Robust estimates of cuticle conductance on stomatous leaf surfaces during the light induction of photosynthesis

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Summary (178 words)

- Cuticle conductance (g_{cw}) can bias calculations of intercellular CO₂ concentration inside the leaf (C_i) when stomatal conductance (g_{sw}) is small.
- We examined how the light induction of photosynthesis impacts calculations by directly measuring C_i along with standard gas exchange in sunflower and tobacco leaves.
- When photosynthesis was induced from dark to saturating light (1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR) the calculated C_i was significantly larger than measured C_i and the difference decreased as g_{sw} increased. This difference could lead to over-estimation of rubisco deactivation by limited CO₂ supply during early induction of photosynthesis. However,

only small differences in C_i were observed during the induction from shade ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR) because g_{sw} was sufficiently large. The induction from dark also allowed robust estimations of g_{cw} when combined with direct C_i measurements. These g_{cw} estimates succeeded in correcting the calculation, suggesting that the cuticle was the major source of error.

- Despite a technical restriction to amphi-stomatous leaves, the presented technique has a potential to provide insights into the cuticle conductance on intact stomatous leaf surfaces.

Key words: Fluctuating light, Gas exchange, *Heliunthus annuus*, Intercellular airspace, *Nicotiana tabacum*, Stomata, Unsaturation

Introduction

Since Moss and Rawlins (1963) first calculated intercellular CO_2 concentration inside leaves (C_i) from the gas exchange of single leaves, the technique has become standard practice in gas exchange studies. The calculations became gained utility when Farquhar et al. (1980) developed a photosynthesis model that uses C_i to estimate substrate availability for ribulose-1,5-bisphosphate (RuBP) carboxylase oxygenase (rubisco). Based on a simple principle that rubisco carboxylation is limited by either of the two substrates, RuBP or CO_2 , the model predicts gas exchange of leaves as well as extrapolates underlying photosynthetic biochemistry (von Caemmerer, 2013). Accordingly, the calculations are the foundation of modern understanding of leaf carbon and water exchange.

However, calculations of C_i have been questioned because uncertainties exist in assumptions (Hanson et al., 2016). C_i is calculated from the relationship between water vapor exiting and CO_2 entering the leaf by assuming both gasses only diffuse through stomata (Moss & Rawlins, 1963). In fact, water also diffuses through the cuticle. By including the cuticular transpiration (or cuticle conductance: g_{cw}) in stomatal transpiration (or stomatal conductance: g_{sw}), the calculation can overestimate the CO_2 that has entered through stomata. Correction for cuticle effects is difficult as standard gas exchange measurements cannot distinguish cuticular and stomatal transpirations. Therefore, g_{cw} is generally measured in isolated cuticles (Kerstiens, 1996; Schuster et al., 2017). The calculations also assume lateral uniformity in physicochemical properties over the leaf surface. Any heterogeneity, such as non-uniform stomatal apertures

(Terashima, 1992; West et al., 2005), potentially bias the calculations as well (Terashima et al., 1988; Buckley, 1997; Meyer & Genty, 1998).

The accuracy of C_i can be validated by directly measuring C_i along with normal gas exchange measurements (Sharkey et al., 1982; Boyer & Kawamitsu, 2011; Tominaga & Kawamitsu, 2015a). In previous studies, amphi-stomatous leaves were put between an open gas exchange system and a closed system. The CO_2 concentration inside the closed system is measured after it equilibrates with the C_i while gas exchange is measured on the other side. Indeed, Sharkey et al. (1982) found that the measured C_i was in close agreement to the value calculated on the other side when stomatal conductance was sufficiently large. Using a similar system, however, the over-estimation of calculated C_i has been demonstrated by a large discrepancy from the measured C_i when g_{sw} is small (Boyer, 2015a; Tominaga & Kawamitsu, 2015b; Tominaga et al., 2018).

C_i is critical for understanding how photosynthesis approaches a steady state during light induction. Within the first minute after a shift from low to high irradiance photosynthesis can be limited by the slow regeneration of RuBP while the Calvin Benson Cycle enzymes are activated (Sassenrath-Cole & Pearcy, 1992). Much of the rest of induction may be (co)-limited by stomatal opening and activation of rubisco. Upon illumination C_i is depleted because stomatal opening is generally slower than activation of photosynthetic enzymes, thereby limiting rubisco carboxylation (Kirshbaum & Pearcy, 1988; Pearcy et al., 1996; Lawson and Blatt, 2014; McAusland et al., 2016; Dean et al., 2018). Meanwhile, rubisco is activated by sequential binding of CO_2 and Mg^{2+} to the active site (Portis, 2003). This process is inhibited by a range of sugar-phosphates bound to the active site in darkness, and promoted by rubisco activase which releases these ligands in light. The reduction of C_i also hinders this activation process, thereby limiting rubisco carboxylation indirectly (Mott & Woodrow, 1993). Therefore, initial g_{sw} at the onset of light induction as well as the rapidity of stomatal opening have a large impact on gas exchange kinetics (Wachendorf & Küppers, 2017; Vialet-Chabrand et al., 2017). Both rapid stomatal regulation (Wang et al., 2014; Lawson & Blatt, 2014; McAusland et al., 2016) and rubisco activation (Yamori et al., 2012; Carmo-Silva & Salvucci, 2013; Soleh et al., 2016; Taylor and Long, 2017) can play significant roles and are novel targets for improving photosynthesis.

Because the initial g_{sw} tends to be small in low irradiance or darkness the C_i may be miscalculated during photosynthetic induction. Here we examine how light induction experiments impact the standard calculations by directly measuring C_i along

with gas exchange in leaves of sunflower (*Helianthus annuus* L.) and tobacco (*Nicotiana tabacum* L.). Our results show when cuticle conductance matters and how it affects data interpretation. Together, we propose a novel technique to estimate intact cuticle conductance using the dual gas exchange system.

