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An improved theory for calculating leaf gas exchange more precisely accounting for small fluxes

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The widely used theory for gas exchange proposed by von Caemmerer and Farquhar (vCF) integrates molar fluxes, mole fraction gradients and ternary effects but does not account for cuticular fluxes, for separation of the leaf surface conditions or for ternary effects within the boundary layer. The magnitude of cuticular conductance to water (g_{cw}) is a key factor for determining plant survival in drought but is difficult to measure and often neglected in routine gas exchange studies. The vCF ternary effect is applied to the total flux without the recognition of different pathways that are affected by it. These simplifications lead to errors in estimations of stomatal conductance, intercellular carbon dioxide concentration (C_i) and other gas exchange parameters. The theory presented here is a more precise physical approach to the electrical resistance analogy for gas exchange, resulting in a more accurate calculation of gas exchange parameters. Additionally, we extend our theory, using physiological concepts, to create a model that allows us to calculate cuticular conductance to water.

Gas exchange between a leaf and the atmosphere occurs simultaneously through two pathways: (1) epidermal cells and cuticular waxes (henceforth referred to as 'cuticle') which are always present at each leaf surface and (2) stomata, which typically control most of the exchange. The gradients that drive the gas exchange are the differences between concentrations inside the leaf and the surfaces of the leaf, and between the leaf surfaces and the external air. The ternary effect applies to the fluxes between the surfaces of the leaf and the external air and to the fluxes crossing the stomata but not to the fluxes crossing the cuticle. However, in the von Caemmerer and Farquhar[1] theory (vCF), the gas exchange is considered as fluxes passing through the stomata and the boundary layer with direct connection between the inside and the external environment, and ternary corrections are applied to the whole flux.

In calculations of gas exchange parameters, measurements of transpiration rate are used to estimate conductances to water and those estimations are widely used to calculate conductances to CO_2 (ref. [2]). Thus, to differentiate the fluxes from the leaf surfaces, it is necessary to define the cuticular conductance to H_2O (g_{cw}) and stomatal conductance to H_2O (g_{sw}). However, it is typically impracticable to identify g_{cw} and g_{sw} separately when the flux of water occurs through both at the same time[3]. Then, as stomata dominate gas exchange, g_{cw} is for convenience commonly treated as being negligible[4].

Overlooking g_{cw} is problematic because, in some cases, it can be as high as 28% of the maximum stomatal conductance[5] or the stomatal conductance to H_2O vapour (g_{sw}) can be low enough to magnify the importance of g_{cw} (ref. [6]). Additionally, cuticular transpiration is important under dry conditions when it contributes to desiccation and death of leaves whose stomata are already closed[7,8]. In practice, the assumption that cuticular conductance is negligible leads to the miscalculation of stomatal water fluxes and CO_2 fluxes deduced from them, including the internal CO_2 concentration (C_i) and all the subsequent calculations that involve these parameters[9] (for example, mesophyll conductance[10]). Thus, cuticular conductance to water is a key unknown when calculating gas exchange parameters more precisely[9,11,12].

Cuticular conductance is often estimated from measurements of leaf conductance when stomata are tending to close, such as in darkness[13], obtaining the 'minimum' leaf conductance. The 'minimum' leaf conductance is, however, the sum of the cuticular conductance and additions from stomata which may be incompletely closed[13]. This situation can be improved by adding abscisic acid (ABA) to promote stomatal closure, so that gas exchange becomes dominated by the cuticle[11]. An additional measurement difficulty is that cuticular conductance changes with leaf water status[6,14]. Boyer[14] observed that when the leaf dries out, epidermal cells shrink, affecting the cuticular conductance to H_2O and CO_2. This highlights the importance of obtaining values of cuticular conductance under the same conditions as those of the experiment being conducted or, better, during the measurements themselves.

Boyer[11] and Tominaga et al.[15] presented a technique to estimate cuticular conductance to water from the difference between two estimations of conductance to CO_2. One from direct measurements of C_i from the abaxial surface ($g_{tc\text{-}m}$) and a second using vCF calculations of total conductance to CO_2 through the adaxial surface ($g_{tc\text{-}vCF}$) (ref. [12]) as $g_{cw} = 1.6 \times (g_{tc\text{-}vCF} - g_{tc\text{-}m})$, on the basis of the tacit assumption of adaxial and abaxial C_i being equal. Equations used by Tominaga[12] and Boyer[11] differ slightly but both are based on the von Caemmerer and Farquhar[1] (vCF) theory. This is a convenient simplification to obtain a g_{cw} value where it is assumed that the difference between the calculated and measured g_{tc} is only due to the vCF theory neglecting cuticular conductance to water. However, the vCF theory does not include any variable describing H_2O and CO_2 fluxes through the cuticle, nor the interaction between cuticular fluxes and stomatal and boundary layer fluxes.

In our study, a theory of gas exchange is developed including cuticular fluxes and their interaction with the other gas exchange parameters, the conditions at the leaf surface and ternary corrections in the boundary layer. To evaluate the theory proposed here, a model to calculate cuticular conductance to water was derived from the theory and a set of experiments was performed to obtain g_{cw} using our model. These experiments were largely performed on a

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system created by S.C. Wong[16,17] that measures adaxial and abaxial gas exchange independently (system 1). Further tests were made using an inhouse setup of two LI-6800s that emulate system 1 dual measurements.

Here, we examine the basis of usual leaf gas exchange calculations, then introduce the main theory proposed for calculation of gas exchange parameters. Finally, we extend the theory by adding physiological assumptions to create a model that allows us to calculate cuticular conductance. The technique and the assumptions made are then examined.

A list of the abbreviations and subscripts used in this paper is shown in Supplementary Note 1.

The basis of current theory of leaf gas exchange

The total conductance to H_2O (g_{tw}) is conventionally estimated from the evaporative flux[2,18] (E_T) by using equations introduced by von Caemmerer and Farquhar[1] (vCF). The vCF theory was the first to combine the use of molar fluxes, mole fraction gradients and ternary corrections (including the effect of the evaporative flux on itself and on CO_2 fluxes) but is silent regarding g_{cw} and gas concentrations at leaf surfaces.

$$g_{\mathrm{tw-vCF}} = \frac{E_T \cdot \left(1 - \frac{w_i + w_a}{2}\right)}{w_i - w_a} = \frac{E_T \cdot (1 - \bar{w})}{\Delta w} \qquad (1)$$

The main assumptions in equation (1) are that: the total gas exchange occurs through a single pathway between the inside of the leaf and the atmosphere; the ternary correction is applied to the whole flux; and the gradient that drives the exchange is that between the internal (w_i) and external ambient (w_a) concentrations. Equation (1) does not separate stomatal, cuticular and boundary layer fluxes for calculating g_{tw}. However, in practice, E_T is the sum of water vapour passing through the stomata (E_s) and the cuticle (E_c)

$$E_T = E_s + E_c \qquad (2)$$

and the last is not affected by ternary effects as are the stomata.

In the electrical resistance analogue to leaf gas exchange, the total conductance to H_2O and CO_2 includes stomatal (g_{sw} and g_{sc}), cuticular (g_{cw} and g_{cc}) and boundary layer (g_{bw} and g_{bc}) conductances, where g_s and g_c operate in parallel and g_b in series with them (Fig. 1b,c)

$$g_{tw} = \frac{1}{\frac{1}{g_{sw} + g_{cw}} + \frac{1}{g_{bw}}} \wedge g_{tc} = \frac{1}{\frac{1}{g_{sc} + g_{cc}} + \frac{1}{g_{bc}}} \qquad (3)$$

Combined cuticular and stomatal conductances to H_2O form the total conductance to H_2O through the surface of the leaf (g_{lw}) that is without the boundary layer

$$g_{lw} = g_{cw} + g_{sw} \qquad (4)$$

In the vCF calculations, g_{cw} is neglected and g_{sw} approximates to g_{lw}. Taking g_{tw} from equation (1) and rearranging equation (3)

$$g_{\mathrm{sw-vCF}} \approx g_{lw} = \frac{1}{\frac{1}{g_{tw}} - \frac{1}{g_{bw}}} = \frac{g_{tw} \cdot g_{bw}}{g_{bw} - g_{tw}} \qquad (5)$$

Then, stomatal conductance to CO_2 (g_{sc}) is calculated using the ratio of H_2O and CO_2 diffusivities in air[19]. The ratio g_{sw}/g_{sc} is taken as a constant value, estimated to be between 1.58 (ref. [20]) and 1.62, commonly taken as 1.6 (ref. [21]). Thus, $g_{sc} = g_{sw}/1.6$ and then taking $g_{\mathrm{sw\text{-}vCF}}$ from equation (5)

$$g_{\mathrm{sc-vCF}} \approx \frac{g_{lw}}{1.6} = \frac{g_{\mathrm{sw-vCF}}}{1.6} \qquad (6)$$

One of the implications of using the result from equation (5) in this way is that the ratios of cuticular and stomatal conductances to H_2O and CO_2 are (incorrectly) considered to be the same. However, estimates of g_{cw}/g_{cc} are one order of magnitude greater than g_{sw}/g_{sc}, varying in a range between 20 and 40 (ref. [14]). Thus, while the assumption of negligible cuticular conductance to H_2O has been a practical one, it can lead to significant overestimates of CO_2 conductance and subsequent parameters.

The composition of, and transport pathways through, the cuticle (epidermis, cell wall, waxes and so on) that generate these differences are non-trivial[22–24] and are not treated here. The cuticle and its components are treated as a single resistance as is common in gas exchange modelling.

