

# A Transgenic Approach to Understanding the Influence of Carbonic Anhydrase on C<sup>18</sup>O Discrimination during C<sub>4</sub> Photosynthesis<sup>1</sup>

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The oxygen isotope composition of atmospheric CO<sub>2</sub> is an important signal that helps distinguish between ecosystem photosynthetic and respiratory processes. In C<sub>4</sub> plants the carbonic anhydrase (CA)-catalyzed interconversion of CO<sub>2</sub> and bicarbonate (HCO<sub>3</sub><sup>-</sup>) is an essential first reaction for C<sub>4</sub> photosynthesis but also plays an important role in the CO<sub>2</sub>-H<sub>2</sub>O exchange of oxygen as it enhances the rate of isotopic equilibrium between CO<sub>2</sub> and water. The C<sub>4</sub> dicot *Flaveria bidentis* containing genetically reduced levels of leaf CA (CA<sub>leaf</sub>) has been used to test whether changing leaf CA activity influences online measurements of C<sup>18</sup>O discrimination (Δ<sup>18</sup>O) and the proportion of CO<sub>2</sub> in isotopic equilibrium with leaf water at the site of oxygen exchange (θ). The Δ<sup>18</sup>O in wild-type *F. bidentis*, which contains high levels of CA relative to the rates of net CO<sub>2</sub> assimilation, was less than predicted by models of Δ<sup>18</sup>O. Additionally, Δ<sup>18</sup>O was sensitive to small decreases in CA<sub>leaf</sub>. However, reduced CA activity in *F. bidentis* had little effect on net CO<sub>2</sub> assimilation, transpiration rates (E), and stomatal conductance (g<sub>s</sub>) until CA levels were less than 20% of wild type. The values of θ determined from measurements of Δ<sup>18</sup>O and the <sup>18</sup>O isotopic composition of leaf water at the site of evaporation (δ<sub>e</sub>) were low in the wild-type *F. bidentis* and decreased in transgenic plants with reduced levels of CA activity. Measured values of θ were always significantly lower than the values of θ predicted from in vitro CA activity and gas exchange. The data presented here indicates that CA content in a C<sub>4</sub> leaf may not represent the CA activity associated with the CO<sub>2</sub>-H<sub>2</sub>O oxygen exchange and therefore may not be a good predictor of θ during C<sub>4</sub> photosynthesis. Furthermore, uncertainties in the isotopic composition of water at the site of exchange may also limit the ability to accurately predict θ in C<sub>4</sub> plants.

The oxygen isotope composition (δ<sup>18</sup>O) of atmospheric CO<sub>2</sub> is an important tool for monitoring variations in the global exchange of CO<sub>2</sub> (Farquhar et al., 1989, 1993; Farquhar and Lloyd, 1993; Yakir and Wang, 1996; Flanagan and Ehleringer, 1998; Yakir and Sternberg, 2000). The use of isotopes in this context relies on the fact that photosynthesis and ecosystem respiration generally have different effects on the isotope composition of atmospheric CO<sub>2</sub> and the δ<sup>18</sup>O of CO<sub>2</sub> in the atmosphere has been used to distinguish between the photosynthetic CO<sub>2</sub> uptake and CO<sub>2</sub> release during respiration (Farquhar et al., 1993; Gillon and Yakir, 2001). Dissolved CO<sub>2</sub> exchanges oxygen molecules with water allowing the CO<sub>2</sub> to take on the isotopic signature of the water that is in a much higher molar concentration. The water in leaves is generally highly enriched in <sup>18</sup>O relative to ground water due to the preferential evaporation of H<sub>2</sub><sup>16</sup>O during leaf tran-

spiration (Yakir and Wang, 1996; Yakir and Sternberg, 2000). Furthermore the exchange of <sup>18</sup>O between CO<sub>2</sub> and water is facilitated in leaves by the presence of carbonic anhydrase (CA), which catalyzes the interconversion of CO<sub>2</sub> and bicarbonate (HCO<sub>3</sub><sup>-</sup>). Thus, the retrodiffusion of CO<sub>2</sub> out of a leaf during photosynthesis drives the <sup>18</sup>O enrichment of atmospheric CO<sub>2</sub>. Root respiration and decomposition of organic material by soil microbes work in the opposite direction, releasing CO<sub>2</sub> depleted in <sup>18</sup>O because it is in equilibrium with unenriched water (Flanagan and Ehleringer, 1998; Yakir and Sternberg, 2000).

