Research Paper

A high throughput gas exchange screen for determining rates of photorespiration or regulation of C₄ activity

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Abstract

Large-scale research programmes seeking to characterize the C₄ pathway have a requirement for a simple, high throughput screen that quantifies photorespiratory activity in C₃ and C₄ model systems. At present, approaches rely on model-fitting to assimilatory responses (A/Ci curves, PSII quantum yield) or real-time carbon isotope discrimination, which are complicated and time-consuming. Here we present a method, and the associated theory, to determine the effectiveness of the C₄ carboxylation, carbon concentration mechanism (CCM) by assessing the responsiveness of V_o/V_c, the ratio of RuBisCO oxygenase to carboxylase activity, upon transfer to low O₂. This determination compares concurrent gas exchange and pulse-modulated chlorophyll fluorescence under ambient and low O₂, using widely available equipment. Run time for the procedure can take as little as 6 minutes if plants are pre-adapted. The responsiveness of V_o/V_c is derived for typical C₃ (tobacco, rice, wheat) and C₄ (maize, Miscanthus, cleome) plants, and compared with full C₃ and C₄ model systems. We also undertake sensitivity analyses to determine the impact of R_LIGHT (respiration in the light) and the effectiveness of the light saturating pulse used by fluorescence systems. The results show that the method can readily resolve variations in photorespiratory activity between C₃ and C₄ plants and could be used to rapidly screen large numbers of mutants or transformants in high throughput studies.

Key words: C₃, C₄, photosynthesis, RuBisCO, oxygenation, carboxylation, carbon concentration mechanism (CCM), Cleome gynandra, rice, maize, wheat, Miscanthus.

Introduction

In most photosynthetic organisms Ribulose Bisphosphate Carboxylase Oxygenase (RuBisCO) catalyses the first key step in carbon assimilation, reacting ribulose-1,5-bisphosphate with CO₂ to produce two molecules of 3-phosphoglycerate (PGA). Oxygen competitively inhibits this reaction and leads to the synthesis of the 2-carbon compound phosphoglycolate, which is recycled to PGA (consuming ATP, and then NADPH) and CO₂ by the photorespiratory cycle (Yoshimura et al., 2004; Sage et al., 2012). The result of photorespiration is a noticeable carbon loss and a consequent metabolic cost for carbon recapture and for the recycling of photorespiratory intermediates (Ehleringer and Pearcy, 1983; Pearcy and Ehleringer, 1984; Eckardt, 2005). Many plants have evolved strategies to reduce photorespiration by increasing the level of CO₂ around RuBisCO, including both crassulacean acid metabolism (CAM) and the C₄ photosynthetic pathway (Dodd et al., 2002; Sage, 2004; Sage et al., 2011; Osborne and Sack, 2012; Griffiths et al., 2013; Owen and Griffiths, 2013). C₄ photosynthesis is most often based on a two-celled carbon concentrating mechanism, where HCO₃⁻ is first fixed into the four-carbon compound oxaloacetic acid (OAA) in the mesophyll by phosphoenolpyruvate carboxylase (PEPC). OAA is then reduced to malate or transaminated to aspartate and the resulting C₄-(amino)acid is shuttled into the bundle sheath (BS), where it is decarboxylated, releasing CO₂ for refixation by RuBisCO (Hibberd and Covshoff, 2010; Bellasio and Griffiths, 2014c).

Although the enzymes catalysing the core C₄ carbon concentration mechanism (CCM) are well characterized (Kanai and Edwards, 1999), many of the genes responsible for the
accompanying anatomical alterations or for generating and maintaining expression of the C₄ cycle genes (Hibberd et al., 2008; Langdale, 2011) have yet to be identified. One approach that is increasingly proving useful to identify candidate genes underlying the C₄ pathway is comparative transcriptomics of samples either undergoing C₃ or C₄ photosynthesis (Bräutigam et al., 2011; Gowik et al., 2011; John et al., 2014), or tissues in the process of inducing the full C₄ system (Li et al., 2010; Pick et al., 2011; Chang et al., 2012; Wang et al., 2013). Because stable transformation of C₄ species is specific limitations (Laisk and Edwards, 2000; von Caemmerer, a priori knowledge of species-specific limitations (Laisk and Edwards, 2000; von Caemmerer, 2000, 2013; Yin and Struik, 2009; Yin et al., 2009; Yin et al., 2011b). ¹³C/¹²C discrimination during photosynthesis (Evans et al., 1986) can also be used, and a comparison with stomatal conductance allows the internal mesophyll conductance, or extent of CCM or PEPC activity, to be resolved (Meyer et al., 2008; Kromdijk et al., 2010; Pengelly et al., 2010; Bellasio and Griffiths, 2014a, b, c). However, this latter technique is sensitive, and requires either off-line sample preparation for mass spectrometric analyses or specialized laser equipment which is not readily available (Table 1).

In this paper we describe a novel method, and present the associated theory, to determine rates of photorespiration from instantaneous rates of RuBisCO carboxylation and oxygenation. The approach compares concurrent gas exchange and pulse-modulated chlorophyll fluorescence measurements under ambient and low O₂. Under these non-photorespiratory conditions assimilation (A) increases, because RuBisCO competitive inhibition from O₂ is reduced. In contrast, (Y(II)) decreases because the demand for NADPH associated with photorespiratory by-product cycling (and reduction) is lower, and cannot entirely be offset by the increase in A. The new method combines developments in approaches using gas exchange (Sharkey, 1988; Long and Bernacchi, 2003; Ripley et al., 2007) and the quantitative interpretation of quantum yield (Yin et al., 2004, 2009, 2011b; Yin and Struijk, 2009, 2012; Bellasio and Griffiths, 2014b). This new method can be performed with off-the-shelf commercial equipment, which is generally available in ecophysiology laboratories.

### Table 1. Comparison between methods screening for activity of a functional CCM

| Method                             | Advantages and limitations                                                                 | Reference                                                                                     |
|------------------------------------|---------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|
| Dry matter isotopic discrimination | *Specialized equipment<br>*Integrates the isotopic signal throughout growth<br>*Cannot resolve transient changes in assimilatory physiology | Cernusak et al. (2013)                                                                       |
| On line isotopic discrimination    | *Laser is no longer commercially available<br>*Maintenance costs of isotope ratio mass spectrometer<br>*Need of highly skilled operator | Evans et al. (1986); Bellasio and Griffiths (2014b); von Caemmerer et al. (2014)             |
| A/C curves                         | *Requires a priori knowledge of the limitations underpinning each part for the A/C curve for correct model fitting<br>*Result may depend on experimental routine | Long and Bernacchi (2003); Yin et al. (2009)                                                 |
| Gas exchange and fluorescence      | *Requires initial response curve for parameterisation<br>*Requires model fitting              | Long and Bernacchi (2003); Martins et al. (2013)                                             |
| O₂ sensitivity of carboxylation efficiency | *Delicate experimental routine                                                            | Laisk et al. (2002); Yin et al. (2009)                                                     |
| Assimilation increase under low O₂ | *Ease of determination<br>*Ignores the effect of changing O₂ concentration on Y(II)        | Sharkey (1988); Ripley et al. (2007)                                                        |
| Gas exchange and fluorescence      | *Rapid (6 minutes)<br>*Wide available equipment<br>*Independent of leaf size<br>*Ease of determination and calculation<br>*Does not require fitting or parameterisation<br>*Assessment under growth conditions | This study                                                                                   |
procedure takes as little as 6 minutes to perform if plants are pre-adapted, making it significantly faster than \( \Delta / C \) curves and potentially useful as a high-throughput approach for assessing \( C_4 \) activity in mutant screens, the progeny from \( C_3-C_4 \) crosses or \( C_3-C_4 \) intermediates.