The cuticular conductance to CO_2 (g_{cc}), is conventionally assumed to be zero. As g_{cc} is an order of magnitude lower than g_{cw} (ref. [14]), this typically only results in a minor error in the calculations. Then, $g_{\mathrm{tc-vCF}}$ is calculated taking g_{sc} from equation (6) and rearranging equation (3)

$$g_{\mathrm{tc-vCF}} \approx \frac{1}{\frac{1}{g_{sc} + g_{cc}} + \frac{1}{g_{bc}}} = \frac{g_{bc} \cdot g_{sc}}{g_{sc} + g_{bc}} \qquad (7)$$

with the main source of error coming from equations (5) and (6).

Direct measurement of $[CO_2]$ in the air flowing over the leaf allows us to determine the net assimilation rate (A_T) (ref. [2]). CO_2 concentration inside the leaf (C_i) is conventionally calculated using the value of $g_{\mathrm{tc-vCF}}$ obtained from equation (7) and

$$C_{\mathrm{i-vCF}} = \frac{\left(g_{tc} - \frac{E}{2}\right) \cdot C_a - A_T}{g_{tc} + \frac{E}{2}} \qquad (8)$$

Equation (8) takes into account the ternary effect[1] (vCF) of the collisions between H_2O and CO_2 molecules when they are passing through stomata in opposing flows. However, the term E in equation (8) should refer only to the portion E_s of the total evaporation which is crossing through stomatal pores. Equation (8) has the same assumptions as discussed for equation (1).

Also, it is important to consider that leaf gas exchange occurs through the adaxial and abaxial leaf faces. The outgoing gases are typically mixed before analysis (Fig. 1a) and the results used to calculate the gas exchange parameters according to the vCF equations (henceforth Gas-Mix vCF). Gas-Mix vCF calculations have the same assumptions discussed above plus the forced presumption of a single C_i in the leaf and single values for other gas exchange parameters.

Deriving more precise theory

Looking at leaf gas exchange more precisely (Fig. 1b,c), E_T is the water flux from the leaf surface, at vapour concentration (w_s), crossing the boundary layer to the atmosphere (w_a). Then, taking into account the ternary effect on the boundary layer transect the following expression is obtained for the humidity at the leaf surface (derived in Supplementary Note 2)

$$w_s = \frac{\frac{E_T}{g_{bw}} \cdot \left(1 - \frac{w_a}{2}\right) + w_a}{1 + \frac{E_T}{2 \cdot g_{bw}}} \qquad (9)$$

The total outward flux of H_2O vapour at the surface of the leaf comes from the stomata and the cuticle. Then, adapting equation (3) for the total conductance to H_2O (g_{tw}) by including the ternary effects and cuticular transpiration and conductance (Supplementary Note 3), the total leaf conductance to water vapour is given by

$$g_{tw} = \frac{1}{\frac{1}{\left[\frac{E_c}{w_i - w_s}\right] + \left[\frac{E_s - E_s \cdot \bar{w}_s}{w_i - w_s}\right]} + \frac{1}{\left[\frac{E_T - E_T \cdot \bar{w}_a}{w_s - w_a}\right]}} = \frac{1}{\frac{w_i - w_s}{E_T - E_s \cdot \left(\frac{w_i + w_s}{2}\right)} + \frac{1}{g_{bw}}} \qquad (10)$$

Equation (10) for g_{tw} is identical to equation (3) with the conductances defined to include their associated ternary effects (see

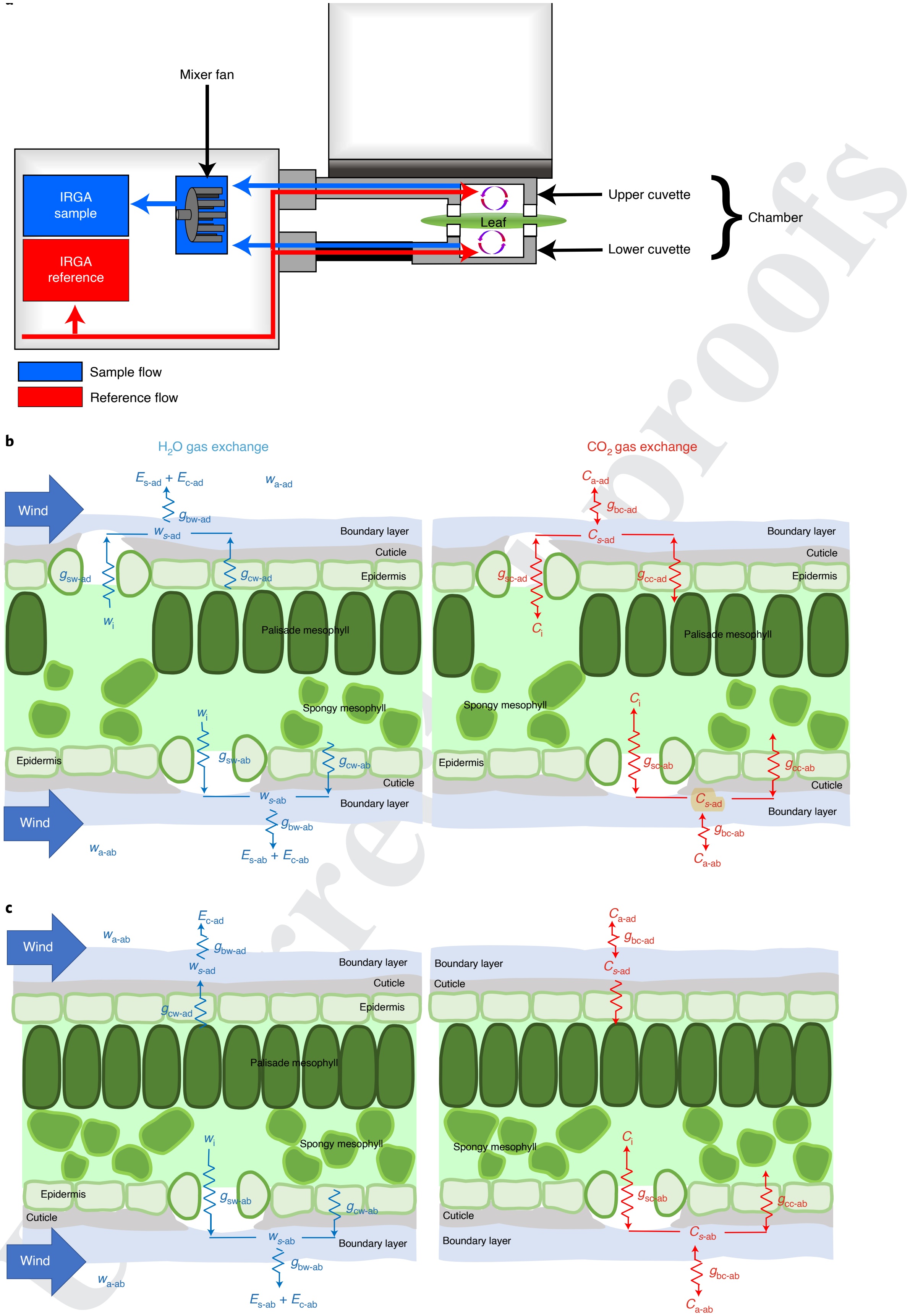

Fig. 1 | a–c, Typical gas exchange analyser setup (**a**) and gas exchange diagram from an amphistomatous leaf (**b**) and a hypostomatous leaf (**c**). IRGA, infrared gas analysis.

Supplementary Notes 3 and 2) but differs from the vCF theory equation (1), which is an approximation.

Analogous to g_{tw} we have total conductance to CO_2, which includes ternary effects, cuticular CO_2 flux (A_c) and stomatal CO_2 flux (A_s)

$$g_{tc} = \frac{1}{\frac{1}{\left[\frac{A_c}{C_s - C_i}\right] + \left[\frac{A_s + E_s \cdot \bar{c}_s}{C_s - C_i}\right]} + \frac{1}{\left[\frac{A_T - E_T \cdot \bar{c}_a}{C_a - C_s}\right]}} \tag{11}$$

Equation (11) is identical to equation (3) for total conductance to CO_2 (g_{tc}) (see Supplementary Notes 4 and 5).

Then, the CO_2 concentration at the leaf surface (C_s) is (Supplementary Note 4)

$$C_s = \frac{g_{bc} \cdot C_a - A_T - \frac{E_T}{2} \cdot C_a}{g_{bc} + \frac{E_T}{2}} \tag{12}$$

This introduces the equation for calculating C_i, taking into account cuticular conductance to CO_2 and ternary corrections through the boundary layer and stomatal pathways (Supplementary Note 5)

$$C_i = \frac{C_s \cdot \left(g_{sc} + g_{cc} - \frac{E_s}{2}\right) - A_T}{g_{sc} + g_{cc} + \frac{E_s}{2}} \tag{13}$$

By analogy with the vCF theory, Ball[25] obtained similar equations for C_s and C_i but did not differentiate between E_T and E_s nor g_{sc} and g_{cc}; and g_{sc} in Ball's derivation is based on the calculations from vCF theory equations (5) and (6).

