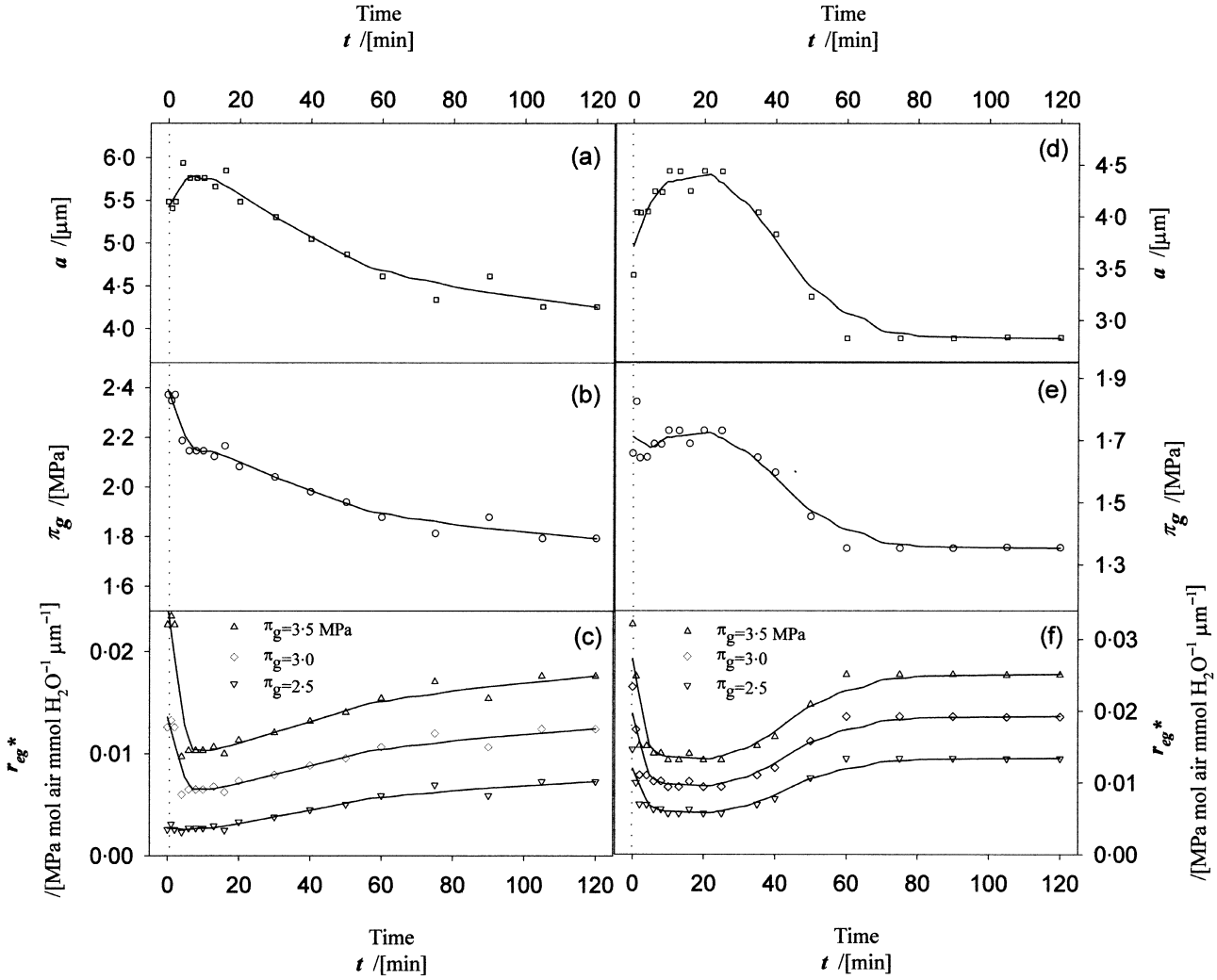

Figure 6. Timecourses, following a step increase in evaporative demand, observed for stomatal aperture (panels a and d) and inferred, using the model, from these aperture data for guard cell osmotic pressure (π_g, panels b and e) and the epidermal/guard cell drawdown factor (r_{eg}*, panels c and f).

