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Direct measurement of intercellular CO₂ concentration in a gas-exchange system resolves overestimation using the standard method

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Abstract

Intercellular CO₂ concentration of leaves (Cᵢ) is a critical parameter in photosynthesis. Nevertheless, uncertainties in calculating Cᵢ arise as stomata close. Here, by modifying the assimilation chamber of a commercial gas-exchange equipment to directly measure Cᵢ, we demonstrate overestimation of calculated Cᵢ (i.e. Cᵢ(c)) without stimulating stomatal closure. Gas exchange was measured on one side of the leaf while measured Cᵢ (Cᵢ(m)) was acquired simultaneously on the other side of the leaf in hypostomatous passion fruit (Passiflora edulis Sims) and amphistomatous sunflower (Helianthus annuus L.) and common bean (Phaseolus vulgaris L.). The adaxial surface showed comparable Cᵢ(c) and Cᵢ(m) in sunflower, whereas in common bean, where the adaxial surface has a low stomatal density, Cᵢ(c) markedly differed from Cᵢ(m) when the stomata remained open. However, the latter discrepancy disappeared when measuring the leaf flipped upside down so that the gas exchange was measured (i.e. Cᵢ was calculated) on the abaxial side, which has a much higher stomatal density. The passion fruit showed the largest discrepancy on the astomatous side, indicating that the cuticle has a large impact on the calculation. Direct measurement of Cᵢ is recommended as a more accurate estimate than the calculation when stomatal gas transport is restricted. Occurrence of overestimation and prospects for direct measurement are discussed.

Keywords: A–Cᵢ curve, amphistomatous, cuticle, Helianthus annuus, hypostomatous, patchy stomatal closure, Passiflora edulis, Phaseolus vulgaris, photosynthesis, stomata.

Introduction

In land plants, leaves constitute the major interface with the atmosphere. Inside leaves, mesophyll cells consume CO₂ during photosynthetic assimilation (A), and consequently the CO₂ concentration in the intercellular airspace (Cᵢ) is lower than in the bulk air outside the leaf (Cₐ). The CO₂ enters leaves by diffusing through stomatal pores on the leaf surface, so Cᵢ essentially indicates the CO₂ substrate available for A (Farquhar et al., 1980). The cells inside leaves are wet, and the stomata allow water vapor to escape by transpiration. Although stomata can close if dehydration becomes excessive, CO₂ entry is also restricted by stomatal closure, thereby diminishing A. Therefore, the response of A to various Cᵢ (i.e. the A–Cᵢ curve) is the foundation for relating photosynthetic biochemistry to prevailing environmental conditions experienced by leaves (Hanson et al., 2016).

In general gas-exchange measurements, Cᵢ is calculated from the relationship between water vapor exiting and CO₂ entering through stomata (Moss and Rawlins, 1963). Instead, Sharkey et al. (1982) measured Cᵢ directly. They put amphistomatous leaves between a chamber and a cup and measured the CO₂...
concentration inside the cup after it had equilibrated. Because no net CO$_2$ exchange occurred through that side of the leaf, the CO$_2$ concentration measured in the cup would be close to the concentration inside the leaf (measured as C$_i$ C$_{i(m)}$). Indeed, the C$_{i(m)}$ was identical to the value calculated (calculated C$_i$ C$_{i(c)}$) on the other side.

Subsequently, close comparisons of C$_{i(m)}$ and C$_{i(c)}$ have highlighted the gradient of intercellular CO$_2$ in the airspace owing to the finite conductance of the airspace (Mott and O’Leary, 1984; Parkhurst et al., 1988). The gradient varies between species, depending on the anatomy of the mesophyll pores (Evans et al., 2009). For species having a fast assimilation rate, such as sunflower (Helianthus annuus L.), the gradient is generally too small to affect C$_{i(m)}$ (Mott and O’Leary, 1984; Boyer and Kawamitsu, 2011). The small effect observed for sunflower leaves was confirmed recently when a direct-measurement system for C$_i$ (Boyer and Kawamitsu, 2011) was incorporated into a commercial open gas-exchange apparatus (Tominaga and Kawamitsu, 2015a). Using a similar system, however, Tominaga and Kawamitsu (2015b) demonstrated that C$_{i(c)}$ markedly differed from C$_{i(m)}$ as stomata began to close after abscisic acid (ABA) was applied to this species. Tracing A–C$_i$ curves, the C$_{i(c)}$ showed an artefactual limitation of photosynthesis, as was previously indicated for ABA-fed leaves (Downton et al., 1988; Terashima et al., 1988; Meyer and Genty, 1998), whereas the C$_{i(m)}$ showed a similar curve to leaves with open stomata. Therefore, these investigators attributed this difference to overestimation of the calculation.

For the calculation, the diffusivity ratio of trace gases (water vapor, CO$_2$) through the leaf surface is considered constant—assuming stomata are the dominant path for both gasses (Moss and Rawlins, 1963, von Caemmerer and Farquhar, 1981; Boyer and Kawamitsu, 2011). Using hypostomatous leaves of grape (Vitis vinifera L.), however, small gas fluxes on the stomatous adaxial side (i.e. cuticle/epidermis) were detected while the opposite stomatous surface was sealed (Boyer et al., 1997; Boyer, 2015a). At the same time, C$_{i(c)}$ was much larger than expected (the photosynthetic CO$_2$ compensation point). Evidently, this discrepancy was a consequence of the cuticle, which transmits water vapor 20–40-fold faster than it transmits CO$_2$, a difference that is considerably larger than the constant 1.6 for stomata. Measuring C$_i$ directly in sunflower, Boyer (2015b) calculated cuticular transpiration on the stomatous surface and suggested that it could significantly overestimate CO$_2$ entry. Likewise, Tominaga and Kawamitsu (2015b) calculated the cuticular conductance of water vapor and reached the same conclusion.

