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Dynamics of stomatal water relations following leaf excision

JULIA E. POWLES[1,2], THOMAS N. BUCKLEY[1]*, ADRIENNE B. NICOTRA[2] & GRAHAM D. FARQUHAR[1]

[1]*Environmental Biology Group and Cooperative Research Centre for Greenhouse Accounting, Research School of Biological Sciences, Institute of Advanced Studies, The Australian National University, Canberra ACT 2601 Australia and* [2]*School of Botany and Zoology, Faculty of Science, The Australian National University, Canberra ACT 2601 Australia*

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 π_g, respectively) from stomatal conductance (g_s) and bulk leaf water potential (ψ_l), using a hydromechanical model of g_s. ψ_l was calculated from cumulative post-excision transpirational water loss using empirical relationships between ψ_l and relative water content obtained on similar leaves. Inferred P_g and P_e both declined immediately after leaf excision. Inferred π_g also declined after a lag period. The kinetics of π_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_2 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 (ψ_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.

Correspondence: Tom Buckley. Fax: +1 435 797 1575; e-mail: tom_buckley@alumni.jmu.edu

*Present address: Biology Department, Utah State University, Logan, UT 84322-5305, USA.

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)	mol m^{-2} s^{-1}
Transpiration rate	E	mol m^{-2} s^{-1}
Turgor pressure: epidermal guard cell	P_e, P_g	MPa
Osmotic pressure: guard cell, epidermal, saturated bulk leaf	π_g, π_e, π_s	MPa
Bulk leaf water potential (at excision, pre-dawn)	ψ_l (ψ_x, ψ_{pd})	MPa
Turgor-conductance scaling factor	χ	mol m^{-2} s^{-1} 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 (π_g) in order to make steady-state π_g proportional to epidermal water status (Darwin 1898; Darwin & Pertz 1911; Stålfelt 1929; Meidner 1986; Buckley, Mott & Farquhar 2003). Tracking of water status by π_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 π_g temporarily decouple g_s from π_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 glasshouse. 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 ψ_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 ψ_l from water loss was validated by measuring ψ_l directly on leaves removed from the chamber at various times after excision. We applied time courses of g_s and ψ_l to a simple model for g_s to infer the dynamics of P_g, P_e and π_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^{-2} s^{-1}, 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_2, bubbled through a humidifier to saturate it with water vapour, passed through a temperature-controlled condenser to regulate inlet humidity, mixed with 12% CO_2 in air using mass flow controllers, and passed through the chamber at a flow rate of 4 L min^{-1}, monitored by a mass flowmeter (Brooks, Hatfield, PA, USA). Inlet CO_2 concentration was kept near ambient (≈ 0.37 mg g^{-1}). The CO_2 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, Helinski, 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_2 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 difference (VPD) was set at 1–2 kPa (held constant during each experiment), and irradiance was increased in steps to 1000 μE m^{-2} s^{-1} 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^{-2}, 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, ψ_l is an empirical function of leaf relative water content (RWC):

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

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 ψ_l to be estimated from E, which is measured by gas exchange. A third assumption was used to infer π_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), χ is a turgor-conductance scaling factor, and P_g and P_e are related to π_g, ψ_l and π_e by standard expressions of water relations:

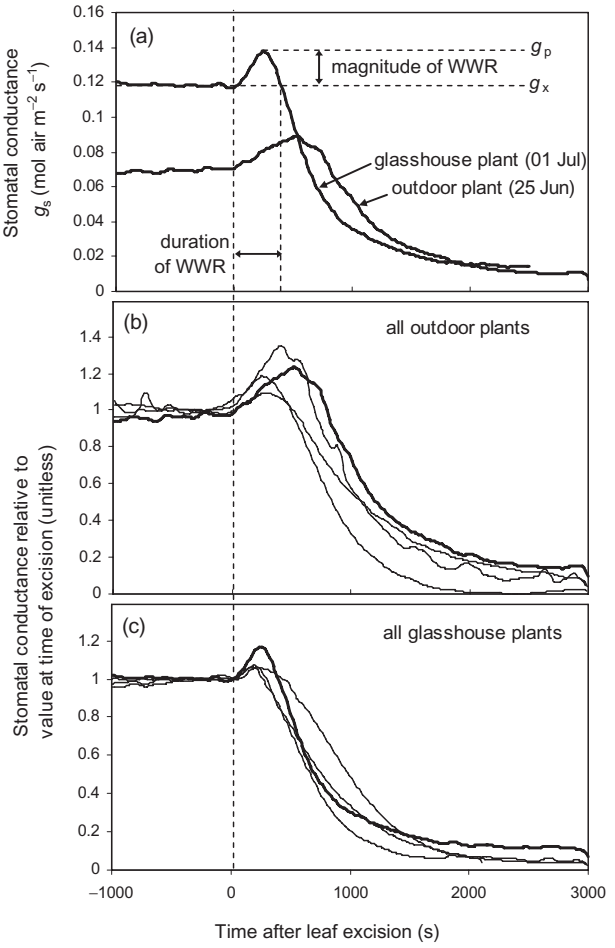

Figure 1. Measured time courses of stomatal conductance to water vapour (g_s) after leaf excision, which occurred at time zero. (a) Two sample traces of absolute stomatal conductance: 25 June (an outdoor plant) and 01 July (a glasshouse plant). (b) All traces for outdoor plants, expressed relative to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

$$P_e = \max(\psi_l + \pi_e, 0), \quad (4)$$

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

Equation 4 assumes that the capacitor tissues represented by ψ_l are in close hydraulic contact with the epidermis (so that ψ_e is in quasi-static equilibrium with ψ_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 π_g during the excision response, we applied Eqns 1–5 to gas exchange measurements of E and g_s as follows. Firstly, ψ_l was estimated from E using Eqns 1–2. Secondly, P_e was inferred from ψ_l using Eqn 4, in conjunction with a value for π_e 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_e, an estimate for the quantity χM (discussed below), and a guess for the value of M (0.5, also discussed below). Finally, π_g was calculated from Eqn 5 using estimated values of P_g and ψ_l.

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

$$g_s = \chi[-M\psi_l + \pi g - (M+1)\pi_e], \quad (6)$$

where P_e and P_g are understood to be non-negative. Immediately after excision, a decline in ψ_l and an increase in g_s occur in concert before π_g begins to change significantly. Hence, the product χM may be estimated from the initial slope of a phase plot of g_s versus ψ_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 π_g adjustment were calculated from inferred π_g time courses after Gaussian smoothing with a 220 s window and a decay constant of 0.01 s^{-2}. Initial and final values of π_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 ψ_l inference method was validated against direct psychrometer measurements of ψ_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_e, P_g and π_g) contains two major assumptions that we could not directly validate: that ψ_l and π_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 (π_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	π_s	MPa	1.00 ± 0.12	1.47 ± 0.12	$2.4{\cdot}10^{-7}$ (a,c)
Stomatal conductance at excision	g_x	mol m^{-2} s^{-1}	0.092 ± 0.022	0.161 ± 0.041	0.0016 (b,d)
Pre-dawn bulk leaf water potential	ψ_{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	$2.7{\cdot}10^{-6}$ (a,d)
Relative size of WWR	–	%	19 ± 10	7.9 ± 6.5	0.033 (b,d)
Absolute size of WWR	–	mol m^{-2} s^{-1}	0.017 ± 0.008	0.011 ± 0.007	0.21 (a,d)
Time to 25% of π_g decrease	t_{25}	min	15.7 ± 3.2	8.2 ± 2.3	0.012 (b,e)
Time from 25 to 75% of π_g decrease	$t_{(25–75)}$	min	8.1 ± 2.4	8.7 ± 2.4	0.74 (a,e)
Time to 75% of π_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 π_s were estimated from the relationship between ψ_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 ψ_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 ψ_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 ψ_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 ψ_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 ψ_l using the regression equation above. Pairwise measurements of RWC and ψ_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 (π_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 (π_g) following excision: t_{25}, $t_{(25–75)}$ and t_{75}, the time required for π_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^{-1}	*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^{-1}	*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^{-1}	*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