To accurately interpret the δ<sup>18</sup>O of atmospheric CO<sub>2</sub> requires an understanding of the isotopic fractionation steps associated with specific processes during leaf gas exchange (Yakir and Sternberg, 2000). The δ<sup>18</sup>O of the water at the site CO<sub>2</sub>-H<sub>2</sub>O oxygen exchange within a leaf (δ<sub>ex</sub>) is the primary factor influencing the δ<sup>18</sup>O of CO<sub>2</sub> diffusing out of a leaf (Farquhar et al., 1993). Additionally, the proportion of CO<sub>2</sub> in isotopic equilibrium with the water at the site of oxygen exchange (θ) will also influence the δ<sup>18</sup>O of the CO<sub>2</sub>. The value of θ is determined by the balance between the gross flux of CO<sub>2</sub> into the leaf and the activity of CA at the site of CO<sub>2</sub>-H<sub>2</sub>O oxygen isotope exchange as this influences the residence time of CO<sub>2</sub> within a leaf and thus the number of hydration reactions per CO<sub>2</sub> molecule (Gillon and Yakir, 2000a, 2000b).

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The CA-catalyzed hydration of  $\text{CO}_2$  to  $\text{HCO}_3^-$  is the first enzymatic step of the  $\text{C}_4$  photosynthetic pathway, a biochemical  $\text{CO}_2$  concentrating mechanism that concentrates  $\text{CO}_2$  around Rubisco in bundle sheath cells (BSC; Hatch, 1987; Kanai and Edwards, 1999). The  $\text{HCO}_3^-$  is subsequently fixed via phosphoenol pyruvate carboxylase into a four-carbon acid that diffuses to the BSC for decarboxylation (Kanai and Edwards, 1999). The majority of CA in a  $\text{C}_4$  plant occurs within the mesophyll cytoplasm and the CA-catalyzed oxygen exchange between  $\text{CO}_2$ - $\text{H}_2\text{O}$  occurs throughout the cytoplasmic space (Ku and Edwards, 1975; Burnell and Hatch, 1988; Hatch and Burnell, 1990). This is in contrast to  $\text{C}_3$  plants where the chloroplasts, which contain the majority of the leaf's CA, are appressed against the cell walls adjacent to the intercellular air space and the sites of water evaporation during transpiration. In  $\text{C}_3$  plants the majority of leaf CA activity is generally thought to be in close proximity to the water at the site of evaporation (Williams et al., 1996; Gillon and Yakir, 2000a). Under such conditions the isotopic signature of water at the site of evaporation ( $\delta_e$ ) is likely similar to the site of  $\text{CO}_2$ - $\text{H}_2\text{O}$  oxygen exchange  $\delta_{\text{ex}}$ ; however, this may not be the case in  $\text{C}_4$  species.

Online measurements of  $\text{C}^{18}\text{OO}$  discrimination ( $\Delta^{18}\text{O}$ ) during gas exchange of leaves have found  $\Delta^{18}\text{O}$  to be much lower in two  $\text{C}_4$  monocots (*Zea mays* and *Sorghum bicolor*) than what is commonly observed for  $\text{C}_3$  species (Gillon and Yakir, 2000a, 2000b). This lower discrimination can be explained by a combination of lower stomatal conductances and low CA activities assuming that the  $\text{CO}_2$ - $\text{H}_2\text{O}$  oxygen exchange takes place in water with the same isotopic signature as the water at the site of evaporation (Gillon and Yakir, 2000b). In a survey of leaf CA activity of species belonging to different functional types it was found that  $\text{C}_4$  dicots contain more CA than their  $\text{C}_4$  monocot counterparts (Gillon and Yakir, 2001). The large amount of CA relative to net rates of  $\text{CO}_2$  assimilation in  $\text{C}_4$  dicots suggests that  $\Delta^{18}\text{O}$  ought to be higher in these plants, but this has not yet been tested (Gillon and Yakir, 2001). In this article we examine the influence changing CA activity has on  $\Delta^{18}\text{O}$  and the proportion of  $\text{CO}_2$  in isotopic equilibrium with leaf water at the site of oxygen exchange ( $\theta$ ) during  $\text{C}_4$  photosynthesis in a  $\text{C}_4$  plant with high levels of CA activity.