Materials and methods

Plant material

Tobacco (*N. tabacum* L. cv. Samsun) and sunflower (*H. annuus* L. cv. Mammoth Russian) plants, 9–11 weeks old and 5–7 weeks old respectively, were used. The tobacco plants were grown in 2.5-L plastic pots, and the sunflower plants were grown in 3.8-L plastic pots during December 2018 to April 2019 under ambient CO₂ at 24.2/19.9 °C day/night temperature; each pot contained a soil mixture (Metro-Mix® 360; Sun Gro Horticulture, Agawam, MA, USA) in a greenhouse located at the University of New Mexico [35.08°N, 106.62°W, 1587 m.a.s.l.]. The plants were automatically watered three times each day and the pots were manually saturated with nutrient solutions containing 357 ppm N (Peters Professional® 20-20-20 General Purpose; ICL Specialty Fertilizers, Summerville, SC, USA) and 195 ppm chelated Fe (Southern Agricultural Insecticides, Inc., Rubonia, FL, USA) twice and once weekly, respectively. Daylength in the greenhouse was extended to 15 h with LED lamps (VRPRx PLUS; Fluence Bioengineering, Inc., Austin, TX, USA) to prevent early flowering. All experiments with these plants were conducted with fully expanded upper leaves.

Direct measurement system incorporated into LI-6800

The direct C_i measurement system has previously been incorporated into a commercial open gas exchange system LI-6400 (LI-COR, Lincoln, NE, USA) and reported in detail elsewhere (Tominaga & Kawamitsu, 2015a). We applied a similar system for a new LI-6800. Instead of customizing a chamber, the manufactured LI-6800 chambers could be slightly modified to include the direct system (Fig. S1). In the present experiments, a large 6 x 6 cm chamber (6800-13; LI-COR) was used to maximize measurement precision, but the following system is applicable to the other chambers (e.g., Fluorometer chamber; 6800-01A; LI-COR). The chamber bottom-half was detached from the manifold and rotated 180°. Then, the flexible bellows originally attached to the bottom chamber were replaced with a solid adapter. The manifold to the bottom chamber was closed by a blanking plate with a rubber plate in between so that air only flows through the top chamber (i.e., open system). All experimental leaves were

large enough to cover the entire chamber area, so the top and bottom chambers were divided by the leaf. Two miniature impeller pumps were connected in parallel to the adapter (inlet and outlet) of the rotated bottom chamber, and smoothly circulated the air to an infrared gas analyzer (IRGA; LI-7000; LI-COR) in a loop. When a sample leaf closed the loop, a water jacketed glass tube (condenser) maintained the dew point slightly lower than the room temperature (checked by the LI-7000), preventing condensation in the IRGA. In the closed loop, CO_2 equilibrated with that inside the stomatal pores ($C_{i(m)}$) was continuously measured while gas exchange through the opposite side of same leaf area was measured with the open system, which automatically calculated C_i ($C_{i(c)}$). Accordingly, both $C_{i(m)}$ and $C_{i(c)}$ were obtained simultaneously for the same leaf area. We configured LI-7000 to the differential mode by leading the air to the reference cell from the LI-6800 reference port (located at the backside of the sensor head) at a constant flow rate of $400 \mu\text{mol s}^{-1}$. The values of CO_2 and water vapor concentrations in the reference air measured by the LI-6800 was exported to the LI-7000 whereas the sample CO_2 concentration measured by the LI-7000 (i.e. $C_{i(m)}$) was exported to and recorded by the LI-6800 along with other gas exchange parameters. By sharing the reference gas and data between the two systems, measurements were accurate and in sync. Plumbing of the closed loop was made mostly with copper tubing to avoid diffusion leaks. The advanced polymer gaskets (6568-512; LI-COR) and a large leaf area to edge ratio minimized the diffusion leaks such that any leaks were too small to be detected for the empty chamber in our experimental conditions, and no corrections were performed.

Light induction

Plants were taken in the morning from the greenhouse to the laboratory. There, whole plants were acclimated in the dark for at least 1 h. Leaves were then clamped and illuminated by the chamber equipped with a large light source (6800-03; LI-COR). We set three levels of irradiances with white LEDs: 50, 200, and $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR) with the spectrum shown in Fig. S2. Measurements were automatically logged every 10 s until reaching a steady state. In all irradiances, full induction usually took less than 1.5 h and 2 h for tobacco and sunflower leaves, respectively. In addition to the induction from the dark, leaves were also subjected to saturating light ($1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR) after being acclimated in shade ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR). Throughout the experiments, incoming air with a flow rate of $700 \mu\text{mol s}^{-1}$, air temperature of 25°C , water vapor of 10 mmol mol^{-1} and CO_2 concentration

of 400 $\mu\text{mol mol}^{-1}$ were held constant to avoid transient artefacts. Leaf temperature increased instantaneously upon illumination and was decreased throughout the induction by cooling from transpiration as stomata opened. Leaf temperature ranges varied among inductions: in tobacco, 24.5 ± 0.2 – 25.1 ± 0.1 °C (50 PAR), 24.4 ± 0.1 – 25.3 ± 0.1 °C (200 PAR), and 26.0 ± 0.1 – 27.4 ± 0.2 °C (1200 PAR), and in sunflower, 24.0 ± 0.1 – 24.7 ± 0.1 °C (50 PAR), 24.1 ± 0.2 – 25.3 ± 0.2 °C (200 PAR), and 25.8 ± 0.6 – 26.9 ± 0.4 °C (1200 PAR), respectively.

Calculations

The intercellular CO_2 concentration inside the leaf (C_i) was calculated from the gas exchange measurements on the adaxial leaf side, according to von Caemmerer and Farquhar (1981) as:

$$C_{i(c)} = \frac{(g_{sc} - 0.5E)C_b - A}{(g_{sc} + 0.5E)} \quad (1)$$

where C_b is the CO_2 concentration out of the leaf in the boundary layer (mol mol^{-1}), A and E are the CO_2 flux ($\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) and water vapor flux ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), respectively, g_{sc} is the stomatal conductance of CO_2 ($\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$). Given that C_b , A , and E are measured variables, g_{sc} is the critical parameter that determines validity of $C_{i(c)}$ (though measurement precision of C_b , A , and E affect the calculation). Then, g_{sc} would be expressed as:

$$g_{sc} = \frac{g_{sw}}{1.6} \quad (2)$$

where g_{sw} is the stomatal conductance of water vapor ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) and 1.6 is the ratio of diffusivities of CO_2 and water vapor in air. Then, g_{sw} was calculated as:

$$g_{sw} = \frac{E + 0.5E(W_b + W_i)}{W_i - W_b} \quad (3)$$

where W_i and W_b are the water vapor concentrations inside the leaf (mol mol^{-1}) and out of the leaf in the boundary layer (mol mol^{-1}), respectively. The W_i was assumed to be saturated at the leaf temperature. However, the g_{sw} includes cuticle conductance of water vapor (g_{cw}) as:

$$g_{sw} = g_{sw}' + g_{cw} \quad (4)$$

where g_{sw}' is the strict sense of stomatal conductance of water vapor, which is only based on water vapor passing through the stomatal pore, and is in parallel with cuticle conductance (Jarvis, 1971).