Arranging equations (9)–(13) with parameters in the form usually measured in gas exchange devices, we obtain (derivation in Supplementary Note 6)

$$C_i = C_s - \frac{A_T + C_s \cdot E_T - C_s \cdot D \cdot g_{cw}}{G_i - \alpha \cdot g_{cw}} \tag{14}$$

where

$$\begin{aligned}
D &= w_i - w_s\\
G_i &= \frac{E_T}{1.6 \cdot (w_i - w_s)} \cdot (1 - \bar{w}_s) + \frac{E_T}{2}\\
\bar{w}_s &= \frac{w_i + w_s}{2}\\
w_s &= \frac{w_a + \left(1 - \frac{w_a}{2}\right) \cdot \frac{E_T}{g_{bw}}}{1 + \frac{E_T}{2 \cdot g_{bw}}}\\
\alpha &= \frac{1 - \bar{w}_s}{1.6} + \frac{D}{2} - \beta\\
\beta &= \frac{g_{cc}}{g_{cw}}
\end{aligned}$$

Then, expanding the term E_s in equation (10)

$$g_{tw} = \frac{1}{\frac{w_i - w_s}{E_T - [E_T - g_{cw-T} \cdot (w_i - w_s)] \cdot \left(\frac{w_i + w_s}{2}\right)} + \frac{1}{g_{bw}}} \tag{15}$$

In equations (14) and (15) the only unknown values are the cuticular conductances to H_2O (g_{cw}) and CO_2 (g_{cc}).

Equations (9)–(15) represent a new way of calculating gas exchange parameters, modified structurally from the widely used vCF theory.

Model for calculating g_{cw}

The omission of g_{cw} in $g_{tw\text{-}vCF}$ is the major cause of a miscalculation (knowing $g_{cw} \neq 0$) in the conventional $C_{i\text{-}vCF}$ calculation. This error in the vCF theory has been used to calculate a correction factor taken as g_{cw} (refs. [11,12]). However, this approach to calculating g_{cw} carries the assumptions made in the vCF theory and assumes that the sole mismatch is from neglecting g_{cw}. Our proposed theory does not involve such assumptions and includes g_{cw} from the outset of the derivation rather than as a correction. If C_i and β are known, equation (14) can be rearranged to solve for g_{cw} directly

$$g_{cw} = \frac{(C_s - C_i) \cdot G_i - (A_T + C_s \cdot E_T)}{(C_s - C_i) \cdot \alpha - C_s \cdot D} \tag{16}$$

However, in most cases C_i is unknown. Here, we propose a model developed from equation (14) that allows us to estimate g_{cw} when C_i is unknown, using adaxial and abaxial leaf gas exchange measurements.

Adding physiological parameters representing the ratio of CO_2 concentrations at the adaxial and abaxial evaporative sites by $\kappa = C_{i\text{-}ad}/C_{i\text{-}ab}$ and the ratio of g_{cw} on the abaxial and adaxial surface by $\gamma = g_{cw\text{-}ab}/g_{cw\text{-}ad}$, we obtain

$$\begin{aligned}
&C_{s-ad} - \frac{A_{T-ad} + C_{s-ad} \cdot E_{T-ad} - C_{s-ad} \cdot D_{ad} \cdot g_{cw-ab} \cdot \gamma}{G_{i-ad} - \alpha_{ad} \cdot g_{cw-ab} \cdot \gamma}\\
&= \left(C_{s-ab} - \frac{A_{T-ab} + C_{s-ab} \cdot E_{T-ab} - C_{s-ab} \cdot D_{ab} \cdot g_{cw-ab}}{G_{i-ab} - \alpha_{ab} \cdot g_{cw-ab}}\right) \cdot \kappa
\end{aligned} \tag{17}$$

where the subscripts ad and ab denote adaxial and abaxial surfaces of the leaf. Equation (17) is then solved for g_{cw} (full derivation in Supplementary Note 7)

$$a \cdot g_{cw-ab}^2 + b \cdot g_{cw-ab} + c = 0 \tag{18}$$

where

$$\begin{aligned}
&a = (C_{s-ad} - C_{s-ab} \cdot \kappa) \cdot \alpha_{ad} \cdot \alpha_{ab} \cdot \gamma + \kappa \cdot C_{s-ab} \cdot D_{ab} \cdot \alpha_{ad} \cdot \gamma\\
&-C_{s-ad} \cdot D_{ad} \cdot \alpha_{ab} \cdot \gamma\\
&b = (C_{s-ad} \cdot D_{ad} \cdot G_{i-ab} \cdot \gamma + (A_{T-ad} + C_{s-ad} \cdot E_{T-ad}) \cdot \alpha_{ab})\\
&-(C_{s-ad} - C_{s-ab} \cdot \kappa) \cdot (\alpha_{ad} \cdot G_{i-ab} \cdot \gamma + G_{i-ad} \cdot \alpha_{ab})\\
&-\kappa \cdot (C_{s-ab} \cdot D_{ab} \cdot G_{i-ad} + (A_{T-ab} + C_{s-ab} \cdot E_{T-ab}) \cdot \alpha_{ad} \cdot \gamma)\\
&c = (C_{s-ad} - C_{s-ab} \cdot \kappa) \cdot G_{i-ad} \cdot G_{i-ab} - (A_{T-ad} + C_{s-ad} \cdot E_{T-ad})\\
&\cdot G_{i-ab} + \kappa \cdot (A_{T-ab} + C_{s-ab} \cdot E_{T-ab}) \cdot G_{i-ad}
\end{aligned}$$

and the lesser positive root is taken.

Our model to calculate g_{cw} is based on using the uneven impact of g_{cw} and g_{cc} on total conductance to H_2O (g_{tw}) and CO_2 (g_{tc}) on each surface of the leaf to find g_{cw} for one surface. The ratio of abaxial and adaxial g_{cw} (γ) distributes the influence of g_{cw} on each surface. However, the actual value of γ is unknown, so for simplicity a value of one is assumed. The rationale for this assumption is discussed and tested later in the paper.

A requirement for using equation (18) is that the experimental setup must provide conditions that allow inference of the value of κ. Most gas exchange measurements assume that $C_{i\text{-}ad} = C_{i\text{-}ab}$, that is $\kappa = 1$, and for usual gas exchange measurements, the assumption is adequate[26–30]. However, κ can vary depending on the environmental conditions and leaf characteristics[31,32].

A compilation of κ values reported for different species under different conditions is shown in Supplementary Note 8. The highest value of κ found was 1.18 ($|\Delta C_i| \approx 11$ ppm), when a *Helianthus annuus* leaf was exposed to <100 ppm of ambient [CO_2]; and the lowest κ value found was 0.88 ($|\Delta C_i| \approx 25$ ppm), when a *Eucalyptus pauciflora* leaf was exposed to 3 h of photo-inhibition. However, under non-stressed light conditions and at CO_2 concentrations near that of the atmosphere, κ values were on average 1.00 ± 0.05. For simplicity, then, our experimental setup was established in conditions and ranges of C_a where κ can be expected to be close to one and we use $\kappa = 1$ in our calculations. To test the impact of κ in the calculations, κ was varied within reasonable bounds (Extended Data Fig. 1). The results of this evaluation are discussed later in the paper.

Cuticular conductance to H_2O

Adaxial and abaxial gas exchange was measured independently on *Capsicum annuum* leaves and equation (18) used with κ set to unity,