The efficient genetic transformation of the  $\text{C}_4$  dicot *Flaveria bidentis* provides the opportunity to manipulate various aspects of the  $\text{C}_4$  photosynthetic pathway, allowing this plant to be used as a model system for understanding the controls and limitation of  $\text{C}_4$  photosynthesis and isotope exchange (Chitty et al., 1994; Furbank et al., 1997; von Caemmerer et al., 1997a; Ludwig et al., 1998; Cousins et al., 2006). Additionally, under steady-state conditions *F. bidentis* has higher stomatal conductance than most other  $\text{C}_4$  plants allowing fractionation factors other than stomatal limited diffusion to influence  $\Delta^{18}\text{O}$ . These attributes make *F. bidentis* an excellent model system to study how changes in  $\text{C}_4$  photosynthesis and CA activity influ-

ence the isotopic exchange of  $\text{CO}_2$ . Here we present data from online measurements of  $\Delta^{18}\text{O}$ , leaf CA activity and the isotope composition of transpired water in *F. bidentis* with genetically modified levels of leaf CA. The antisense suppression of CA activity provides us with the unique opportunity to assess the role of CA in determining  $\Delta^{18}\text{O}$  without modifying environmental conditions. We show that  $\Delta^{18}\text{O}$  in *F. bidentis* leaves, which contain high levels of CA activity, is nevertheless sensitive to small changes in CA activity even when stomatal conductances and  $\text{CO}_2$  assimilation rates are unaffected. These results are discussed in relationship to the isotopic signature of water at the site of exchange and the internal conductance of  $\text{CO}_2$  diffusion from the intercellular airspaces to the site of phosphoenol pyruvate carboxylase reaction.

## ISOTOPE THEORY

The  $\delta^{18}\text{O}$  of water at the sites of evaporation within a leaf ( $\delta_e$ ) can be estimated from the Craig and Gordon model of evaporative enrichment (Craig and Gordon, 1965; Farquhar and Lloyd, 1993)

$$\delta_e = \delta_t + \varepsilon_k + \varepsilon^+ + (\delta_a - \delta_t - \varepsilon_k) \frac{e_a}{e_i} \quad (1)$$

where  $e_a$  and  $e_i$  are the vapor pressures in the atmosphere and the leaf intercellular spaces.  $\delta_a$  and  $\delta_t$  are the isotopic composition of water vapor in the air and transpired by the leaf, respectively. The kinetic fractionation during diffusion of water from leaf intercellular air spaces to the atmosphere ( $\varepsilon_k$ ) can be calculated as (Cernusak et al., 2004)

$$\varepsilon_k(\text{‰}) = \frac{32r_s + 21r_b}{r_s + r_b} \quad (2)$$

where  $r_s$  and  $r_b$  are the stomatal and boundary layer resistance and 32 and 21 are the fractionation factors in parts per mil. The equilibrium fractionation between liquid water and water vapor ( $\varepsilon^+$ ) is calculated as (Cernusak et al., 2004)

$$\varepsilon^+(\text{‰}) = 2.644 - 3.206 \left( \frac{10^3}{T} \right) + 1.534 \left( \frac{10^6}{T^2} \right) \quad (3)$$

where  $T$  is leaf temperature in degrees Kelvin. Under steady-state conditions the value of  $\delta_t$  is equal to the isotopic composition of source water ( $\delta_s$ ), the water taken up by the plant (Harwood et al., 1998). A summary of the symbols used in the text are listed in Table I.

