

## Original Article

Improved method for measuring the apparent CO<sub>2</sub> photocompensation point resolves the impact of multiple internal conductances to CO<sub>2</sub> to net gas exchangeBerkley J. Walker<sup>1,2</sup> & Donald R. Ort<sup>1,2,3</sup><sup>1</sup>Global Change and Photosynthesis Research Unit, USDA/ARS, Urbana, IL 61801, USA, <sup>2</sup>Institute of Genomic Biology and <sup>3</sup>Department of Plant Biology, University of Illinois, Urbana, IL 61801, USA

## ABSTRACT

There is a growing interest in accurate and comparable measurements of the CO<sub>2</sub> photocompensation point ( $\Gamma^*$ ), a vital parameter to model leaf photosynthesis. The  $\Gamma^*$  is measured as the common intersection of several CO<sub>2</sub> response curves, but this method may incorrectly estimate  $\Gamma^*$  by using linear fits to extrapolate curvilinear responses and single conductances to convert intercellular photocompensation points ( $C_i^*$ ) to chloroplastic  $\Gamma^*$ . To determine the magnitude and minimize the impact of these artefacts on  $\Gamma^*$  determinations, we used a combination of meta-analysis, modelling and original measurements to develop a framework to accurately determine  $C_i^*$ . Our modelling indicated that the impact of using linear fits could be minimized based on the measurement CO<sub>2</sub> range. We also propose a novel method of analysing common intersection measurements using slope–intercept regression. Our modelling indicated that slope–intercept regression is a robust analytical tool that can help determine if a measurement is biased because of multiple internal conductances to CO<sub>2</sub>. Application of slope–intercept regression to *Nicotiana tabacum* and *Glycine max* revealed that multiple conductances likely have little impact to  $C_i^*$  measurements in these species. These findings present a robust and easy to apply protocol to help resolve key questions concerning CO<sub>2</sub> conductance through leaves.

**Key-words:** photosynthesis; photorespiration.

## INTRODUCTION

Biochemical models of leaf photosynthesis are an invaluable tool to both predict plant response to climate change and identify potential targets to improve the efficiency of CO<sub>2</sub> fixation (Sage & Kubien 2007; Zhu *et al.* 2008). These models are also used to estimate terrestrial CO<sub>2</sub> exchange at the canopy and earth system scale (Drewry *et al.* 2010; Rogers *et al.* 2014). Models of net gas exchange combine the physiology of CO<sub>2</sub> fixation with CO<sub>2</sub> release from mitochondrial respiration and loss following the oxygenation of ribulose 1·5-bisphosphate carboxylase/oxygenase

(Rubisco) by photorespiration. Leaf photosynthesis models are parameterized using the substrate gas (CO<sub>2</sub> and O<sub>2</sub>) partial pressures, reaction kinetics of Rubisco, stoichiometry of CO<sub>2</sub> release per Rubisco oxygenation ( $\alpha$ ), and rates of daytime mitochondrial respiration ( $R_d$ ; von Caemmerer & Farquhar 1981; von Caemmerer 2000). The CO<sub>2</sub> photocompensation point ( $\Gamma^*$ ), the chloroplastic CO<sub>2</sub> partial pressure at which rates of Rubisco carboxylation equal rates of CO<sub>2</sub> loss from photorespiration, is a key parameter in these models and combines Rubisco specificity ( $S_{c/o}$ ), O<sub>2</sub> partial pressure ( $O$ ) and  $\alpha$  according to

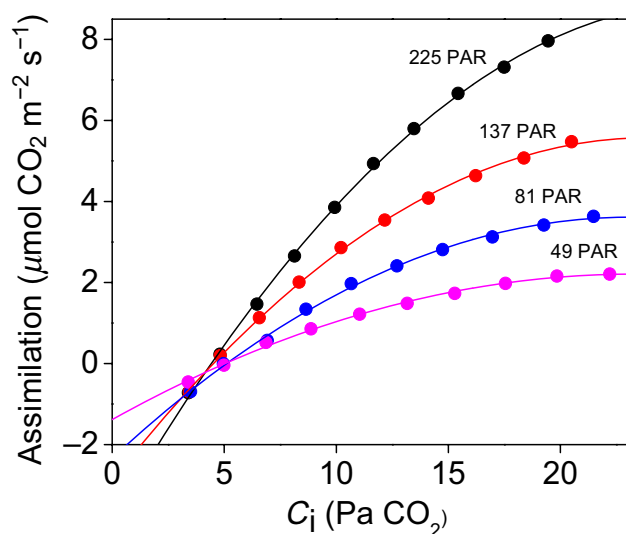
$$\Gamma^* = \frac{\alpha O}{S_{c/o}} \quad (1)$$

$\Gamma^*$  can be estimated from the common intersection of photosynthetic chloroplastic CO<sub>2</sub> response ( $A-C_c$ ) curves measured under sub-saturating irradiances (Fig. 1; Laisk 1977; Brooks & Farquhar 1985). The common intersection is predicted to relate to  $\Gamma^*$  assuming either RuBP-regeneration-limited or Rubisco-limited photosynthesis and has the added benefit of providing an estimate of  $R_d$  at the  $y$ -value of the common intersection (Laisk 1977; von Caemmerer *et al.* 1994). Since this intersection is determined from chloroplastic CO<sub>2</sub> partial pressures, CO<sub>2</sub> conductance from the intercellular air space to the chloroplast must be known to obtain a true estimate of  $\Gamma^*$  since in practice, the photosynthetic response to intercellular CO<sub>2</sub> concentrations ( $A-C_i$ ) is measured (von Caemmerer *et al.* 1994). CO<sub>2</sub> conductance into the chloroplast has traditionally been considered as a single mesophyll conductance ( $g_m$ ) and relates  $\Gamma^*$  to  $C_i^*$  according to

$$\Gamma^* = C_i^* + R_d/g_m \quad (2)$$

where  $C_i^*$  is the CO<sub>2</sub> partial pressure at the intersection.  $\Gamma^*$ , and by extension  $C_i^*$ , has a temperature dependency, usually considered dependent on the temperature response of Rubisco specificity. Currently the measured temperature response of  $\Gamma^*$  or  $C_i^*$  determined from plants grown at a common temperature is used to model the temperature response of photosynthesis in climate change models (Bernacchi *et al.* 2001, 2002; Walker & Cousins 2013; Walker *et al.* 2013).

Correspondence: D. R. Ort. e-mail: d-ort@illinois.edu



**Figure 1.** An example intercellular CO<sub>2</sub> photocompensation point ( $C_i^*$ ) measurement using the common intersection method. Shown is a single replicate of a measurement of the *N. tabacum* CO<sub>2</sub> response of net CO<sub>2</sub> assimilation measured at four sub-saturating light intensities indicated by the PAR values on the plot with lines representing a trinomial fitting function.

Recently, it has been questioned if a single diffusive term is adequate to describe mesophyll conductance. Several variations of a multiple diffusion model account for CO<sub>2</sub> movement through the cell wall and chloroplast separately and allow CO<sub>2</sub> produced from photorespiration in the mitochondria to pass directly to the intercellular space (Tholen & Zhu 2011; Tholen *et al.* 2012; Busch *et al.* 2013; von Caemmerer 2013). These models predict that multiple conductances to CO<sub>2</sub> would increase actual  $C_i^*$  and not produce a common intersection, but it is unclear how much they impact current estimations of  $C_i^*$ .

It has also been questioned if  $C_i^*$  intersections from linear fits can properly be used in common intersection measurements since the CO<sub>2</sub> response of photosynthesis is curvilinear (Gu & Sun 2014). Linear fits are modelled to underestimate  $C_i^*$  and  $R_d$  over a given CO<sub>2</sub> range. Since the initial portions of  $A-C_i$  curves are closer to linear than portions under higher CO<sub>2</sub> (Fig. 1), linear fits are expected to introduce more error when applied over a broad range of CO<sub>2</sub> values, and less error if applied to a narrow range. The impact of specific CO<sub>2</sub> ranges on final  $C_i^*$  values has not yet been shown.

These uncertainties highlight potential issues in using the common intersection method to determine both  $C_i^*$  and  $R_d$  that would compromise current parameterization of biochemical models of leaf photosynthesis and impact modelled CO<sub>2</sub> exchange. In addition to impacting physiological measurements, multiple diffusion paths could also decrease the efficiency of photorespiration if the resistance to CO<sub>2</sub> diffusion was very large through the chloroplast relative to the cell wall. Such a difference in resistances would allow photorespired CO<sub>2</sub> to diffuse from the mitochondria into the intercellular airspace and escape the leaf through the stomata (Tholen & Zhu 2011; Tholen *et al.* 2012; Busch *et al.*

2013; von Caemmerer 2013). These potential issues with traditional common intersection determination of  $C_i^*$  and  $R_d$  suggest that a more sensitive and robust protocol is needed to account for the impact of CO<sub>2</sub> measuring range and the possible significance of multiple conductance pathways and thus conductance values.

To determine the impact of linear fitting and different assumptions of CO<sub>2</sub> conductance to common intersection measurements of  $C_i^*$ , we analysed various reports of  $C_i^*$  published over the last 40 years. In addition to this meta-analysis, we performed additional measurements and modeling of common intersection curves to quantify how estimates of  $C_i^*$  might be biased and determine measurement conditions that limit this bias. We introduce a new method for interpreting common intercept data (slope–intercept regression) that should produce more accurate determinations of  $C_i^*$  and  $R_d$  and can determine if multiple conductances to CO<sub>2</sub> are significant (see Theory below).

## THEORY

The common intersection method determines  $C_i^*$  by averaging the intersection of several  $A-C_i$  curves measured under several sub-saturating irradiances (Fig. 1). In practice, these lines seldom converge on a single point and it is common to remove intersection values or to re-measure until all intersections are within two standard deviations (or some other metric) from the mean (e.g. Weise *et al.* 2015). Determining a common intersection is complicated by the fact that the intersection of two lines is more sensitive to experimental noise when slopes are similar, since small differences in slope can produce large differences in intersections between lines that are approaching parallel. Conversely, the intersection of two lines with very different slopes is more resilient to experimental noise. An important shortcoming of the common intersection method is that all intersections are equally weighted ignoring the fact that some of the intersections are more robust than others. This problem becomes increasingly apparent as more than three irradiances are used to determine  $C_i^*$  and when the slopes of neighboring lines become more similar.