On the other hand, a number of studies have attributed the overestimation of C$_i$ to a heterogeneous stomatal aperture or patchy stomatal closure. This likely occurs in response to acute stimuli (Gunasekera and Berkowitz, 1992), including application of ABA (Downton et al., 1988; Terashima et al., 1988), casting doubt upon the conclusion made about stomatous leaf surfaces. In fact, it is difficult to determine whether a suspicious value for C$_i$ can be attributed to cuticular transport or to stomatal patchiness because both effects may appear simultaneously as stomata close. Another factor of concern is the intercellular CO$_2$ gradient because any direct measurement inevitably limits CO$_2$ entry from one side, thereby increasing the gradient. Finally, in evaluations of the cuticle effect on the calculation (Boyer, 2015b; Tominaga and Kawamitsu, 2015b), CO$_2$ transport through the cuticle was assumed to be negligible; however, this might be an oversimplification.

We therefore conducted experiments to investigate the effect of the cuticle on the calculation without stimulating stomatal closure. Gas exchange was measured separately on the adaxial and abaxial leaf surfaces of common bean (Phaseolus vulgaris L.), for which the stomatal density differs on each side. Simultaneously, C$_{i(m)}$ was acquired for the other side. For comparison with common bean, we measured symmetric amphistomatous leaves of sunflower (Helianthus annuus L.) and hypostomatous leaves of passion fruit (Passiflora edulis Sims).

### Materials and methods

#### Plant material

Common bean (P. vulgaris L. cv. Kentucky Blue) and sunflower (H. annuus L. cv. Hybrid sunflower) plants, each 5–6 weeks old, were sources of amphistomatous leaves, and 1-year-old purple passion fruit (P. edulis Sims) plants were the source of hypostomatous leaves. Passion fruit plants were grown in 12-liter plastic pots, and the bean and sunflower plants were grown in 4-liter plastic pots; each pot contained a soil mixture (soil:peatsand=1:1:1) in a greenhouse located at the University of the Ryukyus, Okinawa, Japan (26°15’N, 127°45’E; elevation 127 m). The growth irradiance depended on solar radiation, and the daily maximum ranged from 300 to 1500 μmol m$^{-2}$ s$^{-1}$ photosynthetically active radiation (PAR) for sunflower and common bean. Passion fruit was grown under a shade net and received approximately 30% less irradiance than the other species. The daily maximum and minimum growth temperatures were, on average, 30.9 °C and 16.2 °C, respectively. Water was supplied whenever the soil surface was dry. All soil was saturated weekly with Hoagland’s nutrient solution composed of 4 mM KNO$_3$, 6 mM Ca(NO$_3$)$_2$·4H$_2$O, 2 mM MgSO$_4$·7H$_2$O, 2 mM KH$_2$PO$_4$, 0.5 μM CuSO$_4$·5H$_2$O, 10 μM MnSO$_4$·H$_2$O, 2 μM ZnSO$_4$·7H$_2$O, 25 μM H$_3$BO$_3$, 0.5 μM H$_2$MoO$_4$, and 0.5 μM Fe(III)·EDTA. All experiments with these plants were conducted with fully expanded leaves.

#### Gas-exchange and direct-measurement systems

The gas-exchange system has been reported in detail elsewhere (Tominaga and Kawamitsu, 2015a), and we used a modified system (the design drawings are available upon request). Briefly, the small cup of the Tominaga and Kawamitsu system was replaced with the bottom half of an assimilation chamber (LI-6400-40; Li-Cor, Lincoln, NE, USA) of a commercial gas-exchange system (LI-6400XT; Li-Cor). Although use of the commercial assimilation chamber has certain advantages (e.g. combined fluorescence measurement), the small chamber is especially prone to diffusion leaks (Rodeghiero et al., 2007). To minimize the effect of leakage and detect small fluxes accurately, we used a larger chamber/cup that could also be attached to the sensor head (Fig. 1). This new laboratory-made chamber/cup with a window diameter of 6 cm encloses a 14-fold larger leaf area (28.3 cm$^2$) than the LI-6400–40 chamber (2 cm$^2$) with the window diameter of 0.8 cm. The chamber window is covered with Propafilm (polypropylene that is coated with Saran; 250–01885, Li-Cor).

The new chamber/cup was replaced with the upper or lower half of the chamber (Fig. 1). A paraffin/lanolin mixture (2:8) was spread evenly (thickness ~1 mm) on the rims of both the chamber and the cup (i.e. the interface). This semi-solid coat sealed the chamber tightly when a leaf was clamped. All experimental leaves were large enough to cover the entire window area so that the upper chamber and lower cup were divided by the leaf. In the cup, CO$_2$ equilibrated with that inside the stomatal pores (C$_{i(m)}$). The equilibrated air was smoothly circulated with a small fan to the infrared gas analyser (IRGA; LI-840A; Li-Cor) in a closed loop.
adjusted to 300 \( \mu \text{mol} \ \text{m}^{-2} \text{s}^{-1} \) between the light source and the chamber so that the irradiance was approximately 1 \( \text{m}^{-2} \) above the chamber. Fine net shades were inserted when the leaf was illuminated with three metal-halide lamps (D-400, Toshiba) located above the chamber. Water circled inside the chamber wall (b) controls air temperature in the cup. A small blower circulates the air in the cup to the gas analyser (LI-840A; Li-Cor) through the inlet and outlet located at the bottom of the cup (c). The round chamber window (28.3 cm\(^2\)) is covered with Propafilm (250-01885, Li-Cor). A leaf-temperature thermocouple (6400-04; Li-Cor) is inserted into the cup and attached to the lower surface of the leaf, as is the case in the standard LI-6400 chamber.