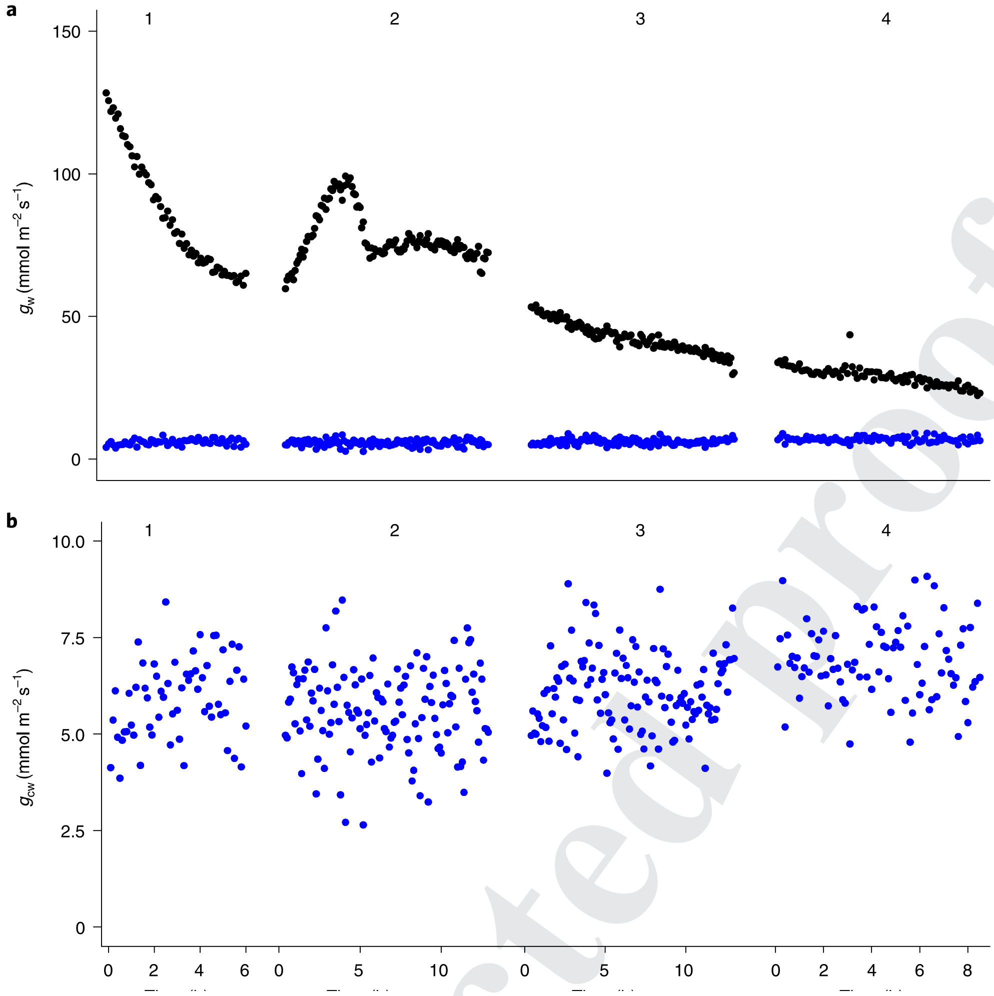

Fig. 2 | Cuticular conductance calculated for single leaf surfaces for four leaves of the same *C. annuum* plant. Four different leaves from the same *C. annuum* plant (1, 2, 3 and 4), with measurements of 6, 13, 13 and 10 h under the same light, [CO$_2$] and air saturation deficit ($n_{(1,2,3,4)} = 60$, 126, 120 and 85). **a**, Total leaf surface conductance ($g_{\text{lw-T}} = g_{\text{lw-ad}} + g_{\text{lw-ad}}$, black dots) and calculated cuticular conductance for a single surface with $\kappa = 1$ (blue dots). **b**, Close-up of the calculated cuticular conductance. Each point is a single measurement repeated every 6 min. The dataset of each leaf was divided into sections of 1 h to analyse changes in the calculated g_{cw}. No significant differences were found between time sections within measurements of each leaf; no significant differences were found between the average values of leaves 1, 2 and 3; leaf 4 showed a significantly higher g_{cw} than the other leaves (statistical analysis: two-sided Student's *t*-test with $P < 0.05$; Supplementary Note 9).

to calculate g_{cw}. Cuticular conductance to water (g_{cw}) of each leaf was found to be constant regardless of changes in evaporation rate and natural stomatal aperture changes (circadian rhythm) during the day (Fig. 2; Methods). These observations are consistent with reports by Hoad et al.[33] and Boyer[11,14] that cuticular conductance to H$_2$O is stable while the leaf retains turgor. Correction of g_{sw} was 5–20% of the total g_{lw} ($g_{\text{lw-T}} = g_{\text{lw-ad}} + g_{\text{lw-ad}}$) for *C. annuum* plants during these experiments.

In a study comparing species, leaf cuticular conductance to H$_2$O was calculated for *C. annuum*, *Ginkgo biloba*, *Gossypium hirsutum* and *Nicotiana tabacum* plants (Fig. 3).

Our results were compared with a common method for measuring adaxial and abaxial minimum conductance, which involves measuring gas exchange after long dark adaptation with and without ABA on the leaf surface[13]. From lengthy experiments conducted on *G. hirsutum* and *C. annuum*, adding ABA and placing the plants in the dark, the minimum conductance ($g_{\text{lw-min}}$ + ABA) for the adaxial surface of the leaf was practically the same as our calculated g_{cw}. The value of $g_{\text{lw-min}}$ + ABA for *C. annuum* was 5.7 mmol m^{-2} s^{-1} and the g_{cw} was 5.6 mmol m^{-2} s^{-1}. For *G. hirsutum* the values were 1.9 and 1.63 mmol m^{-2} s^{-1}, respectively.

Our calculated cuticular conductances were always below the adaxial and abaxial minimum conductance (Table 1) as one would expect assuming a tiny stomatal aperture. In three of these species, the conductance of both leaf surfaces was measured after 12 h of dark adaptation ($g_{\text{lw-min}}$) when stomata had closed. It was found that, in *C. annuum* leaves, the cuticular conductance comprised 94% of $g_{\text{lw-min}}$ ($g_{cw}/g_{\text{lw-min}}$) on the upper surface and 60% on the lower surface (in other words, 6% and 40% of vapour was coming from almost closed stomata, respectively). The corresponding values of $g_{cw}/g_{\text{lw-min}}$ for *G. hirsutum* were 90% and 75%, and in *G. biloba* were 8% in the lower surface and 100% for the astomatous surface, the last largely confirming almost closed stomata as the other water source.

Leaves of *C. annuum* plants grown under water-stress conditions (Fig. 3, *C. annuum* 3-D and 4-D) were also measured after long dark adaptation. The water stress of our *C. annuum* plants was managed by watering them only when the soil water potential reached −0.1 MPa. It was found that $g_{\text{lw-min}}$ for these plants, measured when fully rehydrated, was two to three times less than in well-watered plants and the stomatal component of the water loss was higher. Compared with minimum conductance measured in the dark, the cuticular conductance was 50% of the abaxial surface conductance and 75%

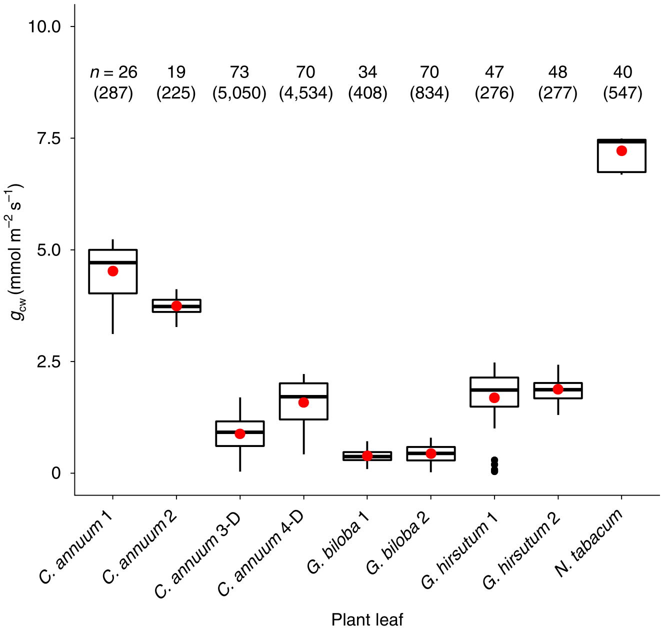

Fig. 3 | Average cuticular conductance calculated for a single leaf surface for leaves of different plants ($\kappa = 1$). *C. annuum* 3-D and 4-D were grown in drought conditions. Red dots are the mean values of the dataset, boxes represent the range of data between the second and third quartiles of frequency (IQ = Q3 − Q2), the horizontal lines in the boxes represent the median of the dataset, vertical lines represent the range of maximum and minimum values excluding outliers and black dots are outliers (outliers are: <Q2 − 1.5*IQ or >Q3 + 1.5*IQ). Each observation (n) used for the calculations is the average of 12 to 72 measurements under stable conditions for a period of 6 min. The total number of observations is shown in parentheses, each leaf being measured for 2–7 h.

for the adaxial surfaces, a lower percentage than in well-watered plants. Other researchers have reported similar results with plants grown in well-watered and drought-stressed conditions[34–37].

Effect of C_a, temperature and humidity on g_{cw} calculation

It was found that simultaneous changes in C_a at both surfaces had no significant effect on the g_{cw} calculation, when plants were in stable gas exchange conditions (Extended Data Fig. 2). Neither temperature nor atmospheric saturation deficit affected our calculations of cuticular conductance to H_2O on well-watered plants (Extended Data Fig. 2).

A stable g_{cw} of about 5 ± 0.5 (mean ± s.d.) mmol m^{-2} s^{-1} for one surface of the leaf ($\gamma = 1$) was found for a *C. annuum* leaf regardless of changes in air temperature. Our measurements were carried out at leaf temperatures in the range 20–29 °C and are consistent with the minor changes in cuticle permeability reported for isolated cuticles within this range[38].

There was no significant change in the calculated g_{cw} in a large range of ambient relative humidity (Extended Data Fig. 2) although other studies on isolated cuticles[39] have reported important variations in the cuticular permeability resulting from changes in relative humidity. These variations seem to be species dependent, with permeabilities at 100% relative humidity between 1.4 and ten times higher than at 0% relative humidity. There is still little information about the mechanism of this variation and the effect that isolation of the cuticular waxes has on the measurements.

Cuticular conductance to CO_2

On an astomatous surface, it should be possible to measure the actual g_{cc}; however, the conductance to CO_2 through the cuticle is usually so low that the signal is too weak and noise is overwhelming. The measurements of assimilation rate through the astomatous surfaces in *G. biloba* leaves were at the limit of the instrumental noise, averaging 0.015 ± 0.018 (mean ± s.d.) µmol m^{-2} s^{-1}. This makes it challenging to estimate a reliable ratio of $g_{cc}/g_{cw} = \beta$ directly.

The literature suggests values for β of between 0.025 and 0.05, depending on the species and growth conditions[6,11,14]. The average positive assimilation rate occurring through the cuticle (A_c) in *G. biloba* leaves means that in our calculations a residual g_{sw} is assigned to the astomatous surfaces if β is set as 0. This residual g_{sw} is due to the calculations where CO_2 must pass through one of the two pathways and $\beta = 0$ mathematically blocks the cuticular pathway. If we estimate a β value that satisfies the condition of obtaining an average g_{sw} for the adaxial surface equal to 0, we obtain $\beta \approx 0.05$. Even though the β value found is within the range suggested in the literature, the measurements of A_c were not precise enough for us to validate our β value.

