

# Dynamics of stomatal water relations following leaf excision

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## ABSTRACT

We examined the stomatal response to leaf excision in an evergreen woody shrub, *Photinia × fraseri*, using a novel combination of gas exchange, traditional water relations and modelling. Plants were kept outdoors in mild winter conditions (average daily temperature range:  $-1$  to  $12$  °C) before being transferred to a glasshouse (temperature range:  $20$ – $30$  °C) and allowed to acclimate for different periods before experiments. ‘Glasshouse plants’ were acclimated for at least 9 d, and ‘outdoor plants’ were acclimated for fewer than 3 d before laboratory gas exchange experiments. The transient stomatal opening response to leaf excision was roughly twice as long in outdoor plants as in glasshouse plants. To elucidate the reason for this difference, we inferred variables of stomatal water relations (epidermal and guard cell turgor pressures and guard cell osmotic pressure:  $P_e$ ,  $P_g$  and  $\pi_g$ , respectively) from stomatal conductance ( $g_s$ ) and bulk leaf water potential ( $\psi_l$ ), using a hydromechanical model of  $g_s$ .  $\psi_l$  was calculated from cumulative post-excision transpirational water loss using empirical relationships between  $\psi_l$  and relative water content obtained on similar leaves. Inferred  $P_g$  and  $P_e$  both declined immediately after leaf excision. Inferred  $\pi_g$  also declined after a lag period. The kinetics of  $\pi_g$  adjustment after the lag were similar in outdoors and glasshouse plants, but the lag period was much longer in outdoor plants. This suggests that the longer transient opening response in outdoor plants resulted from slower induction, not slower execution, of guard cell osmoregulation. We discuss the implications of our results for the mechanism of short-term stomatal responses to hydraulic perturbations, for dynamic modelling of  $g_s$  and for leaf water status regulation.

**Key-words:** epidermal cell; guard cell; stomata; transpiration; turgor pressure.

## INTRODUCTION

The control of gas exchange by leaf stomata has broad implications for the response of terrestrial vegetation to

changes in environmental conditions, including global climate change (Hetherington & Woodward 2003). It is desirable to produce robust models of stomatal behaviour, ideally based on conserved physico-chemical mechanisms operating in and around stomatal guard cells. Great progress has been made in recent years in elucidating the signal transduction pathways by which guard cells respond to changes in light intensity, CO<sub>2</sub> concentration and a variety of compounds such as abscisic acid (Assmann & Shimazaki 1999; McAinsh *et al.* 2000; Assmann & Wang 2001; Hetherington 2001; Schroeder, Kwak & Allen 2001; Zeiger *et al.* 2002; Dodd 2003; Hetherington & Woodward 2003; Vavasseur & Raghavendra 2005). Stomata also respond to short-term changes in hydraulic variables such as humidity (Cowan & Farquhar 1977; Mott & Parkhurst 1991; Cowan 1994; Monteith 1995; Oren *et al.* 1999), xylem hydraulic conductance (Saliendra, Sperry & Comstock 1995; Cochard *et al.* 2002; Brodribb & Holbrook 2003; Brodribb & Holbrook 2004) and soil water status (Raschke 1970; Fuchs & Livingston 1996; Comstock & Mencuccini 1998). However, there is still no consensus regarding the identity of the proximal effector(s) involved in stomatal responses to hydraulic perturbations, nor regarding the biophysical mechanisms by which those effectors induce changes in stomatal conductance ( $g_s$ ; see Table 1 for a list of symbols and units) (Buckley & Mott 2002b; Meinzer 2002; Franks 2004; Buckley 2005).

Some observations suggest that  $g_s$  is regulated by negative feedback from leaf water status. It is clear, for example, that  $g_s$  tends to respond to variations in hydraulic supply and demand in a way that reduces the consequent change in bulk leaf water potential ( $\psi_l$ ):  $g_s$  declines to a new steady-state value when atmospheric humidity, soil water status or xylem hydraulic conductance are reduced. However, additional assumptions are required to make the  $\psi_l$ – $g_s$  feedback hypothesis consistent with what is known about stomatal hydromechanics. Stomatal aperture is determined not only by guard cell turgor pressure ( $P_g$ ), which increases aperture, but also by epidermal turgor pressure ( $P_e$ ), which reduces aperture. The effect of  $P_e$  is greater, so aperture increases if  $P_g$  and  $P_e$  decline by similar amounts (Sharpe, Wu & Spence 1987; Franks, Cowan & Farquhar 1998). The passive effect of water status on  $g_s$  therefore produces positive, not negative, feedback. To produce negative feedback,  $P_g$  must be made more sensitive than  $P_e$  to hydraulic perturbations.

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**Table 1.** Symbols used in this paper

Variable	Symbol	Units
Stomatal conductance (at excision, at peak of transient response)	$g_s$ ( $g_x, g_p$ )	$\text{mol m}^{-2} \text{s}^{-1}$
Transpiration rate	$E$	$\text{mol m}^{-2} \text{s}^{-1}$
Turgor pressure: epidermal guard cell	$P_e, P_g$	MPa
Osmotic pressure: guard cell, epidermal, saturated bulk leaf	$\pi_g, \pi_e, \pi_s$	MPa
Bulk leaf water potential (at excision, pre-dawn)	$\psi_l$ ( $\psi_s, \psi_{pd}$ )	MPa
Turgor-conductance scaling factor	$\chi$	$\text{mol m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$
Net epidermal mechanical advantage	$M$	–
Leaf relative water content	RWC	–
Leaf water content (at saturation, at excision)	$Q$ ( $Q_s, Q_x$ )	mol
Fresh leaf weight (at saturation)	$FW$ ( $FW_s$ )	g
Dry leaf weight	$DW$	g
Pressure-chamber balance pressure reading	$P_b$	MPa

Two alternative hypotheses purport to explain how this happens. One holds that water lost directly from guard cells is replaced by flow through a large hydraulic resistance from epidermal cells to guard cells, which increases the passive sensitivity of  $P_g$  to changes in evaporative demand (Farquhar 1978; Maier-Maercker 1983; Dewar 1995; Dewar 2002; Eamus & Shanahan 2002). However, such a resistance would not cause  $P_g$  and  $P_e$  to differ in their sensitivities to perturbations upstream of the epidermis, such as changes in xylem hydraulic conductance or soil pressurization. The other hypothesis holds that epidermal water status is sensed by guard cells (via an unknown signal transduction process), which modulate their osmotic pressure ( $\pi_g$ ) in order to make steady-state  $\pi_g$  proportional to epidermal water status (Darwin 1898; Darwin & Pertz 1911; Stålfelt 1929; Meidner 1986; Buckley, Mott & Farquhar 2003). Tracking of water status by  $\pi_g$  would amplify the response of  $P_g$  to any change in hydraulic supply and demand in the epidermis.

It is not straightforward how best to evaluate these hypotheses and to work out their subtleties, because most of the variables of stomatal water relations are difficult to measure directly and to control independently by experiment. However, some insight may be gained by studying the transient ‘wrong-way’ response (WWR) that typically precedes, and is in the opposite direction to the steady-state response to hydraulic perturbations. WWRs often occur when any part of the soil–plant–atmosphere hydraulic flow continuum is perturbed, including changes in atmospheric humidity (Cowan & Farquhar 1977; Kappen, Andresen & Losch 1987; Grantz 1990), atmospheric pressure around the root system (Comstock & Mencuccini 1998) and transpiration rate ( $E$ ) elsewhere in the same leaf or plant (Mott, Denne & Powell 1997; Buckley & Mott 2000), and in response to leaf excision (Darwin 1898; Iwanoff 1928; Rufelt 1963; Raschke 1970). The WWR is useful for studying the kinetics of guard cell osmoregulation because differences in the kinetic behaviours of water status and  $\pi_g$  temporarily decouple  $g_s$  from  $\pi_g$ .