regulation of r_{eg}* is not inherently implausible. Regulation of r_{eg}* could be effected by changes in r_{eg}, K or γ. The analysis of Tyree & Yianoulis (1980) suggests that K is relatively constant, and γ is a function of stomatal topology and density. However, at least one mechanism for regulating cell-to-cell hydraulic resistance is known to exist – aquaporins, which are potentially regulatable water and ion channels in cell membranes (Tyerman *et al.* 1999; Johansson *et al.* 2000). Aquaporins are known to exist in guard cells, but their function in these cells has not been ascertained.

CONCLUSION

This paper provides a theoretical analysis of two hypothetical mechanisms by which guard cell turgor pressure may be decoupled from epidermal turgor to overcome the epidermal mechanical advantage and produce a decline in stomatal aperture with decreasing humidity. These decoupling mechanisms are: (a) regulation of guard cell osmotic pressure, and (b) a water potential drawdown from epidermal to guard cells. The analysis has yielded novel insights concerning these two hypotheses. First, the drawdown hypothesis demands that one or more of the factors controlling the gradient in water potential between epidermal and guard cells must vary substantially as stomata respond to humidity. Such changes could, in principle, be effected by aquaporins in the guard cell membrane (Tyerman *et al.* 1999; Johansson *et al.* 2000). Second, the osmoregulation hypothesis predicts a continuous and monotonic change in guard cell osmotic pressure as stomata respond to humidity. Third, the drawdown hypothesis predicts no consistent relationship between the steady-state values of humidity and the putative drawdown-controlling factors, whereas the osmoregulation hypothesis predicts a monotonic steady-state relationship between guard-cell osmotic pressure and

humidity. Both predictions of the osmoregulation hypothesis are consistent with a feedback response of guard-cell osmotic pressure to epidermal water potential or turgor, which is in turn consistent with observed short-term responses of stomatal conductance to xylem cavitation and soil water potential (Buckley & Mott 2001). The predictions of the drawdown hypothesis, however, cannot be explained by a simple hydraulic feedback loop. Mathematical models of stomatal functioning that attempt to predict the stomatal response to humidity should incorporate the mathematical and empirical constraints on this response revealed by recent experiments (Franks *et al*. 1998, 2001) and by our analysis.

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Appendix

Empirical models for aperture and volume versus turgor pressures

Observations of stomata of *Tradescantia virginiana* by Franks *et al*. (1998) allow stomatal aperture (a, μm) to be estimated from empirical functions of epidermal and guard cell turgor pressure (P_e and P_g, respectively, MPa):

$$a = f_1(P_g) - \left(\frac{P_e}{\pi_e}\right)(f_1(P_g) - f_2(P_g)) \quad \text{(A1)}$$

The functions $f_1(P_g)$ and $f_2(P_g)$, which relate stomatal aperture to guard cell turgor at zero and maximum epidermal turgor, respectively (maximum epidermal turgor is considered equal to epidermal osmotic pressure, π_e, MPa) are given by Eqns A2 and A3:

$$f_1(P_g) = a_m[1 - \exp(-P_g/\zeta_1)] \cdot [1 + \exp(-P_g/\zeta_1)]^{-1} \quad \text{(A2)}$$

$$f_2(P_g) = (1-\xi)a_m\left(\left[1 + \exp\left(\left(\hat{P}_g - P_g\right)/\zeta_2\right)\right]^{-1} - \left[1 + \exp\left(\hat{P}_g/\zeta_2\right)\right]^{-1}\right) \quad \text{(A3)}$$

