Carbonic Anhydrase and Its Influence on Carbon Isotope Discrimination during C_4 Photosynthesis. Insights from Antisense RNA in *Flaveria bidentis*[1]

Asaph B. Cousins*, Murray R. Badger, and Susanne von Caemmerer

Molecular Plant Physiology Group (A.B.C., M.R.B., S.V.C.) and Australian Research Council Centre of Excellence in Plant Energy Biology (M.R.B.), Research School of Biological Sciences, Australian National University, Canberra, Australian Capital Territory 2601, Australia

In C_4 plants, carbonic anhydrase (CA) facilitates both the chemical and isotopic equilibration of atmospheric CO_2 and bicarbonate (HCO_3^-) in the mesophyll cytoplasm. The CA-catalyzed reaction is essential for C_4 photosynthesis, and the model of carbon isotope discrimination ($\Delta^{13}C$) in C_4 plants predicts that changes in CA activity will influence $\Delta^{13}C$. However, experimentally, the influence of CA on $\Delta^{13}C$ has not been demonstrated in C_4 plants. Here, we compared measurements of $\Delta^{13}C$ during C_4 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 $\Delta^{13}C$, which was also evident in the leaf dry matter carbon isotope composition ($\delta^{13}C$). Contrary to the isotope measurements, photosynthetic rates were not affected until CA activity was less than 20% of wild type. Measurements of $\Delta^{13}C$, $\delta^{13}C$ of leaf dry matter, and rates of net CO_2 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 CO_2 assimilation; however, reducing leaf CA activity has a relatively large influence on $\Delta^{13}C$, often without changes in net CO_2 assimilation. Our data indicate that the extent of CA activity in C_4 leaves needs to be taken into account when using $\Delta^{13}C$ and/or $\delta^{13}C$ to model the response of C_4 photosynthesis to changing environmental conditions.

Isotope analysis of atmospheric CO_2 is an important tool for monitoring changes in the global exchange of CO_2 (Flanagan and Ehleringer, 1998; Yakir and Sternberg, 2000). However, to interpret the atmospheric CO_2 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 ($\Delta^{13}C$) in C_4 plants have been used for many years to help interpret the response of C_4 plants to changing environmental conditions. However, only recently has the genetic manipulation of the C_4 photosynthetic apparatus provided an opportunity to reexamine the C_4 leaf level models of $\Delta^{13}C$ (von Caemmerer et al., 1997a, 1997b).

Most C_4 plants utilize a compartmentalized CO_2-concentrating mechanism between the mesophyll and bundle sheath cells (BSC) to increase the CO_2 partial pressure (pCO_2) around the site of Rubisco in the BSC. The first enzymatic step in C_4 photosynthesis is the reversible hydration reaction catalyzed by carbonic anhydrase (CA), which converts CO_2 to bicarbonate (HCO_3^-) in the mesophyll cytoplasm. Subsequently, HCO_3^- is fixed via phospho*enol*pyruvate 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 C_4 plants results in a pCO_2 around the site of Rubisco severalfold higher than current atmospheric levels, significantly reducing the rates of photorespiration (Hatch, 1987; Kanai and Edwards, 1999).

The carbon isotope discrimination during C_4 photosynthesis is determined by the fractionation that occurs during diffusion of CO_2 into the leaf, its conversion to HCO_3^- 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 CO_2 is determined by the amount of leakiness (ϕ), defined as the fraction of CO_2 fixed by PEPC that subsequently leaks out of the BSC. If the BSC were gas tight, then all of the CO_2 released into the BSC would be fixed by Rubisco and no fractionation would occur at this step. However, CO_2 can leak out of the BSC, allowing Rubisco to influence the overall discrimination during C_4 photosynthesis (Farquhar, 1983; Peisker and Henderson, 1992).

Differences in the ratio of CO_2 partial pressures between the intercellular airspace and the atmosphere (p_i/p_a) along with ϕ are the main factors attributed to

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* Corresponding author; e-mail asaph.cousins@anu.edu.au; fax 61–2–61255075.

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variation in $\Delta^{13}C$ in C_4 plants (Farquhar, 1983). The ratio p_i/p_a is primarily determined by stomatal conductance, whereas ϕ depends on the physical conductance of the BSC walls and the balance between the C_4 and C_3 cycles. Little change in ϕ 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 ϕ 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 (micromass 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_a, and BSC CO_2 leakiness (ϕ), 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 (ξ; see Fig. 2, legend). Leakiness was calculated by rearranging Equation 2 (see equations in "Materials and Methods") and substituting b_4 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_p/V_h) 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