In the present study, we examined the stomatal response to leaf excision in an evergreen woody shrub,

*Photinia × fraseri*. Preliminary work revealed a fortuitous discovery: that the duration of the WWR of  $g_s$  to leaf excision was greater in plants that were kept outdoors in mild winter conditions than in plants that were kept in a glass-house. The objective of this study was to determine the cause of these differences by characterizing the response kinetics of various stomatal water relations variables following leaf excision in these two groups of plants. To achieve this objective, we modified the protocol of Brodribb & Holbrook (2004), who estimated  $\psi_l$  over time after leaf excision, by applying pressure–volume curves to measurements of cumulative post-excision water loss (assessed by repeated weighing). In our version of the protocol, both water loss and  $g_s$  were measured concurrently and with high temporal resolution (15 s) during the excision response, by enclosing leaves in a gas exchange chamber. The inference of  $\psi_l$  from water loss was validated by measuring  $\psi_l$  directly on leaves removed from the chamber at various times after excision. We applied time courses of  $g_s$  and  $\psi_l$  to a simple model for  $g_s$  to infer the dynamics of  $P_g$ ,  $P_e$  and  $\pi_g$  during the excision response.

## MATERIALS AND METHODS

### Plant material

All experiments used the woody evergreen shrub *P. × fraseri* (common names *Photinia* ‘red tip’, ‘red robin’, or ‘superhedge’, a hybrid cross between *P. glabra* and *P. serrulata*, in the family Rosaceae). This species was chosen primarily because of its sturdy and long petioles, which facilitated the excision protocol and allowed for repeated pressure-chamber measurements. (*Vicia faba* L. seemed a logical choice initially, because parameters of stomatal hydromechanics have been estimated for that species, but its soft petioles proved not to be conducive to repeated pressure-chamber measurements.) *P. × fraseri* has alternate, finely serrated leaves and is characterized by flushes of new crimson foliage which turn deep green within a few weeks. Mature leaves are glossy, robust and hypostomatous. Eighteen-month-old plants were bought from a local

nursery, kept in a semishaded outdoors location (7–17 °C day; –7 to 7 °C night) on the campus of the Australian National University, Canberra, between June and August 2004, and watered daily. Some plants were subsequently transferred into a glasshouse [daytime: 30 °C and 70% relative humidity (RH); night: 20 °C and 90% RH]. Plants used for experiments were grouped according to how long they had acclimated in the glasshouse before measurement: ‘outdoor plants’ were kept in the glasshouse three or fewer days before the experiments, and ‘glasshouse plants’ were kept in the glasshouse for at least 9 d before the experiments.

### Leaf gas exchange

The experiments were performed using a laboratory-based open-flow gas exchange system described previously (e.g. Boyer, Wong & Farquhar 1997; Barbour *et al.* 2000). A single leaf was enclosed in a 22 cm × 18 cm chamber with a glass lid; air was stirred by a tangential fan to give a boundary layer conductance to water vapour of 5 mol m<sup>-2</sup> s<sup>-1</sup>, and leaf temperature was held at 24 °C by circulating water from a water bath through a jacket under the chamber. Leaf temperature was measured with two copper–constantan thermocouples pressed against the lower surface of the leaf. Irradiance was provided by a metal-halide lamp. Compressed air was passed through soda lime columns to remove CO<sub>2</sub>, bubbled through a humidifier to saturate it with water vapour, passed through a temperature-controlled condenser to regulate inlet humidity, mixed with 12% CO<sub>2</sub> in air using mass flow controllers, and passed through the chamber at a flow rate of 4 L min<sup>-1</sup>, monitored by a mass flowmeter (Brooks, Hatfield, PA, USA). Inlet CO<sub>2</sub> concentration was kept near ambient (≈ 0.37 mg g<sup>-1</sup>). The CO<sub>2</sub> partial pressures of incoming and outgoing air were measured with an infra-red gas analyser (IRGA; LI-6251; Li-Cor, Lincoln, NE, USA) operated in absolute mode and calibrated daily.

Previous experiments using this system measured vapour pressures of the incoming and outgoing air with an IRGA and alternated between measurements of incoming and outgoing air every 140 s. To permit data collection at shorter intervals, we measured vapour pressure with a Vaisala integrated RH and air temperature sensor (Humitter 50Y; Vaisala, Helsinki, Finland) and switched from the alternating mode described above to ‘continuous’ mode (recording only the outgoing stream, but every 15 s) 30 min before excision. Subsequent calculations assumed the composition of incoming gas was constant; we checked for drift in the incoming stream by periodically switching back to the alternating mode. CO<sub>2</sub> assimilation rate, transpiration rate ( $E$ ) and  $g_s$  were calculated from expressions given by von Caemmerer & Farquhar (1981).

Sample plants were brought to the laboratory on the afternoon prior to an experiment and kept well watered overnight. In the morning, a fully expanded, mature leaf from the 5th or 6th rank below the apex was sealed in the gas exchange chamber. Leaf-to-air vapour pressure differ-

ence (VPD) was set at 1–2 kPa (held constant during each experiment), and irradiance was increased in steps to 1000 μE m<sup>-2</sup> s<sup>-1</sup> between 0800 and 1000 h using neutral density filters. When gas exchange had reached steady-state, the petiole was excised close to the chamber, and the cut end was covered with parafilm. Gas exchange was measured continuously until stomatal closure occurred or until the leaf was removed to measure water potential (to validate the water potential inference method discussed below).

### Magnitude and duration of transient opening response

To quantify aspects of the initial transient opening response of stomata to leaf excision, we first reduced high-frequency noise in the  $g_s$  signal, using Gaussian smoothing in a moving 180 s window and a decay constant of 0.1 s<sup>-2</sup>, and then estimated the following parameters from the smoothed data: steady-state  $g_s$  before excision ( $g_x$ );  $g_s$  at the peak of the transient response ( $g_p$ ); relative and absolute magnitude of the transient response [ $g_p - g_x$  and  $(g_p - g_x)/g_x$ ]; and duration of the transient response (time that  $g_s > g_x$  after excision). These parameters are illustrated in Fig. 1a.