Discrimination against  $\text{C}^{18}\text{OO}$  ( $\Delta^{18}\text{O}$ ) when water at the site of exchange and  $\text{CO}_2$  are at full isotopic equilibrium ( $\theta = 1$ ) can be predicted (Farquhar and Lloyd, 1993) as

$$\Delta^{18}\text{O} = \frac{a - \varepsilon\Delta_{\text{ea}}}{1 - \varepsilon\Delta_{\text{ea}}} \quad (4)$$

where  $a$  is the diffusional discrimination (7.7‰) and  $\varepsilon$  is calculated as  $p_m/(p_a - p_m)$  where  $p_a$  is the

**Table 1.** Symbols used in the text

Symbol	Description
A	Net CO <sub>2</sub> assimilation
a	Fractionation during diffusion of CO <sub>2</sub> from the mesophyll to the atmosphere (7.7‰)
α	Fractionation factor for <sup>18</sup> O between water and CO <sub>2</sub> at 25°C (41.1‰)
Δ <sub>ea</sub>	<sup>18</sup> O enrichment of CO <sub>2</sub> at the site of exchange compared to the atmosphere when the CO <sub>2</sub> is in full isotopic equilibrium with the water
Δ <sub>ca</sub>	<sup>18</sup> O enrichment of CO <sub>2</sub> at the site of exchange compared to the atmosphere
Δ <sup>13</sup> C	<sup>13</sup> CO <sub>2</sub> isotope discrimination
Δ <sup>18</sup> O	C <sup>18</sup> OO isotope discrimination
δ <sup>18</sup> O	Isotopic ratio of oxygen (‰ relative to VSMOW)
δ <sub>a</sub>	<sup>18</sup> O isotopic composition of water vapor in the air (‰ relative to VSMOW)
δ <sub>c</sub>	The dilution corrected value of δ <sup>18</sup> O
δ <sub>e</sub>	<sup>18</sup> O isotopic composition of water at the site of evaporation in the leaf (‰ relative to VSMOW)
δ <sub>ex</sub>	<sup>18</sup> O isotopic composition of water at the site of CO <sub>2</sub> -H <sub>2</sub> O oxygen exchange (‰ relative to VSMOW)
δ <sub>g</sub>	δ <sup>18</sup> O value of the equilibrating CO <sub>2</sub>
δ <sub>m</sub>	Measured δ <sup>18</sup> O value
δ <sub>t</sub>	<sup>18</sup> O isotopic composition of water transpired by the leaf (‰ relative to VSMOW)
δ <sup>18</sup> O <sub>SAM</sub>	<sup>18</sup> O isotopic composition of water sample (‰ relative to VSMOW)
δ <sub>c</sub> <sup>SAM</sup>	Dilution corrected <sup>18</sup> O isotopic composition of the water sample
δ <sub>c</sub> <sup>S1</sup>	Dilution corrected <sup>18</sup> O isotopic composition of reference water no. 1
δ <sub>c</sub> <sup>S2</sup>	Dilution corrected <sup>18</sup> O isotopic composition of reference water no. 2
δ <sub>SAM</sub> <sup>S1</sup>	The <sup>18</sup> O isotopic composition of reference water no. 1 relative to VSMOW (−6.44‰)
δ <sub>SAM</sub> <sup>S2</sup>	The <sup>18</sup> O isotopic composition of reference water no. 2 relative to VSMOW (−22.83‰)
E	Transpiration rate
e <sub>a</sub>	Vapor pressure in the atmosphere
e <sub>i</sub>	Vapor pressure in the leaf intercellular air spaces
ε	$p_m/(p_a - p_m)$
ε <sub>k</sub>	Kinetic fractionation during diffusion of water from leaf intercellular air spaces to the atmosphere
ε <sub>w</sub>	The equilibrium <sup>18</sup> O fractionation between CO <sub>2</sub> and water (40.17‰ at 30°C)
ε <sup>+</sup>	The equilibrium <sup>18</sup> O fractionation between liquid and vapor water (8.77‰ at 30°C)
F <sub>in</sub>	Gross influx of CO <sub>2</sub> into a leaf
g <sub>s</sub>	Stomatal conductance
g <sub>w</sub>	The internal conductance to the diffusion of CO <sub>2</sub> between the intercellular air space and the site of carboxylation in the mesophyll cytoplasm
k	The ratio of oxygen atoms in the water to the oxygen atoms in CO <sub>2</sub>
k <sub>CA</sub>	The hydration rate constant of CA
k <sub>t</sub>	The number of hydration reactions per CO <sub>2</sub> molecule
pCO <sub>2</sub>	Partial pressure of CO <sub>2</sub>
p <sub>a</sub>	pCO <sub>2</sub> of dry air in the atmosphere
p <sub>e</sub>	pCO <sub>2</sub> of dry air entering the leaf chamber
p <sub>m</sub>	pCO <sub>2</sub> of the mesophyll cytoplasm and site of CO <sub>2</sub> -H <sub>2</sub> O oxygen exchange
p <sub>o</sub>	pCO <sub>2</sub> of dry air leaving the leaf chamber
R <sub>e</sub>	<sup>13</sup> C/ <sup>12</sup> C of the air entering the leaf chamber
R <sub>o</sub>	<sup>13</sup> C/ <sup>12</sup> C of the air leaving the leaf chamber
r <sub>b</sub>	Boundary layer resistance to water vapor diffusion (m <sup>2</sup> s mol <sup>-1</sup> )
r <sub>s</sub>	Stomatal resistance to water vapor diffusion (m <sup>2</sup> s mol <sup>-1</sup> )
θ	The proportion of CO <sub>2</sub> in isotopic equilibrium with water at the site of oxygen exchange
ξ	$p_e/(p_e - p_o)$
τ	Residence time of CO <sub>2</sub> in the leaf cytosol