## Materials and methods

### Plants

Plants of *Miscanthus* (*Miscanthus giganteus*), cleome (*Cleome gynandra*), maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), tobacco (*Nicotiana tabacum* L.), and rice (*Oryza sativa* L.) were grown at the Plant Growth Facility located at the University of Cambridge Botanic Garden in controlled environment growth rooms (Conviron Ltd, Winnipeg, Canada) set at 16 h day length, temperature of 25 \(^\circ\)C/23 \(^\circ\)C (day/night), 40% relative humidity, and photosynthetic photon flux density (PPFD)=300 \( \mu \)mol m \(^{-2}\) s \(^{-1}\). Plants were manually watered daily, with particular care to avoid overwatering.

### Gas exchange measurements with concurrent PSII yield

Measurements were performed with an infra-red gas analyser (IRGA, a LI6400XT, LI-cor, USA), fitted with a 6400–40 leaf chamber fluorimeter. The IRGA was fed with CO2 (through the IRGA gas mixing unit) and ambient air. Gas flow was set at 150 \( \mu \)mol s \(^{-1}\). Reference CO2 was set at 200 \( \mu \)mol mol \(^{-1}\) (Figure 1 and Table 1) or set alternatively at 400, 300, 200, 150, 100, and 50 \( \mu \)mol s \(^{-1}\) (Figure 3). Block temperature was controlled at 35 \(^\circ\)C. The fluorometer was set to multiphase pulse with factory setting, target intensity=10 and ramp depth=40% (Loriaux et al., 2013). A portion of a light-adapted leaf was clamped in the cuvette. The leaf was allowed to reach stable photosynthetic conditions under PPFD=300 \( \mu \)mol m \(^{-2}\) s \(^{-1}\) (factory setting: 90% red, 10% blue). Photosynthesis was measured every 10 s for 30 s (the three values were then averaged) and a multiphase pulse was applied for the determination of \( Y(II) \). A humidified 2% O2/N2 gas (pre-mixed, BOC, Guilford, UK) was switched to supply the inlet of the IRGA. The gas was allowed to completely flush the cuvette (c. 6 min). Photosynthesis was measured every 10 s for 30 s (the three values were then averaged) and a multiphase pulse was applied for the determination of \( Y(II) \). Light was turned off, the inlet was fed with ambient air, the reference CO2 was set at 500 \( \mu \)mol mol \(^{-1}\), similar to the lab CO2 concentration (c. 550 \( \mu \)mol mol \(^{-1}\)) to minimize the errors caused by CO2 leakage (Boesgaard et al., 2013), and flow was set to 40 \( \mu \)mol s \(^{-1}\). Once the cuvette had been flushed, and the signal stabilised (c. 5 min), respiration was measured every 10 s for 2 min (the values were then averaged). \( C_r \) was not adjusted to account for changes in stomatal conductance or for the control of \( C_t \) during this procedure. This avoided the need for IRGA recalibration as the \( Y(II) \) measurements are independent of \( C_t \). The measured \( A \) and \( Y(II) \) under low and ambient \( O_2 \), together with an estimate of \( R_{\text{LIGHT}} \) (see below), were used to determine Rubisco rate of carboxylation (\( V_c \)), Rubisco rate of oxygenation (\( V_o \)), and the rate of photorespiratory CO2 evolution in the light (\( F \)).

### Theory

Rubisco catalyses two reactions: a carboxylase reaction whereby Ribulose Bisphosphate (RuBP) is carboxylated to form two molecules of phosphoglyceric acid (PGA), and an oxygenase reaction whereby RuBP is oxygenated to form one PGA and one glycolate molecule. Each carboxylase event requires 2 NADPH for the reduction of the 2 PGA molecules formed. Each oxygenase event requires 1 NADPH for the reduction of the PGA directly produced by Rubisco, 0.5 NADPH to recycle glycollate, and 0.5 NADPH to reduce the PGA regenerated, which total 2 NADPH (Bellasio and Griffiths, 2014c). The overall NADPH demand, at steady-state, equals the total photosynthetic NADPH production rate \( J_{\text{NADPH}} \) (Yin et al., 2004; Yin and Struik, 2012):

\[
J_{\text{NADPH}} = 2V_c + 2V_o
\]

Where \( J_{\text{NADPH}} \) is the total NADPH produced for photosynthesis, \( V_c \) is Rubisco carboxylation rate, and \( V_o \) is Rubisco oxygenation rate. Notably, this reducing power requirement is the same for all types of photosynthesis, as active types of CCM require additional ATP but not NADPH. In line with von Caemmerer (2000) equation 1 assumes that PGA is entirely reduced, and therefore the small quantity of PGA consumed by respiration (\( \frac{1}{3} R_{\text{LIGHT}} \)) is neglected, in fact under growth light irradiance \( 2V_c +2V_o \geq \frac{1}{3} R_{\text{LIGHT}} \) unless at very low irradiances, see equation 7 in Bellasio and Griffiths (2014c).

Although the carboxylation reaction of Rubisco consumes CO2, the regeneration of glycollate releases 0.5 CO2 for each oxygenase catalytic event. CO2 is also produced by light respiration, a process which is active during photosynthesis to support basal metabolism. The net assimilation rate (\( A \), which is the quantity measured through gas exchange) results from summing the CO2 consumed by Rubisco, the CO2 produced by glycollate regeneration and the CO2 produced by respiration:

\[
A = V_c - \frac{1}{2} V_o - R_{\text{LIGHT}}
\]

Where \( A \) is net CO2 assimilation, \( R_{\text{LIGHT}} \) is respiration in the light and other variables were previously defined. Notably, this equation is universal for all types of photosynthesis (von Caemmerer, 2013).