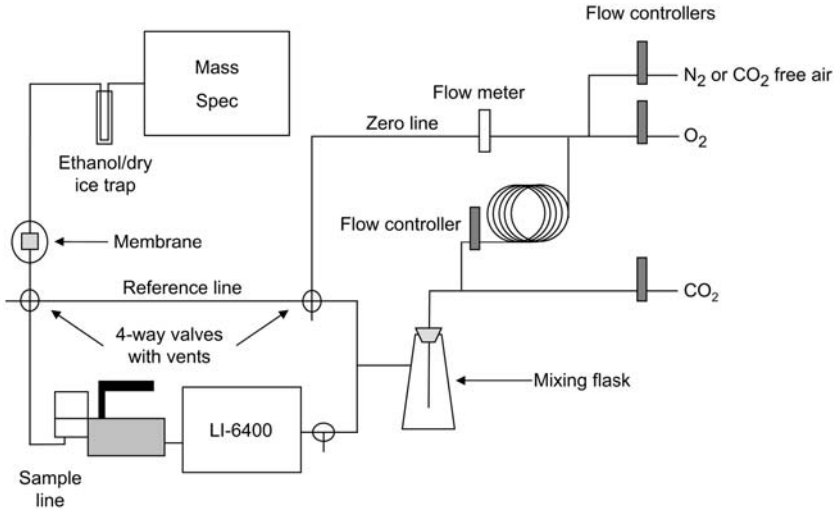

Figure 1. Arrangement of the gas flow controllers, the LI-6400 gas exchange system, and the mass spectrometer system used for simultaneous measurements of leaf gas exchange and carbon isotope discrimination. Switching between gas samples was controlled by a manual four-way valve. The zero and reference readings were made before and after each leaf measurement and averaged during the calculations.

Table I. *Symbols used in the text*

Symbol	Description
A	Net CO_2 assimilation
a	Fractionation during diffusion of CO_2 from the chloroplast to the atmosphere (4.4‰)
a_l	Fractionation of CO_2 diffusion through a liquid (0.7‰)
BSC	Bundle sheath cells
b_3	Combined discrimination of Rubisco, respiration, and photorespiration (see Eq. 4)
b_4	Combined discrimination of PEPC, respiration, and hydration/dehydration of CO_2 (see Eq. 3 and Fig. 4)
b_p	Discrimination by PEPC (2.2‰)
$\Delta^{13}C$	Carbon isotope discrimination
CA	Carbon anhydrase
e	Fractionation during respiration (3‰ or −6‰)
e_s	Fractionation as CO_2 dissolves (1.1‰)
e_b	Equilibrium fractionation factor for the catalyzed hydration/dehydration of CO_2 (−9‰)
f	Discrimination during photorespiration (10‰ or −6.8‰)
ϕ	The fraction of CO_2 fixed by PEPC that subsequently leaks out of the BSC
g_w	The internal conductance to the diffusion of CO_2 between the intercellular air space and the site of carboxylation in the mesophyll cytoplasm
h	Catalyzed fractionation during CO_2 hydration (1.1‰)
k_{CA}	Rate constant of carbonic anhydrase
K_c	Michaelis constant of Rubisco for CO_2
K_o	Michaelis constant of Rubisco for O_2
K_p	Michaelis constant of PEPC for CO_2
M_d	Rate of mitochondrial respiration
M_m	Rate of mitochondrial respiration in the mesophyll cells
M_s	Rate of mitochondrial respiration in the BSC
pCO_2	Partial pressure of CO_2
p_e	pCO_2 of dry air entering the leaf chamber
p_i	pCO_2 of the intercellular airspace
p_m	pCO_2 of the mesophyll cytoplasm
p_o	pCO_2 of dry air leaving the leaf chamber
R_e	$^{13}C/^{12}C$ of the air entering the leaf chamber
R_o	$^{13}C/^{12}C$ of the air leaving the leaf chamber
PFD	Photon flux density
PSII	PSII
ξ	$p_e/(p_e - p_o)$
s	Fractionation during the leakage of CO_2 from the BSC (1.8‰)
V_c	Rate of Rubisco carboxylation
V_{cmax}	Maximal rate of Rubisco carboxylation
V_h	Rate of CO_2 hydration
V_o	Rate of photorespiration
V_p	Rate of PEP carboxylation $(A + M_d)/(1 - \phi)$ or $(p_m V_{pmax})/(p_m + K_p)$
V_{pmax}	Maximal rate of PEPC carboxylation

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_a, $\Delta^{13}C$, and ϕ were higher in the anti-SSu-plants as compared with wild-type plants (Table II). The parameter ϕ was determined from simultaneous gas-exchange and isotope measurements and solving for ϕ in Equation 2. Our measurements of $\Delta^{13}C$ and leaf gas exchange 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} μmol m^{-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 $H_2{}^{16}O$ (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‰, 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_a 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_a 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_a was calculated with a ϕ value of 0.24, and the initial CO_2 carboxylation reaction catalyzed by PEPC relative to the CO_2 hydration by CA (V_p/V_h) was assumed to be either zero or 1 (as indicated in Fig. 4). The b_4 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_a 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),

