Carbonic Anhydrase and Its Influence on Carbon Isotope Discrimination during C₄ Photosynthesis. Insights from Antisense RNA in Flaveria bidentis

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In C₄ plants, carbonic anhydrase (CA) facilitates both the chemical and isotopic equilibration of atmospheric CO₂ and bicarbonate (HCO₃⁻) in the mesophyll cytoplasm. The CA-catalyzed reaction is essential for C₄ photosynthesis, and the model of carbon isotope discrimination (Δ₁³C) in C₄ plants predicts that changes in CA activity will influence Δ₁³C. However, experimentally, the influence of CA on Δ₁³C has not been demonstrated in C₄ plants. Here, we compared measurements of Δ₁³C during C₄ photosynthesis in Flaveria bidentis wild-type plants with F. bidentis plants with reduced levels of CA due to the expression of antisense constructs targeted to a putative mesophyll cytosolic CA. Plants with reduced CA activity had greater Δ₁³C, which was also evident in the leaf dry matter carbon isotope composition (δ¹³C). Contrary to the isotope measurements, photosynthetic rates were not affected until CA activity was less than 20% of wild type. Measurements of Δ₁³C, δ¹³C of leaf dry matter, and rates of net CO₂ assimilation were all dramatically altered when CA activity was less than 5% of wild type. CA activity in wild-type F. bidentis is sufficient to maintain net CO₂ assimilation; however, reducing leaf CA activity has a relatively large influence on Δ₁³C, often without changes in net CO₂ assimilation. Our data indicate that the extent of CA activity in C₄ leaves needs to be taken into account when using Δ₁³C and/or δ¹³C to model the response of C₄ photosynthesis to changing environmental conditions.

Isotope analysis of atmospheric CO₂ is an important tool for monitoring changes in the global exchange of CO₂ (Flanagan and Ehleringer, 1998; Yakir and Sternberg, 2000). However, to interpret the atmospheric CO₂ isotopic signature requires an understanding of the isotopic fractionation steps associated with specific processes during leaf gas exchange (Yakir and Sternberg, 2000). Leaf level models of carbon isotope exchange (Δ₁³C) in C₄ plants have been used for many years to help interpret the response of C₄ plants to changing environmental conditions. However, only recently has the genetic manipulation of the C₄ photosynthetic apparatus provided an opportunity to reexamine the C₄ leaf level models of Δ₁³C (von Caemmerer et al., 1997a, 1997b).

Most C₄ plants utilize a compartmentalized CO₂-concentrating mechanism between the mesophyll and bundle sheath cells (BSC) to increase the CO₂ partial pressure (pCO₂) around the site of Rubisco in the BSC. The first enzymatic step in C₄ photosynthesis is the reversible hydration reaction catalyzed by carbonic anhydrase (CA), which converts CO₂ to bicarbonate (HCO₃⁻) in the mesophyll cytoplasm. Subsequently, HCO₃⁻ is fixed via phosphoenolpyruvate carboxylase (PEPC) into a four-carbon acid that diffuses to the BSC for decarboxylation (Kanai and Edwards, 1999). The specialized biochemistry and leaf anatomy of C₄ plants results in a pCO₂ around the site of Rubisco several-fold higher than current atmospheric levels, significantly reducing the rates of photorespiration (Hatch, 1987; Kanai and Edwards, 1999).

The carbon isotope discrimination during C₄ photosynthesis is determined by the fractionation that occurs during diffusion of CO₂ into the leaf, its conversion to HCO₃⁻ via CA, and the subsequent carboxylation reactions catalyzed by PEPC and Rubisco (Peisker, 1982; Farquhar, 1983; Peisker and Henderson, 1992; von Caemmerer et al., 1997a). The extent to which Rubisco can fractionate against CO₂ is determined by the amount of leakiness (φ), defined as the fraction of CO₂ fixed by PEPC that subsequently leaks out of the BSC. If the BSC were gas tight, then all of the CO₂ released into the BSC would be fixed by Rubisco and no fractionation would occur at this step. However, CO₂ can leak out of the BSC, allowing Rubisco to influence the overall discrimination during C₄ photosynthesis (Farquhar, 1983; Peisker and Henderson, 1992).

Differences in the ratio of CO₂ partial pressures between the intercellular airspace and the atmosphere (pᵢ/pₒ) along with φ are the main factors attributed to...
variation in $\Delta^{13}C$ in C$_4$ plants (Farquhar, 1983). The ratio $p/p_a$ is primarily determined by stomatal conductance, whereas $\phi$ depends on the physical conductance of the BSC walls and the balance between the C$_4$ and C$_3$ cycles. Little change in $\phi$ was determined with gas exchange and $\Delta^{13}C$ measurements in various C$_4$ plants under a variety of environmental conditions (Henderson et al., 1992). However, with the use of antisense technologies, it has been shown that $\Delta^{13}C$ and $\phi$ increase when the capacity of the C$_3$ cycle is reduced relative to the C$_4$ cycle (von Caemmerer et al., 1997a, 1997b). Growth conditions (e.g., elevated CO$_2$ and water stress) have also been reported to influence the balance of the C$_4$ and C$_3$ cycles, leading to an altered isotopic composition of dry matter (Watling et al., 2000; Williams et al., 2001), although the influence on CA was not addressed in these studies.

There is limited research concerning the influence of CA activity on $\Delta^{13}C$ in C$_4$ plants. Recent work indicates that CA activity in wild-type Flaveria bidentis is in excess and does not limit CO$_2$ assimilation under normal conditions (von Caemmerer et al., 2004). F. bidentis lines with reduced levels of CA, due to the expression of antisense constructs targeted to a putative mesophyll cytosolic CA, showed that rates of CO$_2$ assimilation were unaffected by a decrease in CA activity until activity was less than 20% of wild type (von Caemmerer et al., 2004). Although large changes in CA activity had little effect on photosynthetic rates, according to the $\Delta^{13}C$ theory developed by Farquhar in 1983 (see “Materials and Methods”), the decrease in the hydration reaction of CO$_2$ ($V_h$) relative to the rate of PEPC carboxylation ($V_p$) should increase $\Delta^{13}C$ potentially without a corresponding change in the rate of net CO$_2$ assimilation (Farquhar, 1983).

In this article, we use F. bidentis plants with low CA activity to examine the influence of the hydration reaction of CO$_2$ on $\Delta^{13}C$ during C$_4$ photosynthesis. These results are discussed in relation to measurements of $\Delta^{13}C$ made in F. bidentis under various irradiances, as well as plants with reduced levels of Rubisco.

