Carbon isotope discrimination as a diagnostic tool for $C_4$ photosynthesis in $C_3\text{-}C_4$ intermediate species

Hugo Alonso-Cantabrana* and Susanne von Caemmerer
Division of Plant Sciences, Research School of Biology, The Australian National University, Canberra, ACT 0200, Australia

* To whom correspondence should be addressed. E-mail: hugo.alonso@anu.edu.au

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Abstract

The presence and activity of the $C_4$ cycle in $C_3\text{-}C_4$ intermediate species have proven difficult to analyze, especially when such activity is low. This study proposes a strategy to detect $C_4$ activity and estimate its contribution to overall photosynthesis in intermediate plants, by using tunable diode laser absorption spectroscopy (TDLAS) coupled to gas exchange systems to simultaneously measure the CO$_2$ responses of CO$_2$ assimilation ($A$) and carbon isotope discrimination ($\Delta$) under low O$_2$ partial pressure. Mathematical models of $C_3\text{-}C_4$ photosynthesis and $\Delta$ are then fitted concurrently to both responses using the same set of constants. This strategy was applied to the intermediate species Flaveria floridana and F. brownii, and to F. pringlei and F. bidentis as $C_3$ and $C_4$ controls, respectively. Our results support the presence of a functional $C_4$ cycle in F. floridana, that can fix 12–21% of carbon. In F. brownii, 75–100% of carbon is fixed via the $C_4$ cycle, and the contribution of mesophyll Rubisco to overall carbon assimilation increases with CO$_2$ partial pressure in both intermediate plants. Combined gas exchange and $\Delta$ measurement and modeling is a powerful diagnostic tool for $C_4$ photosynthesis.

Key words: Carbon isotope discrimination, $C_3\text{-}C_4$, intermediate photosynthesis, Flaveria, F. brownii, F. floridana.

Introduction

$C_4$ photosynthesis is a highly efficient carbon fixation system characterized by the presence of a biochemical carbon pump with the capacity of increasing the CO$_2$ partial pressure (pCO$_2$) at the site of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) to concentrations higher than ambient air (Hatch et al., 1967; Hatch, 1987; Ehleringer et al., 1991). This increases photosynthetic rates and reduces photospiration, potentially improving nitrogen and water use efficiency (Hibberd et al., 2008; Langdale, 2011). Most $C_4$ species show a common anatomical pattern, called Kranz anatomy, that leads to the separation of enzyme functions in two compartments, the mesophyll and the bundle sheath cell (Brown, 1975). CO$_2$ is first hydrated into bicarbonate in the mesophyll cell cytoplasm in a reversible reaction catalyzed by carbonic anhydrase (CA) (Badger and Price, 1994). Carbon is then fixed by phosphoenol pyruvate carboxylase (PEPC), localized exclusively in the mesophyll, into four-carbon acids that diffuse to the internally adjacent bundle sheath cell, where they are decarboxylated and the released CO$_2$ is refixed by Rubisco.

The most productive crops, such as maize, sorghum and sugar cane, are $C_4$ plants, exemplifying the higher efficiency of this system over the $C_3$ photosynthetic pathway present in most plant species, including major crops like wheat and rice. For this reason, there is currently a strong interest in implementing the advantages of $C_4$ photosynthesis in to $C_3$
crops with the aim of increasing yield, to keep pace with the food needs of a growing world population (von Caemmerer et al., 2012; Karki et al., 2013; Leegood, 2013). This kind of approach is boosting research on genetic, biochemical and physiological aspects of C₄ photosynthesis. However, the initial phases of these initiatives are not expected to produce fully functional C₄ plants, but plants showing incomplete C₄ phenotypes like those observed in C₃-C₄ intermediate species, which have been considered remnants of the evolution from C₃ ancestors to C₄ plants (Rawsthorne, 1992; Sage et al., 2011). They show Kranz or Kranz-like leaf anatomy, but the activity of C₄-related enzymes, such as PEPC, is lower compared to strict C₄ plants, and enzyme compartmentation is incomplete, with Rubisco and PEPC present in both the mesophyll and the bundle sheath cells (Cheng et al., 1988; Brown and Hattersley, 1989; Byrd et al., 1992). These factors reduce the efficiency of the carbon concentrating mechanism. In intermediate plants, a photosynthetic CO₂ pump, also known as the C₂ cycle or glycine shuttle, transports glycine formed during mesophyll photosynthesis to the bundle sheath where it is decarboxylated and the CO₂ re-fixed, thus increasing overall CO₂ assimilation rate and reducing the effect of photorespiration (Monson et al., 1984; Sage et al., 2012; Schulze et al., 2013; Keerberg et al., 2014). The genus Flaveria has been the focus of numerous studies in the past because it comprises C₃, C₄ and C₃-C₄ intermediate species, the latter showing different degrees of C₄ activity (Ku et al., 1983; McKown et al., 2005).

The C₄ cycle contribution to growth has been difficult to quantify in intermediate species. In these plants, a steeper initial slope in the CO₂ response of the CO₂ assimilation rate compared to a strict C₄ plant is expected. However, this trait is also affected by Rubisco content and its kinetic properties, so conclusions are not straightforward (von Caemmerer, 2000; von Caemmerer and Quick, 2000). Another important manifestation of C₄ activity in intermediate species is a reduction of the O₂ sensitivity of CO₂ assimilation and the compensation point (Γ) due to a proportion of Rubisco being contained in the bundle sheath (BS) and thus not in direct contact with air (Byrd and Brown, 1989; Dai et al., 1996). With the photosynthetic pump causing a similar effect, separating and quantifying the contribution of each biochemical pathway through this approach is not possible. The C₄ cycle activity relative to overall photosynthesis in intermediates has been estimated in the past by metabolite profiling, but recent reports indicate that metabolite accumulation is strongly dependent on the leaf zone sampled and its developmental stage (Monson et al., 1986; Leegood and von Caemmerer, 1994; Wang et al., 2014).

In order to develop a deeper understanding of the physiology of both natural and artificial C₃-C₄ intermediates, better tools are needed to evaluate the contribution of C₄ photosynthesis to overall assimilation. One signature of the activity of PEPC as the initial CO₂ fixation enzyme is a change in carbon isotopic discrimination (Δ) during photosynthesis. Whereas Rubisco has a strong preference for the lighter isotope, ¹²C, over the heavier isotope, ¹³C, PEPC is less discriminating, which causes an important difference in the biochemical fractionation between C₃ and C₄ plants (O’Leary, 1981; Farquhar, 1983). Incomplete C₄ photosynthesis in C₃-C₄ intermediates is also reflected in Δ, with both PEPC and mesophyll Rubisco acting as the initial CO₂ fixing enzymes and their relative activities determining the resulting Δ. Mathematical models describing CO₂ assimilation and isotopic discrimination in these plants have been previously developed (von Caemmerer and Hubick, 1989; von Caemmerer, 1992). However, attempts to characterize Flaveria intermediate species by studying carbon-isotope ratios in dry matter resulted in C₄-like profiles, and were interpreted as having little or no contribution of the C₄ system to plant growth, which was in contradiction to results from metabolite analysis (Monson et al., 1988; Byrd et al., 1992).

Tunable diode laser (TDL) absorption spectroscopy allows relatively rapid measurements of Δ concurrently with gas exchange, and has been used to analyze and compare C₃ and C₄ species (Tazoe et al., 2011; von Caemmerer et al., 2014). The present work uses this technique, combined with mathematical modeling, as a tool to determine the presence and contribution of C₄ photosynthesis in C₃-C₄ intermediate plants. An updated mathematical model of carbon isotope discrimination for C₃-C₄ intermediate species is proposed, which considers the effect of mesophyll conductance and allows the calculation of the biochemical fractionation. The strategy was applied to the study of Flaveria bidentis (C₃), F. pringlei (C₃), F. floridana (C₃-C₄) and F. brownii (C₄-like). F. floridana has been described as a C₃ plant with elevated PEPC activity, but it was unclear if a C₄ cycle is actually contributing to total carbon assimilation in this species (Monson et al., 1986, 1988; Leegood and von Caemmerer, 1994; Dai et al., 1996). F. brownii, on the other hand, was initially considered a C₄ species, but later experiments proved incomplete enzyme compartmentation, with a small proportion of Rubisco activity present in the mesophyll cells, and it was then reclassified as a C₃-like intermediate species (Holaday et al., 1984; Monson et al., 1987; Moore et al., 1989). In the present study, concurrent Δ and gas exchange measurement and modeling allowed the detection and estimation of the C₄ cycle in the intermediate species, proving itself as a powerful diagnostic tool for C₄ photosynthesis.