An additional issue with the calculation explained above is that it was made assuming $\gamma = 1$ and technically we could make the same calculation assigning any reasonable $\beta > 0$ and correcting the g_{sw} by varying γ. Further in the paper, γ is estimated as 1.06 for *G. biloba* leaves (adaxial surface with lower conductance) using measurements of minimum conductance in the dark. Using $\gamma = 1.06$ and recalculating β to make adaxial $g_{sw} = 0$, we obtain an insignificantly higher β that still approximates to $\beta \approx 0.05$ but again with insufficient precision of measurement of A_c.

Correcting our calculations using $\beta = 0.05$ in *G. biloba* leaves has no significant influence on g_{cw} estimation, on C_i or on any other gas exchange parameter calculation during positive net assimilation rate. Nor does the value of β impact the calculations made in the other species used in our experiments. However, knowing that $g_{cc} \neq 0$, β values within the range reported in the literature are appropriate in the absence of a known β.

Elucidating the model

To calculate g_{cw} using our model (equation (18)), the values of A_T, E_T plus g_{lw} and g_{lc} for the adaxial and abaxial surface are balanced using a mediated κ value. Thus, to calculate g_{cw} the mathematics require a difference between leaf surfaces in at least one of these parameters, as usually happens. However, this also implies that the calculation relies on the precision of [H_2O] and [CO_2] measurements from the upper and lower cuvettes and any failure in the precision between cuvette measurements results in a miscalculation of g_{cw}. Our experiments showed that the main possible source of error in our model and method is the drift in the equipment used to calculate H_2O and CO_2 balances (Supplementary Note 10).

During our experiments the calculated g_{cw} was stable with a reliable value whenever the experimental setup allowed inference of κ. The impact of κ in the calculation using equation (17) depends on the magnitude of g_{lc} on each surface. In the case of a hypostomatous leaf, the astomatous surface has a very low CO_2 conductance in comparison with the surfaces with stomata. Conventionally, internal [CO_2] is defined as the [CO_2] calculated in the evaporative site[31] but, in the astomatous surface, internal [CO_2] and C_s are physically separated. This physical separation offers great resistance to CO_2 diffusion, creating the conditions where the value of κ has a small impact on our calculations.

It is possible to evaluate the latter concept from our measurements made in hypostomatous *G. biloba* leaves. Our first approach was to estimate g_{cw} assuming $\kappa = 1$. Later, we imposed $\kappa \approx 0.9$ equivalent to the ΔC_i found by Parkhurst et al.[29] when only one surface of an amphistomatous leaf was fed with CO_2. The change did not affect the calculated value of g_{cw}. We evaluated κ in a range from 0.9 to 1.1 and found insignificant variation in the calculation of g_{cw} in *G. biloba* leaves (Extended Data Fig. 1). In contrast, the same analysis in an amphistomatous leaf of *N. tabacum* showed a big impact in the same range.

Table 1 | Comparison of measured minimum conductance to H_2O, calculated g_{cw} using equation (18), the dark-g_{sw} calculated as $g_{lw\text{-}min} - g_{cw}$, Dark-$g_{sw}$ ratio and stomatal density ratio

Plant	Surface	$g_{lw\text{-}min}$ (mmol m^{-2} s^{-1})	g_{cw} (mmol m^{-2} s^{-1})	Dark-g_{sw} (mmol m^{-2} s^{-1})	Dark-g_{sw} (%)	Stomatal density (%)
C. annuum	Adaxial	8.5	5.6	2.9	26	10
	Abaxial	13.8	5.6	8.2	74	90
G. biloba[a]	Adaxial	0.5	0.35	0.15	6	0
	Abaxial	2.75	0.35	2.4	94	100
G. biloba[b]	Adaxial	0.5	0.49	0.01	0	0
	Abaxial	2.75	0.49	2.26	100	100
G. hirsutum	Adaxial	2.5	1.63	0.88	17	25
	Abaxial	6	1.63	4.38	83	75

[a] Calculation assuming $\beta = 0$. [b] Calculated using $\beta = 0.05$.

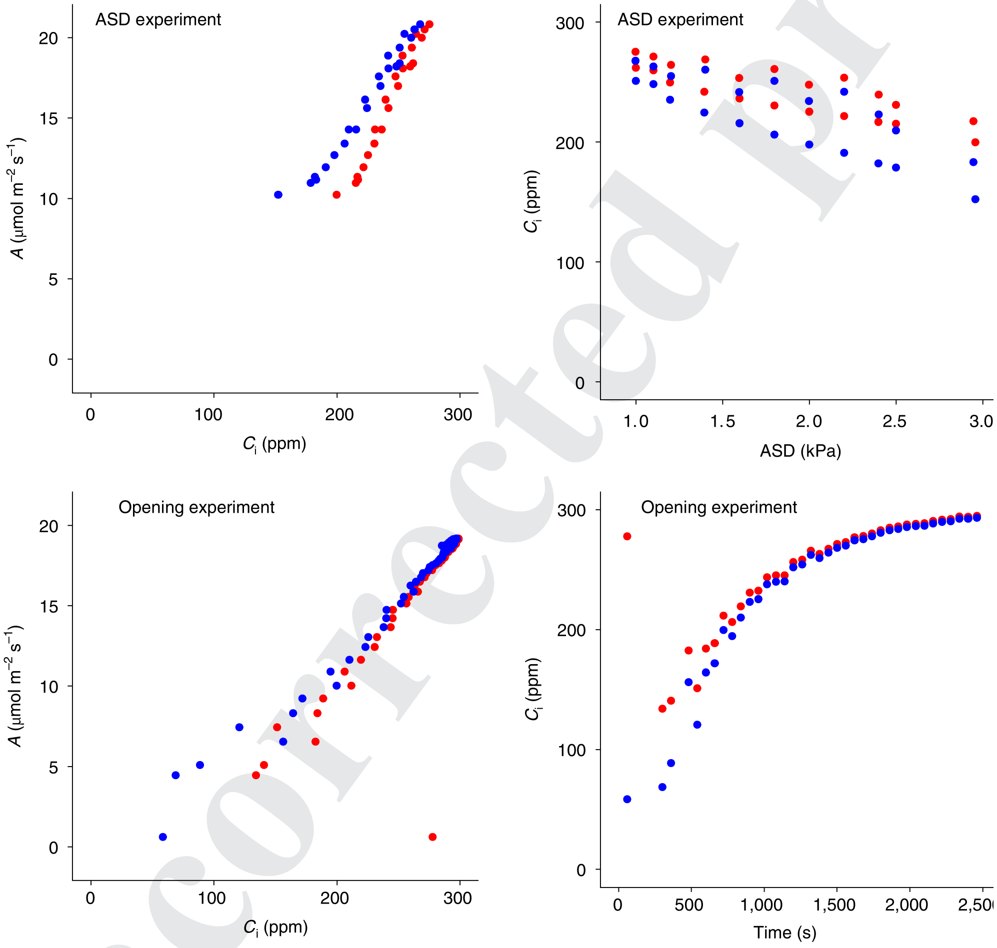

Fig. 4 | Gas exchange parameters calculated using equations (9)–(15) and the vCF theory for experiments changing ASD and stomatal aperture from dark to light (opening) on *C. annuum* plants. Red dots show the results of using the Gas-Mix vCF calculation and the blue dots the calculations using equations (9)–(15) accounting for cuticular gas fluxes. Each point in the ASD experiment is a single measurement taken under stable leaf gas exchange conditions. Each point in the opening experiment is a single measurement taken every 1 min; initial measurements are more spaced due to the rapid change of gas exchange conditions and subsequent IRGA matches for pairing the sample and reference IRGA.

In a general analysis, the value of the parameter κ affects the calculated relationship between g_{lw}, g_{sw} and g_{cw}; the ternary correction on the CO_2 fluxes; and the CO_2 gradients between the stomatal cavity and the leaf surface. In the calculations made in this study, we assume that due to our experimental setup the $[CO_2]$ at the sites of evaporation is the same for both sides of a leaf ($\kappa = 1$). This is likely to be the case when there is negligible resistance to CO_2 diffusion inside the leaf and the ambient conditions are equal in both surfaces but not necessarily restricted to that condition (Supplementary Note 8). On the other hand, gradients across a leaf can be of the order of 20 ppm or more if A_T at one surface is constrained to zero[29]. Additionally, the assumption of $\kappa = 1$ may not hold when the plant

