RESEARCH PAPER

Carbon isotope discrimination as a diagnostic tool for C₄ photosynthesis in C₃-C₄ 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

Received 22 October 2015; Revised 9 December 2015; Accepted 11 December 2015

Editor: Christine Raines, University of Essex

Abstract

The presence and activity of the C₄ cycle in C₃-C₄ intermediate species have proven difficult to analyze, especially when such activity is low. This study proposes a strategy to detect C₄ 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 CO₂ responses of CO₂ assimilation (A) and carbon isotope discrimination (Δ) under low O₂ partial pressure. Mathematical models of C₃-C₄ photosynthesis and Δ 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 C₃ and C₄ controls, respectively. Our results support the presence of a functional C₄ cycle in F. floridana, that can fix 12–21% of carbon. In F. brownii, 75–100% of carbon is fixed via the C₄ cycle, and the contribution of mesophyll Rubisco to overall carbon assimilation increases with CO₂ partial pressure in both intermediate plants. Combined gas exchange and Δ measurement and modeling is a powerful diagnostic tool for C₄ photosynthesis.

Key words: Carbon isotope discrimination, C₃-C₄, intermediate photosynthesis, Flaveria, F. brownii, F. floridana.

Introduction

C₄ photosynthesis is a highly efficient carbon fixation system characterized by the presence of a biochemical carbon pump with the capacity of increasing the CO₂ partial pressure (pCO₂) 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 photorespiration, potentially improving nitrogen and water use efficiency (Hibberd et al., 2008; Langdale, 2011). Most C₄ 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). CO₂ 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 CO₂ is refixed by Rubisco.

The most productive crops, such as maize, sorghum and sugar cane, are C₄ plants, exemplifying the higher efficiency of this system over the C₃ 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 C₄ photosynthesis in to C₃
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 glycolate shuttle, transports glycolate formed during mesophyll photorespiration to the bundle sheath where it is decarboxylated and the CO2 refixed, 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 later 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 C3 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 C3-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 30 1 pots in a garden soil mix fertilized with Osmocote (Scotts, Australia) in a greenhouse 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,
Concurrent gas exchange and $\Delta$ 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; Griffis 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, Thermoline 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 μbar $pCO_2$. The desired $pCO_2$ was achieved as described above and supplied to the Li-Cors 6400. Reference $pCO_2$ was changed stepwise to 392, 980, 686, 490, 294, 196, 98, 49 and 392 μbar and measurements were made every 4 min for at least 30 min at each $pCO_2$. Dark respiration ($R_d$) was measured at the end of an $A/C_i$ curve at 392 μbar $pCO_2$ and 20 μbar $pO_2$ by switching off the Li-Cor lamp. Three or four plants from each species were analyzed. $\Delta$ was calculated as previously described (Evans et al., 1986; Evans and von Caemmerer, 2013).

Mesophyll conductance ($g_{mm}$) was calculated for $F. pringlei$ from concurrent gas exchange and $\Delta$ measurements at the above range of reference $pCO_2$ and 19 μbar $pO_2$, 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 $CO_2$ response of $g_{mm}$ found in $F. pringlei$, and scaled the absolute value at ambient $pCO_2$ to obtain the best fit of the $A$ and $\Delta$ models for the observed results (see Results section).

Mathematical models

The overall rate of net $CO_2$ assimilation ($A$) for C₃-C₄ intermediate plants was previously described (von Caemmerer, 1992, 2013):

$$A = A_i + A_m$$

(1)

where $A_m$ is the assimilation in the mesophyll and $A_i$ is the assimilation in the bundle sheath, which are defined as:

$$A_m = V_p + \beta F_m - L$$

(2)

$$A_i = V_m - R_m - (1 - \beta) F_m$$

(3)

so:

$$A = V_m - R_m - F_m + V_p - L$$

(4)

where $V_p$ is PEPC carboxylation and $\beta$ is the fraction of the $CO_2$ 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 $CO_2$ out of the bundle sheath, and can be expressed as:

$$L = \phi(V_p + \beta F_m)$$

(5)

and

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

(6)

where $\phi$ (leakiness) is the ratio of the leak rate of $CO_2$ out of the bundle sheath and the supply rate of $CO_2$ to the bundle sheath ($V_p + \beta F_m$). When $pCO_2$ 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):

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

(7)

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

(8)

$$C_m = C_i = \frac{A}{g_m}$$

(9)

where $C_m$ and $C_i$ are mesophyll and intercellular $pCO_2$, respectively. $K_i$ and $K_o$ are the Michaelis-Menten constants for $CO_2$ and $O_2$, respectively, expressed as a partial pressure. Although the $pCO_2$ 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 ($C_i$) was assumed in both compartments (von Caemmerer, 2000, 2013; Tholen and Zhu, 2011).

