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SPECIAL ISSUE REVIEW PAPER

Stand aside stomata, another actor deserves centre stage: the forgotten role of the internal conductance to CO_2 transfer

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Abstract

Internal conductance describes the movement of CO_2 from substomatal cavities to sites of carboxylation. Internal conductance has now been measured in approximately 50 species, and in all of these species it is a large limitation of photosynthesis. It accounts for somewhat less than half of the decrease in CO_2 concentrations from the atmosphere to sites of carboxylation. There have been two major findings in the past decade. First, the limitation due to internal conductance (i.e. C_i–C_c) is not fixed but varies among species and functional groups. Second, internal conductance is affected by some environmental variables and can change rapidly, for example, in response to leaf temperature, drought stress or CO_2 concentration. Biochemical factors such as carbonic anhydrase or aquaporins are probably responsible for these rapid changes. The determinants of internal conductance remain elusive, but are probably a combination of leaf anatomy, morphology, and biochemical factors. In most plants, the gas phase component of internal conductance is negligible with the majority of resistance resting in the liquid phase from cell walls to sites of carboxylation. The internal conductance story is far from complete and many exciting challenges remain. Internal conductance ought to be included in models of canopy photosynthesis, but before this is feasible additional data on the variation in internal conductance among and within species are urgently required. Future research should also focus on teasing apart the different steps in the diffusion pathway (intercellular spaces, cell wall, plasmalemma, cytosol, and chloroplast envelope) since it is likely that this will provide clues as to what determines internal conductance.

Key words: Economics, internal conductance, mesophyll conductance, nitrogen, photosynthesis, stomatal conductance, transfer conductance, transpiration, water.

Introduction

The first step in the Calvin cycle involves fixation of CO_2 by the enzyme Rubisco. Rubisco has a poor affinity for CO_2 and thus atmospheric concentrations of CO_2 are subsaturating for photosynthesis of C_3 plants (Fig. 1). Hence, photosynthesis is limited by CO_2 concentration even in the best case scenario in which the concentration of CO_2 at the sites of carboxylation in the chloroplast is the same as atmospheric. The actual situation is much worse than this, with the concentration of CO_2 at the sites of carboxylation, on average, 50% of atmospheric concentrations (Table 1). Hence, photosynthesis of C_3 plants is limited, not only by low atmospheric CO_2 concentrations, but also by the large drawdown in CO_2 concentrations from the atmosphere to the sites of carboxylation. In real terms, this means that the rates of photosynthesis are as much a function of the drawdown in CO_2 concentration from the atmosphere to the sites of carboxylation, as they are a function of the amounts and activities of enzymes (Fig. 1).

Two resistances dominate the pathway from the atmosphere to the sites of carboxylation. The first is very well known, it is diffusion of CO_2 from the atmosphere (with CO_2 concentration C_a) to substomatal cavities (with CO_2 concentration C_i) via the stomata. Stomata act like tiny valves that rapidly change their aperture so as to control the loss of water from leaves. Leaves have small water reserves compared with the flux of water via transpiration and would be rapidly dehydrated if it were not for

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mechanisms that control cellular water availability and the rate of water loss (Slatyer, 1967). Closing stomata is the most direct means by which plants prevent cellular water loss. The CO_2 required for photosynthesis diffuses from the atmosphere into the substomatal cavities via the stomata, the same points at which the majority of water exits, and thus stomatal closure simultaneously slows transpiration and the diffusion of CO_2 into the substomatal cavity (Gaastra, 1959). Stomatal conductance is easily determined and variation in stomatal conductance among and within species has been exhaustively studied (Lange *et al*., 1971; Wong *et al*., 1979; Schulze, 1986; Franks and Farquhar, 1999). The average drawdown in the CO_2 concentrations from atmosphere to substomatal cavities (C_a–C_i) of light-saturated leaves is 123±5 μmol mol^{-1} when C_a is 360 μmol mol^{-1} (Table 1).

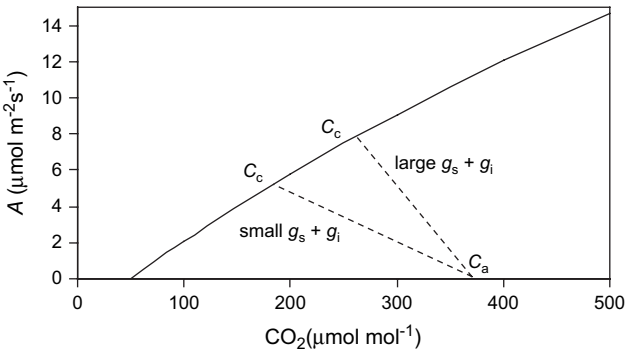

Fig. 1. The response of net photosynthesis (A) to CO_2 concentration. The curved solid line shows the biochemical response of net photosynthesis to CO_2 concentration. The rate of net photosynthesis is a function of the CO_2 concentration in the chloroplasts (C_c). The decrease in CO_2 concentration from the atmosphere (C_a) to chloroplasts (C_c) is a function of the sum of stomatal and internal conductances (dashed lines). Note that plants with the same biochemical capacity for photosynthesis can have quite different rates of photosynthesis if the sum of stomatal+internal conductances differ. Data are for a plant with V_{cmax}=50 μmol m^{-2} s^{-1}, J=100 μmol m^{-2} s^{-1}, and R_d=0.6 μmol m^{-2} s^{-1}.

This article considers the second, less-well-known step in the diffusion pathway, that is, from the substomatal cavities to the sites of carboxylation (C_c). This additional step is commonly described as the internal conductance (g_i=A/(C_i–C_c)). Internal conductance has been reviewed several times in the past decade (Evans and von Caemmerer, 1996; Evans and Loreto, 2000; Evans *et al*., 2004; Terashima *et al*., 2005, 2006) and what is presented here builds on these earlier reviews. Rather than dealing with every aspect of internal conductance, this review attempts to answer several key questions:

(i) What is internal conductance and what should we call it?

(ii) How is internal conductance estimated?

(iii) How does internal conductance affect CO_2 diffusion, photosynthesis, and leaf economics?

(iv) Does the limitation due to internal conductance differ among species?

(v) Does internal conductance affect interpretation and fitting of A/C_i responses to the biochemical model of Farquhar *et al*. (1980)?