Instead of estimating from g_{sw} (Eq. (3)), we also calculated g_{sc} from the directly measured C_i ($C_{i(m)}$) (Boyer and Kawamitsu, 2011) as:

$$g_{sc}' = \frac{A}{C_b - C_{i(m)}} \quad (5)$$

209 where g_{sc} was directly calculated from the measured CO_2 gradients across the adaxial
210 leaf surface.

211 According to the manufacturer's calibration, boundary layer conductance of
212 CO_2 ($1.5 CO_2 \text{ mol m}^{-2} \text{ s}^{-1}$) and that of water vapor ($2.1 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) were estimated
213 from the constant fan speed of 14,000 rpm, and used for calculating the C_b and W_b ,
214 respectively. A summary of parameters referred to within the text is shown with
215 accompanying units in Table 1.

216

217 *Stomatal kinetics*

218 To get a sense of 'rapidity' of stomatal openings and increase of g_{sw} in the
219 leaves used here, we estimated kinetic parameters for the temporal response of g_{sw}
220 according to a sigmoidal model (McAusland et al., 2016; Vialet-Chabrand et al., 2017):

$$221 \quad g_{sw} = (G_{steady} - G_0)e^{-e^{\left(\frac{\lambda-t}{k_i}+1\right)}} + G_0 \quad (6)$$

222 where G_0 and G_{steady} are the g_{sw} ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) at the time, t (min), for the
223 beginning of light induction ($t = 0$) and at the steady state, respectively, k_i (min) is the
224 time constants for the increase of g_{sw} and λ is the initial lag time (min). The parameter
225 values were estimated with using a curve-fitting routine in Microsoft Excel provided
226 elsewhere (Supplemental File S1 in Vialet-Chabrand et al., 2017). The maximum slope
227 of g_{sw} increase (Sl_{max} , $\text{mmol m}^{-2} \text{ s}^{-2}$) was calculated based on the previously described
228 parameters as:

$$229 \quad Sl_{max} = \frac{(G_{steady}-G_0)}{e \cdot 60 k_i} \quad (7)$$

230 Our estimates only reflect the adaxial stomata while they usually represent both adaxial
231 and abaxial stomata in amphi-stomatous leaves (e.g., McAusland et al., 2016).

232

233 *Rubisco activation kinetics*

234 The activation of rubisco would limit photosynthesis within approximately the
235 initial 10 min of the increase in PAR (Woodrow & Mott, 1989,1992). This phase can be
236 found as a linear portion of a semilogarithmic plot of normalized photosynthesis to a
237 constant C_i (A^*) versus time (t) (Woodrow & Mott, 1989), expressed as:

$$238 \quad \ln (A_f^* - A^*) = -at + b \quad (8)$$

239 where a and b are the slope and intercept of the regression for this linear portion, A_f^* is
240 the A^* in the final steady state. The relaxation time for the rubisco phase (τ) is then
241 defined from the slope a as:

$$\tau = \frac{-1}{a} \quad (9)$$

In the induction experiments from dark to saturating light, we estimated τ with using $C_{i(c)}$ or $C_{i(m)}$, according to Woodrow and Mott (1989). A was normalized to a C_i of 250 $\mu\text{mol mol}^{-1}$ by assuming a linear relationship between A and C_i , that passed through the CO_2 compensation point of 50 $\mu\text{mol mol}^{-1}$. The maximum A^* was used as A_f^* during each induction. a was determined by linear regression of the data for 3 min (i.e., 18 points).

249

250 *Stomatal density*

251 Nail polish was spread at three points on each upper and lower side of ten
252 leaves. The dried polish was striped with clear tape and attached to a glass slide (a
253 replica of the epidermis). Microphotographs (0.60 mm^2) of the replicates were taken with
254 a digital camera (AxioCam HRc; Carl Zeiss, Göttingen, Germany) attached to a
255 microscope (Axioskop 2 Mot Plus; Carl Zeiss, Göttingen, Germany). Stomata were
256 counted in each replicate and averaged to calculate stomatal density for each side of
257 the leaves.

258

259 *Intercellular CO_2 conductance and gradients*

260 Vertical gradients of CO_2 exist in a leaf because CO_2 has to further diffuse
261 through the mesophyll airspace once inside the stomata (Parkhurst et al. 1988). In our
262 measurement system, C_i gets lower towards abaxial side because CO_2 is only supplied
263 from the adaxial side and more CO_2 is consumed as it diffuses deeper into the leaf
264 (Boyer and Kawamitsu, 2011). In one-dimensional model, the CO_2 gradient is
265 expressed as:

$$\text{Intercellular } \text{CO}_2 \text{ gradient} = \frac{A}{g_{ias}} \quad (10)$$

267 where g_{ias} is the intercellular conductance of CO_2 ($\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$). We determined g_{ias}
268 anatomically from the fraction of mesophyll volume occupied by intercellular airspace
269 (f_{ias}) and the effective diffusion path length (ΔL_{ias}) (Syvertsen et al., 1995) as:

$$g_{ias} = \frac{D_a f_{ias}}{\Delta L_{ias} \varsigma} \quad (11)$$

271 where ς is the tortuosity factor and D_a is the diffusion coefficient for CO_2 in the gas
272 phase ($1.51 \cdot 10^{-5} \text{ m}^2 \text{ s}^{-1}$). We used mesophyll thickness as ΔL_{ias} and a constant ς of 1.57
273 (Tosens et al., 2012). The f_{ias} and the mesophyll thickness between the two epidermal

layers were measured from microscopic observations of leaf sections with the same equipment described above.

Statistics

The number of replications and the statistical tests, if applicable, are presented in figure legends for each experiment. The results are given as means with SDs unless otherwise indicated.

Results

Leaf anatomy and intercellular conductance to CO₂

Sunflower leaves had more stomata than tobacco leaves while their stomatal ratios were similar (Table 2). For adaxial sides on which gas exchange was measured, the mean stomatal density was $49 \pm 4 \text{ mm}^{-2}$ in tobacco and $118 \pm 9 \text{ mm}^{-2}$ in sunflower, respectively. In both species, adaxial side had fewer stomata than abaxial side with the mean stomatal ratio (adaxial/abaxial) of 0.6-0.7. Intercellular conductance of CO₂ (g_{ias}) was determined from anatomical properties of leaf sections (Table 2). Sunflower leaves trended toward having a smaller fraction of airspace (0.27 ± 0.01) than tobacco leaves (0.35 ± 0.05) whereas both leaves had a comparable mesophyll thickness of around 200 μm . There were no significant differences in the mean g_{ias} between tobacco ($617 \pm 87 \text{ mmol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) and sunflower ($538 \pm 135 \text{ mmol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$).