When the rate of PEP regeneration is limiting, $V_p = V_{pe}$, where $V_{pe}$ is a constant. $V_{m,max}$ is the maximum Rubisco carboxylation in the mesophyll, and $V_{p,max}$ is the maximum PEPC carboxylation (Table 1). When RuBP becomes limiting, $V_m$ in eq. 6 can be given by an electron transport limited rate ($W_i$), 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 $g_{mm}$, was modified to incorporate the ternary effects of transpiration rate as suggested by Farquhar and Cernusak (2012):

$$\Delta = \frac{1}{1-t} a' + \frac{1+t}{1-t} (a_i + b_i - \Delta_{bio}) - \frac{A}{g_m} C_a$$

(10)

where $a_i$ is the fractionations during diffusion in water and $b_i$ is the fractionation as $CO_2$ enters solution. The term $t = \frac{(1+a')E}{2g_{aw}}$, where $E$ denotes the transpiration rate and $g_{aw}$ the total conductance to $CO_2$ 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:

$$a' = \frac{a_b (C_a - C_i) + a(C_i - C_l)}{(C_a - C_l)}$$

(11)

where $a$ is the fractionation during diffusion in air, $a_b$ is the fractionation during diffusion in the boundary layer, and $C_a$, $C_i$, $C_l$ are the $pCO_2$ 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 C₄ and *F. floridana* as a strict C₃ species, values were assigned to obtain the best fitting without considering measured enzyme activities.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Definition</th>
<th>F. pringlei</th>
<th>F. bidentis</th>
<th>F. brownii</th>
<th>F. brownii (strict C₄)</th>
<th>F. floridana</th>
<th>F. floridana (strict C₃)</th>
<th>Origin of the value</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Fractionation during diffusion in air (‰)</td>
<td>4.4</td>
<td>4.4</td>
<td>4.4</td>
<td>4.4</td>
<td>4.4</td>
<td>4.4</td>
<td>Farquhar (1983)</td>
</tr>
<tr>
<td>aₐ</td>
<td>Fractionation during diffusion through the boundary layer (‰)</td>
<td>2.9</td>
<td>2.9</td>
<td>2.9</td>
<td>2.9</td>
<td>2.9</td>
<td>2.9</td>
<td>Griffiths et al. (2007)</td>
</tr>
<tr>
<td>abs</td>
<td>Leaf absorptance</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>von Caemmerer (2000)</td>
</tr>
<tr>
<td>aₙ</td>
<td>Fractionation during diffusion in water (‰)</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>Griffiths et al. (2007)</td>
</tr>
<tr>
<td>β</td>
<td>Fraction of the photosynthesized CO₂ released in the bundle sheath</td>
<td>1.0</td>
<td>1.0</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>Assigned</td>
</tr>
<tr>
<td>bₐ</td>
<td>Fractionation during carboxylation by Rubisco (‰)</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>Roeske and O'Leary (1984)</td>
</tr>
<tr>
<td>bₐ</td>
<td>Combined fractionation by the C₄ cycle (‰)</td>
<td>na</td>
<td>−5.7</td>
<td>−5.7</td>
<td>−5.7</td>
<td>−5.7</td>
<td>na</td>
<td>O'Leary (1981)</td>
</tr>
<tr>
<td>bₐ</td>
<td>Fractionation during CO₂ dissolution in water (‰)</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>von Caemmerer (1992)</td>
</tr>
<tr>
<td>c</td>
<td>gₚ scaling constant</td>
<td>0.666ᵇ</td>
<td>0.8ᵇ</td>
<td>0.666ᵇ</td>
<td>0.666ᵇ</td>
<td>0.78ᵇ</td>
<td>0.78ᵇ</td>
<td>Calculated as ( e = \delta_{13}^{C_{\text{cylinder}}} - \delta_{13}^{C_{\text{atmosphere}}} )</td>
</tr>
<tr>