**RESULTS**

**Carbon Isotope Discrimination**

**Light Response Curves**

In the mass spectrometric gas-exchange system used here for online $\Delta^{13}C$ measurements, the leaf chamber gas outlet of a LI-6400 gas-exchange system (LI-COR) was directly coupled to a mass spectrometer (micro-mass ISOPRIME; Micromass Ltd.) via a gas-permeable silicone membrane (Fig. 1). This allowed the measurement of the $^{13}C/^{12}C$ ratio of the CO$_2$ in the airstream without prior purification of that CO$_2$. We measured rates of net CO$_2$ assimilation and $\Delta^{13}C$ in F. bidentis wild-type plants in response to photon flux density (PFD) to test our online systems with previously published values of $\Delta^{13}C$ from C$_4$ plants (Henderson et al., 1992). A summary of the symbols used in the text are shown in Table I. Net CO$_2$ assimilation increased with PFD to near-saturating rates (Fig. 2a). However, there was little change in $\Delta^{13}C$, $p_i/p_o$ and BSC CO$_2$ leakiness ($\phi$), except at the two lowest light levels (Fig. 2, b–d). There was more uncertainty in the $\Delta^{13}C$ measurements made at low light because of the higher ratio of the rate of CO$_2$ entry into the chamber to the rate of net CO$_2$ assimilation by the leaf (ϕ; see Fig. 2, legend). Leakage was calculated by rearranging Equation 2 (see equations in “Materials and Methods”) and substituting $b_i$ with Equation 3, with the assumption that the initial CO$_2$ carboxylation reaction catalyzed by PEPC to the rate of CO$_2$ hydration by CA ($V_h/V_p$) was zero. These gas-exchange and $\Delta^{13}C$ measurements are similar to those previously reported for Amaranthus edulis and Zea mays under similar measurement conditions (Henderson et al., 1992).

**Rubisco Small Subunit Plants**

Net CO$_2$ assimilation in F. bidentis plants with reduced levels of Rubisco caused by antisense RNA constructs targeted to the nuclear-encoded gene for
Our measurements of $\Delta^{13}C$ are similar to previously published values by von Caemmerer et al. (1997b). The comparison of our results to previously published $\Delta^{13}C$ values shows that our system can accurately and consistently monitor the influence of both environmental conditions and perturbations to the C$_4$ photosynthetic apparatus on instantaneous carbon isotope discrimination.

### CA Plants

Carbon isotope discrimination ($\Delta^{13}C$) increased as CA activity decreased in the F. bidentis plants containing the antisense RNA constructs targeted to the putative cytosolic CA (anti-CA plants; Fig. 3). CA activity, reported here as a rate constant ($k_{CA}$ μm$^{-2}$ s$^{-1}$ Pa$^{-1}$), was determined on leaf extracts using mass spectrometry to measure the rates of $^{18}$O$_2$ exchange from doubly labeled $^{13}$C$^{18}$O$_2$ to H$_2$O$^{18}$O (see "Materials and Methods"). Interestingly, $\Delta^{13}C$ was more sensitive than net CO$_2$ assimilation to changes in CA activity as $\Delta^{13}C$ increased in some anti-CA plants, whereas net CO$_2$ assimilation remained similar to wild-type plants (Fig. 3). In these anti-CA plants with reduced CA activity and wild-type rates of net CO$_2$ assimilation, $\Delta^{13}C$ increased 1% to 2% above which is a large shift for C$_4$ photosynthesis (Fig. 3c, inset; Table III). In the anti-CA plants, net CO$_2$ assimilation rates and $p_i/p_o$ were similar to wild-type plants, except when CA activities were less than 20% of wild type (Fig. 3, a and b). Anti-CA plants with extremely low levels of CA activity (<5% of wild type) and low rates of net CO$_2$ assimilation had extremely high values of $\Delta^{13}C$ (Fig. 3c).

Nearly all of the measured values of $\Delta^{13}C$ fall within the theoretical relationship of $\Delta^{13}C$ to $p_i/p_o$ as predicted from the model of C$_4$ carbon isotope discrimination developed by Farquhar (1983; Fig. 4; see "Materials and Methods"). Only in the anti-CA plants with extremely low CA activity do the measured values of $\Delta^{13}C$ fall outside the predicted values (Fig. 4). The theoretical relationship of $\Delta^{13}C$ and $p_i/p_o$ was calculated with a $\phi$ value of 0.24, and the initial CO$_2$ carboxylation reaction catalyzed by PEPC relative to the CO$_2$ hydration by CA ($V_{CA}/V_p$) was assumed to be either zero or 1 (as indicated in Fig. 4). The $b_1$ parameter, which is the combined fractionation associated with PEPC, respiration, and the isotopic equilibrium during the dissolution of CO$_2$ and conversion to HCO$_3^-$, used in these calculations was determined with either the CA catalyzed (solid lines) or the spontaneous uncatalyzed (dotted lines) CO$_2$ and HCO$_3^-$ hydration and dehydration fractionation factors (see "Materials and Methods").

To characterize the influence of CA activity on $\Delta^{13}C$, independent of changes in net CO$_2$ assimilation, we pooled the data of anti-CA plants with reduced CA activity and wild-type-like photosynthetic rates. $\Delta^{13}C$ was higher in the anti-CA plants compared to the wild-type plants, whereas $p_i/p_o$ was unchanged (Table III). The in vivo CA activity ($CA_{leaf}$), which is the product of $k_{CA}$ and the pCO$_2$ in the mesophyll cytoplasm ($p_m$),

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### Table I. Symbols used in the text

