Deriving C₄ photosynthesis parameters by fitting intensive A/Cᵢ curves

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ABSTRACT

Measurements of photosynthetic assimilation rate as a function of intercellular CO2 ($A/C_i$ curves) are widely used to estimate photosynthetic parameters for C3 species, yet few parameters have been reported for C4 plants, because of a lack of estimation methods. Here, we extend the framework of widely-used estimation methods for C3 plants to build estimation tools by exclusively fitting intensive $A/C_i$ curves (6-8 more sampling points) for C4 using three versions of photosynthesis models with different assumptions about carbonic anhydrase processes and ATP distribution. We use simulation-analysis, out-of-sample tests, existing in vitro measurements and chlorophyll-fluorescence-measurements to validate the new estimation methods. Of the five/six photosynthetic parameters obtained, sensitivity analyses show that maximal-Rubisco-carboxylation-rate, electron-transport-rate, maximal-PEP-carboxylation-rate and carbonic-anhydrase were robust to variation in the input parameters, while day-respiration and mesophyll-conductance varied. Our method provides a way to estimate carbonic anhydrase activity, a new parameter, from $A/C_i$ curves, yet also shows that models that do not explicitly consider carbonic anhydrase yield approximate results. The two photosynthesis models, differing in whether ATP could freely transport between RuBP and PEP regeneration processes yielded consistent results under high light, but they may diverge under low light intensities. Modeling results show selection for Rubisco of low specificity and high catalytic rate, low leakage of bundle sheath and high PEPC affinity, which may further increase C4 efficiency.

Kew words: $A/C_i$ curves, C4, estimation method, nonlinear curve fitting, photosynthesis parameters, $V_{cmax}$, electron transport, PEP carboxylation rate, carbonic anhydrase
Abbreviations: $a$, light absorptance of leaf; $A_c$, Rubisco carboxylation assimilation rate; $AEE$, RuBP carboxylation and PEPc carboxylation limitation assimilation; $AET$, RuBP regeneration and PEP regeneration limitation assimilation; $A_g$, gross CO$_2$ assimilation rate per unit leaf area; $A_j$, RuBP regeneration assimilation rate; $A_n$, net CO$_2$ assimilation rate per unit leaf area; $ATE$, RuBP carboxylation and PEPc regeneration limitation assimilation; $ATT$, RuBP regeneration and PEPc regeneration limitation assimilation; $\alpha$, the fraction of O$_2$ evolution occurring in the bundle sheath; $c$, scaling constant for temperature dependence for parameters; $CaL$, Lower boundary CO$_2$ under which assimilation is limited by RuBP carboxylation and PEPc carboxylation; $CaH$, Higher boundary CO$_2$ above which assimilation is limited by RuBP regeneration and PEPc regeneration; $C_{bs}$, bundle sheath CO$_2$ concentration; $C_i$, intercellular CO$_2$ concentration; $C_m$, mesophyll CO$_2$ concentration; $\Delta H_a$, energy of activation for temperature dependence for parameters; $\Delta H_d$, energy of deactivation for temperature dependence for parameters; $\Delta S$, entropy for temperature dependence for parameters; $\phi_{PSII}$, quantum yield; $\gamma^*$ (25), the specificity of Rubisco at 25°C; $g_{bs}$, bundle sheath conductance for CO$_2$; $g_{bso}$, bundle sheath conductance for O$_2$; $g_m$, mesophyll conductance for CO$_2$; $I$, light intensity; $J_{max}(25)$, maximum rate of electron transport at 25°C; $K_c(25)$, Michaelis-Menten constant of Rubisco activity for CO$_2$ at 25°C; $K_o(25)$, Michaelis-Menten constants of Rubisco activity for O$_2$; $K_p(25)$, Michaelis-Menten constants of PEP carboxylation for CO$_2$; $O_{bs}$, O$_2$ concentration in the bundle sheath cells; $Q_{10}$ for $K_p$, temperature sensitivity parameter for $K_p$; $R$, the molar gas constant; $R_d$, daytime respiration; $R_{dbs}$, daytime respiration in bundle sheath cells; $R_{dm}$, daytime respiration in mesophyll cells; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate; $T_k$, leaf absolute temperature; $V_c$, velocity of Rubisco.
carboxylation; $V_{c\text{max}}(25)$, maximal velocity of Rubisco carboxylation at 25°C; $V_p$, PEP carboxylation; $V_{pc}$, PEPc reaction rate; $V_{p\text{max}}(25)$, maximal PEP carboxylation rate at 25°C; $V_{pr}$, PEP regeneration rate; $x$, the maximal ratio of total electron transport could be used for PEP carboxylation.
1. INTRODUCTION

Key photosynthetic parameters allow for the assessment of how biochemical and biophysical components of photosynthesis affect net carbon assimilation in response to environmental changes, phenotypic/genotypic differences, and genetic modification. The changes in net assimilation \( A_n \) that occur along with the changes of intercellular CO2 concentration \( C_i \) —or \( A/C_i \) curves— are widely used to estimate photosynthetic parameters for C3 species. In particular, the method by Sharkey et al. (2007), based on the C3 photosynthesis model of Farquhar et al. (1980; FvCB model), has been one of the most widely used tools since it is based exclusively on \( A/C_i \) curves, which are easy to measure in both lab and field conditions.

Fewer estimates of photosynthetic parameters have been reported for C4 species, as there has been a lack of accessible C4 estimation methods. Several recent studies, however, used \( A/C_i \) curves to estimate photosynthesis parameters based on the C4 photosynthesis model of von Caemmerer (2000) (Ubierna et al., 2013; Bellasio et al., 2015). These studies use partial \( A/C_i \) curves; measuring assimilation rates for only a few CO2 concentrations coupled with ancillary measurements of chlorophyll fluorescence and/or 2% O2. While these estimation methods lead to estimates of photosynthetic parameters, the additional measurements they require make estimation more cumbersome for field work or large-scale sampling.