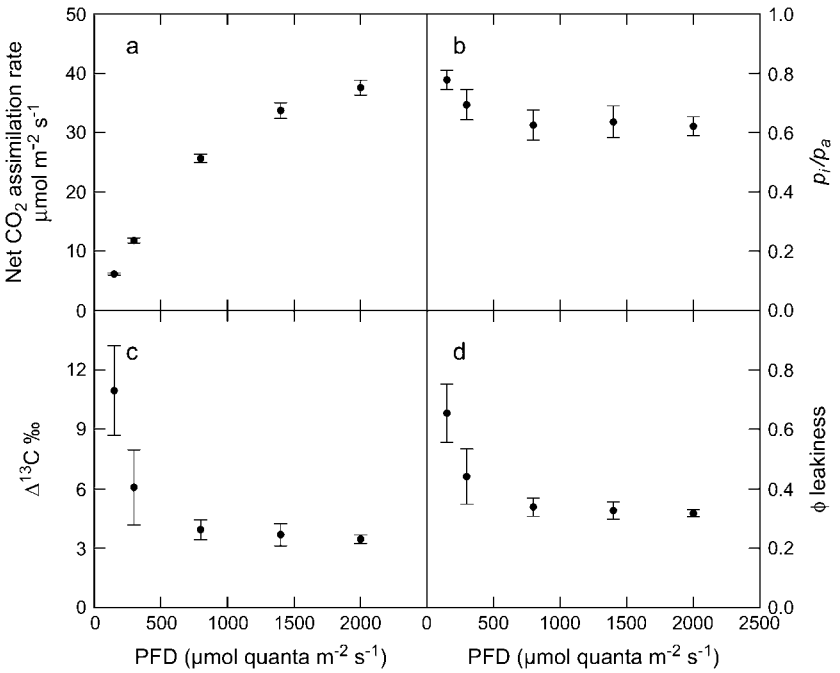

Figure 2. a, Net CO_2 assimilation rate. b, Ratio of intercellular to ambient CO_2 partial pressures (p_i/p_a). c, Carbon isotope discrimination ($\Delta^{13}C$). d, Bundle sheath leakiness to CO_2 (ϕ) as a function of PFD (μmol quanta m^{-2} s^{-1}). Measurements were made at a pCO_2 of 52 Pa, a pO_2 of 4.8 kPa, and a leaf temperature of 30°C. Shown are the means ± the SE of measurements made on three to five leaves from two *F. bidentis* wild-type plants. Values for ξ (Eq. 1) were 29.9 ± 0.75, 15.7 ± 0.54, 7.11 ± 0.24, 5.5 ± 0.23, and 4.9 ± 0.17 at PFDs of 150, 300, 800, 1,400, and 2,000 μmol quanta m^{-2} s^{-1}. ϕ was calculated from Equation 5, assuming $V_p/V_h = 0$.

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 ϕ.

It should be noted that the absolute value of V_p/V_h determined this way is largely influenced by ϕ 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 ϕ 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 ϕ 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 $(A + M_d)/(1 - \phi)$ (von Caemmerer, 2000). The parameter M_d is the daytime rate of mitochondrial respiration assumed to be 2 μmol 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 ϕ, although neither

Table II. *CA rate constant (k_{CA}), net CO_2 assimilation rate (A), ratio of intercellular to atmospheric CO_2 partial pressure (p_i/p_a), online $\Delta^{13}C$ discrimination, and leakiness of CO_2 out of the BSCs (ϕ) in the anti-SSu plants from the primary transformant 136-13*

For calculation of ϕ, the V_p/V_h ratio was assumed to be zero. Measurements were made at a pCO_2 of 52 Pa, a pO_2 of 4.8 kPa, a PFD of 2,000 μmol quanta m^{-2} s^{-1}, and a leaf temperature of 30°C. $n = 4$ for the wild-type plants.

	k_{CA}	A	p_i/p_a	$\Delta^{13}C$	Leakiness ϕ
	mol m^{-2} s^{-1} Pa^{-1}	*µmol m^{-2} s^{-1}*		‰	
136-13-11#1	52	17.0	0.67	6.1	0.43
136-13-12#4	88	19.5	0.64	6.5	0.45
136-13-12#3	62	27.9	0.72	6.0	0.42
136-13-11#2	81	29.9	0.71	5.4	0.39
Wild type	69 ± 2	37.6 ± 2.6	0.55 ± 0.02	2.5 ± 0.4	0.25 ± .02