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>$A$</td>
<td>Net CO$_2$ assimilation</td>
</tr>
<tr>
<td>$a$</td>
<td>Fractionation during diffusion of CO$_2$ from the chloroplast to the atmosphere (4.4% at 500 μm$^{-1}$)</td>
</tr>
<tr>
<td>$a_i$</td>
<td>Fractionation of CO$_2$ diffusion through a liquid (0.7% at 500 μm$^{-1}$)</td>
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<tr>
<td>BSC</td>
<td>Bundle sheath cells</td>
</tr>
<tr>
<td>$b_j$</td>
<td>Combined discrimination of Rubisco, respiration, and photorespiration (see Eq. 4)</td>
</tr>
<tr>
<td>$b_i$</td>
<td>Combined discrimination of PEPC, respiration, and hydration/dehydration of CO$_2$ (see Eq. 3 and Fig. 4)</td>
</tr>
<tr>
<td>$b$</td>
<td>Discrimination by PEPC (2.2% at 500 μm$^{-1}$)</td>
</tr>
<tr>
<td>$\Delta^{13}C$</td>
<td>Carbon isotope discrimination</td>
</tr>
<tr>
<td>CA</td>
<td>Carbon anhydrase</td>
</tr>
<tr>
<td>$e$</td>
<td>Fractionation during respiration (3% or -6% at 500 μm$^{-1}$)</td>
</tr>
<tr>
<td>$e_i$</td>
<td>Fractionation as CO$_2$ dissolves (1.1% at 500 μm$^{-1}$)</td>
</tr>
<tr>
<td>$e_o$</td>
<td>Equilibrium fractionation factor for the catalyzed hydration/dehydration of CO$_2$ (9% at 500 μm$^{-1}$)</td>
</tr>
<tr>
<td>$i$</td>
<td>Discrimination during photorespiration (10% or -6.8% at 500 μm$^{-1}$)</td>
</tr>
<tr>
<td>$\phi$</td>
<td>The fraction of CO$_2$ fixed by PEPC that subsequently leaks out of the BSC</td>
</tr>
<tr>
<td>$g_w$</td>
<td>The internal conductance to the diffusion of CO$_2$ between the intercellular air space and the site of carboxylation in the mesophyll cytoplasm</td>
</tr>
<tr>
<td>$h$</td>
<td>Catalyzed fractionation during CO$_2$ hydration (1.1% at 500 μm$^{-1}$)</td>
</tr>
<tr>
<td>$k_{CA}$</td>
<td>Rate constant of carbonic anhydrase</td>
</tr>
<tr>
<td>$K_r$</td>
<td>Michaelis constant of Rubisco for CO$_2$</td>
</tr>
<tr>
<td>$K_p$</td>
<td>Michaelis constant of Rubisco for O$_2$</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis constant of PEPC for CO$_2$</td>
</tr>
<tr>
<td>$M_d$</td>
<td>Rate of mitochondrial respiration</td>
</tr>
<tr>
<td>$M_m$</td>
<td>Rate of mitochondrial respiration in the mesophyll cells</td>
</tr>
<tr>
<td>$M_b$</td>
<td>Rate of mitochondrial respiration in the BSC</td>
</tr>
<tr>
<td>$p$</td>
<td>Partial pressure of CO$_2$</td>
</tr>
<tr>
<td>$p_{CA}$</td>
<td>pCO$_2$ of dry air entering the leaf chamber</td>
</tr>
<tr>
<td>$p_i$</td>
<td>pCO$_2$ of the intercellular airspace</td>
</tr>
<tr>
<td>$p_m$</td>
<td>pCO$_2$ of the mesophyll cytoplasm</td>
</tr>
<tr>
<td>$p_o$</td>
<td>pCO$_2$ of dry air leaving the leaf chamber</td>
</tr>
<tr>
<td>$R_a$</td>
<td>13C$^{13}$C$^{12}$C of the air entering the leaf chamber</td>
</tr>
<tr>
<td>$R_b$</td>
<td>13C$^{12}$C of the air leaving the leaf chamber</td>
</tr>
<tr>
<td>PFD</td>
<td>Photon flux density</td>
</tr>
<tr>
<td>PSII</td>
<td>PSII</td>
</tr>
<tr>
<td>$\xi$</td>
<td>$p_i/(p_{CA} - p_o)$</td>
</tr>
<tr>
<td>$s$</td>
<td>Fractionation during the leakage of CO$_2$ from the BSC (1.8% at 500 μm$^{-1}$)</td>
</tr>
<tr>
<td>$V_{i}$</td>
<td>Rate of Rubisco carboxylation</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>Maximal rate of Rubisco carboxylation</td>
</tr>
<tr>
<td>$V_{i}$</td>
<td>Rate of CO$_2$ hydration</td>
</tr>
<tr>
<td>$V_o$</td>
<td>Rate of photorespiration</td>
</tr>
<tr>
<td>$V_p$</td>
<td>Rate of PEP carboxylation $(A + M_j)/(1 - \phi)$ or $(p_m V_{max} - p_m + K_p)$</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>Maximal rate of PEPC carboxylation</td>
</tr>
</tbody>
</table>

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the small subunit of Rubisco (anti-SSu plants) had rates between 40% to 80% of wild-type plants (Table II). Additionally, the ratio of $p_i/p_o$, $\Delta^{13}C$, and $\phi$ were higher in the anti-SSu-plants as compared with wild-type plants (Table II). The parameter $\phi$ was determined from simultaneous gas-exchange and isotope measurements and solving for $\phi$ in Equation 2. Our measurements of $\Delta^{13}C$ and leaf gas exchange
was significantly less in the anti-CA relative to the wild-type plants (Table III). The value of \( p_m \) was calculated with an internal conductance to the diffusion of CO\(_2\) between the intercellular airspace and the site of carboxylation in the mesophyll cytoplasm (\( g_w \)) of 10 mol m\(^{-2}\) s\(^{-1}\) Pa\(^{-1}\). The ratio of \( V_p/V_h \) determined from the online measurements of \( \Delta^{13}C \) was approximately 6 times greater in the anti-CA plants than in the wild-type plants (Table III). It appears that a rather large decrease in leaf CA activity in \( F. \) bidentis can maintain the chemical equilibrium between CO\(_2\) and HCO\(_3^-\) needed to sustain photosynthesis, but limits the isotopic equilibrium causing \( \Delta^{13}C \) to increase without changes in \( \phi \).

It should be noted that the absolute value of \( V_p/V_h \) determined this way is largely influenced by \( \phi \) and slightly by \( g_w \). For example, changing \( g_w \) from 6 to 10 mol m\(^{-2}\) s\(^{-1}\) Pa\(^{-1}\) shifts calculations of \( V_p/V_h \) from 0.08 to 0.07 and 0.55 to 0.46 for wild-type and anti-CA plants, respectively. However, changing \( \phi \) from 0.24 to 0.10, assuming a constant \( g_w \) of 10 mol m\(^{-2}\) s\(^{-1}\) Pa\(^{-1}\), causes \( V_p/V_h \) to increase from 0.07 to 0.61 in the wild-type plants and from 0.46 to 0.99 in the anti-CA plants (Table III). In the anti-CA plants, which have a reduced capacity to concentrate CO\(_2\) within the BSC, it is predicted from the C\(_4\) photosynthetic model that \( \phi \) will decrease relative to the wild-type plants (see below), which would increase the difference of \( V_p/V_h \) between the wild-type and the anti-CA plants. \( V_p/V_h \) can also be approximated from gas exchange and in vitro CA activity as \( V_p/CA_{leaf} \) where \( CA_{leaf} \) is calculated as \( k_{CA}p_m \) and \( V_p \) is calculated as \( (A + M_{\phi})(1 - \phi) \) (von Caemmerer, 2000). The parameter \( M_{\phi} \) is the daytime rate of mitochondrial respiration assumed to be 2 \( \mu \)mol m\(^{-2}\) s\(^{-1}\). The ratio of \( V_p/V_h \) determined from the in vitro assays of CA activity was approximately 4 times greater in the anti-CA plants than in the wild-type plants (Table III). The absolute value of \( V_p/V_h \) calculated in this manner is also influenced by changes in \( g_w \) and \( \phi \), although neither

![Figure 2](https://www.plantphysiol.org/)

Table II. CA rate constant \( k_{CA} \), net CO\(_2\) assimilation rate \( A \), ratio of intercellular to atmospheric CO\(_2\) partial pressure \( p/p_i \), online \( \Delta^{13}C \) discrimination, and leakage of CO\(_2\) out of the BSCs \( \phi \) in the anti-SSu plants from the primary transformant 136-13