The parameters ζ_1 and ζ_2 (both MPa) control the curvature of a versus P_g; a_m is the maximum aperture (μm); ξ is the unitless proportion by which maximum stomatal aperture is reduced at full epidermal turgor (π_e, MPa) relative to zero epidermal turgor; $\hat{P}_g$ pis the value of guard cell turgor (MPa) at which the curvature of a versus P_g becomes negative at high epidermal turgor (when $P_e = \pi_e$); and the second term in parentheses in Eqn A3 ensures the pore is closed when both guard cell and epidermal turgor are zero. The data of Franks *et al*. (2001) allow guard cell volume (V_g, μm^3) to be inferred from P_g, using an empirical model of the form:

$$V_g = V_g(P_g) = c_1 P_g^2 + c_2 P_g + c_3 \quad \text{(A4)}$$

where the terms c_1, c_2 and c_3 are constants for a given guard cell pair. Values for all of the parameters in Eqns A1–A4 are given in Table 1, and our parameter estimation procedures are discussed below under the heading *Parameter estimation*. We assume the relationship between P_g and V_g is not affected by P_e; no data exist to test this hypothesis, which is applied here for simplicity. Note this is equivalent to specifying a value of unity for Cowan's (1977) variable σ.

Mathematical development

We assumed that the water potential of the epidermal cells (ψ_e, MPa) is in equilibrium with the water potential of other parallel evaporating sites (Nonami *et al*. 1990), and that this common water potential is determined by a balance between xylem supply (liquid-phase flow from a source at potential ψ_s [MPa] through a resistance r_{se} [MPa (mmol H_2O m_{leaf}^{-2} s^{-1})$^{-1}$]) and transpiration (vapour-phase flow at a rate E [mmol H_2O m_{leaf}^{-2} s^{-1}]) from inner surfaces of cells in the leaf. E is defined as the product of conductance (g, mol air m_{leaf}^{-2} s^{-1}) and the evaporative gradient (D, mmol H_2O mol^{-1}air; formally, the mole fraction gradient of water vapour from leaf to atmosphere). Stomatal conductance (g_s, mol air m_{leaf}^{-2} s^{-1}) is assumed to be proportional to aperture by a constant factor γ (mol air m_{leaf}^{-2} s^{-1} μm^{-1}), and the boundary layer resistance is considered zero for simplicity ($g = g_s$). Collectively, these assumptions (Eqn A5) imply that ψ_e is a decreasing linear function of the rate of water loss (γaD), with slope given by $-r_{se}$ (Eqn A6):

$$E = \frac{(\psi_s - \psi_e)}{r_{se}} = gD = \gamma aD \quad \text{(A5)}$$

$$\psi_e = \psi_s - r_{se}\gamma aD \quad \text{(A6)}$$

Equation A6 can also be expressed in terms of epidermal turgor pressure (P_e) and epidermal osmotic pressure (π_e, MPa, assumed constant in the simulations presented here), for the purposes of predicting water flow across isothermal phase boundaries:

Table 1. Parameter values

Parameter name	Symbol	Value
Maximum stomatal aperture	a_m	18·11 μm[a]
a versus P_g curvature parameter, low P_e	ζ_1	1·598 MPa[a]
a versus P_g curvature parameter, high P_e	ζ_2	0·7694 MPa[a]
Relative reduction in a_m by high P_e	ξ	0·509 (unitless)[a]
Inflection point in a versus P_g at high P_e	$\hat{P}_g$	2·015 MPa[a]
Polynomial constants for V_g versus P_g	c_1	–88·469 μm^3 MPa^{-2} [b]
Polynomial constants for V_g versus P_g	c_2	726·846 μm^3 MPa^{-1} [b]
Polynomial constants for V_g versus P_g	c_3	4813·476 μm^3[b]
Source water potential	ψ_s	0 MPa[a]
[source-to-epidermis resistance]·[aperture to conductance scaling factor]	$r_{se}\cdot\gamma$	0·001309 mol air $mmol^{-1}$ H_2O μm^{-1}[c]
Epidermal osmotic pressure	π_e	0·5252 MPa[c]
Gas constant	R	8·31441 MPa μm^3 $pmol^{-1}$ K^{-1}
Leaf temperature	T	298 K

Sources in bracketed superscripts to the right of numerical values: [a] estimated from the data of Franks *et al*. (1998) and [b] Franks *et al*. 2001); see *Parameter estimation* in the Appendix for details. [c] Measurements by the authors; see *Materials and Methods* for details.