As a result of the lower irradiance, the PAR used in a previous study (Kawamitsu, 2015) was less than the 800 \( \mu \text{mol} \ \text{m}^{-2} \text{s}^{-1} \) supplied to the LI-6400 console. According to Kawamitsu (2015), the ratio of 300–400 \( \mu \text{mol} \ \text{m}^{-2} \text{s}^{-1} \) to the LI-6400-40 chamber had a better seal. Because the leakage can cause gas fluxes that affect both assimilation and transpiration, which are measured on a leaf-area basis, the apparent fluxes (leak rate divided by leaf area) were significantly decreased in the larger chamber as compared with the previous value of 0.09 \( (C^\text{a}=280 \ \mu \text{mol} \ \text{mol}^{-1}) \) measured previously (Tominaga and Kawamitsu, 2015b). All gas-exchange measurements were carried out at a leaf temperature of 25 ± 0.1 °C, a leaf to air vapor pressure deficit of <1.5 kPa, and a constant gas flow rate of 500 \( \mu \text{mol s}^{-1} \). Common bean and sunflower leaves responded by slowly closing their stomata when the \( \text{CO}_2 \) concentration was somewhat higher than the ambient level, which often required a longer measurement time. To help decrease the measurement time, the ambient \( \text{CO}_2 \) concentration was increased to no more than 700 \( \mu \text{mol mol}^{-1} \).

**Diffusion leaks**

The upper chamber and lower cup were separated by clamping aluminum foil (instead of the leaf in Fig. 1C) using the paraffin/lanolin coat. For the open gas-exchange system, a test for diffusion leaks was carried out by estimating \( \text{CO}_2 \) and the water vapor diffusion molar flow rate, \( K_{\text{CO}_2} \) and \( K_{\text{H}_2\text{O}} \), respectively, according to Rodeghiero et al. (2007). To maintain the concentration gradients of \( \text{CO}_2 \) and water vapor between the outside and inside of the chamber, the sensor head was enclosed by a semi-closed cylinder in which air containing 400 \( \mu \text{mol mol}^{-1} \) \( \text{CO}_2 \) and 20 mmol \( \text{mol}^{-1} \) water vapor (monitored with the LI-840A) flowed continuously at a rate of 15 l min\(^{-1} \) whereas dry air with 30 \( \mu \text{mol mol}^{-1} \) \( \text{CO}_2 \) was supplied to the chamber from the console. \( K_{\text{CO}_2} \) and \( K_{\text{H}_2\text{O}} \) were estimated as 0.21 ± 0.079 and 0.71 ± 0.13 \( \mu \text{mol s}^{-1} \), respectively, values that were comparable to those determined for the smaller LI-6400-40 chamber (Rodeghiero et al., 2007; Tominaga and Kawamitsu, 2015a), suggesting a similar rate of diffusion leak on a chamber basis. Accounting for the larger chamber interface (i.e. circumference of the chamber window), the new chamber had a better seal. Because the leakage can cause gas fluxes that can affect both assimilation and transpiration, which are measured on a leaf-area basis, the apparent fluxes (leak rate divided by leaf area) were significantly decreased in the larger chamber as compared with the previous

**Gas-exchange measurements**

After reaching steady state photosynthesis at the ambient \( \text{CO}_2 \) concentration of 300–400 \( \mu \text{mol} \ \text{m}^{-2} \text{s}^{-1} \), the concentration was changed stepwise, allowing \( C^\text{a} \) to vary. \( \text{CO}_2 \) concentration was regulated by mixing pure \( \text{CO}_2 \) supplied to the LI-6400 console with \( \text{CO}_2 \)-free air humidified with a dew-point generator (LI-610; Li-Cor). Each leaf was illuminated with three metal-halide lamps (D-400, Toshiba) located approximately 1 m above the chamber. Fine net shades were inserted between the light source and the chamber so that the irradiance was adjusted to 300 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) PAR, which was less than the 800 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) PAR used in a previous study (Tominaga and Kawamitsu, 2015a,b). We reduced the irradiance to control leaf temperature as the light source containing a broad thermal ray otherwise warmed the leaf. As a result of the lower irradiance, the \( A \) for sunflower leaves was below saturation and yielded a lower \( A/C^\text{a} \) ratio of 0.064 \( (C^\text{a}=220 \ \mu \text{mol} \ \text{mol}^{-1}) \) than the