<table>
<thead>
<tr>
<th></th>
<th>( k_{CA} )</th>
<th>( A )</th>
<th>( p/p_i )</th>
<th>( \Delta^{13}C )</th>
<th>Leaks. ( \phi )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mol m(^{-2}) s(^{-1}) Pa(^{-1})</td>
<td>( \mu )mol m(^{-2}) s(^{-1})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>136-13-11#1</td>
<td>52</td>
<td>17.0</td>
<td>0.67</td>
<td>6.1</td>
<td>0.43</td>
</tr>
<tr>
<td>136-13-12#4</td>
<td>88</td>
<td>19.5</td>
<td>0.64</td>
<td>6.5</td>
<td>0.45</td>
</tr>
<tr>
<td>136-13-12#3</td>
<td>62</td>
<td>27.9</td>
<td>0.72</td>
<td>6.0</td>
<td>0.42</td>
</tr>
<tr>
<td>136-13-11#2</td>
<td>81</td>
<td>29.9</td>
<td>0.71</td>
<td>5.4</td>
<td>0.39</td>
</tr>
<tr>
<td>Wild type</td>
<td>69 ± 2</td>
<td>37.6 ± 2.6</td>
<td>0.55 ± 0.02</td>
<td>2.5 ± 0.4</td>
<td>0.25 ± 0.02</td>
</tr>
</tbody>
</table>

For calculation of \( \phi \), the \( V_p/V_i \) ratio was assumed to be zero. Measurements were made at a \( pCO_2 \) of 52 Pa, a \( pCO_2 \) of 4.8 kPa, a PFD of 2,000 \( \mu \)mol quanta m\(^{-2}\) s\(^{-1}\), and a leaf temperature of 30°C. \( n = 4 \) for the wild-type plants.
Dry Matter $\delta^{13}C$

Leaf dry matter $\delta^{13}C$, the ratio of $^{13}C/^{12}C$ of the sample relative to the standard Vienna Pee Dee Belemnite (VPDB), was lower in plants with low levels of CA and correlated with increases in $\Delta^{13}C$ (Fig. 5). Leaf $\delta^{13}C$ was determined on plants germinated and grown in a glasshouse. After collecting an entire leaf for $\delta^{13}C$, the three plants with very low CA and photosynthetic rates were transferred after several weeks to the 1% CO$_2$ growth cabinets before leaf gas-exchange measurements were made. Otherwise, the leaf opposite to the one used for gas exchange was sampled for $\delta^{13}C$.

Photosynthetic and Carbon Isotope Discrimination Models

It has recently been shown that low leaf CA activity in F. bidentis reduces the capacity of the C$_4$ cycle by limiting the rate of PEPC carboxylation of HCO$_3^-$ ($V_p$) (von Caemmerer et al., 2004). Here, we use the C$_4$ photosynthetic model developed by Berry and Farquhar (1978) and von Caemmerer (2000) to predict the response of net CO$_2$ assimilation, bundle sheath $p$CO$_2$, photorespiration ($V_p$) and $\phi$ to changes in the activity of PEPC due to a limitation in CA activity. In the C$_4$ photosynthetic model, the CA-mediated hydration/dehydration reaction of CO$_2$ within the mesophyll cytoplasm has not been incorporated. However, manipulating $V_p$ within the model simulates the effect of changing CA activity and leads to a diminished ability to concentrate CO$_2$ within the BSC, which decreases both the photosynthetic rate and $\phi$ (Fig. 6, a and b).

The outputs from the C$_4$ photosynthetic model, specifically the rates of Rubisco carboxylation ($V_c$), $V_o$, $V_p$, $\phi$, and the $p$CO$_2$ in the BSC, were then incorporated into the model of C$_4$ carbon isotope discrimination ($\Delta^{13}C$) developed by Farquhar (1983). The $\Delta^{13}C$ model was used to determine which photosynthetic parameters would influence $\Delta^{13}C$ consistent with our experimental data and to demonstrate the influence of $\phi$ on $\Delta^{13}C$ independent of changes in $V_p/V_o$. The model in Figure 6 included sufficient CA activity to keep $V_p/V_o$ close to zero as $V_p$ changes and $p_i/p_o$ were held constant at 0.4. As shown in Figure 6c, when $\phi$ and the $p$CO$_2$ in the BSC are low, $\Delta^{13}C$ decreases as the ability of Rubisco to fractionate is reduced. Additionally, the $\Delta^{13}C$ model accounts for the effects of fractionation during respiration ($e$) and photorespiration ($f$); however, there is uncertainty in the specific values of factors $e$ and $f$ in the model (Gillon and Griffiths, 1997; Ghashghaie et al., 2003). Therefore, to test the influence of these parameters on the $\Delta^{13}C$ model, various values of $e$ (3%o versus $-6.8%o$) and $f$ ($-6.8%o$ versus 10%o) were used. Even at low CO$_2$ assimilation rates, relatively large changes in $e$ and $f$ had only a small influence on $\Delta^{13}C$ (Fig. 6c).

Figure 3. Net CO$_2$ assimilation rate, the ratio of intercellular to ambient $p$CO$_2$ ($p_i/p_o$), and carbon isotope discrimination ($\Delta^{13}C$) as a function of the rate constant of leaf CA ($k_{CA}$, $\mu$mol m$^{-2}$ s$^{-1}$ Pa$^{-1}$). The inset in c shows the expanded scale of $\Delta^{13}C$ where net CO$_2$ assimilation is relatively constant. Each point represents a measurement made on a different plant grown in a glasshouse at ambient CO$_2$, or in a growth cabinet at 1% CO$_2$; wild-type plants grown at ambient CO$_2$ (□); anti-CA plants grown at ambient CO$_2$ (■); wild-type grown at 1% CO$_2$ (○, △); and anti-CA plants grown at 1% CO$_2$ (◆). Measurements were made at 2,000 $\mu$mol quanta m$^{-2}$ s$^{-1}$, leaf temperature of 30°C, and an inlet CO$_2$ concentration of either 38 Pa in air (△, ◆) or 52 Pa of CO$_2$ in a 90.5 kPa of N$_2$ and 4.8 kPa of O$_2$ gas mixture (□, ○). The lines represent the best fit for all measurements (wild-type and anti-CA plants) made at either 38 or 52 Pa of CO$_2$. The ratio of intercellular to ambient $p$CO$_2$ ($p_i/p_o$), and carbon isotope discrimination ($\Delta^{13}C$) as a function of the rate constant of leaf CA ($k_{CA}$, $\mu$mol m$^{-2}$ s$^{-1}$ Pa$^{-1}$).
DISCUSSION

Carbon isotope discrimination increased in the short term during leaf gas exchange (Δ^{13}C) and the carbon isotope composition of leaf dry matter (δ^{13}C) decreased in transformants containing reduced levels of leaf CA. As was previously reported (von Caemmerer et al., 2004), leaf CA activity appears to be in excess to maintain steady-state rates of net CO₂ assimilation in F. bidentis at high light. A nearly 80% decrease in leaf CA activity was needed before net CO₂ assimilation was affected when measured at a CO₂ partial pressure (pCO₂) of 52 Pa (Fig. 3a). The CA activity required to maintain wild-type-like photosynthetic rates increased when measurements were conducted at a lower pCO₂ of 38 Pa (Fig. 3a). This is in agreement with previously published work where reduced leaf cytosolic CA activity affected the initial slope of the CO₂ response curve in F. bidentis when the rate of CO₂ hydration limited the supply of HCO₃⁻ for PEPC carboxylation (von Caemmerer et al., 2004).