### Theory

We inferred the dynamics of stomatal water relations variables after leaf excision, using a theoretical framework based on several assumptions. First,  $\psi_l$  is an empirical function of leaf relative water content (RWC):

$$\psi_l = f(\text{RWC}). \quad (1)$$

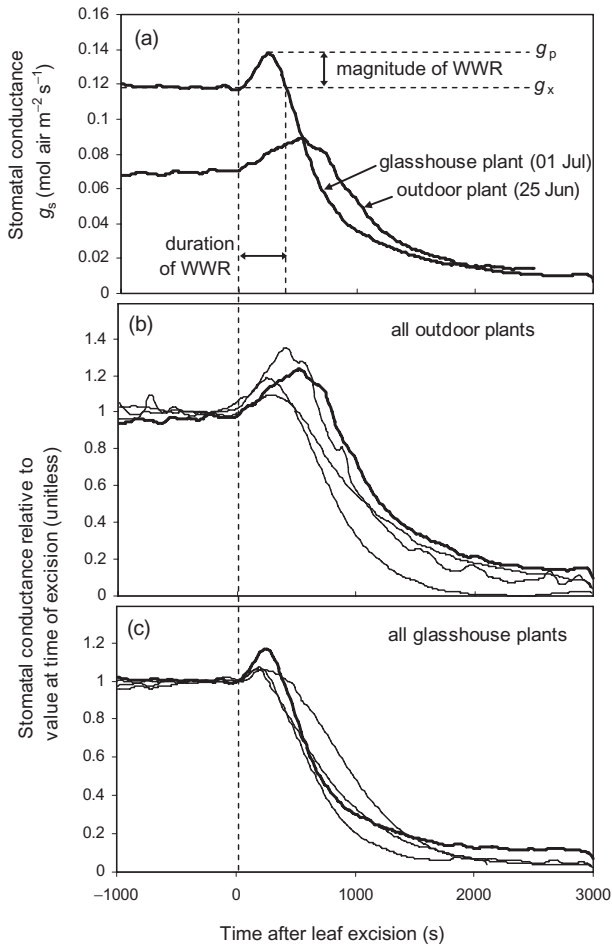
The function  $f$  is often taken as a composite of two lines, one of which applies above the point of turgor loss and the other below, and whose slopes are termed ‘capacitances’. In this study, we were less concerned with estimating capacitance values than with maximizing the empirical accuracy of the function  $f$ , so we used polynomial regressions instead. RWC is the ratio of leaf water content ( $Q$ ) to saturated leaf water content ( $Q_s$ ). When a leaf is excised in air,  $Q$  changes at a rate equal to  $E$ :  $dQ/dt = -E(t)$ , so  $Q(t)$  equals the water content at excision ( $Q_x$ ) minus the integral of  $E$  since excision:

$$Q(t) = Q_x - \int_0^t E(\tilde{t}) d\tilde{t} \quad (2)$$

Equations 1 and 2 permit  $\psi_l$  to be estimated from  $E$ , which is measured by gas exchange. A third assumption was used to infer  $\pi_g$  from  $g_s$ :  $g_s$  was assumed to be a linear combination of  $P_g$  and  $P_e$ , floored at zero (e.g. Sharpe *et al.* 1987):

$$g_s = \max\{\chi[P_g - (M + 1)P_e], 0\}, \quad (3)$$

where  $M$  is the net mechanical advantage of the epidermis ( $M + 1$  is conventionally denoted  $m$  and simply called the mechanical advantage),  $\chi$  is a turgor-conductance scaling factor, and  $P_g$  and  $P_e$  are related to  $\pi_g$ ,  $\psi_l$  and  $\pi_e$  by standard expressions of water relations:



**Figure 1.** Measured time courses of stomatal conductance to water vapour ( $g_s$ ) after leaf excision, which occurred at time zero. (a) Two sample traces of absolute stomatal conductance: 25 June (an outdoor plant) and 01 July (a glasshouse plant). (b) All traces for outdoor plants, expressed relative to stomatal conductance at excision ( $g_x$ ). (c) All traces for glasshouse plants, expressed relative to  $g_s$  at excision. In (b) and (c), the time courses from 25 June and 01 July are shown in bold.

$$P_c = \max(\psi_l + \pi_c, 0), \quad (4)$$

$$P_g = \max(\psi_l + \pi_g, 0). \quad (5)$$

Equation 4 assumes that the capacitor tissues represented by  $\psi_l$  are in close hydraulic contact with the epidermis (so that  $\psi_c$  is in quasi-static equilibrium with  $\psi_l$ ). Similarly, Eqn 5 assumes that guard and epidermal cells are in quasi-static equilibrium with one another.

### Inference of stomatal water relations

To infer the dynamics of  $\pi_g$  during the excision response, we applied Eqns 1–5 to gas exchange measurements of  $E$  and  $g_s$  as follows. Firstly,  $\psi_l$  was estimated from  $E$  using Eqns 1–2. Secondly,  $P_c$  was inferred from  $\psi_l$  using Eqn 4, in conjunction with a value for  $\pi_c$  estimated from the pressure–volume analysis (discussed below). Thirdly,  $P_g$  was estimated from  $g_s$  using Eqn 3, in conjunction with inferred

values of  $P_c$ , an estimate for the quantity  $\chi M$  (discussed below), and a guess for the value of  $M$  (0.5, also discussed below). Finally,  $\pi_g$  was calculated from Eqn 5 using estimated values of  $P_g$  and  $\psi_l$ .

The procedure described above requires estimates for several parameters:  $\chi$ ,  $M$ ,  $\pi_c$ ,  $Q_s$ ,  $Q_x$ , the polynomial coefficients in Eqn 1 and the value of  $\psi_l$  at leaf excision ( $\psi_x$ ). Estimation of  $Q_s$ ,  $Q_x$ , the polynomial coefficients, and  $\psi_x$  is discussed below under pressure–volume analysis. We assumed that  $\pi_c$  is similar to bulk leaf osmotic pressure at saturation ( $\pi_c \approx \pi_s$ ). One constraint on the remaining parameters,  $\chi$  and  $M$ , may be found by combining Eqns 3–5 to give

$$g_s = \chi[-M\psi_l + \pi_g - (M+1)\pi_c], \quad (6)$$

where  $P_c$  and  $P_g$  are understood to be non-negative. Immediately after excision, a decline in  $\psi_l$  and an increase in  $g_s$  occur in concert before  $\pi_g$  begins to change significantly. Hence, the product  $\chi M$  may be estimated from the initial slope of a phase plot of  $g_s$  versus  $\psi_l$  after excision:

$$(\partial g_s / \partial \psi_l)_i = -\chi M, \quad (7)$$

where the subscript ‘i’ denotes ‘initial’. We supplied the final constraint by making an arbitrary guess for the value of  $M$ . Most estimates of  $M$  in the literature are between 0.2 and 1.1, averaging around 0.6 (Meidner & Edwards 1975; Cooke *et al.* 1976; Edwards, Meidner & Sheriff 1976; Meidner & Bannister 1979; Buckley *et al.* 2003). Within this range, large  $M$  leads to large inferred  $P_g$  values (e.g. for  $M = 1.0$ ,  $P_g$  inferred from our data is as large as 12.2 MPa). We therefore used a low value within this range ( $M = 0.5$ ) for the simulations presented in Table 2 and Figs 2 and 3, and we quantified the sensitivity of our results to uncertainty in the value of  $M$  by repeating the simulations at different  $M$ -values between 0.3 and 1.0 (Table 3).

Kinetic properties of  $\pi_g$  adjustment were calculated from inferred  $\pi_g$  time courses after Gaussian smoothing with a 220 s window and a decay constant of 0.01 s<sup>-2</sup>. Initial and final values of  $\pi_g$  were calculated as the average of smoothed inferred values for 5 min before leaf excision and for 5 min prior to the end of the experiment, respectively.

### Validation

The  $\psi_l$  inference method was validated against direct psychrometer measurements of  $\psi_l$  in eight experiments (three outdoor and five glasshouse plants), each terminated at a different time after excision. In each case, three leaf disks were taken from the sample leaf and equilibrated in separate psychrometer chambers for 16 h before measurement. (Psychrometer measurements are described below.) The method for inferring stomatal water relations parameters ( $P_c$ ,  $P_g$  and  $\pi_g$ ) contains two major assumptions that we could not directly validate: that  $\psi_l$  and  $\pi_s$  are representative of epidermal water potential and osmotic pressure, respectively, and that guard and epidermal cells are in quasi-static hydraulic equilibrium. These assumptions are evaluated in the Discussion.