$$P_e = \pi_e + \psi_s - r_{se}\gamma aD \tag{A7}$$

This equation emerges from standard water relations, given the assumptions listed above. An independent expression for P_e can be obtained by solving the empirical/mechanical equation (Eqn A1) of Franks *et al*. (1998) that relates aperture to P_e and P_g:

$$P_e = \pi_e \frac{(f_1 - a)}{(f_1 - f_2)} \tag{A8}$$

The existence of two independent expressions for P_e allows its elimination, removing one degree of freedom. We equate the right-hand sides of Eqns A7 and A8 and solve for aperture:

$$a = \frac{f_2(P_g) - f_1(P_g)\dfrac{\psi_s}{\pi_e}}{1 - (f_1(P_g) - f_2(P_g))\dfrac{r_{se}\gamma D}{\pi_e}} = a(P_g, D) \tag{A9}$$

Equation A9 shows that the theoretical hydraulic relationship between a, P_e and D (Eqn A7) reduces the empirical mechanical relationship between a, P_e and P_g (Eqn A1) to a semi-empirical hydromechanical function allowing a to be inferred from only P_g and D (Eqn A9); (P_g and D are considered the only dependent variables because all other terms in Eqn A9 are assumed constant). The hydraulics and mechanics of guard cells also represent an independent set of theoretical and empirical constraints on D, P_g and a, and link these variables to ψ_e, guard cell water potential (ψ_g, MPa), the hydraulic resistance between guard and epidermal cells (r_{eg}, MPa (mmol H_2O m^{-2} $s^{-1})^{-1}$), guard cell volume (V_g), and guard cell osmotic content (n_g, pmol). One fundamental theoretical relationship among these variables is the equality of ψ_g with $P_g - \pi_g$. A gradient/resistance model of water flow provides another constraint: at steady-state; any flow from epidermal to guard cells (F, mmol H_2O m_{leaf}^{-2} s^{-1}) is balanced by evaporation from guard cells, and is determined by r_{eg} and the difference between ψ_g and ψ_e. Defining the rate of evaporation from guard cells (E_g, mmol H_2O m_{leaf}^{-2} s^{-1}) as a fraction (K, unitless) of the total rate of transpiration, E, the steady-state flow through the guard cell evaporating site is given by Eqn A10:

$$F = E_g = KE = K\gamma aD = \frac{(\psi_e - \psi_g)}{r_{se}} \tag{A10}$$

The magnitude of K is a key factor distinguishing the two hypotheses under discussion in this study: the n_g-regulation hypothesis does not require any drawdown between ψ_e and ψ_g, so K can be assumed to equal zero, but the r_{eg}-regulation hypothesis does require a drawdown, and therefore positive F and K. However, for the purposes of this study, neither K nor the aperture/conductance scaling factor, γ, need to be considered in isolation from r_{eg}, because these three terms only appear as a product ($K{\cdot}r_{eg}{\cdot}\gamma$) in the critical equations. In solving Eqn A10 for guard cell water potential or turgor pressure, the product of K, r_{eg} and γ can thus be replaced by a single variable, r_{eg}*:

$$\begin{aligned}\psi_g &= \psi_e - r_{eg}K\gamma aD = \psi_s - r_{se}\gamma aD - r_{eg}K\gamma aD \\ &= \psi_s - r_{se}\gamma aD - r_{eg}{}^*aD\end{aligned} \tag{A11}$$

