Tracing Photosynthetic Response Curves with Internal CO₂ Measured Directly

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In this study, a system to measure leaf internal CO₂ (Cᵢ) was incorporated into an open gas exchange system (LI-COR, Lincoln, NE, USA). The Cᵢ was directly measured with a cup attached to the abaxial surface of sunflower (Helianthus annuus L.) leaves with open stomata while normal CO₂ and water vapor exchange through the same section of adaxial surface was simultaneously detected. The potential problems in the system, namely bulk air flow through the leaf, diffusion leaks, and change in the CO₂ gradient inside the leaf, were examined with the aim to apply the system to measure net photosynthesis at various Cᵢ (i.e. A–Cᵢ curves). A micro blower constantly circulated the air in a loop without pressure pulses or bulk air movement through the leaf. The measured Cᵢ (Cᵢ₀) generally followed the external CO₂ as much as the calculated one (Cᵢ₀). There was close agreement between the Cᵢ₀ and the Cᵢ, particularly at low Cᵢ, and the diffusion leak hardly affected the relationship between the two. Despite possible alterations of leaf properties by cup attachment, the direct measurement is expected to cast a new light on leaf gas exchange.

Keywords: A–Cᵢ curve, amphistomatous, gas diffusion, Helianthus annuus L., internal CO₂ gradient, LI-6400

INTRODUCTION

For photosynthesis, CO₂ is critical as a substrate for the reaction. The CO₂ diffuses from the outside to the intercellular airspace through stomatal pores on the surface of the leaf. From there, it diffuses in the liquid phase to the tercellular airspace through stomatal pores on the surface of the leaf. Stomatal closure is the most direct means of regulation, and closure prevents excessive cellular water loss. But inward CO₂ diffusion is physically restricted because water vapor and CO₂ share common diffusion pathways via the stomata. Consequently, closure leads to a decrease in the internal CO₂ concentration (Cᵢ) and rate of assimilation (A).

The photosynthetic CO₂ response curve, namely A–Cᵢ curve, has been widely used to assess the photosynthetic capacity because it eliminates gas phase diffusion or stomatal conductance (gₛ) in which changes in the curve are considered indicators of non-stomatal limitation on photosynthesis (Boyer, 1971; Farquhar and Sharkey, 1982; Graan and Boyer, 1981). In most studies, Cᵢ is routinely calculated from the outward diffusive behavior of water vapor (Moss and Rawlins, 1963; Jarmar, 1974; von Caemmerer and Farquhar, 1981). When stomata are open, the calculation appears reasonably accurate (Sharkey et al., 1982). Sharkey et al. (1982) measured Cᵢ directly along with the standard gas exchange parameters. Later, Boyer and Kawamitsu (2011) incorporated the Cᵢ measurement of Sharkey et al. (1982) into a gas exchange system. They experimentally measured the A–Cᵢ curve in sunflower, determined the effect of stomatal closure, and confirmed the hindrance of water vapor on entry of CO₂.

Direct Cᵢ measurement has a potential advantage when stomata close, which increases the cuticle influence (Boyer et al., 1997; Meyer and Genty, 1998) or patchiness of stomatal closure (Terashima et al., 1988; Mott, 1995; Buckley et al., 1997), these are considered problems for the A–Cᵢ analysis, which usually depends on calculated Cᵢ and thus the diffusive behavior of water vapor. Lauer and Boyer (1992) and Boyer and Kawamitsu (2011) considered the direct method to be a robust approach for tracing A–Cᵢ curves. In this study, we incorporated a direct Cᵢ measurement system into the LI-6400 open gas exchange system (LI-COR, Lincoln, NE, USA). The applicability of the system for A–Cᵢ curve measurement was tested in the sunflower (Helianthus annuus L.) leaf. We took special care to minimize bulk air flow through the leaf (Lauer and Boyer, 1992; Boyer and Kawamitsu, 2011), diffusion leaks (Flexas et al., 2007; Rodeghiero et al., 2007), and changes in the CO₂ gradient across the leaf mesophyll (Mott and O’Leary, 1984; Parkhurst et al., 1988; Parkhurst and Mott, 1990) that can be important for the measurement.