Theoretically, it is possible to estimate photosynthetic parameters by exclusively fitting \( A/C_i \) curves to a C4 photosynthesis model. In this paper, we propose the method to estimate C4 photosynthesis parameters using only \( A/C_i \) curves.
There are several potential problems with $A/C_i$-based estimation methods for $C_3$ plants that carry over to existing $C_4$ methods (Gu et al. 2010); it is therefore important to develop a $C_4$ estimation method with improvements to solve the general problems and drawbacks outlined below. First, the structure of the FvCB model makes it easy to be over-parameterized. Second, a general shortcoming for the estimation methods is that they require an artificial assignment of the RuBP regeneration and Rubisco carboxylation limitation states to parts of the $A/C_i$ curves (Xu and Baldocchi, 2003; Ethier et al., 2006; Ubierna et al., 2013; Bellasio et al., 2015), which has turned out to be problematic (Type I methods) (Gu et al. 2010). These methods assume constant transition points of limitation states for different species. Furthermore, Type I methods tend to minimize separate cost functions of different limitation states instead of minimizing a joint cost function. Some recent estimation methods for $C_3$ species ameliorate these problems by allowing the limitation states to vary at each iterative step of minimizing the cost function (Type II methods; Dubois et al., 2007; Miao et al., 2009; Yin et al., 2009; Gu et al., 2010). However, for these type II methods, additional degrees of freedom in these “auto-identifying” strategies can lead to over-parameterization if limitation states are allowed to change freely for all data points. Gu et al. (2010) also pointed out that existing Type I and Type II methods fail to check for inadmissible fits, which happen when estimated parameters lead to an inconsistent identification of limitation states from the formerly assigned limitation states. More specifically to $C_4$, the recently developed $C_4$ estimation methods artificially assign limitation states for $A/C_i$ curves (Ubierna et al., 2013; Bellasio et al., 2015) and also did not check for inadmissible fits.

We developed methods to estimate photosynthetic parameters for $C_4$ species based solely on
fitting intensive $A/C_i$ curves to a $C_4$ photosynthesis model (von Caemmerer, 2000). The intensive $A/C_i$ curves ($A/C_i$ curves with 6-8 more sampling points than the common $A/C_i$ for $C_3$ species) are important for two reasons: First, at low $C_i$, the slope of $A/C_i$ is very steep and the assimilation rate saturates quickly. Second, $C_4$ species have more photosynthetic parameters as the carbon concentrating mechanism adds complexity. Additionally, carbonic anhydrase catalyzes the first reaction step for $C_4$ photosynthesis (Jenkins et al., 1989), and it has been commonly assumed to not limit CO$_2$ uptake in estimation methods and $C_4$ models (von Caemmerer, 2000; Yin et al., 2011b). Recent studies, however, showed evidence of potential limitation by carbonic anhydrase (von Caemmerer et al., 2004; Studer et al., 2014; Boyd et al., 2015; Ubierna et al., 2017).

Therefore, first, we built estimation methods using two different fitting procedures of Sharkey et al. (2007) and Yin et al. (2011b) without considering carbonic anhydrase activity. Then, we add carbonic anhydrase limitation into the estimation method. We can also use this approach to examine how the carbonic-anhydrase-limitation assumption impacts parameter estimation, and whether the modeling of $C_4$ photosynthesis can be simplified by omitting it. All together, our method estimates five to six photosynthesis parameters: (1) maximum carboxylation rate allowed by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) ($V_{c_{\text{max}}}$), (2) rate of photosynthetic electron transport ($J$), (3) day respiration ($R_d$), (4) maximal PEP carboxylation rate ($V_{p_{\text{max}}}$), (5) mesophyll conductance ($g_m$), and optionally (6) the rate constant for carbonic anhydrase hydration activity ($k_{CA}$). These approaches yield the following improvements to eliminate common problems occurring in the previous $C_3$ and $C_4$ estimation methods: avoiding over-parameterization, maximizing joint cost function, freely
determining transition points instead of assigning in advance, and checking for inadmissible fits. Second, since both RuBP regeneration and PEP regeneration need ATP (Hatch, 1987), we also examine two different assumptions about ATP distribution between RuBP regeneration and PEP regeneration in C4 photosynthesis models. Third, we validate the estimation methods in four independent ways, using: (i) simulation tests using A/C_i curves generated using our model with known parameters and adding random errors, (ii) out of sample test, (iii) existing in vitro measurements and (iv) Chlorophyll fluorescence measurement. Finally, we used the C4 photosynthesis model to perform sensitivity analyses and simulation analyses for important physiological input parameters. These analyses allow us to illustrate the underlying physiological significance of these parameters to the ecology and evolution of the C4 photosynthesis pathway.

2. MATERIALS and METHODS

2.1 C4 Mechanism

The CO2 concentrating mechanism of C4 pathway increases CO2 in the bundle sheath cells to eliminate photorespiration. Like the C3 pathway, the diffusion of CO2 starts from the ambient atmosphere through stomata into intercellular spaces, and then into the mesophyll cells. In the mesophyll cells, the first step is the hydration of CO2 into HCO_3^- by carbonic anhydrase. PEPC, then, catalyze HCO_3^- and PEP into C4 acids and the C4 acids are transported to the bundle sheath cells. In the bundle sheath cell, C4 acids are decarboxylated to create a high CO2 environment for the C3 photosynthetic cycle, and PEP is regenerated. All the modeling equations and mechanistic processes used for our estimation method are from von Caemmerer (2000), Hatch and Burnell (1990), Boyd et al. (2015) and Ubierna et
Given the two limitation states of C₄ cycle (PEP carboxylation ($V_{pc}$) and PEP Regeneration ($V_{pr}$)), and two limitation states of C₃ cycle (RuBP carboxylation ($A_c$) and RuBP Regeneration ($A_j$)) in the C₄ photosynthesis model, there are four combinations of limitation states (as Yin et al., 2011b, Fig. 1): RuBP carboxylation and PEP carboxylation limited assimilation (AEE), RuBP carboxylation and PEP regeneration limited assimilation (ATE), RuBP regeneration and PEP carboxylation limited assimilation (AET) and RuBP regeneration and PEP regeneration limited assimilation (ATT). Since the C₄ cycle operates before the C₃ cycle and provides substrates for the C₃ cycle, the determination process of $A_n$ is as follows:

\[
\text{If } (V_{pc} < V_{pr}), \ A_c = AEE, \ A_j = AET, \ other\text{wise } A_c = AT E, \ A_j = ATT
\]

\[
A_n = \min (A_c, A_j), \tag{2}
\]

which we used for our estimation method.

### 2.2 Plant Material

We performed intensive $A/C_i$ curves on nine different C₄ species to develop and examine the efficacy of our estimation tools: *Zea mays* L., *Eragrostis trichodes* (Nutt.) Alph. Wood, *Andropogon virginicus* L., *Schizachyrium scoparium* (Michx.) Nash, *Panicum virgatum* L., *Panicum amarum* Elliott, *Setaria faberi* Herrm., *Sorghastrum nutans* (L.) Nash and *Tripsacum dactyloides* (L.) L. The intensive $A/C_i$ curves contain more sample points under more CO₂ concentrations than the default curve used for C₃ species. Here we set the CO₂ concentrations as 400, 200, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325,
350, 400, 500, 600, 700, 800, 1000, 1200, 1400 ppm under light intensity of 1500 μmolm^{-2}s^{-1} (light intensity encountered by the plants in greenhouse). At each point, data was recorded when the intercellular CO2 concentration equilibrated within 2-5 minutes. The datasets were obtained using a standard 2 x 3 cm² leaf chamber with a red/blue LED light source of LI-6400 (LI-COR Inc., Lincoln, NE, USA). If the stomatal conductance of a species does not decrease quickly at high CO2, then the sample points at the high CO2 level can be increased. Fluorescence was measured along with A/C_i curves for seven C_4 species (CO2 concentration is similar with above). After each change of CO2 concentration and A reached steady state, the quantum yield was measured by multiphase flash using a 2 cm² fluorescence chamber head (Bellasio et al., 2014). All the measurements are conducted at 25°C and VPD is controlled at 1-1.7kPa. The cuvette was covered by Fun-Tak to avoid and correct for the leakiness (Chi et al., 2013).