Materials and methods

Plant material and growth conditions

Flaveria bidentis was propagated from seeds and F. pringlei, F. brownii and F. floridana were propagated from cuttings (Brown and Hattersley, 1989; Whitney et al., 2011). Plants were grown in 301 pots in a garden soil mix fertilized with Osmocote (Scotts, Australia) in a glasshouse under natural light conditions, at 28/18°C day/night temperatures, respectively. Pots were watered daily.

Responses of CO₂ assimilation rate and CO₂ compensation point to O₂ partial pressure

Two Li-Cor 6400XTs (Li-Cor, USA) were used to measure CO₂ assimilation at a range of reference pCO₂ (388, 0, 24, 48, 73, 97, 145, 194, 291, 388, 485, 582 and 776 µbar). N₂ and O₂ were mixed in different ratios by mass flow controllers (Omega Engineering Inc., USA) to generate a range of O₂ partial pressures (pO₂: 20, 50, 100,
200 and 300 mbar) supplied to the LI-6400s. Response curves of CO₂ assimilation rate (A) to intercellular pCO₂ (Ci), A/Ci curves, were repeated sequentially at each pO₂. The measurements were made at 25°C, a flow rate of 500 μmol s⁻¹ and 1500 μmol quanta m⁻² s⁻¹, inside a growth cabinet at 25°C. Four plants from each species were analyzed. The compensation point (Γ) was calculated from the A/Ci curves at each pO₂, as the intercellular CO₂ concentration where net CO₂ assimilation is zero.

To study the inhibitory effect of O₂ on assimilation rate, we compared the CO₂ assimilation rate at a reference pCO₂ of 380 μbar at each pO₂.

**Concurrent gas exchange and Δ measurements and calculations of mesophyll conductance**

Two Li-Cor 6400XTs (Li-Cor, USA) coupled to a tunable-diode laser absorption spectroscope (TDLAS, model TGA100A, Campbell Scientific, Inc., USA) as described in Tazoe et al. (2011) were used for concurrent measurements of gas exchange and carbon isotope discrimination (Bowling et al., 2003; Griffiths et al., 2004; Pengelly et al., 2012; Evans and von Caemmerer, 2013). Plants were transferred from the glasshouse to a growth cabinet with fluorescence lights (TRIL1175, Thermolino Scientific Equipment, Australia) at 25°C and one young fully expanded leaf was placed in each of the 6 cm² leaf chambers. Measurements were made at a leaf temperature of 25°C, a flow rate of 200 μmol s⁻¹, 1500 μmol quanta m⁻² s⁻¹ and 20 mbar pO₂. The desired pO₂ was achieved as described above and supplied to the Li-Cors 6400. Reference pCO₂ was changed stepwise to 392, 980, 686, 490, 294, 196, 98, 49 and 39 μbar and measurements were made every 4 min for at least 30 min at each pCO₂. Dark respiration (Rd) was measured at the end of an A/Ci curve at 392 μbar pCO₂ and 20 mbar pO₂, by switching off the Li-Cor lamp. Three or four plants from each species were analyzed. Δ was calculated as previously described by Evans et al., 1986; Evans and von Caemmerer, 2013.

Mesophyll conductance (gₘ) was calculated for *F. pringlei* from concurrent gas exchange and Δ measurements at the above range of reference pCO₂ and 19 mbar pO₂, applying the equations previously described and including the ternary effects of transpiration rate (Farquhar and Cernusak, 2012; Evans and von Caemmerer, 2013). This method is only valid for C₃ species. For intermediate and C₄ species, we assumed the same CO₂ response of gₘ found in *F. pringlei*, and scaled the absolute value at ambient pCO₂ to obtain the best fit of the A and Δ models for the observed results (see Results section).

**Mathematical models**

The overall rate of net CO₂ assimilation (A) for C₄-C₃ intermediate plants was previously described (von Caemmerer, 1992, 2013):

\[ A = A_l + A_m \]  

where \( A_m \) is the assimilation in the mesophyll and \( A_l \) is the assimilation in the bundle sheath, which are defined as:

\[ A_l = V_p + \beta F_m - L \]  

\[ A_m = V_m - R_m - (1 - \beta) F_m \]  

so:

\[ A = V_m - R_m - F_m + V_p - L \]  

where \( V_p \) is PEPC carboxylation and \( \beta \) is the fraction of the CO₂ produced from photorespiration in the mesophyll (\( F_m \)) that is released in the bundle sheath. For simplification, bundle sheath respiration and photorespiration are not taken into account in eq. 4. The term \( L \) is the leak rate of CO₂ out of the bundle sheath, and can be expressed as:

\[ L = \phi (V_p + \beta F_m) \]  

and

\[ A = V_m - R_m - F_m + V_p - \phi (V_p + \beta F_m) \]  

where \( \phi \) (leakiness) is the ratio of the leak rate of CO₂ out of the bundle sheath and the supply rate of CO₂ to the bundle sheath (\( V_p + \beta F_m \)). When pO₂ is low, \( F_m \) can be considered 0. \( V_m \) and \( R_m \) are Rubisco carboxylation and day respiration in the mesophyll, respectively, \( V_p \) and \( V_m \) are calculated as described in von Caemmerer (2000):

\[ V_m = \frac{C_m V_{m, max}}{C_m + K_i (1 + \frac{O_i}{K_o})} \]  

\[ V_p = \frac{C_m V_{p, max}}{C_m + K_p} \]  

and

\[ C_m = \frac{A}{g_m} \]  

where \( C_m \) and \( C_i \) are mesophyll and intercellular pCO₂, respectively, \( K_i \) and \( K_o \) are the Michaelis-Menten constants for CO₂ and O₂, respectively, expressed as a partial pressure. Although the pCO₂ in the cytosol (site of PEPC carboxylation) and the chloroplast (site of Rubisco carboxylation) of the mesophyll cell are presumably different due to diffusional limitations, the same value (\( C_p \)) was assumed in both compartments (von Caemmerer, 2000, 2013; Tholen and Zhu, 2011).

When the rate of PEP regeneration is limiting, \( V_p = V_{p, max} \), where \( V_{p, max} \) is the maximum Rubisco carboxylation in the mesophyll, and \( V_p \) can be given by an electron transport limited rate (\( W_f \)), as previously described (von Caemmerer, 2000, 2013).

Theory developed by Farquhar et al. (1982) and Farquhar (1983) showed that photosynthetic carbon isotope discrimination can be described by equations having diffusion and biochemistry dependent terms. The equation of \( \Delta \) presented by Griffiths et al., 2007, which takes into account the effect of \( g_{aw} \), was modified to incorporate the ternary effects of transpiration rate as suggested by Farquhar and Cernusak (2012):

\[ \Delta = \frac{1}{1-t} \left( 1 + t (a_l + b_l - \Delta_{bio}) \right) \frac{A}{g_m C_a} + \frac{1}{1-t} (1 + t) \Delta_{bio} - a_l \frac{C_m}{C_a} \]  

where \( a_l \) is the fractionations during diffusion in water and \( b_l \) is the fractionation as CO₂ enters solution. The term \( t = \frac{(1+a')E}{2g_{aw}} \), where \( E \) denotes the transpiration rate and \( g_{aw} \) the total conductance to CO₂ diffusion including boundary layer and stomatal conductance. The symbol \( a' \) denotes the combined fractionation during diffusion in the boundary layer and in air, and is calculated as:

\[ a' = \frac{a_h (C_a - C_l) + a_l (C_l - C_i)}{(C_a - C_l)} \]  

where \( a \) is the fractionation during diffusion in air, \( a_h \) is the fractionation during diffusion in the boundary layer, and \( C_a, C_l, C_i \) are the pCO₂ in the air, leaf surface and intercellular space respectively.
Table 1. Values assigned to variables for model fitting purposes

When fitting *F. brownii* as a strict C$_4$ and *F. floridana* as a strict C$_3$ species, values were assigned to obtain the best fitting without considering measured enzyme activities.