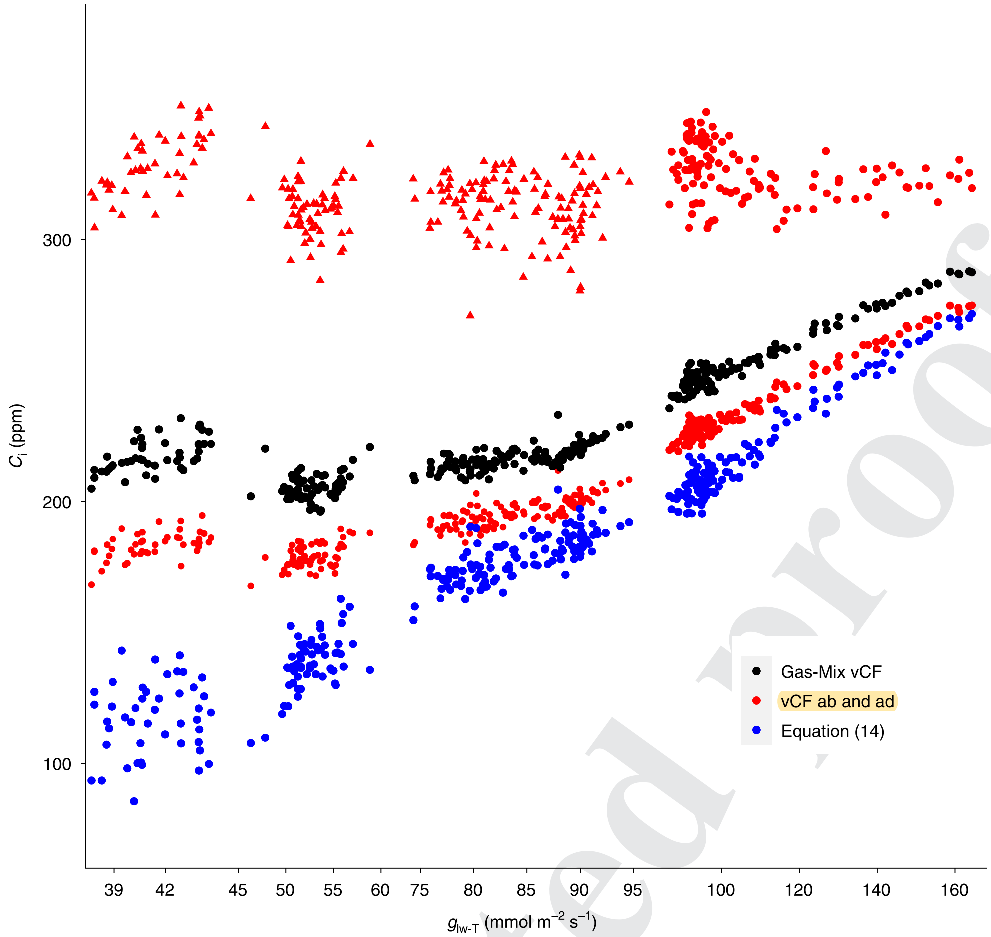

Fig. 5 | Comparison of C_i calculation using vCF, Gas-Mix vCF and equation (14) calculations. CO$_2$ concentration inside a *C. annuum* leaf using equation (8) (vCF) for the adaxial (red triangles) and abaxial (red circles) faces of a leaf and the Gas-Mix vCF calculation using a mixture of the gases from both faces of the leaf (black circles). Blue circles are the calculation of C_i using equation (14) with $\kappa = 1$. The total conductance to H$_2$O through both surfaces of the leaf ($g_{\mathrm{lw\text{-}T}}$) is displayed as the *x* axis: sections are different periods of the day; each section has a different scale in the *x* axis to make them readable. One point is a single measurement, taken every 6 min. Further analysis of Fig. 5 in Supplementary Note 11.

species has thick leaves or if there is an internal or external physical impediment to free diffusion in the leaf[26,27]. For instance, see the contrast between *Xanthium strumarium* and *Zea mays* reported by Farquhar and Raschke[32]. In our experimental setup, we used a combination of plants and environmental conditions where it is reasonable to assume that $\kappa \approx 1$ and we tested the possible error arising from this assumption (Extended Data Fig. 1).

Another aspect that might affect the calculations is the difference between the adaxial and abaxial g_{cw}. Although the two leaf surfaces might have different cuticular structures, direct values of cuticular conductance from the astomatous surfaces have been used as an approximation of the cuticular conductance of the opposite surface[5,6]. We took this initial approach assigning $\gamma = 1$ for equation (18) because the actual difference is unknown. However, a recent study[40] found that the adaxial and abaxial cuticles of a hypostomatous leaf have different evaporative rates under similar environmental conditions. This might indicate significant differences in g_{cw} for the adaxial and abaxial surfaces.

To address this, we used dark measurements to obtain the $g_{\mathrm{lw\text{-}min}}$ of the adaxial and abaxial surfaces. Then, the calculated g_{cw} and $g_{\mathrm{lw\text{-}min}}$ were used to isolate the dark-g_{sw} from both surfaces. Ratios of stomatal conductance in the dark were compared with the stomatal density ratios and used to test whether differences in $g_{\mathrm{lw\text{-}min}}$ between the abaxial and adaxial surfaces were explained by stomatal densities (Table 1). It was found that a clear majority of the differences in the adaxial and abaxial $g_{\mathrm{lw\text{-}min}}$ were explained by stomatal density imbalances.

The difference between the stomatal density ratio and the $g_{\mathrm{lw\text{-}min}}$ ratio might be explained by uneven stomatal closure on the two surfaces, differences in adaxial and abaxial g_{cw} or a mixture of these two factors. To evaluate the impact of differences in adaxial and abaxial g_{cw}, we considered the extreme case where the whole difference between these ratios is due to uneven g_{cw} in the adaxial and abaxial surfaces. We found values of γ ($g_{\mathrm{cw\text{-}ab}}/g_{\mathrm{cw\text{-}ad}}$) of 1.18, 1.09 and 1.06 for *C. annuum*, *G. hirsutum* and *G. biloba*, respectively. Using equation (18) and these γ values to recalculate the total g_{cw} ($g_{cw\text{-}T} = g_{cw\text{-}ad} + g_{cw\text{-}ab}$), it was found that $g_{\mathrm{cw\text{-}T}}$ of our plants was increased by 12.5% in *C. annuum*, 5% in *G. hirsutum* and 3% in *G. biloba*. The other gas exchange parameters were not significantly affected when corrected by γ. An analysis of the impact of γ values of between 0.7 and 1.4 is presented in (Extended Data Fig. 3).

In the model presented in equation (18) we included the ratio of abaxial to adaxial g_{cw} (γ), which is usually unknown. This introduces a level of uncertainty to the calculations of total cuticular conductance to water ($g_{cw\text{-}T} = g_{cw\text{-}ad} + g_{cw\text{-}ab}$); however, within reasonable range, γ has a minor effect in the calculation of other gas exchange parameters. Our theory gives a tool to evaluate the differences between adaxial and abaxial g_{cw} and the future direction of our research will focus on it.

Theory insights

The gas exchange mathematical theory derived in equations (9)–(15) incorporates two new variables in the calculation of gas exchange: g_{cw} and g_{cc}. The variable g_{cc} can be expressed in terms of g_{cw} using the parameter β as presented in equation (14) and discussed in the section Cuticular conductance to CO_2.

The theory can be applied to any gas exchange measurement, independently of whether the intention is to estimate g_{cw} or not. It calculates gas exchange parameters from normal gas exchange measurements provided g_{cw} is known. For instance, using the default setup of a LI-6800, we performed experiments involving air saturation deficit (ASD) and stomatal opening in response to light using *C. annuum* leaves where the g_{cw} was determined in advance. Then, we calculated all the gas exchange parameters using equations (9)–(15) (Fig. 4).

In the examples shown in Fig. 4, the $A-C_i$ relation obtained using equations (9)–(15) is lower than that calculated with the widely used Gas-Mix vCF calculation. These results are consistent with the corrections made by Tominaga et al.[15] using direct measurements to estimate C_i. Calculations made using equations (9)–(15) show more consistency in the $A-C_i$ trend, especially when stomata are not fully open. Similar results were found in experiments conducted in a gas exchange system able to measure adaxial and abaxial surfaces independently (Fig. 5). The vCF equations overestimate C_i, which results in in miscalculation of other parameters calculated from it such as mesophyll conductance. Overestimation of C_i under ambient [CO_2] ($C_a \approx 400\,\mu mol\,mol^{-1}$) causes underestimation of the mesophyll conductance, for example in calculations based on isotope discrimination techniques[41] such as those by Tazoe et al.[42] or Busch et al.[43].

The proposed physiological model to estimate cuticular conductance (equation (18)) is valuable when the actual C_i is unknown and reasonable assumptions can be made about the relationship between adaxial and abaxial C_i values (κ). The application of the model requires a gas exchange system able to measure adaxial and abaxial surfaces independently such as those used by us, Mott and O'Leary[27], Sharkey et al.[44], Wong et al.[17], Parkhurst et al.[29] and Farquhar and Raschke[32].

Another example of the use of our theory to calculate g_{cw} is where C_i is known and then substituted in equation (16). For instance, C_i can be estimated in the way proposed by Sharkey et al.[44] or with the method used by Tominaga and Kawamitsu[12] to estimate g_{cw} but using equation (16) instead. Then all the gas exchange parameters can be calculated as before, using equations (9)–(15).

Conclusion

The theory developed here adds a more detailed characterization of leaf gas exchange considering the two pathways of transport from inside the leaf to the atmosphere. This innovation adds more detail to gas exchange calculations, including the interaction of cuticular fluxes with the other gas exchange parameters and the effect of ternary corrections in the boundary layer. Also, it was shown that calculations allow a more precise estimation of gas exchange parameters in normal gas exchange measurements without any special setup.

Additionally, we accurately estimated the cuticular conductance to H_2O for different species using the model derived from our theory. It was shown that atmospheric conditions did not affect our results of cuticular conductance. Differing stomatal densities on adaxial and abaxial surfaces facilitate the use of our instrumentation and experimental setup to estimate cuticular conductance to H_2O vapour.

Methods

Gas exchange devices. Two LI-6800 gas exchange analysers (LI-COR) were used simultaneously to evaluate the gas exchange from the adaxial and abaxial surfaces of the leaf. The upper and lower cuvettes of a fluorescence system head of an LI-6800 were separated; leaving only the upper cuvette connected to the gas analyser system (Extended Data Fig. 4, LI-6800 no. 1). The lower cuvette was connected to the other LI-6800 system (Extended Data Fig. 4, LI-6800 no. 2): its reference and sample gas were isolated after the fan mixer. The connection was made using an adaptor created inhouse (Extended Data Fig. 4) by P. Groeneveld. In addition, experiments were carried out on a gas exchange analysis system (system 1) built by S. C. Wong, which uses two LI-6251 gas analysers (LI-COR) to measure the [H_2O] and [CO_2] of the upper and lower cuvettes independently. System 1 is constantly measuring a reference gas of known concentrations obtaining the actual absolute values from each LI-6251 on each measurement. During all the experiments the pressures of both cuvettes were kept equal, using the pressure and flow control included in the LI-6800s and the independent flow control in system 1.