<td>e</td>
<td>Fractionation during mitochondrial respiration</td>
<td>2.91</td>
<td>3.54</td>
<td>3.51</td>
<td>3.51</td>
<td>3.72</td>
<td>3.72</td>
<td>von Caemmerer (2003)</td>
</tr>
<tr>
<td>F</td>
<td>Correction coefficient for spectral quality</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>Assigned (von Caemmerer (2000), eq. 5.17)</td>
</tr>
<tr>
<td>Jₜ</td>
<td>Total electron transport rate (µmol electrons m⁻² s⁻¹)</td>
<td>120</td>
<td>400</td>
<td>440</td>
<td>700</td>
<td>250</td>
<td>0</td>
<td>Assigned (von Caemmerer (2000), eq. 5.17)</td>
</tr>
<tr>
<td>Jₘ</td>
<td>Electron transport rate allocated to mesophyll C₃ cycle</td>
<td>120</td>
<td>0</td>
<td>40</td>
<td>0</td>
<td>200</td>
<td>240</td>
<td>Assigned (von Caemmerer (2000), eq. 5.17)</td>
</tr>
<tr>
<td>Kₐ</td>
<td>Rubisco Michaelis–Menten constant for CO₂ (µbar)</td>
<td>359</td>
<td>605</td>
<td>383</td>
<td>383</td>
<td>395</td>
<td>395</td>
<td>Kubien et al. (2008)</td>
</tr>
<tr>
<td>Kₒ</td>
<td>Rubisco Michaelis–Menten constant for O₂ (µbar)</td>
<td>528 000</td>
<td>507 000</td>
<td>300 000</td>
<td>300 000</td>
<td>544 000</td>
<td>544 000</td>
<td>Kubien et al. (2008)</td>
</tr>
<tr>
<td>Kₚ</td>
<td>PEPC Michaelis–Menten constant for PEP (µbar)</td>
<td>n.a.</td>
<td>80</td>
<td>80</td>
<td>n.a.</td>
<td>80</td>
<td>n.a.</td>
<td>von Caemmerer (2000)</td>
</tr>
<tr>
<td>Rₜ</td>
<td>Mitochondrial respiration (µmol m⁻² s⁻¹)</td>
<td>0.6</td>
<td>0.4</td>
<td>1.3</td>
<td>1.3</td>
<td>1.7</td>
<td>1.7</td>
<td>Measured in the dark in this work</td>
</tr>
<tr>
<td>s</td>
<td>Fractionation during leakage (‰)</td>
<td>n.a.</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
<td>n.a.</td>
<td>von Caemmerer (1992)</td>
</tr>
<tr>
<td>Vₘₙ, max</td>
<td>Maximum Rubisco carboxylation rate in the mesophyll (µmol m⁻² s⁻¹)</td>
<td>60ᵃ</td>
<td>0ᵇ</td>
<td>15ᵇ</td>
<td>0ᵇ</td>
<td>90ᵃ</td>
<td>130ᵇ</td>
<td>ᵃ, measured in this work; ᵇ, assigned</td>
</tr>
<tr>
<td>Vₘₜ, max</td>
<td>Maximum PEP carboxylation rate (µmol m⁻² s⁻¹)</td>
<td>0ᵃ</td>
<td>90ᵃ</td>
<td>80ᵃ</td>
<td>80ᵃ</td>
<td>15ᵃ</td>
<td>0ᵇ</td>
<td>ᵃ, measured in this work; ᵇ, assigned</td>
</tr>
<tr>
<td>Vₘₜ</td>
<td>PEP regeneration rate (µmol m⁻² s⁻¹)</td>
<td>0</td>
<td>36</td>
<td>32</td>
<td>50</td>
<td>8</td>
<td>0</td>
<td>Assigned</td>
</tr>
<tr>
<td>φ</td>
<td>Leakiness</td>
<td>n.a.</td>
<td>0.28</td>
<td>0.21</td>
<td>0.3</td>
<td>0.40</td>
<td>n.a.</td>
<td>Assigned from model fitting</td>
</tr>
<tr>
<td>θ</td>
<td>Empirical curvature factor</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>Ubierna et al. (2011)</td>
</tr>
</tbody>
</table>

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:

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

(12)

Because $g_m$ was obtained from combined measurement of $\Delta$ and gas exchange in the C$_3$ 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$_4$ 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. floridana, 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:

$$\frac{R_s}{R_p} = 1 + \frac{(b - \frac{fF_m + eR_m}{A}) + A}{A}[(b - s)\phi] + \frac{(b - b_s)\frac{V_p - f^\beta F_m}{V_p + \beta F_m}}{A} - \frac{fF_s + eR_s}{A}\phi$$