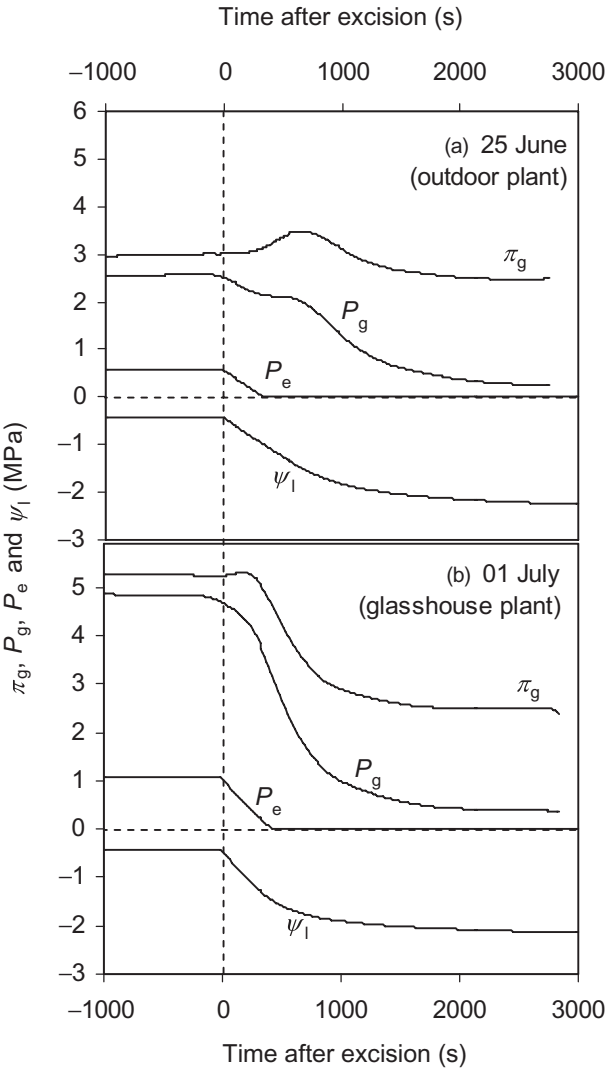

Figure 2. Sample inferred time courses of water relations parameters (bulk leaf water potential, ψ_l; epidermal turgor pressure, P_e; guard cell turgor pressure, P_g; and guard cell osmotic pressure, π_g) after leaf excision, for an outdoor plant (a, 25 June) and a glasshouse plant (b, 01 July). The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

classical water relations (e.g. Tyree & Hammel 1972). There is no turgor pressure at low water potentials, so osmotic pressure (π) 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 $V = V_s$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, π_s can be estimated from the slope of a line (the osmotic line) relating $-\psi_l$ to 1/RWC at low ψ_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 ψ_l value to successively larger ψ_l values, and calculated π_s as the average slope of all lines having $r^2 > 0.90$. These π_s estimates were validated against direct psychrometric measurements of π_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 (ψ_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, ψ_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 ψ_x was estimated from Eqn 1. The average ψ_x was -0.42 ± 0.17 MPa. Initial relative water content was calculated from ψ_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