We propose a variation on the common intercept method that we term the slope–intercept regression as a way to appropriately analyse common intercept data while giving more weight to intersections that are more robust. The value of  $x$  at the intersection ( $C_i^*$ ) of the linear portion of two  $A-C_i$  curves can be calculated from their slopes ( $m_1$  and  $m_2$ ) and  $y$ -intercepts ( $b_1$  and  $b_2$ ) as

$$C_i^* = \frac{b_1 - b_2}{m_2 - m_1} \quad (3)$$

and the value of  $y$  at the intersection ( $R_d$ ) as

$$R_d = mC_i^* + b \quad (4)$$

Traditionally the  $C_i^*$  and  $R_d$  values for each combination of line intersections is calculated graphically or with these equations and averaged together, with each line pairing given equal weight.

The slope–intercept regression analysis begins by plotting the  $m$  values on the  $x$ -axis and the  $b$  values on the  $y$ -axis from each individual  $A$ - $C_i$  curve. A linear regression is made of this plot producing a standard linear equation except that the  $x$  and  $y$  values represent the  $m$  and  $b$  from individual  $\text{CO}_2$  response measurements. The slope of this regression ( $m_{\text{reg}}$ ) is therefore

$$m_{\text{reg}} = \frac{b_2 - b_1}{m_2 - m_1} \quad (5)$$

which, when multiplied by  $-1$  and rearranged, is equal to  $C_i^*$  (Eqn 3). The  $y$ -intercept of this line also represents  $R_d$ , as can be seen when the slope–intercept regression line is expressed using negative  $C_i^*$  for slope (Eqn 5) and the  $y$ -intercept of the regression ( $b_{\text{reg}}$ ) as

$$b = -C_i^*m + b_{\text{reg}} \quad (6)$$

that when combined with Eqn 4 produces

$$R_d = b_{\text{reg}} \quad (7)$$

This method will have an advantage over simply averaging intercepts from each combination of  $A$ - $C_i$  curves since the regression weights values of  $m$  that are further apart more strongly in its determination of  $C_i^*$  than those that are close together. It should be noted that a variation of the slope–intercept regression technique is often used in computer-assisted image processing and can be applied to more complicated curves within images (Duda and Hart, 1972).

This method could also help determine if multiple conductances to  $\text{CO}_2$  across the cell wall and chloroplast impact measurements of  $C_i^*$  using the common intersection method. The slope–intercept regression of individual  $A$ - $C_i$  curves would not be linear assuming multiple  $\text{CO}_2$  conductances between the cell wall and chloroplast since  $C_i^*$  (the slope of this relationship) is predicted to increase when rates of photosynthesis and photorespiration are high (Tholen & Zhu 2011; Tholen *et al.* 2012; Busch *et al.* 2013; von Caemmerer 2013). This condition would produce a curvilinear instead of linear relationship, with the degree of curvature increasing as more photorespired  $\text{CO}_2$  is lost from the mitochondria into the intercellular airspace when the ratio of chloroplastic to cell wall increases.

We used a brute-force modelling approach to determine the impact of multiple internal conductances to the relationship between the slope and intercept of the initial portions of  $A$ - $C_i$  curves measured under sub-saturating irradiances. To determine slope and intercept values, we assumed six  $\text{CO}_2$  partial pressures in the mesophyll ( $C_m$ , 25, 35, 45, 65 and 75 Pa  $\text{CO}_2$ ) and solved for  $C_c$  using a quadratic solution of a RuBP regeneration-limited model accounting for multiple conductances, expressed here as resistances (equation A29 in von Caemmerer 2013)

$$C_c = \frac{\left(C_m - \frac{r_c J}{4} - 2\Gamma^*\right) + \sqrt{\left(C_m - \frac{r_c J}{4} - 2\Gamma^*\right)^2 + 4(C_m 2\Gamma^*)}}{2} \quad (8)$$

where  $r_c$  represents the chloroplastic resistance to  $\text{CO}_2$  transfer and  $J$  represents the rate of electron transfer. Net  $\text{CO}_2$  assimilation ( $A$ ) was then determined assuming RuBP regeneration-limitation and cell wall resistance to  $\text{CO}_2$  transfer ( $r_w$ ) according to

$$A = (C_i - C_m)/r_w \quad (9)$$

To determine how slope–intercept regression is impacted by different resistances to  $\text{CO}_2$  transfer, we assumed four different scenarios ( $r_c = 0$  and  $r_w = 0.3$ ;  $r_c = 0.06$  and  $r_w = 0.24$ ;  $r_c = 0.15$  and  $r_w = 0.15$ ;  $r_c = 0.3$  and  $r_w = 0$   $\text{m}^2 \text{s Pa mol}^{-1}$ ). Different slopes and intercepts were generated by varying  $J$  and the model used the  $R_d$  value from the slope–intercept regression (Eqn 7) and a  $\Gamma^*$  value that gave a best fit with the lowest measured irradiance slope and intercept.

This paper uses measured and modelled data to evaluate slope–intercept analysis and demonstrates that is a superior way to interpret common intercept data using both measured and modelled datasets. We also use slope–intercept analysis to explore if multiple intercellular conductances to  $\text{CO}_2$  significantly impact measurements of  $C_i^*$  in *Nicotiana tabacum* and *Glycine max*.

## MATERIAL AND METHODS

### Literature reports of common intercept values

Reported values of  $C_i^*$  and  $\Gamma^*$  were gathered from an extensive literature search using standard search tools (SCOPUS, Web of Science and Google Scholar) for ‘ $\text{CO}_2$  compensation point’, ‘Day respiration’ and ‘Laisk method’. We additionally searched individual journal archives and papers referencing papers likely to contain reported values. If necessary,  $C_i^*$  and  $\Gamma^*$  values were converted to the SI units of Pa from  $\mu\text{mol}$  or  $\mu\text{bar}$   $\text{CO}_2$  using the elevation of the measurement location to determine atmospheric pressure.  $\Gamma^*$  values were converted to  $C_i^*$  using reported values of  $g_m$  and  $R_d$  (Eqn 2) and temperature corrected to 25 °C using a derivation of the temperature response of *N. tabacum* (Bernacchi *et al.* 2001). The  $\text{CO}_2$  range used in the measurement, species, and species functional type was recorded for statistical analysis. Species names listed are as categorized by the United States Department of Agriculture Natural Resources Conservation Service PLANTS Database using the Cronquist taxonomic system for flowering plants using the information available from the primary publication (Cronquist 1981; USDA 2015). A full list of non-abbreviated species names used is presented as Supporting Information Table S2.

### Growth conditions

*N. tabacum* were sown in covered germination flats containing potting soil (Sunshine Mix #1 LC1, SunGro Horticulture, Agawam, MA, USA). After 2–3 weeks, plants were transplanted to 2 L pots and further grown for an additional 2–3 weeks (4–6 weeks total) until large enough for gas exchange. *G. max* were sown in 3 L pots with a top dressing

of Osmocote (Scotts Miracle-Gro, Marysville, OH, USA). Both *N. tabacum* and *G. max* plants were grown in a climate-controlled cabinet (Conviron, Winnipeg, Manitoba, Canada) with day/night cycles of 11/13 h and 25/23 °C under an irradiance of 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Plants were watered as needed and fertilized weekly (Peters 20-20-20, J.R. Peters, Allentown, PA, USA).

### Measurements of $C_i^*$

The youngest fully expanded leaves of 30–40-day-old plants were used for gas exchange for *N. tabacum* and *G. max*. Gas exchange was performed using a Li-Cor 6400 XT modified to reach low  $\text{CO}_2$  partial pressures using a 6  $\text{cm}^2$  chamber with a red/blue light source (Li-Cor Biosciences, Lincoln, NE, USA; Li-Cor Biosciences 2010). To determine irradiances that would result in an even distribution of photosynthetic rates for  $C_i^*$  determinations, the photosynthetic light response of each species was first measured at 20 Pa  $\text{CO}_2$ . *N. tabacum* was measured under irradiances of 225, 137, 80 and 49  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and *G. max* under 300, 175, 120, 80 and 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . For each light intensity, assimilation was measured at five intercellular  $\text{CO}_2$  partial pressures ranging from 3.5 to 9.5 Pa  $\text{CO}_2$ .

Accounting for  $\text{CO}_2$  leakage during gas exchange measurements is essential for determining accurate assimilation rates when measurement  $\text{CO}_2$  is below atmospheric partial pressures (Long and Bernacchi, 2003; Flexas *et al.* 2007). Assimilation measurements were corrected for  $\text{CO}_2$  leakage between the chamber and surrounding atmosphere according to the manufacturer's instruction. To validate this correction method, leaves were measured in the dark under identical  $\text{CO}_2$  measuring regimes as used during the common intersection measurements. The photosynthetic rate in the dark (dark respiration) should not be sensitive to changes in  $\text{CO}_2$ , so measured differences were attributed to gasket leakage. This gasket leakage for each  $\text{CO}_2$  partial pressure was then used to correct common intersection measurements. Both methods of leak correction produced similar values of  $C_i^*$  and  $R_d$ . Values corrected according to the manufacturer's instruction were presented.

Prior to  $C_i^*$  determinations using the common intersection method, plants were acclimated at 39 Pa  $\text{CO}_2$  until photosynthesis reached steady state to activate Rubisco. After each change in irradiance, a measurement was made at 39 Pa  $\text{CO}_2$  to maintain Rubisco activation. The  $C_i^*$  and  $R_d$  values were determined both from the average  $x$ - and  $y$ -intersection values (common intersection) and the regression values of the slopes and  $y$ -intercepts from each irradiance (slope–intercept regression, see Theory).

### Sensitivity analysis of $C_i^*$ and $R_d$ to $\text{CO}_2$ measuring range

The impact of using linear fits to calculate common intersection values of  $C_i^*$  and  $R_d$  was determined using standard biochemical models of Rubisco- and RuBP-regeneration-limited photosynthesis (von Caemmerer & Farquhar 1981;

von Caemmerer 2000).  $C_i^*$  and  $R_d$  were defined as the averaged intersection of three  $A$ - $C_i$  curves modelled assuming Rubisco limitation at the highest irradiance and RuBP-regeneration at the lower irradiances. Constant values of  $K_c$ ,  $K_o$ ,  $\Gamma^*$  and  $R_d$  (25.9 Pa, 17.9 kPa, 3.86 Pa and 1  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) were used with a  $V_{\text{cmax}}$  at the highest irradiance of 68.5  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . We selected values of rates of electron transport for the lower two light intensities to give an even distribution of slopes among each modelled irradiance for each  $\text{CO}_2$  measuring range since a researcher would select light intensities that produce an even spread of slopes. These  $J$  values ranged between 67 and 21  $\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$ . The model was solved for six evenly spaced chloroplastic  $\text{CO}_2$  partial pressures ( $C_c$ ) for each  $\text{CO}_2$  range. The  $\text{CO}_2$  range started at 35 Pa  $\text{CO}_2$  and ended at an uppermost value that started at 50 and increased to 235 Pa  $\text{CO}_2$  by increments of 10–40 Pa  $\text{CO}_2$ . These  $C_c$  partial pressures were converted to  $C_i$  partial pressures assuming a single linear pathway  $g_m$  value of 3.33  $\text{mol m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$ . The partial  $A$ - $C_i$  curves were then fitted using a linear model and  $C_i^*$  and  $R_d$  determined as the averaged value for each combination of intersections of the three lines.