Table 1. *Summary of light-saturated rate of photosynthesis at ambient CO_2 (A_{max}), internal conductance (g_i) and the drawdown from substomatal cavities to sites of carboxylation (C_i-C_c) in 50 C_3 species*

Data are for fully-developed, non-senescent leaves of plants that are neither salt- nor water-stressed. Measurements were made at ambient CO_2 concentrations (360–400 μmol mol^{-1}) between 20 °C and 30°C. In cases where C_i–C_c was not reported, it was calculated from published A and g_i: C_i–C_c=A/g_i. Data are based on the literature review of Ethier and Livingston (2004), updated with recently published data: (De Lucia *et al*., 2003; Loreto *et al*., 2003; Pons and Welschen, 2003; Hanba *et al*., 2004; Warren, 2004; Bernacchi *et al*., 2005; Grassi and Magnani, 2005; Niinemets *et al*., 2005; Ethier *et al*., 2006; Flexas *et al*., 2006*b*; Warren *et al*., 2006; Yamori *et al*., 2006). Input data were mean values for each species/treatment combination, rather than individual measurements. Differences among functional groups were assessed by one-way ANOVA and numbers with different letters are significantly different at P <0.05 (LSD *post hoc* test). Data are means with one standard error in parentheses. (*) <0.05; (**) <0.01; (***) <0.001; NS, not significant.

	n (g_i)[a]	Species (g_i)[b]	n (g_s)[c]	Species (g_s)[d]	A_{max} (μmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	g_i (mol m^{-2} s^{-1})	C_a–C_i (μmol mol^{-1})	C_i–C_c (μmol mol^{-1})	C_a–C_c (μmol mol^{-1})
Angiosperms										
Herbaceous dicot	27	9	16	8	18 (1) a	0.27 (0.04) a	0.29 (0.02) a	126 (13)	68 (6) a	197 (13)
Herbaceous monocot	20	3	9	2	24 (3) b	0.23 (0.03) ab	0.35 (0.04) b	111 (11)	77 (7) ab	189 (15)
Woody deciduous	77	17	28	13	11.2 (0.5) c	0.17 (0.02) bc	0.14 (0.01) c	136 (7)	88 (3) b	214 (7)
Woody evergreen	51	20	38	15	9.7 (0.6) c	0.21 (0.02) ab	0.11 (0.01) c	109 (9)	91 (4) c	202 (9)
Gymnosperms										
Evergreen	6	4	6	6	8.6 (0.9) c	0.09 (0.01) c	0.12 (0.03) c	162 (15)	91 (18) abc	253 (27)
P ANOVA					0.000	0.02	0.000	NS	0.004	NS
Grand mean	181				13.1 (0.5)	0.20 (0.01)	0.178 (0.009)	123 (5)	85 (2)	207 (5)

[a] Number of replicates for g_i, A_{max}, and C_i–C_c.
[b] Number of species for g_i, A_{max}, and C_i–C_c.
[c] Number of replicates for g_s, C_a–C_i, and C_a–C_c.
[d] Number of species for g_s, C_a–C_i, and C_a–C_c.

(vi) Can internal conductance respond rapidly to environmental variables?

(vii) Does internal conductance acclimate to environmental variables?

(viii) What mechanisms determine internal conductance?

(ix) What are priority areas for future research?

What is internal conductance and what should we call it?

Internal conductance (g_i) is referred to throughout the text, but one must be aware that over the past 20 or so years internal conductance has also been referred to as transfer conductance, (cell) wall conductance (g_w) and mesophyll conductance (g_m). Other authors prefer to think in terms of resistance rather than conductance, giving rise to such terms as internal resistance, transfer resistance, and mesophyll resistance. There is currently no consensus as to the best name for internal conductance; however, use of mesophyll conductance ought to be discouraged because mesophyll conductance has historically been used to describe several different parameters including the initial slope of an A/C_i curve, a parameter that is a function of biochemical activity and CO_2 diffusion within leaves.

Internal conductance is a much more complex pathway than is implied when one simply mentions the start and end points for the CO_2 molecules. It involves CO_2 moving through the complex intercellular air spaces of the mesophyll until it reaches a cell wall, at the cell wall it enters the liquid phase and diffuses across the cell wall, plasmalemma, cytosol, and chloroplast envelope before finally reaching the chloroplast stroma where it is carboxylated by Rubisco. The common measurement techniques do not permit measurements of these individual steps, and thus these discrete (and disparate) steps are lumped together and described by a single conductance. When interpreting internal conductance it must be remembered that it is a property of multiple resistances in series, and that any (or all) of these might be the cause of some observed property (e.g. the temperature response).

How is internal conductance estimated?

Early attempts at estimating internal conductance were beset by a variety of problems and thus internal conductance was to a large extent left in the 'too hard' basket. There were attempts to model the drawdown from C_i to C_c by considering path lengths for diffusion, areas for diffusion, and diffusion coefficients (Rackham, 1966; Nobel, 1991), but this approach was problematic because diffusion co-efficients are not generally known for plant cells and thus estimates were to a large extent a 'best guess'.

The past 20 years have seen the development of a number of methods to estimate internal conductance. The most popular methods involve simultaneous measurement of gas exchange with instantaneous carbon isotope discrimination (Evans *et al*., 1986), or gas exchange with chlorophyll fluorescence (Bongi and Loreto, 1989; Di Marco *et al*., 1990). These methods will only be described briefly because they were described in some detail by their originator(s) (Evans *et al*., 1986; Bongi and Loreto, 1989; Di Marco *et al*., 1990), and all have been reviewed recently (Warren, 2006*a*).

Estimation of internal conductance from carbon isotope discrimination is based on different diffusion and carboxylation rates of $^{12}CO_2$ and $^{13}CO_2$. $^{13}CO_2$ diffuses more slowly through the boundary layer (2.9‰) and stomata (4.4‰), slower through the liquid phase (1.8‰), and is carboxylated much more slowly than $^{12}CO_2$ (27–30‰) (Farquhar *et al*., 1982, 1989; Evans *et al*., 1986). The average 'discrimination' against $^{13}CO_2$ in photosynthesis depends on the relative weights of the boundary layer, stomatal, internal, and carboxylation resistances in the catena. Average discriminations are between −20‰ and −30‰ in C_3 plants, indicating the overriding effect of discrimination due to carboxylation and explaining why discrimination is proportional to C_c. Estimation of internal conductance from instantaneous carbon isotope discrimination requires simultaneous measurement of gas exchange and isotope discrimination.

A combination of chlorophyll fluorescence and gas exchange can be used to estimate internal conductance via two slightly different methods (Bongi and Loreto, 1989; Di Marco *et al*., 1990). Chlorophyll fluorescence estimates the electron transport rate, typically considering that a proportion of this rate is going to alternative electron acceptors and the majority is used in photosynthesis and photorespiration. The relative proportions of photosynthesis and photorespiration are a function of the substrate concentrations (i.e. CO_2 and O_2 in the chloroplast) and the relative specificity of Rubisco for CO_2 and O_2. Photorespiration can be estimated from the (measured) rates of electron transport and photosynthesis. C_c can be calculated from the rate of photorespiration and specificity of Rubisco, which then allows calculation of internal conductance.