2.3 Estimation Protocol

We implemented the estimation methods using the non-linear curve-fitting routine in MS Excel (Supplementary Material I, II, III) and independently in R (“C4Estimation”) to get solutions that minimize the squared difference between observed and predicted assimilation rates (A). Five (or six when considering carbonic anhydrase) parameters will be estimated by fitting the A/C_i curve: V_{cmax}, J, R_d, V_{pmax}, g_m, and k_{CA}. Other input parameters for C_4 are in Table S1.

**Input data sets and preliminary calculations.** The input data sets are the leaf temperature during measurements, atmosphere pressure, two CO2 bounds (CaL and CaH discussed in the
following section), and the assimilation rates ($A$) and the $C_i$s (in ppm) in the $A/C_i$ curve.

Also, reasonable initial values of output parameters need to be given in the output section to initiate the non-linear curve fitting (Supplementary Material IV). $C_i$ will be adjusted from the unit of ppm to the unit of Pa inside the program as suggested by Sharkey et al. (2007).

**Estimating limitation states.** We set upper and lower limits to the value of $C_i$ between which the assimilation rates are freely determined by limitation states. Also, we can avoid over-parameterization by pre-assigning limitation states at the lower and upper ends of the $C_i$ range. We assumed that under very low $C_i$ ($CaL$), CO$_2$ is the limiting substrate; thus, $V_p$ is limited by $V_{pc}$ and $A$ is given by $A_c$ (AEE); under very high $C_i$ ($CaH$) electron transport is limiting, thus, $V_p$ is limited by $V_{pr}$ and $A$ is given by $A_j$ (ATT) (Fig. 1). The points between $CaL$ to $CaH$ are freely determined by AEE, ATE, AET or ATT from eq. (16) and (17) to minimize the cost function. We suggest setting $CaL$ as 10 Pa initially, then adjusting based on the preliminary results. The points of constant $A$ at high $C_i$ end can initially be set as being limited by ATT primarily (based on the three points, we can $CaH$) or use 65 Pa as the first trial. The range of freely determined points can be adjusted by users by setting appropriate $CaL$ and $CaH$. In the column of “Estimate Limitation”, whether the data points are limited by AEE (represented by ”1”), ATT (represented by ”4”) or freely vary (represented by ”0”), all the assignments of “1”, “4” and “0” are determined automatically by the given values of $CaL$ and $CaH$. One can input “-1” to disregard a data pair. Users can adjust limitation states according to how many points and the range of $C_i$ they have in their $A/C_i$ curves.
We assume different processes in the C₄ photosynthesis are coordinated with each other and co-limit the assimilation rate (Sharkey et al., 2007; Yin et al., 2011b; Ubierna et al., 2013; Bellasio et al., 2015). Thus, the estimation parameters allow the limitation states to be compactly clustered with each other (Fig. 1). However, if there were only a few points under CaL, the estimation results will depend heavily on the given initial values and unbalanced results would be more likely. Fig. S1 shows an example of unbalanced estimation results by deleting some points under 10 Pa or setting a very low CaL: in the estimation results, Aᵣ is limited by AEE at very low Cᵢ and is mostly limited by Aᵣ (shown by AET and ATT) in the C₃ cycle. In this case, Aᵣ (shown by AEE and ATE) has a clear redundancy at higher Cᵢ. Unbalanced results happened when there are not enough constraints points under CaL or above CaH. Such results explain why intensive A/Cᵢ curves are preferred, especially more measuring points under the lower end and higher end of Cᵢ. However, existing A/Cᵢ data with 14 points might be used in the current estimation method if there are at least four points below CaL and three points above CaH.
Fig. 1 An introduction of how our estimation methods assign transition points between limitation states. AEE represents RuBP carboxylation, and PEP carboxylation limited assimilation rate, ATT represents RuBP regeneration and PEP regeneration limited assimilation rate. Transition states indicate assimilation could be limited by AEE, ATT, ATE (RuBP carboxylation and PEP regeneration) and AET (RuBP regeneration and PEP carboxylation). Our algorithm allows the transition states to be freely limited by the above four conditions from a lower bound (CaL, 10 Pa for instance) and a higher bound (CaH, 65 Pa for example), indicated by the dashed vertical lines in the figure.

Estimation algorithm and fitting procedures. The objective of our estimation methods is to minimize the following joint cost function (eq. 3 and 4) by varying the above five or six output parameters ($V_{cmax}$, $J$, $R_d$, $V_{pmax}$, $g_m$, and $k_{CA}$):

\[
f = \sum_{i=1}^{n} (A_i - A_{mi})^2.\tag{3}
\]

\[
A_i = [If(C_i \leq CaL), AEE; If(C_i \geq CaH), ATT; IF(CaL \leq C_i \leq CaH), \min(A_{ci}, A_{ji})] \tag{4}
\]

$n$ is the total number of observations, $A_{ci}$ is determined by AEE and ATE and $A_{ji}$ is determined by AET and ATT from eq. (1). $A_{mi}$ is the observed net assimilation rate. In this calculation, we take Michaelis-Menten constant of Rubisco activity for CO$_2$ ($K_c$), Michaelis-Menten constant of Rubisco activity for O$_2$ ($K_o$), the specificity of Rubisco ($\gamma^*$), Michaelis-Menten constants of PEP carboxylation for CO$_2$ or HCO$_3^-$ ($K_p$), the fraction of O$_2$ evolution occurring in the bundle sheath ($\alpha$) and bundle sheath conductance ($g_{bs}$) as given (input parameters), similar to Sharkey et al. (2007). We conduct further sensitivity analyses in the following section to determine the effects of variability of these inputs parameters on the estimation results.
We used two fitting procedures in the current study: one was from Sharkey et al. (2007), which is an implicit minimization of error (Supplementary Material I, III), and the other one was based on the explicit calculations given by Yin et al. (2011b) (Supplementary Material II). For the method of Sharkey et al. (2007), "estimated" $A_n$ was calculated using the above equations and observed $A_n$ values. We call them "estimated", because when we calculate $A_n$, observed $A_n$ is used to calculate intermediate parameters, for example, the CO$_2$ concentration in mesophyll cells ($C_m$), the CO$_2$ concentration in bundle sheath ($C_{bs}$), which we then use to calculate $A_c$ and $A_j$. The objective function is to minimize the sum of square errors between "estimated" $A_n$ and observed $A_n$ (Simulation Error in Supplementary Material I, III). For the model without carbonic anhydrase, Yin et al. (2011b) gave explicit solutions for AEE, ATE, AEE, and ATT). “Explicit” here means the assimilation rates are totally calculated by the estimated parameters without calculating the intermediates with observed $A_n$. These calculations give us the real estimation error of our fitting procedure for models without carbonic anhydrase and thus provide a validation for the goodness of fit (“True Error” in Supplementary Material I-III).