Applying Eqn A9–A11 and rearranging yields:

$$a = \frac{\psi_s + \pi_g - P_g}{r_{eg}\gamma D + r_{eg}{}^*D} \tag{A12}$$

It is more illuminating in the present context to express π_g in terms of guard cell osmotic content, n_g, because, whereas π_g is influenced by a suite of hydraulic factors, n_g is directly controlled by metabolic processes. Recognizing that V_g can be inferred from P_g using the data of Franks *et al*. (2001) (Eqn A4), and that π_g is a simple function of V_g and n_g in ideal dilute solutions, π_g can be expressed as a function of n_g and P_g (Eqn A13):

$$\pi_g = \frac{n_g RT}{V_g(P_g)} = \pi_g(n_g, P_g) \tag{A13}$$

Equation A13 allows π_g to be replaced by n_g in Eqn A12, yielding Eqn. A12b:

$$\begin{aligned}a &= \frac{\psi_s + \left(\dfrac{n_g RT}{c_1 P_g^2 + c_2 P_g + c_3}\right) - P_g}{r_{se}\gamma D + r_{eg}{}^*D} \\ &= a(\psi_s, P_g, D, r_{se}\gamma, r_{eg}{}^*, n_g)\end{aligned} \tag{A12b}$$

We now have seven variables (a, ψ_s, P_g, D, r_{se}, r_{eg}*, and n_g) and two constraints among these variables (Eqns A9 and A12b), leaving five degrees of freedom. To perform the semi-empirical simulations presented in this study, we closed the system by using adding four empirical constraints (measurements of *a, D*, and ψ_s, and estimates of $r_{se} \cdot \gamma$, in intact plants; details are provided in the *Materials and Methods* section in the main text) and one hypothetical constraint, the latter representing either the n_g- or r_{eg}-regulation mechanism for the humidity response. To represent the guard cell osmotic regulation hypothesis, r_{eg}* was set equal to zero, and to represent the drawdown hypothesis, π_g was held constant at each of three values (2·5, 3·0 and 3·5 MPa). (These choices of hypothetical constraints are explained in the next section of the Appendix, where the mathematical criteria for each hypothesis are developed.) The remaining variable (n_g or r_{eg}*, respectively) was then determined by iterative solution of Eqns A9 and A12b, as described below, and plotted against time and other system variables to show the inferred behaviour of the hypothetical control variable during the stomatal response to humidity.

Care is needed in implementing Eqns A9 and A12b in a numerical simulation, because, although P_e is not explicitly constrained to be non-negative in the preceding derivation, P_e remains implicit in Eqns A9 and A12b. In conditions where plasmolysis occurs, P_e will implicitly become negative unless it is explicitly calculated by the computer code and constrained to be non-negative; note that in this case, epidermal osmotic pressure must also implicitly be allowed to increase with further decreases in epidermal water potential. However, when P_e is zero, Eqn A9 may be

replaced by Eqn A2, so the code never needs to change π_e explicitly.

Derivation of the mathematical criteria for the mechanism of the feedback humidity response

Stomatal aperture declines as the evaporative demand increases. Under most conditions, this also causes transpiration rate (E) to increase with D, consistent with evidence that stomata respond directly to the rate of water loss from leaves (Mott & Parkhurst 1991). This is often described as a feedback response of g to E, or of E to D, which contrasts with occasional reports of an apparent 'feedforward' response in which E declines with increasing D at high values of D (Farquhar 1978; Monteith 1995; Franks *et al.* 1997). In the following analysis, we consider only the 'feedback' domain of the response, because the mathematical criteria for producing a decline of aperture with increasing E are much simpler.

To produce the observed steady-state and transient 'wrong-way' responses of stomatal aperture to humidity, we must express these responses mathematically, in the context of the epidermal mechanical advantage. Formally, changes in epidermal turgor pressure must be overcompensated by changes in guard cell turgor to cause a decline in aperture with transpiration rate. The magnitude of this overcompensation is given by the ratio of the sensitivities of aperture to epidermal and guard cell turgors:

$$a = a(P_g, P_e) \Rightarrow da = \frac{\partial a}{\partial P_g} dP_g + \frac{\partial a}{\partial P_e} dP_e \quad \text{(A14)}$$

$$m \equiv -\frac{\partial a}{\partial P_e} \Big/ \frac{\partial a}{\partial P_g} = m(P_g, P_e) \quad \text{(A15)}$$