MATERIALS AND METHODS

Plant material

Sunflower (Helianthus annuus L.) plants were grown in a glasshouse located in the Department of Agriculture, University of the Ryukyus, Okinawa, Japan (26°15’N, 127°45’E; altitude 127 m). In December 2013, seeds were germinated in a fertilized seeding soil with 380, 290 and 340 mg l⁻¹ of N:P:K (Takii & Co., Ltd., Kyoto, Japan). After
10 d, seedlings were transplanted and grown in 4-L plastic pots containing a soil mixture consisting of 1:1:1 soil:peat:sand. The plants were automatically watered three times each d and were fertilized weekly with 500 ml of Hoagland’s nutrient solution. Fluorescent light was supplemented when photosynthetic photon flux density (PPFD) above the plants fell below 800 µmol m$^{-2}$ s$^{-1}$. Daylength in the glass-house was extended to 15 h to prevent flowering. The day and night temperatures ranged from 17–24 °C and 13–22°C, respectively. Only fully expanded leaves (130–180 cm$^2$) from 7–8 weeks old plants were used.

**Gas exchange systems**

To measure internal CO$_2$ concentration directly, we made a small acrylic chamber (cup) which can be incorporated into a commercially available open gas exchange system (LI-6400XT; LI-COR, Lincoln, NE, USA). The cup was specially designed for the bottom half of an integrated fluorescence chamber head (LI-6400-40; LI-COR), having a round airspace with 2 mm depth surrounded by the black neoprene gasket (6400-41; LI-COR) which shares the same leaf area (2 cm$^2$) with the upper half (Fig. 1). In the cup, the CO$_2$ equilibrated with that in the stomatal pores adjacent to the airspace (Sharkey et al., 1982; Lauer and Boyer, 1992; Boyer and Kawamitsu, 2011). The cup was connected in a closed loop with an IRGA (LI-840A; LI-COR) and a micro blower (109P0412H309; Sanyo Denki Co., Ltd., Osaka, Japan) which allows the air to gently circulate around the loop (ca. 300 ml min$^{-1}$) (Fig. 2). The water jacketed Y-shape glass tube (condenser) connected in the loop and a bubble seal to its lower end ensured atmospheric pressure and free of condensation in the loop path and the cup. Pressures in the loop were also monitored continuously in the optical cell of the Li-840A. The blower generated a pressure differential of about 0.04 kPa without any pulses. The condensation occurred slowly in the condenser but not the other parts of tubing or cup. The condensate naturally dripped through the inner wall of the condenser to the surface of the water seal and did not affect the measurement. The cup was located opposite the blower in the closed loop so as to prevent bulk air movement through the leaf (Boyer and Kawamitsu, 2011). The approximate total volume of the closed system was 100 ml (including LI-840A) with a total path length of 1.8 m. Leaf temperature was measured with a fine 0.13 mm chromel-constantan thermocouple (CHCO-005; Omega engineering, Stanford, CT, USA) appressed to the underside of the leaf by the flexed stainless wire in the cup (Fig. 1). The sensor was connected to the chamber head so that the leaf temperature was led to the LI-6400 console in the usual manner. While the bottom cup measured CO$_2$ equilibrated with that in the intercellular spaces of the leaf (C$_{cu}$) the upper half measured standard gas exchange parameters including calculated internal CO$_2$ (C$_{cu}$). In the LI-6400 gas exchange system, the correction of the two IRGAs (for reference/sample), known as ‘match’, is essential for the measurement precision. We retained this feature by bypassing the cup to the exhaust, which enabled the two IRGAs to be matched dur-
ing measurements (Fig. 1). CO₂ concentration was regulated with pure CO₂ in a tank connected to the LI-6400 console and CO₂-free air primarily passed through soda lime. Humidity was controlled by a dew point generator (LI-610; LI-COR) with the CO₂-free air. We modified the system to attain low CO₂ concentration (< 50 μmol mol⁻¹) according to LI-COR (2010). Both IRGAs for LI-6400 and LI-840A were calibrated using the same standard gases. For LI-6400 calibration was performed with 0 and 400 μmol CO₂ mol⁻¹ air whereas for LI-840A additional 2000 μmol CO₂ mol⁻¹ air was used for the higher CO₂ range.