Carbon Isotope Discrimination and CA Activity

According to the C₄ photosynthetic model (von Caemmerer, 2000), a limitation in the supply of cytosolic HCO₃⁻ will lead to a decrease in the initial CO₂ carboxylation reaction catalyzed by PEPC and reduce the capacity of the C₄ pump to concentrate CO₂ within the BSC. A reduced pCO₂ in the BSC leads to a decrease in the rate of net CO₂ assimilation as well as a lower BSC CO₂ leakiness (φ). In the model of C₄ carbon isotope discrimination, the main factors that influence Δ^{13}C are changes in the intercellular to ambient CO₂ partial pressures (p/pₐ) and φ (Farquhar, 1983). When the ratio of PEPC carboxylation to the hydration reaction of CO₂ (Vₚ/Vₜ) is near zero (i.e. CA activity is high relative to PEPC carboxylation), the C₄ carbon isotope model predicts that Δ^{13}C will decrease as φ decreases (Fig. 6). The Δ^{13}C modeling illustrates that when the front end of the C₄ cycle is diminished (either by reduced CA and/or PEPC activity or anything else), φ decreases and Δ^{13}C associated with φ also decreases (Fig. 6). However, in the anti-CA plants, which potentially reduced the ability to concentrate CO₂ in the BSC, Δ^{13}C increased, which cannot be explained in the model by decreases in φ, but can be explained by changes in Vₚ/Vₜ.

Due to the high levels of mesophyll cytoplasmic CA activity in C₄ plants, it is generally assumed that CO₂ and HCO₃⁻ are in close chemical equilibrium. Under such conditions, the ratio of Vₚ/Vₜ in Equation 3 approaches zero and can be omitted from the calculation of b₄. However, when Vₚ/Vₜ tends away from zero, Equation 3 can be expressed with the fractionation factors provided in “Materials and Methods” as b₄ = -5.7 + 7.9 Vₚ/Vₜ at 25°C. The b₄ fractionation factor becomes more positive as Vₚ/Vₜ increases and Δ^{13}C increases even without changes in p/pₐ and φ. As shown in Figure 4, varying Vₚ/Vₜ between 0 and 1 can

<table>
<thead>
<tr>
<th>A</th>
<th>p/pₐ</th>
<th>CAₙod</th>
<th>Δ^{13}C</th>
<th>Vₚ/Vₜ</th>
<th>( g_{an} = 10 )</th>
<th>( \phi = 0.24 )</th>
<th>( g_{an} = 6 )</th>
<th>( \phi = 0.24 )</th>
<th>( g_{an} = 10 )</th>
<th>( \phi = 0.1 )</th>
<th>( g_{an} = 6 )</th>
<th>( \phi = 0.1 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CA</td>
<td>42 ± 0.6</td>
<td>0.48 ± 0.02</td>
<td>228 ± 37</td>
<td>4.4 ± 0.2</td>
<td>Δ^{13}C*</td>
<td>0.46 ± 0.06</td>
<td>0.55 ± 0.07</td>
<td>0.99 ± 0.08</td>
<td>1.1 ± 0.08</td>
<td>0.29 ± 0.04</td>
<td>0.40 ± 0.04</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>Wild type</td>
<td>40 ± 0.1</td>
<td>0.47 ± 0.02</td>
<td>774 ± 48</td>
<td>3.3 ± 0.2</td>
<td>Δ^{13}C*</td>
<td>0.29 ± 0.04</td>
<td>0.40 ± 0.04</td>
<td>0.24 ± 0.03</td>
<td>0.30 ± 0.04</td>
<td>0.07 ± 0.07</td>
<td>0.08 ± 0.08</td>
<td>0.61 ± 0.06</td>
</tr>
</tbody>
</table>

Table III. Net CO₂ assimilation rate (A), the ratio of intercellular to ambient pCO₂ (p/pₐ), CAₙod calculated as \( \left( k_{p,\text{CA}} \right) \Delta^{13}C \), and Vₚ/Vₜ for F. bidentis wild-type plants and anti-CA plants with low CAleaf activity and wild-type-like net CO₂ assimilation rates.

Measurements were made at a pCO₂ of 52 Pa, a pO₂ of 4.8 kPa, PFD of 2,000 μmol quanta m⁻² s⁻¹, and a leaf temperature of 30°C. Vₚ/Vₜ was estimated either by online Δ^{13}C measurements* using Eqs. 3 and 5 (“Materials and Methods”) or estimated from Vₚ/CAₙod**, where Vₚ was calculated as \( (A + M_d)/(1 - \phi) \) and CAₙod was assumed to be either 10 or 6 μmol m⁻² s⁻¹ Pa⁻¹, and φ was set at either 0.24 or 0.1. M₉ is the daytime rate of respiration assumed to be 2 μmol m⁻² s⁻¹, n = 7 and 5 for anti-CA and wild-type plants, respectively.

Figure 4. Carbon isotope discrimination (Δ^{13}C) as a function of the ratio of intercellular to ambient pCO₂ (p/pₐ). The white symbols are wild-type plants and the black symbols are anti-CA plants. Other symbols and measurement conditions are as described in Figure 3. The lines represent the theoretical relationship of Δ^{13}C and p/pₐ, where φ = 0.24, the ratio of the PEPC carboxylation to the CO₂ hydration reaction (Vₚ/Vₜ) is either 0 or 1, and the b₄ parameter is calculated with the catalyzed (solid lines, b₄ = -5.7 + 7.9 Vₚ/Vₜ) and uncatalyzed (dotted lines, b₄ = -4.5 + 12.5 Vₚ/Vₜ) CO₂ and HCO₃⁻ hydration and dehydration fractionation factors. gₐ₂ was assumed to be large such that pₐ = pₐ.
photosynthetic rate (see Fig. 3c; Table III). Gillon and Yakir (2001) reported even lower CA activity for a number of C4 grasses, which correspond to the low CA activities we measured in anti-CA plants shown in Figure 3c, inset. The values of $V_p/V_h$ estimated from the CA activity and net CO2 assimilation from this article indicate that $\Delta^{13}C$ will differ in C4 plants that have been reported to contain a range of CA_leaf activity (2–529 $\mu$mol m$^{-2}$ s$^{-1}$) with generally similar photosynthetic rates (Gillon and Yakir, 2001). However, a

Variation in the Ratio of PEPC Carboxylation to CO2 Hydration by CA

The large change in $V_p/V_h$ without changes in photosynthesis in the anti-CA plants (Table III) indicates that $\Delta^{13}C$ is more sensitive to a reduction in CA activity than net CO2 assimilation. The influence of $V_p/V_h$ on $\Delta^{13}C$ is predicted by the C4 photosynthetic model for carbon isotope discrimination developed by Farquhar (1983), and here we demonstrate the influence of CA activity on $\Delta^{13}C$ in a C4 plant. In wild-type F. bidentis plants, CA appears to be in excess for supporting photosynthesis and $V_p/V_h$ approaches zero. However, it has been reported that CA activity in most C4 species is only just sufficient to support photosynthetic rates, especially in C4 monocots (Hatch and Burnell, 1990; Gillon and Yakir, 2000, 2001), and the influence of $V_p/V_h$ on $\Delta^{13}C$ may be greater in these C4 species. For example, we measured (A.B. Cousins, M. R. Badger, and S. von Caemmerer, unpublished data) CA activity in Z. mays as 266 ± 22 $\mu$mol m$^{-2}$ s$^{-1}$, which is similar to our anti-CA plants with wild-type

![Figure 5.](image)

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have a large impact on $\Delta^{13}C$, especially when $p_i/p_o$ is high. Nearly all the variation in $\Delta^{13}C$ in the anti-CA plants can be explained by changes in $V_p/V_h$ (Fig. 4). Only when CA activity and photosynthetic rates decline dramatically do changes in $V_p/V_h$ and $p_i/p_o$ not accurately predict $\Delta^{13}C$ (see below for further discussion).