The variant on this approach is to focus solely on points at high C_i at which photosynthesis is limited by RuBP regeneration and electron transport is invariant. Chlorophyll fluorescence is used to establish when rates of electron transport are invariant. Internal conductance can be calculated because the response of photosynthesis to CO_2 depends on C_c and the CO_2/O_2 specificity of Rubisco (Bongi and Loreto, 1989).

Instantaneous carbon isotope discrimination and the two chlorophyll fluorescence methods are the most popular means of estimating internal conductance, but there are

also a number of other methods. These other methods include one based on the difference between the chloroplastic (Γ^*) and intercellular (C_i^*) photocompensation points (von Caemmerer and Evans, 1991; Peisker and Apel, 2001), and another based on the reduction in initial slope of an A/C_i curve from its theoretical maximum (Evans and Terashima, 1988). Neither of these methods requires a mass spectrometer or chlorophyll fluorescence system. Photoacoustic measurements may be used to estimate oxygen diffusion through cells, and by inference the liquid-phase component of internal conductance to CO_2 (Gorton *et al*., 2003). Finally, internal conductance can be estimated from its effect on the curvature of an A/C_i curve using nothing more than A/C_i data (Ethier and Livingston, 2004; Ethier *et al*., 2006; Sharkey *et al*., 2007).

One promising approach that has received little attention is the possibility of using discrimination against ^{18}O of CO_2 to provide an estimate of internal conductance to the chloroplast surface (Peltier *et al*., 1995; Gillon and Yakir, 2000). ^{18}O discrimination in CO_2 is a function of oxygen exchange between ^{18}O-enriched water and CO_2 in the chloroplast, a process catalysed by carbonic anhydrase (CA). A proportion of this ^{18}O-labelled CO_2 escapes back to the atmosphere, resulting in an effective discrimination against $C^{18}OO$ during photosynthesis. Discrimination against ^{18}O allows one to estimate the CO_2 concentration at the sites of CO_2–H_2O equilibrium. It is commonly argued that the majority of CA activity resides within the chloroplast (Everson, 1970), and thus discrimination against ^{18}O may indicate the concentration of CO_2 at the chloroplast surface (Gillon and Yakir, 2000). A series of recent studies have shown that CA is not restricted to the chloroplast and is also found in the cytoplasm, plasma membrane, and mitochondria (Moroney *et al*., 2001; Fabre *et al*., 2007), which may have implications for the use of ^{18}O as an indicator of CO_2 concentration at the chloroplast surface. Nevertheless, if it is accepted that ^{18}O estimates CO_2 at the chloroplast surface, combined analysis of discrimination against ^{13}C and ^{18}O, can be used to determine conductance from the substomatal cavity to the chloroplast surface and from the chloroplast surface to sites of carboxylation.

How does internal conductance affect CO_2 diffusion, photosynthesis and leaf economics?

It is now known that internal conductance is finite and C_c is significantly less than C_i in all of the species so far examined (Evans *et al*., 1986; Lloyd *et al*., 1992; Epron *et al*., 1995; Warren *et al*., 2003). Among 50 species, the mean drawdown from C_i to C_c under saturating light at a C_a of around 360 μmol mol^{-1} is in the order of 85 μmol mol^{-1} (Table 1), versus 123 μmol mol^{-1} for the drawdown from C_a to C_i due to stomatal conductance. Hence, internal conductance accounts for around 40% of the decrease in CO_2 concentration between the atmosphere and sites of carboxylation.

Given that internal conductance results in a large decrease in CO_2 concentrations, it would be reasonable to expect that it has an equally large effect on photosynthesis. A growing number of studies are reporting relative stomatal and internal limitations, and in most cases the relative limitations due to internal and stomatal conductances are of a similar size in well-watered plants (Epron *et al*., 1995; Warren *et al*., 2003; Yamori *et al*., 2006). Typical relative limitations due to stomatal conductance or internal conductance are 20–40%. Hence, internal conductance is a very large limitation of photosynthesis, typically as large as that due to stomatal conductance.

Internal conductance is not only as large a limitation to photosynthesis as stomatal conductance, but it also affects aspects of leaf economics that are normally attributed to stomata and biochemical activity. Variation in C_i-C_c among species results in different rates of photosynthesis in plants with the same biochemical activity and stomatal conductance. Hence, finite internal conductance reduces rates of photosynthesis per unit nitrogen (Lloyd *et al*., 1992; Poorter and Evans, 1998; Warren and Adams, 2006) and per unit water lost (Evans and von Caemmerer, 1996).

Does the limitation due to internal conductance differ among species?

Among species there is a positive relationship between rates of photosynthesis and internal conductance (von Caemmerer and Evans, 1991; Harley *et al*., 1992*a*; Loreto *et al*., 1992; Epron *et al*., 1995; Warren *et al*., 2003), but there is wide variation in this relationship (Fig. 2). For example, at an A_{max} of 10 μmol m^{-2} s^{-1}, internal conductance varies between 0.06 and 0.31 mol m^{-2} s^{-1}. This 5-fold variation in internal conductance is much greater than can be attributed to the precision of internal conductance estimates which is in the order of 20% (Warren, 2006*b*). Therefore, a large proportion of variation in internal conductance is not explained by A_{max} or by the poor precision of estimation techniques. When the relationship of internal conductance with A_{max} is broken down further, it is apparent that relationships vary among species (Hanba *et al*., 2001) and may be weak or non-existent within species (Warren, 2004).

Because the relationship of internal conductance with photosynthesis is not fixed, there is large variation among species in the relative limitation due to internal conductance (e.g. as indicated by C_i–C_c) (Table 1; see also Warren and Adams, 2006). At any chosen g_i, values of C_i–C_c differ by >50 μmol mol^{-1} among species (Fig. 3). In addition to the large variability in C_i–C_c at any given internal conductance, there is a systematic trend insofar as

C_i–C_c is greater for species with small internal conductance. Hence, while it is possible to calculate a mean drawdown from C_i to C_c (e.g. 85 μmol mol^{-1}; Table 1), this is largely misleading given the known variability among species.

It has been claimed that one of the reasons for slow photosynthesis and poor nitrogen-use efficiency of sclerophytes is that the limitation due to internal conductance is greater than in sclerophytes (Lloyd *et al*., 1992; Warren and Adams, 2004), perhaps due to the thicker cell walls required for long-lived stress-tolerant leaves. Analysis of data current to 2007 shows that C_i–C_c is indeed larger, on average, in sclerophytes than non-sclerophytes (Figs 3, 4).