**Table 2.** Comparison of physiological measurements, properties of the wrong-way stomatal response to leaf excision (WWR), and inferred kinetic properties of guard cell osmotic pressure ( $\pi_g$ ) adjustment following leaf excision (assuming the net mechanical advantage,  $M$ , equals 0.5), for outdoor plants and glasshouse plants

Variable	Symbol	Units	Outdoor plants	Glasshouse plants	$P$ -value (notes)
Bulk leaf osmotic pressure at saturation	$\pi_s$	MPa	1.00 ± 0.12	1.47 ± 0.12	2.4·10 <sup>-7</sup> (a,c)
Stomatal conductance at excision	$g_x$	mol m <sup>-2</sup> s <sup>-1</sup>	0.092 ± 0.022	0.161 ± 0.041	0.0016 (b,d)
Pre-dawn bulk leaf water potential	$\psi_{pd}$	MPa	-0.231 ± 0.037	-0.196 ± 0.029	0.093 (b,c)
Duration of 'wrong-way response' (WWR)	–	min	10.7 ± 1.4	5.4 ± 1.2	2.7·10 <sup>-6</sup> (a,d)
Relative size of WWR	–	%	19 ± 10	7.9 ± 6.5	0.033 (b,d)
Absolute size of WWR	–	mol m <sup>-2</sup> s <sup>-1</sup>	0.017 ± 0.008	0.011 ± 0.007	0.21 (a,d)
Time to 25% of $\pi_g$ decrease	$t_{25}$	min	15.7 ± 3.2	8.2 ± 2.3	0.012 (b,e)
Time from 25 to 75% of $\pi_g$ decrease	$t_{(25-75)}$	min	8.1 ± 2.4	8.7 ± 2.4	0.74 (a,e)
Time to 75% of $\pi_g$ decrease	$t_{75}$	min	23.8 ± 2.8	16.7 ± 3.9	0.032 (b,e)

Notes: (a) two-tailed  $t$ -test assuming equal variances; (b) two-tailed  $t$ -test assuming unequal variances; (c)  $n = 8$  and 12 for outdoor and glasshouse plants, respectively; (d)  $n = 7$  and 8 for outdoor and glasshouse plants, respectively; (e)  $n = 4$  for both outdoor and glasshouse plants.

### Water relations parameters estimated by pressure–volume analysis

Parameters for pressure–volume curves (Eqn 1) and values of  $\pi_s$  were estimated from the relationship between  $\psi_l$  and RWC as follows. Branches or leaves were excised underwater and rehydrated for up to 24 h to establish a water potential close to zero. A fully hydrated leaf was weighed to determine saturated fresh weight ( $FW_s$ ) and then placed in a pressure chamber (EMS, Santa Barbara, CA, USA) to estimate leaf balance pressure ( $P_b$ ). Successive pairwise measurements of  $FW$  and  $P_b$  were acquired as the leaf slowly desiccated on the laboratory bench, until  $P_b$  exceeded the measuring range of the pressure chamber (–4.0 MPa). Leaves were then dried completely (verified by repeated weighing) to determine dry weight ( $DW$ ). Absolute water content ( $Q$ ) was calculated as  $Q = (FW - DW)/M_w$  (where  $M_w$  is the molar mass of water) and RWC was calculated as  $Q/Q_s$ , where  $Q_s$  is saturated water content  $[(FW_s - DW)/M_w]$ .

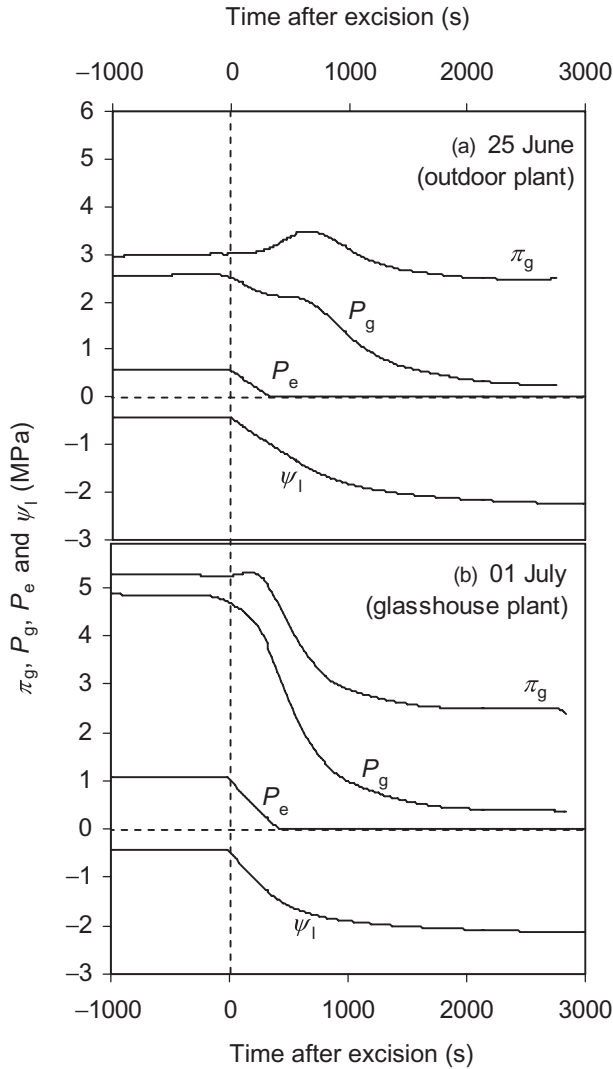
We compared the pressure chamber and psychrometer estimates of  $\psi_l$  by performing paired measurements on

identical leaf samples across a range of RWC values. We found that  $P_b$  was linearly and reproducibly related to  $\psi_l$  (measured by the psychrometer), but with a slope far from unity:  $\psi_l = 0.6136(-P_b) + 0.0794$  MPa ( $r^2 = 0.9646$ ,  $n = 17$ ). The reason for the difference between  $-P_b$  and  $\psi_l$  is unclear. While some past comparisons of the two methods in the range of 0 to –2 MPa have found good agreement (Boyer 1967; Blum, Sullivan & Eastin 1973; Bennett, Cortes & Lorens 1986), others reported marked deviations (Kaufmann 1968; Barrs *et al.* 1970; Wilson *et al.* 1979; Turner, Spurway & Schulze 1984), and the validity of  $P_b$  as an estimate of  $\psi_l$  has been challenged on theoretical grounds (Canny & Roderick 2005; Roderick & Canny 2005). We feel that the psychrometer represents the more direct of the two measurements of water potential per se, so we converted all values of  $-P_b$  to  $\psi_l$  using the regression equation above. Pairwise measurements of RWC and  $\psi_l$  were grouped separately for outdoor plants and glasshouse plants, and polynomial functions (2nd- and 3rd-order, respectively) were fitted to each data set.