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

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

Thus:

$$\Delta_{bio} = \frac{(b - \frac{fF_m + eR_m}{A}) + A}{A}[(b - s)\phi] + \frac{(b - b_s)\frac{V_p - f^\beta F_m}{V_p + \beta F_m}}{A} - \frac{fF_s + eR_s}{A}\phi$$

(13)

The factor $b_s$ is the Rubisco fractionation, and $b_s$ is the combined fractionation of PEP 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 $R_s$ are the mitochondrial respiration rates in the mesophyll and the bundle sheath in the light, respectively. It was assumed that $R_c=R_m+R_s$ and $R_{c}=0.5R_s$. The factors $F_m$ and $F_s$ are the photorespiration rates derived from Rubisco oxygenation in the mesophyll and the bundle sheath, respectively. When $pO_2$ is low, $F_m$ and $F_s$ are close to 0, so equation 13 simplifies to:

$$\Delta_{bio} = \frac{(b - \frac{eR_m}{A}) + A}{A}[(b - s)\phi] + \frac{(b - b_s)\frac{V_p - f^\beta F_m}{V_p + \beta F_m}}{A} - \frac{eR_s}{A}\phi$$

(14)

The parameter $e$ needs to account for differences between the isotope 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 $\delta^{13}$C in the CO$_2$ cylinder used during the experiments and $\delta^{13}$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).

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 μbar reference CO$_2$, an irradiance of 1500 μmol 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 μbar at 19 mbar O$_2$ to 53 μbar at 285 μbar 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 $pO_2$. In these plants barely changed with $pO_2$, and ranged from 0.2 to 1.2 μbar.

The effect of CO$_2$ 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 μbar (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. floridana was intermediate between C$_3$ and C$_4$ species (2.3–18 μbar; 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 μbar or lower, despite the important differences in the enzyme compartmentation between these two species (Fig. 1a). Only at 290 μbar O$_2$ the inhibition of photosynthesis was higher for F. floridana, with a reduction of a 22%, compared to that in F. brownii (15% inhibition).

Stomatal conductance and C$_i$ 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. bidentis* (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 Cᵣ, respectively (Fig. 3a). The initial slope of the A/Cᵣ 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 C₄ 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 Cᵣ, 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 Cᵣ, but Δ was lower than in *F. pringlei* across the whole experimental range, ranging from 12.2% to 18.6%.

The response of Cᵣ/CO₂ concentration was parallel to that of Δ in *F. pringlei* and *F. floridana*, reflecting the strong dependence of Δ on the ratio Cᵣ/CO₂ in C₃ species and also in...
F. floridana (Fig. 3c). The initial decrease of $\Delta$ in F. pringlei is also caused by a drop in $C_i/C_a$, which is in turn driven by a reduction of stomatal conductance with increasing $C_i$ when $C_i$ is lower than 200 mbar.

In F. bidentis, as expected from a C_4 plant, discrimination was low (2–4‰) and decreased slightly with increasing $C_i$. $\Delta$ in F. brownii was similar to F. bidentis at $C_i$ under 95 mbar (3.5–2.6‰), but above that the value of $\Delta$ increased with increasing $C_i$, to a maximum of 6.1‰.

Measured $\Delta$ is shown with respect to $C_i/C_a$ 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 C_3 plants, with $\Delta$ and $C_i/C_a$ generally lower in F. floridana. In F. bidentis, the result was as predicted by a theoretical CO_2 response of $\Delta$ for a C_4 plant when $\phi = 0.25$, whereas F. brownii only fitted the expected response at low $C_i/C_a$, with $\Delta$ higher than predicted at high $C_i/C_a$.

**Modeling CO_2 assimilation rate and carbon isotope discrimination in C_3-C_4 intermediate species**

In order to evaluate the contribution of the C_4 cycle to overall photosynthesis in the intermediate species F. floridana and F. brownii, the mathematical models proposed here for $\Delta$ and $\Delta$ responses to $C_i$ (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 C_3 and C_4 species (see Supplementary Fig. S2).

Table 1 shows the values assigned for fitting purposes and their source. Rubisco $K_C$ and $K_O$ (Michaelis–Menten constants for CO_2 and O_2, respectively) in the four Flaveria
species analyzed here have been previously reported (Kubien et al., 2008), and $V_{c,max}$ and $V_{p,max}$ are from our own in vitro experiments. We assigned reasonable values for maximum electron transport ($J_{max}$). 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 $V_{m,max}$ (maximum rate of Rubisco carboxylation in the mesophyll) value. When $V_{m,max}$ equals the $V_{c,max}$ observed in vitro, all Rubisco is in the mesophyll. A lower assigned $V_{m,max}$ indicates that part of the Rubisco activity is contained in the bundle sheath cells.