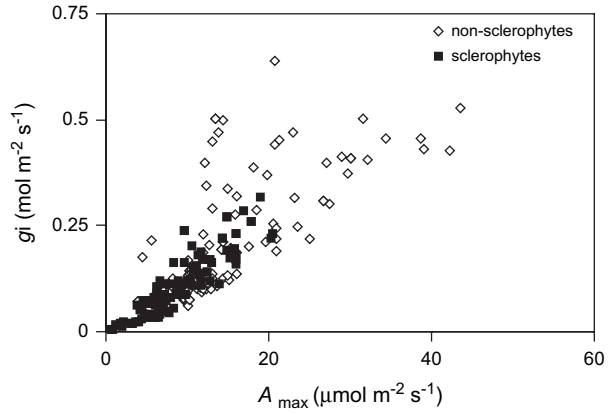

Fig. 2. The relationship between light-saturated rate of net photosynthesis (A_{max}) and internal conductance (g_i) in non-sclerophytes (open symbols) and sclerophytes (closed symbols). Data are based on the literature review of Ethier and Livingston (2004), updated with recently published data (see Table 1 for details). Input data were mean values for each species/treatment combination, rather than individual measurements. n=112 non-sclerophytes, 114 sclerophytes.

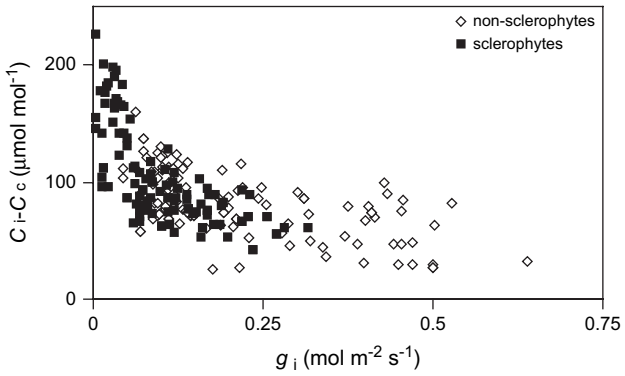

Fig. 3. Relationship between the drawdown in CO_2 concentrations from substomatal cavities to sites of carboxylation (C_i–C_c) and internal conductance (g_i) in non-sclerophytes (open symbols) and sclerophytes (closed symbols). Data are based on the literature review of Ethier and Livingston (2004) updated with recently published data (see Table 1 for details). Input data were mean values for each species/treatment combination, rather than individual measurements. n=112 non-sclerophytes, 114 sclerophytes.

Moreover, internal conductance (C_i–C_c) rather than stomatal conductance (C_a–C_i) is the cause of smaller concentrations of CO_2 at the sites of carboxylation and thus contributes to the slower photosynthesis of sclerophytes than sclerophytes (Fig. 4). There are also small yet significant differences in C_i–C_c between herbaceous and woody species and even between woody deciduous species and woody evergreen species (Table 1). Once again, it is worth bearing in mind that these differences among functional groups are rather small in comparison to the large variation within functional groups (e.g. Fig. 3 and see also Ethier and Livingston, 2004).

Does internal conductance affect interpretation and fitting of A/C_i responses to the biochemical model of Farquhar *et al.* (1980)?

The significant drawdown from C_i to C_c has implications for fitting of gas exchange data to the biochemical model of Farquhar and co-workers (Farquhar *et al*., 1980). The biochemical model states that the rate of net photosynthesis is limited by the maximum rate of Rubisco-limited carboxylation (V_{cmax}) or RuBP regeneration limited rates of electron transport (J), with the limitation shifting from V_{cmax} to J as CO_2 concentration increases. The traditional calculation of V_{cmax} and J from A/C_i curves assumes that there is no drawdown from C_i to C_c (i.e. internal conductance is infinitely large) (Long and Bernacchi, 2003). One consequence of the drawdown from C_i to C_c is that V_{cmax} and J calculated from C_i are not solely biochemical parameters, as is assumed, but also contain 'information' pertaining to internal conductance (Epron *et al*., 1995; Ethier and Livingston, 2004). Calculating V_{cmax} and J on a C_i basis leads to substantial underestimation of the 'true' V_{cmax} or J, if the same kinetic constants are used. In the case of V_{cmax}, the true V_{cmax} is,

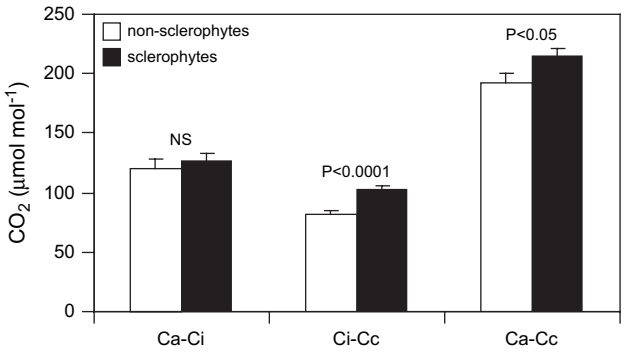

Fig. 4. Mean draw-downs in CO_2 concentrations from atmosphere (C_a) to substomatal cavities (C_i) to sites of carboxylation (C_c) in non-sclerophytes and sclerophytes. Data are based on the literature review of Ethier and Livingston (2004), updated with recently published data (see Table 1 for details). Input data were mean values for each species/treatment combination, rather than individual measurements. Data are means. Error bars are 1 SE (n=112 non-sclerophytes, 114 sclerophytes).

on average, 1.6 times V_{cmax} on a C_i basis (Fig. 5). The picture is not so clear for J because there are fewer data, but it is nevertheless apparent that C_i-based J is smaller than C_c-based J (Fig. 5).

The drawdown from C_i to C_c affects the choice of kinetic constants used to calculate V_{cmax}, but if the right kinetic constants are used it is possible to estimate V_{cmax} correctly from C_i or C_c. Choice (and interpretation) of kinetic constants (K_c, K_o, Γ^*) for C_c-based V_{cmax} is comparatively straightforward, one may use either *in vitro* values (Jordan and Ogren, 1984), or *in vivo* values determined on a C_c basis (von Caemmerer *et al*., 1994; Bernacchi *et al*., 2002). For C_i-based V_{cmax} the appropriate kinetic constants are a function of the relationship between internal conductance and photosynthetic capacity (V_{cmax}) (i.e. the drawdown from C_i to C_c). If these 'apparent' kinetic constants are known then V_{cmax} can be correctly estimated from C_i.