### Simulations of common intercept measurements

To compare the accuracy of different methods of determining  $C_i^*$  and  $R_d$  values from common intersection data, we simulated a number of initial slopes of  $A$ - $C_i$  curves assuming different numbers of light intensities. The simulation began by first generating an ideal common intersection measurement consisting of lines that intersected at the parameterized  $C_i^*$  and  $R_d$  values with evenly spaced slopes. To represent experimental data, we then selected a random slope from a normal distribution with a mean equal to our ideal values and a standard deviation based on the measured variation in *N. tabacum*. The simulation used the experimental relationship between slope and standard deviation to parameterize the variance since our experimental data indicated that the standard deviation varied with slope. These simulated experimental data were determined for each line to produce a single replicate of simulated experimental data. The  $C_i^*$  and  $R_d$  for each of these replicates was then determined in one of three ways: (1) as the average of all combinations of intersections, (2) as the average of all combinations of intersections following outlier removal defined as intercept values more or less than two standard deviations from the original average or (3) using slope–intercept regression. This simulation was performed 1 000 000 times using 2–8 separate lines with evenly spaced slopes to represent the impact of using additional light intensities. Modelling was performed using R (R Core Team 2013) and is available as Supporting Information Table S2.

### Impact of low $\text{CO}_2$ to photosynthesis

We performed additional gas exchange to determine the impact of low  $\text{CO}_2$  partial pressures to Rubisco activation



and optimize measuring regimes. Gas exchange was performed using the Li-Cor 6400 XT on fully expanded leaves of 30–40-day-old *N. tabacum* by first acclimating leaves at 40 Pa CO<sub>2</sub> and an irradiance of 390 μmol m<sup>-2</sup> s<sup>-1</sup> until they reached steady-state assimilation and conductance values. Following acclimation, the CO<sub>2</sub> partial pressure was changed to either 1, 6, 8, 10 or 120 Pa CO<sub>2</sub> for 7 min, then returned to 40 Pa CO<sub>2</sub> for 20 min. This cycle was repeated on the same leaf with increasing periods of time at the lowered CO<sub>2</sub> (12, 17 and 22 min). Data were recorded every minute and the infrared gas analysers were matched following each CO<sub>2</sub> transition. Presented values were normalized by dividing the measured assimilation rate by the assimilation rate immediately before transition to lower CO<sub>2</sub>.

### Statistics

A one-way ANOVA for measurement temperature, CO<sub>2</sub> measuring range and species type was used to test significance ( $P < 0.05$ ) of interactions within compiled  $C_i^*$  values. One-way ANOVA was also performed to determine significant differences between measurements of  $C_i^*$  and  $R_d$ . All ANOVA were followed with a Tukey's post-hoc test and determined using statistical software (OriginPro 9.0, OriginLab, Northampton, MA, USA).

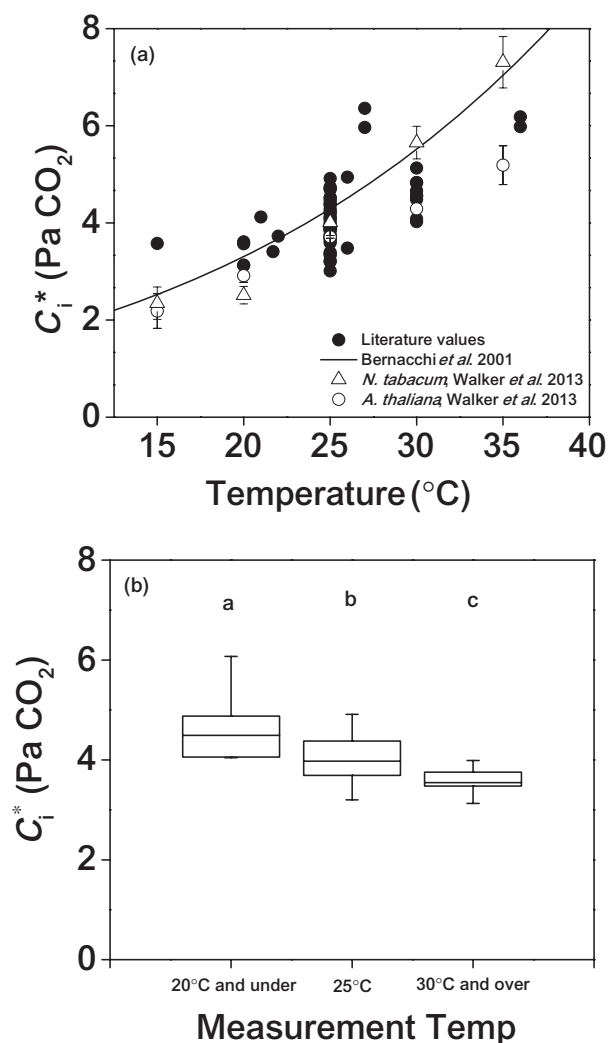
## RESULTS

### Literature $C_i^*$ values varied significantly with measurement temperature

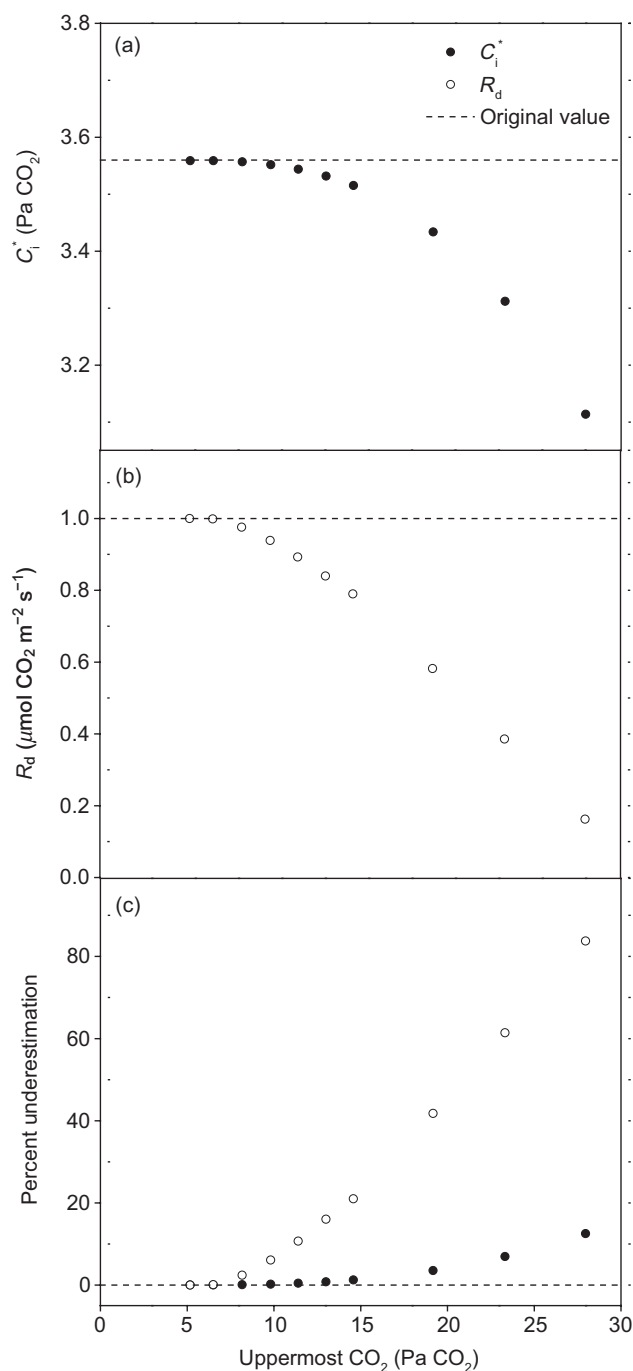
To determine the variation in literature values of  $C_i^*$ , we compared values from 34 published and unpublished sources (Fig. 2 and Supporting Information Table S1). The temperature response of  $C_i^*$  measured in *N. tabacum* was more pronounced than raw  $C_i^*$  values reported in the literature (Fig. 2a).  $C_i^*$  normalized to 25 °C using the temperature response of *N. tabacum* ranged from an average of 3.49 Pa CO<sub>2</sub> in *Oryza* species to 5.57 Pa CO<sub>2</sub> in *Acer rubrum*. Interestingly, the only significant correlation in values of  $C_i^*$  was with measurement temperature despite all values being normalized to using the temperature response of *N. tabacum* (Fig. 2b). Temperature-normalized  $C_i^*$  values measured at lower temperatures (20 °C or lower) were significantly higher than measurements made at 25 °C. This trend continued with  $C_i^*$  values measured at higher temperatures (30 °C or higher). There were no significant correlations between literature  $C_i^*$  and the CO<sub>2</sub> range used in the measurement or species type (data not shown).