| Variable          | Definition                                                                 | F. *pringlei* | F. *bidentis* | F. *brownii* | F. *brownii* (strict C$_4$) | F. *floridana* | F. *floridana* (strict C$_3$) | Origin of the value |
|-------------------|-----------------------------------------------------------------------------|---------------|---------------|---------------|-----------------------------|----------------|-----------------------------|-------------------|
| a                 | Fractionation during diffusion in air (‰)                                  | 4.4           | 4.4           | 4.4           | 4.4                         | 4.4            | 4.4                         | Farquhar (1983)   |
| $a_0$             | Fractionation during diffusion through the boundary layer (‰)              | 2.9           | 2.9           | 2.9           | 2.9                         | 2.9            | 2.9                         | Griffiths et al. (2007) |
| abs               | Leaf absorptance                                                          | 0.8           | 0.8           | 0.8           | 0.8                         | 0.8            | 0.8                         | von Caemmerer (2003) |
| $a_1$             | Fractionation during diffusion in water (‰)                                | 0.7           | 0.7           | 0.7           | 0.7                         | 0.7            | 0.7                         | Griffiths et al. (2007) |
| $\beta$           | Fraction of the photorespired CO$_2$ released in the bundle sheath         | 1             | 1             | 1             | 1                           | 1              | 1                           | Assigned          |
| $b_1$             | Fractionation during carboxylation by Rubisco (‰)                         | 29            | 29            | 29            | 29                          | 29             | 29                          | Roeske and O’Leary (1984) |
| $b_2$             | Combined fractionation by the C$_4$ cycle (‰)                              | na            | −5.7          | −5.7          | −5.7                        | na             | na                          | O’Leary (1981) |
| $b_3$             | Fractionation during CO$_2$ dissolution in water (%)                       | 1.1           | 1.1           | 1.1           | 1.1                         | 1.1            | 1.1                         | von Caemmerer (1992) |
| $c$               | $g_m$ scaling constant                                                    | 0.666$^a$     | 0.8$^b$       | 0.666$^b$     | 0.666$^b$                   | 0.78$^b$       | 0.78$^b$                     | Calculated as $\delta^{13}C_{cylinder} - \delta^{13}C_{atmosphere}$ on Caemmerer (2003) |
| $e$               | Fractionation during mitochondrial respiration                             | 2.91          | 3.54          | 3.51          | 3.51                        | 3.72           | 3.72                        | Assigned | von Caemmerer (2000), eq. 5.17 |
| $F$               | Correction coefficient for spectral quality                               | 0.15          | 0.15          | 0.15          | 0.15                        | 0.15           | 0.15                        | Assigned | von Caemmerer (2000), eq. 5.17 |
| $J_t$             | Total electron transport rate (µmol electrons m$^{-2}$ s$^{-1}$)           | 120           | 400           | 440           | 700                         | 250            | 0                           | Assigned | von Caemmerer (2000), eq. 5.17 |
| $J_n$             | Electron transport rate allocated to mesophyll C$_3$ cycle                | 120           | 0             | 40            | 0                           | 200            | 240                         | Assigned | von Caemmerer (2000), eq. 5.17 |
| $K_C$             | Rubisco Michaelis–Menten constant for CO$_2$ (µbar)                       | 359           | 605           | 383           | 383                         | 395            | 395                         | Kubiien et al. (2008) |
| $K_O$             | Rubisco Michaelis–Menten constant for O$_2$ (µbar)                         | 528 000       | 507 000       | 300 000       | 300 000                     | 544 000        | 544 000                     | Kubiien et al. (2008) |
| $K_P$             | PEPC Michaelis–Menten constant for PEP (µbar)                              | n.a.          | 80            | 80            | n.a.                        | 80             | n.a.                        | Bauwe (1986) |
| $R_d$             | Mitochondrial respiration (µmol m$^{-2}$ s$^{-1}$)                         | 0.6           | 0.4           | 1.3           | 1.3                         | 1.7            | 1.7                         | Measured in the dark in this work |
| s                 | Fractionation during leakage (‰)                                           | n.a.          | 1.8           | 1.8           | 1.8                         | n.a.           | n.a.                        | von Caemmerer (1992) |
| $V_{m, max}$      | Maximum Rubisco carboxylation rate in the mesophyll (µmol m$^{-2}$ s$^{-1}$) | 60$^a$        | 0$^b$         | 15$^b$         | 0$^b$                       | 90$^a$         | 130$^b$                     | $^a$, measured in this work; $^b$, assigned |
| $V_{c, max}$      | Maximum PEP carboxylation rate (µmol m$^{-2}$ s$^{-1}$)                    | 0$^a$         | 90$^a$        | 80$^a$        | 80$^a$                      | 15$^a$         | 0$^b$                      | $^a$, measured in this work; $^b$, assigned |
| $V_{Pr}$          | PEP regeneration rate (µmol m$^{-2}$ s$^{-1}$)                             | 0             | 36            | 32            | 50                          | 8              | 0                           | Assigned |
| $\phi$            | Leakiness                                                                  | n.a.          | 0.28          | 0.21          | 0.3                         | 0.40           | n.a.                        | Assigned from model fitting |
| $\theta$          | Empirical curvature factor                                                 | 0.1           | 0.1           | 0.1           | 0.1                         | 0.1            | 0.1                         | Ubierna et al. (2011) |

n.a., not applicable.
The biochemical fractionation, $\Delta_{bio}$, is the integrated net biochemical discrimination, and depends on the biochemistry of net CO$_2$ uptake (Griffiths et al., 2007).

When $\Delta$ and $g_m$ are known, $\Delta_{bio}$ can be solved from equation 10, resulting in:

$$
\Delta_{bio} = \frac{\Delta - \frac{1}{1-t} \left( C_a - C_m \right) - \frac{1+t}{1-t} \frac{A}{g_m} \left( C_a - C_m \right)}{1 + \frac{1+t}{1-t} \frac{A}{g_m} \left( C_a - C_m \right)}
$$

(12)

Because $g_m$ was obtained from combined measurement of $\Delta$ and gas exchange in the C$_4$ species $F.$ pringlei, $\Delta$ and $g_m$ are not independent and we could not estimate $\Delta_{bio}$ from eq. 12. For the intermediate and C$_3$ species, $g_m$ was calculated independently of the $\Delta$ measurements as described in the Materials and Methods section, so $\Delta_{bio}$ could be estimated from eq. 12 for $F.$ floridanana, $F.$ brownii and $F.$ bidentis.

For modeling purposes, or when $\Delta$ is unknown, $\Delta_{bio}$ can be derived from von Caemmerer’s (1992) equation A17:

$$
\frac{R}{R_p} = 1 + \left( b_h - \frac{fF_m + eR_m}{A} \right) \frac{A}{b_h} \phi + \frac{\left( b_h - b_i \right) V_p - f \beta F_m}{V_p + \beta F_m} \frac{f F_i + e R_i}{A} \phi
$$

where $R_i$ and $R_p$ are the molar abundance ratios of $^{13}$C/$^{12}$C in the intercellular space and the photosynthetic product, respectively.

$$
\Delta_{bio} = \frac{R}{R_p} - 1
$$

Thus:

$$
\Delta_{bio} = \left( b_h - \frac{fF_m + eR_m}{A} \right) \frac{A}{b_h} \phi + \frac{\left( b_h - b_i \right) V_p - f \beta F_m}{V_p + \beta F_m} \frac{f F_i + e R_i}{A} \phi
$$

(13)

The factor $b_p$ is the Rubisco fractionation, and $b_i$ is the combined fractionation of PEPC carboxylation and the preceding isotope equilibrium during dissolution of CO$_2$ and conversion to bicarbonate; $s$ is the fractionation during leakage of CO$_2$ out of the bundle sheath; $e$ is the fractionation during mitochondrial respiration; $f$ is the fractionation during photorespiration; $R_m$ and $R_i$ are the mitochondrial respiration rates in the mesophyll and the bundle sheath in the light, respectively. It was assumed that $R_c = R_m + R_i$ and $R_a = R_i = 0.5 R_c$. The factors $F_m$ and $F_i$ are the photorespiration rates derived from Rubisco oxygenation in the mesophyll and the bundle sheath, respectively. When $pO_2$ is low, $F_m$ and $F_i$ are close to 0, so equation 13 simplifies to:

$$
\Delta_{bio} = \left( b_h - \frac{e R_m}{A} \right) \frac{A}{b_h} \phi + \frac{\left( b_h - b_i \right) V_p - f \beta F_m}{V_p + \beta F_m} \frac{f F_i + e R_i}{A} \phi
$$

(14)

The parameter $e$ needs to account for differences between the isotopic composition of CO$_2$ during plant growth and during the measurements, because the substrates used during respiration are most likely carbohydrates assimilated before the experiment (Wingate et al., 2007). No fractionation during mitochondrial respiration was assumed in this work, so $e$ was calculated as the difference between $^{13}$C in the CO$_2$ cylinder used during the experiments and $^{13}$C in the atmosphere during growth conditions ($e = \delta^{13}$C$_{cylinder} - \delta^{13}$C$_{atmosphere}$) (Tazoe et al., 2009; Pengelly et al., 2010). In this work, $\delta^{13}$C$_{cylinder}$ was between −4.12‰ and −5.14‰, and $\delta^{13}$C$_{atmosphere}$ was assumed to be −8‰ (Table 1).