Note that the mechanical advantage, formally defined by Eqn A15, is highly sensitive to both P_g and P_e (Fig. 1b). From Eqns A14 and A15, the total dependence of aperture on transpiration rate, E, can be expressed in differential form as:

$$\frac{da}{dE} = \frac{\partial a}{\partial P_g}\left(\frac{dP_g}{dE} - m\frac{dP_e}{dE}\right) \quad \text{(A16)}$$

Noting that $E = \gamma aD$, with γ assumed constant in the present study, we can rewrite Eqn A16 as:

$$\frac{da}{d[aD]} = \frac{\partial a}{\partial P_g}\left(\frac{dP_g}{d[aD]} - m\frac{dP_e}{d[aD]}\right) \quad \text{(A16b)}$$

This transformation will clarify later steps in the derivation, by preserving the linkage of K, r_{eg} and γ in the variable r_{eg}*, and of r_{se} and γ as a product (which was estimated as a single parameter; see *Materials and Methods*). Therefore, since $\partial a/\partial P_g$ is always positive (Sharpe *et al.* 1987; Franks *et al.* 1998) in Fig. 1a, the parenthetical term in Eqn. A16b must be negative if aperture is to decline with increasing transpiration rate:

$$\frac{da}{d[aD]} < 0 \quad implies \quad \frac{dP_g}{d[aD]} < m\frac{dP_e}{d[aD]} \quad \text{(A17)}$$

The derivatives in Eqn. A17 are obtained by differentiating Eqns A11 and A7:

$$\frac{dP_g}{d[aD]} = \frac{d\psi_g}{d[aD]} + \frac{d\pi_g}{d[aD]} = -\left(r_{se}\gamma + \frac{d[r_{se}\gamma]}{d[\ln aD]}\right) - \left(r_{eg}^* + \frac{dr_{eg}^*}{d[\ln aD]}\right) + \frac{d\pi_g}{d[aD]} \quad \text{(A18)}$$

$$\frac{dP_e}{d[aD]} = \frac{d\psi_e}{d[aD]} + \frac{d\pi_e}{d[aD]} = \frac{d\psi_e}{d[aD]} = -r_{se}\gamma - \frac{d[r_{se}\gamma]}{d[\ln aD]} \quad \text{(A19)}$$

By combining Eqns A17, A18 and A19, we identify the following general criterion:

$$\left[-\frac{d\pi_g}{d[aD]} + \left(r_{eg}^* + \frac{dr_{eg}^*}{d[\ln aD]}\right)\right] \Big/ \left(r_{se}\gamma + \frac{d[r_{se}\gamma]}{d[\ln aD]}\right) > (m-1) \quad \text{(A20)}$$

Equation A20 states that, in order for guard cell turgor to overcome the residual mechanical advantage ($m - 1$), at least one of the following two quantities must always be sufficiently large: (i) a metabolically controlled decline in guard cell osmotic pressure ($-d\pi_g/d[aD]$), or (ii) an epidermal-guard cell drawdown factor (r_{eg}*) and its sensitivity to relative changes in transpiration rate (dr_{eg}*/$d[\ln aD]$). These requirements are weighted inversely by the hydraulic supply resistance to the epidermis and its sensitivity to normalized transpiration (the denominator on the left side of Eqn A20).

Equation A20 can be greatly simplified under conditions where transpiration does not substantially alter hydraulic supply, either by causing source water potential (ψ_s) to decrease or by drawing down leaf water potential enough to cause cavitation in the hydraulic supply pathway to the epidermis, decreasing r_{se}. If ψ_s and r_{se} are constant, then from Eqn A5,

$$\frac{dE}{d\psi_e} = -\frac{1}{r_{se}} = \frac{dE}{dP_e} \Rightarrow \left(-r_{se}\frac{dE}{dP_e}\right) = \left(-r_{se}\gamma\frac{d[aD]}{dP_e}\right) = 1 \quad \text{(A21)}$$