### Modelled impact of uppermost CO<sub>2</sub> range on common intersection measurements

We next modelled the impact of CO<sub>2</sub> measuring range on common intersection determination of  $C_i^*$  and  $R_d$  assuming a single linear mesophyll conductance pathway (Fig. 3). Both  $C_i^*$  and  $R_d$  were close to the values used to parameterize the

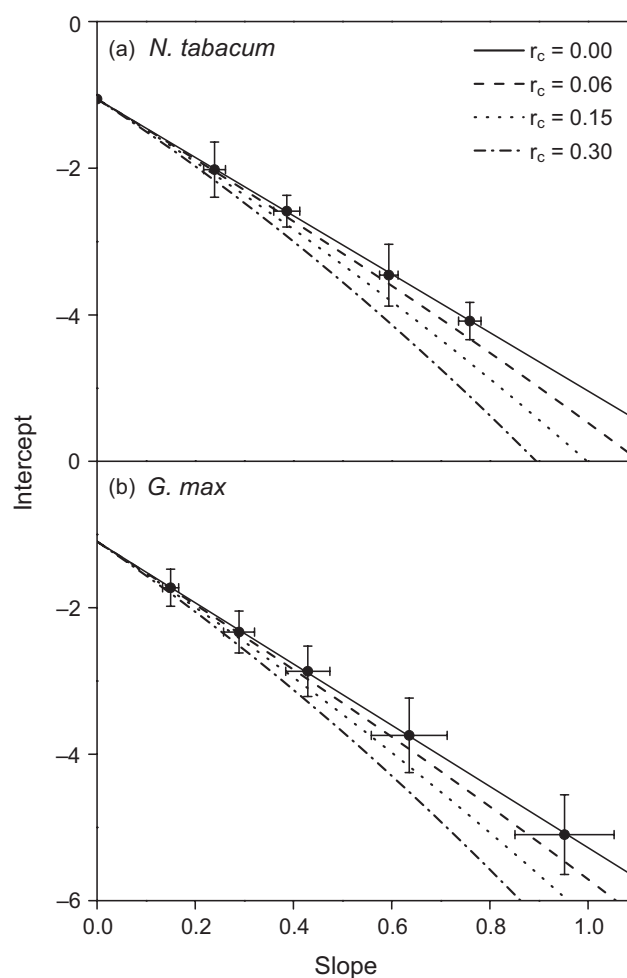


**Figure 2.** The intercellular CO<sub>2</sub> photocompensation point ( $C_i^*$ ) as reported in the literature according to measurement temperature (a) and a categorical boxplot (b). The temperature response (a) shows absolute literature values with both the temperature response of Bernacchi et al. 2001 (solid line) and temperature response of *N. tabacum* and *A. thaliana* from Walker et al. 2013. The boxplot values (b) were normalized from measurement temperature to 25 °C using the temperature response reported in Bernacchi et al. 2001. Boxes depict a 75% confidence interval with whiskers indicating the 95% interval. Significant ( $p < 0.05$ ) interaction of  $C_i^*$  with measuring range according to ANOVA analysis is indicated by different letters above each plot. Data was obtained from a variety of reports (Laisk, 1977; Kirschbaum and Farquhar, 1984; Brooks and Farquhar, 1985; Jacob and Lawlor, 1993; Berry et al., 1994; Villar et al., 1994; von Caemmerer et al., 1994; Balaguer et al., 1996; Häusler et al., 1996; Bunce, 1998; Evans and Loreto, 2000; Bernacchi et al., 2001; Peisker and Apel, 2001; Igamberdiev et al., 2004; Pons and Westbeek, 2004; Guo et al., 2005; Galmés et al., 2006; Schneiderei et al., 2006; Warren and Dreyer, 2006; Flexas et al., 2007; Kebeish et al., 2007; Weston et al., 2007; Li et al., 2009; Tomaz et al., 2010; Cano et al., 2011; Cousins et al., 2011; Tholen and Zhu, 2011; Douthe et al., 2012; Gilbert et al., 2012; Tholen et al., 2012; Busch et al., 2013; Giuliani et al., 2013; Sage et al., 2013; Walker et al., 2013; Walker and Cousins, 2013; Walker et al., 2014; Weise et al., 2015).



**Figure 3.** Impact of uppermost CO<sub>2</sub> partial pressure on estimates of  $C_i^*$  (a) and  $R_d$  (b) using the common intersection method. Values of  $C_i^*$  (a) and  $R_d$  used in the modelled parameterization are shown with dotted lines. Common intersection measurements were modelled assuming three light intensities as outlined in the Materials and Methods. Also shown are the percent underestimations under increasing uppermost CO<sub>2</sub> partial pressures (c).

model when the uppermost CO<sub>2</sub> value was low, but decreased as the uppermost value of CO<sub>2</sub> increased (Fig. 3a,b). When uppermost values of CO<sub>2</sub> were below 10 Pa CO<sub>2</sub>,  $C_i^*$  was underestimated by less than 1% and  $R_d$  by less than 10%



**Figure 4.** Slope–intercept regression analysis of common intercept measurements in *N. tabacum* (a) and *G. max* (b). Slope and intercept measurements from the initial slope of photosynthetic CO<sub>2</sub> response were determined under 4–5 sub-saturating irradiances. Lines represent the modelled relationship between slope and intercept assuming multiple internal resistances to CO<sub>2</sub> and increasing chloroplast CO<sub>2</sub> resistance ( $r_c$ ). Means of  $n = 4 \pm 5$  SD are shown. The model assumed  $\Gamma^*$  and  $R_d$  were 4.11 Pa and 1.1  $\text{m}^2 \text{ s}^{-1}$  for *N. tabacum* and 4.31 Pa and 1.1  $\mu\text{mol m}^{-2} \text{ s}^{-1}$  for *G. max*. Slopes and intercepts were determined using a linear fit of six mesophyll CO<sub>2</sub> partial pressures between 2.5 and 7.5 Pa and total (See Theory).

(Fig. 3c). These percent underestimations did not change significantly when models were repeated with  $R_d$  and  $g_m$  values either half or double that assumed in the presented data (data not shown).

### Slope–intercept regression in *N. tabacum* and *G. max*

The relationship between the slope and intercept of common intercept measurements in both *N. tabacum* and *G. max* were strongly linear (Fig. 4). The average coefficient of determination ( $R^2$ ) for a linear model was  $0.997 \pm 0.001$  and  $0.999 \pm 0.000$  for *N. tabacum* and *G. max*. These linear models

	<i>N. tabacum</i>		<i>G. max</i>	
	$C_i^*$ (Pa CO <sub>2</sub> )	$R_d$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	$C_i^*$ (Pa CO <sub>2</sub> )	$R_d$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )
Common intersection	3.94 ± 0.04	1.07 ± 0.35	4.06 ± 0.23	1.03 ± 0.21
Slope-int. reg.	4.00 ± 0.07	1.04 ± 0.33	4.08 ± 0.24	1.02 ± 0.22

CO<sub>2</sub> gas exchange in *N. tabacum* and *G. max* were measured under 4–5 sub-saturating light intensities and intercellular CO<sub>2</sub> partial pressures ranging from 3.5–9.5 Pa CO<sub>2</sub>.  $C_i^*$  and  $R_d$  were determined as the averaged  $x$  and  $y$  value for the intersections of each light intensity (common intersection) or from the regression of the slope and intercept of all the light intensities together (slope-int. reg.). Means of  $n = 4–5 \pm \text{SD}$  are shown. There were no significant differences between method of determining  $C_i^*$  or  $R_d$  within species according to ANOVA analysis and Tukey post-hoc test.

produced  $C_i^*$  and  $R_d$  values similar to those determined using the averaged intersection of each  $A-C_i$  curve (Table 1). The modelled relationship between the slope and the  $y$ -intercept became increasingly non-linear as the ratio of  $r_c$  to  $r_w$  increased.

We additionally used the modelled relationship between the slope and intercept of  $A-C_i$  curves in response to  $r_c$  to  $r_w$  in *G. max* to determine how different resistances would impact apparent  $C_i^*$  and  $R_d$  measurements using slope–intercept regression (Table 2). We found that increasing the ratio of  $r_c$  to  $r_w$  increased apparent  $C_i^*$  values by up to 45% and decreased  $R_d$  by up to 30%.

### Modelled simulation of common intercept interpretation using three different analysis techniques

The impact of determining  $C_i^*$  and  $R_d$  from common intersection measurements was simulated using the measured variation of *N. tabacum*  $A-C_i$  measurements and number of light intensities between two and eight (Fig. 5). When two light intensities were used, all methods of determining  $C_i^*$  and  $R_d$  produced the same values and variation. When three or more light intensities were used, values of  $C_i^*$  and  $R_d$  varied little with additional measuring light intensities and approached the modelled input when determined using averaged intercepts following outlier removal or the slope–intercept method. When all the intercepts were averaged with no outlier removal,  $C_i^*$  and  $R_d$  showed high variation,

	$r_c = 0, r_w = 0.3$	$r_c = 0.06, r_w = 0.24$	$r_c = 0.15, r_w = 0.15$	$r_c = 0.3, r_w = 0$
$C_i^*$ Pa CO <sub>2</sub>	4.19 (0%)	4.67 (11%)	5.26 (25%)	6.07 (45%)
$R_d$ $\mu\text{mol m}^{-2} \text{s}^{-1}$	1.1 (0%)	1.0 (9%)	0.9 (19%)	0.8 (30%)

A slope–intercept regression was made on five modelled electron transport rates (9, 16, 24, 36 and 55  $\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$ ) with different assumed CO<sub>2</sub> resistances through the cell wall ( $r_c$ ) and chloroplast ( $r_w$ ). The model assumed  $\Gamma^*$  and  $R_d$  were 4.31 Pa and 1.1  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Parenthesis indicate the percent error in apparent  $C_i^*$  and  $R_d$  relative to values assuming complete refixation of photorespired CO<sub>2</sub> ( $r_c = 0, r_w = 0.3$ ). Slopes and  $y$ -intercepts were determined using a linear fit of 6 mesophyll CO<sub>2</sub> partial pressures between 2.5 and 7.5 Pa and total (See Theory).

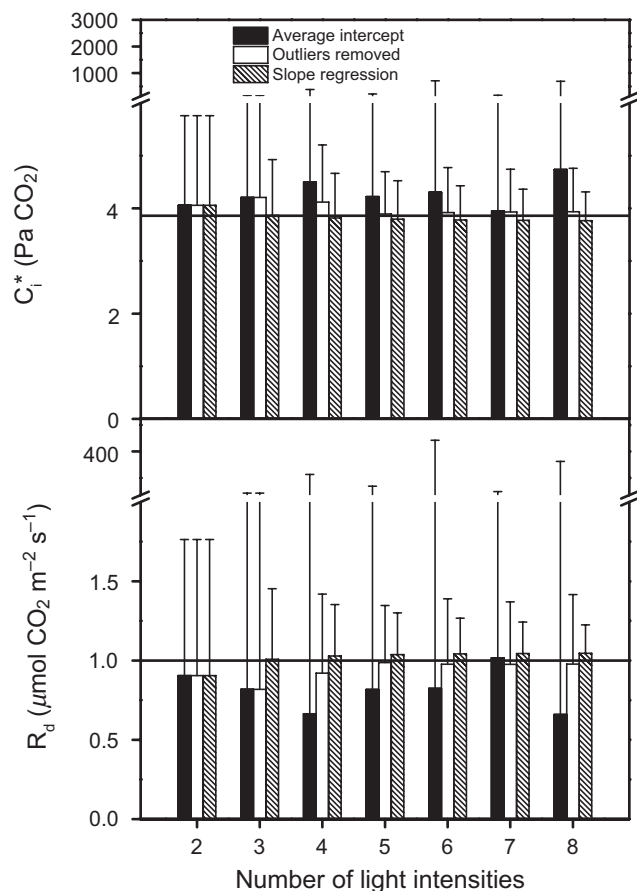
**Table 1.** Comparison of  $C_i^*$  and  $R_d$  determined from CO<sub>2</sub> assimilation curves measured under sub-saturating illumination using two different methods

regardless of light intensities used or even after simulating 1 000 000 common intersection measurements. The simulated variation of  $C_i^*$  and  $R_d$  determined using averaged intersections following outlier removal or the slope–intercept method decreased with increasing number of light intensities, but additional light intensities above five had only marginal improvement in decreasing variation. The slope–intercept regression produced the least variation at every light intensity above two out of all the methods to determine  $C_i^*$  and  $R_d$  and involved no outlier removal.