In vitro enzyme activity assays

Leaf discs (0.5 cm$^2$) were collected from the leaves used for gas exchange experiments and frozen in liquid nitrogen immediately after the experiment. Soluble protein was extracted by grinding one frozen leaf disc in a cold Tenbroek homogenizer with 0.5 ml extraction buffer [50 mM HEPES, 1 mM EDTA, 0.1% (v/v) Triton X-100, 10 mM DTT, 1% (v/v) PVP, 1% (v/v) protease inhibitor cocktail (Sigma), pH 7.8]. Extracts were centrifuged at 13,000 rpm for 30 s. Spectrophotometric assays were performed to determine Rubisco and PEPC activities as described in Pengelly et al. (2010).

CA activity was measured in the same extract used for PEPC and Rubisco activity measurements, using a membrane inlet mass spectrometer to measure the rates of $^{18}$O exchange from labeled $^{13}$C$^{18}$O$_2$ to H$_2^{18}$O at 25°C with a subsaturating total carbon concentration of 1 mM (Badger and Price, 1989; von Caemmerer et al., 2004; Cousins et al., 2008). The hydration rates were calculated from the enhancement in the rate of $^{18}$O loss over the uncatalyzed rate, and the nonenzymatic first-order rate constant was applied at pH 7.4 ($k_i = 6.22 \times 10^{-11}$/[H$^+$]$+3.8 \times 10^{-2}$=0.0396), appropriate for the mesophyll cytosol, at a CO$_2$ concentration of 8 mM, which is approximately the CO$_2$ concentration in the mesophyll of $F.$ bidentis (Jenkins et al., 1989; von Caemmerer et al., 2004). When CA is in the chloroplast, which is typically the case in C$_1$ plants like $F.$ pringlei, our calculations underestimate its $in planta$ activity by ~10% due to the effect of the higher chloroplastic pH on $k_i$ ($k_i = 0.0442$ at pH 8).

Results

$O_2$ response of CO$_2$ assimilation rate and compensation point

The effect of $pO_2$ on CO$_2$ assimilation rate and the compensation point ($\Gamma$) was measured at 380 $\mu$bar reference CO$_2$, an irradiance of 1500 $\mu$mol quanta m$^{-2}$ s$^{-1}$ and 25 °C (Fig. 1).

In $F.$ pringlei, increasing $pO_2$ caused a decrease in CO$_2$ assimilation rate, a response typical of a C$_3$ plant. Consistent with this, the $\Gamma$ increased with increasing $pO_2$, ranging from 5.6 $\mu$bar at 19 $\mu$bar O$_2$ to 53 $\mu$bar at 285 $\mu$bar O$_2$.

In the C$_4$ species $F.$ bidentis, the effect of oxygen was very small, with only a 5% decrease in CO$_2$ assimilation rate at the highest tested $pO_2$. $\Gamma$ in these plants barely changed with $pO_2$, and ranged from 0.2 to 1.2 $\mu$bar.

The effect of $O_2$ on $\Gamma$ in $F.$ brownii was also very small and similar to the C$_4$ species $F.$ bidentis, ranging from 1.3 to 3.1 $\mu$bar (Fig. 1b). However, the inhibitory effect of $O_2$ on CO$_2$ assimilation rate was more pronounced, and resulted in an intermediate response of CO$_2$ assimilation rate to increasing $pO_2$ (Fig. 1a).

The $O_2$ response of $\Gamma$ in $F.$ floridanana was intermediate between C$_3$ and C$_4$ species (2.3–18 $\mu$bar; Fig. 1b), as has been previously shown (Ku et al., 1991). However, in our experiments the inhibitory effect of $O_2$ on photosynthesis was smaller than that previously reported by these authors and strikingly similar to that in $F.$ brownii when $pO_2$ was 200 $\mu$bar or lower, despite the important differences in the enzyme compartmentation between these two species (Fig. 1a). Only at 290 $\mu$bar O$_2$ the inhibition of photosynthesis was higher for $F.$ floridanana, with a reduction of a 22%, compared to that in $F.$ brownii (15% inhibition).

Stomatal conductance and C$_4$ increased slightly with $pO_2$, with the exception of $F.$ bidentis, which remained stable, and...
were considerably higher in the C₃ species *F. pringlei* at any pO₂ (Supplementary Fig. S1 at JXB online).

**Rubisco, PEPC and CA activity**

*In vitro* Rubisco, PEPC and CA activities were analyzed in extracts from the same leaves on which the concurrent gas exchange and Δ measurements were made (Fig. 2). Rubisco activity was higher in *F. floridana* (average of 74.9 μmol m⁻² s⁻¹), followed by *F. pringlei* (60.5 μmol m⁻² s⁻¹), *F. brownii* (49.2 μmol m⁻² s⁻¹) and *F. bidentis* (39.7 μmol m⁻² s⁻¹). PEPC activity was lowest in *F. pringlei* (2.9 μmol m⁻² s⁻¹) and, notably, four times higher in *F. floridana* (13.8 μmol m⁻² s⁻¹). *F. brownii* showed a PEPC activity closer to that of *F. bidentis* (79.3 and 91.8 μmol m⁻² s⁻¹ respectively). CA activity was similar and high in *F. bidentis* and *F. brownii* (1278.7 and 1464.5 μmol m⁻² s⁻¹ respectively), and lower in *F. pringlei* and *F. floridana* (614.9 and 623.6 μmol m⁻² s⁻¹ respectively).

The relative activity of PEPC to Rubisco was lowest in *F. pringlei* and highest in *F. bidentis* (Fig. 2b). *F. floridana* showed a PEPC:Rubisco ratio 3.4 times greater than the C₃ species, and *F. brownii* was closer to the C₄ species.

**CO₂ assimilation rate and carbon isotope discrimination**

Measurements of carbon isotope discrimination concurrently with gas exchange were performed under a range of CO₂ concentrations at 19 mbar O₂ on 3–4 plants from each species (Fig. 3). At this low pO₂, photorespiration is greatly reduced and the effect of the C₂ cycle is negligible. Thus, small differences in the level of C₄ activity or mesophyll Rubisco activity are easier to detect.

*F. pringlei* and *F. bidentis* showed the typical C₃ and C₄ response of CO₂ assimilation rate to increasing C₃, respectively (Fig. 3a). The initial slope of the A/Ci curve in *F. floridana* was closer to that in the C₃ species, *F. pringlei*, whereas that of *F. brownii* was more similar to that of the C₃ species, *F. bidentis*, although in both intermediate species the sharp saturation typical of the C₄ species was missing. The maximum apparent assimilation rates in both intermediates were higher than those of the C₃ and C₄ species.

Carbon isotope discrimination measured over the defined range of pCO₂ provided clear differences between the four species (Fig. 3b). Δ was greatest in *F. pringlei* at any C₃, ranging from 16‰ to 24.4‰. Discrimination in *F. floridana* followed a similar trend than that in the C₃ species, with Δ generally increasing with C₃, but Δ was lower than in *F. pringlei* across the whole experimental range, ranging from 12.2‰ to 18.6‰. The response of C/ΔC to CO₂ concentration was parallel to that of Δ in *F. pringlei* and *F. floridana*, reflecting the strong dependence of Δ on the ratio C/ΔC in C₃ species and also in...
Fig. 3. Concurrent measurements of (a, d) CO₂ assimilation rate, A, (b, e) carbon isotope discrimination, Δ, and (c, f) the ratio of intercellular to ambient CO₂, Cᵢ/Cₐ, as a function of intercellular CO₂ (Cᵢ) in F. pringlei, F. floridana, F. brownii and F. bidentis. Values represent averages and standard error of 4 replicates. Measurements were made at 19 mbar Cᵢ, a leaf temperature of 25°C and an irradiance of 1500 μmol m⁻² s⁻¹.

F. floridana (Fig. 3c). The initial decrease of Δ in F. pringlei is also caused by a drop in Cᵢ/Cₐ, which is in turn driven by a reduction of stomatal conductance with increasing Cᵢ when Cᵢ is lower than 200 μbar.

In F. bidentis, as expected from a C₄ plant, discrimination was low (2–4‰) and decreased slightly with increasing Cᵢ. Δ in F. brownii was similar to F. bidentis at Cᵢ under 95 μbar (3.5–2.6‰), but above that the value of Δ increased with increasing Cᵢ, to a maximum of 6.1‰.