The parenthetical quantity in Eqn A21 can be multiplied by the first term in Eqn A20 to give

$$-\frac{d\pi_g}{d[aD]} = r_{se}\gamma\frac{d\pi_g}{dP_e} \quad \text{(A22)}$$

Invariance of r_{se} also eliminates the second term in the denominator of Eqn A20, which, together with Eqn A22, simplifies Eqn A20 to

$$\frac{d\pi_g}{dP_e} + \left(r_{eg}^* + \frac{dr_{eg}^*}{d[\ln E]}\right) \Big/ [r_{se}\gamma] > (m-1) \quad \text{(A20b)}$$

The hypothesis that the humidity response is due solely to regulation of guard cell osmotic pressure, π_g (with no need for a water potential gradient between epidermal and guard

cell) is best represented by the assumption that r_{eg}* is zero, which can be interpreted in two ways: no evaporation occurs from guard cells ($K = 0$) or the resistance between epidermal and guard cells is negligible ($r_{eg} = 0$). This hypothesis reduces the criterion to Eqn. A20c, which states that changes in guard cell osmotic pressure must outpace changes in epidermal turgor by a factor equal to the residual mechanical advantage ($m - 1$):

$$\frac{d\pi_g}{dP_e} > (m-1) \qquad \text{(A20c)}$$

The alternative hypothesis, that the humidity response is due solely to non-zero r_{eg} (with no assistance from active regulation of guard cell osmotic pressure), is best represented by the assumption that π_g is constant ($d\pi_g = 0$). In this case, the criterion reduces to Eqn. A20d,

$$\left(r_{eg}^* + \frac{dr_{eg}^*}{d[\ln aD]}\right) \Big/ [r_{se}\gamma] > (m-1) \qquad \text{(A20d)}$$

which states that the sum of the epidermal-guard cell hydraulic drawdown factor (r_{eg}*) and its sensitivity to relative changes in transpiration rate, both expressed relative to the source-to-epidermis hydraulic resistance factor ($r_{se} \cdot \gamma$), must exceed the residual mechanical advantage.

Iterative solution procedure

For the model output shown in Figs 4 and 5, the system was solved by specifying values for D and either n_g (Fig. 4) or r_{eg}*(Fig. 5), and adjusting P_g incrementally until the ratio of values for ψ_e specified by two independent functions (Eqns A9 and A12b) was within 10^{-4} of unity. If an increment in P_g overshot the target value, the increment was reversed and the step size cut in half. To infer π_g from empirical measurements of aperture (Fig. 6), P_e was determined from Eqn A8 using the measured values for a and D, and P_g was incremented as described above until the estimate of aperture given by Eqn A12b agreed with the empirically measured aperture with a rational precision of 10^{-4}. Hypothetical dynamics of r_{eg}* were inferred from aperture data (Fig. 6) by the same method used to infer n_g, except that Eqn A12 was used instead of Eqn. A12b, and a constant value of π_g (2·5, 3·0 or 3·5 MPa) was imposed.

Parameter estimation for Eqs A1–A4

Franks *et al*. (1998) did not present data relating a and P_g at low P_e for *V. faba*, so we estimated this relationship from other data given in their paper. First, we fit Eqn A3 to the a versus P_g data given for high P_e (Fig. 1 from Franks *et al*. 1998) using the 'solver' feature in Microsoft Excel to identify values for $\hat{P}_g$, ζ_2, and $[(1 - \xi)a_m]$. Second, we assumed that the ratio $[(1 - \xi)$ in Eqn A3] of maximum apertures (a_m) at high and low P_e (0·491 for *T. virginiana* in Franks *et al*. 1998), and the *ratio* of the curvature parameters (*dx* in Franks *et al*. 1998; ζ_2 and ζ_1 in Eqns. A2 and A3) at high and low P_e (0·30 for *T. virginiana* in Franks *et al*. 1998) were the same for *V. faba* as for *T. virginiana*.

Guard cell pressure versus volume data were presented by Franks *et al*. (2001) for three guard cells of *V. faba*. We chose the data set with the highest resolution in P_g, doubled all volumes so that the model described in this paper represents a pair of guard cells, and fit a second-order polynomial ($r^2 = 0{\cdot}992$) to those data. These parameters are given in Table 1.