Mesophyll conductance ($g_m$) for $F. pringlei$ 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 $g_m$ decreases from $0.62 \pm 0.1$ to $0.33 \pm 0.03$ 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 $g_m$ in $F. pringlei$ is described by the polinomial function $g_m = 10^{-6}C_i^2 - 0.0013C_i + c$, where $c=0.666$. In $C_4$ and $C_3$-$C_4$ intermediate species, $g_m$ cannot be obtained from concurrent gas exchange and $\Delta^{13}C$ measurements, so the same CO$_2$ dependence of $g_m$ was assumed for $F. bidentis$, $F. brownii$ and $F. floridana$, 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 $g_m$ are shown in Fig. 6. Methods for obtaining $g_m$ in $C_4$ and $C_3$-$C_4$ 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 $F. pringlei$ and $F. bidentis$ (Supplementary Fig. S2).
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 cycle contribution 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 if some C4 activity, specified by a *V* _p,max_ 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 (*V* _m,max_ = 15 μmol m⁻² s⁻¹; observed in vitro *V* _m,max_ = 50 μmol m⁻² s⁻¹). A comparison of Δ and Δ _bio_ highlights the fact that CO2 diffusion processes have a large influence on Δ (Figs 3, 7). Δ _bio_ was calculated from eq. 12 using gas exchange and Δ measured values. Calculation of Δ _bio_ factors out the contribution from CO2 diffusion and shows that the biochemical fractionations are different in the species analyzed. In *F. floridana*, Δ _bio_ was high and increasing with C_i. In *F. brownii*, Δ _bio_ also increased with increasing C_i, whereas in the C4 species *F. bidentis* Δ _bio_ generally decreases with C_i.

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

**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 C_i. In *F. brownii*, almost all carbon was fixed by Rubisco in the bundle sheath at very low C_i, but up to 25% of fixation occurred via Rubisco in the mesophyll at high C_i. In *F. floridana*, the maximum estimated contribution of the bundle sheath photosynthesis via the C4 cycle was 21% at very low C_i and it dropped to 12% at the highest C_i analyzed.

**Fig. 7.** Biochemical fractionation (Δ _bio_ ) as a function of intercellular CO2 (C_i) in *F. floridana*, *F. brownii* and *F. bidentis*. Δ _bio_ was calculated from eq. 12 using the combined gas exchange and Δ measurements shown in Fig. 3. Δ _bio_ could not be calculated for *P. pringlei* because g_m is obtained from Δ measurements in this species, so both factors are not independent. Values represent averages and standard error of four replicates.

**Fig. 8.** CO2 response of the estimated contribution of the C4 cycle and the mesophyll C3 cycle in the intermediate species *F. floridana* and *F. brownii*, expressed as a percent of total CO2 assimilation rate, under low pO2.
Discussion

Effect of O2 on carbon assimilation and compensation point

The oxygen responses of CO2 assimilation and the compensation point have been used in the past as a tool to identify and characterize C3-C4 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 CO2 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 C2 and C4 cycles from studies on the O2 response of CO2 assimilation, as both cycles contribute to reduce the negative effect of photorespiration in carbon assimilation and the compensation point. Moreover, the efficiency of the C2 cycle varies between different intermediate species, as does the contribution of the C4 cycle (Cheng et al., 1988; Keerberg et al., 2014).

In this work, the O2 response of carbon assimilation, and especially \( \Gamma \), in F. brownii was very close to that of the C4 species F. bidentis. A highly efficient C3 cycle would have a greater impact on the O2 sensitivity of \( \Gamma \) than on carbon assimilation and that, combined with high in vitro PEPC and CA activities at the same level as the C4 species F. bidentis, eliminates the effect of \( \rho \)O2 on \( \Gamma \) almost completely (Cheng et al., 1988; Ku et al., 1991). Previous studies initially classified F. brownii as a C4 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 \( \rho \)O2.

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 C4 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 C4 cycle in this plant is small. In our experiments, the O2 sensitivity of \( \Gamma \) in F. floridana is intermediate, and the O2 response of CO2 assimilation rate is remarkably close to that of F. brownii.