Plant material. Five species were used in our experiments: *C. annuum*, *N. tabacum*, *H. annuus*, *G. hirsutum* and *G. biloba*. All the plants, except *G. biloba*, were kept in a greenhouse under natural light conditions with a temperature of 28 °C during the day and 20 °C at night. *G. biloba* plants were kept in a shadehouse. They were watered twice a day, except those plants under water-stress treatments (Data collection). *C. annuum* plants were germinated in a germination chamber and transplanted to their final pots after the third true leaf was fully expanded. All the other species were grown in their final pots inside the greenhouse.

We took advantage of two characteristics of *C. annuum*: (1) *C. annuum* has a significant difference in the stomatal distributions between the adaxial and abaxial surfaces of the leaf; in our plants, the distribution was about 10% adaxial, 90% abaxial. (2) *C. annuum* plants also possess a strong circadian rhythm in their stomatal behaviour, which was used to measure the effect of different natural variations of stomatal conductance on cuticular conductance calculations under non-stressed conditions.

In addition, experiments to determine the cuticular conductance of other species with different stomatal distributions were performed. The plants were *N. tabacum*, which has a 40/60% distribution of stomatal density on the adaxial and abaxial surface of the leaf; *G. hirsutum*, which has 25/75% distribution; *G. biloba*, which has 0/100%; and *H. annuus*, which has ~50/50% distribution.

Data collection. To evaluate the feasibility of using equation (18) to calculate g_{cw}, two experiments were performed on *C. annuum* plants using system 1 under light conditions of $1{,}000\,\mu mol\,m^{-2}\,s^{-1}$: first following the diurnal rhythm over 24 h at 450 ppm of CO_2 and second with 30 min of measurement under 220, 380, 420 and 880 ppm of CO_2. Similar experiments were performed on all the plant species mentioned above using the system with two LI-6800s following the diurnal rhythm over 24 h, setting atmospheric [CO_2] at 400 ppm.

Our results were compared with minimum leaf surfaces conductance after 12 h in the dark with and without ABA application. The ABA was prepared at 10^{-4} M in water. The ABA was applied to the adaxial surfaces of the leaf using a spray.

The 24-h experiments were performed to evaluate the effect of air temperature in the range 20–28 °C and atmospheric ASD of 0.8–1.8 kPa on our calculations. Additionally, experiments with cycles of temperature and relative humidity were performed setting the air temperature at 19, 25 and 35 °C; and atmospheric relative humidity at 40 and 80%.

A set of *C. annuum* plants was grown under water deficit, being watered only when the soil matric potential reached −0.1 MPa, as measured with Decagon MPS-6 devices (Decagon Devices). Two MPS-6 were placed at different depths in each plant pot. Plants were grown under this condition from the third true leaf stage until maturity when the measurements were performed. They were then watered to ensure fully turgid conditions during the test.

Another two sets of experiments were conducted on well-watered *C. annuum* plants using the system of two LI-6800s to evaluate the impact of changes in stomatal conductance on estimates of g_{cw}. The first involved 11 changes in ASD, from 1 to 3 kPa, and then decreasing from 3 to 1 kPa, waiting until stomatal conditions were stable at each point. The second consisted of changing the irradiance from 0 to $1{,}000\,\mu mol\,m^{-2}\,s^{-1}$, recording the gas exchange parameters every minute.

To analyse how C_i calculation is affected during all these experiments, C_i was also calculated following the typical method in most gas exchange experiments, which involves effectively mixing the gases from both surfaces (cuvettes) and calculating as per the vCF theory[1].

Additional experiments were made using the normal setup of a LI-6800 (single chambers) on the same plants for which the cuticular conductance to water was previously determined.

The data analysis was performed using the software RStudio v.1.3.959.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All generated and analysed data from this study are included in the published article, its Supplementary Information and in the GitHub repository (https://github.com/PlantPhysiologist/When_small_fluxes_matter-calculating-leaf-gas-exchange). Source data are provided with this paper.

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Author contributions

D.M.A., H.S-W. and G.D.F. conceived the study. D.M.A. undertook the experimental work and data analysis. D.M.A. and G.D.F. carried out the modelling. D.M.A. wrote the manuscript with help from all authors.

Competing interests

The authors declare no competing interests.

Additional information

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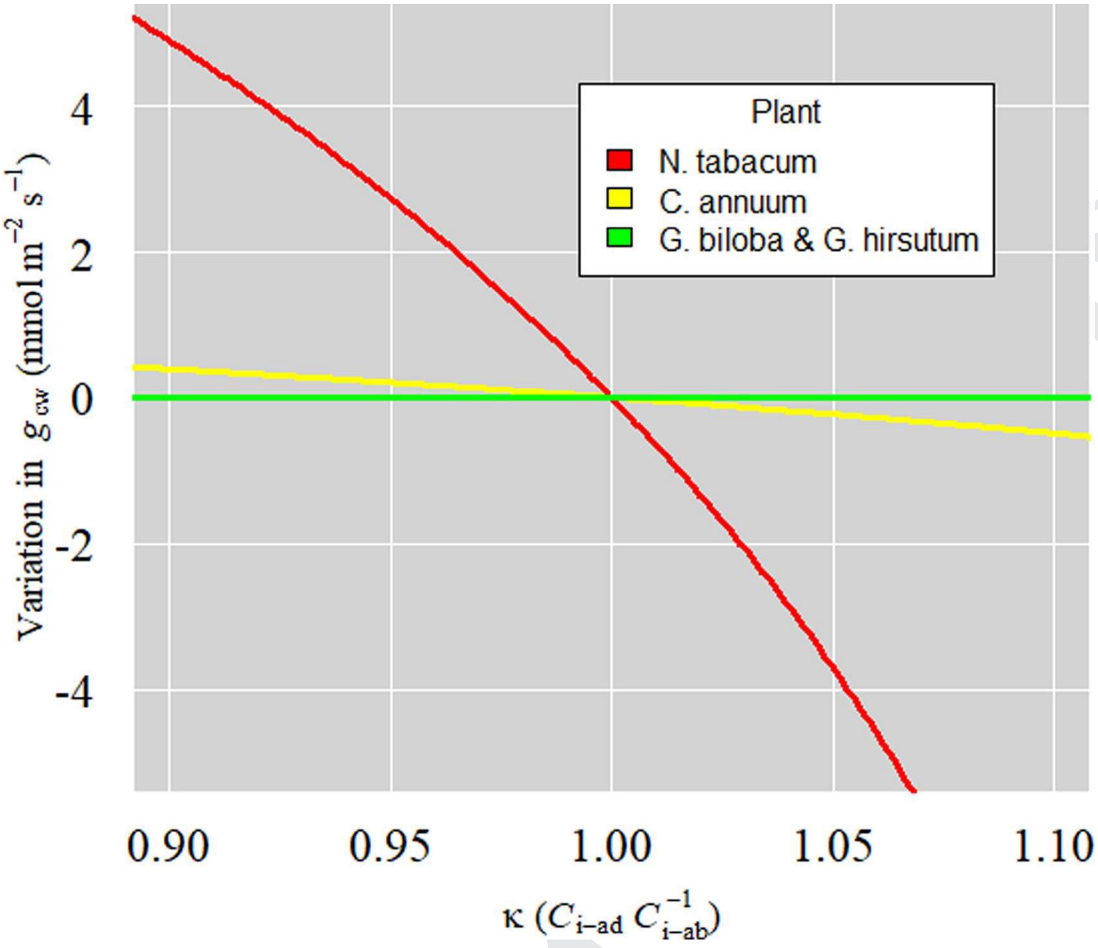
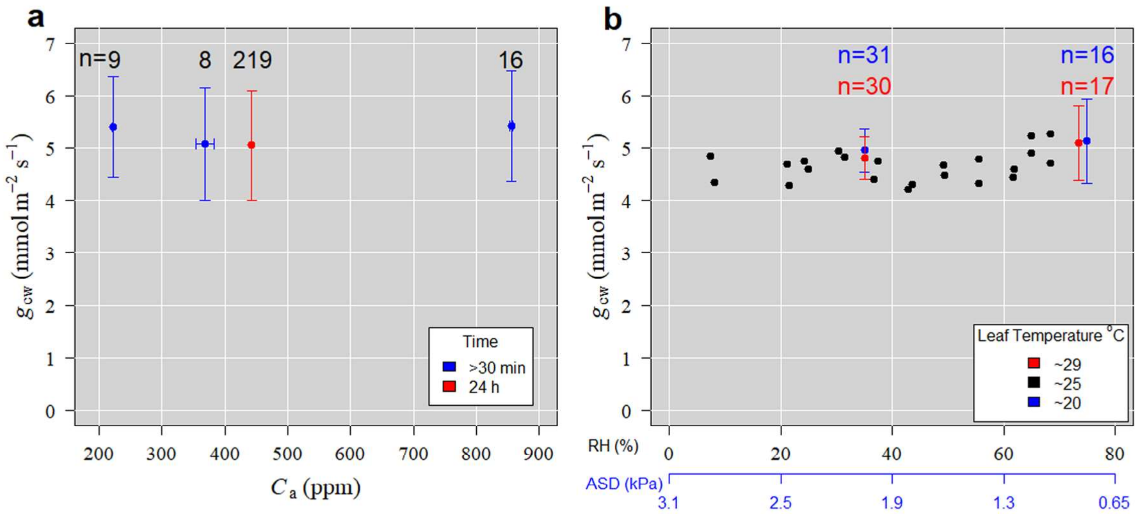
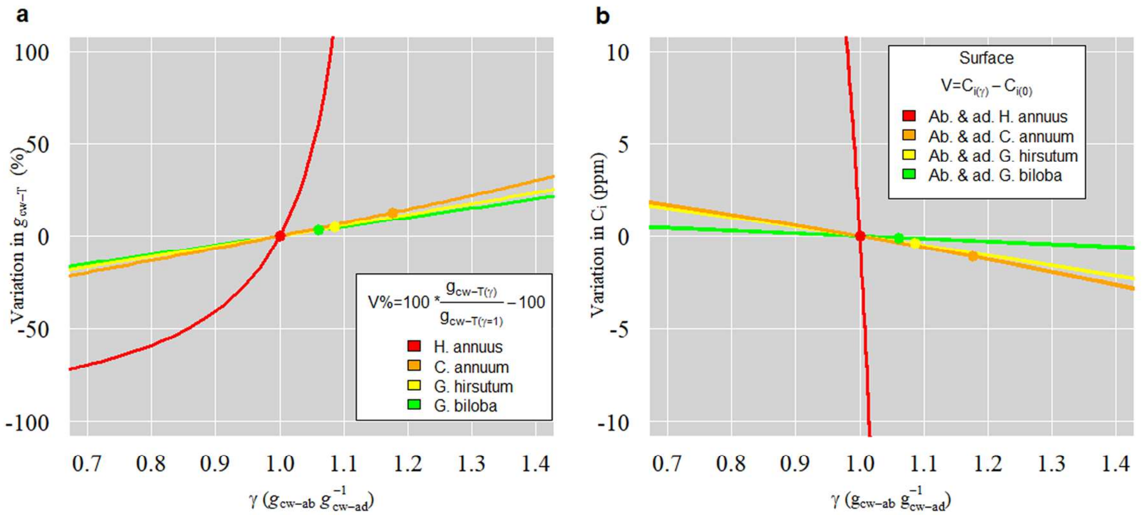