**Fig. 1.** The lab-made chamber/cup for direct measurement of intercellular \( \text{CO}_2 \) concentration (C). View from (A) front side and (B) back side, and (C) schematic diagram of the chamber/cup clamped on a leaf (represented by the green horizontal line). The brass chamber/cup was specially designed for the sensor head of the Li-Cor LI-6400XT open gas-exchange system. The leaf separates the cup from the chamber. The leaf-clamp seal is ensured by a coating of paraffin/lanolin. Mixing air passes through the upper chamber, allowing the leaf surface to exchange gases that are exhausted through the bypass (a). Water circulated inside the chamber wall (b) controls air temperature in the cup. A small blower circulates the air in the cup to the gas analyser (LI-840A; Li-Cor) through the inlet and outlet located at the bottom of the cup (c). The round chamber window (28.3 cm\(^2\)) is covered with Propafilm (250-01885, Li-Cor). A leaf-temperature thermocouple (6400-04; Li-Cor) is inserted into the cup and attached to the lower surface of the leaf, as is the case in the standard LI-6400 chamber.
system. The CO₂ and water vapor concentrations outside the chamber were monitored by open-path IRGA (LI-7500; Li-Cor) set around the leaves, and were subsequently used to correct for leaks as described in Rodeghiero et al. (2007). The apparent fluxes in the open system were estimated to be <0.03 μmol m⁻² s⁻¹ for CO₂ and <0.001 mmol m⁻² s⁻¹ for water vapor throughout the measurements. In most of our experiments, such small fluxes changed the calculation of $C_i$ by only <1%. The CO₂ leakage in the closed system increases or decreases the equilibrium of $C_{i(m)}$ depending on the concentration gradient relative to the outside. To detect the leakage, we monitored the change of CO₂ concentration in the empty cup ($C_{i(up)}$) after partially enclosing the exprited air containing a high concentration of CO₂ (~1800 μmol mol⁻¹) (see Supplementary Fig. S2). The leak was indicated by the $C_{i(up)}$ decreasing slowly at a rate of 10.8 μmol mol⁻¹ h⁻¹ while the outside CO₂ concentration ($C_{i(out)}$) was constant at ~400 μmol mol⁻¹. Based on this leak rate and the known volume of the closed system (137 ml), the rate of CO₂ diffusion leak was estimated to be 0.0054 μmol m⁻² s⁻¹ on a leaf-area basis and should be even lower when $C_{i(out)}$ is closer to $C_{i(m)}$ (i.e. 400–500 μmol mol⁻¹) in our experiments. The CO₂ leakage occurred too slowly to affect the equilibrium, and thus no correction was applied to the $C_{i(m)}$.

Stomatal density and size

After gas-exchange measurements, nail polish was thinly spread at several points on both the upper and lower leaf surfaces within the measured leaf area. After waiting for the polish to dry, the polish was stripped with clear double-sided tape and attached to a glass slide (i.e. a replica of the epidermis). Microphotographs of the replica were taken with a digital camera (Eclipse 80i; Nikon, Tokyo, Japan) attached to a microscope (Eclipse 80i; Nikon, Kawasaki, Japan). For each side of the leaves, 20 microphotographs were taken at random. We counted the number of stomata in each microphotograph (0.64–1.82 mm²) to calculate stomatal density. Mean stomatal size was calculated based on the length of the long axis of the stomata.

Calculations

Realizing that departing vapor behaved like entering CO₂, Moss and Rawlins (1963) first calculated $C_i$ ($C_{i(up)}$) from measured variables:

$$C_{i(up)} = C_i - 1.6 \frac{A}{E} (w_i - w_a)$$

where $C_i$ is the CO₂ concentration in the air (mol mol⁻¹), $A$ is the assimilation rate for CO₂ moving into the leaf through stomata (μmol m⁻² s⁻¹), $E$ is the transpiration rate for water vapor moving out of the leaf through stomata (μmol m⁻² s⁻¹), and $w_i$ and $w_a$ are the water vapor concentrations in the leaf and outside the leaf, respectively (mol mol⁻¹). The only other factor needed was the ratio of the diffusivity of water vapor to that of CO₂, which is ~1.6 in air (Jarvis, 1971; Massman, 1998) assuming that stomata are the dominant path for both gases. Von Caemmerer and Farquhar (1981) rigorously extended the Moss–Rawlins relation to account for interactions between water vapor and CO₂:

$$C_{i(m)} = C_i - 1.6 \frac{A + CE}{E - \bar{w}E} (w_i - w_a)$$

where $\bar{C}$ and $\bar{w}$ ( $\bar{C}=(C_i + C_{i(up)})/2$ and $\bar{w}=(w_i + w_a)/2$) are interaction terms. This equation became the norm for determining $C_i$ and is routinely used in commercial gas-exchange units including the LI-6400. Note that both $C_i$ and $w_a$ were implicitly located in the boundary layer of the leaf surface in this study. The $C_i$ and $w_a$ were, respectively, estimated with the boundary layer conductance of water vapor (1.38 mol m⁻² s⁻¹) and that of CO₂ (1.01 mol m⁻² s⁻¹) estimated from the ratio of diffusivity in the ‘convective air’ (1.37) rather than ‘still air’ (1.6) for the stomatal pore (Ball, 1987). Von Caemmerer and Farquhar (1981) expressed the diffusion property of water vapor in Eq. (2) as a form of conductance ($g_w$):

$$g_w = \frac{E - \bar{w}E}{w_i - w_a}$$

In contrast to Eq. (2), the $C_{i(m)}$ already reflected the interactions of CO₂ and water vapor (Boyer and Kawamitsu, 2011):

$$C_{i(m)} = C_i - 1.6 \frac{A}{E - \bar{w}E} (w_i - w_a)$$

where the only difference from Eq. (2) is the absence of the term $\bar{C}$. Boyer and Kawamitsu (2011) first expressed conductance of CO₂ ($g_c$) in terms of directly measured variables:

$$g_c = \frac{A}{C_i - C_{i(m)}}$$

In this study, the conductance of water vapor ($g_w$) and of CO₂ ($g_c$) was derived from the directly measured variables using Eqs (3) and (5), respectively.