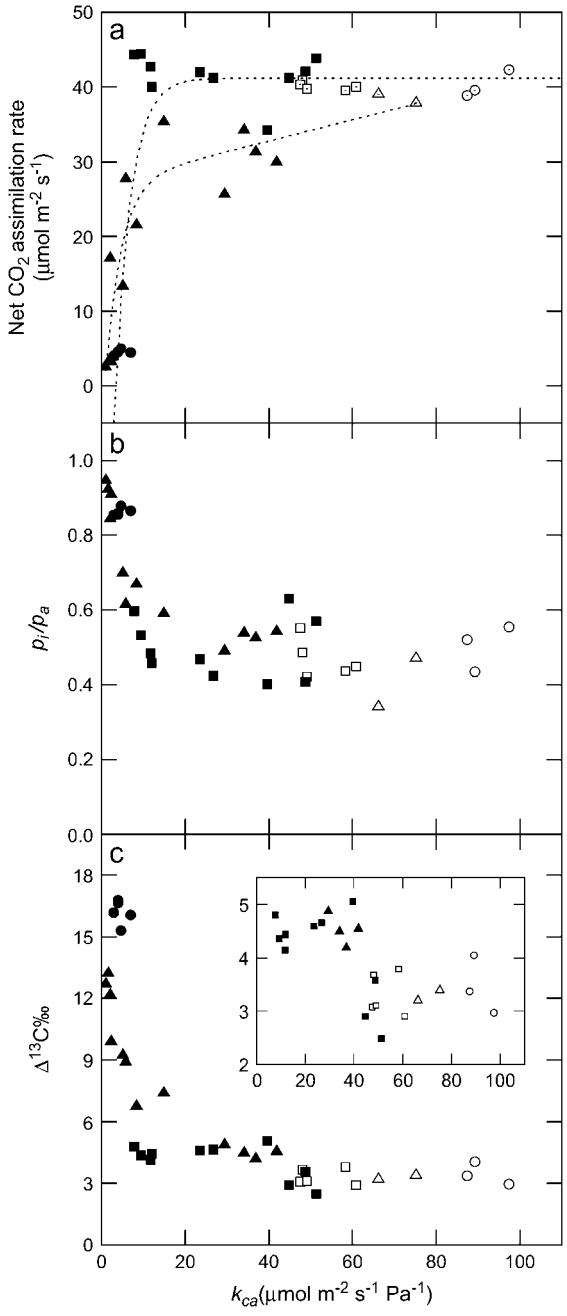

Figure 3. Net CO_2 assimilation rate, the ratio of intercellular to ambient pCO_2 (p_i/p_a), and carbon isotope discrimination ($\Delta^{13}C$) as a function of the rate constant of leaf CA (k_{CA} μmol m^{-2} s^{-1} Pa^{-1}). The inset in c shows the expanded scale of $\Delta^{13}C$ where net CO_2 assimilation 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 μmol 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.

parameter has a large influence on the relative changes of V_p/V_h between wild-type and anti-CA 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 pCO_2, photorespiration (V_o), and ϕ 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 ϕ (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, ϕ, and the pCO_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 ϕ on $\Delta^{13}C$ independent of changes in V_p/V_h. The model in Figure 6 included sufficient CA activity to keep V_p/V_h close to zero as V_p changes and p_i/p_a were held constant at 0.4. As shown in Figure 6c, when ϕ and the pCO_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‰ versus −6‰) and f (−6.8‰ versus 10‰) 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).

Table III. *Net CO_2 assimilation rate (A), the ratio of intercellular to ambient pCO_2 (p_i/p_a), CA_{leaf} calculated as ($k_{CA}p_m$), $\Delta^{13}C$, and V_p/V_h for F. bidentis wild-type plants and anti-CA plants with low CA_{leaf} activity and wild-type-like net CO_2 assimilation rates*

Measurements were made at a pCO_2 of 52 Pa, a pO_2 of 4.8 kPa, PFD of 2,000 μmol quanta m^{-2} s^{-1}, and a leaf temperature of 30°C. V_p/V_h was estimated either by online $\Delta^{13}C$ measurements* using Eqs. 3 and 5 (''Materials and Methods'') or estimated from V_p/CA_{leaf}**, where V_p was calculated as $(A + M_d)/(1 - \phi)$ and CA_{leaf}. g_w 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. M_d 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.

	A	p_i/p_a	CA_{leaf}	$\Delta^{13}C$		V_p/V_h			
						$g_w = 10$ $\phi = 0.24$	$g_w = 6$ $\phi = 0.24$	$g_w = 10$ $\phi = 0.1$	$g_w = 6$ $\phi = 0.1$
	μmol m^{-2} s^{-1}		μmol m^{-2} s^{-1}	‰					
Anti-CA	42 ± 0.6	0.48 ± 0.02	228 ± 37	4.4 + 0.2	$\Delta^{13}C$*	0.46 ± 0.06	0.55 ± 0.07	0.99 ± 0.08	1.1 ± 0.08
					In vitro**	0.29 ± 0.04	0.40 ± 0.04	0.24 ± 0.03	0.30 ± 0.04
Wild type	40 ± 0.1	0.47 ± 0.02	774 ± 48	3.3 ± 0.2	$\Delta^{13}C$*	0.07 ± 0.07	0.08 ± 0.08	0.61 ± 0.06	0.62 ± 0.08
					In vitro**	0.07 ± 0.01	0.09 ± 0.01	0.06 ± 0.01	0.07 ± 0.01

DISCUSSION

Carbon isotope discrimination increased in the short term during leaf gas exchange ($\Delta^{13}C$) and the carbon isotope composition of leaf dry matter ($\delta^{13}C$) 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 CO_2 assimilation in *F. bidentis* at high light. A nearly 80% decrease in leaf CA activity was needed before net CO_2 assimilation was affected when measured at a CO_2 partial pressure (pCO_2) of 52 Pa (Fig. 3a). The CA activity required to maintain wild-type-like photosynthetic rates increased when measurements were conducted at a lower pCO_2 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 CO_2 response curve in *F. bidentis* when the rate of CO_2 hydration limited the supply of HCO_3^- for PEPC carboxylation (von Caemmerer et al., 2004).