Bulk osmotic pressure at saturation ( $\pi_s$ ) was estimated for each sample leaf using the 'osmotic line' method of

**Table 3.** Sensitivity of inferred results to the value of the unknown parameter  $M$  (net mechanical advantage, unitless), which was assumed equal to 0.5 for the simulations described in Table 2 and shown in Figs 2 and 3. 'od' and 'gh' refer to outdoor plants and glasshouse plants, respectively. Sensitivities are shown for the maximum inferred value of guard cell turgor pressure ( $P_g$ ) during the experiment, and for three parameters describing the kinetics of adjustment in guard cell osmotic pressure ( $\pi_g$ ) following excision:  $t_{25}$ ,  $t_{(25-75)}$  and  $t_{75}$ , the time required for  $\pi_g$  to complete the first 25%, the middle 50%, and the first 75% of its eventual total decline

	$M$	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
max $P_g$ /MPa	od	2.1 ± 0.2	2.6 ± 0.3	3.1 ± 0.4	3.6 ± 0.4	4.1 ± 0.5	4.5 ± 0.6	5.0 ± 0.6	5.5 ± 0.7
	gh	3.9 ± 0.5	4.8 ± 0.7	5.7 ± 0.9	6.6 ± 1.0	7.6 ± 1.2	8.5 ± 1.4	9.4 ± 1.5	10.4 ± 1.7
$t_{25}$ min <sup>-1</sup>	od	20.1 ± 3.9	17.6 ± 4.2	15.7 ± 3.2	14.9 ± 2.9	14.4 ± 2.7	14.1 ± 2.6	13.9 ± 2.5	13.7 ± 2.5
	gh	8.3 ± 2.8	8.2 ± 2.6	8.2 ± 2.3	8.2 ± 2.2	8.1 ± 2.1	8.1 ± 2.1	8.0 ± 2.0	8.0 ± 2.0
$t_{(25-75)}$ min <sup>-1</sup>	od	6.2 ± 2.5	7.5 ± 2.5	8.1 ± 2.4	8.3 ± 2.4	8.5 ± 2.4	8.6 ± 2.4	8.5 ± 2.3	8.7 ± 2.3
	gh	8.4 ± 2.7	8.6 ± 2.6	8.7 ± 2.4	8.6 ± 2.4	8.6 ± 2.2	8.6 ± 2.2	8.7 ± 2.1	8.7 ± 2.1
$t_{75}$ min <sup>-1</sup>	od	26.3 ± 3.3	25.2 ± 3.3	23.8 ± 2.8	23.2 ± 2.7	22.9 ± 2.6	22.7 ± 2.7	22.4 ± 2.7	22.4 ± 2.6
	gh	16.7 ± 4.6	16.7 ± 4.2	16.7 ± 3.9	16.7 ± 3.8	16.7 ± 3.6	16.7 ± 3.6	16.7 ± 3.5	16.7 ± 3.5



**Figure 2.** Sample inferred time courses of water relations parameters (bulk leaf water potential,  $\psi_l$ ; epidermal turgor pressure,  $P_e$ ; guard cell turgor pressure,  $P_g$ ; and guard cell osmotic pressure,  $\pi_g$ ) after leaf excision, for an outdoor plant (a, 25 June) and a glasshouse plant (b, 01 July). The vertical dotted line indicates the time of excision, and the horizontal dotted lines indicate the  $x$ -axis ( $y = 0$ ).

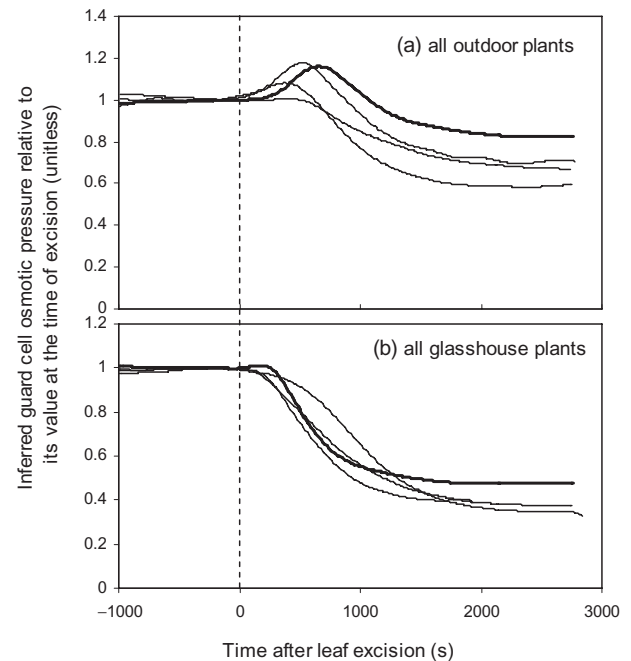
classical water relations (e.g. Tyree & Hammel 1972). There is no turgor pressure at low water potentials, so osmotic pressure ( $\pi$ ) equals  $-\psi_l$ . However,  $\pi = nRT/V$ , where  $R$  is the gas constant,  $T$  is temperature,  $n$  is leaf osmotic content and  $V$  is the volume of water in the leaf, and  $V = V_s \text{RWC}$ , where  $V_s$  is the leaf water volume at saturation. It follows that in the absence of turgor,  $-\psi_l = \pi = \pi_s \cdot (1/\text{RWC})$ . Thus,  $\pi_s$  can be estimated from the slope of a line (the osmotic line) relating  $-\psi_l$  to  $1/\text{RWC}$  at low  $\psi_l$ . To identify which data points should be included in the osmotic line for each leaf, we fitted lines to data subsets extending from the lowest measured  $\psi_l$  value to successively larger  $\psi_l$  values, and calculated  $\pi_s$  as the average slope of all lines having  $r^2 > 0.90$ . These  $\pi_s$  estimates were validated against direct psychro-

metric measurements of  $\pi_s$  on five leaves that had been frozen in liquid nitrogen to eliminate turgor; inferred values and direct measurements were not statistically different (Welch two-sample  $t$ -test assuming unequal variances, d.f. = 4,  $P = 0.94$ ).

Water content at the time of excision ( $Q_x$ ) was calculated by adding the total water loss after of excision (determined from gas exchange) to the water content at the end of the experiment (determined from  $FW$  and  $DW$ ). Sample leaves were rehydrated with the intent of calculating  $Q_s$ ; however, we found that complete rehydration was not possible for excised leaves that had been permitted to transpire for more than a few minutes. Thus,  $Q_s$  was calculated on the basis of an assumed initial water potential at the time of excision ( $\psi_x$ ), estimated for eight leaves that had been enclosed in the gas exchange chamber but removed at the time of excision. For three of these leaves,  $\psi_x$  was measured directly by thermocouple psychrometry (see below); the other five leaves were rehydrated (which was possible because these leaves were not significantly dehydrated) and  $\psi_x$  was estimated from Eqn 1. The average  $\psi_x$  was  $-0.42 \pm 0.17$  MPa. Initial relative water content was calculated from  $\psi_x$  and Eqn 1, permitting calculation of  $Q_s$  from  $Q_x$ .

### Thermocouple psychrometry

Water potential measurements were made on leaf samples taken with a hole-punch, using a Wescor HR-33T Dew



**Figure 3.** Inferred time courses of guard cell osmotic pressure ( $\pi_g$ ) expressed relative to the values of  $\pi_g$  at the time of excision, with results compiled from all experiments on (a) outdoor plants and (b) glasshouse plants. The time courses from 25 June and 01 July are shown in bold for comparison with Figs 1 and 2.

Point Microvoltmeter equipped with a C-52 Sample Chamber (Wescor, Logan, UT, USA), calibrated with a series of KCl solutions with osmotic pressures from 0.0 to 5.0 MPa. All measurements were made in hygrometric (automatic dew-point temperature depression) mode.