Measured Δ is shown with respect to Cᵢ/Cₐ in Fig. 4. The theoretical lines assume infinite mesophyll conductance, which explains why both F. pringlei and F. floridana fell below the theoretical response for C₃ plants, with Δ and Cᵢ/Cₐ generally lower in F. floridana. In F. bidentis, the result was as predicted by a theoretical CO₂ response of Δ for a C₄ plant when φ = 0.25, whereas F. brownii only fitted the expected response at low Cᵢ/Cₐ, with Δ higher than predicted at high Cᵢ/Cₐ.

Modeling CO₂ assimilation rate and carbon isotope discrimination in C₃–C₄ intermediate species

In order to evaluate the contribution of the C₄ cycle to overall photosynthesis in the intermediate species F. floridana and F. brownii, the mathematical models proposed here for Δ and Δ responses to Cᵢ (eqs 6 and 10, respectively) were fitted concurrently to the observed results (Fig. 5). By simultaneously fitting both models using the same set of parameters, the accuracy of the predictions increases because some combinations of assigned constants that may result in a good fit for one of the models are unacceptable for the other. For comparison, the same strategy was also applied to the C₃ and C₄ species (see Supplementary Fig. S2).

Table 1 shows the values assigned for fitting purposes and their source. Rubisco Kₐ and K₀ (Michaelis–Menten constants for CO₂ and O₂, respectively) in the four Flaveria
species analyzed here have been previously reported (Kubien et al., 2008), and \( V_{\text{c,max}} \) and \( V_{\text{p,max}} \) are from our own \textit{in vitro} experiments. We assigned reasonable values for maximum electron transport (\( J_{\text{max}} \)). Leakiness (\( \phi \)) was assigned so that the sum of the squares of the variances between the measured and modeled \( A \), and between the measured and modeled \( \Delta \), was minimum. The distribution of Rubisco between the mesophyll and the bundle sheath in the intermediate species can be adjusted in the models by the assigned \( V_{\text{m,max}} \) (maximum rate of Rubisco carboxylation in the mesophyll) value. When \( V_{\text{m,max}} \) equals the \( V_{\text{c,max}} \) observed \textit{in vitro}, all Rubisco is in the mesophyll. A lower assigned \( V_{\text{m,max}} \) indicates that part of theRubisco activity is contained in the bundle sheath cells.

Mesophyll conductance (\( g_{\text{m}} \)) for \textit{F. pringlei} was calculated from concurrent gas exchange and carbon isotope discrimination measurements at 19 mbar \( O_2 \) and a range of reference \( pCO_2 \) as previously described (Tazoe et al., 2011; Farquhar and Cernusak, 2012; Evans and von Caemmerer, 2013). Results show that \( g_{\text{m}} \) decreases from 0.62±0.1 to 0.33±0.03 mol m\(^{-2}\) s\(^{-1}\) bar\(^{-1}\) with increasing \( C_i \) when atmospheric \( pCO_2 \) is lower than ambient, and then remains stable at higher \( pCO_2 \) (Fig. 6). The CO\(_2\) dependence of \( g_{\text{m}} \) in \textit{F. pringlei} is described by the polinomial function \( g_{\text{m}}=10^{-6}C_i^{-2}+0.0013-C_i+c \), where \( c=0.666 \). In \( C_4 \) and \( C_{37}C_4 \) intermediate species, \( g_{\text{m}} \) cannot be obtained from concurrent gas exchange and \( \Delta^{13}C \) measurements, so the same \( CO_2 \) dependence of \( g_{\text{m}} \) was assumed for \textit{F. bidentis}, \textit{F. brownii}, and \textit{F. floridana}, and the constant \( c \) was calculated from model fitting so that the sum of variances between the measured and modeled \( A \), and between the measured and modeled \( \Delta \), was minimum (Table 1). The resulting \( g_{\text{m}} \) are shown in Fig. 6. Methods for obtaining \( g_{\text{m}} \) in \( C_4 \) and \( C_{37}C_4 \) intermediate species, based on \( ^{18}O \) discrimination measurements, are currently being developed (S. von Caemmerer, unpublished results).

The \( A \) and \( \Delta \) responses to increasing \( C_i \) predicted with this strategy were reasonably close to the measured values for \textit{F. pringlei} and \textit{F. bidentis} (Supplementary Fig. S2).

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**Table 1**

| Species | \( V_{\text{c,max}} \) (mol m\(^{-2}\) s\(^{-1}\) bar\(^{-1}\)) | \( V_{\text{p,max}} \) (mol m\(^{-2}\) s\(^{-1}\) bar\(^{-1}\)) | \( g_{\text{m}} \) (mol m\(^{-2}\) s\(^{-1}\) bar\(^{-1}\)) |
|---------|---------------|----------------|--------------|
| \textit{F. pringlei} | 0.62±0.1 | 0.33±0.03 | 10^{-6}C_i^{-2}+0.0013-C_i+c |
| \textit{F. brownii} | 0.62±0.1 | 0.33±0.03 | 10^{-6}C_i^{-2}+0.0013-C_i+c |
| \textit{F. bidentis} | 0.62±0.1 | 0.33±0.03 | 10^{-6}C_i^{-2}+0.0013-C_i+c |
| \textit{F. floridana} | 0.62±0.1 | 0.33±0.03 | 10^{-6}C_i^{-2}+0.0013-C_i+c |

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**Fig. 5**. Comparison between modeled and measured responses of CO\(_2\) assimilation rate, \( A \), and carbon isotope discrimination, \( \Delta \), to variation in intercellular \( pCO_2 \). \( C_i \) in the \( C_3-C_4 \) intermediate species \textit{F. floridana} and \textit{F. brownii}. Measured \( A \) (a) and \( \Delta \) (b) as a function of \( C_i \) in \textit{F. floridana} (empty triangles), compared with the modeled responses predicted by \( C_3-C_4 \) photosynthetic model assuming an active \( C_4 \) cycle (solid lines) or no \( C_3 \) cycle activity (dashed lines). Measured \( A \) (c) and \( \Delta \) (d) as a function of \( C_i \) in \textit{F. brownii} (white squares), compared with the modeled responses using the \( C_3-C_4 \) models assuming Rubisco activity in the mesophyll cells (solid lines) or a strict compartmentalization of Rubisco in the bundle sheath cells (dashed lines). Parameters used for model simulations are presented in Table 1.

**Fig. 6**. Response of mesophyll conductance (\( g_{\text{m}} \)) to changes in atmospheric \( pCO_2 \). In \textit{F. pringlei}, \( g_{\text{m}} \) was calculated from concurrent gas exchange and \( \Delta \) measurements made at 19 mbar \( pCO_2 \). The values for \textit{F. floridana}, \textit{F. brownii} and \textit{F. bidentis} were assigned assuming the same response of \( g_{\text{m}} \) to \( C_i \) as observed in \textit{F. pringlei}, scaled from model fitting.
In an exercise to prove the predictive value of these models for the presence of low levels of activity of the C₄ component, we attempted to fit the models for *F. floridana* under two different premises. In one case, we assumed a certain level of effective C₄ fractionation to overall carbon assimilation (Fig. 5a, b, solid lines). In the second case, we considered no C₄ activity and values were assigned to obtain the best possible fitting ignoring the measured enzyme activities (Fig. 5a, b, dashed lines). The models could only be fitted to the measured values of Δ and Δbio if some C₄ activity, specified by a Vₚₙₘₐₓ close to our in vitro measurements, was assumed.

A similar approach was used with *F. brownii*. In one case, the models were fitted assuming the presence of Rubisco in the mesophyll, and in the other case the model was fitted as if it were a strict C₄ plant (Fig. 5c, d). The predicted responses approached the measured values only if ~30% of Rubisco activity was located in the mesophyll (Vₚₙₘₐₓ = 15 μmol m⁻² s⁻¹; observed in vitro Vₚₙₘₐₓ = 50 μmol m⁻² s⁻¹).

A comparison of Δ and Δbio highlights the fact that CO₂ diffusion processes have a large influence on Δ (Figs 3, 7). Δbio was calculated from eq. 12 using gas exchange and Δ measured values. Calculation of Δbio factors out the contribution from CO₂ diffusion and shows that the biochemical fractionations are different in the species analyzed. In *F. floridana*, Δbio was high and increasing with Cᵢ; in *F. brownii*, Δbio also increased with increasing Cᵢ, whereas in the C₄ species *F. bidentis* Δbio generally decreases with Cᵢ.