### Impact of measurement CO<sub>2</sub> on net photosynthesis

We next determined how net photosynthetic CO<sub>2</sub> assimilation is impacted during long periods of leaf exposure to low CO<sub>2</sub>. This control is important since it is possible for a leaf to remain for long periods at very low CO<sub>2</sub> depending on measuring regime during a common intercept measurement. Net CO<sub>2</sub> assimilation at 40 Pa CO<sub>2</sub> showed no decrease after exposure to 8 Pa CO<sub>2</sub> or greater for up to 22 min (Fig. 6a). Following 7 min exposure to 1 or 6 Pa CO<sub>2</sub> there was similarly no impact to net CO<sub>2</sub> assimilation at 40 Pa CO<sub>2</sub> (Fig. 6). Following a 12 min exposure to 1 or 6 Pa CO<sub>2</sub>, there was a transient decrease in net CO<sub>2</sub> assimilation that returned to starting values after around 5 min. Net CO<sub>2</sub> assimilation took much longer to recover following a 17 min exposure to 1 or 6 Pa CO<sub>2</sub> and took the most time following a 22 min exposure.

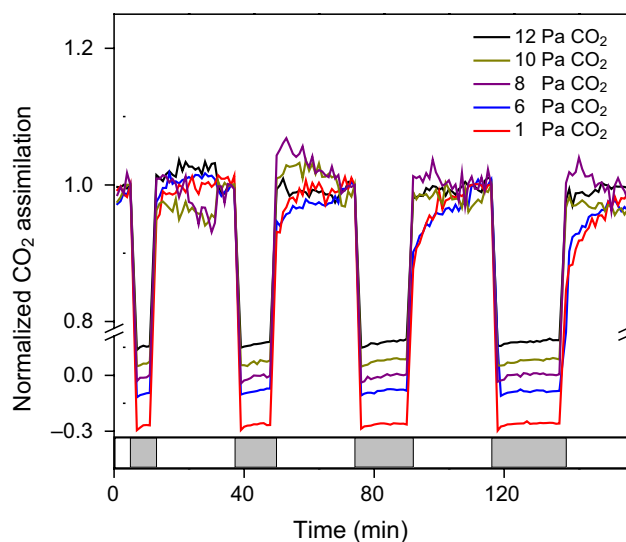
**Table 2.** Impact of multiple conductances on apparent  $C_i^*$  and  $R_d$  measured using slope–intercept regression



**Figure 5.** Simulations of  $C_i^*$  (a) and  $R_d$  (b) values determined using the common intersection method and interpreted using all averaged intersections, averaged intersections following outlier removal and using slope–intercept regression. Simulations were performed by producing an ‘ideal’ common intersection measurement and then introducing random variation to the initial slopes using experimental standard deviations. These simulations were performed for 2–8 light intensities with the parameterized values indicated by a solid line. Means of  $n = 1\,000\,000 \pm$  SD simulations are shown.

### Rubisco specificity does not correlate with $C_i^*$ in meta-analysis

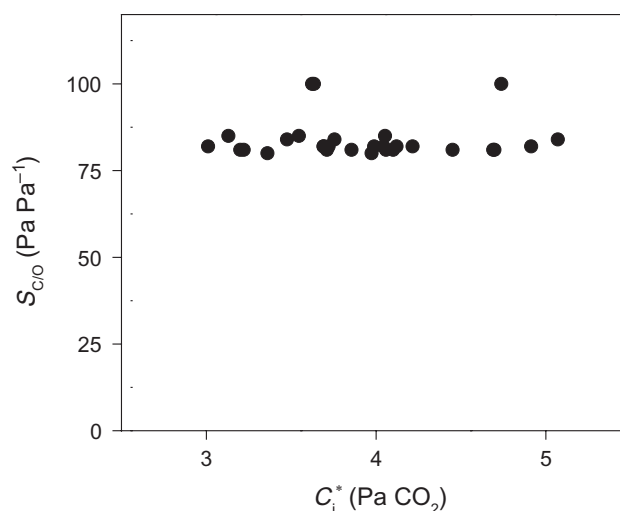
To determine if species-specific factors explained differences in literature  $C_i^*$  values, we compared  $C_i^*$  values with reported Rubisco specificity ( $S_{C/O}$ ) measured at 25 °C. There was no correlation between  $S_{C/O}$  and  $C_i^*$  in the six species for which both values were available in the literature despite  $C_i^*$  ranging from 2.80 to 5.31 Pa CO<sub>2</sub> (Fig. 7). Reported  $S_{C/O}$  in these species were fairly similar and centred around 80 Pa Pa<sup>-1</sup>. The two species that had higher values around 100 Pa/Pa (*Triticum aestivum* and *Limonium gibertii*) were reported from different lab groups than the other values.  $\Gamma^*$  derived from specificity values in plants representing adaptation to broad habitats ranged from the xerophytic *L. gibertii* Sennen (3.41 Pa) to the more mesophytic *Helleborus foetidus* L. (4.25 Pa; Supporting Information Fig. S2).



**Figure 6.** Impact of low CO<sub>2</sub> partial pressures on net CO<sub>2</sub> assimilation. Net CO<sub>2</sub> assimilation was measured in *N. tabacum* after plants had reached steady-state assimilation and conductance values at 40 Pa CO<sub>2</sub> and an irradiance of 390  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . CO<sub>2</sub> was then decreased to between 1 and 12 Pa CO<sub>2</sub> for 5 min and then returned to 40 Pa CO<sub>2</sub> for 25 min. This cycle was repeated with exposure to low CO<sub>2</sub> for 10, 15 and 20 min as indicated by the shaded portion of the lower bar. CO<sub>2</sub> assimilation was normalized to the value measured one minute before the drop to low CO<sub>2</sub> for each cycle. Means of  $n = 4\text{--}5$  are shown.

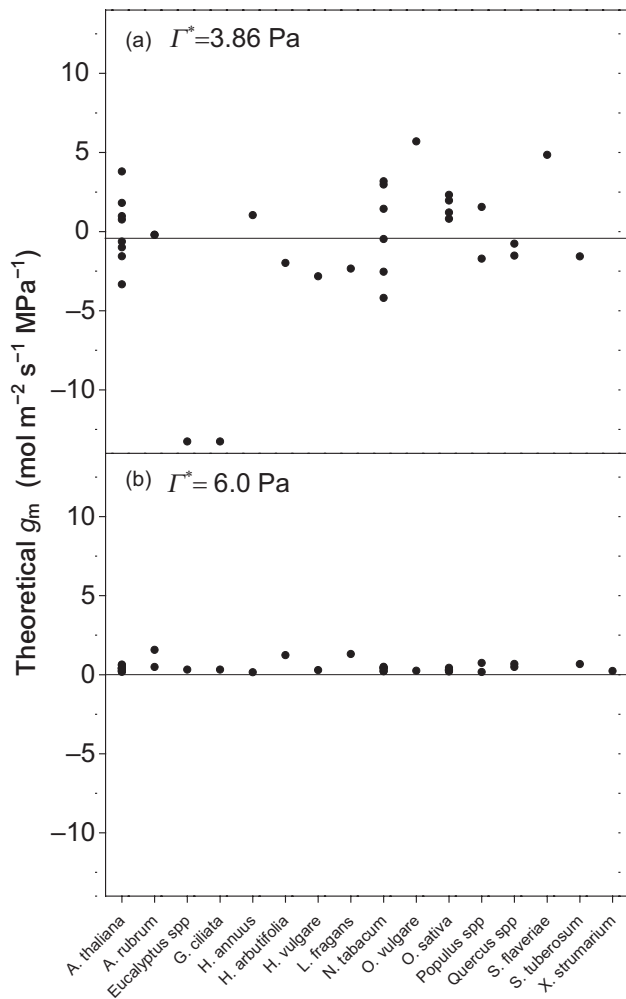
### Response of modelled $C_i^*$ measurements to assumptions of internal CO<sub>2</sub> conductances

We used a modelling approach to quantify how changes in assumptions of the conductance of CO<sub>2</sub> from the intercellular



**Figure 7.** Rubisco specificity for reaction with CO<sub>2</sub> relative to O<sub>2</sub> ( $S_{C/O}$ ) plotted against the intercellular CO<sub>2</sub> photocompensation point ( $C_i^*$ ).  $S_{C/O}$  values were used from (Whitney *et al.* 2011) for  $C_i^*$  values reported in Fig. 1. Shown are  $C_i^*$  values from six species with reported  $S_{C/O}$ .





**Figure 8.** The theoretical mesophyll conductance ( $g_m$ ) necessary to make reported  $C_i^*$  values equal to a constant photocompensation point ( $\Gamma^*$ ) value of 3.86 Pa (a) and 6.0 Pa (b). The theoretical  $g_m$  was calculated for measurements of  $C_i^*$  with a reported rate of day respiration ( $R_d$ ) according to Eqn 2. Shown are 37 observations where  $R_d$  was recorded with three values being off the axis in panel a.

space to the chloroplast would impact the common intersections of  $\text{CO}_2$  response curves. Since the variation in reported  $S_{c/0}$  was minimal for reported  $C_3$  species, we first determined what mesophyll conductance ( $g_m$ ) would be required to explain the differences between reported  $C_i^*$  assuming a constant  $\Gamma^*$  (Fig 8, Eqns 1 & 2). We found that  $g_m$  would need to vary widely among species and individual measurements and include impossible negative values to explain the variation in  $C_i^*$  assuming  $\Gamma^*$  was equal to 3.86 Pa (8a). Since net assimilation is negative at the common intersection,  $\Gamma^*$  would need to be greater than  $C_i^*$  to result in positive  $g_m$  (Eqn 2). If  $\Gamma^*$  were equal to 6.00 Pa, the differences between 90% of reported  $C_i^*$  values could be explained with a positive  $g_m$  (Fig. 8b).