**Checking inadmissible fits.** We made it possible to check the inadmissible fits for limitation states in our estimation method. After the estimation process finishes, the limitation states based on the estimated parameters will be calculated in the last column. If the calculated limitation states are inconsistent with the assigned ones in the estimation method, one needs to readjust the assignment of the “Estimate Limitation” (adjust $CaL$ or $CaH$) and rerun the estimation method, until they are consistent with each other.
3. RESULTS

3.1 Estimation results and assumptions

Estimation methods based on assumptions with and without carbonic anhydrase yield similar results (Supplementary material V). In Supplementary material III, carbonic anhydrase indeed shows limitation to $V_{pc}$, which confirms its potential role as a limiting step in the C₄ cycle. However, $V_{pc}$ calculated from CO₂ are only a little higher than $V_{pc}$ calculated from HCO₃⁻, which resulted in the similar estimation results. In addition, the estimation errors and true errors from Yin’s equations are quite small (average<1), and also similar between models with and without carbonic anhydrase.

Estimation methods based on the two equations of different assumptions about electron transport between RuBP regeneration and PEP regeneration yield consistent parameter estimates and assimilation- CO₂ response curves (Fig. 2), but there were minor differences. The second assumption that ATP, resulting from electron transport, is freely allocated between PEP carboxylation-regeneration and RuBP regeneration leads to a bump at low CO₂ when estimating ATE. The two assumptions produce different ATE under low CO₂; but this is largely inconsequential because, under low CO₂, assimilation is usually limited by AEE.
Assimilation-CO₂ response curves (A/C_i) generated using C₄ photosynthesis of two different assumptions about electron transport. Photosynthetic parameters (V̇_c,max, J, R_d, V̇_p,max, and g_m) are the same for both assumptions. AET_e’ Assumption1 and ATT_e’ Assumption1 represent results of the assumption that no matter how much electron transport is used for PEP carboxylation/regeneration, a certain amount (xJ) is confined for this use. AET_e’ Assumption2 and ATT_e’ Assumption2 represent results of the assumption that electron transport can be freely distributed between PEP carboxylation/regeneration and RuBP regeneration. Parameters are estimated from A/C_i curve of T. dactyloides under the light intensity of 1500 μmol m⁻² s⁻¹. AEE and ATE are the same for both assumptions.

3.2 Sensitivity analysis

The parameters K_c, K_o, γ, K_p, α, and g_ba can vary among species in nature (Cousins et al., 2010) and it is therefore important to know how sensitive our results are to variation in these parameters. We conducted a sensitivity analysis for variation in these parameters on the
estimated $V_{cmax}$, $J$, $R_d$, $V_{pmax}$, $g_m$ and $k_{CA}$ (Fig. 3). This analysis shows all the estimated
parameters are robust under the variation of $\alpha$ (Fig. 3A) and showed little variation
responding to the change of $\gamma^c$ (Fig. 3E) and $K_o$ (Fig. 3C); however, the estimated
parameters are dependent on the other input parameters to different extents (Fig. 3B, D, F).
We calculate the average percentage change of estimated parameters along with the 50 %
decrease and 100 % increase of the input parameters. $V_{cmax}$ showed some medium extent of
sensitivity for $K_c$, $K_p$, and $g_{bs}$ with the average percentage change of 23.11, 7.54 and 17.69 %
respectively. $J$ is robust in the variations of $K_c$ and $g_{bs}$ (the average change is less than 2%)
and with a medium 6.96 % change for $K_p$. $k_{CA}$ is robust in the variations of $K_c$, $K_p$, and $g_{bs}$
(average change less than 5%). $V_{pmax}$ is sensitive for $K_p$ with the average change of 27.34%,
moderately sensitive to the change of $g_{bs}$ with 4.01 % and 13.38% change and is robust for
$K_c$, $R_d$ is sensitive to $K_c$, $K_p$, and $g_{bs}$ with the change of 6.73, 43.88 and 13.38%. $g_m$ is
strongly sensitive to $K_c$, $K_p$, and $g_{bs}$ with the average percentage changes of 22.95, 107.04
and 23.19 %. This results suggest that $V_{cmax}$, $J$, $V_{pmax}$, and $k_{CA}$ estimated using our method
are relatively robust.
Fig. 3 Sensitivity analysis of six estimation parameters to the variation in six input parameters using the model with carbonic anhydrase. Relative changes in the estimated $V_{cmax}$, $J$, $R_d$, $g_m$, $V_{pmax}$, and $k_{CA}$.
in response to the relative change of six input parameters [(A) $\alpha$, (B) $K_p$, (C) $K_o$, (D) $K_c$, (E) $\gamma^*$ and (F) $g_{bs}$] from the initial values in Table S1. The relative change of estimated parameters refers to the ratio of estimated values at a changed input parameter to the estimated value at the initial value of that input parameter. The symbols represent the average change of the nine C$_4$ species and error bars represent standard error.