The Δ and Δ responses to Cᵢ could be modeled assuming a constant gₘ without important differences (data not shown). However, the calculation of biochemical fractionation (Δbio) from eq. 12 is dependent on gₘ, and thus the dependence of gₘ on Cᵢ must have an effect on Δbio. To show the magnitude of this effect, the Cᵢ response of Δbio was calculated from eq. 12 and the gas exchange and Δ measurements, assuming either variable gₘ, assigned as previously explained in this section, or constant gₘ calculated as the average of the variable gₘ values obtained for each species (see Supplementary Fig. 83). As a reference, the Cᵢ response of Δbio was calculated from eq. 14 (modelled Δbio) after fitting the models for the Cᵢ responses of Δ and Δ using variable gₘ.

**Estimation of the C₄ (bundle sheath) photosynthesis contribution to total photosynthesis**

The relative contribution of the bundle sheath to total photosynthesis in the intermediate species was estimated from $A_i$ in eq. 2, after fitting the models to our observed results (Fig. 8). Because the experiments were performed under low O₂, photorespiration is greatly reduced and it can be assumed that all the CO₂ assimilated in the bundle sheath is transported by the C₄ cycle. The contribution of the bundle sheath to total photosynthesis in both *F. floridana* and *F. brownii* decreased with increasing Cᵢ. In *F. brownii*, almost all carbon was fixed by Rubisco in the bundle sheath at very low Cᵢ, but up to 25% of fixation occurred via Rubisco in the mesophyll at high Cᵢ. In *F. floridana*, the maximum estimated contribution of the bundle sheath photosynthesis via the C₄ cycle was 21% at very low Cᵢ and it dropped to 12% at the highest Cᵢ analyzed.
Discussion

Effect of $O_2$ on carbon assimilation and compensation point

The oxygen responses of $CO_2$ assimilation and the compensation point have been used in the past as a tool to identify and characterize $C_3$-$C_4$ intermediate species (Sayre and Kennedy, 1977; Monson et al., 1984; Dai et al., 1996; Vogan et al., 2007). As only mesophyll Rubisco is exposed to air oxygen, its effect on $CO_2$ assimilation and $\Gamma$ decreases with increasing proportions of the enzyme allocated to the bundle sheath. However, it is difficult to separate and quantify the effects of the $C_2$ and $C_4$ cycles from studies on the $O_2$ response of $CO_2$ assimilation, as both cycles contribute to reduce the negative effect of photorespiration in carbon assimilation and the compensation point. Moreover, the efficiency of the $C_2$ cycle varies between different intermediate species, as does the contribution of the $C_4$ cycle (Cheng et al., 1988; Keerberg et al., 2014).

In this work, the $O_2$ response of carbon assimilation, and especially $\Gamma$, in $F. brownii$ was very close to that of the $C_4$ species $F. bidentis$. A highly efficient $C_3$ cycle would have a greater impact on the $O_2$ sensitivity of $\Gamma$ than on carbon assimilation and that, combined with high in vitro PEPC and CA activities at the same level as the $C_4$ species $F. bidentis$, eliminates the effect of $pO_2$ on $\Gamma$ almost completely (Cheng et al., 1988; Ku et al., 1991). Previous studies initially classified $F. brownii$ as a $C_4$ species, but it was later demonstrated that the enzyme compartmentation is incomplete in this plant (Monson et al., 1987; Ku et al., 1991). The small proportion of Rubisco present in the mesophyll is reflected in the sensitivity of assimilation rate to $pO_2$.

CA activity in $F. floridana$ is similar to $F. pringlei$ but PEPC activity is four times higher (13.8 $\mu$mol m$^{-2}$ s$^{-1}$), consistent with Ku et al. (1991) and supporting the hypothesis of an active $C_4$ cycle. However, PEPC activity is still low when compared with $F. bidentis$ (91.8 $\mu$mol m$^{-2}$ s$^{-1}$), indicating that the activity of the $C_4$ cycle in this plant is small. In our experiments, the $O_2$ sensitivity of $\Gamma$ in $F. floridana$ is intermediate, and the $O_2$ response of $CO_2$ assimilation rate is remarkably close to that of $F. brownii$.

Previous studies have reported a $C_3$-like $O_2$ response in $F. floridana$ (Dai et al., 1996; Monson et al., 1986), which differs from our observations. Although the reason for this discrepancy is not known, it must be noted that $O_2$ sensitivity measurements are affected by variation of parameters like temperature or stomatal conductance between measurements at different $pO_2$. These interactions increase the difficulty of estimating the activity of the $C_4$ cycle from $O_2$ response experiments.

Signature of $C_4$ photosynthesis in the $CO_2$ response of $\Delta$ in intermediate species

The different $CO_2$ responses of $\Delta$ in the intermediate $C_3$-$C_4$ species, relative to the $C_3$ or $C_4$ species, can be attributed to the different ratios of PEPC/Rubisco activity in the mesophyll. The lower $\Delta$ observed in $F. floridana$, relative to $F. pringlei$, is partially attributable to a lower $C_i/C_a$, but their different $\Delta_{bio}$ indicates an influence of the PEPC to Rubisco ratio, especially at low $C_i$.

Interestingly, $F. brownii$ and $F. bidentis$ show similar $\Delta$ at low $C_i$, but it increases in $F. brownii$ with increasing $pCO_2$ instead of decreasing as in the $C_4$ plant. This particular response can be attributed to the activity of the small fraction of Rubisco in the mesophyll that would have a stronger influence at high $pCO_2$. In $F. floridana$, Rubisco is abundant in the mesophyll but PEPC activity is low, and as a consequence the greatest effect of the $C_4$ cycle activity is observed at very low $pCO_2$, with a greater reduction of $\Delta$ compared to the $C_3$ species. Both results indicate that the contribution of mesophyll Rubisco to overall assimilation is more important under high $pCO_2$, and of the $C_4$ cycle at low $pCO_2$. The fact that environmental conditions affect the contribution of $C_4$ photosynthesis may explain ambiguous results on previous analyses of dry matter $\Delta^{13}C$ in $F. floridana$ and other intermediates, which showed $C_3$-like ratios (Monson et al., 1988; Byrd et al., 1992). $\Delta^{13}C$ is a result of carbon discrimination during the leaf growth, thus it integrates the effect of variable environmental conditions. In the online experiments presented here, instantaneous discrimination is measured under controlled conditions, highlighting their influence. By performing the analyses under low $pO_2$, the effect of photorespiration and subsequent refixation through the $C_2$ cycle is greatly reduced, emphasizing the differences in biochemical fractionation caused by the presence of $C_4$ activity.

Although the $CO_2$ response of $\Delta$ is also influenced by different relative activities of mesophyll Rubisco and PEPC, the effect of each enzyme in this case is difficult to separate. The greater initial slope of the $A/C_i$ curve in $F. floridana$, compared with $F. pringlei$, reflects the slightly greater PEPC activity detected in our in vitro assays, but could also be attributed to higher Rubisco activity. In the same sense, the initial slope of the $A/C_i$ curve in $F. brownii$ and $F. bidentis$ are similar and typically $C_4$, whereas their $\Delta$ are different.

Concurrent model fitting reveals $C_4$ activity in $F. floridana$

The strategy to evaluate the contribution of the $C_4$ cycle to total carbon assimilation in intermediate species presented in this work is based on concurrently measuring and model-fitting the $CO_2$ responses of carbon assimilation and discrimination.