**Leak test**

To test the diffusion leak in the closed loop, 0.2 ml of either 1 or 5% CO₂ was injected into the closed loop from the water seal of the condenser. Instead of a leaf an aluminum foil was clamped by the chamber to isolate the loop from the open gas exchange system. This amount of injection did not cause a detectable increase in pressure in the closed loop. The CO₂ injection was also conducted for intact leaves to examine the response of photosynthesis towards equilibration.

Diffusion leaks may also occur in an open gas exchange system (LI-COR, 2008). We estimated diffusion molar flow rate of CO₂ (Kₐ) and water vapor (Kᵥ) according to Flexas et al. (2007) and Rodeghiero et al. (2007). Previous studies (Lauer and Boyer, 1992; Boyer and Kawamitsu, 2011) have preferentially used paraffin-/lanolin (P/L) coat to prevent diffusion leaks in the gas exchange systems. Accordingly, we tested the effects of the P/L (2:8) as well as Vaseline (Unilever, Rotterdam, The Netherlands) and a high vacuum grease (Dow Corning P/L (2:8) as well as Vaseline (Unilever, Rotterdam, The Netherlands) and a high vacuum grease (Dow Corning Toray Co., Ltd., Tokyo, Japan) on the leaks. The CO₂ and water vapor concentration outside the chamber was allowed to fluctuate but monitored during all the measurements by using an open path IRGA (LI-7500; LI-COR) set around the chamber head.

**A–C Curve measurement**

Assimilation rate (A) at various Cᵣ for intact leaves was made with either the cup or the standard bottom half of the assimilation chamber. Photosynthesis and Cᵣ became steady within 40–60 min after clamping on the leaf at an ambient CO₂ concentration (Cᵣ) of around 400 μmol mol⁻¹. Thereafter, the photosynthesis response to varying Cᵣ was measured. The Cᵣ was lowered stepwise down to 30 μmol mol⁻¹ and then returned to 400 μmol mol⁻¹ to reestablish the initial steady-state value of photosynthesis. The Cᵣ was then increased stepwise up to 1400–2000 μmol mol⁻¹. Measurements consisted of 8–10 measurements for each curve. When steady-state photosynthesis and Cᵣ were achieved at each Cᵣ, standard gas exchange parameters were determined.

Photosynthesis was measured at PPDF of 800 μmol m⁻² s⁻¹, which was about 80–90 % of saturated A, to prevent photo-inhibition during often prolonged measurements. All measurements were carried out at a leaf temperature of 25°C and leaf to air vapor pressure difference (VPD) of 1.0–2.0 kPa, using a constant flow rate of 250 μmol s⁻¹. In the early morning, plants were taken from the glasshouse to the laboratory (room temperature of 25°C). There, plants were illuminated with fluorescent lamps that delivered PPDF of 150–400 μmol m⁻² s⁻¹ at leaf height. The plants were acclimated under the light at least 1 h before the measurements started.

**Photosynthesis parameters**

The equations for standard gas exchange parameters are essentially those derived by von Caemmerer and Farquhar (1981). CO₂ was fed to both surfaces of the leaf (free leaf) with the standard bottom chamber whereas CO₂ was fed only to the upper surface for the cup-attached leaf. Accordingly, Cᵣ(c) was determined for the free leaf while both Cᵣ(c) and Cᵣ(u) was obtained for the cup-attached leaf. For the latter configuration, we halved the boundary layer conductance, assuming that the boundary layer was symmetrically distributed between the two surfaces in the configuration with the standard bottom chamber.

**RESULTS**

**CO₂ injection to the closed loop**

With an aluminum foil clamped onto the cup, it was possible to test for leaks in the closed loop. At several s after a CO₂ injection a chromatographic peak appeared in both 1% and 5% CO₂ (Fig. 3). The CO₂ equilibrated at a higher concentration within 10–20 s after the injection and remained steady thereafter. The increase in CO₂ was fairly proportional to the concentration of added CO₂ (1:5), i.e., the CO₂ increased by 17 and 86 μmol mol⁻¹ after 1% and 5% CO₂ injection, respectively. These results confirmed no apparent diffusion leaks through the closed loop.