3.3 Physiological significance for assimilation rate of the input parameters

In addition to the sensitivity analysis, we performed a simulation analysis to illustrate the physiological importance of input parameters further, and to indicate further the importance of physiological properties in maintaining the efficiency of C$_4$ photosynthesis pathway. We chose the estimation parameter set of $T$. dactyloides as an example, held photosynthetic parameters constant $V_{cmax}$ (28 $\mu$mol m$^{-2}$ s$^{-1}$), $J$ (134 $\mu$mol m$^{-2}$ s$^{-1}$), $R_d$ (0.78 $\mu$mol m$^{-2}$ s$^{-1}$), $g_m$ (30.00 $\mu$mol m$^{-2}$ s$^{-1}$ Pa$^{-1}$) and $V_{pmax}$ (41.91 $\mu$mol m$^{-2}$ s$^{-1}$), while changing the values of $\alpha$, $\gamma^*$, $g_{bs}$, and $K_p$ (as half or twice of the original parameters) to see their effects on the assimilation rate, $C_{bs}$ and the O$_2$ concentration in bundle sheath ($O_{bs}$) (Fig. 4, Table 1). Using photosynthetic parameter sets of other species to perform the simulation analysis yielded similar results (data not shown). The change of $\alpha$ did not lead to changes in assimilation rate (Fig. 4A) and led to small changes in $O_{bs}$ (Table 1). The decrease of $\gamma^*$ to half of the current value led to a small change of $C_{bs}$ and assimilation rate (less than 0.5 $\mu$mol m$^{-2}$ s$^{-1}$) while doubling $\gamma^*$ led to a larger, but still not significant, change (less than 1 $\mu$mol m$^{-2}$ s$^{-1}$) (Fig. 4B, Table 1). Importantly, the changes of assimilation rates were less than 0.3 $\mu$mol m$^{-2}$ s$^{-1}$ when $C_i$ was less than 20 Pa, which is the regular range of $C_i$ under current ambient CO$_2$. However, the change of $g_{bs}$ significantly changed the assimilation rate.
and \(C_{bs}\) (Fig. 4C, Table 1). The change of \(K_p\) significantly affected the assimilation rate and \(C_{bs}\) to a large degree under low \(C_i\) (Fig. 4D, Table 1).

**Table 1** The average change of percentage of CO\(_2\) concentration (\(C_{bs}\)) and O\(_2\) concentration at bundle sheath (\(O_{bs}\)) compared to the reference value of \(\alpha_0, \gamma^*0, g_{bs0}\) and \(K_p\). Simulation results are obtained by using the original parameter set of *T. dactyloides* with \(V_{cmax} = 28 \ \mu\text{mol m}^{-2} \text{s}^{-1}, J = 134 \ \mu\text{mol m}^{-2} \text{s}^{-1}, R_d = 0.78 \ \mu\text{mol m}^{-2} \text{s}^{-1}, g_m = 30.00 \ \mu\text{mol m}^{-2} \text{s}^{-1} \ \text{Pa}^{-1}\) and \(V_{pmax} = 41.91 \ \mu\text{mol m}^{-2} \text{s}^{-1}\). The values represent average change of percentage of 21 values from 0-120 Pa of intercellular CO\(_2\) (\(C_i\)) (data show mean (standard error)).

| Parameters          | \(\alpha = 0\) | \(\alpha = 2 \alpha 0\) | \(\gamma^* = 0.5 \gamma^*0\) | \(\gamma^* = 2 \gamma^*0\) |
|---------------------|-----------------|-----------------|-----------------|-----------------|
| Chang of \(C_{bs}\) (%) | -0.91(0.06)     | 0.97(0.06)     | -2.96(0.28)     | 5.05(0.49)      |
| Chang of \(O_{bs}\) (%) | -6.07(0.30)     | 6.01(0.30)     | 0.07(0.01)      | -0.21(0.02)      |

| Parameters          | \(g_{bs} = 0.5 g_{bs0}\) | \(g_{bs} = 2 g_{bs0}\) | \(K_p = 0.5 K_p0\) | \(K_p = 2 K_p0\) |
|---------------------|--------------------------|--------------------------|-------------------|-------------------|
| Chang of \(C_{bs}\) (%) | 56.99(3.03)               | -29.48(0.41)             | 43.12(10.75)      | -36.57(4.07)      |
| Chang of \(O_{bs}\) (%) | 6.77(0.29)               | -3.41(0.16)              | 0.91(0.18)        | -1.18(0.14)       |
Fig. 4 Simulation results of assimilation rate along with different intercellular CO₂ concentration (Cᵢ) with the known photosynthetic parameters, but with the change of (A) α, (B) γ*, (C) gₘ and (D) Kₚ.

The original data set are $V_{\text{cmax}} = 28 \, \mu\text{mol m}^{-2} \text{s}^{-1}$, $J = 134 \, \mu\text{mol m}^{-2} \text{s}^{-1}$, $R_d = 0.78 \, \mu\text{mol m}^{-2} \text{s}^{-1}$, $g_m = 30.00 \, \mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$ and $V_{\text{pmax}} = 41.91 \, \mu\text{mol m}^{-2} \text{s}^{-1}$. The reference value of changing parameters at 25°C: $\alpha(25) = 0.15$, $\gamma^*(25) = 0.000244$, $g_m(25) = 0.0295$ and $K_p(25) = 8.55 \, \text{Pa}$.

3.4 Validating the estimation methods

In order to test our estimation methods, we first conducted a simulation test with manipulated error terms. We use the estimated results of the nine species as known parameters (the known values in Fig. 5) to generate new datasets using the C₄
photosynthesis equations based the first assumption of electron transport and adding error terms to the assimilation rates. The error terms were randomly drawn from a normal distribution of mean zero and standard deviation of 0.1 or 0.2 in an effort to simulate the inevitable random errors in the real measurements. Estimating simulated data sets gave us an idea about how likely we can capture the real parameters of the species given unavoidable errors in measurements. The results show that robust estimation results for $V_{cmax}$, $J$, $V_{pmax}$, and $R_d$ can be obtained (Fig. 5A, B, C, D). However, some estimation results of $g_m$ and $k_{CA}$ show some deviation from the real values (Fig. 5E, F).

To test whether our estimation method could give accurate predictions across typical prediction scenarios, (CO$_2$ ranging from 20 Pa to 60 Pa), we performed out of sample tests for our nine target species. To perform these tests, we removed five points of CO$_2$ concentrations between 20 and 60 Pa range out of the A/C$_i$ curves and used the rest of the A/C$_i$ curves to estimate parameters. And then we used these parameters to predict the assimilation rate under the CO$_2$ concentrations we took out before and calculated the estimation errors. In general, the estimation errors for all our species were small (Table 2).

### Table 2
Out of sample test results. Five measured points from 20 Pa-60 Pa were taken out when we conducted the estimation process. Then the calculated assimilation rates under these five CO$_2$ concentrations were compared with the measured ones. The data shows estimated error between the calculated and measured assimilation rates (data show mean (standard error)).

| Species          | A. virginicus | Z. mays   | E. trichodes | P. virgatum | P. amarum |
|------------------|---------------|-----------|--------------|-------------|-----------|
| Model without CA | 0.069(0.036)  | 0.150(0.056) | 0.035 (0.017) | 0.193(0.063) | 0.055(0.034) |
| Model with CA    | 0.066(0.043)  | 0.154(0.057) | 0.111 (0.058) | 0.195 (0.061) | 0.054(0.033) |
| Species       | S. scoparium | S. faberi | S. nutans | T. dactyloides |
|---------------|--------------|-----------|-----------|---------------|
| Model without CA | 0.023(0.010) | 0.114(0.055) | 0.258(0.080) | 0.199(0.090) |
| Model with CA  | 0.105(0.034) | 0.068(0.040) | 0.263(0.133) | 0.200(0.090) |
Fig. 5 Simulation tests for the estimated parameters ((A) $V_{cmax}$, (B) $J$, (C) $V_{pmax}$, (D) $R_d$, (E) $g_m$ and (F) $k_{CA}$) using estimation methods with and without carbonic anhydrase reaction (With CA and Without CA). Datasets are generated by adding random errors for the modeling results using the known photosynthesis parameters of nine species. These known photosynthesis parameters are the true values in the x-axis and are used to compare with the newly estimation parameters. The small error refers to error term randomly chosen with mean 0 and standard deviation of 0.1 and the bigger error refers to error term with randomly chosen mean 0 and standard deviation of 0.2.