**Results**

**Stomatal density and size**

In leaves of the common bean, the adaxial side had significantly fewer stomata (9 ± 5 mm⁻²) than the abaxial side (274 ± 31 mm⁻²), whereas in sunflower the stomata were distributed more evenly on both sides (Table 1). On the adaxial side, the stomatal density in sunflower (221 ± 24 mm⁻²) is much higher than in common bean. The stomatal ratio (adaxial/abaxial) was 0.03 for common bean leaves and 0.85 for sunflower. Stomatal size did not differ significantly between the two sides in sunflower, whereas in the common bean the adaxial surface had 33% larger stomata than the abaxial surface. Passion fruit leaves, 

| Table 1. Stomatal density and size in passion fruit, sunflower, and common bean |
|---------------------------------|-------------------------------|-----------------|-----------------|-----------------|
| Stomatal density (mm⁻²)         | Adaxial                      | Abaxial         | $P$             | Ratio (adaxial/abaxial) |
| Passion fruit                   | 0 (n=80)                     | 209 ± 26 (n=80) | <0.001          | 0.85             |
| Sunflower                       | 188 ± 25 (n=80)               | 221 ± 24 (n=80) | <0.001          | 0.032            |
| Common bean                     | 9 ± 5 (n=160)                 | 274 ± 31 (n=160) |                |                  |
| Long axis of stomata (μm)       | Passion fruit                 | 10.7 ± 1.3 (n=40) | 0.583           | 1.02             |
| Sunflower                       | 12.4 ± 1.8 (n=40)             | 12.2 ± 2.7 (n=40) |                |                  |
| Common bean                     | 10.9 ± 1.7 (n=80)             | 8.2 ± 1.3 (n=80) | <0.001          | 1.33             |

The values are the mean ±SD.
which were observed to bear stomata only on the abaxial surface, had a stomatal density (209 ± 26 mm⁻²) comparable to that of sunflower.

**Hypostomatous leaves**

We placed the cup on the abaxial stomatous side while photosynthesis and transpiration were measured simultaneously in the chamber on the adaxial stomatous side of passion fruit leaves. Soon after clamping the leaf, both assimilation rate (A) and conductance of CO₂ (gₐ) became too small to be detected on the stomatous side (Fig. 2A). In contrast, both transpiration rate (E) and conductance of water vapor (gₙ) were detectably large on the same side (Fig. 2B). These results demonstrated that the cuticle loses more water vapor than the amount of entering CO₂. On the stomatous side, photosynthesis diminished the amount of CO₂ inside the cup until equilibrium was reached (Fig. 2C). Because CO₂ scarcely entered from the stomatous surface (Fig. 2A), the CO₂ concentration at equilibrium (Cᵢ(m)) ought to be equivalent to the photosynthetic CO₂ compensation point where no net flux occurs in the leaf. In agreement with this supposition, Cᵢ(m) equilibrated to ~43 μmol mol⁻¹ at a leaf temperature (Tₑ) of 25 °C. In contrast, Cᵢ calculated from the transpiration (Cᵢ(c)) remained high, differing substantially from Cᵢ(m). E on the stomatous surface denotes cuticular transpiration (Eₐcut), and on average Eₐcut was 4.25 μmol m⁻² s⁻¹ at a Tₑ of 25 °C. While keeping the ambient vapor concentration (wᵦ) constant, the vapor concentration inside the leaf (wᵣ) was raised stepwise by heating the leaf to 29 °C and subsequently to 33 °C (see Supplementary Fig. S3). The difference of the vapor concentration gradients across the cuticle (wᵣ−wᵦ) increased from 21 mmol mol⁻¹ (at Tₑ of 25 °C) to 29 and 39 mmol mol⁻¹, respectively. Eₐcut correlated positively and linearly with the vapor concentration gradients (Fig. 3).

We calculated the cuticular conductance of water vapor (gₐcut) on the stomatous surface according to gₐcut = Eₐcut/(wᵦ − wᵣ) (Boyer et al. 1997). The gₐcut was 0.21 mmol m⁻² s⁻¹ at each leaf temperature (Table 2) and also coincided well with the slope of the regression in Fig. 3. The constant gₐcut indicated that the cuticle was stable at physiological temperatures (25–33 °C), supporting the previous study of isolated cuticles (Riederer and Schreiber, 2001). On the stomatous side, Cᵢ(m) closely followed leaf temperature (Supplementary Fig. S3). The value and temperature dependence of Cᵢ(m) (Table 2) were similar to those of the intercellular CO₂ photocompensation point (Cᵢ*) for Cₐ species (Walker and Ort, 2015). In this experiment, the smaller wᵦ compared with wᵣ during a rapidly balanced state caused leakage of water vapor into the chamber. The effect on Eₐcut was estimated to be greatest (7.6–13.3% of the apparent flux) when Eₐcut was small at 25 °C and reached a minimum (4.6–6.0%) when Eₐcut was high at 33 °C.