Attaching the cup to the lower leaf surface inevitably restricts the CO₂ supply from one surface, and enlarges gradients of CO₂ inside the leaf depending on the intercellular conductance to CO₂. The effect of this restriction was detected by monitoring CO₂ depletion for Cᵣ(u) together with
photosynthesis parameters when CO₂ was injected into the cup (Fig. 4). After a 5% CO₂ injection, A dropped solely because CO₂ diffused from the cup into the assimilation chamber. The stomata were open as indicated by the constant gₛ of about 210 mmol m⁻² s⁻¹ (i.e. approximately 70% of the maximum gₛ for the cup attached leaves) during the measurement. The A gradually recovered as Cᵢ was depleted and returned to the original level around 15 min after the injection.

Leak test for open gas exchange system

We tested further for leaks in the open gas exchange system by coating the gaskets with several sealing materials over the range of the A–Cᵢ curve measurement (Fig. 5A). Any leak would appear as an apparent ‘net photosynthesis’. The diffusion leak increased linearly as Cᵢ increased, i.e., the gradient of CO₂ between inside and outside of the chamber increased. The apparent ‘net photosynthesis’ reached up to 2.5 μmol m⁻² s⁻¹ at the highest Cᵢ (2000 μmol mol⁻¹). The similarity in responses regardless of the coating and the kind of sealing materials suggested either no effects of the coating or the absence of the leak from the sealed part (i.e. the gaskets). Accordingly, no coating material was used in all the subsequent experiments.

The effects of clamping the leaf and attaching the cup were tested (Fig. 5B) and gave comparable responses among the chamber with the aluminum foil, the killed leaf (i.e. photosynthetically inactive leaf) and the empty chamber (Fig. 5A, B). For the chamber monitoring the cup, the aluminum foil was expected to halve the diffusion leak through the gaskets. Nevertheless, the apparent ‘net photosynthesis’ was reduced only slightly (Fig. 5B). This suggested that the gaskets did not account for the observed leak. Finally, we determined K__CO₂ and K__H₂O by creating either negative or positive concentration gradients of CO₂ and H₂O between inside and outside of the chamber (Fig. 6). An empty chamber with the standard bottom half was used for this experiment because the leak was not affected by cup-attachment or the clamped leaf (Fig. 5). No matter whether the gradient was inwardly or outwardly directed, the same leakage occurred (Fig. 6). K__CO₂ and K__H₂O had mean values ranging from 0.17–0.24 μmol s⁻¹ and 1.5–2.3 μmol s⁻¹, respectively. Average values of 0.21 μmol s⁻¹ for K__CO₂ and 2.0 μmol s⁻¹ for K__H₂O were used for leak corrections in the subsequent A–Cᵢ curve measurements.

A–Cᵢ curve

The Cᵢ measuring system gave continuous results while other features of gas exchange were monitored.
Typical direct C measurement cycle during A–C curve measurement is shown in Fig. 7. After clamping on the leaf, at an ambient CO2 concentration (Ca) of around 400 μmol mol−1, photosynthesis and CAUC became steady within 40–60 min depending on the leaf. During this time, water vapor concentration in the closed loop also became steady at around 26 mmol mol−1, i.e., somewhat lower than saturated humidity at the room temperature (25°C). In general, the CAUC and CAUC followed the change in CO2 concentration in a similar manner. When Ca changed stepwise, steady-state photosynthesis and CAUC were achieved within 10–20 min as we observed with the injection test (Fig. 3). Mostly, the CAUC became steady as fast as the CAUC. We conducted the ‘match’ of gas analyzer calibrations at each Ca step after the steady-state photosynthesis before moving to the next step. A slight increase in the CAUC by approximately 2 μmol mol−1 was observed during ca. 30 s match mode, possibly associated with either altered flow rate/pressure or bulk air movement through the leaf during the mode. After coming back from the mode, the CAUC decreased to the steady value again within a minute. Accordingly, the data were taken a little while after the match was completed so that the potential bulk air movement was neglected.