Carbon Isotope Discrimination and CA Activity

According to the C_4 photosynthetic model (von Caemmerer, 2000), a limitation in the supply of cytosolic HCO_3^- will lead to a decrease in the initial CO_2 carboxylation reaction catalyzed by PEPC and reduce the capacity of the C_4 pump to concentrate CO_2 within the BSC. A reduced pCO_2 in the BSC leads to a decrease in the rate of net CO_2 assimilation as well as a lower BSC CO_2 leakiness (ϕ). In the model of C_4 carbon isotope discrimination, the main factors that influence $\Delta^{13}C$ are changes in the intercellular to ambient CO_2 partial pressures (p_i/p_a) and ϕ (Farquhar, 1983). When the ratio of PEPC carboxylation to the hydration reaction of CO_2 (V_p/V_h) is near zero (i.e. CA activity is high relative to PEPC carboxylation), the C_4 carbon isotope model predicts that $\Delta^{13}C$ will decrease as ϕ decreases (Fig. 6). The $\Delta^{13}C$ modeling illustrates that when the front end of the C_4 cycle is diminished (either by reduced CA and/or PEPC activity or anything else), ϕ decreases and $\Delta^{13}C$ associated with ϕ also decreases (Fig. 6). However, in the anti-CA plants, which potentially reduced the ability to concentrate CO_2 in the BSC, $\Delta^{13}C$ increased, which cannot be explained in the model by decreases in ϕ, but can be explained by changes in V_p/V_h.

Due to the high levels of mesophyll cytoplasmic CA activity in C_4 plants, it is generally assumed that CO_2 and HCO_3^- are in close chemical equilibrium. Under such conditions, the ratio of V_p/V_h in Equation 3 approaches zero and can be omitted from the calculation of b_4. However, when V_p/V_h tends away from zero, Equation 3 can be expressed with the fractionation factors provided in ''Materials and Methods'' as $b_4 = -5.7 + 7.9\ V_p/V_h$ at 25°C. The b_4 fractionation factor becomes more positive as V_p/V_h increases and $\Delta^{13}C$ increases even without changes in p_i/p_a and ϕ. As shown in Figure 4, varying V_p/V_h between 0 and 1 can

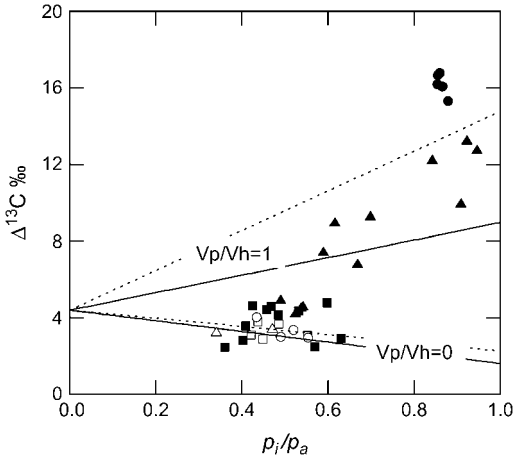

Figure 4. Carbon isotope discrimination ($\Delta^{13}C$) as a function of the ratio of intercellular to ambient pCO_2 (p_i/p_a). The white symbols are wild-type plants and the black symbols are anti-CA plants. Other symbols and measurement conditions are as described in Figure 3. The lines represent the theoretical relationship of $\Delta^{13}C$ and p_i/p_a, where $\phi = 0.24$, the ratio of the PEPC carboxylation to the CO_2 hydration reaction (V_p/V_h) is either 0 or 1, and the b_4 parameter is calculated with the catalyzed (solid lines, $b_4 = -5.7 + 7.9\ V_p/V_h$) and uncatalyzed (dotted lines, $b_4 = -4.5 + 12.5\ V_p/V_h$) CO_2 and HCO_3^- hydration and dehydration fractionation factors. g_w was assumed to be large such that $p_i = p_m$.

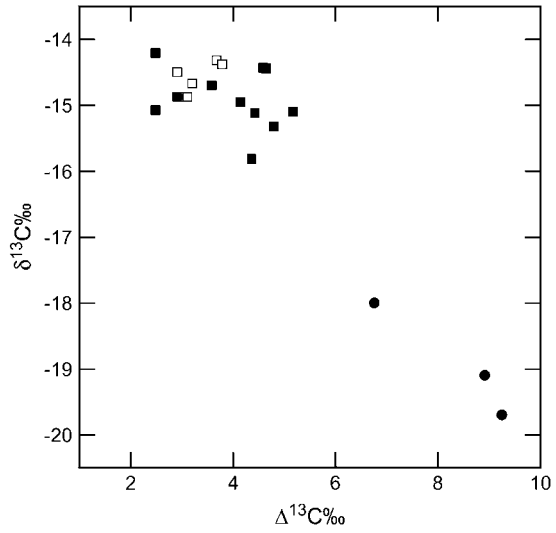

Figure 5. Leaf dry matter $\delta^{13}C$, determined on the entire leaf opposite to the one used for gas exchange and enzyme analysis, plotted against changes in online carbon isotope discrimination ($\Delta^{13}C$). The white symbols are wild-type plants and the black symbols are anti-CA plants. All plants were germinated and grown in a glasshouse under ambient atmospheric CO_2 conditions. The plants with extremely low $\delta^{13}C$ values (●) were transferred to the 1% CO_2 growth cabinets after tissue was collected for $\Delta^{13}C$ analysis.

have a large impact on $\Delta^{13}C$, especially when p_i/p_a 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_a not accurately predict $\Delta^{13}C$ (see below for further discussion).