Light induction from dark to saturating irradiance

When dark-adapted leaves were clamped with the chamber under saturating irradiance (1200 PAR), both assimilation and conductance increased slowly (Fig. 1a,b). Meanwhile, CO₂ concentration in the closed loop was rapidly consumed by photosynthesis until reaching an equilibrium with the airspace inside the leaf ($C_{i(m)}$ in Fig. 1c). Until then, the $C_{i(m)}$ was higher than the actual C_i and the g_{sc}' derived from the $C_{i(m)}$ was incorrect (shown with grey background in Fig. 1). The equilibrium state was indicated by the parallel changes in the g_{sw} and g_{sc}' as both changes should commonly reflect the stomatal behavior (inset in conductance of Fig. 1b). The equilibrium was generally expected to occur within 5 min after clamping on the leaf in both species. The $C_{i(c)}$ was significantly larger than the $C_{i(m)}$, and the difference became largest when the $C_{i(m)}$ was around the minimum (Fig. 1c). The $C_{i(m)}$ decreased to $96 \pm 15 \mu\text{mol mol}^{-1}$ in tobacco and $76 \pm 11 \mu\text{mol mol}^{-1}$ in sunflower, whereas the minimum $C_{i(c)}$ was $137 \pm 19 \mu\text{mol mol}^{-1}$ and $150 \pm 10 \mu\text{mol mol}^{-1}$, respectively. Therefore, rubisco underwent much

lower C_i than was predicted from the calculation. Because rubisco activity can be inferred from A relative to C_i within the early induction phase, these differences in the minimum C_i would predict a different activation status of rubisco. Based on the $C_{i(m)}$ or $C_{i(c)}$, we estimated the relaxation time (τ) primarily limited by the rate of rubisco activation as illustrated in Fig. S3. The τ estimated from the $C_{i(c)}$ (5.0 ± 2.7 min and 7.1 ± 2.8 min in tobacco and sunflower, respectively) were significantly longer than those estimated from the $C_{i(m)}$ (2.0 ± 1.0 min and 3.3 ± 2.0 min, respectively) (Fig. 2). The difference between the $C_{i(c)}$ and $C_{i(m)}$ diminished as the conductance increased (Fig. 1c).

Light induction from shade to saturating irradiances

In light induction experiments, leaves are often pre-acclimated to shade, rather than to the darkness. We also looked at the induction from shade (50 PAR) (Fig. 3). While the induction from dark to shade similarly resulted in a large discrepancy between the $C_{i(c)}$ and $C_{i(m)}$ as dark to saturating light, the subsequent induction from shade to saturating irradiance caused less discrepancies (Fig. 3c). However, tobacco $C_{i(c)}$ and $C_{i(m)}$ remained different at saturating light (insets of Figs. 1c & 2c). A large difference between the minimum $C_{i(c)}$ and $C_{i(m)}$ was also evident in the induction from the dark to 200 PAR (data not shown). Therefore, the calculations primarily deviated from the direct measurements only in the induction from the darkness. For those dark-adapted leaves, the initial g_{sw} right after the induction was as small as 9.4 ± 6.6 mmol H_2O m^{-2} s^{-1} and 9.4 ± 6.8 mmol H_2O m^{-2} s^{-1} for g_{sw} in tobacco and sunflower, respectively. On the other hand, when the photosynthesis reached a steady-state in the shade, g_{sw} increased to as much as 61 ± 20 mmol m^{-2} s^{-1} in tobacco, and 190 ± 56 mmol m^{-2} s^{-1} in sunflower. These results are consistent with the previous direct measurements showing the over-estimation of $C_{i(c)}$ mostly when g_{sw} is small (Tominaga & Kawamitsu, 2015b, Boyer, 2015a; Tominaga et al., 2018), and these initial g_{sw} in the shade-acclimated leaves must have been sufficiently large to avoid over-estimations.

Kinetics of stomatal openings

Kinetic parameters for temporal response of g_{sw} during the light induction were summarized in Table 3. In general, increase of g_{sw} was sigmoidal in tobacco whereas it was multiple stepwise in sunflower (Figs. 1b & 3b). As a result, the smooth sigmoidal function (Eq. 6) was less fit in sunflower showing the higher normalized root-mean-square error (NRMSE). Time constant of stomatal openings (k_i) was shorter in the induction from dark to shade (0 to 50 PAR), suggesting that stomata reached

steady-state more quickly with partial openings. On the other hand, the lower Sl_{max} indicated the slower increase of g_{sw} in the shade. In the induction from dark to saturating light (0 to 1200 PAR), average Sl_{max} was higher in sunflower ($0.139 \text{ mmol m}^{-2} \text{ s}^{-2}$) than in tobacco ($0.047 \text{ mmol m}^{-2} \text{ s}^{-2}$) despite the similar k_i (approx. 20 min) because of the larger steady-state g_{sw} with the higher stomatal density (Table 2). These Sl_{max} values were within or close to the high end of reported values for C_3 forbs displaying the similar growth form and guard cell type (McAusland et al., 2016). Hence, it follows that over-estimations of $C_{i(c)}$ in the above experiments occurred in leaves with moderate to rapid g_{sw} responses. Shade acclimation (50 to 1200 PAR) neither shortened the k_i nor increased the Sl_{max} (Table 3).

Estimation and correction of cuticle conductance

The standard calculations use the diffusivity ratio of water vapor to CO_2 , which is a constant value of 1.6 in air (Sharkey & Farquhar, 1982; LI-COR, 2019) while assuming that stomata are the dominant path for both gasses (Eq. (2)). Note that Massman (1998) reported uncertainty (maximum deviations after removing outliers) of $\pm 7\%$ and $\pm 5\%$ for the respective diffusivities, indicating $\pm 12\%$ uncertainty (± 0.19) in the mean ratio of 1.58. We tested this assumption that $g_{sw} = 1.6g_{sc}'$ by comparing the g_{sw} with the g_{sc}' during the initial light induction from the dark ($g_{sc}' < 20 \text{ mmol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) (Fig. 4). The g_{sw} was linearly related to the g_{sc}' , indicating a tight stomatal regulation on the g_{sw} in this early induction phase. However, the g_{sw} was consistently larger than the assumption of $g_{sw} = 1.6g_{sc}'$ (broken lines in Fig. 4). Given little CO_2 transport through the cuticle (Boyer, 2015a), a zero g_{sc}' would be obtained with complete stomatal closure. Thus, the g_{sw} with complete stomatal closure can be estimated by extrapolating the regression line to the intersection with the y-axis. This intersection is consistently above the origin (Fig. 4), indicating that water transport continues even if stomata close completely. We considered this residual g_{sw} as cuticle conductance for water vapor (g_{cw}).