We tried to compare our estimation methods with in vitro measurements or other estimation methods using isotopic analysis, especially for Zea. Our estimation results for Zea obtained similar $V_{cmax}$ with the in vitro estimated Rubisco activity of Pinto et al. (2014); however, the estimated value for $V_{pmax}$ is a little lower than the in vitro PEPC activity measurement with a difference of around 10 μmol m$^{-2}$ s$^{-1}$. For species of the Panicum family with NAD-ME subtype, $P$. virgatum and $P$. amarum in the current study and $P$. coloratum in Pinto et al. (2014), the estimated $V_{cmax}$ and $V_{pmax}$ are quite consistent with the in vitro measurements. Ubierna et al. (2017) reported the $g_m$ for Zea ranged from 1.69 ± 0.17 to 8.19 ± 0.80 μmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ using $^{18}$O and in vitro $V_{pmax}$. Our estimation method fitted a $g_m$ for Zea of 7.34 μmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$, which falls into the range of their measurements. Barbour et al. (2016) reported a little lower mesophyll conductance for Zea using $^{18}$O measurements.

3.5 Validating transition point range

We used chlorophyll fluorescence measurements from seven C$_4$ species to test whether the upper and lower boundary CO$_2$ concentrations, $CaL$ and $CaH$, are reasonable (Table 3). The apparent quantum efficiency of PSII electron transport was calculated with $\Delta F/F_{m'} = (F_{m'} -$
Fluorescence analysis (Baker et al. 2007) is a powerful tool for identifying the limitation states of C₃ species (Sharkey et al. 2007). If Chlorophyll fluorescence is increasing with increasing CO₂, Aₙ is limited by Rubisco carboxylation limited; when Chlorophyll fluorescence stays constant with increasing CO₂, Aₙ is limited by RuBP regeneration. For C₄ species, however, the situation is more complicated. Since Vₚ could be limited by Vₚᵣ and Vₚₛ (eq. (9)). Part of the RuBP carboxylation limited condition and RuBP regeneration limited condition for the C₃ cycle will mix together, leading to a linear increase of fluorescence with increasing of CO₂, but of a small slope (Fig. S2). Thus, we can only obtain two boundaries of CO₂ concentrations. Below the lower boundary, A and fluorescence increases with increasing Cᵢ with a steep slope and A is RuBP carboxylation and PEP carboxylation limited (AEE); above the higher boundary, A and fluorescence is relatively constant along with the increase of Cᵢ and A is RuBP regeneration and PEP regeneration limited (ATT). We measured fluorescence to test whether the upper and lower boundary CO₂ concentrations, Caₐ and Caₜ, are reasonable. It seems all the Caₐ are above 14 Pa and all the Caₜ are below 65 Pa (Table 3). These results suggest that 10Pa-65Pa is a reasonable range for the transitional point.

**Table 3** CO₂ concentration boundaries result for assimilation-limited conditions from fluorescence measurements for seven species. Low: CO₂ concentration under which assimilation rate increases greatly with increasing CO₂ (potentially assimilation is limited by PEP carboxylation and RuBP carboxylation). High: CO₂ concentration above which assimilation rate no longer increases with increasing CO₂ (potentially assimilation is limited by PEP regeneration and RuBP regeneration). Data show the mean (standard error).
The photosynthetic parameters from the estimation method are good indicators for the biochemical and biophysical mechanisms underlying the photosynthesis processes of plants. Together with photosynthesis models, they can provide powerful information for evolutionary and ecological questions in both physiological and ecosystem response to natural environmental variation and climate change, to illustrate evolutionary trajectory of C4 pathway, as well as in efforts to improve crop productivity (Osborne & Beerling, 2006; Osborne & Sack, 2012; Heckmann et al., 2013). Photosynthetic parameters represent different physiological traits, and comparison of these parameters within a phylogenetic background could help us to understand the further divergence of lineages and species through evolutionary time. Additionally, the response of productivity and carbon cycle of vegetation towards the future climate change depends heavily on photosynthesis parameter estimation as input parameters.

4. DISCUSSION

Each of the two different fitting procedures has advantages and disadvantages. Yin’s method (Supplementary material II) uses the explicit calculation of assimilation rate and consequently gives lower estimation error. However, it needs a more accurate assignment of limitation states, especially at the lower end. Thus, Yin’s method will be preferable if one
has additional support (e.g. fluorescence measurement) to define the limitation states; otherwise, the Yin’s method may give unbalanced results (Fig. 3). However, Sharkey’s method (Supplementary material I) usually can avoid unbalanced results even without ancillary measurements. Thus, it is better to use both procedures to support each other to find more accurate results. For example, one can first use Sharkey’s method to get estimation results and limitation states, and then use them as initial values for Yin’s method.

Our estimation methods yielded similar results when using models with and without carbonic anhydrase reaction processes. Although carbonic anhydrase activity may well be a limiting step for C4 cycle (von Caemmerer et al., 2004; Studer et al., 2014; Boyd et al., 2015; Ubierna et al., 2017), its limitation did not greatly affect assimilation rates in this study. Including the carbonic anhydrase reaction makes the model more complex and difficult to get an explicit solution; therefore, the model without carbonic anhydrase could be used as a simplified form yielding flawed but ‘nearly correct’ predicted values as a part of larger models. However, carbonic anhydrase limitation of C4 photosynthesis needs the further assessment from physiological or biochemical perspectives, and our estimation method provides another way to derive carbonic anhydrase parameters, which were comparable with in vitro measurements (Boyd et al., 2015). In addition, our results for models with and without carbonic anhydrase activity support the proposition of Cousins et al. (2007) that carbonic anhydrase activity may not be a limiting factor for A/Ci curves of C4 plants.

Our results show that despite a clear difference between the assumptions of how the
products of electron transport are distributed, the results were similar and comparable with studies using different models under measurements of high light intensity. The bump in the second model happens in AET. In AET, assimilation is limited by RuBP regeneration and PEP carboxylation; therefore, PEP regeneration is not reaching $V_{pr}$, and the extra electron transport in PEP regeneration could be freely assigned to RuBP regeneration. This effect will weaken as PEP carboxylation increases. However, under lower photosynthetic photon flux density, assimilation rate will be limited more by electron transport, and the separate assumptions concerning electron transport may start to show divergent results.