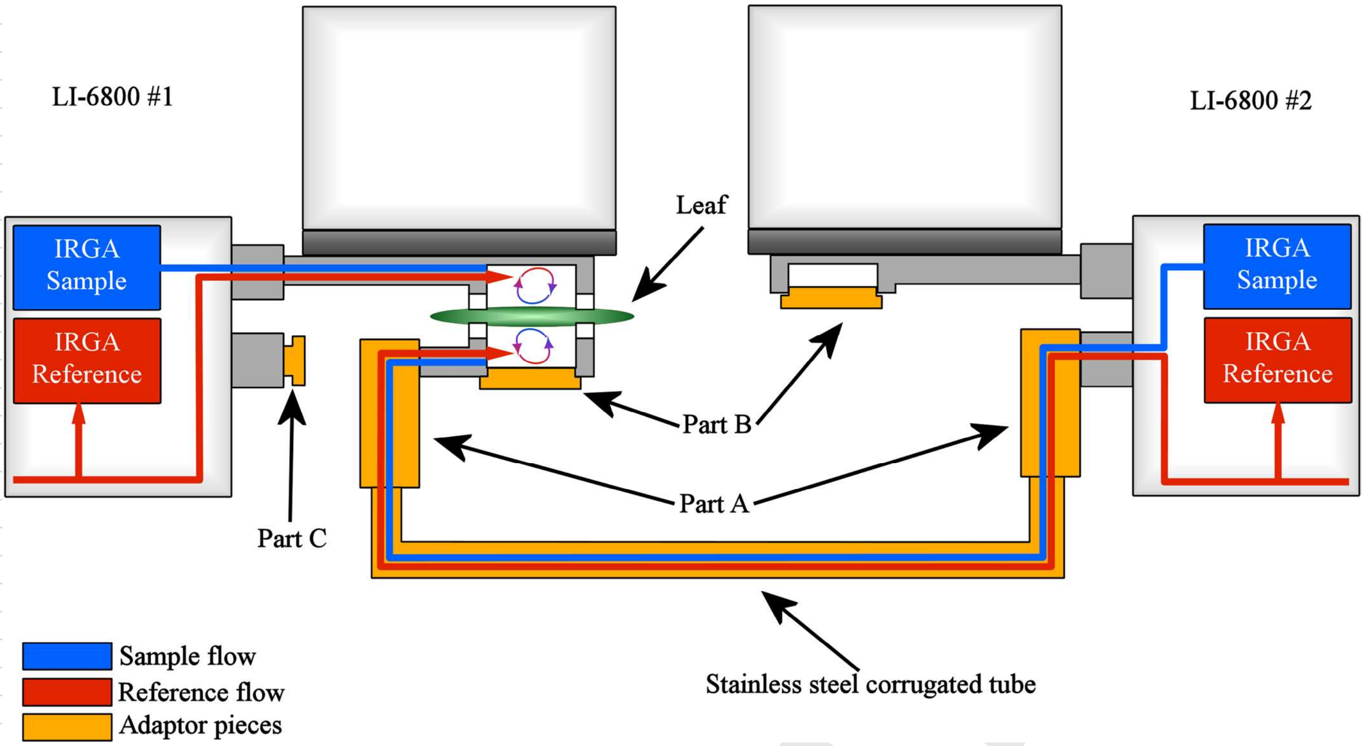
Extended Data Fig. 1 | Effect of κ on cuticular conductance calculations. Variation of g_{cw} calculation if the [CO_2] at the evaporative sites of the adaxial and abaxial surfaces of the leaf differ (Variation = $g_{cw}(\kappa)$ - $g_{cw}(1)$). Differences in [CO_2] in the evaporative site of the adaxial and abaxial surfaces impact gas exchange measurements and calculations to different degrees depending on the external measured values. For the purposes of our study, the impact on the g_{cw} calculation was tested by keeping measurements of external gas exchange parameters constant and changing κ.



Extended Data Fig. 2 | Average g_{cw} for a *C. annuum* leaf under different atmospheric conditions (assuming $\kappa = 1$). a, g_{cw} measured under different atmospheric CO$_2$ concentrations (C_a) over 30 minutes after achieving stable conditions (blue dots) and an average of 24 hours under light at constant [CO$_2$] following a diurnal cycle of atmospheric conditions (red dot); and (**b**) at different temperatures of the leaf and varying relative humidity (RH) also expressed as atmospheric saturation deficits (ASD) for the black dots. In panel (**a**), each blue point gives the average and standard deviation of 8 to 16 measurements (n); the red point gives the average and standard deviation of 239 measurements (n), one measurement being taken every 4 minutes. In panel (**b**), each red and blue dot gives the average and standard deviation of 16 to 31 measurements (n), one measurement taken every 2 minutes; black dots are single measurements under stable gas exchange conditions for at least 25 minutes. The measurements were made from low to high ASD and later from high to low ASD. Graphs (**a**) and (**b**) are different leaves. Cuticular conductance (g_{cw}) is expressed as the value for one surface and therefore needs to be doubled for the whole leaf ($\gamma = 1$).



Extended Data Fig. 3 | Effect of possible differences in abaxial and adaxial leaf cuticular conductance. Impact of unequal adaxial and abaxial g_{cw} in (**a**) the total cuticular conductance ($g_{cw\text{-}T} = g_{cw\text{-}ad} + g_{cw\text{-}ab}$) calculated for a leaf and in (**b**) the C_i calculation, depending on the proportion of abaxial over adaxial cuticular conductance (γ) using as a reference $g_{cw\text{-}T}$ calculated with γ=1. A single measurement under stable gas exchange conditions was selected for calculating the variation of each species using $\kappa = 1$. The dots show the values using our dark measurements to estimate γ for each species. Zhang et al.[40] measured weight loss via cuticle of detached leaves and they concluded that astomatous surfaces have about 30% lower evaporation than the cuticle of the surfaces with stomata. It is difficult to extrapolate these evaporation values to g_{cw} values in growing leaves because the boundary layer conductance and leaf temperature were not provided, and the leaves had already been detached for one hour. Bearing this in mind, we used dark measurements to estimate inequalities between the adaxial and abaxial cuticular conductance, which can be used to quantify a possible g_{cw} difference between the adaxial and adaxial surfaces. Then, assuming equal stomatal closure on the adaxial and abaxial surface, in *G. biloba* only 6% of the difference in g_{lw} was not explained by the stomatal density differences, 8% for *G. hirsutum* and 15% for *C. annuum*; the abaxial and adaxial g_{lw} of *H. annuus* were practically the same in the dark. Using equation (18), it was found that significant variations in γ can have a big impact on the calculated total cuticular conductance ($g_{cw\text{-}T}$= $g_{cw\text{-}ad}$+$g_{cw\text{-}ab}$). The magnitude of this impact depends on how much γ affects the w_i -w_s gradient.



Extended Data Fig. 4 | Diagram of the connection of two LI-6800. Part A: Flanges on the lower cuvette and the second LI-6800. Part B: Lid of the upper cuvette, the lid of the lower cuvette includes two cavities to insert two thermocouples. Part C: Flange to block the flow of gases from and to the lower cuvette.