Variation in the Ratio of PEPC Carboxylation to CO_2 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 CO_2 assimilation. The influence of V_p/V_h on $\Delta^{13}C$ is predicted by the C_4 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 C_4 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 C_4 species is only just sufficient to support photosynthetic rates, especially in C_4 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 C_4 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 μmol m^{-2} s^{-1}, which is similar to our anti-CA plants with wild-type photosynthetic rate (see Fig. 3c; Table III). Gillon and Yakir (2001) reported even lower CA activity for a number of C_4 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 CO_2 assimilation from this article indicate that $\Delta^{13}C$ will differ in C_4 plants that have been reported to contain a range of CA_{leaf} activity (2–529 μmol m^{-2} s^{-1}) with generally similar photosynthetic rates (Gillon and Yakir, 2001). However, a

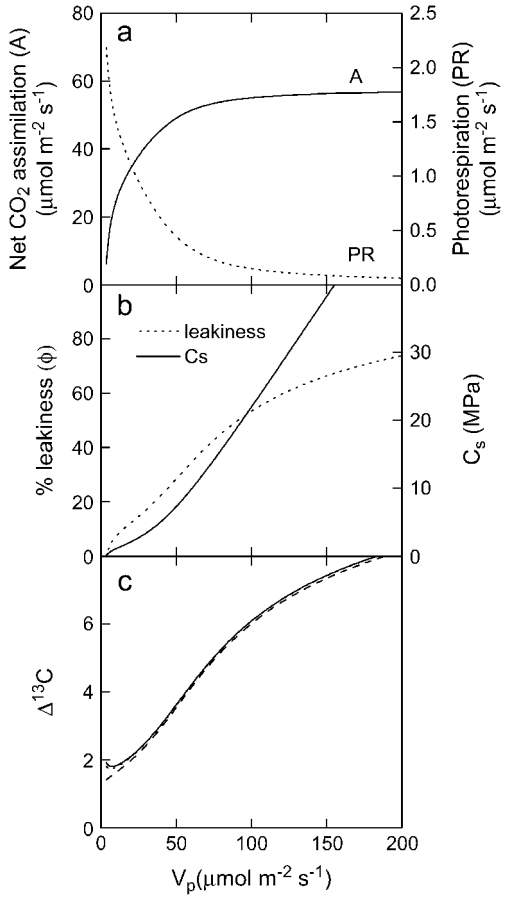

Figure 6. Modeling the response of net CO_2 assimilation (a); the pCO_2 in the BSC and BSC CO_2 leakiness, ϕ (b); and $\Delta^{13}C$ (c) in response to changes in PEPC activity (V_p). The C_4 model used a V_{cmax} of 60 μmol m^{-2} s^{-1}, a bundle sheath conductance to CO_2 per leaf area of 0.03 μmol m^{-2} s^{-1} Pa^{-1}, K_m of PEPC for CO_2 (K_p) of 8 Pa, K_m of Rubisco for CO_2 (K_c) and O_2 (K_o) of 65 Pa and 45 kPa, fraction of PSII in the BSC 0.2 and Rubisco specificity of 2,590 Pa/Pa in the gas phase, and mitochondrial respiration was 2 μmol m^{-2} s^{-1}, one-half of which was assumed to occur in the mesophyll. The pO_2 in the mesophyll was assumed to be 20 kPa. Carbon isotope discrimination was calculated using the C_4 photosynthetic model output and a constant p_i/p_a of 0.4. V_h was set at 2,000 μmol m^{-2} s^{-1} and V_p/V_h varied between 0.001 and 0.1, causing only a 0.3‰ shift in $\Delta^{13}C$ at a constant ϕ. The lines for $\Delta^{13}C$ represent models determined with Equation 2 by substituting the b_4 and b_3 factors with Equations 3 and 4, respectively. The lines for $\Delta^{13}C$ represent models using different fractionation factors for respiration (3‰ dotted line and −6‰ solid and dashed lines) and photorespiration (10‰ dashed line and −6.8‰ dotted and solid lines).

systematic investigation of the influence of V_p/V_h on $\Delta^{13}C$ in a range of C_4 species needs to be conducted.