The problem with estimating V_{cmax} from A/C_i data and apparent kinetic constants is that apparent kinetic constants are not in fact 'constant', but instead differ among and within species. This problem has been discussed at length (Ethier and Livingston, 2004; Warren and Dreyer, 2006), but is perhaps best understood by using a simple example. Widely used C_i-based kinetic constants (apparent K_c, apparent K_o, C_i^*) (von Caemmerer *et al*., 1994; Bernacchi *et al*., 2001) are only appropriate for plants that have the same relationship between internal conductance and photosynthetic capacity (i.e. same C_i–C_c). This is problematic given that there is wide variation among species in C_i–C_c (Table 1; Fig. 3) and thus C_i-based kinetic constants are not universally applicable.

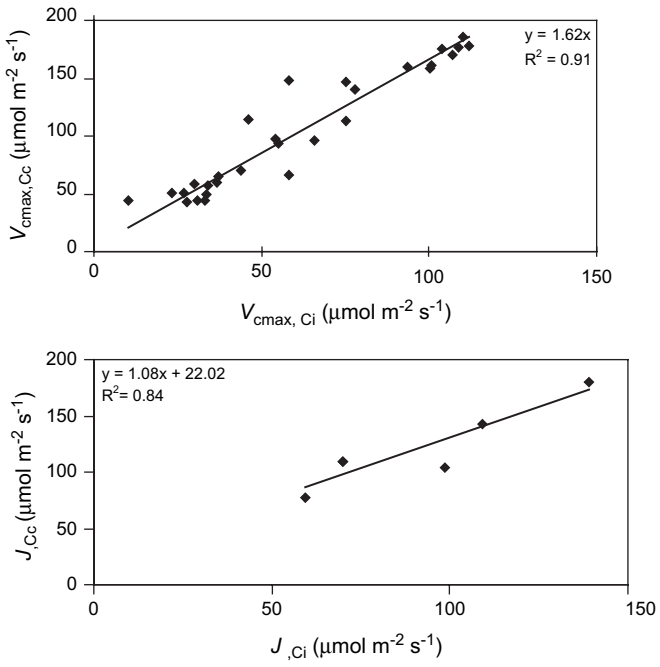

Fig. 5. The relationship between V_{cmax} and J calculated on a C_i basis with V_{cmax} and J calculated on a C_c basis. V_{cmax} data were from Epron *et al*. (1995), Piel *et al*. (2002), Warren *et al*. (2003, 2007), Grassi and Magnani (2005), Ethier *et al*. (2006), and Flexas *et al*. (2006*b*). J data were from Epron *et al*. (1995), Piel *et al*. (2002), and Flexas *et al*. (2006*b*). Input data were mean values for each species/treatment combination, rather than individual measurements. Note that C_i-based and C_c-based V_{cmax} and J were calculated with the same kinetic constants.

Can internal conductance respond rapidly to environmental variables?

One of the largest revelations in the past decade has been the recognition that internal conductance is not only a constitutive property of leaf anatomy, but also exhibits short-term responses (minutes) and longer-term acclimation (days) to environmental variables (Table 2). Comparatively little is known about the rate of change in internal conductance, compared with the voluminous literature examining how quickly stomatal conductance changes (Valladares *et al*., 1997; Tausz *et al*., 2005). Nevertheless, it would appear that internal conductance can change significantly within 5 or 10 min (e.g. in response to CO_2 concentration or water stress) (Flexas *et al*., 2007). What follows is a quick summary of the short-term responses of internal conductance to environmental variables. The mechanisms and interpretation of short-term responses and longer-term acclimation are probably different and thus longer-term acclimation is presented in a separate, later section.

Water and salt stress

A large number of papers on a variety of species have shown that water and salt stress cause reductions in stomatal conductance and internal conductance (Cornic *et al*., 1989; Roupsard *et al*., 1996; Delfine *et al*., 1999; Flexas *et al*., 2002; Loreto *et al*., 2003) (Fig. 6). It is probable that this effect of water and salt stress on internal conductance is ubiquitous, and, in general, reductions in internal conductance are of a similar size to reductions in stomatal conductance. Reductions in internal and stomatal conductances may well be proportional, but it remains unclear if the relationship between internal and stomatal conductances (under water or salt stress) is linear, as observed in olives (Centritto *et al*., 2003), non-linear as found for field-grown grapevines (Flexas *et al*., 2002), or if it varies among species or functional groups.

An interesting implication of drought- or salt-induced reductions in internal conductance is that they may, at least partially, explain non-stomatal limitations of photosynthesis. Non-stomatal limitations are reductions in photosynthesis that are not explained by stomatal conductance. In practice, non-stomatal limitations are implied from reductions in photosynthesis at a common C_i, or a reduction in the slope of the A/C_i relationship. Normally,

Table 2. *Effect of environmental variables on instantaneous internal conductance (g_i) and stomatal conductance (g_s)*

Variable	Effect on g_i	Effect on g_s	Examples
Soil waterstress	Decrease	Decrease	Cornic *et al*., 1989; Roupsard *et al*., 1996; Delfine *et al*., 2001; Flexas *et al*., 2002; Warren *et al*., 2004; Terashima *et al*., 2006
Salinity	Decrease	Decrease	Delfine *et al*., 1998; Delfine *et al*., 1999; Loreto *et al*., 2003; Parida *et al*., 2004
Leaf-to air vapour pressure deficit	No effect (?)[a]	Decrease	C Warren, unpublished data
Temperature	3-fold increase from 10 °C to 30 °C, may plateau from 20–35 °C, may decrease at temperatures >35 °C		Bernacchi *et al*., 2002; Warren and Dreyer, 2006; Yamori *et al*., 2006; Warren, 2007
Elevated CO_2	Decrease	Decrease	Centritto *et al*., 2003; During, 2003; Flexas *et al*., 2007
Subambient CO_2	Increase	Increase	Centritto *et al*., 2003; During, 2003; Flexas *et al*., 2007
Light intensity	Not known	Increase	

[a] Tested for one species only.