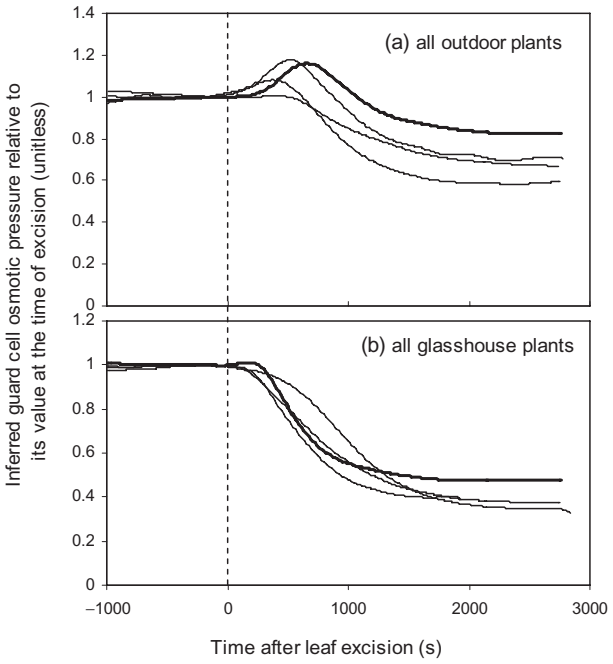

Figure 3. Inferred time courses of guard cell osmotic pressure (π_g) expressed relative to the values of π_g at the time of excision, with results compiled from all experiments on (a) outdoor plants and (b) glasshouse plants. The time courses from 25 June and 01 July are shown in bold for comparison 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 ψ_l are shown in Fig. 4 for outdoor and glasshouse plants. The polynomial functions that best fitted these data were: $\psi_l = 48.5 \cdot RWC^2 - 74.8 \cdot RWC + 26.4$ ($r^2 = 0.960$, $n = 58$) for outdoor plants, and $\psi_l = 83.5 \cdot RWC^3 - 177.02 \cdot RWC^2 + 125.08 \cdot RWC - 31.656$ ($r^2 = 0.976$, $n = 177$) for glasshouse plants.

Bulk leaf osmotic pressure at saturation (π_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_x, Table 2) than glasshouse plants. Pre-dawn water potential (ψ_{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 (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).

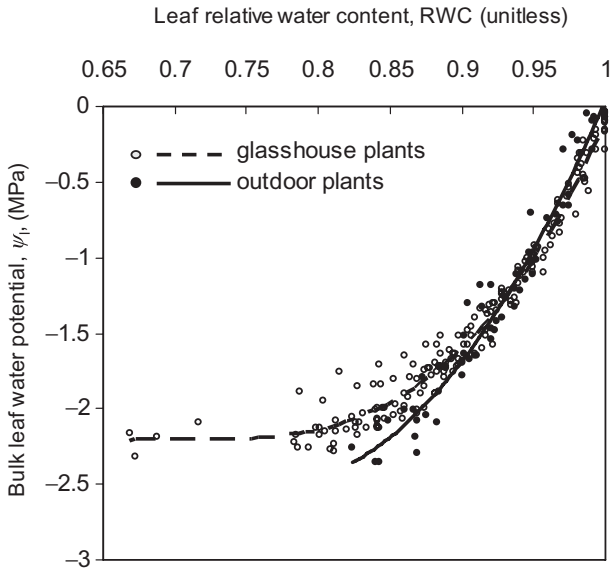

Figure 4. Relationship between leaf relative water content (RWC) and water potential (ψ_l) measured as described under pressure–volume analysis in the main text. Broken line and open symbols: glasshouse plants (acclimated in a glasshouse for ≥9 d prior to measurements). Solid line and closed symbols: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

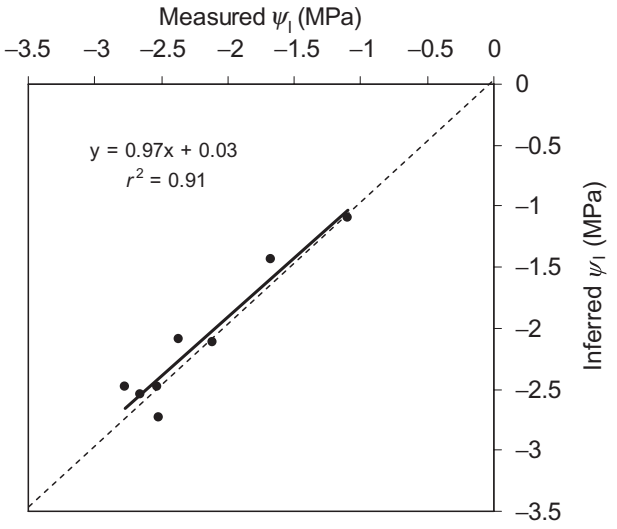

Figure 5. Comparison of measured and inferred values of bulk leaf water potential (ψ_l) at the end of five experiments that were terminated early to validate the ψ_l inference technique. Measured values were acquired with a thermocouple psychrometer, and inferred values were acquired as described in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

Inference of water potential from gas exchange

Bulk leaf water potential (ψ_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 ψ_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 ψ_l values were linearly related with a slope near unity [(inferred ψ_l) = 0.97 × (measured ψ_l) + 0.03 MPa; $n = 8$, $r^2 = 0.91$; Fig. 5]. Inferred time courses of ψ_l are discussed below and presented in Fig. 2.