![Figure 6.](image)
systematic investigation of the influence of \( V_f/V_h \) on \( \Delta^{13}C \) in a range of \( C_4 \) species needs to be conducted.

Changes in \( V_f/V_h \) and its influence on \( \Delta^{13}C \) also have important implications for interpreting physiological processes responsible for changes in \( \Delta^{13}C \) and \( \delta^{13}C \) during \( C_4 \) photosynthesis, particularly in response to changing environmental conditions. Water stress, reduced nitrogen availability, and atmospheric CO2 availability have all been reported to increase \( \Delta^{13}C \) in \( C_4 \) plants by 1 to \( 3 \% \) (Meinzer et al., 1994; Ranjith et al., 1995; Buchmann et al., 1996; Saliendra et al., 1996; Meinzer and Saliendra, 1997; Meinzer and Zhu, 1998; Watling et al., 2000). Variation in \( \Delta^{13}C \) in these reports has been interpreted as changes in either \( p_f/p_s \) and/or \( \phi \), with the apparent assumption that \( V_f/V_h \) remains close to zero in all treatments. However, there are a few reports in the literature that suggest CA activity in \( C_4 \) plants is largely dependent on the internal CO2 partial pressures, conditions that influence CO2 availability, such as water stress and growth under elevated atmospheric CO2, will also alter \( V_f/V_h \) and thus influence measured \( \Delta^{13}C \). Because leaf CA activity in \( C_4 \) plants is largely dependent on the internal CO2 partial pressures, conditions that influence CO2 availability, such as water stress and growth under elevated atmospheric CO2, will also alter \( V_f/V_h \). C4 photosynthesis generally operates near CO2-saturating conditions at current atmospheric CO2 such that a reduction in \( p_i \) due to stomatal closure will cause \( V_f/V_h \) to increase. However, under such conditions, the ratio of \( p_f/p_s \) also decreases and the influence of \( V_f/V_h \) on \( \Delta^{13}C \) decreases as shown in Figure 4. Alternatively, it has been shown that, under well-watered conditions, \( C_4 \) photosynthesis generally does not respond to increases in atmospheric pCO2 (MeLeod and Long, 1999; Ghannoun et al., 2000; Wall et al., 2001; Ainsworth and Long, 2005; Leakey et al., 2006). However, because \( p_f/p_s \) is generally constant with changing atmospheric pCO2 and CA has a very high \( K_m \) for HCO3−, an increase in CO2 availability will increase \( V_f/V_h \), whereas PEPC is generally saturated around ambient pCO2 and \( V_f \) will not change. This raises the possibility that growth under future atmospheric CO2 conditions will alter \( \Delta^{13}C \) regardless of other environmental changes if CA is limiting.

Both online and in vitro measurements of \( V_f/V_h \) indicated that changes in CA activity have a significant influence on \( \Delta^{13}C \) without changes in \( p_f/p_s \) and \( \phi \). It must be noted that, although changes in \( \Delta^{13}C \) have a subtle effect on estimates of \( V_f/V_h \) variation in \( \phi \) can lead to large shifts in the absolute values of \( V_f/V_h \) when determined from the \( \Delta^{13}C \) measurements. There are no direct means of measuring \( \phi \), but it can be estimated using \( \Delta^{13}C \) measurements when \( V_f/V_h \) is assumed to be close to zero. The use of antisense technology targeted toward the \( C_4 \) PEPC enzyme would provide a range of \( V_f/V_h \) values and would allow an estimate of \( \phi \) when \( V_f/V_h \) was known to be close to zero.

Low CA and Photosynthetic Mutants

In the majority of CA plants, the increase in \( \Delta^{13}C \) can be explained by changes in the ratio of \( V_f/V_h \) and \( p_f/p_s \) (Fig. 4). However, this explanation does not hold true for plants with very low leaf CA activity and photosynthetic rates. Potentially, the amount of direct fixation of atmospheric CO2 in the BSC, leakage of HCO3− from the BSC, as well as photorespiration and respiration would influence \( \Delta^{13}C \) especially when net CO2 assimilation is inhibited. Theoretically, CO2 assimilation by direct diffusion of CO2 from the atmosphere into the BSC would increase the \( \Delta^{13}C \) as the exchange of CO2 between the atmosphere and the BSC would allow Rubisco to fractionate against the heavier carbon isotope. However, a low conductance of CO2 diffusion across the BSC (\( g_w \ mmol m^{-2} s^{-1} \ Pa^{-1} \)) is an essential component of the \( C_4 \) CO2-concentrating mechanism and limits the amount of direct fixation of CO2 under ambient CO2 concentrations (Jenkins et al., 1988; Brown and Byrd, 1993; He and Edwards, 1996; von Caemmerer, 2000; Kiirats et al., 2002). Therefore, even when the initial carboxylation reaction of the \( C_4 \) pump is limited by low CA activity or low light, there would be little, if any, direct fixation of CO2 in the BSC and minimal influence on \( \Delta^{13}C \).

Alternatively, because \( ^{13}C \) concentrates in HCO3− and Rubisco preferentially fixes \( ^{12}C \), leakage of HCO3− out of the BSC would change the fractionation factor associated with CO2 leakage from the BSC (from Eq. 2). However, with the relatively low CA activity in the BSC, it is unlikely that CO2 and HCO3− would be in full isotopic equilibrium and there would be little influence on \( s \) (Farquhar, 1983; von Caemmerer et al., 1997a; Ludwig et al., 1998). Additionally, the influence of respiration and photorespiration on the modeled value of \( \Delta^{13}C \) will increase as the rates of net CO2 assimilation decrease. However, changing the fractionation effect of respiration (\( \epsilon \) in Eqs. 3 and 4) and photorespiration (\( \delta \) in Eq. 4) to a range of values reported in the literature (Ghashghaie et al., 2003) had only a slight influence on the modeled \( \Delta^{13}C \) even at low photosynthetic rates (Fig. 6c).