Mathematical modeling has proved to be a powerful tool to get a deeper insight into the biochemical and physiological basis of the observed responses of carbon assimilation and discrimination, and it has been used to estimate parameters such as the maximum carboxylase activity of Rubisco in vivo ($V_{C_{max}}$) and $g_m$, in $C_3$, species, or $V_{p_{max}}$ and leakiness in $C_4$ systems (Tazoe et al., 2011; Ubierna et al., 2011; Walker et al., 2013; Sharwood and Whitney, 2014). However, in most cases there is more than one unknown variable in the equations that represent those responses. This is especially problematic in intermediate species, where the number of factors affecting those responses is greater than in $C_3$ or $C_4$.
plants. By concurrently fitting the CO₂ responses of $A$ and $\Delta$ in each experiment with the same set of constants, the range of values that can be assigned to these variables to obtain a satisfactory fitting is reduced. In this work, the activities of photosynthetic enzymes were analyzed in vitro to further reduce the number of unknowns, providing more accurate predictions. This method confirmed the presence of Rubisco activity in the mesophyll of F. brownii, which was already known (Cheng et al., 1988), but more interestingly indicated that F. floridana harbors an active C₄ cycle. This C₄ activity causes a change in the biochemical fractionation, compared to F. pringlei, which is evident at any $C_3$ analyzed. This is consistent with the increased activity of PEPC and previous observations based on $^{14}$CO₂ pulse-chase experiments (Monson et al., 1986; von Caemmerer and Hubick, 1989). It is important to note that other studies based on δ¹³C analyses, metabolite dynamics and $O_2$ response of carbon assimilation and $\Gamma$ were unable to conclusively prove a contribution of the C₄ cycle to overall photosynthesis in F. floridana, and the presence of a futile C₄ cycle was proposed where most or all the CO₂ released in the bundle sheath is not fixed and leaks back to the mesophyll (Monson et al., 1988; Leegood and von Caemmerer, 1994; Dai et al., 1996). However, other authors have already indicated that in F. floridana the C₄ cycle may contribute up to 50% of the total CO₂ fixation (Ku et al., 1991). In this work, the contribution of the mesophyll and the bundle sheath Rubisco to overall carbon assimilation was calculated for F. brownii and F. floridana. In both intermediate species, the contribution of the C₃ cycle, or bundle sheath Rubisco, is highest at very low pCO₂, and decreases with increasing pCO₂. This reflects the lower apparent $K_s$ of PEPC compared to that of Rubisco (Bauwe, 1986; Kubien et al., 2008).

An improved equation describing CO₂ response of $\Delta$ in intermediate species

An equation describing photosynthetic carbon isotope discrimination ($\Delta$) that is applicable for C₃, C₄ and C₃-C₄ photosynthesis is provided and applied in this study. It allows the calculation of the biochemical fractionation occurring for the different photosynthetic pathways as a function of $C_i$ and takes into account $g_m$ and the ternary effects of transpiration rate. The biological relevance of $g_m$ and its influence on $\Delta$, has been reported extensively and incorporated in mathematical models for C₃ species (Evans et al., 1986; von Caemmerer and Evans, 1991; Tazoe et al., 2011). When mesophyll conductance is considered in C₃ species, $C_c$ (pCO₂ at the site of Rubisco) can be estimated and is lower than $C_i$, and this affects the estimates of Rubisco carboxylations. The same applies in intermediate species, where assimilation and discrimination by mesophyll Rubisco is dependent on the concentration of CO₂ diffusing from the intercellular space. For model fitting purposes, the calculated $C_c$ was used as the available CO₂ for both PEPC and mesophyll Rubisco in the case of the intermediate species. The models presented in this work assume that pCO₂ is the same in the cytosol and the chloroplast.

The effect of pCO₂ on $g_m$ has been studied by other authors, with results depending on the species analyzed. Whereas previous results showed that $g_m$ is not affected by pCO₂ in wheat (Tazoe et al., 2009), other authors reported an inverse correlation in several C₃ species (Flexas et al., 2007; Tazoe et al., 2011). We observed that $g_m$ is dependent on pCO₂ in the C₃ F. pringlei, and assumed that the same is true for the C₄ and intermediate species analyzed. Although the effect of using either constant or variable $g_m$ on the models of the CO₂ responses of carbon assimilation and discrimination has only a minor effect at low $C_i$, it is important for the calculation of $\Delta_{bio}$ and thus the contribution of the C₃ and C₄ cycles to overall carbon assimilation, especially at low $C_i$. The fact that $\Delta_{bio}$ is similar when calculated using either constant or variable $g_m$ in F. brownii and F. bidentis reflects the lower relevance of $g_m$ when the CO₂ concentrating mechanism is expressed at high levels.

Conclusion

Concurrent $\Delta$ and gas exchange measurements and modeling provide a powerful diagnostic tool for C₄ photosynthesis. Performing the measurements under controlled environmental conditions, especially low pO₂, allows the detection and estimation of the C₄ cycle activity in the C₃-C₄ intermediate species even when it is low. This approach confirmed the presence of active Rubisco in the mesophyll of F. brownii, and revealed a contribution of the C₄ cycle to total carbon assimilation in F. floridana. However, the carbon isotope signal is complex and not all its components are well understood, so some caution is required. We show for example that a CO₂ dependence of $g_m$ affects the calculation of the biochemical fractionation, and thus the contribution of the C₄ cycle to overall CO₂ assimilation.

Supplementary data

Supplementary data are available from JXB online.

Figure S1. Responses of $C_i$ and stomatal conductance to changes in atmospheric pO₂.

Figure S2. Models of CO₂ response of assimilation rate and carbon isotope discrimination in the C₃ and C₄ species.

Figure S3. Effect of assuming constant or variable $g_m$ in the calculation of the biochemical fractionation.

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References

Badger MR, Price GD. 1989. Carbonic anhydrase activity associated with the Cyanobacterium Synechococcus PCC7942. Plant Physiology 89, 51–60.
Brown RH, Hattersley PW. 1991. Leaf anatomy of C₄-C₅ species as related to evolution of C₅ photosynthesis. Plant Physiology 91, 1543–1550.

Brown WV. 1975. Variations in anatomy, associations, and origins of Kranz tissue. American Journal of Botany 61, 395–402.

Byrd GT, Brown RH. 1989. Environmental effects on photosynthesis of C₃-C₄ species: I. Influence of CO₂ and O₂ during growth on photosynthetic characteristics and leaf anatomy. Plant Physiology 90, 1022–1028.

Byrd GT, Brown RH, Bouton JH, Bassett CL, Black CC. 1991. Degree of C₄ photosynthesis in C₅ and C₃-C₄ Flaveria species and their hybrids: I. CO₂ assimilation and metabolism and activities of phosphoenolpyruvate carboxylase and NADP-malic enzyme. Plant Physiology 100, 939–946.

Cheng S-H, Moore BD, Edwards GE, Ku MSB. 1988. Photosynthesis in Flaveria brownii, a C₄-like species: leaf anatomy, characteristics of CO₂ exchange, compartmentation of photosynthetic enzymes, and metabolism of ¹⁴CO₂. Plant Physiology 87, 867–873.

Cousins AB, Badger MR, von Caemmerer S. 2008. C₄ photosynthetic isoype exchange in NAD-ME- and NADP-ME-type grasses. Journal of Experimental Botany 59, 1695–1703.

Dai Z, Ku MB, Edwards G. 1996. Oxygen sensitivity of photosynthesis and photoprotection in different photosynthetic types in the genus Flaveria. Planta 189, 563–571.

Ehlertinger JR, Sage RF, Flanagan LB, Pearcy RW. 1991. Climate change and the evolution of C₂ photosynthesis. Trends in Ecology & Evolution 6, 95–99.

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. Functional Plant Biology 13, 281–292.

Evans JR, von Caemmerer S, Setchell BA, Hudson GS. 1994. the relationship between CO₂ transfer conductance and leaf anatomy in transgenic tobacco with a reduced content of Rubisco. Functional Plant Biology 21, 475–495.

Evans JR, von Caemmerer S. 2013. Temperature response of carbon isotope discrimination and mesophyll conductance in tobacco. Plant, Cell & Environment 36, 745–756.

Farquhar GD. 1983. On the Nature of carbon isotope differentiation in C₄ species. Functional Plant Biology 10, 205–226.

Farquhar GD, O’Leary MH, Berry J. 1982. On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. Functional Plant Biology 9, 121–137.

Farquhar GD, Cernusak LA. 2012. Ternary effects on the gas exchange of isotopologues of carbon dioxide. Plant, Cell & Environment 35, 1221–1231.

Flexas J, Diaz-Espejo A, Galmés J, Kaldenhoff R, Medrano H, Ribas-Carbo M. 2007. Rapid variations of mesophyll conductance in response to changes in CO₂ concentration around leaves. Plant, Cell & Environment 30, 1284–1298.

Griffis TJ, Baker JM, Sargent SD, Tanner BD, Zhang J. 2004. Measuring field-scale isotopic CO₂ fluxes with tunable diode laser absorption spectroscopy and micrometeorological techniques. Agricultural and Forest Meteorology 124, 15–29.

Griffiths H, Cousins AB, Badger MR, von Caemmerer S. 2007. Discrimination in the dark. Resolving the interplay between metabolic and physical constraints to phosphoenolpyruvate carboxylase activity during the crassulacean acid metabolism cycle. Plant Physiology 143, 1055–1067.

Hatch MD. 1987. C₃ photosynthesis – a unique blend of modified biochemistry, anatomy and ultrastructure. Biochimica et Biophysica Acta 895, 81–106.