## DISCUSSION

### Proper selection of $\text{CO}_2$ range and use of slope–intercept regression can improve the accuracy and precision of $C_i^*$ determinations

Our meta-analysis indicates the need for a consistent and robust protocol for measuring and interpreting  $C_i^*$  using the common intersection method. Literature-reported  $C_i^*$  showed greater variation than was found in a previous comparison of  $\Gamma^*$  values from only 12 different reports using the common intercept method (Fig. 2, Evans & Loreto 2000). This variation did not correlate with the  $\text{CO}_2$  range used during the measurement, despite modelling which suggests that  $C_i^*$  values should decrease when  $\text{CO}_2$  measuring ranges increase (Fig. 3, Gu and Sun 2014). The insensitivity of  $C_i^*$  values to measuring range suggests that literature reports of  $C_i^*$  were not significantly biased by assuming linear fits or that other factors introduced more variation. This lack of  $\text{CO}_2$  range bias could be explained if researchers selected  $\text{CO}_2$  ranges that produced linear fits and common intersections for each species measured.

Modelling of common intersections under different ranges of  $\text{CO}_2$  suggests that errors attributed to assumptions of linear  $A-C_i$  curves can be effectively minimized by using low measurement  $\text{CO}_2$  partial pressure during the measurement (Fig. 3). By limiting the uppermost intercellular  $\text{CO}_2$  partial pressure to under 10 Pa,  $C_i^*$  is underestimated by only 1% and  $R_d$  by 10% (Fig. 3c). These percent underestimations are additionally insensitive to parameterized  $g_m$  or  $R_d$  values and are consistent with past predictions (Gu & Sun 2014), but quantify what the underestimation would be. This 1% underestimation would likely be within the experimental noise for gas exchange, indeed only one measurement from our meta-analysis had a standard deviation 1% of the average value and the average was 8% (Supporting Information Table S1). Given this small underestimation, it seems appropriate to use linear slopes to determine common intersections provided the uppermost intercellular  $\text{CO}_2$  partial pressure used is under 10 Pa. However, since the impact of assuming linear relationships of  $A-C_i$  curves bias  $R_d$  much more than  $C_i^*$ , care should be taken with common intersection measurements used to quantify  $R_d$  in situations where absolute values are critical (Fig. 2b,c, Harley *et al.* 1992; Gu and Sun 2014).

This modelling further suggests that while all the linear fits of  $A-C_i$  curves are not expected to intersect mathematically, their individual intersections can be very close to curvilinear intersections provided the uppermost  $\text{CO}_2$  partial pressure is kept below  $\sim 10$  Pa. The intersection of these  $A-C_i$  curves is also less sensitive to uppermost  $\text{CO}_2$  range when the initial slopes are evenly spaced. This could explain the smaller modelled impact of linear fits that we found as compared with previous work, where two of the three initial slopes were similar (fig. 4 in Gu and Sun 2014). We therefore recommend that light intensities are selected, which give evenly spaced initial slopes.

We next examined how common intersection measurements are impacted by assumptions of intracellular

resistances to CO<sub>2</sub> using slope–intercept regression. Slope–intercept regression provides sensitive and quantitative analysis of the impact of multiple internal resistances to CO<sub>2</sub> on common intercept measurements. When  $r_c$  is large relative to  $r_w$ , there is a curvilinear relationship between the slope and y-intercept of  $A-C_i$  curves measured at sub-saturating light intensities (see Theory, Fig. 4). This curvilinear relationship was not apparent in *N. tabacum* or *G. max*, suggesting that the effective  $r_w$  was larger than  $r_c$  and that calculation of  $\Gamma^*$  could be made assuming simple linear resistance to CO<sub>2</sub> (Eqn 2).

It should be noted that this curvilinear relationship is subtle at low  $r_c$  to  $r_w$  ratios and is therefore probably not sensitive enough to directly measure small differences in  $r_c$  and  $r_w$  through fitting of 4–5 points. The slight curvilinearity is large enough, however, to significantly increase apparent  $C_i^*$  values (Table 2). Therefore, while slope–intercept regression of common intersection data may not be able to quantify small differences in the ratios of  $r_c$  to  $r_w$ , it should be able to detect larger differences. Additionally, since small differences impact  $C_i^*$ , larger apparent  $C_i^*$  values from slope–intercept regression could indicate leakage of photorespired CO<sub>2</sub> into the intercellular airspace. Slope–intercept regression of common intersection data thus provides a new tool to evaluate the partitioning between  $r_c$  and  $r_w$  and determine if more simple linear resistance pathways are adequate to describe CO<sub>2</sub> exchange.

Slope–intercept regression was more precise and accurate in determining  $C_i^*$  and  $R_d$  from  $A-C_i$  curves modelled under sub-saturating light intensities (Fig. 5). Slope–intercept regression yielded accurate values of  $C_i^*$  and  $R_d$  with less variance compared with taking the average of all intersections or taking the average of intersections following outlier removal. Slope–intercept regression also had the least variance for all measurements using more than two light intensities. It is interesting that even after 1 000 000 simulated measurements, the standard deviation of raw values with no outlier removal is expected to be very large (~300–900 for  $C_i^*$  and ~400 for  $R_d$ ). Such a large standard deviation could be a result of how the simulation imposed error on generated data, but the qualitative differences between the variations in interpreting common intersection data using simple averaging versus slope–intercept regression is the same regardless of how much error is assumed in the gas exchange. Additionally, this variability in averaging all intersections may explain why despite careful technique, common intersection measurements often fail to produce common intersections, forcing either re-measurement or removal of ‘outlier’ data. Analysis using slope–intercept regression obviates the removal of data and results in more precise measurements of  $C_i^*$  and  $R_d$  by effectively weighting the intersections of lines with similar slopes (see Theory). These simulations also demonstrate that 4–5 light intensities are optimal for  $C_i^*$  and  $R_d$  measurements and that further light intensities are not expected to greatly improve precision.

It is unclear why previous measurements of common intersections in *N. tabacum* yielded large differences in the intersection of high versus low-light  $A-C_i$  curves (Fig. 3 in Tholen *et al.* 2012). The discrepancy between our data and Tholen *et al.* (2012) may be explained by differences in leaf ages used in the measurement, since older leaves may have a decrease

in the surface area of the intercellular airspace covered by chloroplasts and a larger effective ratio of  $r_c$  to  $r_w$  (Busch *et al.* 2013). Differences in the intersections of high- versus low-light  $A-C_i$  curves could also be explained through progressive deactivation of Rubisco during the measurement of high-light  $A-C_i$  curves (see below).

Appropriate selection of CO<sub>2</sub> measuring ranges may minimize the impact of progressive Rubisco deactivation to common intersection measurements and  $A-C_i$  curves in general. Rubisco does not appear to deactivate under moderately low CO<sub>2</sub> partial pressures ( $C_i$  of 14 Pa CO<sub>2</sub>, Cen & Sage 2005), but does deactivate near the CO<sub>2</sub> compensation point (Caemmerer & Edmondson 1986). Our findings indicated that the impact of CO<sub>2</sub> partial pressure on Rubisco activation state could be time dependent (Fig. 6). There was no apparent deactivation of Rubisco evident from the net CO<sub>2</sub> assimilation rate at 40 Pa CO<sub>2</sub> following 5 min exposure to between 1 and 12 Pa CO<sub>2</sub>. Rubisco appeared to deactivate slightly following ten minutes of exposure to 1 and 6 Pa CO<sub>2</sub> but net CO<sub>2</sub> exchange returned to starting values after less than 5 min upon return to 40 Pa CO<sub>2</sub>. After 15 and 20 min of exposure to 1 and 6 Pa CO<sub>2</sub>, Rubisco appeared to take much longer to reactivate. Exposure to 8 Pa CO<sub>2</sub> and above had no effect on Rubisco activation state even after 20 min (Fig. 6). These findings indicate that common intersection measurements should not remain below ~8 Pa CO<sub>2</sub> for longer than ~10 min to maintain Rubisco activation state. To balance the need to measure under low CO<sub>2</sub> to minimize the impact of using linear fits with the danger of Rubisco deactivation, we suggest starting each light intensity at 40 Pa CO<sub>2</sub> and then measuring at 5–6 CO<sub>2</sub> partial pressures between 3 and 10 Pa to determine the initial slope and y-intercept. We also recommend returning to 40 Pa CO<sub>2</sub> before changing the light intensity to determine if Rubisco deactivation occurred at the lower CO<sub>2</sub> Pa. These recommendations are based on measurements in *N. tabacum* and could vary based on interspecies differences in Rubisco activation.

### The temperature response of literature $C_i^*$ values is different than that commonly used to parameterize leaf models of photosynthesis

The differences in the temperature response of  $C_i^*$  between literature values and that of *N. tabacum* could be due to *N. tabacum* having an unusually high temperature response as compared with other species (Fig. 2b). The increased temperature response of  $C_i^*$  could be due to differences in the temperature response of  $g_m$ , since *N. tabacum* has among the largest temperature response and absolute values of  $g_m$  compared to eight other species (von Caemmerer & Evans 2014). Larger  $g_m$  values result in increased  $C_i^*$  assuming constant  $S_{c/o}$  and  $R_d$  (Eqns 1 & 2). Alternatively, the temperature response of  $C_i^*$  in *N. tabacum* could be due to either an unusually decreased temperature sensitivity of  $S_{c/o}$  or a decreased sensitivity of  $R_d$  relative to other species. It is interesting that the literature temperature response of  $C_i^*$  closely resembles the response of *A. thaliana*, suggesting that species other than tobacco might more broadly represent the

temperature response of  $C_i^*$  for modelling (Fig. 2b). Clearly, more work is needed confirming the temperature response of  $C_i^*$ ,  $\Gamma^*$  and  $g_m$  across ecologically and agronomically important species to determine the most appropriate temperature functions to use in leaf scale or earth system models.

Alternatively, since the *N. tabacum* temperature response was originally determined and since confirmed by growing plants in a controlled environment with day temperatures between 23 and 25 °C and then temporarily moving plants to higher or lower temperatures for measurement of  $C_i^*$  (Bernacchi *et al.* 2001; Walker & Cousins 2013; Walker *et al.* 2013). This approach would not account for species-specific differences in the temperature response of  $C_i^*$ , the absolute values of  $C_i^*$  or an acclimation of  $C_i^*$  that could occur during longer-term exposure to elevated temperature.