As previously mentioned, Equation 3 simplifies to \( b_k = -5.7 + 7.9 \ V_f/V_h \) at 25°C when the catalyzed fractionation values for \( e_k \) of \( -9.0 \% \) and \( h \) of 1.1 \% are used. However, if the interconversion of CO2 and HCO3− occurs via the spontaneous uncatalyzed reaction, \( e_k \) and \( h \) become \( -7.8 \% \) and \( 6.9 \% \), respectively, and Equation 3 is \( b_k = -4.5 + 12.5 \ V_f/V_h \), causing the \( b_k \) value to become larger, leading to an increase in \( \Delta^{13}C \) (Fig. 4). The catalyzed and uncatalyzed values of \( e_k \) and \( h \) are taken from previously published work on the hydration and dehydration of CO2 and HCO3− (Mook et al., 1974; Marlíer and O’Leary, 1984; Paneth and O’Leary, 1985). The proportion of catalyzed to uncatalyzed hydration/dehydration reactions may have an influence on the \( \Delta^{13}C \) when the photosynthetic rates are extremely low, such as in the anti-CA plants with extremely low CA activity, but it would have little, if any, influence in wild-type plants.
Carbon Isotope Discrimination Increases at Low Light

The response of $\Delta^{13}C$ in C$_4$ plants to various light levels has not been well characterized, but is an important factor to consider when interpreting dry matter $\delta^{13}C$ of plants exposed to different light environments or leaves within a canopy. The increase in $\Delta^{13}C$ and estimated values of $\phi$ in F. bidentis (Fig. 2) is similar to earlier reports that showed that $\Delta^{13}C$ generally increases as the PFD decreases (Henderson et al., 1992; Peisker and Henderson, 1992; Tazoe et al., 2005). Buchmann et al. (1996) also showed that $\Delta^{13}C$, calculated from leaf $\delta^{13}C$ values, in a number of C$_4$ plants was greater at low PFD. The low conductance of CO$_2$ diffusion across the BSC needed for C$_4$ photosynthesis would limit the direct fixation of CO$_2$ by Rubisco, even under low light, and its influence on $\Delta^{13}C$ should be minimal. However, it has been demonstrated with the C$_4$ photosynthetic model that $\phi$ increases at low PFD as more electron transport is needed for recycling of photorespired CO$_2$ (von Caemmerer, 2000). The predicted change in $\phi$ at low PFD by the C$_4$ photosynthetic model is consistent with our current experimental evidence, as well as earlier unpublished results (Henderson et al., 1992). The evidence from both online and dry matter isotope measurements indicates that growth light conditions need to be considered when interpreting carbon isotope discrimination in C$_4$ plants.

CONCLUSION

CA activity in wild-type F. bidentis appears to be in excess to maintain net CO$_2$ assimilation; however, reducing leaf CA activity had a relatively large influence on $\Delta^{13}C$, often without changes in net CO$_2$ assimilation. The influence of CA activity on $\Delta^{13}C$ was also evident in the leaf dry matter $\delta^{13}C$. The model of $\Delta^{13}C$ developed by Farquhar (1983) predicted the influence of changes in PEPC carboxylation relative to the hydration reaction of CO$_2$ ($V_p/V_o$) on $\Delta^{13}C$, except when photosynthetic rates and CA activity were dramatically reduced. It will be important to take the extent of CA activity in C$_4$ leaves into account when using $\Delta^{13}C$ and/or $\delta^{13}C$ to model leaf level and global C$_4$ photosynthesis in response to changing environmental influences. The influence of environmental conditions on leaf CA activity, $V_p/V_o$, and thus on $\Delta^{13}C$ warrants further investigation.

Additionally, the amount of CA activity in a leaf plays an important role in determining C$^{18}$O discrimination during C$_4$ photosynthesis because CA enhances the rate of oxygen exchange between CO$_2$ and leaf H$_2$O and thus determines the extent of isotopic equilibrium. The anti-CA plants will be used to test whether changing leaf CA activity influences C$^{18}$O discrimination under similar environmental conditions and whether high CA activity, relative to photosynthetic rates, corresponds to complete isotopic equilibrium between CO$_2$ and leaf H$_2$O as predicted.

MATERIALS AND METHODS

Growth Conditions

Flaveria bidentis plants were previously transformed with antisense RNA constructs targeted to either the nuclear-encoded gene for the small subunit of Rubisco (anti-Su plants) or a putative cytosolic CA (anti-CA plants; Furbank et al., 1996; von Caemmerer et al., 1997b, 2004). The segregating T$_2$ generations of anti-CA primary transformants with photosynthetic rates similar to wild type were grown during the summer months in a glasshouse under natural light conditions (27°C d/18°C night temperatures). Anti-CA and anti-Su plants (segregating T$_2$ generation from primary transformant 136-1) with low photosynthetic capacities and wild-type plants were grown under 1% CO$_2$ in a controlled environment growth cabinet and at a photosynthetic PFD of 400 mol quanta m$^{-2}$ s$^{-1}$ at plant height and air temperature of 27°C during the day and 18°C at night with a 14-h daylength. Three plants with very low CA and photosynthetic rates were germinated and grown for several weeks in the glasshouse. Subsequently, these plants were transferred to the 1% CO$_2$ growth cabinets before leaf gas-exchange measurements were made. Plants were grown in 5-L pots in garden mix with 2.4 to 4 g Osmocote/L soil (15/48/10.8/12 N/P/K/Mg + trace elements: B, Cu, Fe, Mn, Mo, Zn; Scotts Australia Pty Ltd.) and watered daily.

Gas-Exchange Measurements

Plants from either the glasshouse or growth cabinet were transferred to the gas-exchange system, where one of the uppermost fully expanded leaves was placed into the leaf chamber of the LI-6400 and allowed to equilibrate at a leaf temperature of 30°C and 2,000 mol quanta m$^{-2}$ s$^{-1}$ for a minimum of 1.5 h. Air entering the leaf chamber was prepared by using mass flow controllers (MKS Instruments) to obtain a CO$_2$ mix of 90.5 kPa of dry nitrogen and 4.8 kPa oxygen (Fig. 1). A portion of the nitrogen/oxygen mixture was used to zero the mass spectrometer to correct for N$_2$O and other contaminants contributing to the 44 and 45 peaks. Pure CO$_2$ (6$^{13}$C $= -29.0_{\text{vppm}}$, VPDB) was added to the remaining airstream to obtain a CO$_2$ partial pressure of approximately 52 Pa. Alternatively, some measurements were made by mixing pure CO$_2$ with CO$_2$-free air and using the CO$_2$-free air as a zero.

The different gas mixtures had no apparent influence on leaf gas exchange or $^{13}C$ isotope discrimination. Low oxygen (4.8 kPa) was used to minimize contamination of the 46 peak caused by the interaction of O$_2$ and N$_2$ to produce NO, with the mass spectrometer source element. This was important when looking at C$^{18}$O discrimination (A.B. Cousins, M.R. Badger, and S. von Caemmerer, unpublished data). The CO$_2$ used during the gas-exchange measurements had a similar isotopic signature to the CO$_2$ in the high CO$_2$ growth cabinet. This minimized the influence of respired CO$_2$ on the $\Delta^{13}C$ measurements in plants with low photosynthetic rates.