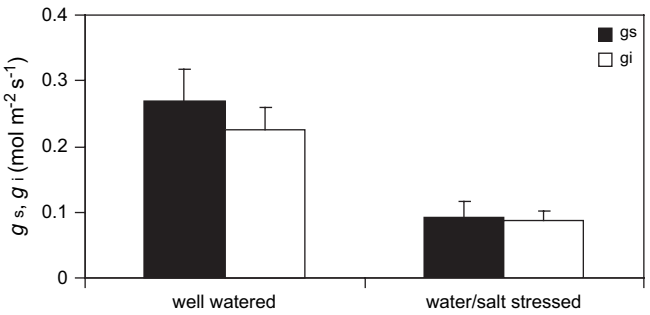

Fig. 6. The mean effect of drought and/or salt stress on stomatal conductance and internal conductance in nine different species. Data are from Delfine *et al*. (1999, 2001, 2002), Flexas *et al*. (2002, 2006*a*), Centritto *et al*. (2003), Parida *et al*.(2004), Warren *et al*. (2004), and Grassi and Magnani (2005). Input data were mean values for each species/treatment combination, rather than individual measurements. Water- and salt-stressed data are for the most extreme treatments. Error bars are 1 SE (n=12 measurements, n=9 species).

reductions in the slope of the A/C_i relationship are purported to be '*prima facie* evidence of inhibition of A by altered metabolism' (Boyer, 1971; Quick *et al*., 1992; Lawlor, 2002). However, non-stomatal limitations may indicate reduced internal conductance rather than an effect on mesophyll metabolism. In support of this idea, several authors have shown that non-stomatal limitations of photosynthesis can, in fact, be explained by reduced internal conductance (Flexas *et al*., 2002; Warren *et al*., 2004; Grassi and Magnani, 2005).

Temperature

Temperature is one source of variation in internal conductance that has received little attention, despite these data being critical for photosynthesis models, the correct determination of V_{cmax}, and understanding the major limitations of photosynthesis (Bernacchi *et al*., 2002). The temperature response of internal conductance was first measured in *Nicotiana tabacum* (Bernacchi *et al*., 2002), but has since been measured in *Quercus canariensis* (Warren and Dreyer, 2006), *Spinacia oleracea* (Yamori *et al*., 2006), and *Eucalyptus regnans* (Warren, 2007). All of these studies have shown that internal conductance increases from 10–20 °C (Fig. 7). At temperatures greater than 20 °C the temperature responses of internal conductance differ among species.

One consequence of the differing temperature responses of internal conductance is that different mathematical models are required to describe the data (Table 3). The shape of temperature responses is simply so divergent that no single equation can fit all four species well. *Nicotiana tabacum* and *Quercus canariensis* are described well by an Arrhenius-type equation with a de-activation term. However, in the case of *Q. canariensis* a better and more parsimonious fit can be found with a 3-parameter log-normal, the same equation which gave an excellent fit to data of *S. oleracea*. Additional data from other species are required before generalizations can be made about the shape of the temperature response of internal conductance. For example, it may be the case that the temperature response varies among functional groups of plants, but until there are many more data a clear trend will not emerge.

Carbon dioxide concentration

At least two studies have shown that elevated CO_2 causes internal conductance to decrease and sub-ambient CO_2 causes internal conductance to increase (Table 2) (During, 2003; Flexas *et al*., 2007), similar trends to what is commonly observed with stomatal conductance. During (2003), for example, reported a 6-fold decrease in internal conductance as CO_2 concentration increased 50 μmol to 2000 μmol mol^{-1}. Using a somewhat different approach, Centritto *et al*. (2003) showed that internal conductance

measured at a reference CO_2 concentration of 350 μmol mol^{-1} is affected by exposure to sub and supra-ambient CO_2 concentrations.

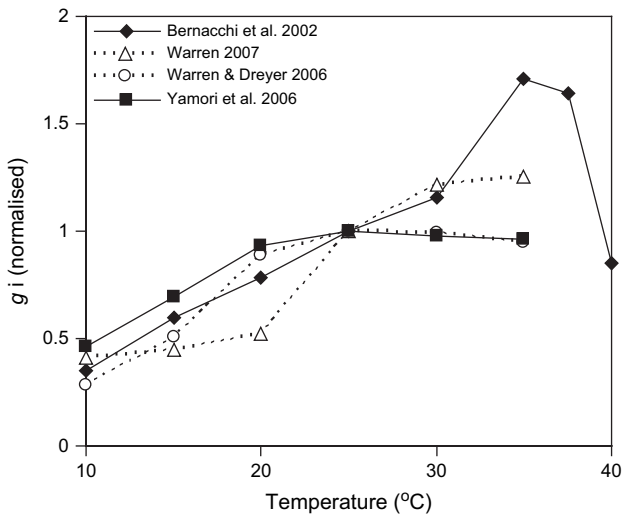

Fig. 7. The temperature response of internal conductance in four species. Data are for *Nicotiana tabacum* (Bernacchi *et al*., 2002), *Quercus canariensis* (Warren and Dreyer, 2006), *Spinacia oleracea* (Yamori *et al*., 2006), and *Eucalyptus regnans* (Warren, 2007). Some studies using the variable *J* method also used alternative methods (Bernacchi *et al*., 2002; Warren and Dreyer, 2006; Warren, 2007), but for the sake of consistency results are only shown of the variable *J* method. In *E. regnans* data are the mean of plants acclimated to high and low temperature while in the case of *S. oleracea* data are only shown for plants acclimated to high temperatures. Internal conductance was normalised to 25 °C and data points are means. Curve fits to these data are shown in Table 3.

Does internal conductance acclimate to environmental variables

Leaf age

Studies with herbaceous and woody plants have found that internal conductance increases during leaf development up to the point of full leaf expansion, and then declines as leaves age and/or senesce (Loreto *et al*., 1994; Hanba *et al*., 2001; Niinemets *et al*., 2005; Ethier *et al*., 2006). In general, changes in internal conductance are correlated with changes in photosynthesis, i.e. photosynthesis and internal conductance increase to full leaf expansion and then both decrease thereafter (Loreto *et al*., 1994; Hanba *et al*., 2001; Niinemets *et al*., 2005; Ethier *et al*., 2006).