For the definition of gross assimilation \( (GA = A + R_{\text{LIGHT}}) \), equation 2 can be rearranged:

\[
V_c = GA + \frac{1}{2} V_o
\]

Equation 1 and 3 can be combined to give:

\[
V_o = J_{\text{NADPH}} - \frac{2GA}{3}
\]

The rate of photorespiratory CO2 evolution, \( F \) can be calculated as: (von Caemmerer, 2013)

\[
F = \frac{1}{2} V_o
\]

Under low O2, \( V_o \) can be approximated to \( =0 \), hence, from equation 4:

\[
J_{\text{NADPH Low O2}} = 2GA_{\text{Low O2}}
\]

Which is valid when \( V_o \neq 0 \). NADPH is produced through linear electron flow. Independently from where this reaction is located (e.g. in mesophyll cells), electrons are invariably extracted from water by PSII (Yin and Struik, 2012), therefore \( J_{\text{NADPH}} \) is proportional to \( Y(II) \) (Yin and Struik, 2012). This allows \( J_{\text{NADPH}} \) to be calculated under photosynthetic conditions using the information derived under non-photorespiratory conditions, and can be expressed as (Bellasio and Griffiths, 2014b):

\[
J_{\text{NADPH}} = J_{\text{NADPH Low O2}} \frac{Y(II)}{Y(II)_{\text{Low O2}}}
\]

Where \( J_{\text{NADPH}} \) and \( Y(II) \) refer to ambient O2 conditions. Equation 7 has been validated in \( C_3 \) and \( C_4 \) plants (Yin et al., 2009, 2011b; Bellasio and Griffiths, 2014b, c) but it is worth noting that equation 7 is a mathematical simplification and holds true when: (i) photorespiration is negligible under non-photorespiratory conditions, which
is a widely used simplification; (ii) \( R_{\text{LIGHT}} \) does not vary between low and ambient \( O_2 \)—this is also a fair assumption because any \( O_2 \) effect is generally negligible (Badger, 1985; Gupta et al., 2009); (iii) the allocation to alternative sinks (non-assimilatory and non-photorespiratory) is proportional to \( Y(II) \). This is the normal case in \( C_4 \) plants where the relationship between \( Y(II) \) and \( Y(CO_2) \) has a null intercept (Edwards and Baker, 1993). When that is not the case, for instance when the allocation to alternative sinks is constant, equation 7 would also hold true if the allocation to alternative sinks is small compared with \( Y(II) \). This is the normal case in \( C_3 \) plants (Valentini et al., 1995; Martins et al., 2013). Should the allocation to alternative sinks be large, equation 7 would still hold true mathematically when \( \frac{Y(II)}{Y(II)_{\text{Low } O_2}} \) is close to the unity. The implications for method accuracy are detailed in the discussion.

Equation 3, 4, 6, and 7 can be combined to obtain:

\[
\frac{V_o}{V_C} = \frac{2GA_{\text{low } O_2}Y(II)}{Y(II)_{\text{Low } O_2}} - 2GA \frac{Y(II)}{Y(II)_{\text{Low } O_2}} + 2GA
\]

(8)

Which expresses the RuBisCO rate of oxygenation relative to carboxylation. The influence on the quality of \( R_{\text{LIGHT}} \) estimate on \( V_o/V_C \) is described in the discussion, together with the other factors influencing the results.

Modelling \( C_3 \) and \( C_4 V_o/V_C \)

The data obtained for tobacco and maize were compared with a simulated \( V_o/V_C \) based on the validated von Caemmerer models for \( C_3 \) and \( C_4 \) photosynthesis. Briefly, for tobacco, the response of \( A \) to \( C_i \) was modelled using the quadratic equation (Table 3, equation 9) proposed by Ethier and Livingston (2004), which takes into account mesophyll conductance to \( CO_2 \). The \( CO_2 \) concentration at the site of carboxylation \( C_C \) was then calculated through the supply function of mesophyll (equation 10), and, finally \( V_o/V_C \) was simulated from the kinetic properties of RuBisCO and the ratio between \( C_C \) and the \( O_2 \) concentration at the site of carboxylation (equation 11). For maize (Table 4), firstly we simulated the responses of \( V_P \) and \( A \) to decreasing \( C_i \), using the equations for the enzyme-limited model for \( C_4 \) photosynthesis (equation 12 and 16, respectively). These were used to simulate the \( CO_2 \) and \( O_2 \) concentration in the bundle sheath (equation 13 and 14, respectively), the ratio of which, together with RuBisCO specificity, was used to simulate \( V_o/V_C \) (equation 15 and 17).

Results

Figure 1 displays a typical primary data profile for a \( C_3 \) tobacco leaf, showing the interaction between steady state assimilation (\( A \)) and quantum yield of PSII, \( Y(II) \), during the transition from ambient to low \( O_2 \) (21 to 2% \( O_2 \)), with hatched areas indicating the steady state conditions under which readings were taken to derive \( V_o/V_C \). Under non-photorespiratory conditions, \( A \) increases because of the lower competitive inhibition of \( O_2 \), whereas \( Y(II) \) decreases owing to the lower NADPH demand for photorespiratory by-product recycling and reduction. The experimental conditions were deliberately chosen to minimize reductions of quantum yield at saturating light (relatively low PPFD of 300 \( \mu \text{mol} \text{ m}^{-2} \text{ s}^{-1} \)), and enhance photorespiratory responses to low \( O_2 \) partial pressure (measurements at 200 \( \mu \text{mol} \text{ mol}^{-1} \text{ CO}_2 \)) (Fig. 1 and Table 2). Subsequently, \( V_o/V_C \) was measured on \( C_3 \) tobacco and \( C_4 \) maize using different \( CO_2 \) concentrations in the reference gas: 400, 300, 200, 150, 100, and 50 \( \mu \text{mol} \text{ mol}^{-1} \) (Fig. 2) and results were compared with simulated values of \( V_o/V_C \) generated with the validated von Caemmerer \( C_3 \) and \( C_4 \) models. To facilitate the comparison, data were plotted against the substomatal \( CO_2 \) concentration \( C_i \). As expected, under decreasing \( C_i \), \( V_o/V_C \) becomes progressively higher in tobacco but it is only marginally affected in maize. The measured data track the trend and magnitude of the theoretical curves in \( C_3 \), whereas we could not capture the theoretical increase in \( V_o/V_C \) expected when \( C_i \) was close to zero. This may be due to errors in the determination of \( C_i \) at very low stomatal conductance or to the simplifications used to resolve equation 7. Our data slightly underestimate \( V_o/V_C \) derived using pulsed of \( ^{13} \text{C} \) enriched \( CO_2 \) (Busch et al., 2013), which, however, lay above the curve simulated with the von Caemmerer \( C_3 \) model (see Fig. 2).

Additional measurements were undertaken with the IRGA, including a recalibration procedure to account for the changing sensitivity to water vapour pressure after the transition to low \( O_2 \), but stomatal conductance was reduced on average by 1% and internal \( CO_2 \) concentration, \( C_i \), by 3 \( \mu \text{mol} \text{ mol}^{-1} \) (data not shown). In the subsequent sections, primary data for \( V_o/V_C \) determinations using this new method (calculated from equation 4) are initially presented for three representatives of \( C_3 \) and \( C_4 \) species. We then undertake a systematic error analysis of the method, to include the impact of biological and environmental variables. These include physiological components (\( R_{\text{LIGHT}} \) and \( F_m' \)), as well as light intensity and \( CO_2 \) concentration used during experimentation.