To see the impact of cuticle conductance, we corrected $C_{i(c)}$ by calculating the strict sense of stomatal conductance to water vapor (g_{sw}'), as described in Eq.(4), and substituting it for the g_{sw} . The initial extrapolated g_{sw}' right after the induction from dark was $6.8 \pm 6.5 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ and $6.1 \pm 6.4 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ in tobacco and sunflower, respectively, that are $63 \pm 21\%$ and $56 \pm 22\%$ of the initial g_{sw} . The corrected $C_{i(c)}$ ($C_{i(c),cut}$) followed the $C_{i(m)}$ more closely than the $C_{i(c)}$ and the discrepancy became much smaller (Fig. 5a,b), suggesting that the discrepancy in the early induction was

mostly attributable to the contribution of cuticular water loss. In contrast, the g_{cw} marginally corrected the $C_{i(c)}$ as the g_{sw} increased, and there remained the discrepancy even when the g_{sw} was maximum, especially in tobacco (insets of Fig. 5a,b).

The g_{cw} did not show a particular trend in response to the irradiance, with the mean across all irradiances of $2.65 \pm 1.10 \text{ mmol m}^{-2} \text{ s}^{-1}$ in tobacco and $3.27 \pm 1.93 \text{ mmol m}^{-2} \text{ s}^{-1}$ in sunflower, respectively (Fig. 6a). Because of the strict linearity of the regressions (Fig. 4), the standard error of each g_{cw} estimate (i.e., y-intercept) was less than $0.1 \text{ mmol m}^{-2} \text{ s}^{-1}$ in both species, that is much smaller than the variations among leaves (Fig. 6a). The slope of the regression was mostly within or slightly above the uncertainty interval of 1.58, except for the inductions in shade (Fig. 6b). The slower photosynthesis in the shade (Table S1) would delay the equilibrium in the closed system, and perhaps affected the slope. The slope value was greater in sunflower than in tobacco across the irradiances (Fig. 6b).

Effect of intercellular CO₂ gradients

Because intercellular conductance is finite, vertical gradients of C_i must be present in a leaf. In our measurement system, the $C_{i(m)}$ should be the lowest C_i as the CO₂ is only supplied from the opposite side, and the CO₂ gradients would be attributable to the residual difference of $C_{i(c),cut}$ from $C_{i(m)}$. Assuming that the $C_{i(c)}$ represents the value just inside the stomatal pore or ‘stomatal cavity’ on the adaxial stomata (Sharkey et al., 1982; Parkhurst et al; 1988; Buckley et al., 2017), we subtracted the gradients estimated with the mean g_{ias} (Table 2) according to Eq. (10) from the $C_{i(c),cut}$. The gradient reduced the $C_{i(c)}$ when the g_{sw} was relatively large ($C_{i(c),cut} + grad$ in Fig. 7a,b). The gradients slightly overcorrected the $C_{i(c)}$ in tobacco (insets of Fig. 7a,b), and these mismatches were adjustable by approximately doubling the g_{ias} (data not shown). In sunflower the $C_{i(c)}$ was already close to or even lower than the $C_{i(m)}$ by several-ppm when the stomata were open (Figs. 1c & 2c). Then, the gradients greatly overcorrected the $C_{i(c)}$ (Fig. 7a,b), and the g_{ias} had to be unrealistically large—more than 10X larger than the anatomical g_{ias} ($>5000 \text{ mmol m}^{-2} \text{ s}^{-1}$)—in order to match the $C_{i(c),cut} + grad$ with the $C_{i(m)}$. Therefore, the over-correction would rather indicate that $C_{i(c)}$ was indicated deeper inside the leaf than the adaxial stomatal cavity and/or that $C_{i(c)}$ was underestimated.

Discussion

In this study, the intercellular CO₂ concentration inside leaves was directly measured as well as calculated from the standard gas exchange measurement. A commercially

available open system was minimally modified to include the direct system, and simultaneous measurements on the same leaf area allowed a close comparison. The standard calculations for the gas exchange measurements were validated under a square-wave light pattern that is often employed for induction studies. Over-estimations of C_i were observed in the dark-adapted leaves whose stomata started out closed. This raises a potential problem for interpreting gas exchange kinetics during non-steady state photosynthesis because C_i is an essential indicator of dynamic limitations (Kirshbaum & Pearcy, 1988; Pearcy et al., 1996). In our experiments, over-estimation of C_i was profound most at around its minimum during the early induction phase (Fig. 1c). Within this timescale photosynthesis is primarily limited by the rate at which rubisco is converted from an inactive to an active form (Woodrow & Mott, 1989, 1992). We found that the over-estimation of the minimum C_i delayed the relaxation period of the rubisco phase (Fig. 2). This suggests that rubisco activation status derived from A vs C_i relationship is potentially underestimated while the rubisco actually experiences lower CO_2 concentrations than the calculation indicates. In other words, one would be fooled as if rubisco is decarbamylated at the higher $C_{i(c)}$ than the actual C_i . Our study demonstrated that the cuticular water loss was responsible for the calculation error and should be taken into account in the induction measurements.

Importantly, the g_{cw} hardly affected the induction calculation when stomata were partially opened in the shade prior to the full induction (cf. $C_{i(c)}$ & $C_{i(c),cut}$ in Fig. 5b). In the induction from shade in *Alocasia macrorrhiza* (giant taro), Kirshbaum and Pearcy (1988) also realized that the A/C_i slope (at the initial 1 min) declined when the initial g_{sw} was suppressed under high CO_2 . While stomata would partially open in fairly low irradiance under the current atmospheric CO_2 level (Valladares et al., 1997), the initial g_{sw} varies greatly among species in both shade (McAusland et al., 2016; Deans et al., 2018) and darkness (Wachendorf & Küppers, 2017). The initial g_{sw} is confined in and likely scaled to the maximum g_{sw} among taxa in vascular plants (McAusland et al., 2016, Deans et al., 2019). Because greater stomatal conductance appears to evolve through the vascular plant lineages (Flanks & Beerling, 2009; McElwain et al., 2016), ferns and gymnosperms may have smaller initial g_{sw} than angiosperms. Together, ‘sluggish’ stomatal openings could exacerbate the calculation by prolonging the cuticle effect. In general, graminoids with dumbbell-shaped guard cells exhibit much faster light response of g_{sw} than the other vascular plants with elliptic guard cells (Franks & Farquhar, 2007; Vico et al., 2011; McAusland et al., 2016). In the elliptic guard cells, both stomatal opening and g_{sw} increase are slower with lower maximum g_{sw} (McAusland

et al., 2016), and gymnosperm opens stomata more slowly than angiosperm (Vico et al., 2011). Besides genetic variations, water stress decreases the initial g_{sw} (Allen & Pearcy, 2000; Wachendorf & Küppers, 2017) and so drought conditions should be prone to the error.