Temporal trends in internal conductance and photosynthesis through a leaf's lifespan are broadly similar, but results differ as to whether the limitation due to internal conductance is constant throughout a leaf's lifespan. Several recent papers have asked the question of whether internal conductance is the cause of decreasing photosynthesis and rates of photosynthesis per unit N in ageing foliage (Ethier *et al*., 2006; Warren, 2006*b*). In two evergreen conifers it was argued that, in ageing foliage, internal conductance scaled with photosynthetic capacity and thus did not contribute to age-related declines in photosynthesis (Ethier *et al*., 2006; Warren, 2006*b*). By contrast, experiments with broadleaf evergreens and deciduous trees showed that the photosynthetic limitation imposed by internal conductance increased with leaf age

Table 3. *Mathematical fits to the temperature response of internal conductance in four species (Fig. 7)*

Some studies using the variable *J* method also used alternative methods (Bernacchi *et al*., 2002; Warren and Dreyer, 2006; Warren, 2007), but for the sake of consistency results are only shown of the variable *J* method. Curves were fitted to mean values. In *E. regnans* data are the mean of plants acclimated to high and low temperature while in the case of *S. oleracea* data are only shown for plants acclimated to high temperatures. Three curve fits were used: Arrhenius equations with and without de-activation terms [scaling constant (c), enthalpy of activation (H_a), de-activation (H_d), and entropy (Δ_s) Harley *et al*., 1992*b*] and a three-parameter log normal ($g_i = -0.5 \times (\ln(T\ °C/T_{max}\ °C)/y)^2$ (Warren and Dreyer, 2006). Standard errors of fits are shown in parentheses, while the goodness of fit (R^2) is shown in the final column. Please note that fits to data were in many cases weak and unreliable, such poor fits are marked by an X in the final column.

Author	Species	Method	c	H_a	Δ_s	H_d	R^2
Arrhenius with de-activation term							
Bernacchi *et al*., 2002	*Nicotiana tabacum*	Variable *J*	16 (2)	41 (4)	0.5 (0.1)	383 (98)	0.98
Warren and Dreyer, 2006	*Quercus canariensis*	Variable *J*	57 (15)	136 (37)	0.11 (0.02)	74 (11)	0.89
Yamori *et al*., 2006	*Spinacia oleracea*	$\delta^{13}C$	64 (452)	149 (1048)	0.1 (0.7)	65 (460)	0.99 X
Warren, 2007	*Eucalyptus regnans*	Variable *J*	22 (651)	53 (1366)	0.02 (0.3)	15 (196)	0.91 X
Combined data of four species		N/A	12 (2)	30 (4)	1 (15)	996 (12370)	0.77 X
Arrhenius without de-activation term							
Bernacchi *et al*., 2002	*Nicotiana tabacum*	Variable *J*	9 (4)	23 (10)			0.55 X
Warren and Dreyer, 2006	*Quercus canariensis*	Variable *J*	9 (4)	23 (9)			0.67 X
Yamori *et al*., 2006	*Spinacia oleracea*	$\delta^{13}C$	6 (2)	15 (6)			0.67 X
Warren, 2007	*Eucalyptus regnans*	Variable *J*	14 (3)	36 (7)			0.89
Combined data of 4 species		N/A	10 (2)	25 (4)			0.64 X
Three parameter log-normal			y	T_{max}°C			
Bernacchi *et al*, 2002	*Nicotiana tabacum*	Variable *J*	0.9 (0.8)	35 (27)			0.38 X
Warren and Dreyer, 2006	*Quercus canariensis*	Variable *J*	0.61 (0.05)	28 (2)			0.98
Yamori *et al*., 2006	*Spinacia oleracea*	$\delta^{13}C$	0.82 (0.05)	28 (1)			0.99
Warren, 2007	*Eucalyptus regnans*	Variable *J*	0.8 (0.4)	36 (15)			0.72 X
Combined data of four species		N/A	0.8 (0.2)	35 (8)			0.61 X

and was partially responsible for age-related decreases in photosynthesis (Hanba *et al*., 2001; Miyazawa and Terashima, 2001; Niinemets *et al*., 2005, 2006).

The effect of age on internal conductance has been related to anatomical traits such as the surface area of chloroplasts facing the substomatal cavity (Hanba *et al*., 2001). These anatomical changes might be a true effect of leaf age (e.g. senescence and/or remobilization of nutrients to younger leaves) or partly driven by the environmental variables that change with leaf age. For example, young expanding foliage is on the periphery of canopies and thus receives full sunlight, but as foliage ages it occupies successively more shaded locations as new foliage is added to the outside of the canopy. Leaf temperatures, VPD, and CO_2 concentrations may also change as foliage ages and moves from the outside to the inside of the canopy. At the same time there may be a loss of hydraulic conductivity (Melcher *et al*., 2003). Hence, age is a complex factor and it may well be the case that differing responses of internal conductance to age are because changes in the underlying environmental factors differ among species, or that species respond differently to the underlying factors.

Light environment

A handful of experiments have shown that internal conductance acclimates to the light environment. Differences in internal conductance between sun and shade leaves are in the same direction as those in photosynthesis and stomatal conductance (Warren *et al*., 2007). That is, sun leaves are characterized by greater internal and stomatal conductances and faster rates of photosynthesis than shade leaves (Terashima *et al*., 2006). Internal conductance is approximately twice as large in sun leaves than shade leaves of *Fagus sylvatica* (Warren *et al*., 2007) and *Juglans regia* (Piel *et al*., 2002), which is much larger than the modest 20–35% differences reported for *Prunus persica* and *Citrus paradisi* (Lloyd *et al*., 1992) and *Pseudotsuga menziesii* (Warren *et al*., 2003). The reason the difference between sun and shade leaves is large in some species but small in others is that the size of differences is a function of the size of differences in photosynthesis, stomatal conductance, and leaf anatomical and morphological traits (Lloyd *et al*., 1992; Warren *et al*., 2007). The net result of this scaling of internal conductance with photosynthesis is that the relative limitation due to internal conductance generally does not vary between sun and shade leaves (Warren *et al*., 2007).

Acclimation to other environmental variables

Generally speaking, little is known about acclimation of internal conductance to the myriad other environmental variables. Two experiments have examined whether internal conductance acclimates to growth temperature, but these gave conflicting results (Yamori *et al*., 2006; Warren, 2007), while two experiments examining acclimation to CO_2 concentration found that effects on internal conductance were species and experiment-dependent (Singsaas *et al*., 2004; Bernacchi *et al*., 2005). Given the profound effect of internal conductance on photosynthesis and the prevalence of prolonged drought stress and salt stress it is imperative that we know whether internal conductance acclimates to these and other environmental variables. Unfortunately, little is known about acclimation of internal conductance to long-term differences in drought or salt stress.

What mechanisms determine internal conductance?

Most available evidence suggests that the bulk of the resistance to CO_2 movement is in the liquid phase. The gas phase conductance can be estimated by contrasting gas exchange in normal air with air in which the nitrogen has been replaced by helium ('helox') in which CO_2 diffuses 2.3 times more quickly. Parkhurst and Mott (1990), for example, found that rates of photosynthesis of six amphistomatous species were, on average, 2% faster in helox than air; whereas photosynthesis of five hypostomatous species were 12% faster in helox than air. What this means is that gas phase conductance is a negligible limitation in amphistomatous leaves and even in hypostomatous leaves the limitation is modest.