Variability between and within populations

Table 2 demonstrates that the method clearly discriminates between \( C_3 \) species, possessing a functional CCM, and \( C_4 \) species with higher rates of photorespiration. \( V_o/V_C \) ranged from 0.0435 to 0.0852 for the representative \( C_4 \) species, with
Table 2. Example of variability within populations and between populations displayed by plants with different pathways of assimilation

\( V_{O}/V_{C} \) was measured on species (Miscanthus, Cleome gynandra, maize, wheat, tobacco, and rice) under photosynthetic photon flux density (PPFD) of 300 \( \mu \)mol m\(^{-2}\) s\(^{-1}\), and \( C_{a}=200 \) \( \mu \)mol mol\(^{-1}\).

| Population          | \( n \) | Mean \( V_{O}/V_{C} \) | Standard deviation | Coefficient of variation |
|---------------------|---------|-------------------------|--------------------|--------------------------|
| Miscanthus          | 7       | 0.0504                  | 0.0091             | 18%                      |
| Cleome gynandra     | 5       | 0.0852                  | 0.0046             | 5.4%                     |
| Maize               | 4       | 0.0435                  | 0.0074             | 17%                      |
| Wheat               | 3       | 0.522                   | 0.071              | 14%                      |
| Tobacco             | 4       | 0.533                   | 0.030              | 5.5%                     |
| Rice                | 4       | 0.569                   | 0.037              | 6.5%                     |

Fig. 2. \( V_{O}/V_{C} \) measured under different CO2 concentrations in the substomatal cavity \( (C_{i}) \) obtained by imposing reference CO2 concentrations of 400, 300, 200, 150, 100, and 50 \( \mu \)mol mol\(^{-1}\) for \( C_{1} \) tobacco (triangles) and \( C_{4} \) maize (squares). Data are compared with simulated \( V_{O}/V_{C} \) using the validated von Caemmerer \( C_{3} \) and \( C_{4} \) models (lines, see also Table 3 and 4). With decreasing \( C_{i} \), \( V_{O}/V_{C} \) gets progressively higher in tobacco but it is only marginally affected in maize. CO2 concentration can therefore be used to control the resolution of the method. All data shown, \( n=4 \).

Accuracy of \( R_{LIGHT} \) estimates

To account for the extent that \( R_{LIGHT} \) affected the measurement of \( V_{O}/V_{C} \), a sensitivity analysis was used to determine how \( R_{LIGHT} \) influences \( V_{O}/V_{C} \) (Fig. 3). To do so, equation 8 was calculated for a realistic dataset \( (R_{LIGHT}=1 \) \( \mu \)mol m\(^{-2}\) s\(^{-1}\), \( V_{O}/V_{C}=0.2 \) and \( Y(II)=0.65 \)) at variable assimilation values. Then, test values for \( V_{O}/V_{C} \) were calculated after \( R_{LIGHT} \) was varied to 2 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) (+100%), 1.5 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) (+50%), 1.2 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) (+20%), 0.8 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) (-20%), 0.5 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) (-50%), 0 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) (-100%, \( GA=A \)). The deviation from the set \( V_{O}/V_{C} \) value (0.2) represented the effect of errors in the evaluation of \( R_{LIGHT} \) on \( V_{O}/V_{C} \). Figure 3 shows that \( V_{O}/V_{C} \) was relatively insensitive to \( R_{LIGHT} \): for assimilation rates higher than 4 \( \mu \)mol m\(^{-2}\) s\(^{-1}\), \( R_{LIGHT} \) values which differed \( \pm 50\% \) resulted in an error lower than 4% in relative terms. \( R_{LIGHT} \) overestimation resulted in a lower error than \( R_{LIGHT} \) underestimation. For these reasons there is generally no need for a high quality estimate of \( R_{LIGHT} \).

Accuracy of \( F_{m}' \) measurements

Equations 7 and 8 require the photochemical yield of PSII, \( Y(II) \). This is determined according to the formula of Genty (Genty et al., 1989; Maxwell and Johnson, 2000; Kramer et al., 2004), whereby \( Y(II) \) is calculated as the difference between the light-saturated chlorophyll fluorescence signal \( (Fm') \) minus the chlorophyll fluorescence signal measured during photosynthesis \( (F_{s}) \), expressed as relative to \( Fm' \). Key to this technique is achieving full saturation of PSII in the determination of \( Fm' \) (Earl and Ennahli, 2004; Loriaux et al., 2006; Harbinson, 2013; Loriaux et al., 2013). Sub-saturating light pulses result in the underestimation of \( Fm' \); however, the degree of underestimation depends not only on the saturating pulse spectra and intensity, but also on the species, the growth light intensity, and the light intensity used during the measurements (Earl and Ennahli, 2004).

Here, we show how a given \( Fm' \) underestimation influences the values for \( V_{O}/V_{C} \) (Fig. 4). To do so, equation 8 was set to physiologically realistic conditions \( (R_{LIGHT}=1 \) \( \mu \)mol m\(^{-2}\) s\(^{-1}\), \( V_{O}/V_{C}=0.2 \), and \( A=5 \) \( \mu \)mol m\(^{-2}\) s\(^{-1}\)\)), at different \( Y(II) \) values. Underestimates of \( Fm' \) were then introduced by multiplying the realistic \( Fm' \) value by, successively, 0.99 (–1%), 0.98 (–2%), 0.97 (–3%), and 0.95 (–5%). The difference between the two values represented the effect of \( Fm' \) underestimation on \( V_{O}/V_{C} \). Figure 4 shows that \( V_{O}/V_{C} \) was sensitive to \( Fm' \) underestimation; for instance the relative error of \( V_{O}/V_{C} \) was c. 20% when \( Y(II) \) was 0.15 and \( Fm' \) was underestimated by 3%. The error increased hyperbolically at decreasing \( Y(II) \), and increased proportionally as the \( Fm' \) underestimation was increased.

Light intensity and CO2 concentration used for experimentation

High light intensities (e.g. PPFD>1000 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)) result in a low PSII yield, which may potentially amplify the systematic error from any \( Fm' \) underestimation (see above). Similarly, small \( Y(II) \) could potentially lead to \( V_{O}/V_{C} \) underestimation when the allocation to alternative sinks is significant (see description...
of equation 7). Further, high light conditions require longer timescales to reach stable photosynthetic conditions. On the other hand, depending on growth conditions, low light intensities (e.g. <100 μmol m⁻² s⁻¹) might lead to low assimilation rates, which could amplify the systematic errors in the estimation of \( R_{\text{LIGHT}} \) (see Fig. 3 and above). For these reasons, intermediate light intensities represent the best solution, whereby \( Y(II) \) and \( A \) are both high. For instance, values at the top end of the linear region of the light response curve would be ideal. These generally correspond to the growth light intensity.