Differences in the temperature response or acclimation of  $S_{c/o}$  could also explain some of the variation in  $C_i^*$  found in the literature among species (Eqns 1 and 2). Such acclimation would result in plants grown at higher temperatures having decreased  $C_i^*$  values when measured near their growth temperature. Indeed, measurement temperature was within 2 °C of growth temperature for 70% of  $C_i^*$  values with reported growth temperature (Supporting information Table S1). There is some evidence that  $S_{c/o}$  acclimates to growth temperature by differential expression of Rubisco small subunit genes, which would result in decreased  $C_i^*$  at high temperatures (Cavanagh & Kubien 2014). This differential expression could explain the observation that the temperature response of *in vitro*  $\Gamma^*$  in spinach leaves is decreased in plants grown under elevated temperature (Yamori *et al.* 2006). Such an acclimation response of  $S_{c/o}$  is consistent with the differences seen between literature values primarily measured near growth temperatures and those reported in (Bernacchi *et al.* 2001; Fig. 2a).

Differences in  $S_{c/o}$  among species could also explain differences in  $C_i^*$  values, although there was no significant interaction between species and  $C_i^*$  values normalized to 25 °C. This lack of interaction could be explained by improper temperature response normalization functions or noisy measurement technique. Alternatively, this variation could be due to differences among species due to adaptation of  $S_{c/o}$  (Sage 2002; Galmés *et al.* 2005). When  $S_{c/o}$  measured in a diversely adapted group of species were converted to  $\Gamma^*$ , there was a less variation than we observed in the literature (Fig. 2, Supporting Information Fig. S1). It is also possible that methodological differences between labs could explain the lack of correlation between  $S_{c/o}$  and  $C_i^*$ , but we would still expect to see some correlation since most of the reported  $S_{c/o}$  and measurements came from the same group. These observations suggest that typical variation in  $S_{c/o}$  is not large enough to significantly impact measurements of  $C_i^*$  and do not explain the variation in reported values.

To understand if differences in internal conductance to CO<sub>2</sub> could explain the variation in  $C_i^*$ , we modelled the impact of different linear conductance pathways to measurements of  $C_i^*$ . The range of mesophyll conductances needed to make literature  $C_i^*$  values equal a common  $\Gamma^*$  value of

3.86 Pa are larger than what has been reported in the literature and include impossible negative conductances (Fig. 6a; Warren 2008; Niinemets *et al.* 2009). To result in only positive conductances,  $\Gamma^*$  needs to be greater than the assumed  $C_i^*$  assuming a single mesophyll conductance (Eqn 2).  $\Gamma^*$  would need to be 6.0 Pa to be greater than 90% of the literature reported  $C_i^*$  measurements, which is almost twice as large as what is predicted from a wide range of  $S_{c/o}$  values in higher C3 plants (Fig. 8b, Supporting Information Fig. S1). These findings suggest that differences in  $g_m$  do not alone explain the variation in observed  $C_i^*$  assuming a single diffusive path of CO<sub>2</sub>. These analysis on previous data confirm that much of the variation reported in  $C_i^*$  is not due to physiological factors (differences in  $S_{c/o}$  or  $g_m$ ), but to methodological issues with the measurement. Such potential for methodological error highlights the importance of adapting a robust and consistent protocol (e.g. slope–intercept analysis) for measuring  $C_i^*$ .

It should be noted that it is possible for  $\Gamma^*$  to be less than  $C_i^*$  assuming multiple diffusive resistances if  $r_c$  is much larger than  $r_w$  (Eqns 4 & 5; Tholen *et al.* 2012). Assuming a simple linear diffusion path, physical modelling of CO<sub>2</sub> resistance based on leaf anatomy suggests  $r_w$  could account for 50% or more of total resistance and the contribution of  $r_c$  ranges significantly (Evans *et al.* 2009). Assuming RuBP-limited photosynthesis and, a J value of 75  $\mu\text{mol electrons e}^- \text{m}^{-2} \text{s}^{-1}$ , and an  $R_d$  of 2,  $r_c$  would need to be ~3 times greater than  $r_w$  for  $\Gamma^*$  to be less than  $C_i^*$  (Eqn 5). We found no evidence of such a large ratio of  $r_c$  to  $r_w$  in our slope–intercept measurements for *N. tabacum* and *G. max* but it is possible this ratio is variable in other species. Additionally, to explain the differences in reported  $C_i^*$  values, the ratio of  $r_w$  relative to  $r_c$  would need to be highly variable within species since both positive and negative  $g_m$  values were required to explain a common  $\Gamma^*$  value in both *A. thaliana* and *N. tabacum* (Fig. 6a).

## CONCLUSIONS

Slope–intercept regression provides a robust and precise analysis tool for determining  $C_i^*$  and  $R_d$  from CO<sub>2</sub> gas exchange. Slope–intercept analysis can help determine if multiple resistances within the cell to CO<sub>2</sub> should be considered and what their impact would be. This analysis offers significant improvement over traditional intersection averaging and is less sensitive to experimental noise. We found that biases introduced by using linear fits to interpret curvilinear data could be minimized by measuring with CO<sub>2</sub> ranges under 10 Pa and that predicted variation is optimal when 4–5 light intensities are used. Our meta-analysis shows significant variation in reported  $C_i^*$  values and evidence that commonly used temperature response functions of  $C_i^*$ , and by extension  $\Gamma^*$ , needs to be re-examined for species diversity and possible acclimation.

## ACKNOWLEDGMENTS

Jessica Ayers for technical assistance during common intersection measurements and Susanne von Caemmerer for

helpful thoughts on the modelling and discussion points. Thomas Sharkey also provided insight for technical considerations for the common intersection method and helped spawn this study in the first place. We would also like to thank two anonymous reviewers for thoughtful comments that greatly broadened the scope of our analysis and discussion. This research was supported via subcontract by the Bill and Melinda Gates Foundation (OPP1060461) titled 'RIPE-Realizing Increased Photosynthetic Efficiency for Sustainable Increases in Crop Yield'.