Changes in V_p/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 CO_2 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_i/p_a and/or ϕ, with the apparent assumption that V_p/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 also influenced by environmental conditions, including nitrogen status, atmospheric CO_2 availability, and salt stress (Cervigni et al., 1971; Burnell et al., 1990; Brownell et al., 1991), implying that environmental conditions may also alter V_p/V_h and thus influence measured $\Delta^{13}C$. Because leaf CA activity in C_4 plants is largely dependent on the internal CO_2 partial pressures, conditions that influence CO_2 availability, such as water stress and growth under elevated atmospheric CO_2, will also alter V_p/V_h. C_4 photosynthesis generally operates near CO_2-saturating conditions at current atmospheric pCO_2 such that a reduction in p_i due to stomatal closure will cause V_p/V_h to increase. However, under such conditions, the ratio of p_i/p_a also decreases and the influence of V_p/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 pCO_2 (McLeod and Long, 1999; Ghannoum et al., 2000; Wall et al., 2001; Ainsworth and Long, 2005; Leakey et al., 2006). However, because p_i/p_a is generally constant with changing atmospheric pCO_2 and CA has a very high K_m for HCO_3^-, an increase in CO_2 availability will increase V_h, whereas PEPC is generally saturated around ambient pCO_2 and V_p will not change. This raises the possibility that growth under future atmospheric CO_2 conditions will alter $\Delta^{13}C$ regardless of other environmental changes if CA is limiting.

Both online and in vitro measurements of V_p/V_h indicated that changes in CA activity have a significant influence on $\Delta^{13}C$ without changes in p_i/p_a and ϕ. It must be noted that, although changes in g_w have a subtle effect on estimates of V_p/V_h, variation in ϕ can lead to large shifts in the absolute values of V_p/V_h when determined from the $\Delta^{13}C$ measurements. There are no direct means of measuring ϕ, but it can be estimated using $\Delta^{13}C$ measurements when V_p/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_p/V_h values and would allow an estimate of ϕ when V_p/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_p/V_h and p_i/p_a (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 CO_2 in the BSC, leakage of HCO_3^- from the BSC, as well as photorespiration and respiration would influence $\Delta^{13}C$ especially when net CO_2 assimilation is inhibited. Theoretically, CO_2 assimilation by direct diffusion of CO_2 from the atmosphere into the BSC would increase the $\Delta^{13}C$ as the exchange of CO_2 between the atmosphere and the BSC would allow Rubisco to fractionate against the heavier carbon isotope. However, a low conductance of CO_2 diffusion across the BSC (g_w mmol m^{-2} s^{-1} Pa^{-1}) is an essential component of the C_4 CO_2-concentrating mechanism and limits the amount of direct fixation of CO_2 under ambient CO_2 concentrations (Jenkins et al., 1989; 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 CO_2 in the BSC and minimal influence on $\Delta^{13}C$.

Alternatively, because ^{13}C concentrates in HCO_3^- and Rubisco preferentially fixes ^{12}C, leakage of HCO_3^- out of the BSC would change the fractionation factor associated with CO_2 leakage from the BSC (s from Eq. 2). However, with the relatively low CA activity in the BSC, it is unlikely that CO_2 and HCO_3^- 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 CO_2 assimilation decrease. However, changing the fractionation effect of respiration (e in Eqs. 3 and 4) and photorespiration (f 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 $b_4 = -5.7 + 7.9\ V_p/V_h$ at 25°C when the catalyzed fractionation values for e_b of −9.0‰ and h of 1.1‰ are used. However, if the interconversion of CO_2 and HCO_3^- occurs via the spontaneous uncatalyzed reaction, e_b and h become −7.8‰ and 6.9‰, respectively, and Equation 3 is $b_4 = -4.5 + 12.5\ V_p/V_h$, causing the b_4 value to become larger, leading to an increase in $\Delta^{13}C$ (Fig. 4). The catalyzed and uncatalyzed values of e_b and h are taken from previously published work on the hydration and dehydration of CO_2 and HCO_3^- (Mook et al., 1974; Marlier 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 ϕ in *F. bidentis* (Fig. 2) are 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 ϕ increases at low PFD as more electron transport is needed for recycling of photorespired CO_2 (von Caemmerer, 2000). The predicted change in ϕ at low PFD by the C_4 photosynthetic model is consistent with our current experimental evidence, as well as earlier published 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_h) 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_h, and thus on $\Delta^{13}C$ warrants further investigation.

Additionally, the amount of CA activity in a leaf plays an important role in determining $C^{18}OO$ discrimination during C_4 photosynthesis because CA enhances the rate of oxygen exchange between CO_2 and leaf H_2O and thus determines the extent of isotopic equilibrium. The anti-CA plants will be used to test whether changing leaf CA activity influences $C^{18}OO$ 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_2O 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-SSu plants) or a putative cytosolic CA (anti-CA plants; Furbank et al., 1996; von Caemmerer et al., 1997b, 2004). The segregating T_1 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-SSu plants (segregating T_2 generation from primary transformant 136-13) with low photosynthetic capacities and wild-type plants were grown under 1% CO_2 in a controlled environment growth cabinet at a photosynthetic PFD of 400 μmol 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 (15/4.8/10.8/1.2 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 μmol 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 gas 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_2O and other contaminates contributing to the 44 and 45 peaks. Pure CO_2 ($\delta^{13}C = -29‰$; 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 use to minimize contamination of the 46 peak caused by the interaction of O_2 and N_2 to produce NO_2 with the mass spectrometer source element. This was important when looking at $C^{18}OO$ 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 fed to the inlet of the LI-6400 console and a flow rate of 200 μmol 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 or by placing a tee in the match valve line, allowing flow to both 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 trap and a thin, gas-permeable silicone membrane, which was housed in a temperature-controlled cuvette. Masses 44 and 45 were monitored continuously and the carbon isotope discrimination during CO_2 exchange, $\Delta^{13}C$, was calculated from the ratio of mass 45 to 44 in the reference air, determined before and after each sample measurement, entering the chamber (R_e), and the composition of the sample air leaving the leaf chamber (R_o) as described by Evans et al. (1986):