CO₂ concentration in the cuvette (\( C_\ell \)) can be used to manipulate photorespiration. Figure 2 shows the measured and predicted \( V_{O/2}/V_C \) of \( C_3 \) and \( C_4 \) plants under different CO₂ concentrations. Because of the CCM, \( V_{O/2}/V_C \) is low in maize, even at low \( C_\ell \), whereas in wheat \( V_{O/2}/V_C \) increases hyperbolically as decreasing \( C_\ell \). This contrasting behaviour allows the resolution of the method to be manipulated by changing the CO₂ concentration in the background gas. However, decreasing CO₂ concentration is disadvantageous because: (i) low \( C_\ell \) results in quenching of PSII yield, which may potentially amplify the systematic error determined by \( Fm' \) underestimation (see above); (ii) low \( Y(II) \) would amplify the magnitude of \( V_{O/2}/V_C \) underestimation owing to the partitioning of \( Y(II) \) to alternative sinks (see description of equation 7); (iii) under low \( C_\ell \), more time is required to reach stable photosynthetic conditions, which result in lower throughput; (iv) low \( C_\ell \) increases the driving force for diffusion from outside of the cuvette, which may constitute a potential source of error, especially when assimilation is low (Boesgaard et al., 2013). For these reasons the optimal \( C_\ell \) will depend on the purpose of the analysis, and on the desired resolution and speed.

**Discussion**

This method is based upon the difference in net assimilation (\( A \)) and photosystem II yield (\( Y(II) \)) observed when the gas supplied to an actively photosynthesizing leaf is switched from ambient \( O_2 \) to low \( O_2 \). The goal was to develop a relatively quick, readily available method, which could be used to screen large numbers of transiformants, \( C_5-C_4 \), \( C_2 \), or photorespiratory refixation variants (Busch et al. 2013; Oakley et al., 2014) in a given population of plants. The data show that the method readily distinguishes between \( V_{O/2}/V_C \) for typical \( C_3 \) and \( C_4 \) plants (Table 2), and, given the low coefficients of variation, should detect more subtle variations in \( C_\ell \) repressation or activation within a screen. It would then be possible to subject plants identified in this way to a more detailed, conventional gas exchange or stable isotope screen, to identify contributory morphological, metabolic or genetic factors. In the subsequent discussion, we explore the theoretical and practical limitations underpinning the accuracy of the method, and improvements that could be instituted to enhance the outputs, if high sample throughput was not a primary limitation.

Other methods have been proposed to determine the contribution of photorespiration in vivo through gas exchange measurements. The method proposed by Ripley et al. (2007) uses only the increase in assimilation under non-photorespiratory conditions, and therefore ignores the effect on \( Y(II) \). In our work we observed that \( Y(II) \) is generally influenced by changes in \( O_2 \) concentration (Figure 1), even in \( C_4 \) plants (see Fig. 2 in Bellasio and Griffiths, 2014b); therefore it is important to take into account the feedback from assimilation on photosystem II yield. Long and Bernacchi (Long and Bernacchi, 2003) proposed a comprehensive method to determine the partitioning of total electron transport rate between photorespiratory and assimilatory demand. Their protocol requires an initial light or \( A/C_\ell \) response so as to fit a linear relationship between quantum yield for CO₂ fixation \( Y(CO_2) \) and quantum yield of photosystem II, \( Y(II) \).

In comparison, the simple method that we have proposed requires no previous parameterization, no curve fitting, and no knowledge of the underpinning physiology or biochemical constants. It is also independent of leaf area, as when
deriving \( V_O/V_C \) from equation 8, both the numerator and the denominator are proportional to leaf area, a huge advantage for small or dissected leaves. The likelihood of triose phosphate limitation (Sharkey, 1988) is minimized under the relatively low light intensities and low \( C_C \), which are optimal for this protocol. The determination of \( V_O/V_C \) could take as little as c. 6 min, although the complete routine was longer (c. 40 min) as leaves were allowed to acclimate before measurement of both assimilation and dark respiration. Therefore, the run time can be minimized by measuring assimilation under growth conditions (e.g. at growth light intensity and \( CO_2 \) concentration), and either measuring respiration after all plants have been collectively dark–adapted, or estimating it separately (see below).

**Other factors affecting accuracy of \( V_O/V_C \) determination**

As shown in Fig. 3, the estimation of \( R_{\text{LIGHT}} \) is important when calculating gross assimilation using eqn. 8 (\( GA = A + R_{\text{LIGHT}} \)) at low assimilation rates. \( R_{\text{LIGHT}} \) can be determined with several methods; for instance, by linear regression of \( A \) versus irradiance (under very low irradiance e.g. <150 μmol photons m\(^{-2}\) s\(^{-1}\)), by linear regression of \( A \) versus irradiance multiplied by \( Y(II) \) [under moderate irradiance, e.g. <400 μmol photons m\(^{-2}\) s\(^{-1}\) (Yin et al., 2011a)], by non-linear regression [throughout the light response curve (Prioul and Chartier, 1977; Dougherty et al., 1994)] or assumed to equal dark respiration [e.g. (Kromdijk et al., 2010; Ubierna et al., 2013)]. These methods do not necessarily yield the same \( R_{\text{LIGHT}} \) values, and so, the degree of similarity between different \( R_{\text{LIGHT}} \) estimates depends on the species and growth conditions. For instance, in Cocklebur (Xanthium strumarium L., Asteraceae), \( R_{\text{LIGHT}} \) was significantly different from dark respiration (Tcherkez et al., 2008), whereas in maize \( R_{\text{LIGHT}} \) is generally non-significantly different from dark respiration (C. Bellasio, unpublished data). The most suitable method to estimate \( R_{\text{LIGHT}} \) should therefore be evaluated on a case-by-case basis (see Bellasio and Griffiths, 2014a), and for a uniform population (e.g. one species or set of transformants in a growth chamber), \( R_{\text{LIGHT}} \) could be estimated on a subset of individuals, with one of the methods described above. If dark respiration is used as a proxy, the quality of the estimate can be increased using large chambers and low flow rates. In a diverse population, \( R_{\text{LIGHT}} \) could be estimated by measuring

**Table 3. Model for \( C_3 \) photosynthesis**

| Symbol | Definition/calculation | Equation | Values/Units/References |
|--------|------------------------|----------|-------------------------|
| \( A \) | Net Assimilation \( A = \frac{-b + \sqrt{b^2 - 4ac}}{2a} \) where: \( a = \frac{1}{g_m} \); \( b = (V_{\text{Cmax}} - R_{\text{LIGHT}}) + C_C + K_C(1 + \frac{O}{K_O}) \); \( c = R_{\text{LIGHT}} \left( C_C + K_C(1 + \frac{O}{K_O}) \right) \) \(-V_{\text{Cmax}} \left( G - \Gamma^* \right) \) | (9) | Ethier and Livingston (2004) |
| \( C_C \) | CO\(_2\) partial pressure at the site of carboxylation \( C_C = G - \frac{A}{g_m} \) | (10) | μbar |
| \( C_i \) | CO\(_2\) concentration in the intercellular spaces as calculated by the IRGA. | | μmol mol\(^{-1}\) (LI-cor 6400 manual equation 1–18) |
| \( g_m \) | Mesophyll conductance to CO\(_2\). | 0.25 m\(^2\) s\(^{-1}\) bar\(^{-1}\) (Ethier and Livingston, 2004) |
| \( K_C \) | RuBisCO Michaelis-Menten constant for CO\(_2\). | 319.3 μbar (Ethier and Livingston, 2004) |
| \( K_O \) | RuBisCO Michaelis-Menten constant for O\(_2\). | 277,100 μbar (Ethier and Livingston, 2004) |
| \( O \) | O\(_2\) partial pressure at the site of carboxylation | 200,000 μbar |
| \( R_{\text{LIGHT}} \) | Respiration in the light | | |
| \( V_{\text{Cmax}} \) | Maximum RuBisCO carboxylation rate | 34.7 μmol m\(^{-2}\) s\(^{-1}\) (Ethier and Livingston, 2004) |
| \( V_O/V_C \) | | 13.25 μmol m\(^{-2}\) s\(^{-1}\) (Ethier and Livingston, 2004) |
| \( \Gamma^* \) | CO\(_2\) compensation point in absence of dark respiration | 44 μbar |
dark respiration on each individual plant after the measurements in the light.