## REFERENCES

- Balaguer L., Afif D., Dizengremel P. & Dreyer E. (1996) Specificity factor of ribulose biphosphate carboxylase/oxygenase of *Quercus robur*. *Plant Physiology and Biochemistry* **34**, 879–883.
- Bernacchi C.J., Pimentel C. & Long S.P. (2003) In vivo temperature response functions of parameters required to model RuBP-limited photosynthesis. *Plant, Cell & Environment* **26**, 1419–1430.
- Bernacchi C.J., Portis A.R., Nakano H., von Caemmerer S. & Long S.P. (2002) Temperature response of mesophyll conductance. Implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis *in vivo*. *Plant Physiology* **130**, 1992–1998.
- Bernacchi C.J., Singsaas E.L., Pimentel C., Portis A.R. & Long S.P. (2001) Improved temperature response functions for models of Rubisco-limited photosynthesis. *Plant, Cell & Environment* **24**, 253–259.
- Berry J., Collatz G., Guy R. & Fogel M. (1994) The compensation point: can a physiological concept be applied to global cycles of carbon and oxygen. *Regulation of Atmospheric CO<sub>2</sub> and O<sub>2</sub> by Photosynthetic Carbon Metabolism*, pp. 234–248. Oxford University Press, Oxford.
- Brooks A. & Farquhar G.D. (1985) Effect of temperature on the CO<sub>2</sub>/O<sub>2</sub> specificity of ribulose-1, 5-bisphosphate carboxylase/oxygenase and the rate of respiration in the light. *Planta* **165**, 397–406.
- Bunce J.A. (1998) The temperature dependence of the stimulation of photosynthesis by elevated carbon dioxide in wheat and barley. *Journal of Experimental Botany* **49**, 1555–1561.
- Busch F.A., Sage T.L., Cousins A.B. & Sage R.F. (2013) C<sub>3</sub> plants enhance rates of photosynthesis by reassimilating photorespired and respired CO<sub>2</sub>. *Plant, Cell & Environment* **36**, 200–212.
- Caemmerer S. & Edmondson D. (1986) Relationship between steady-state gas exchange, *in vivo* ribulose biphosphate carboxylase activity and some carbon reduction cycle intermediates in *Raphanus sativus*. *Australian Journal of Plant Physiology* **13**, 669–688.
- Cano F.J., Sánchez-Gómez D., Gascó A., Rodríguez-Calcerrada J., Gil L., Warren C.R. & Aranda I. (2011) Light acclimation at the end of the growing season in two broadleaved oak species. *Photosynthetica* **49**, 581–592.
- Cavanagh A.P. & Kubien D. (2014) Can phenotypic plasticity in Rubisco performance contribute to photosynthetic acclimation? *Photosynthesis Research* **119**, 203–214.
- Cen Y.-P. & Sage R.F. (2005) The regulation of Rubisco activity in response to variation in temperature and atmospheric CO<sub>2</sub> partial pressure in sweet potato. *Plant Physiology* **139**, 979–990.
- Cousins A.B., Walker B.J., Pracharoenwattana I., Smith S.M. & Badger M.R. (2011) Peroxisomal hydroxypyruvate reductase is not essential for photorespiration in *Arabidopsis* but its absence causes an increase in the stoichiometry of photorespiratory CO<sub>2</sub> release. *Photosynthesis Research* **108**, 91–100.
- Cronquist A. (1981) *An Integrated System of Classification of Flowering Plants*. Columbia University Press.
- Doutche C., Dreyer E., Brendel O. & Warren C.R. (2012) Is mesophyll conductance to CO<sub>2</sub> in leaves of three *Eucalyptus* species sensitive to short-term changes of irradiance under ambient as well as low O<sub>2</sub>? *Functional Plant Biology* **39**, 435–448.
- Drewry D.T., Kumar P., Long S., Bernacchi C., Liang X.Z. & Sivapalan M. (2010) Ecohydrological responses of dense canopies to environmental variability: 1. Interplay between vertical structure and photosynthetic pathway. *Journal of Geophysical Research* **115**, G04022.
- Duda R.O. & Hart P.E. (1972) Use of the Hough transformation to detect lines and curves in pictures. *Communications of the ACM* **15**, 11–15.
- Evans J. & Loreto F. (2000) Acquisition and diffusion of CO<sub>2</sub> in higher plant leaves. In *Photosynthesis: Physiology and Metabolism* (eds R.C. Leegood, T.D. Sharkey & S. von Caemmerer), pp. 321–351. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Evans J.R., Kaldenhoff R., Genty B. & Terashima I. (2009) Resistances along the CO<sub>2</sub> diffusion pathway inside leaves. *Journal of Experimental Botany* **60**, 2235–2248.
- Flexas J., Ortuño M.F., Ribas-Carbo M., Diaz-Espejo A., Flórez-Sarasa I.D. & Medrano H. (2007) Mesophyll conductance to CO<sub>2</sub> in *Arabidopsis thaliana*. *The New Phytologist* **175**, 501–511.
- Galmés J., Flexas J., Keys A.J., Cifre J., Mitchell R.A.C., Madgwick P.J., ... Parry M.A.J. (2005) Rubisco specificity factor tends to be larger in plant species from drier habitats and in species with persistent leaves. *Plant, Cell & Environment* **28**, 571–579.
- Galmés J., Medrano H.L. & Flexas J. (2006) Acclimation of Rubisco specificity factor to drought in tobacco: discrepancies between *in vitro* and *in vivo* estimations. *Journal of Experimental Botany* **57**, 3659–3667.
- Gilbert M.E., Pou A., Zwieniecki M.A. & Holbrook N.M. (2012) On measuring the response of mesophyll conductance to carbon dioxide with the variable J method. *Journal of Experimental Botany* **63**, 413–425.
- Giuliani R., Koteyeva N., Voznesenskaya E., Evans M.A., Cousins A.B. & Edwards G.E. (2013) Coordination of leaf photosynthesis, transpiration, and structural traits in rice and wild relatives (Genus *Oryza*). *Plant Physiology* **162**, 1632–1651.
- Gu L. & Sun Y. (2014) Artefactual responses of mesophyll conductance to CO<sub>2</sub> and irradiance estimated with the variable J and online isotope discrimination methods. *Plant, Cell & Environment* **37**, 1231–1249.
- Guo S., Schinner K., Sattelmacher B. & Hansen U.-P. (2005) Different apparent CO<sub>2</sub> compensation points in nitrate- and ammonium-grown *Phaseolus vulgaris* and the relationship to non-photorespiratory CO<sub>2</sub> evolution. *Physiologia Plantarum* **123**, 288–301.
- Harley P., Loreto F., Di Marco G. & Sharkey T. (1992) Theoretical considerations when estimating the mesophyll conductance to CO<sub>2</sub> flux by analysis of the response of photosynthesis to CO<sub>2</sub>. *Plant Physiology* **98**, 1429–1436.
- Häusler R.E., Kleines M., Uhrig H., Hirsch H.-J. & Smets H. (1999) Overexpression of phosphoenolpyruvate carboxylase from *Corynebacterium glutamicum* lowers the CO<sub>2</sub> compensation point (Γ\*) and enhances dark and light respiration in transgenic potato. *Journal of Experimental Botany* **50**, 1231–1242.
- Igamberdiev A., Mikkelsen T., Ambus P., Bauwe H., Lea P. & Gardeström P. (2004) Photorespiration contributes to stomatal regulation and carbon isotope fractionation: a study with barley, potato and *Arabidopsis* plants deficient in glycine decarboxylase. *Photosynthesis Research* **81**, 139–152.
- Jacob J. & Lawlor D.W. (1993) Extreme phosphate deficiency decreases the *in vivo* CO<sub>2</sub>/O<sub>2</sub> specificity factor of ribulose 1,5-bisphosphate carboxylase-oxygenase in intact leaves of sunflower. *Journal of Experimental Botany* **44**, 1635–1641.
- Kebeish R., Niessen M., Thiruveedhi K., Bari R., Hirsch H.-J., Rosenkranz R., ... Peterhansel C. (2007) Chloroplastic photorespiratory bypass increases photosynthesis and biomass production in *Arabidopsis thaliana*. *Nature Biotechnology* **25**, 593–599.
- Kirschbaum M. & Farquhar G. (1984) Temperature dependence of whole-leaf photosynthesis in *Eucalyptus pauciflora* Sieb. Ex Spreng. *Functional Plant Biology* **11**, 519–538.
- Laisk A. (1977) Kinetics of photosynthesis and photorespiration in C<sub>3</sub> plants. Nauka, Moscow (in Russian).
- LI-COR Biosciences (2010) Modification of LI-6400/LI-6400XT to control at low [CO<sub>2</sub>]. LI-COR application note Application note 7.
- Li Y., Gao Y., Xu X., Shen Q. & Guo S. (2009) Light-saturated photosynthetic rate in high-nitrogen rice (*Oryza sativa* L.) leaves is related to chloroplastic CO<sub>2</sub> concentration. *Journal of Experimental Botany* **60**, 2351–2360.
- Niinemets U., Wright I.J. & Evans J.R. (2009) Leaf mesophyll diffusion conductance in 35 Australian sclerophylls covering a broad range of foliage structural and physiological variation. *Journal of Experimental Botany* **60**, 2433–2449.
- Peisker M. & Apel H. (2001) Inhibition by light of CO<sub>2</sub> evolution from dark respiration: comparison of two gas exchange methods. *Photosynthesis Research* **70**, 291–298.
- Pons T.L. & Westbeek M.H.M. (2004) Analysis of differences in photosynthetic nitrogen-use efficiency between four contrasting species. *Physiologia Plantarum* **122**, 68–78.



- R Core Team (2013) *R: A Language and Environment for Statistical Computing*, R Foundation for Statistical Computing, Vienna, Austria. (URL: <http://www.R-project.org/>).
- Rogers A., Medlyn B.E. & Dukes J.S. (2014) Improving representation of photosynthesis in Earth System Models. *The New Phytologist* **204**, 12–14.
- Sage R.F. (2002) Variation in the  $k_{cat}$  of Rubisco in C<sub>3</sub> and C<sub>4</sub> plants and some implications for photosynthetic performance at high and low temperature. *Journal of Experimental Botany* **53**, 609–620.
- Sage R.F. & Kubien D.S. (2007) The temperature response of C<sub>3</sub> and C<sub>4</sub> photosynthesis. *Plant, Cell & Environment* **30**, 1086–1106.
- Sage T.L., Busch F.A., Johnson D.C., Friesen P.C., Stinson C.R., Stata M., . . . Sage R.F. (2013) Initial events during the evolution of C<sub>4</sub> photosynthesis in C<sub>3</sub> species of *Flaveria*. *Plant Physiology* **163**, 1266–1276.
- Schneidereit J., Häusler R.E., Fiene G., Kaiser W.M. & Weber A.P.M. (2006) Antisense repression reveals a crucial role of the plastidic 2-oxoglutarate/malate translocator DiT1 at the interface between carbon and nitrogen metabolism. *The Plant Journal* **45**, 206–224.
- Tholen D., Ethier G., Genty B., Pepin S. & Zhu X.-G. (2012) Variable mesophyll conductance revisited: theoretical background and experimental implications. *Plant, Cell & Environment* **35**, 2087–2103.
- Tholen D. & Zhu X.-G. (2011) The mechanistic basis of internal conductance: a theoretical analysis of mesophyll cell photosynthesis and CO<sub>2</sub> diffusion. *Plant Physiology* **156**, 90–105.
- Tomaz T., Bagard M., Pracharoenwattana I., Linden P., Lee C.P., Carroll A.J., . . . Millar A.H. (2010) Mitochondrial malate dehydrogenase lowers leaf respiration and alters photorespiration and plant growth in Arabidopsis. *Plant Physiology* **154**, 1143–1157.
- USDA (2015) The PLANTS Database. <http://plants.usda.gov>.
- Villar R., Held A.A. & Merino J. (1994) Comparison of methods to estimate dark respiration in the light in leaves of two woody species. *Plant Physiology* **105**, 167–172.
- von Caemmerer S. (2000) *Biochemical Models of Leaf Photosynthesis*, Vol. 2, CSIRO, Collingwood.
- von Caemmerer S. (2013) Steady-state models of photosynthesis. *Plant, Cell & Environment* **36**, 1617–1630.
- von Caemmerer S., Evans J., Hudson G. & Andrews T. (1994) The kinetics of ribulose-1, 5-bisphosphate carboxylase/oxygenase in vivo inferred from measurements of photosynthesis in leaves of transgenic tobacco. *Planta* **195**, 88–97.
- von Caemmerer S. & Evans J.R. (2014) Temperature responses of mesophyll conductance differ greatly between species. *Plant, Cell & Environment* **38**, 629–637.
- von Caemmerer S. & Farquhar G.D. (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* **153**, 376–387.
- Walker B., Ariza L.S., Kaines S., Badger M.R. & Cousins A.B. (2013) Temperature response of in vivo Rubisco kinetics and mesophyll conductance in *Arabidopsis thaliana*: comparisons to *Nicotiana tabacum*. *Plant, Cell & Environment* **36**, 2108–2119.
- Walker B. & Cousins A. (2013) Influence of temperature on measurements of the CO<sub>2</sub> compensation point: differences between the Laisk and O<sub>2</sub>-exchange methods. *Journal of Experimental Botany* **64**, 1893–1905.
- Walker B.J., Strand D.D., Kramer D.M. & Cousins A.B. (2014) The response of cyclic electron flow around photosystem I to changes in photorespiration and nitrate assimilation. *Plant Physiology* **165**, 453–462.
- Warren C. & Dreyer E. (2006) Temperature response of photosynthesis and internal conductance to CO<sub>2</sub>: results from two independent approaches. *Journal of Experimental Botany* **57**, 3057–3067.
- Warren C.R. (2008) Stand aside stomata, another actor deserves centre stage: the forgotten role of the internal conductance to CO<sub>2</sub> transfer. *Journal of Experimental Botany* **59**, 1475–1487.
- Weise S., Carr D., Bourke A., Hanson D.T., Swarthout D. & Sharkey T. (2015) The arc mutants of Arabidopsis with fewer large chloroplasts have a lower mesophyll conductance. *Photosynthesis Research* 1–10.
- Weston D.J., Bauerle W.L., Swire-Clark G.A., Moore B. & Baird W.V. (2007) Characterization of Rubisco activase from thermally contrasting genotypes of *Acer rubrum* (Aceraceae). *American Journal of Botany* **94**, 926–934.
- Whitney S.M., Houtz R.L. & Alonso H. (2011) Advancing our understanding and capacity to engineer nature's CO<sub>2</sub>-sequestering enzyme, Rubisco. *Plant Physiology* **155**, 27–35.
- Yamori W., Suzuki K., Noguchi K., Nakai M. & Terashima I. (2006) Effects of Rubisco kinetics and Rubisco activation state on the temperature dependence of the photosynthetic rate in spinach leaves from contrasting growth temperatures. *Plant, Cell and Environment* **29**, 1659–1670.
- Zhu X.-G., Long S.P. & Ort D.R. (2008) What is the maximum efficiency with which photosynthesis can convert solar energy into biomass? *Current Opinion in Biotechnology* **19**, 153–159.