**Amphistomatous leaves**

The adaxial side of leaves was set up to allow gas exchange, and we measured A–Cᵢ curves in sunflower—the species previously assessed with a smaller chamber and cup (Tomimaga and Kawamitsu, 2015a,b). The adaxial stomata remained open during the experiments as indicated by the relatively constant gₐ (Fig. 4A). A was rapidly balanced with Cᵢ(m) at each Cᵢ. As expected for open stomata (gₐ = 178 ± 24 mmol m⁻² s⁻¹), the calculation coincided well with the direct measurement for this species (Fig. 4B). It was clear that the larger cup facilitated a response of Cᵢ(m) as fast as Cᵢ(c). We then carried out the experiment using common bean leaves, for which the stomatal
ratio contrasts with that of sunflower leaves (Table 1). With few stomata on the adaxial surface, gs was <20 mmol m⁻² s⁻¹ (Fig. 5A). A sensitive response of A to CO₂ indicates open stomata. Comparison with gs suggested that gs became noisy when Ci was small (see Supplementary Fig. S4) due to the detection limit of A but not the Cᵢ(ᵢᵣ) (see Discussion). The gradient between Ci and Cᵢ(ᵢᵣ) appropriately directed the sub-tissue CO₂ flux, demonstrating the accuracy of the measurement, e.g. Cᵢ(ᵢᵣ) was always lower than the Cᵢ when A is positive and vice versa. The Cᵢ(ᵢ) differed largely from the Cᵢ(ᵢᵣ) from the outset (Fig. 5B). The effect of leak corrections on the Cᵢ(ᵢ) was <4%. To further investigate the cause of this discrepancy, we flipped the leaf so that CO₂ could readily diffuse into the abaxial side having much greater stomatal density (Fig. 6). The gs was 222 ± 15 mmol m⁻² s⁻¹, and the average CO₂ conductance ratio (abaxial/adaxial) was 0.073. This value was more than double that of the stomatal ratio (0.032), suggesting that the stomata on the adaxial surface, although fewer in number, opened wider perhaps because of their larger size (Table 1). It took longer for Cᵢ(ᵢᵣ) to equilibrate for the flipped leaves because the CO₂ in the cup diffused more slowly through the adaxial side with few stomata (cf. Figs 5 and 6). At equilibrium, the Cᵢ(ᵢᵣ) and Cᵢ(ᵢ) were similar (Fig. 6B).

**Diffusivity ratio of water vapor and CO₂ of the leaf surface**

After plotting A against Cᵢ(ᵢᵣ) and Cᵢ(ᵢ) for the experiments described above, suppressed A–Cᵢ curves were then derived from Cᵢ(ᵢ), for the common bean leaves, for which gas exchange was measured on the adaxial side (Fig. 7A). The similar result for this species was also observed previously using the small chamber/cup system (see Supplementary Fig. S5). Evidently, the large discrepancy between the Cᵢ(ᵢ) and Cᵢ(ᵢᵣ) was not induced by patchy stomatal closure because the adaxial stomata remained open (Fig. 5). Assuming that this discrepancy is created by the intercellular CO₂ gradients (i.e. Cᵢ(ᵢᵣ)–Cᵢ(ᵢ)), flipping the leaf should impose the gradient to a similar degree to achieve the same A while keeping the intercellular conductance unchanged. In fact, this was not true (abaxial in Fig. 7A), and even comparable Cᵢ(ᵢ) and Cᵢ(ᵢᵣ) suggested minute gradients. Consequently, the calculation overestimated Cᵢ by neglecting cuticular gas transport since other possibilities were likely ruled out.

Upon measurement of leaf gas exchange, calculations essentially rely on the diffusivity ratio (water vapor/CO₂) for stomata [1.6 in Eqs (1) and (2)], assuming that stomata are the only path for both water vapor and CO₂. To measure the impact of
Direct measurement of intercellular CO$_2$ concentration in leaves

The cuticle on the calculations, the diffusivity ratio through the leaf surface ($D_{w/c}$) was estimated from directly measured variables based on Eq. (4):

$$C_i - C_{i(m)} = D_{w/c} \frac{A}{E - \frac{A}{E}} (w_i - w_e)$$

(6)

where $D_{w/c}$, instead of a theoretical constant 1.6 in Eq. (4), indicates the slope of the regression line for this relationship in Fig. 7B. For sunflower leaves, $D_{w/c}$ was 1.58 ± 0.05. For common bean, values of $D_{w/c}$ were 2.44 ± 0.37 and 1.79 ± 0.12 for the adaxial and abaxial side, respectively.

**Discussion**

Our results demonstrate that the cuticle is an essential contributor to the large discrepancy between $C_i(c)$ and $C_{i(m)}$, supporting the conclusion reached with different species using different approaches (Boyer, 2015b; Tominaga and Kawamitsu, 2015b). In those previous experiments, ABA was fed to sunflower leaves to close stomata, thereby simulating the astomatous leaf surface. Although the stomatal closure is imperfect, the calculation overestimates $C_i$ whereas the direct measurement is unaffected by the open/closed status of stomata. Here, we employed common bean leaves with few stomata on one side, i.e. instead of manually inducing stomatal closure, and we also observed a large discrepancy (Figs 5 and 7A), as seen with stomatal closure in sunflower leaves (Boyer, 2015b; Tominaga and Kawamitsu, 2015b). These stomata remained open even wider than those on the opposite, more stomatous surface, rejecting the possibility of patchy stomatal closure. Moreover, the discrepancy was not a consequence of the intercellular CO$_2$ gradient because it disappeared for the flipped leaf with the intercellular conductance unchanged. These results were consistent with the evidence for passion fruit that clearly shows that cuticular gas transport is not negligible (Fig. 2).

**Implications of the direct measurements**

Instead of diffusing through pores, at the outer cuticle layer both CO$_2$ and water vapor diffuse through solid wax, which creates most of the resistance to cuticular gas transport (Šantrůček et al., 2004). Consequently, the cuticle has a much smaller conductance than stomata despite covering most of the leaf surface (Table 2), and therefore the impact on the calculation is not substantial unless the stomatal gas transport diminishes in stomatous surfaces.