Cuticle conductance also varies among leaves in a species (Fig. 6a) and potentially more among species (Kerstiens, 1996; Schuster et al., 2017). Furthermore, intact cuticle conductance may change with leaf water status (Boyer et al., 1997; Burghardt & Riederer, 2003; Boyer, 2015b). Therefore, a correction seems problematic unless g_{cw} for each intact leaf is known. Indeed, the average g_{cw} could result in over-correction of C_i in our experiments (data not shown). Although, in principle, the cuticle conductance can be easily included in the calculations for C_i ($C_{i(c),cut}$), accurate measurement of g_{cw} is essential for a reliable correction.

Estimation and correction of cuticle conductance

Due to technical difficulty in the measurement of cuticular transpiration on a stomatous leaf surface/cuticle (e.g., Šantrůček et al., 2004), cuticle conductance has been studied exclusively in hypostomatous species with using the astomasous leaf surface/cuticle (Kerstiens, 1996; Schuster et al., 2017). Kirshbaum and Pearcy (1988) first estimated the g_{cw} on the stomatous leaf surface from the above induction measurements in giant taro. They determined the g_{cw} that could match the two initial A/C_i slopes observed with a low and high g_{sw} in each leaf, and reported the mean of $2.7 \pm 0.3 \text{ mmol m}^{-2} \text{ s}^{-1}$ ($\pm \text{SE}$, $n=7$). As they noted, however, the method assumes that the activation of rubisco is independent from the initial g_{sw} , which would not hold (Mott & Woodrow, 1993). In the previous experiments using similar dual gas-exchange systems (Boyer 2015a, Tominaga & Kawamitsu, 2015b), g_{cw} or cuticular transpiration was estimated from a deviation of the calculation from the direct measurement. Unlike the Kirshbaum and Pearcy method, these estimations can be made from a single measurement without assuming the rubisco activation status, thereby increasing the throughput. Nevertheless, these methods still assumed that g_{cw} is solely responsible for their measurement discrepancies, and potentially neglected other factors, such as intercellular CO_2 gradients. In contrast, our intercept method extrapolates g_{cw} at virtually complete stomatal closure when the CO_2 gradient becomes negligibly small with $A \approx 0$ (Fig. 4). Furthermore, the linear relationship allowed a simple extrapolation for a correction that improved the agreement between calculated and measured values (Fig. 5).

484 The regression slope was slightly larger than 1.58, the modelled diffusivities
 485 ratio of CO₂ and water vapor in air (Massman, 1998), but mostly within the uncertainty
 486 interval (Fig. 6b). A potential explanation for the larger slope is a non-uniform stomatal
 487 opening that breaks the assumptions of lateral uniformity over the leaf surface: uniform
 488 stomatal aperture, leaf temperature and metabolic capacity, and in the leaf: uniform CO₂
 489 distribution (Terashima et al., 1988; Meyer & Genty, 1998; West et al., 2005). Because
 490 non-uniformity may be more obvious in heterobaric leaves in which bundle-sheath
 491 extensions laterally disconnects the airspace (Terashima, 1992), the larger slope in
 492 sunflower than tobacco (Fig. 5b) might be due to the greater heterobaricity (Morison &
 493 Lawson, 2007; also noticeable in our microscopic observation). It would be interesting to
 494 compare the thermal and fluorescence imaging over the leaf surface (e.g., West et al.,
 495 2005) during induction experiments among leaves with a wide range of heterobaricity.

496

497 *Intercellular CO₂ gradients and unsaturation in the airspace*

498 In tobacco, the intercellular CO₂ gradient seems to explain the discrepancy
 499 between the $C_{i(c)}$ and $C_{i(m)}$ when stomata are open and g_{cw} has little impact on the
 500 calculation (Fig. 7). The slight over-correction of $C_{i(c)}$ may be due to under-estimation of
 501 the anatomical g_{ias} . However, the much larger over-correction of $C_{i(c)}$ in sunflower would
 502 indicate that the $C_{i(c)}$ was located deeper than the stomatal cavity (i.e., closer to the $C_{i(m)}$
 503 than we expected). This suggests that internal evaporating surface where humidity is
 504 saturated (W_i) occurs deeper than the stomatal cavity, and that g_{sw} includes the diffusion
 505 in the intercellular airspace. Also, this notion could explain the discrepancy in the
 506 over-correction between the two species. Because closer arrangements of stomata
 507 should decrease the lateral diffusion distance of CO₂ inside the leaf, the pathlength ratio
 508 of water vapor to CO₂ per stoma may increase with increasing stomatal density. In
 509 sunflower leaves having the 2.4X as many stomata (Table 2), a larger pathlength ratio
 510 might cause the deeper C_i .

511 It is also possible that the over-correction is manifested by under-estimated $C_{i(c)}$.
 512 In calculating the g_{sw} (Eq. 3), W_i is assumed to be saturated, and if not the g_{sw} is more or
 513 less underestimated. We observed a progressive under-estimation of $C_{i(c)}$ when those
 514 tobacco and sunflower leaves were detached and rapidly desiccated (MS#2). There, the
 515 relative humidity in leaf airspace was estimated to be less than 80% of saturation in both
 516 species as the leaves dried up. Likewise, Cernusak et al. (2018) estimated that the
 517 relative humidity could be as low as 77% and 87% in *Pinus edulis* and *Juniperus*
 518 *monosperma* even when the leaves opened stomata and actively photosynthesized.