Hatch MD, Slack CR, Johnson HS. 1967. Further studies on a new pathway of photosynthetic carbon dioxide fixation in sugar-cane and its occurrence in other plant species. The Biochemical Journal 102, 417–422.

Henderson SA, von Caemmerer S, Farquhar GD. 1992. Short-term measurements of carbon isotope discrimination in several C₄ species. Functional Plant Biology 19, 283–288.

Hibbard JD, Sheehy JE, Langdale JA. 2008. Using C₄ photosynthesis to increase the yield of rice–rational and feasible. Current Opinion in Plant Biology 11, 228–231.

Holaday AS, Lee K, Chollet R. 1984. C₃-C₄ Intermediate species in the genus Flaveria: leaf anatomy, ultrastructure, and the effect of O₂ on the CO₂ compensation concentration. Planta 160, 25–32.

Jenkins CL, 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.

Karki S, Rizal G, Quick W. 2013. Improvement of photosynthesis in rice (Oryza sativa L.) by inserting the C₄ pathway. Rice 6, 28.

Keerberg O, Pärnik T, Ivanova H, Bassüner B, Bauwe H. 2014. C₄ photosynthesis generates about 3-fold elevated leaf CO₂ levels in the C₃-C₄ intermediate species Flaveria pubescens. Journal of Experimental Botany 65, 3649–3656.

Ku MSB, Wu J, Dai Z, Scott RA, Chu C, Edwards GE. 1991. Photosynthetic and photoresponsive characteristics of Flaveria species. Plant Physiology 96, 518–529.

Kubien DS, Whitney SM, Moore PV, Jesson JK. 2008. The biochemistry of Rubisco in Flaveria. Journal of Experimental Botany 59, 1767–1777.

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

Leegood RC. 2013. Strategies for engineering C₄ photosynthesis. Journal of Plant Physiology 170, 378–388.

Leegood RC, von Caemmerer S. 1994. Regulation of photosynthetic carbon assimilation in leaves of C₃-C₄ intermediate species of Moricandia and Flaveria. Planta 192, 232–238.

McKown AD, Moncalvo JM, Dengler NG. 2005. Phylogeny of Flaveria (Asteraceae) and inference of C₃ photosynthesis evolution. American Journal of Botany 92, 1911–1928.

Monson RK, Edwards GE, Ku MSB. 1989. C₃-C₄ intermediate photosynthesis in plants. BioScience 34, 563–574.

Monson RK, Moore BD, Ku MSB, Edwards GE. 1986. Co-function of C₃- and C₄-photosynthetic pathways in C₃, C₄ and C₃-C₄ intermediate Flaveria species. Planta 168, 493–502.

Monson RK, Schuster WS, Ku MSB. 1987. Photosynthesis in Flaveria brownii AM Powell: a C₄-Like C₃-C₄ intermediate. Plant physiology 85, 1063–1067.

Monson RK, Teeri JA, Ku MSB, Gurveitch J, Mets LJ, Dudley S. 1988. Carbon-isotope discrimination by leaves of Flaveria species exhibiting different amounts of C₃ and C₄ cycle-co function. Planta 174, 145–151.

Moore BD, Ku MSB, Edwards GE. 1989. Expression of C₄-like photosynthesis in several species of Flaveria. Plant, Cell & Environment 12, 541–549.

O’Leary MH. 1981. Carbon isotope fractionation in plants. Phytochemistry 20, 553–567.

Pengelly JJL, Sirault XRR, Tazoe Y, Evans JR, Furbank RT, von Caemmerer S. 2010. Antisense reduction of NADP-malic enzyme in Flaveria bidentis reduces flow of CO₂ through the C₄ cycle. Plant Physiology 160, 1070–1080.

Pengelly JDL, 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.

Rawsthorne S. 1992. C₃-C₄ intermediate photosynthesis: linking physiology to gene expression. The Plant Journal 2, 267–274.

Roeske CA, O’Leary MH. 1984. Carbon isotope effects on enzyme-catalyzed carboxylation of ribulose bisphephosphate. Biochemistry 23, 6275–6284.

Sage RF, Christin PA, Edwards EJ. 2011. The C₃ plant lineages of planet Earth. Journal of Experimental Botany 62, 3155–3169.
A diagnostic tool for \( \text{C}_4 \) photosynthesis

Sage RF, Sage TL, Kocacinar F. 2012. Photorespiration and the evolution of \( \text{C}_4 \) photosynthesis. Annual Review of Plant Biology 63, 19–47.

Sayre RT, Kennedy RA. 1977. Ecotypic differences in the \( \text{C}_3 \) and \( \text{C}_4 \) photosynthetic activity in Mollugo verticillata, a \( \text{C}_3–\text{C}_4 \) intermediate. Planta 134, 257–262.

Schulze S, Mallmann J, Burscheidt J, Koczor M, Streubel M, Bauwe H, Gowik U, Westhoff P. 2013. Evolution of \( \text{C}_4 \) photosynthesis in the genus Flaveria: establishment of a photorespiratory \( \text{CO}_2 \) pump. The Plant Cell 25, 2522–2535.

Sharwood RE, Whitney SM. 2014. Correlating Rubisco catalytic and sequence diversity within \( \text{C}_3 \) plants with changes in atmospheric \( \text{CO}_2 \) concentrations. Plant, Cell & Environment 37, 1981–1984.

Tazoe Y, von Caemmerer S, Badger MR, Evans JR. 2009. Light and \( \text{CO}_2 \) do not affect the mesophyll conductance to \( \text{CO}_2 \) diffusion in wheat leaves. Journal of Experimental Botany 60, 2291–2301.

Ubierna N, Sun W, Cousins AB. 2011. The efficiency of \( \text{C}_4 \) photosynthesis under low light conditions: assumptions and calculations with \( \text{CO}_2 \) isotope discrimination. Journal of Experimental Botany 62, 3119–3134.

Vogan PJ, Frohlich MW, Sage RF. 2007. The functional significance of \( \text{C}_2–\text{C}_4 \) intermediate traits in Heliotropium L. (Boraginaceae): gas exchange perspectives. Plant, Cell & Environment 30, 1337–1345.

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

von Caemmerer S, Evans JR. 1991. Determination of the average partial pressure of \( \text{CO}_2 \) in chloroplast from leaves of several \( \text{C}_3 \) plants. Australian Journal of Plant Physiology 18, 287–305.

von Caemmerer S, Ghannoum O, Pengelly JIL, Cousins AB. 2014. Carbon isotope discrimination as a tool to explore \( \text{C}_4 \) photosynthesis. Journal of Experimental Botany 65, 3459–3470.

von Caemmerer S, Hubick KT. 1989. Short-term carbon-isotope discrimination in \( \text{C}_3–\text{C}_4 \) intermediate species. Planta 178, 475–481.

von Caemmerer S, Quick WP. 2000. Rubisco: physiology in vivo. In: Leegood R, Sharkey T, von Caemmerer S, eds. Photosynthesis , Vol. 9: Springer Netherlands, 85–113.

von Caemmerer S, Quick WP, Furbank RT. 2012. The development of \( \text{C}_4 \) rice: current progress and future challenges. Science 336, 1671–1672.

von Caemmerer S, Quinn V, Hancock NC, Price GD, Furbank RT, Ludwig M. 2004. Carbonic anhydrase and \( \text{C}_4 \) photosynthesis: a transgenic analysis. Plant, Cell & Environment 27, 697–703.

Walker B, Ariza LS, Kaines S, Badger MR, Cousins AB. 2013. Temperature response of in vivo Rubisco kinetics and mesophyll conductance in Arabidopsis thaliana: comparisons to Nicotiana tabacum. Plant, Cell & Environment 36, 2108–2119.

Wang L, Czedik-Eysenberg A, Mertz RA, et al. 2014. Comparative analyses of \( \text{C}_4 \) and \( \text{C}_3 \) photosynthesis in developing leaves of maize and rice. Nature Biotechnology 32, 1158–1165.

Whitney SM, Sharwood RE, Orr D, White SJ, Alonso H, Galmés J. 2011. Isoleucine 309 acts as a \( \text{C}_4 \) catalytic switch that increases ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) carboxylation rate in Flaveria. Proceedings of the National Academy of Sciences, USA 108, 14688–14693.

Wingate L, Seibt U, Moncrieff JB, Jarvis PG, Lloyd JON. 2007. Variations in \( \text{^{13}}\text{C} \) discrimination during \text{CO}_2 exchange by Picea sitchensis branches in the field. Plant, Cell & Environment 30, 600–616.