Estimation of χM

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

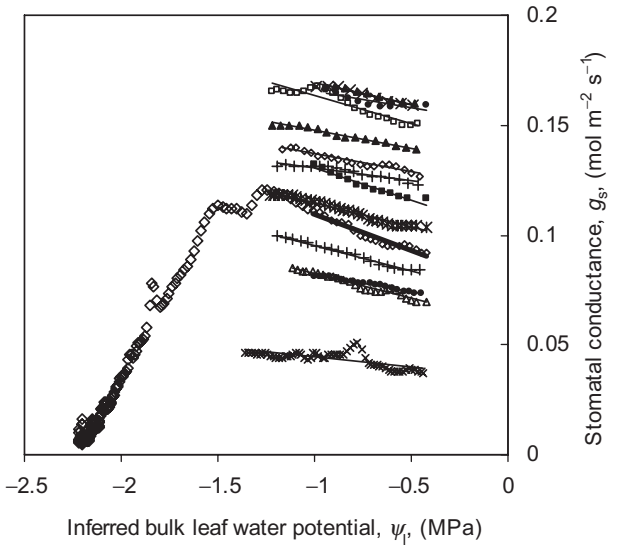

Figure 6. Phase plots of stomatal conductance (g_s) and bulk leaf water potential (ψ_l) after leaf excision. The temporal sequence of points is from right to left. Data and linear regressions are shown for the initial linear phase of the g_s versus ψ_l relationship for all excision experiments in which ψ_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 ± 0.0027 mol m^{-2} s^{-1} 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 ψ_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 ψ_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 mol m^{-2} s^{-1} MPa^{-1}, respectively. Using our estimate of 0.5 for M, this gives an average value for χ of 0.037 mol m^{-2} s^{-1} 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 ψ_l, P_e, P_g and π_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 ψ_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 π_g after excision. The time that it took for π_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 π_g decline ($t_{(25–75)} = t_{75} - t_{25}$) was similar in both groups of plants (Table 2). All of the inferred π_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 π_g adjustment. However, the maximum inferred values of P_g increased strongly at higher M, from a mean of 5.7 ± 0.9 MPa to 10.4 ± 1.7 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.* × *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.* × *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 ψ_l after leaf excision was linear and had a conserved, negative slope (Fig. 6). Furthermore, in all experiments, inferred values of π_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 π_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 π_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 π_g begins in earnest was much longer in outdoor plants, but that the halftime for the subsequent exponential decline in π_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 π_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 π_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 π_g in relation to local water status (any change in π_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 ψ_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 π_g, guard cell water potential would have to decline during the excision response by an amount approaching the initial magnitude of π_g. That magnitude, however, was generally much greater than the magnitude of ψ_l late in the excision response ($\approx$ 3–6 MPa for π_g versus 2–3 MPa for ψ_l; see Fig. 2). This generates a contradiction: guard cell water potential first lags behind the decline in ψ_l because guard cells are downstream from the bulk of leaf tissue in the transpiration stream, but guard cell water potential later overtakes ψ_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 π_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 π_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, π_g varied monotonically in time during the humidity response, and with humidity in the steady-state. The authors concluded that π_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 π_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 π_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 π_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 ψ_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 ψ_l transiently to decline farther than it would in the steady-state, they may also permit ψ_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 ψ_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 π_g assumed epidermal and guard cells were hydraulically quasi-static with respect to ψ_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 π_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 ψ_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 π_e is similar to π_s, and that π_e is constant during the excision response. The latter assumption is supported by earlier experiments in which π_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 π_e and π_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 π_s is a reliable proxy for π_e. Whereas inferred values of π_g remained roughly constant during the lag period after excision in glasshouse plants, inferred π_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 π_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 π_s measured in outdoor plants – then the inferred increase in π_g was greatly reduced or eliminated in all cases (not shown). It is also possible that the inferred π_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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