The gas mixtures were led to the inlet of the LI-6400 console and a flow rate of 200 mol s$^{-1}$ was maintained over the leaf. The remaining airstream was vented or used to determine the isotopic composition of air entering the leaf chamber (Fig. 1). The efflux from the leaf chamber was measured by either replacing the match valve line with a line connected directly to the mass spectrometer and the match valve simultaneously. Gas-exchange parameters were determined by the LI-6400 and pCO$_2$ leaving the chamber was subsequently corrected for the dilution of CO$_2$ by water vapor (von Caemmerer and Farquhar, 1981).

Isotopic Measurements

The efflux from the leaf chamber and the gas mix supplied to the LI-6400 system was linked to a mass spectrometer through an ethanol/dry ice water system was linked to a mass spectrometer through an ethanol/dry ice water
symbols used in the text is listed in Table I. Zero values for the 44 and 45 peaks were determined before and after the sample measurements were subtracted from both the sample and reference measurements prior to determining the mass ratios. The zero values were typically 1% of the 44 and 45 peaks at 4.8 kPa oxygen and 2% at 20 kPa oxygen.

Calculations of Carbon Isotope Discrimination

The model of C₄ carbon isotope discrimination (Δ¹³C) of Farquhar (1983) was used to determine which factors in the model would influence Δ¹³C consistent with our experimental data. The simplified model predicts that:

\[ \Delta^{13}C = a + (b_1 + b_2 - s - d)\frac{f}{p_w} \]

where \( a \) (4.4‰) is the fractionation during diffusion of CO₂ and s (1.8‰) is the fractionation during CO₂ leakage from the BSCs. The combined fractionation of PEPC, respiration, and the isotopic equilibrium during dissolution of CO₂ and conversion to HCO₃⁻ (\( b_1 \)) is calculated as (Farquhar, 1983):

\[ b_1 = (b_2 + c_3 + c_4)(1 - V_e/V_i) + (c + b)V_e/V_i - eM_i/V_p \]

where \( b_2 \) (2.2‰) is the fractionation by PEPC (O’Leary, 1981), \( c_3 \) (1.1‰) is the fractionation as CO₂ dissolves (O’Leary, 1984), and \( c_4 \) (−9.5‰) is the equilibrium fractionation factor of the catalyzed hydration/dehydration reactions of CO₂ and HCO₃⁻ (Mook et al., 1974). Alternatively, during the hydration/dehydration reactions, the uncatalyzed equilibrium fractionation factor \( c_3 \) = −7.8‰ (Marlière and O’Leary, 1984). The fractionation when CO₂ and HCO₃⁻ are not at equilibrium is dependent on the rate of CO₂ hydration (\( V_e \)), the rate of PEPC (\( V_p \)), \( c_3 \) and the catalyzed fractionation during CO₂ hydration (\( b_2 \)). The catalyzed hydration reaction has a fractionation factor of 1.1‰ (calculated by summing the catalyzed CO₂ and HCO₃⁻ equilibrium fractionation factor −9.0‰ and the catalyzed dehydrogenation fraction 10.1‰; Mook et al., 1974; Paneth and O’Leary, 1985), whereas the uncatalyzed reaction has a 6.9‰ fractionation factor (Marlière and O’Leary, 1984). The fractionation attributed to mitochondrial respiration is \( c \) at a rate of mesophyll CO₂ release of \( M_m \).

The combined fractionation of Rubisco (30‰) respiration, and photorespiration (\( b_3 \)) can be calculated as:

\[ b_3 = 30 - s - c(M_m + M_t)/V_i - fV_e/V_i \]

where \( V_i \) is the rate of Rubisco carboxylation reaction, \( M_t \) is the rate of BSC mitochondrial respiration, \( V_e \) is the rate of photorespiration, and \( f \) is the discrimination of photorespiration (Farquhar, 1983).

Equation 2 assumes that the internal conductance to the diffusion of CO₂ between the intercellular airspace and the site of carboxylation in the mesophyll cytoplasm (\( g_c \)) is large, such that \( p_i \) is equal to the pCO₂ at the site of PEPC carboxylation (\( p_e \)). If \( g_c \) is low, then Equation 2 can be modified to:

\[ \Delta^{13}C = a + (b_2 + (b_3 - s - d)\frac{f}{p_w}) + A/(g(p_g)p_e)(e + a - b - (b_3 - s - d)) \]

where \( A \) is the net rate of CO₂ assimilation and \( a \) (0.7‰) is the fractionation of CO₂ diffusion through a liquid (O’Leary, 1984).

CA Activity Measurements

CA activity was measured on leaf extracts using mass spectrometry to measure the rates of ¹³CO₂ exchange from doubly labeled ¹³C¹⁸O₂ to H₂¹⁸O (Badger and Price, 1989; von Caemmerer et al., 2004). Measurements of leaf extracts were made at 25°C with a subsaturating total carbon concentration of 1 mM. The hydration rates were calculated from the enhancement in the rate of ¹⁸O loss over the uncatalyzed rate. We then applied this factor to the nonenzymatic first-order rate constant calculated at pH 7.4 appropriate for the mesophyll cytosol (Furbank et al., 1989) and report the CA activity as a first-order rate constant \( k_{e} (\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}) \). \( k_{e}\) then gives the in vivo CA activity at that particular cytosolic pCO₂. Leaf samples were collected after the gas-exchange measurements on the same leaf material and subsequently frozen in liquid nitrogen and stored at −80°C.

Dry Matter ¹³C

The opposite leaf to the one used during gas exchange was collected and oven dried at 70°C, and ground with a mortar and pestle. A subsample of ground tissue was weighed and the isotopic composition determined by combustion in a Carlo Erba elemental analyzer; the CO₂ was analyzed by mass spectrometry. δ was calculated as \[ \frac{(R_{\text{sample}} - R_{\text{standard}})}{R_{\text{standard}}} \times 1000 \], where \( R_{\text{sample}} \) and \( R_{\text{standard}} \) are the ¹³C/¹²C of the sample and the standard VPDB, respectively. Dry matter δ¹³C was determined on glasshouse-grown plants only because there were large fluctuations in the carbon isotopic composition of the air in the growth cabinets.

Photosynthetic Model

The C₄ photosynthetic model developed by Berry and Farquhar (1978) and von Caemmerer (2000) was used to predict the response of net CO₂ assimilation, bundle sheath pCO₂, pH, photorespiration, and δ to changes in the amount of PEPC activity (\( V_p \)). Manipulating \( V_p \) within the photosynthesis model was used to simulate the effect of changes in CO₂ hydration rates (\( V_e \)). The outputs from the C₄ photosynthetic model, specifically the rates of Rubisco carboxylation (\( V_e \)), the pCO₂ in the BSC, were incorporated into the model of C₄ carbon isotope discrimination (Δ¹³C) developed by Farquhar (1983). The Δ¹³C model was used to determine which photosynthetic parameters would influence Δ¹³C consistent with our experimental data.

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LITERATURE CITED


Paneth A, O’Leary G (1985) The fractionation during diffusion of CO₂ and HCO₃⁻ (calculated as (Farquhar, 1983): $\Delta^{13}C = a + (b_2 + b_3 - s - d)\frac{f}{p_w} + (c + b)V_e/V_i - eM_i/V_p$).