## RESULTS

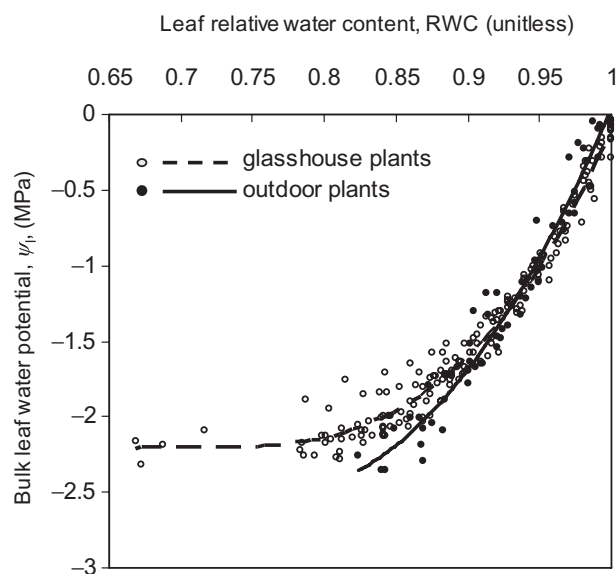
### Water relations and gas exchange properties of outdoor and glasshouse plants

The measured relationships between RWC and  $\psi_l$  are shown in Fig. 4 for outdoor and glasshouse plants. The polynomial functions that best fitted these data were:  $\psi_l = 48.5 \cdot \text{RWC}^2 - 74.8 \cdot \text{RWC} + 26.4$  ( $r^2 = 0.960$ ,  $n = 58$ ) for outdoor plants, and  $\psi_l = 83.5 \cdot \text{RWC}^3 - 177.02 \cdot \text{RWC}^2 + 125.08 \cdot \text{RWC} - 31.656$  ( $r^2 = 0.976$ ,  $n = 177$ ) for glasshouse plants.

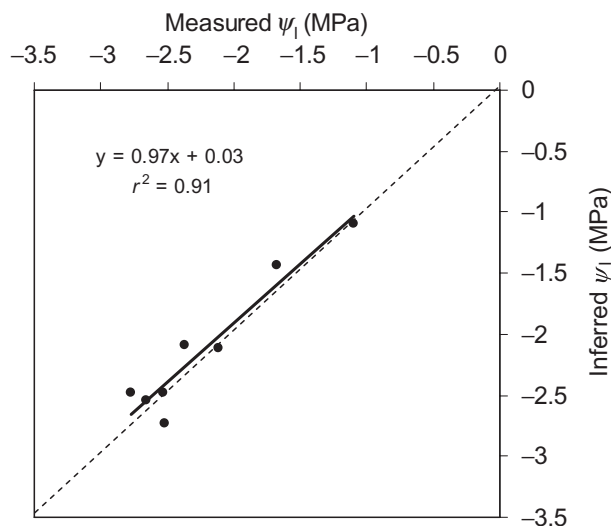
Bulk leaf osmotic pressure at saturation ( $\pi_s$ , Table 2) was significantly lower in outdoor plants than in glasshouse plants (details given in Table 2). Outdoor plants also had lower steady-state stomatal conductance before leaf excision ( $g_s$ , Table 2) than glasshouse plants. Pre-dawn water potential ( $\psi_{pd}$ , Table 2) was slightly but not significantly more negative in outdoor plants than in glasshouse plants.

### Observed dynamics of $g_s$ following leaf excision

Stomatal conductance ( $g_s$ ) followed a similar qualitative trend after leaf excision in all experiments:  $g_s$  initially increased, then decreased more substantially, and finally approached a minimum value close to zero after 2–4 h



**Figure 4.** Relationship between leaf relative water content (RWC) and water potential ( $\psi_l$ ) measured as described under pressure–volume analysis in the main text. Broken line and open symbols: glasshouse plants (acclimated in a glasshouse for  $\geq 9$  d prior to measurements). Solid line and closed symbols: outdoor plants (acclimated in a glasshouse for  $\leq 3$  d prior to measurements).



**Figure 5.** Comparison of measured and inferred values of bulk leaf water potential ( $\psi_l$ ) at the end of five experiments that were terminated early to validate the  $\psi_l$  inference technique. Measured values were acquired with a thermocouple psychrometer, and inferred values were acquired as described in the text. Inferred and measured  $\psi_l$  values were linearly related with a slope close to unity [(inferred  $\psi_l$ ) =  $0.97 \times$  (measured  $\psi_l$ ) + 0.03 MPa;  $n = 8$ ,  $r^2 = 0.91$ ]. The regression line is solid; a dotted 1:1 line is shown for reference.

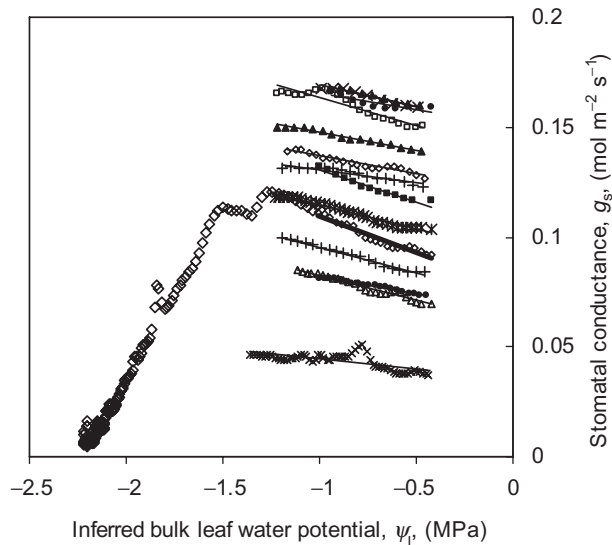
(Fig. 1). In some cases, the final approach towards zero was preceded by a single brief oscillation. Both the duration and the relative magnitude of the initial WWR of  $g_s$  following leaf excision differed between outdoor and glasshouse plants, as is evident from inspection of the traces in Fig. 1 and from the compiled results of formal quantification of the WWR (Table 2). The WWR was longer and of larger relative magnitude in outdoor plants than in glasshouse plants, but the absolute magnitude of the WWR did not differ significantly between the two groups of plants (Table 2).

### Inference of water potential from gas exchange

Bulk leaf water potential ( $\psi_l$ ) was inferred from cumulative transpiration after leaf excision using Eqns 1 and 2, as described in Materials and methods. These inferences were validated against direct measurements of  $\psi_l$  on eight leaves removed from the chamber at a range of times following leaf excision in other experiments (of these eight leaves, three were from outdoor plants and five were from glasshouse plants). Inferred and measured  $\psi_l$  values were linearly related with a slope near unity [(inferred  $\psi_l$ ) =  $0.97 \times$  (measured  $\psi_l$ ) + 0.03 MPa;  $n = 8$ ,  $r^2 = 0.91$ ; Fig. 5]. Inferred time courses of  $\psi_l$  are discussed below and presented in Fig. 2.

### Estimation of $\chi M$

As described in Materials and methods and shown by Eqn 7, one constraint on the parameters in Eqn 3 (which



**Figure 6.** Phase plots of stomatal conductance ( $g_s$ ) and bulk leaf water potential ( $\psi_l$ ) after leaf excision. The temporal sequence of points is from right to left. Data and linear regressions are shown for the initial linear phase of the  $g_s$  versus  $\psi_l$  relationship for all excision experiments in which  $\psi_l$  could be estimated; the entire data time course is also shown for one experiment for reference (large open diamonds; for comparison with Figs 1 and 2, this is the 25 June experiment, which used an outdoor plant). The slope of the initial linear phase provides an estimate of the quantity  $(-\chi M)$ , a product of two parameters. The average slope among these lines was  $-0.0186 \pm 0.0027 \text{ mol m}^{-2} \text{ s}^{-1} \text{ MPa}^{-1}$ .

relates  $g_s$  to  $P_e$  and  $P_g$ ) may be estimated from the initial slope of the trend between  $g_s$  and water potential after leaf excision. Figure 6 presents a sample phase plot of  $g_s$  versus  $\psi_l$ , showing the linear trend that obtains for a period after excision. The initial linear phase is also shown for all other excision experiments in which  $\psi_l$  could be estimated. The minimum, average, maximum and standard deviation of the slopes of these lines were  $-0.0151$ ,  $-0.0186$ ,  $-0.0225$  and  $0.0027 \text{ mol m}^{-2} \text{ s}^{-1} \text{ MPa}^{-1}$ , respectively. Using our estimate of 0.5 for  $M$ , this gives an average value for  $\chi$  of  $0.037 \text{ mol m}^{-2} \text{ s}^{-1} \text{ MPa}^{-1}$ .