The photosynthetic parameters from the estimation method used together with photosynthesis models can provide information and inspiration about the evolutionary and physiological importance of different aspects of the C₄ syndrome (Osborne & Sack, 2012; Heckmann et al., 2013), which can be investigated by empirical measurements. Several examples emanate from our simulation analysis: (1) $\alpha$ represents the fraction of O₂ evolution from photosynthesis occurring in the bundle sheath cells (eq. (4)) and any $\alpha > 0$ means that O₂ will accumulate in the bundle sheath cells, due to low $g_{bs}$. Both the sensitivity analysis and the simulation analysis showed the change of $\alpha$ did not affect the estimated parameters and assimilation rates, because the high $C_{bs}$ created by C₄ carbon concentrating mechanism overcame any increase of $O_{bs}$ and did not lead to high photorespiration. Thus, the compartmentation of O₂ evolution may not have played an important role in the evolution of C₄ photosynthesis. (2) A lower Rubisco specificity factor ($\gamma^*$;eq. (11)) means lower specificity for O₂, higher specificity for CO₂, and lower photorespiration. In C₃ species, selection for Rubisco with lower specificity to O₂ and high specificity of CO₂ can
increase the carbon gain. However, there is a trade-off between the specificity of Rubisco for CO₂ and its catalytic rate (Savir et al., 2010; Studer et al., 2014). Based on this trade-off, we can hypothesize that since C₄ elevates CO₂ around Rubisco relative to the O₂ concentration, maintaining low specificity might be optimal, in order to get high catalytic rate of the enzyme to reach higher assimilation rate as shown by the empirical measurements of Sage (2002) and Savir et al. (2010). Our simulation analysis showed the increase of specificity for CO₂ (decrease of $\gamma^*$) did not increase the assimilation rate much, which indicates the selection upon Rubisco specificity in C₄ plants should be relaxed. (3) $g_{bs}$ represents CO₂ leakage from bundle sheath to the mesophyll cell, and changes in $g_{bs}$ significantly change the assimilation rate and $C_{bs}$. Therefore, avoiding CO₂ leakage was of great importance for the evolution and efficiency of C₄ photosynthesis pathway (Brown and Byrd, 1993; Ubierna et al., 2013; Kromdijk et al., 2014).

Although we have shown that parameter estimation can be achieved solely with $A/C_i$ curves, it is easy to combine our methods with ancillary measurements to yield more accurate estimation results by defining the parameters as estimated or known or add additional constraints (Supplementary Material IV). Yin et al. (2011b) proposed a method to obtain $R_d$ from the fluorescence-light curve, since the method used for C₃ species, the Laisk method, is inappropriate (Yin et al., 2011a). Additional measurement of dark respiration could be an approximation for $R_d$ or could help to provide a constraint for estimating $R_d$ in our estimating method. Ubierna et al. (2017) discussed the estimation method of $g_m$ using instantaneous carbon isotope discrimination. With external measurement results, one can change estimated parameters (such as $R_d$, $g_m$ and $J$) as input parameters, instead of output.
parameters, in this curve fitting method (Supplementary material IV). Additional methods, such as in vitro measurements (Boyd et al., 2015; Pedomo et al., 2015) and membrane inlet mass spectrometry (Cousins et al., 2010) of $V_{\text{cmax}}$, $V_{\text{pmax}}$, and carbonic anhydrase activity can also provide potential parameter values. Furthermore, if some output parameters are determined in the external measurements, one can also relax the input parameters (such as $g_{\text{bs}}$) and make them estimated parameters (Supplementary material IV).

5. Conclusion

We have developed new, accessible estimation tools for extracting C$_4$ photosynthesis parameters from intensive A/$C_\text{i}$ curves. Our estimation method is based on an established estimation protocol for C$_3$ plants and makes several improvements upon C$_4$ photosynthesis models. External measurements for specific parameters will increase the reliability of estimation methods and are summarized independently. We developed estimation methods with and without carbonic anhydrase activity. The comparison of these two methods allows for an estimation of carbonic anhydrase activity, and further shows that the method that did not consider carbonic anhydrase activity was a sufficient simplification for C$_4$ photosynthesis. We tested two assumptions related to whether the electron transport is freely distributed between RuBP regeneration and PEP regeneration or certain proportions are confined to the two mechanisms. They show similar results under high light, but they may diverge under low light intensities. Simulation test, out of sample test, fluorescence analysis, and sensitivity analysis confirmed that our methods gave robust estimation especially for $V_{\text{cmax}}$, $J$, and $V_{\text{pmax}}$. 
Author contributions

HZ, EA and BH conceived the ideas, designed methodology, analyzed the data and led the writing of the manuscript; HZ collected the data; HZ and BH coordinate the study. All the authors contributed to the critical review of the manuscript and approved its final version.

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REFERENCES

Baker NR, Harbinson J, Kramer DM. 2007. Determining the limitations and regulation of photosynthetic energy transduction in leaves. Plant, Cell and Environment 30, 1107–1125.

Barbour MM, Evans JR, Simonin KA, Von Caemmerer S 2016. Online CO₂ and H₂O oxygen isotope fractionation allows estimation of mesophyll conductance in C₄ plants, and reveals that mesophyll conductance decreases as leaves age in both C₄ and C₃ plants. New Phytologist 210, 875-889.

Bellasio C, Beerling DJ, Griffiths H. 2015. Deriving C₄ photosynthetic parameters from combined gas exchange and chlorophyll fluorescence using an Excel tool: theory and practice. Plant, Cell and Environment doi: 10.1111/pce.12626.

Bellasio C, Burgess SJ, Griffiths H, Hibberd JM. 2014. A high throughput gas exchange screen for determining rates of photorespiration or regulation of C₄ activity. Journal of experimental botany 65, 3769-3779.

Boyd RA, Gandin A, Cousins AB. 2015. Temperature responses of C₄ photosynthesis: biochemical analysis of Rubisco, phosphoenolpyruvate carboxylase, and carbonic anhydrase in Setaria viridis. Plant Physiology 169, 1850–1861.

Brown RH, Byrd GT. 1993. Estimation of bundle sheath cell conductance in C₄ species and O₂ insensitivity of photosynthesis. Plant Physiology 103, 1183-1188.

von Caemmerer S. 2000. Biochemical models of photosynthesis. In Techniques in Plant Sciences p. 196. CSIRO Publishing, Colingwood, Australia.

Von Caemmerer S, Quinn V, Hancock NC, Price GD, Furbank RT, Ludwig M. 2004. Carbonic anhydrase and C₄ photosynthesis: a transgenic analysis. Plant, Cell & Environment 27, 697-703.