As shown in Fig. 3, errors in the determination of $Fm'$ suggest that techniques such as the multiphase flash (Loriaux et al., 2013), or initial checks to ensure that the saturating pulse is saturating (see Bellasio and Griffiths, 2014b) are normally appropriate for this method. However, the use of our method is possible without a multiphase flash. Firstly, the underestimation of $Fm'$ introduces a systematic error, i.e. comparable plants will normally show similar $V_o/V_C$, other approaches could: (i) increase the saturating pulse intensity; (ii) reduce the distance between light source or fibre-optic probe and leaf (in some systems); (iii) decrease actinic light intensity (as shown in this study) to maximise $Y(II)$; and (iv) CO$_2$ concentration can be increased, in order to maximise $Y(II)$.

**IRGA recalibration, matching $Y(II)$, $C_p$ and consideration of mesophyll conductance**

As mentioned in the results, a slight effect on stomatal conductance and $C_p$ (under low O$_2$) could have been caused by not recalibrating the IRGA upon switching background gas (Bunce, 2002). Although that recalibration could have increased $C_i$ and gS accuracy (under low O$_2$), this procedure

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**Table 4. Model for $C_4$ photosynthesis**

| Symbol | Definition/calculation | Equation | Values/Units/References |
|--------|-------------------------|----------|-------------------------|
| $A$    | Net Assimilation $A = \frac{-b - \sqrt{b^2 - 4ac}}{2a}$ where: $a = 1 - \frac{\alpha K_C}{0.047 K_O}$; $b = \left( (V_R - K_R + g_{BS} C_M) + (V_{Cmax} - R_{LIGH}) + g_{BS} K_C \left( 1 + \frac{Q_A}{K_C} \right) + \frac{a}{0.047} \left( \gamma V_{Cmax} + R_{LIGH} K_C \right) \right)$; $c = (V_{Cmax} - R_{LIGH}) (V_R - K_R + g_{BS} C_M) - (V_{Cmax} g_{BS} \gamma C_M + R_{LIGH} g_{BS} K_C \left( 1 + \frac{Q_A}{K_C} \right))$ | (12) Equation 4.21 in (von Caemmerer, 2000) |  |
| $C_{BS}$ | CO$_2$ concentration in the bundle sheath $C_{BS} = \frac{\gamma O_{BS} + K_C \left( 1 + \frac{Q_A}{K_C} \right) A + R_{LIGH} V_{Cmax}}{1 - A + R_{LIGH} V_{Cmax}}$ | (13) Equation 4.11 in (von Caemmerer, 2000) |  |
| $C_M$ | CO$_2$ partial pressure in M (at the site of PEP carboxylation) $C_M = C_i$ | $\mu$bar |  |
| $C_i$ | CO$_2$ concentration in the intercellular spaces as calculated by the IRGA | $\mu$bar |  |
| $g_{BS}$ | Bundle sheath conductance to CO$_2$ |  |  |
| $K_C$ | RuBisCO Michaelis-Menten constant for CO$_2$ |  |  |
| $K_O$ | RuBisCO Michaelis-Menten constant for O$_2$ |  |  |
| $K_P$ | PEPC Michaelis-Menten constant |  |  |
| $O_{BS}$ | O$_2$ mol fraction in the bundle sheath cells (in air at equilibrium) $O_{BS} = O_M + \frac{aA}{0.047 g_{BS}}$ | (14) Equation 4.16 in (von Caemmerer, 2000) |  |
| $O_M$ | O$_2$ partial pressure in the mesophyll cells (in air at equilibrium) |  |  |
| $R_{LIGH}$ | Respiration in the light, assumed to equal dark respiration |  |  |
| $R_M$ | Mesophyll non photorespiratory CO$_2$ production in the light $R_M = 0.5 R_{LIGH}$ | $\mu$mol m$^{-2}$ s$^{-1}$ (von Caemmerer, 2000; Kromdijk et al., 2010; Ubierna et al., 2013) |  |
| $V_{Cmax}$ | Maximum RuBisCO carboxylation rate |  |  |
| $V_{O/C}$ | $V_O = 2B^+ - C_{BS}$ | (15) Equation 4.8 in (von Caemmerer, 2000) |  |
| $V_P$ | PEP Carboxylation rate $V_P = \frac{C_M V_{Cmax}}{C_M + K_P}$ | (16) Equation 4.17 in (von Caemmerer, 2000) |  |
| $V_{\text{max}}$ | Maximum PEPC carboxylation rate |  |  |
| $\alpha$ | Fraction of PSII active in BS cells |  |  |
| $\gamma^+$ | Half of the reciprocal of the RuBisCO specificity |  |  |
| $\Gamma^+$ | CO$_2$ compensation point in absence of dark respiration $\Gamma^+ = \gamma O_{BS}$ | (17) Equation 4.9 in (von Caemmerer, 2000) |  |
is liable to introduce operator error and extend the time taken for measurements; further, there are theoretical reasons why we need not account for these processes while carrying out such a simple comparative screen. Firstly, the data used to calculate equation 8 are measured by the CO2 channel of the IRGA and the fluorometer, which are both unaffected by the background gas. Secondly, the effect of $C_i$ on $A$ (under low O2) is, for the greatest part, accounted by the feedback on $Y(II)$. Although $C_i$ decreases under low O2, there is a strong feedback between assimilation and $Y(II)$, and therefore $Y(II)$ decreases proportionally. In fact, the relationship between gross assimilation (or, better, between $Y(CO_2)$, which is $GA$ divided by PPFD) and $Y(II)$ is strictly linear (Edwards and Baker, 1993; Valentini et al., 1995; Martins et al., 2013). In C4 plants, this linear relationship has generally a zero intercept, (Edwards and Baker, 1993); therefore, for C4 plants, there is no need for curve fitting and the relationship can be correctly estimated with a single point. In C3 systems this relationship is still linear but the intercept is, although generally small, not zero. The intercept, which is the magnitude of engagement of alternative sinks, can be estimated by linear curve fitting, although several data points are required (Valentini et al., 1995; Martins et al., 2013). Using the complete fitting of the $Y(CO_2)/Y(II)$ relationship, however, did not improve the estimate of $V_o/V_C$ (data not shown): the complete curve fitting correctly estimates the intercept, but the datapoints are taken under conditions which differ from those under which $V_o/V_C$ is measured.