The cuticle effect appears because cuticle transports more water vapor than CO$_2$ (Boyer et al., 1997; Boyer, 2015a), as can be measured in the astomatous gas exchange (Fig. 2). The effect was still detectable for the stomatous leaf surface having $D_{w/c}$ larger than 1.6 (Fig. 7B). Those $D_{w/c}$ values were much less than those of 20–40 for the astomatous side of grape leaves (Boyer et al., 1997; Boyer, 2015a) because the stomata still accounted for gas exchange to some extent. Similarly, $D_{w/c}$ was 5.5 when the cuticular transpiration was estimated to be 5–16% of the total transpiration for sunflower leaves fed with ABA (Boyer, 2015b).
To what extent does stomatal closure bring uncertainty to the calculation of $C_i$? According to Eq. (6), we estimated $D_{w/c}$ for the $A-C_i$ curve measurements in sunflower leaves fed with ABA (Fig. 4 in Tominaga and Kawamitsu, 2015b) and plotted it against various values of 'stomatal conductance' ($g_w$) (Fig. 8A). The $D_{w/c}$ generally appeared to rise sharply as the $g_w$ decreased from 100 mmol m$^{-2}$ s$^{-1}$. This trend is reasonable because cuticular conductance remains while stomatal conductance is reduced by stomatal closure. In addition, the $D_{w/c}$ varied at a given $g_w$ in different leaves as stomata closed (shown as different symbols in Fig. 8A), indicating variable cuticular conductance among leaves of a single plant (Kerstiens, 1996). This is further supported by the $g_w$–$D_{w/c}$ relationship for the adaxial side of common bean having the largest $D_{w/c}$ despite the highest $g_w$ (Fig. 8B). The variation may be attributable to a lateral non-uniformity of cuticular conductance—the thinner cuticle of the guard cells likely has higher conductance than the cuticle on the surrounding epidermal cells (Šantrůček et al., 2004). The cuticular conductance varied on the stomatous side (Fig. 8) perhaps with the properties of guard-cells/stomata (e.g. number, size, density), whereas it was relatively constant without stomata (Table 2). This possibility can be tested by analysing the $g_w$–$D_{w/c}$ relationships for leaves having various stomatal properties.

When using the regular assimilation chamber with gas flowing both adaxially and abaxially, $C_i$ is calculated from the sum of fluxes on both sides. Therefore, hypostomatous or asymmetric amphistomatous leaves are not necessarily prone to the overestimation because the gas exchange occurring mostly on the stomatous side dilutes the gas exchange through the cuticle (as shown in the $A-C_i$ measurements with a regular Li-Cor head set-up in Supplementary Fig. S5). However, the cuticular gas transport increases with the doubled cuticle. So, it is expected that the $D_{w/c}$ illustrated in Fig. 8A departs from 1.6 more when gas-exchange measurements are configured normally.

In $A-C_i$ curve measurements, ABA caused either no depression (Raschke and Hedrich, 1985) or moderate depression in the broad bean, *Vicia faba* (Terashima et al., 1988). Likewise, *Xanthium strumarium* often showed significant depression (Fischer et al., 1986; Daley et al., 1989), but not always (Dubbe et al., 1978; Mott and Takemoto, 1989). Contrasting results were also observed during experiments (Raschke and Hedrich, 1985; Mott, 1995). These reported experimental inconsistencies were not necessarily attributable to stomatal patchiness but perhaps to the extent of stomatal closure and/or variable cuticular conductance.

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**Fig. 7.** Data from Figs 2–4 using sunflower and common bean plotted as (A) $A-C_i$ curves and (B) relationships derived from the measured variables in Eq. (6). In (A), closed circles denote $C_{i(m)}$ and open circles denote $C_{i(c)}$. Note that $C_{i(m)}$ and $C_{i(c)}$ are on top of each other for adaxial leaf side of sunflower and abaxial leaf side of common bean. The slope in (B) indicates the diffusivity ratio through the leaf surface (water/CO$_2$).
Stomatal patchiness may affect the calculation as lateral gas diffusion is obstructed in the intercellular airspace (Terashima et al., 1988; Terashima, 1992). This is a trait of heterobaric leaves in which bundle-sheath extensions span the gap between the upper and the lower epidermis. Homobaric leaves lacking this barrier, on the other hand, have the interconnected airspace. All our measurements so far have been limited to heterobaric leaves. However, because of a similar arrangement, the cuticle does affect the calculation regardless of the internal anatomy.

**Considerations of the direct measurement**

We technically altered the amphistomatal to hypostomatal leaves by attaching the cup on one side, thereby increasing the CO₂ gradients (Parkhurst et al., 1988; Boyer and Kawamitsu, 2011). The $C_{\text{m}}$ reflects an equilibrium with the concentration just beneath the interior of epidermis on the cup-attached side. On the other hand, the site for the $C_{\text{m}}$ may be peristomata beneath the chamber-attached side (Sharkey et al., 1982; Mott and O’Leary, 1984; Parkhurst et al., 1988), or it may be deeper inside the leaf (Nonami and Schulze, 1989; Brodribb et al., 2007). In either case, the $C_{\text{m}}$ represents the CO₂ that has diffused through a somewhat longer path. This has been previously indicated by the $C_{\text{m}}$ being slightly but consistently lower than the $C_{\text{m}}$ in sunflower leaves with open stomata (Tomina and Kawamitsu, 2015a,b; Boyer, 2015a,b). In contrast, the $C_{\text{m}}$ in the present experiments was almost identical to the $C_{\text{m}}$ in the sunflower leaves (Figs 4 and 7A), suggesting little gradient. Because the gradients must be created by the mesophyll assimilation activity (i.e. $A$), this is likely due to slower assimilation under sub-saturating irradiance ($300 \mu\text{mol m}^{-2} \text{s}^{-1}$). For the flipped leaves illuminated from the abaxial side, even slower mesophyll activity (indicated by the smaller $A-C_{\text{m}}$ slope) should have further contributed to the minor gradients (Fig. 7A).