### Inferred dynamics of stomatal water relations after leaf excision

To generate hypotheses to explain why the WWR to leaf excision differed between outdoor and glasshouse plants, we inferred the dynamics of  $\psi_l$ ,  $P_e$ ,  $P_g$  and  $\pi_g$  in the experiments described above. Figure 2 shows these inferred time courses for two sample excision experiments: one outdoor plant (25 June) and one glasshouse plant (01 July). In all cases, inferred  $P_e$  declined to zero in less than 8 min. Inferred  $\psi_l$  declined steadily, slowing gradually as stomatal closure reduced water loss. Inferred  $P_g$  also declined steadily after excision, generally following a roughly sigmoidal time course. The most distinct difference between outdoor and glasshouse plants, however, was the dynamic behaviour of  $\pi_g$  after excision. The time that it took for  $\pi_g$

to complete 25% and 75% of its eventual total decline ( $t_{25}$  and  $t_{75}$ , respectively) was much longer in outdoor plants than in glasshouse plants, but the time required for the middle 50% of the  $\pi_g$  decline ( $t_{(25-75)} = t_{75} - t_{25}$ ) was similar in both groups of plants (Table 2). All of the inferred  $\pi_g$  time courses are compiled and presented on a relative basis in Fig. 3 to permit comparison between outdoor and glasshouse plants.

### Sensitivity analysis for the parameter $M$

The results described above were based on an arbitrarily assumed value of 0.5 for the parameter  $M$ , the net mechanical advantage of the epidermis. To assess the effect of uncertainty in  $M$ , we repeated all simulations for  $M$ , which ranged from 0.3 to 1.0 (mod 0.1). The qualitative results (Table 3) were unaffected: outdoor plants had a longer lag time for, but not a slower rate of  $\pi_g$  adjustment. However, the maximum inferred values of  $P_g$  increased strongly at higher  $M$ , from a mean of  $5.7 \pm 0.9 \text{ MPa}$  to  $10.4 \pm 1.7 \text{ MPa}$  for glasshouse plants. The fact that such large  $P_g$  values have never been observed, to our knowledge, might suggest that  $M$  in  $P. \times fraseri$  is closer to the low end of this range.

### DISCUSSION

This study examined pressure–volume relations and stomatal responses to leaf excision in an evergreen shrub ( $P. \times fraseri$ ) and compared the behaviour of plants that had been kept in a glasshouse for at least 9 d before measurement ('glasshouse' plants) or had been kept outdoors and transferred to a glasshouse three or fewer days before measurement ('outdoor' plants). A transient opening response (WWR) was always observed for  $g_s$  following leaf excision. That is,  $g_s$  initially increased after excision before subsequently declining towards zero (Fig. 1). The initial trend between measured  $g_s$  and inferred  $\psi_l$  after leaf excision was linear and had a conserved, negative slope (Fig. 6). Furthermore, in all experiments, inferred values of  $\pi_g$  eventually began to decline exponentially after a variable lag period, whereas  $P_g$  and  $P_e$  declined immediately after excision. Together, these results are consistent with the hypothesis that stomatal responses to leaf excision involve two distinct phases: an initial 'hydropassive' phase during which  $\pi_g$  is constant or changes only passively (as a result of water loss from guard cells), followed by a 'hydroactive' phase that involves metabolic reduction of  $\pi_g$  (Darwin 1898; Darwin & Pertz 1911; Stålfelt 1929; Ehret & Boyer 1979; Meidner 1986; Buckley *et al.* 2003).

The duration of the WWR was substantially longer in outdoor plants than in glasshouse plants (Fig. 1). Our analysis of the dynamics of stomatal water relations during these excision responses suggests that the lag period between the time of leaf excision and the time when the hydroactive response of  $\pi_g$  begins in earnest was much longer in outdoor plants, but that the half-time for the subsequent exponential decline in  $\pi_g$  did not differ between outdoor and glasshouse plants. This suggests that the induc-



tion, rather than the execution, of guard cell osmoregulation is slower in the outdoor plants. In contrast, most dynamic models of  $g_s$  have predicted WWRs and stomatal oscillations by assuming a longer time constant for  $\pi_g$  adjustment than for passive hydraulic adjustments (Rand *et al.* 1981; Haefner, Buckley & Mott 1997; Jarvis *et al.* 1999), as opposed to a long lag time for  $\pi_g$ . Cowan & Farquhar (1977) introduced a lag time but did not discuss whether or how it affected the predicted dynamics of  $g_s$ . It is unclear what effect the distinction between a slow time constant and a long lag may have on the stability of stomatal control, but the matter would appear to warrant further study, and future efforts to model  $g_s$  dynamically should consider this distinction.

An alternative view on stomatal hydraulics holds that a large water potential gradient exists between guard and epidermal cells. According to that view,  $M$  is overcome – and right-way hydraulic responses are achieved – by changes in the magnitude of this gradient, not by active adjustment of  $\pi_g$  in relation to local water status (any change in  $\pi_g$  is strictly passive). There are numerous arguments against this hypothesis (Buckley 2005). One is that it is difficult to explain the archetypal two-phase response to hydraulic perturbations with this hypothesis. The correct pattern would result if  $P_g$  responded very slowly to changes in  $\psi_l$ , but our analysis indicated that both  $P_e$  and  $P_g$  began to decline immediately after excision (Fig. 2). Furthermore, for this hypothesis to explain the observed decline in  $g_s$  to near zero without active down-regulation of  $\pi_g$ , guard cell water potential would have to decline during the excision response by an amount approaching the initial magnitude of  $\pi_g$ . That magnitude, however, was generally much greater than the magnitude of  $\psi_l$  late in the excision response ( $\approx 3$ – $6$  MPa for  $\pi_g$  versus  $2$ – $3$  MPa for  $\psi_l$ ; see Fig. 2). This generates a contradiction: guard cell water potential first lags behind the decline in  $\psi_l$  because guard cells are downstream from the bulk of leaf tissue in the transpiration stream, but guard cell water potential later overtakes  $\psi_l$  despite this fact.

A related study (Buckley & Mott 2002a) combined a model with stomatal aperture time courses measured with a microscope to infer the dynamics of  $\pi_g$  for single pairs of guard cells during humidity responses. That study also inferred values for the effective resistance between guard and epidermal cells needed to explain the observed stomatal responses if  $\pi_g$  were assumed constant. The inferred resistance changed dramatically during the humidity responses – first decreasing, then increasing and finally stabilizing at a value that was larger than the initial value in some cases and smaller in others. In contrast,  $\pi_g$  varied monotonically in time during the humidity response, and with humidity in the steady-state. The authors concluded that  $\pi_g$  regulation was a more parsimonious explanation than a varying water potential gradient between guard and epidermal cells for the observed responses. Assmann & Gershenson (1991) likewise concluded that an exponential decay model suggesting metabolic adjustment of  $\pi_g$  best described the kinetics of stomatal adjustment to changes in

VPD, and Grantz & Zeiger (1986) found that the humidity response was kinetically similar to the light response, which is known to involve  $\pi_g$  adjustment.