$$\Delta = \frac{-\xi(R_e/R_o - 1)}{1+\xi(R_e/R_o - 1)}, \quad (1)$$

where $\xi = p_e/(p_e - p_o)$, and p_e and p_o are the CO_2 partial pressures of dry air entering and leaving the leaf chamber, respectively. A summary of the

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 C_4 carbon isotope discrimination ($\Delta^{13}C$) of Farquhar (1983) was used to determine which factors in the model would influence $\Delta^{13}C$ consistent with our experimental data. The simplified model predicts that:

$$\Delta^{13}C = a + (b_4 + (b_3 - s)\phi - a)p_i/p_a, \quad (2)$$

where a (4.4‰) is the fractionation during diffusion of CO_2 and s (1.8‰) is the fractionation during CO_2 leakage from the BSCs. The combined fractionation of PEPC, respiration, and the isotopic equilibrium during dissolution of CO_2 and conversion to HCO_3^- (b_4) is calculated as (Farquhar, 1983):

$$b_4 = (b_p + e_s + e_b)(1 - V_p/V_h) + (e_s + h)V_p/V_h - eM_m/V_p, \quad (3)$$

where b_p (2.2‰) is the fractionation by PEPC (O'Leary, 1981), e_s (1.1‰) is the fractionation as CO_2 dissolves (O'Leary, 1984), and e_b (−9‰) is the equilibrium fractionation factor of the catalyzed hydration/dehydration reactions of CO_2 and HCO_3^- (Mook et al., 1974). Alternatively, during the hydration/dehydration reactions, the uncatalyzed equilibrium fractionation factor $e_b = -7.8$‰ (Marlier and O'Leary, 1984). The fractionation when CO_2 and HCO_3^- are not at equilibrium is dependent on the rate of CO_2 hydration (V_h), the rate of PEPC (V_p), e_s, and the catalyzed fractionation during CO_2 hydration (h). The catalyzed hydration reaction has a fractionation factor of 1.1‰ (calculated by summing the catalyzed CO_2 and HCO_3^- equilibrium fractionation factor −9.0‰ and the catalyzed dehydration fractionation factor 10.1‰; Mook et al., 1974; Paneth and O'Leary, 1985), whereas the uncatalyzed reaction has a 6.9‰ fractionation factor (Marlier and O'Leary, 1984). The fractionation attributed to mitochondrial respiration is e at a rate of mesophyll CO_2 release of M_m.

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

$$b_3 = 30 - s - e(M_m + M_s)/V_c - fV_o/V_c, \quad (4)$$

where V_c is the rate of Rubisco carboxylation reaction, M_s is the rate of BSC mitochondrial respiration, V_o 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 CO_2 between the intercellular airspace and the site of carboxylation in the mesophyll cytoplasm (g_w) is large, such that p_i is equal to the pCO_2 at the site of PEPC carboxylation (p_c). If g_w is low, then Equation 2 can be modified to:

$$\Delta^{13}C = a + (b_4 + (b_3 - s)\phi - a)p_i/p_a + A/(g_w p_a)(e_s + a_l - b_4 - (b_3 - s)\phi), \quad (5)$$

where A is the net rate of CO_2 assimilation and a_l (0.7‰) is the fractionation of CO_2 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 $^{18}O_2$ exchange from doubly labeled $^{13}C^{18}O_2$ to $H_2{}^{16}O$ (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 ^{18}O 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_{CA} (mol m^{-2} s^{-1} Pa^{-1}). $k_{CA}p_m$ then gives the in vivo CA activity at that particular cytosolic pCO_2. 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 $\delta^{13}C$

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 CO_2 was analyzed by mass spectrometry. δ was calculated as $[(R_{sample} - R_{standard})/R_{standard}]1{,}000$, where R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$ of the sample and the standard VPDB, respectively. Dry matter $\delta^{13}C$ 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 C_4 photosynthetic model developed by Berry and Farquhar (1978) and von Caemmerer (2000) was used to predict the response of net CO_2 assimilation, bundle sheath pCO_2, p_i/p_a, photorespiration, and ϕ 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 CO_2 hydration rates (V_h). The outputs from the C_4 photosynthetic model, specifically the rates of Rubisco carboxylation (V_c), V_o, V_p, ϕ, and the pCO_2 in the BSC, were 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.

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