The larger chamber has advantages over the smaller chamber owing to the shorter length (circumference) of the gasket relative to the enclosed leaf area. The leak effect should be reduced because gaseous pores between the rubber gaskets and leaf are the major path for leakage in commercial chambers (Flexas et al., 2007). Diffusion leak could be further suppressed by using semi-solid materials for the gaskets. Consequently, our lab-made chamber enabled commercial open gas-exchange equipment to measure in vivo cuticle properties (Fig. 3). Nevertheless, the $g_{\text{w}}$ derived from the $C_{\text{m}}$ became erratic as $C_{\text{i}}$ decreased (Figs 5A and 6A). As $C_{\text{i}}$ decreases, $g_{\text{w}}$ becomes increasingly sensitive to the small $A$, for which the noise/signal ratio increases accordingly whereas the CO₂ gradient ($C_{\text{i}}-C_{\text{w}}$) decreases [Eq. (5)]. On the other hand, the water vapor gradient ($w_{\text{i}}-w_{\text{w}}$) is relatively stable, and thus the calculation of $g_{\text{w}}$ [Eq. (3)] is insensitive to change in $C_{\text{i}}$ (see Supplementary Fig. S4). The signal noise in $A$ also affects the estimation of $D_{\text{w/c}}$ [Eq. (6)]. Thus, $D_{\text{w/c}}$ is recommended to be estimated from multiple measurements when $g_{\text{w}}$ is stable (Fig. 7B), or otherwise discarded in the region where $A$ is small (as implemented in Fig. 8).

In clamp-on chambers, gas-exchange measurements may be affected by the edge—respiratory CO₂, originating from the leaf areas below the gaskets, laterally diffuses through the intercellular airspace to the leaf areas inside the chamber (Jahnke and Krewitt, 2002; Pieruschka et al., 2005). Because this internal CO₂ supply tends to increase $C_{\text{i}}$ around the edge while reducing the apparent $A$ there (Pieruschka et al., 2006), both measured flux and $C_{\text{m}}$ can be affected. The effect is detectable in homobaric leaves but not heterobaric leaves with negligible lateral diffusion (Pieruschka et al., 2005, 2006). As with leakage through the gasket, the larger chamber/cup helps to avoid the intercellular leakage by decreasing the edge-to-area ratio of the enclosed leaf part.

**Implications for the photosynthesis model parameters**

The impact of the cuticle carries over to the critical parameters in photosynthetic models (Hanson et al., 2016). Because mesophyll conductance ($g_{\text{m}}$) describes assimilatory CO₂ drawdown from intercellular spaces to chloroplast ($C_{\text{i}}$; i.e. $g_{\text{m}}=A/(C_{\text{i}}-C_{\text{w}})$), overestimation of $C_{\text{i}}$ immediately leads to underestimation of

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**Fig. 8.** Relationships between conductance of water vapor ($g_{\text{w}}$) and diffusivity ratio through the leaf surface ($D_{\text{w/c}}$) estimated in A–C curve measurements for adaxial side of (A) sunflower leaves fed with 10 μM ABA and (B) common bean leaves. Different symbols represent replications with different leaves. Data shown in (A) are representative analysis of the previous experiments (Fig. 4 in Tominaga and Kawamitsu, 2015a). Data are removed when $A <\pm 1.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ in (A) and $A <\pm 0.4 \mu\text{mol m}^{-2} \text{s}^{-1}$ in (B) due to incorrect $D_{\text{w/c}}$ (see Discussion). In (B), vertical and horizontal bars indicate ±SD ($n=3$).
Conclusions and prospects

Overestimation of $C_i$ is a general problem in leaf gas-exchange measurements. Obviously, there should be caution when stomatal closure is stimulated, e.g. under drought and salinity (Flexas et al., 2004). Current techniques cannot distinguish cuticular gas transport from measured gas exchange on stomatos leaf surfaces. This makes the overestimation unpredictable. Assuming cuticular conductance to CO$_2$ (i.e. cuticular conductance to CO$_2$ is zero), $g_w$ can be estimated on the stomatos surface and used for correction (Boyer, 2015a; Tominaga and Kawamitsu, 2015b). However, the correction would be obstructed by the variability among leaves (Fig. 8). We conclude that direct measurements are a better option—especially when stomatal gas transport is restricted (Boyer, 2015a,b; Tominaga and Kawamitsu, 2015a,b; Hanson et al., 2016).

Given climate change will likely increase water scarcity worldwide, it is urgent to predict plant response to water deficit that primarily affects photosynthesis (Hanson et al., 2016). To achieve this goal, it is essential to accurately estimate the photosynthetic model parameters in response to stressful environments. However, uncertainties in the calculation of $C_i$ have affected interpretation of gas-exchange measurements (Flexas et al., 2004), thereby preventing scientists from exploring a broad range of species and environmental conditions. The direct measurement may now be a solution. To play safe, the volume of the closed system ought to be reduced until the time lag is negligible.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Measurement of the volume of the closed system.

Fig. S2. Leak test in the closed system.

Fig. S3. Traces of temperature responses of cuticular transpiration and $C_i$.

Fig. S4. Comparisons of $g_c$ with $g_w$.

Fig. S5. Plotting of $A–C_i$ curves with our previous system.

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