At an ambient CO2 of 400 μmol mol−1, the average CAUC was 268 ± 13 μmol mol−1, and was lower than the CAUC by, on average, 10 μmol mol−1. The difference (CAUC–CAUC) became smaller as the Ca decreased (inset of Fig. 7). The CAUC versus CAUC relationship corresponding to Fig. 7 and shown in Fig. 8 indicates that the slope of the relationship was slightly but consistently larger than 1. The leak correction hardly affected this relationship. These results probably reflect changes in the internal gradient when the cup was attached (Sharkey et al., 1982; Parkhurst et al., 1988; Parkhurst and Mott, 1990). To see this effect on an A–C,
curve, we compared these data with the curve for a free leaf with open stomata (Fig. 9A). The $A - C_i$ curves obtained in the same leaf were substantially the same regardless of cup attachment. The $C_i$ was always lower at each comparable $C_a$ step in the cup-attached leaves than in the free leaves because of a reduction in $g_s$, resulting in the lower $A$ especially in the lower $C_i$ region (Fig. 9B). For the cup-attached leaf, the maximum $g_s$ (often observed at the lowest $C_a$) ranged from 229 to 314 mmol m$^{-2}$ s$^{-1}$ with an average of 285 mmol m$^{-2}$ s$^{-1}$ which was approximately 70% of that in the free leaves with open stomata.

**DISCUSSION**

The entire system constructed here resembled the one developed by Sharkey et al. (1982) but incorporated the direct measurement of $C_i$ (i.e. the closed loop) from Boyer and Kawamitsu (2011). One of the critical features of this system was the micro blower which constantly circulated the air in the loop with minimal pressure. This avoided pulses that might cause variation or bulk air flow through the leaf. Lauer and Boyer (1992) used fluid movement in the loop to circulate the air smoothly but did not measure gas exchange by the leaf. In the present work, the smooth and continuous air movement in the loop led to stable and fast responses in the equilibrated CO$_2$. This helped to retain the fast response and environmental control of the LI-6400 system. Although a slight change in the $C_{iso}$ occurred during the match mode the effect can be simply avoided by waiting extra minutes for the $C_{iso}$ to recover.

**Effects of leaks**

As the system depended on the accurate measurement of trace gases, diffusion leaks could be problematic whenever pressure or CO$_2$ concentration gradients existed between the inside and outside of the chamber or closed loop. The effect may become large as the chamber size decreases because leaks are a larger fraction of the measured photosynthesis as projected leaf area decreases (LI-COR, 2008). The $C_i$ measured directly was little affected by leakage as indicated by the CO$_2$ injection. By keeping the projected leaf area small (2 cm$^2$) our system had a smaller cup /loop volume than in previous systems (Sharkey et al., 1982; Lauer and Boyer, 1992; Boyer and Kawamitsu, 2011). This may have helped to prevent leakage while maintaining a response as fast as the similar system measuring a larger leaf area (Boyer and Kawamitsu, 2011).

For the open gas exchange system, the diffusion leak of CO$_2$ was readily detected as the apparent ‘net photosynthesis’ at various CO$_2$ concentrations (Flexas et al., 2007). Unexpectedly, there was only a marginal effect on leakage caused by the isolation of the cup from the open
gas exchange flow. Assuming that the leakage was larger at the interface between the gaskets than through the gasket itself (Flexas et al., 2007), it may be reasonable that the leakage remained when CO₂ diffused from the interface between the upper gasket and the aluminum foil instead of the lower gasket. However, coating the interface of the gaskets with various materials did not diminish the leaks. The maximum or minimum apparent ‘net photosynthesis’ with the empty chamber was almost half of the one found by Flexas et al. (2007), suggesting that the basal leakage was relatively small in our experiment. This was also supported by the lower K_{mij} and K_{sim} found in this experiment than in Rodeghiero et al. (2007) with a comparable setup. Accordingly, we suspect the leakage in this experiment arose somewhere other than the gasket. However, the leakage was not negligible and could not be eliminated (Fig. 5). Therefore, it was necessary to estimate leakage and correct A - C data in this open gas exchange system (Flexas et al., 2007; Rodeghiero et al., 2007).