### Ecological implications of variable WWR kinetics

Other experiments have also found a large degree of variation in the kinetics of stomatal responses to light, which are known to involve guard cell osmotic adjustment (Woods & Turner 1971; Saxe 1979; Kirschbaum, Gross & Pearcy 1988; Meidner 1990; Tinoco-Ojanguren & Pearcy 1992; Mott, Shope & Buckley 1999; Buckley & Mott 2000). It is well established that the rate of stomatal opening can, in many conditions, be the dominant limitation on photosynthetic induction in light flecks. Allen & Pearcy (2000a,b) found that photosynthetic induction was slower at lower initial conductances (pre-light fleck). When initial conductance was high,  $g_s$  began to increase almost immediately after illumination, but when initial  $g_s$  was low, the stomatal response was preceded by a lag time on the order of 5 min (e.g. figure 1b & e in Allen & Pearcy 2000a). However, most of this trend occurred across a narrow range of quite low initial  $g_s$  values, so the lag may have resulted from parts of the leaf still having been in the ‘*Spannungsphase*’ – the period during which  $\pi_g$  and  $P_g$  have begun to increase after illumination, but before  $P_g$  has increased enough to overcome epidermal backpressure (Stålfelt 1929). The variable lag times reported in the present study are unrelated to the *Spannungsphase*, because they preceded stomatal closure, not opening. Nonetheless, they may influence carbon–water balance: to the extent that photosynthesis remains induced during dark periods, rapid stomatal closure after light flecks can be undesirable (for review, see Pearcy *et al.* 1994).

Our analysis suggested that  $P_e$  declined to zero in less than 8 min after leaf excision in all cases. It is unclear whether loss of epidermal turgor would have occurred in response to a more moderate and repeatable hydraulic insult, such as xylem cavitation or a change in ambient humidity. The available data do suggest steady-state that  $P_e$  can decline dramatically – by nearly two-thirds in some cases – across a physiological range of evaporative gradient (Shackel & Brinkmann 1985; Nonami, Schulze & Ziegler 1990; Mott & Franks 2001), and is it likely that  $P_e$  declines further still while  $E$  is elevated during the WWR following a reduction in humidity. Klein *et al.* (1996) concluded that  $P_e$  was close to zero throughout the day in *V. faba*. If, as suggested by those results and by our data, the water lost during WWRs is of the same order as the leaf’s initial water content, then variations in WWR duration may determine whether loss of epidermal turgor occurs in the course of normal leaf functioning. This possibility is supported by data of Brodribb & Holbrook (2003), who found that stomata remained open at bulk leaf water potentials low enough to cause leaf turgor loss, and that substantially lower  $\psi_l$  was required to induce total stomatal closure.

Cowan (1972) has discussed the possibility that the dual-feedback control mechanism believed to underlie both

WWRs and stomatal oscillations – positive feedback from passive water loss and negative feedback from guard cell osmoregulation – might serve an adaptive function by exploring the space of possible steady-states in order to find the optimal state (i.e. that which balances water loss and carbon gain with diurnally varying conditions as needed to maximize daily carbon gain for the available transpirable water supply; Cowan & Farquhar 1977). Furthermore, because WWRs and oscillations allow  $\psi_l$  transiently to decline farther than it would in the steady-state, they may also permit  $\psi_l$  to cross the cavitation threshold transiently. The resulting reduction in xylem hydraulic conductance can provide a kind of feedforward control (Oren *et al.* 1999; Buckley & Mott 2002b) by informing stomata of the proximity of the cavitation threshold. Variation in WWR length could therefore help to explain ‘apparent feedforward’ and isohydric behaviour (Buckley 2005). It may also play a role in defining cavitation safety margins, because the magnitude of the transient deviation of  $\psi_l$  below steady-state should depend on the duration of WWRs. Finally, because the relative time constants for hydraulic and osmotic adjustments are major determinants of the tendency for  $g_s$  to oscillate (Cowan 1972; Farquhar & Cowan 1974; Cowan & Farquhar 1977; Rand *et al.* 1981), osmoregulatory lag time should affect the stability of the stomatal control system. The variation reported here in lag time could therefore also help to explain why oscillations and patchy  $g_s$  are so difficult to replicate in different leaves despite similar experimental conditions (Mott & Buckley 2000).

### Assumptions of the analysis

Our procedure for inferring  $\pi_g$  assumed epidermal and guard cells were hydraulically quasi-static with respect to  $\psi_l$  and to one another. That is, changes in bulk leaf, epidermal and guard cell water potentials occurred simultaneously. It is therefore possible that the inferred delay in  $\pi_g$  adjustment was not caused by a delayed metabolic response to a change in water status, but instead by a delayed response of epidermal water status to the change in bulk leaf water status. The fact that  $g_s$  began to increase immediately after excision implies that  $P_e$  declined immediately, consistent with quasi-stasis between  $P_e$  and  $\psi_l$ , at least on the time scale of our measurements ( $\approx 15$  s). Our data do not rule out the possibility that guard cell water potential and turgor ( $P_g$ ) respond slowly. However, there are two reasons to doubt this. First,  $P_g$  was inferred from  $g_s$  and  $P_e$  using Eqn 3, which contains no assumptions about guard cell hydraulic kinetics, and these inferred  $P_g$  values also began to decline immediately after leaf excision. Second, recent experiments found that the halftime for adjustment of guard cell volume following a change in local water potential in epidermal peels of *V. faba* was typically much less than 1 min, unless the peels were pre-treated with membrane trafficking inhibitors (J.C. Shope and K.A. Mott, unpublished results).

Two other untested assumptions of our analysis are that  $\pi_e$  is similar to  $\pi_s$ , and that  $\pi_e$  is constant during the excision

response. The latter assumption is supported by earlier experiments in which  $\pi_e$  was found to vary only slightly with  $E$  (Meidner & Edwards 1975; Frensch & Schulze 1988; Nonami *et al.* 1990). The former assumption, however, is neither supported nor refuted by any evidence of which we are aware. If  $\pi_e$  and  $\pi_s$  differ, it seems more likely that  $\pi_e < \pi_s$ , because mesophyll cells comprise a large fraction of the total cellular volume in most broad leaves and they often contain substantial stores of osmotically active photosynthate.

It bears mentioning that one curious feature of our results could be explained by a failure of the assumption that  $\pi_s$  is a reliable proxy for  $\pi_e$ . Whereas inferred values of  $\pi_g$  remained roughly constant during the lag period after excision in glasshouse plants, inferred  $\pi_g$  increased substantially during this period in outdoor plants (Fig. 3). Mathematically, this increase can be explained by the fact that inferred  $P_e$  declined to zero long before the WWR had ended, requiring elevated  $\pi_g$  to explain the still-elevated  $g_s$ . If, however, we delayed the loss of epidermal turgor in the analysis by assuming  $\pi_e = 1.5$  MPa for outdoor plants – instead of 1.0 MPa, the average value of  $\pi_s$  measured in outdoor plants – then the inferred increase in  $\pi_g$  was greatly reduced or eliminated in all cases (not shown). It is also possible that the inferred  $\pi_g$  increase was real and resulted from the concentration of solutes in the guard cell because of volume loss before the induction of active osmotic efflux. We are unable to distinguish these alternatives on the basis of our data.

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