519

520 *Applications*

521 One could do a direct C_i measurement simply on one leaf side, but the greatest
522 value is pairing with calculated values on the other side for an accurate paired
523 measurement of water fluxes. In that case a correction of the direct measurement is
524 needed for the vertical CO_2 gradient especially in leaves with lower g_{ias} (e.g., thicker
525 leaves). Anatomical measure of g_{ias} may be used for this correction. The dual
526 gas-exchange system allows a robust estimation of cuticle conductance for water vapor
527 on intact stomatous leaf surfaces in amphi-stomatous species, giving an new
528 opportunity to study cuticular physiology in connection with stomatal physiology. The
529 technique, owing to a high throughput (<10 min/ sample), will also allow for exploring a
530 large number of plant species. A disadvantage is a current limitation of the approach to
531 amphi-stomatous leaves. In hypo-stomatous leaves CO_2 moves through the
532 astomatous side so slowly that the closed system requires much longer times to reach
533 equilibrium. The applicability also depends on the stomatal behavior. The estimation of
534 g_{cw} would be more accurate as stomatal conductance diminishes, although this is aided
535 by simply conducting induction responses from dark-adapted leaves. In addition to g_{cw} ,
536 location and/or unsaturation of W_i deserves more investigations as both have wide
537 implications for gas exchange and water transport in leaves.

538

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549

550 **Author contributions**

551 JT planned and designed the research. JT and JRS performed experiments and
552 analyzed data. JT and DTH wrote the manuscript.

553

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698 lesser extent, of steady–state photosynthesis at high temperature. *The Plant*
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700

701 Tables

702 Table 1. A summary of parameters referred to within the text.

Parameter	Definition	Units
A	Net CO ₂ assimilation rate	$\mu\text{mol m}^{-2} \text{s}^{-1}$
A^*	A normalized to a C_i of 250 mol mol ⁻¹	$\mu\text{mol m}^{-2} \text{s}^{-1}$
A_i^*	Maximum A^* in a step increase in PAR	$\mu\text{mol m}^{-2} \text{s}^{-1}$
C_b	Boundary layer CO ₂ concentration	$\mu\text{mol mol}^{-1}$
C_i	Intercellular CO ₂ concentration	$\mu\text{mol mol}^{-1}$
$C_{i(c)}$	Calculated C_i	$\mu\text{mol mol}^{-1}$
$C_{i(m)}$	Directly measured C_i	$\mu\text{mol mol}^{-1}$
$C_{i(c),cut}$	$C_{i(c)}$ corrected for g_{cw}	$\mu\text{mol mol}^{-1}$
$C_{i(c),cut + grad}$	$C_{i(c)}$ corrected for g_{cw} and intercellular CO ₂ gradient	$\mu\text{mol mol}^{-1}$
D_a	Diffusion coefficient for CO ₂ in gas phase	$\text{m}^2 \text{s}^{-1}$
Intercellular gradient	CO ₂ Vertical CO ₂ concentration gradient along the mesophyll cells in intercellular airspace	$\mu\text{mol mol}^{-1}$
ΔL_{ias}	Effective diffusion path length of CO ₂ in intercellular airspace	μm
E	Transpiration rate	$\text{mmol m}^{-2} \text{s}^{-1}$
f_{ias}	Fraction of mesophyll volume occupied by intercellular airspace	$\text{m}^3 \text{m}^{-3}$
g_{cw}	Cuticle conductance to water vapor	$\text{mmol m}^{-2} \text{s}^{-1}$
g_{sc}	Stomatal conductance to CO ₂ estimated from g_{sw}	$\text{mmol m}^{-2} \text{s}^{-1}$
g_{sc}'	Stomatal conductance to CO ₂ estimated from $C_{i(m)}$	$\text{mmol m}^{-2} \text{s}^{-1}$
G_{steady}	Predicted steady-state g_{sw} in a step increase in PAR	$\text{mmol m}^{-2} \text{s}^{-1}$
g_{sw}	Stomatal and cuticular conductance to water vapor	$\text{mmol m}^{-2} \text{s}^{-1}$
g_{sw}'	Stomatal conductance to water vapor	$\text{mmol m}^{-2} \text{s}^{-1}$
g_{ias}	Intercellular conductance to water vapor	$\text{mmol m}^{-2} \text{s}^{-1}$
G_0	Predicted initial g_{sw} in a step increase in PAR	$\text{mmol m}^{-2} \text{s}^{-1}$
k_i	Time constant for G_0 to achieve G_{steady}	min
PAR	Photosynthetically active radiation	$\mu\text{mol m}^{-2} \text{s}^{-1}$
ζ	Tortuosity factor in intercellular airspace	
SI_{max}	Maximum rate of g_{sw} increase in a step increase in PAR	$\text{mmol m}^{-2} \text{s}^{-2}$
W_b	Boundary layer water vapor concentration	mmol mol^{-1}
W_i	Intercellular water vapor concentration	mmol mol^{-1}
λ	Initial lag in the response of g_{sw} to a step increase in PAR	min
τ	Relaxation time for the rubisco phase which photosynthesis is limited by rubisco activation	min

703

Table 2. Stomatal density and anatomical properties of tobacco and sunflower leaves.

	Tobacco	Sunflower	<i>P</i>
Stomatal density (mm ⁻² , n=10)			
Adaxial	49±4	118±9	<0.0001
Abaxial	76±16	186±20	<0.0001
Ratio (adaxial/abaxial)	0.67±0.15	0.64±0.08	0.55
Anatomical properties (n=3)			
<i>f_{ias}</i>	0.35±0.05	0.27±0.01	0.06
Mesophyll thickness (μm)	225±53	199±3	0.49
<i>g_{ias}</i> (mmol m ⁻² s ⁻¹)	617±70	538±35	0.18

The means ± SDs are compared by using either Student's or Welch's *t* test.

Table 3. Parameters of the dynamic model of *g_{sw}* increase during light induction experiments in tobacco and sunflower.