There was a reasonably close agreement between the measured and calculated C, as was confirmed in the previous study by Sharkey et al. (1982). The correction for the leakage hardly affected the relationship between the two, suggesting that the estimated leakage had little influence on the calculated C over the entire range of CO₂.

Effects of cup attachment on CO₂ environment in leaves

The measurement of C was tested in sunflower leaves having stomata on the both surfaces (i.e. amphistomatous leaves). Attaching the cup on the lower surface inevitably caused a decrease in g, although the effect may not be the same for each surface (g was maintained at 70% of the free leaf). It may be associated with the g ratio (upper/ lower) for the two surfaces (Mott and O’Leary, 1984) and/or stomatal adjustment to cup attachment observed in sunflower leaves (Boyer and Kawamitsu, 2011).

The cup-attached leaf should increase the CO₂ gradients in the leaf because it diffuse the diffusion path of the free leaf (Parkhurst et al., 1988; Parkhurst and Mott, 1990; Boyer and Kawamitsu, 2011). It seems likely that the gradient or intercellular conductance would limit photosynthesis in leaves which have stomata only on one surface (i.e. hypostomatous leaves) (Parkhurst and Mott, 1990; Evans and Loreto, 2000). We technically altered the amphistomatous leaves by attaching the cup on one surface. The slightly but consistently lower C_{mij} than C_{sim} may be evidence of the finite intercellular conductance to CO₂. In return, one can briefly estimate the intercellular conductance by solving 0.5A/(C_{sim} - C_{mij}), according to Sharkey et al. (1982). In this study, the ambient data (i.e. A = 25 μmol m⁻² s⁻¹ and C_{sim} - C_{mij} = 10 μmol mol⁻¹) estimate the intercellular conductance to be approximately 1.2 mol m⁻² s⁻¹, a value similar to 1 mol m⁻² s⁻¹ determined in X. strumarium (Sharkey et al., 1982). These values are 4-5X greater than the stomatal conductance and, in effect, probably too large to be detected as a measurable difference in the A - C curve as was seen in Fig. 9A and ones in other amphistomatous species (Mott and O’Leary, 1984; Parkhurst and Mott, 1990). Furthermore, the gap between the C_{wij} and the C_{sim} became a few μmol mol⁻¹ as the CO₂ concentration decreased whereas the gap becomes less influential on A as substrate CO₂ becomes saturated at the site of carboxylation (see, Fig. 9). This may imply small effects of the gradient on A - C curve measurement for the leaves with high intercellular conductance. On the other hand, the large effect should be readily detected by the gap with the direct measurement, and the estimated intercellular conductance will be used for calculating average C, in leaves, if needed.

Detecting internal CO₂ directly

Like other systems for measuring C, directly (Sharkey et al., 1982; Mott and O’Leary, 1984; Lauer and Boyer, 1992; Boyer and Kawamitsu, 2011), our system is only applicable to amphistomatous leaves but not to hypostomatous leaves because the system blocks CO₂ through one surface. Despite this constraint, the direct measurement of internal CO₂ system may facilitate the A - C measurement especially when stomatal closure brings about the uncertainty in calculation of C because the directly measured C is free from the assumptions needed for the calculations (Boyer and Kawamitsu, 2011). The uncertainty especially arises from the patchy stomatal closure (Terasshima et al., 1988) or the cuticle where the ratio of diffusivities for water vapor and CO₂ differs from stomatal one (Boyer et al., 1997). Given patchy stomatal closure, internal CO₂ may not be distributed uniformly due to the limited lateral diffusion, and the calculated C can be no longer reliable (Terasshima et al., 1988; Terasshima, 1992). With closed stomata, cuticle still allows water to move across but not CO₂ as much, which has a large impact on calculating C (Boyer et al., 1997).

In either case, the C relying on water vapor is potentially overestimated, tracing apparent non-stomatal limitation on photosynthesis with the A - C curve (Terasshima et al., 1988; Mott, 1995; Buckley et al., 1997; Meyer and Genty, 1998). As for internal CO₂ gradient, these unappreciated players in the calculation are expected to be detected in the difference between measured and calculated C for leaves when stomata close.

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