Previous studies have reported a C4-like O2 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 O2 sensitivity measurements are affected by variation of parameters like temperature or stomatal conductance between measurements at different \( \rho \)O2. These interactions increase the difficulty of estimating the activity of the C4 cycle from O2 response experiments.

Signature of C4 photosynthesis in the CO2 response of $\Delta$ in intermediate species

The different CO2 responses of \( \Delta \) in the intermediate C3-C4 species, relative to the C3 or C4 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/\( \delta \)C, but their different \( \Delta_{\text{bio}} \) indicates an influence of the PEPC to Rubisco ratio, especially at low \( \delta \)C.

Interestingly, F. brownii and F. bidentis show similar \( \Delta \) at low \( \delta \)C, but it increases in F. brownii with increasing \( \rho \)CO2 instead of decreasing as in the C4 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 \( \rho \)CO2. In F. floridana, Rubisco is abundant in the mesophyll but PEPC activity is low, and as a consequence the greatest effect of the C4 cycle activity is observed at very low \( \rho \)CO2, with a greater reduction of \( \Delta \) compared to the C3 species. Both results indicate that the contribution of mesophyll Rubisco to overall assimilation is more important under high \( \rho \)CO2, and of the C4 cycle at low \( \rho \)CO2. The fact that environmental conditions affect the contribution of C4 photosynthesis may explain ambiguous results on previous analyses of dry matter \( \delta^{13} \)C in F. floridana and other intermediates, which showed C3-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, instant discrimination is measured under controlled conditions, highlighting their influence. By performing the analyses under low \( \rho \)O2, the effect of photorespiration and subsequent refixation through the C2 cycle is greatly reduced, emphasizing the differences in biochemical fractionation caused by the presence of C4 activity.

Although the CO2 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/\( \delta \)C 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/\( \delta \)C curve in F. brownii and F. bidentis are similar and typically C4, whereas their \( \Delta \) are different.

Concurrent model fitting reveals C4 activity in F. floridana

The strategy to evaluate the contribution of the C4 cycle to total carbon assimilation in intermediate species presented in this work is based on concurrently measuring and model-fitting the CO2 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 (\( V_{c_{\text{max}}} \)) and \( g_m \) in C3 species, or \( V_{p_{\text{max}}} \) and leakiness in C4 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 C3 or C4.
plants. By concurrently fitting the CO2 responses of A and Δ 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 in any C4 analyzed. This is consistent with the increased activity of PEPC and previous observations based on 14CO2 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 O2 response of carbon assimilation and Γ 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 C4 cycle, or bundle sheath Rubisco, is highest at very low pCO2, and decreases with increasing pCO2. This reflects the lower apparent Kc of PEPC compared to that of Rubisco (Bauwe, 1986; Kubien et al., 2008).

An improved equation describing CO2 response of Δ in intermediate species

An equation describing photosynthetic carbon isotope discrimination (Δ) 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 Ci and takes into account gm and the ternary effects of transpiration rate. The biological relevance of gm and its influence on Δ 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, Cc (pCO2 at the site of Rubisco) can be estimated and is lower than Ci, 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 Cc 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 gm has been studied by other authors, with results depending on the species analyzed. Whereas previous results showed that gm is not affected by pCO2 in wheat (Tazoe et al., 2009), other authors reported an inverse correlation in several C4 species (Flexas et al., 2007; Tazoe et al., 2011). We observed that gm 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 gm on the models of the CO2 responses of carbon assimilation and discrimination has only a minor effect at low Ci, it is important for the calculation of Δbiog and thus the contribution of the C4 and C3 cycles to overall carbon assimilation, especially at low Ci. The fact that Δbiog is similar when calculated using either constant or variable gm in F. brownii and F. bidentis reflects the lower relevance of gm when the CO2 concentrating mechanism is expressed at high levels.

Conclusion

Concurrent Δ 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 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 gm 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.

Supplementary Figure S1. Responses of Ci and stomatal conductance to changes in atmospheric pO2.

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

Supplementary Figure S3. Effect of assuming constant or variable gm in the calculation of the biochemical fractionation.

Acknowledgments

We thank Soumi Bala for expert technical assistance with plant culture, biochemical assays, TDL and gas exchange measurements. We thank the High Resolution Plant Phenomics Centre (CSIRO, Australia) for the use of their TDL for some experiments. This research was supported by the Bill and Melinda Gates Foundation's funding for the C4 Rice consortium and by the Australian Research Council Centre of Excellence for Translational Photosynthesis (CE140100015).

References