PAR (μmol m ⁻² s ⁻¹)	Species	n	NRMSE	<i>k_i</i> (min)	λ (min)	<i>S_{lmax}</i> (mmol m ⁻² s ⁻²)	<i>G₀</i> (mmol m ⁻² s ⁻¹)	<i>G_{steady}</i> (mmol m ⁻² s ⁻¹)
0 to 50			**					***
	Tobacco	6	0.018 ± 0.006 ^{bc}	11.3 ± 1.2 ^b	1.9 ± 1.0 ^a	0.034 ± 0.004 ^b	3.0 ± 1.8 ^b	64.4 ± 8.5 ^b
	Sunflower	4	0.057 ± 0.010 ^A	15.5 ± 3.8 ^B	2.1 ± 1.7 ^A	0.092 ± 0.029 ^{AB}	7.3 ± 7.3 ^B	188.5 ± 26.3 ^C
0 to 200					*	***		***
	Tobacco	9	0.032 ± 0.002 ^{ab}	17.8 ± 1.1 ^a	0.3 ± 0.2 ^{ab}	0.050 ± 0.004 ^a	6.6 ± 6.4 ^b	149.7 ± 7.7 ^a
	Sunflower	8	0.057 ± 0.007 ^A	18.0 ± 1.7 ^{AB}	5.4 ± 2.1 ^A	0.134 ± 0.013 ^A	0.0 ± 0.0 ^B	369.6 ± 16.6 ^B
0 to 1200						***		***
	Tobacco	12	0.035 ± 0.004 ^a	20.0 ± 1.1 ^a	0.0 ± 0.0 ^b	0.047 ± 0.003 ^{ab}	12.8 ± 11.4 ^b	165.4 ± 9.9 ^a
	Sunflower	7	0.048 ± 0.007 ^A	18.8 ± 2.2 ^{AB}	1.9 ± 1.3 ^A	0.139 ± 0.013 ^A	7.1 ± 2.9 ^B	410.7 ± 28.9 ^{AB}
50 to 1200			**	**		**	**	***
	Tobacco	9	0.012 ± 0.002 ^c	19.1 ± 1.2 ^a	0.8 ± 0.5 ^{ab}	0.041 ± 0.004 ^{ab}	54.3 ± 16.7 ^a	178.7 ± 11.4 ^a
	Sunflower	4	0.032 ± 0.007 ^A	27.1 ± 1.3 ^A	0.0 ± 0.0 ^A	0.066 ± 0.002 ^B	190.9 ± 24.7 ^A	484.1 ± 34.9 ^A

Means ± SEs. Different letters in each species indicate significant (*P* < 0.05) difference among the irradiance according to Tukey-Kramer test, whereas star(s) in each experiments indicate significant (*P* < 0.05) difference between the species according to *t* test (***: *P* < 0.001, **: *P* < 0.01, *: *P* < 0.05). Note that McAusland et al. (2016) reported 6.9-23.4 min and 0.04-0.13 mmol m⁻² s⁻¹ for the range of *k_i* and *S_{lmax}*, respectively, in the light induction from 100 to 1000 PAR for several C₃ forbs.

Figure legends

Fig. 1. Changes in the standard gas exchange parameters during the light induction of photosynthesis from dark. Assimilation rate (A) (a), stomatal conductances to water vapor (g_{sw} , blue line) and CO_2 (g_{sc}' , orange line) (b), and calculated intercellular CO_2 concentration ($C_{i(c)}$, blue line) and measured intercellular CO_2 concentration ($C_{i(m)}$, orange line) (c) after clamping a dark-adapted leaf of tobacco (left) and sunflower (right) with the dual gas-exchange chamber under saturating irradiance of $1200 \mu mol m^{-2} s^{-1}$ PAR. The g_{sw} was derived from the standard calculation based on the water vapor flux, whereas the g_{sc}' was derived from the direct measurement based on the CO_2 flux (see Calculations). In (b), initial change (0-10 min) is zoomed in the inset. The x-axis ticks in the inset graph represent 2 min intervals. Pre-equilibrium time for the $C_{i(m)}$ was shown with grey background. Representative experiments from twelve and seven replications in tobacco and sunflower, respectively.

Fig. 2. Relaxation time (τ) for the rubisco phase at the minimum $C_{i(m)}$ (orange) or $C_{i(c)}$ (blue). Means $\pm$ SDs ($n=12$ and 7 in tobacco and sunflower, respectively) were compared by using t test (***: $P<0.001$ **: $P<0.01$).

Fig. 3. Light induction experiments from dark to a shade ($50 \mu mol m^{-2} s^{-1}$ PAR) and to a saturating irradiance ($1200 \mu mol m^{-2} s^{-1}$ PAR). A (a), g_{sw} (blue line) and g_{sc}' (orange line) (b), and $C_{i(c)}$ (blue line) and $C_{i(m)}$ (orange line) (c). Measurements prior to the equilibrium were removed. Representative experiments from six and four replications in tobacco and sunflower, respectively.

Fig. 4. Relationships between g_{sw} and g_{sc}' during initial phase ($g_{sc}' < 20 mmol CO_2 m^{-2} s^{-1}$) of the light induction from the dark (< 10 min). Data for Figs. 1 and 3 are shown. Pre-equilibrium section was excluded as described in the text. Broken lines indicate the relationship for the diffusivity ratio of water vapor to CO_2 of 1.6 ($g_{sw} = 1.6 g_{sc}'$). Cuticle conductance to water (g_{cw}) was extrapolated to be y-intercept of the regression line.

Fig. 5. Correction for cuticle conductance in light induction experiments. Data for Figs. 1 and 3 are shown in (a) and (b), respectively. The $C_{i(c)}$ was corrected ($C_{i(c),cut}$

green line) by estimating the strict sense of stomatal conductance to water vapor (g_{sw}') with the estimated g_{cw} at each measurement, according to Eq. (4).

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Fig. 6 Summary of g_{cw} (a) and slope (b) estimated from the regression line for the g_{sw} versus g_{sc}' relationship at various irradiances. Means $\pm$ SDs [n=6 and 4 (50 PAR), n=9 and 8 (200 PAR), and n= 12 and 7 (1200 PAR), in tobacco and sunflower, respectively]. In (b), the modeled diffusivity ratio of water vapor to CO₂ in air (1.58) and its uncertainty intervals ($\pm$ 12%) (Massman, 1998) were shown with a horizontal red line and pale red color, respectively. Different letters in each species indicate significant ($P < 0.05$) difference among the irradiance according to Tukey-Kramer test, whereas star(s) in each experiment indicate significant ($P < 0.05$) difference between the species according to t test (***: $P < 0.001$ **: $P < 0.01$, *: $P < 0.05$).

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Fig. 7. Effect of intercellular CO₂ gradients on the calculation in light induction experiments. Data for Figs. 1 and 3 are shown in (a) and (b), respectively. The $C_{i(c)}$ was corrected for CO₂ gradients after the correction for cuticle effect ($C_{i(c),cut+grad}$, purple line) by subtracting the CO₂ gradient estimated with the mean g_{ias} (Table 1) from the $C_{i(c),cut}$. Comparisons are $C_{i(m)}$ (orange line).

770

771

Tobacco

Sunflower

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