Another way to improve the estimate of $V_o/V_C$ would be to adjust $C_a$ under low O2 so as to match $Y(II)$ measured under ambient O2 with $Y(II)$ measured under low O2. Alternatively, $C_a$ could be manipulated to deliver $C_i$ under low O2, which matches that under ambient. The advantage would be that the measured data would then probably fit the predicted C3 and C4 models more precisely when $C_i$ is limiting (see Fig. 2, Tables 3 and 4). However, these operations do not improve the capacity to screen between C3 and C4 photosynthesis and the additional manipulations increase time and likelihood of errors. We also note that such improvements would allow this method to be used to calculate the CO2 concentration at the site of carboxylation ($C_c$) in C3 plants through equation 11 (Table 3), as well as mesophyll conductance via equation 10, using $C_c$, and the values for assimilation and $C_i$ measured under ambient conditions.

Conclusion

In this paper a simple method, and associated theory, have been presented, which allow the determination of both the oxygenation ($V_o$) and carboxylation ($V_C$) rate of RuBisCO and the rate of photosynthetic CO2 evolution ($F$) based on gas exchange and variable chlorophyll fluorescence under ambient and low O2. This may be of particular interest for high throughput screening to identify C4 mutants lacking a fully functional CCM, C3 variants, or populations of C3–C4 hybrids (Oakley et al., 2014).

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References

Badger MR. 1985. Photosynthetic oxygen exchange. Annual Review of Plant Physiology 36, 27–53.

Bellasio C, Fini A, Ferrini F. 2014. Evaluation of a high throughput starch analysis optimised for wood. Plos ONE 9, e88645.

Bellasio C, Griffiths H. 2014a. Acclimation of $C_4$ metabolism to low light in mature maize leaves could limit energetic losses during progressive shading in a crop canopy. Journal of Experimental Botany 65, 3725–3736.

Bellasio C, Griffiths H. 2014b. Acclimation to low light by $C_4$ maize: Implications for bundle sheath leakiness. Plant Cell and Environment 37, 1046–1058.

Bellasio C, Griffiths H. 2014c. The operation of two decarboxylases (NADP-ME and PEPC), transamination and partitioning of $C_4$ metabolic processes between mesophyll and bundle sheath cells allows light capture to be balanced for the maize $C_4$ pathway. Plant Physiology 164, 466–480.

Boesgaard KS, Mikkelsen TN, Ro-Poulsen H, Ibrom A. 2013. Reduction of molecular gas diffusion through gaskets in leaf gas exchange cuvettes by leaf-mediated pores. Plant, Cell and Environment 36, 1352–1362.

Bräutigam A, Kajala K, Wullenweber J et al. 2011. An mRNA blueprint for $C_4$ photosynthesis derived from comparative transcriptomics of closely related $C_3$ and $C_4$ species. Plant Physiology 155, 142–156.

Bunce J. 2002. Sensitivity of infrared water vapor analyzers to oxygen concentration and errors in stomatal conductance. Photosynthesis Research 71, 273–276.

Busch FA, Sage TL, Cousins AB, Sage RF. 2013. $C_3$ plants enhance rates of photosynthesis by reassimilating photosynthesized and respirated CO2. Plant, Cell and Environment 36, 200–212.

Cernusak LA, Ubierna N, Winter K, Holtum JAM, Marshall JD, Farquhar GD. 2013. Environmental and physiological determinants of carbon isotope discrimination in terrestrial plants. New Phytologist 200, 950–965.

Chang Y-M, Liu W-Y, Shih AC-C, Shen M-N, Lu C-H, Lu M-YJ, Yang H-W, Wang T-Y, Chen SC-C, Chen SM. 2012. Characterizing regulatory and functional differentiation between maize mesophyll and bundle sheath cells by transcriptomic analysis. Plant Physiology 160, 165–177.

Dodd AN, Borland AM, Haslam RP, Griffiths H, Maxwell K. 2002. Grassulacaean acid metabolism: plastic, fantastic. Journal of Experimental Botany 53, 569–580.

Dougherty RL, Bradford JA, Coyne PI, Sims PL. 1994. Applying an empirical model of stomatal conductance to three $C_4$ grasses. Agricultural and Forest Meteorology 67, 269–290.

Earl H, Ennahl SI. 2004. Estimating photosynthetic electron transport via chlorophyll fluorometry without Photosystem II light saturation. Photosynthesis Research 82, 177–186.

Eckardt NA. 2005. Photorespiration revisited. The Plant Cell 17, 2139–2141.

Edwards GE, Baker NR. 1993. Can CO2 assimilation in maize leaves be predicted accurately from chlorophyll fluorescence analysis. Photosynthesis Research 37, 89–102.

Ehleringer J, Pearcy RW. 1983. Variation in quantum yield for CO2 uptake among $C_3$ and $C_4$ plants. Plant Physiology 73, 555–559.

Ethier GJ, Livingston NJ. 2004. On the need to incorporate sensitivity to CO2 transfer conductance into the Farquhar-von Caemmerer-Berry leaf photosynthesis model. Plant, Cell and Environment 27, 137–153.

Evans JR, Sharkey TD, Berry JA, Farquhar GD. 1986. Carbon isotope discrimination measured concurrently with gas-exchange to investigate CO2 diffusion in leaves of higher plants. Australian Journal of Plant Physiology 13, 281–292.
Genty B, Briantais JM, Baker NR. 1989. The relationship between the quantum yield of photosynthetic electron-transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta 990, 87–92.

Gowik U, Bräutigam A, Weber KL, Weber AP, Westonford N. 2011. Evolution of C4 photosynthesis in the genus Flaveria: How many and which genotypes does it take to make C4? The Plant Cell Online 23, 2087–2105.

Griffiths H, Weller G, Toy LFM, Dennis RJ. 2013. You’re so vein: bundle sheath physiology, phylogeny and evolution in C3 and C4 plants. Plant, Cell and Environment 36, 249–261.

Gupta KJ, Zabala A, Van Dongen JT. 2009. Regulation of respiration when the oxygen availability changes. Physiologia Plantarum 137, 383–391.

Harbinson J. 2013. Improving the accuracy of chlorophyll fluorescence measurements. Plant, Cell and Environment 36, 1751–1754.

Hibberd JM, Covshoff S. 2010. The regulation of gene expression required for C4 photosynthesis. Annual Review of Plant Biology 61, 181–207.

Hibberd JM, Sheehy JE, Langdale JA. 2008. Using C4 photosynthesis to increase the yield of rice—rational and feasibility. Current Opinion in Plant Biology 11, 228–231.

John CR, Smith-Unna RD, Woodfield H, Hibberd JM. 2014. Evolutionary convergence of cell specific expression in independent lineages of C4 grasses. Plant Physiology doi: 10.1104/pp.113.238667.

Kanai R, Edwards GE. 1999. The biochemistry of C4 photosynthesis. In: Sage RF, Monson RK, eds. C4 plant biology. San Diego: Academic Press, 49–88.