Chi Y, Xu M, Shen R, Yang Q, Huang B, Wan S. 2013. Acclimation of foliar respiration and photosynthesis in response to experimental warming in a temperate steppe in northern China. PLoS One, 8(2):e56482.

Cousins AB, Baroli I, Badger MR, Ivakov A, Lea PJ, Leegood RC, Von Caemmerer S. 2007. The Role of Phosphoenolpyruvate Carboxylase during C₄ Photosynthetic Isotope Exchange and Stomatal Conductance. Plant Physiology 145, 1006-1017.

Cousins AB, Ghannoum O, von Caemmerer S, Badger MR. 2010. Simultaneous determination of Rubisco carboxylase and oxygenase kinetic parameters in Triticum aestivum and Zea mays using membrane inlet mass spectrometry. Plant, Cell and Environment 33, 444–452.
Dubois JB, Fiscus EL, Booker FL, Flowers MD, Reid CD. 2007. Optimizing the statistical estimation of the parameters of the Farquhar-von Caemmerer-Berry model of photosynthesis. New Phytologist 176, 402–414.

Ethier GJ, Livingston NJ, Harrison DL, Black TA, Moran JA. 2006 Low stomatal and internal conductance to CO₂ versus Rubisco deactivation as determinants of the photosynthetic decline of ageing evergreen leaves. Plant, Cell and Environment 29, 2168-2184.

Farquhar GD, von Caemmerer S, Berry JA. 1980 A biochemical model of photosynthetic carbon dioxide assimilation in leaves of 3-carbon pathway species. Planta 149, 78-90.

Genty B, Briantais J, Baker N. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta 990, 87–92.

Gu L, Pallardy SG, Tu K, Law BE, Wullschleger SD. 2010. Reliable estimation of biochemical parameters from C₃ leaf photosynthesis-intercellular carbon dioxide response curves. Plant, Cell and Environment 33, 1852-1874.

Hatch MD. 1987. C₄ photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure. Biochimica et Biophysica Acta 895, 81-106.

Hatch MD, Burnell JN. 1990. Carbonic anhydrase activity in leaves and its role in the first step of C₄ photosynthesis. Plant Physiology 93, 825–828.

Heckmann D, Schulze S, Denton A, Gowik U, Westhoff P, Weber AP, Lercher MJ. 2013. Predicting C₄ photosynthesis evolution: Modular, individually adaptive steps on a Mount Fuji fitness landscape. Cell 153, 1579-1588.

Jenkins CLD, Furbank RT, Hatch MD. 1989. Mechanism of C₄ photosynthesis. A model describing the inorganic carbon pool in bundle-sheath cells. Plant Physiology 91, 1372–1381.

Kromdijk J, Ubierna N, Cousins AB, Griffiths H. 2014. Bundle-sheath leakiness in C₄ photosynthesis: a careful balancing act between CO₂ concentration and assimilation. Journal of experimental botany 65, 3443-3457.

Miao ZW, Xu M, Lathrop RG, Wang YF. 2009. Comparison of the A-C₃ curve fitting methods in determining maximum ribulose 1.5-bisphosphate carboxylase/oxygenase carboxylation rate, potential light saturated electron transport rate and leaf dark respiration. Plant, Cell and Environment 32, 1191-1204.

Osborne CP, Beerling DJ. 2006. Nature’s green revolution: the remarkable evolutionary rise of C-4 plants. Philosophical Transactions of The Royal Society B 361, 173-194.
Osborne CP, Sack L. 2012. Evolution of C4 plants: a new hypothesis for an interaction of CO2 and water relations mediated by plant hydraulics. Philosophical Transactions of The Royal Society B 367, 583-600.

Pedomo JA, Cavanagh AP, Kubien DS, Galmes J. 2015. Temperature dependence of in vitro Rubisco kinetics in species of Flaveria with different photosynthetic mechanisms. Photosynthesis Research 124, 67–75.

Pinto H, Sharwood RE, Tissue DT, Ghannoum O. 2014. Photosynthesis of C3, C3–C4, and C4 grasses at glacial CO2. Journal of Experimental Botany 65, 3669-3681.

Sage RF. 2002. Variation in the kcat of Rubisco in C3 and C4 plants and some implications for photosynthetic performance at high and low temperature. Journal of Experimental Botany 53, 609-620.

Savir Y, Noor E, Milo R, Tlusty T. 2010. Cross-species analysis traces adaptation of Rubisco toward optimality in a low-dimensional landscape. Proceedings of the National Academy of Sciences 107, 3475-3480.

Sharkey TD, Bernacchi CJ, Farquhar GD, Singsaas EL. 2007. Fitting photosynthetic carbon dioxide response curves for C3 leaves. Plant, Cell and Environment 30, 1035–1040.

Studer RA, Christin PA, Williams MA, Orengo CA. 2014. Stability-activity tradeoffs constrain the adaptive evolution of Rubisco. Proceedings of the National Academy of Science of the United States of America 111, 2223–2228.

Ubierna N, Gandin A, Boyd RA, Cousins AB. 2017. Temperature response of mesophyll conductance in three C4 species calculated with two methods: 18O discrimination and in vitro Vpmax. New Phytologist 214, 66-80.

Ubierna N, Sun W, Kramer DM, Cousins AB. 2013. The efficiency of C4 photosynthesis under low light conditions in Zea mays, Miscanthus X giganteus and Flaveria bidentis. Plant, Cell and Environment 36, 365-381.

Xu LK, Baldocchi DD. 2003 Seasonal trends in photosynthetic parameters and stomatal conductance of blue oak (Quercus douglasii) under prolonged summer drought and high temperature. Tree Physiology 23, 865-877.

Yin X, Struik PC, Romero P, Harbinson J, Evers JB, Van Der Putten PEL, Vos JAN. 2009. Using combined measurements of gas exchange and chlorophyll fluorescence to estimate parameters of a biochemical C3 photosynthesis model: a critical appraisal and a new integrated approach applied to leaves in a wheat (Triticum aestivum) canopy. Plant, Cell and Environment 32, 448-464.
Yin X, Sun Z, Struik PC, Gu J. 2011a. Evaluating a new method to estimate the rate of leaf respiration in the light by analysis of combined gas exchange and chlorophyll fluorescence measurements. Journal of Experimental Botany 62, 3489-3499.

Yin XY, Sun ZP, Struik PC, Van der Putten PEL, Van Ieperen W, Harbinson J. 2011b. Using a biochemical C₄ photosynthesis model and combined gas exchange and chlorophyll fluorescence measurements to estimate bundle-sheath conductance of maize leaves differing in age and nitrogen content. Plant, Cell and Environment 34, 2183-2199.