A major influence on our understanding of what limits internal conductance has been studies, over the past decade, showing that internal conductance can change rapidly (e.g. due to drought or leaf temperature). It used to be thought that internal conductance was constant over periods of 1 d (Evans and von Caemmerer, 1996), which went hand-in-hand with views that leaf anatomy and morphology were the principal determinants of internal conductance (Lloyd *et al*., 1992; Evans *et al*., 1994; Syvertsen *et al*., 1995). In a very general sense, it is known that the potential for CO_2 diffusion in the liquid phase is a function of cell wall thickness (Nobel, 1991; Miyazawa and Terashima, 2001) and the surface area of mesophyll cells or chloroplasts exposed to the intercellular air spaces (Laisk *et al*., 1970; Nobel *et al*., 1975; Evans *et al*., 1994). Now that it is known internal conductance can change more rapidly than leaf anatomy and morphology, the search has turned to the biochemical factors that also determine internal conductance.

It is also now known that liquid-phase conductance is not solely physical diffusion but also has a biochemical component. There are two promising candidates to fill the role: carbonic anhydrase and aquaporins. Carbonic anhydrase in plants exists in three different classes (α, β, γ) with the β class being the most abundant. β-carbonic anhydrase is approximately 5% of the protein in the

chloroplast stroma (Fabre *et al*., 2007). The first experiments showing a role of carbonic anhydrase in CO_2 movement were experiments with tobacco in which antisense technology was used to reduce the amounts of β-carbonic anhydrase to 1–10% of wild-type plants (Majeau *et al*., 1994; Price *et al*., 1994). In plants with reduced amounts of β-carbonic anhydrase the concentration of CO_2 in the chloroplast was decreased (Price *et al*., 1994). An analogous reduction in internal conductance and amounts of carbonic anhydrase was reported in leaves of Zn-deficient rice (Sasaki *et al*., 1998). These results provide evidence that β-carbonic anhydrase plays a role in CO_2 transport (Price *et al*., 1994).

Aquaporins have been implicated in CO_2 movement in a number of recent studies (Terashima and Ono, 2002; Uehlein *et al*., 2003; Hanba *et al*., 2004; Flexas *et al*., 2006*b*). Aquaporins are the most abundant proteins in plant plasma membranes, they predominantly transfer H_2O, but studies with animals have shown that some aquaporins can transfer H_2O and CO_2 (Nakhoul *et al*., 1998). In plants, it also seems that at least some aquaporins are involved with transporting CO_2. Terashima and Ono (2002) showed that $HgCl_2$, a non-specific inhibitor of aquaporins, reduced internal conductance. Experiments with metabolic inhibitors are open to criticism, but there is also evidence from experiments with transgenics that make a case for aquaporins. Overexpression of barley aquaporin HvPip2;1 in transgenic rice increased internal conductance by 40% compared with control leaves (Hanba *et al*., 2004). Leaf anatomy and morphology were also significantly affected, which makes it difficult to interpret whether the effect of aquaporins on internal conductance is actually due to effects on leaf anatomy and morphology. Experiments with antisense tobacco depleted in NtAQP1 and NtAQP1 overexpressing tobacco also found that changes in aquaporin content were related to changes in internal conductance (Flexas *et al*., 2006*b*). In tobacco, there were no effects on leaf anatomy and morphology, but there were differences in the A/C_c responses of photosynthesis between control and transgenic tobacco. This indicates that transgenics and controls were not 'identical' and there were effects on photosynthesis that were unrelated to internal conductance. Hence, it is possible that the differences in internal conductance were not entirely due to aquaporins, but were also a function of whatever was causing the differences in the A/C_c response.

There need not be any controversy regarding the relative roles of leaf anatomy versus biochemical factors, the two are not mutually exclusive and it is probable that both are involved in the liquid-phase component of internal conductance. As argued by Terashima *et al*. (2005), liquid-phase conductance is a function of the surface area of chloroplast appressed to intercellular spaces, cell wall thickness, and biochemical factors. A challenge that remains is to determine which biochemical factors underpin the liquid phase of internal conductance and how this interacts with anatomical factors to determine internal conductance. Additional experiments are required to determine the relative roles of carbonic anhydrase and aquaporins in liquid-phase conductance. For example, experiments are required on all the isoforms of β-carbonic anhydrase, and for both carbonic anhydrase and aquaporins experiments are needed on other species. In all cases, greater emphasis must be placed on teasing apart effects due to carbonic anhydrase or aquaporins from unavoidable (?) pleiotropic effects.

What are priority areas for future research?

The internal conductance story is intriguing and far from complete. There are many unanswered questions and significant challenges. The next decade will hopefully see answers to some of the following questions:

(i) Can we tease apart the different steps in the diffusion pathway so as to improve our mechanistic understanding?

(ii) Is internal conductance correlated with or functionally related to transpiration (e.g. via aquaporins)?

(iii) Which environmental variables does internal conductance acclimate to?

(iv) Which of the many carbonic anhydrases are involved with internal conductance?

(v) What are the relative roles of carbonic anhydrase(s) versus aquaporins?

(vi) Can we increase internal conductance via conventional plant breeding or molecular methods, and will this translate into faster photosynthesis and growth?

Conclusions

For photosynthesis to occur CO_2 must diffuse from the atmosphere to sites of carboxylation. Stomatal conductance describes the first step in the diffusion path from the atmosphere to substomatal cavities, while internal conductance describes the second part of the diffusion path from substomatal cavities to sites of carboxylation. The past five or so decades have seen several thousand papers published on stomatal conductance (versus fewer than 200 on internal conductance). We now have a pretty good idea as to how stomatal conductance affects photosynthesis and is affected by environmental variables. It is now widely accepted that stomatal conductance is one of the primary determinants of photosynthesis and is thus incorporated into most models of canopy and ecosystem CO_2 exchange.

While our knowledge of internal conductance is still in its infancy, we now know that it ought to be sharing centre stage with stomatal conductance. Internal conductance is approximately as large a limitation of photosynthesis and internal conductance is affected by at least some environmental variables. For these reasons, internal conductance ought to be incorporated into models of canopy and ecosystem CO_2 exchange alongside stomatal conductance.

Like stomatal conductance, the limitation of photosynthesis due to internal conductance varies among and with in species. This finding gives some hope that it might be possible to select for plants with greater internal conductance, and thus ameliorate the large limitation internal conductance poses to photosynthesis, water-use efficiency, and rates of photosynthesis per unit nitrogen. Recent experiments with transgenic plants over-expressing aquaporins give some hope that it may be possible to increase internal conductance via molecular means while less dramatic gains might also be achieved by plant breeding.

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