Kramer D, Johnson G, Kiirats O, Edwards G. 2004. New fluorescence parameters for the determination of QA redox state and excitation energy fluxes. Photosynthesis Research 79, 209–218.

Kromdijk J, Griffiths H, Scheepers HE. 2010. Can the progressive increase of C4 bundle sheath leakiness at low PFD be explained by incomplete suppression of photorespiration? Plant, Cell and Environment 33, 1935–1948.

Laish A, Edwards GE. 2000. A mathematical model of C4 photosynthesis: The mechanism of concentrating CO2 in NADP-malic enzyme type species. Photosynthesis Research 66, 199–224.

Laish A, Oja V, Rasulov B, Ramma H, Eichelmann H, Kasparova I, Pettau H, Padu E, Vapaavuori E. 2002. A computer-operated routine apparatus in leaves. Plant, Cell and Environment 25, 923–943.

Langdale JA. 2011. C4 cycles: Past, present, and future research on C4 photosynthesis. The Plant Cell Online 23, 3879–3892.

Li P, Ponnonna L, Gandotra N, Wang L, Si Y, Tausta SL, Kebronn TH, Provart N, Patel R, Myers CR. 2010. The developmental dynamics of the maize leaf transcriptome. Nature Genetics 42, 1060–1067.

Long SP, Bernacchi CJ. 2003. Estimating the rate of photorespiration in leaves. Journal of Experimental Botany 54, 2393–2401.

Loriaux S, Burns R, Welles J, McDermit D, Genty B. 2006. Determination of maximal chlorophyll fluorescence using a multiphase single flash of sub-saturating intensity. American Society of Plant Biologists Annual Meeting. Boston, MA.

Loriaux SD, Avenson TJ, Welles JM, McDermit DK, Eckles RD, Riensche B, Genty B. 2013. Closing in on maximum yield of chlorophyll fluorescence using a single multiphase flash of sub-saturating intensity. Plant, Cell and Environment 36, 1755–1770.

Martins SCV, Calmés J, Molins A, DaMatta FM. 2013. Improving the estimation of mesophyll conductance to CO2 on the role of electron transport rate correction and respiration. Journal of Experimental Botany 64, 3285–3298.

Maxwell K, Johnson GN. 2000. Chlorophyll fluorescence—a practical guide. Journal of Experimental Botany 51, 659–668.

Meyer M, Seibt U, Griffiths H. 2008. To concentrate or ventilate? Carbon acquisition, isotope discrimination and physiological ecology of early land plant life forms. Philosophical Transactions of the Royal Society B: Biological Sciences 363, 2767–2778.

Oakley JC, Sultmanis S, Stinson CR, Sage TL, Sage RF. 2014. Comparative studies of C3 and C4 Atriplex hybrids in the genomics era: physiological assessments. Journal of Experimental Botany 7, 3637–3647.

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: Biological Sciences 367, 583–600.

Owen NA, Griffiths H. 2013. A system dynamics model integrating physiology and biochemical regulation predicts extent of crassulacean acid metabolism (CAM) phases. New Phytologist 200, 1116–1131.

Peary RW, Ehleringer J. 1984. Comparative ecophysiology of C3 and C4 plants. Plant, Cell and Environment 7, 1–13.

Pengelly JJL, Sirault XRR, Tazoe Y, Evans JR, Furbank RT, von Caemmerer S. 2010. Growth of the C4 dicot Flaveria bidentis:photosynthetic acclimation to low light through shifts in leaf anatomy and biochemistry. Journal of Experimental Botany 61, 4109–4122.

Pick TR, Brautigam A, Schulte U et al. 2011. Systems analysis of a maize leaf developmental gradient refines the current C4 model and provides candidates for regulation. Plant Cell 23, 4208–4220.

Proulx JL, Chartier P. 1977. Partitioning of transfer and carboxylation components of intracellular resistance to photosynthetic CO2 fixation: A critical analysis of the methods used. Annals of Botany 41, 789–800.

Ripley BS, Gilbert ME, Ibrahim DG, Osborne CP. 2007. Drought constraints on C4 photosynthesis: stomatal and metabolic limitations in C3 and C4 subspecies of Allostephos semiflava. Journal of Experimental Botany 58, 1351–1363.

Sage RF. 2004. The evolution of C4 photosynthesis. New Phytologist 161, 341–370.

Sage RF, Christin P-A, Edwards EJ. 2011. The C4 plant lineages of planet Earth. Journal of Experimental Botany 62, 3155–3169.

Sage RF, Sage TL, Kocacinar F. 2012. Photorespiration and the evolution of C4 photosynthesis. Annual Review of Plant Biology 63, 19–47.

Sharkey TD. 1988. Estimating the rate of photorespiration in leaves. Physiologia Plantarum 73, 147–152.

Tcherkez G, Bligny R, Gout E, Mahe A, Hodges M, Cornic G. 2008. Respiratory metabolism of illuminated leaves depends on CO2 and O2 conditions. Proceedings of the National Academy of Sciences, USA 105, 797–802.

Ubierna N, Sun W, Kramer DM, Cousins AB. 2013. The efficiency Of C4 photosynthesis under low light conditions in Zea Mays, Macarthius × Giganteus and Flaveria Bidentis. Plant, Cell and Environment 36, 365–381.

Valentini R, Epron D, De Angelis P, Matteucci G, Dreyer E. 1995. In situ estimation of net CO2 assimilation, photosynthetic electron flow and photorespiration in Turkey oak (Q. cerris L) leaves: diurnal cycles under different levels of water supply. Plant, Cell and Environment 18, 631–640.

von Caemmerer S. 2000. Biochemical models of leaf Photosynthesis. Collingwood: CSIRO Publishing.

von Caemmerer S. 2013. Steady-state models of photosynthesis. Plant, Cell and Environment 36, 1617–1630.

von Caemmerer S, Ghannoum O, Pengelly JJL, Cousins AB. 2014. Carbon isotope discrimination as a tool to explore C4 photosynthesis. Journal of Experimental Botany.

Wang P, Kelly S, Fouracres JP, Langdale JA. 2013. Genome-wide transcript analysis of early maize leaf development reveals gene cohorts associated with the differentiation of C3 Kranz anatomy. The Plant Journal 75, 655–670.

Yin X, Struik PC. 2009. C3 and C4 photosynthesis models: An overview from the perspective of crop modelling. Nijas-Wageningen Journal of Life Sciences 57, 27–38.

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, 445–464.

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 X, Van Oijen M, Schapendonk A. 2004. Extension of a biochemical model for the generalized stoichiometry of electron transport limited C3 photosynthesis. Plant, Cell and Environment 27, 1211–1222.
Yin XY, Struik PC. 2012. Mathematical review of the energy transduction stoichiometries of C₄ leaf photosynthesis under limiting light. *Plant, Cell and Environment* **35**, 1290–1312.

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**, 2185–2199.

Yoshimura Y, Kubota F, Ueno O. 2004. Structural and biochemical bases of photorespiration in C₄ plants: quantification of organelles and glycine decarboxylase. *Planta* **220**, 307–317.