CO<sub>2</sub> partial pressure ( $p\text{CO}_2$ ) in air and  $p_m$  is the  $p\text{CO}_2$  at the site of leaf CO<sub>2</sub>-H<sub>2</sub>O oxygen exchange in the mesophyll cytosol. The <sup>18</sup>O enrichment of CO<sub>2</sub> compared to the atmosphere at the site of exchange in full oxygen isotope equilibrium with the water was calculated as (Cernusak et al., 2004)

$$\Delta_{ea} = \frac{\delta_e(1 + \varepsilon_w) + \varepsilon_w - \delta_a}{1 - \delta_a} \quad (5)$$

where the equilibrium fractionation between water and CO<sub>2</sub> ( $\varepsilon_w$ ) can be calculated as (Cernusak et al., 2004)

$$\varepsilon_w(\text{‰}) = \left( \frac{17,604}{T} \right) - 17.93 \quad (6)$$

where  $T$  is leaf temperature in degrees Kelvin. The proportion of CO<sub>2</sub> in isotopic equilibrium with water

at the site of oxygen exchange can be calculated from (Gillon and Yakir, 2000a)

$$\theta = \frac{\Delta_{\text{ca}} + a/(\varepsilon + 1)}{\Delta_{\text{ea}} + a/(\varepsilon + 1)} \quad (7)$$

where  $\Delta_{\text{ca}}$  is the oxygen isotope composition of  $\text{CO}_2$  at the site of exchange during photosynthesis determined by

$$\Delta_{\text{ca}} = \frac{\Delta^{18}\text{O} - a}{(1 + \Delta^{18}\text{O})\varepsilon} \quad (8)$$

where  $\Delta^{18}\text{O}$  is the discrimination against  $\text{C}^{18}\text{OO}$  as defined above.

There has been much interest in the role CA plays in enhancing isotopic equilibrium ( $\theta$ ) because it has a significant influence on the atmospheric  $\text{CO}_2$  isotope signature. It has been suggested that the extent of  $\theta$  in a leaf can be determined by in vitro CA assays coupled with the unidirectional flux of  $\text{CO}_2$  into the leaf (Gillon and Yakir, 2000a, 2000b, 2001) from the equation initially developed by Mills and Urey (1940)

$$\theta = 1 - e^{(-\text{CA}_{\text{leaf}}/F_{\text{in}})/3} \quad (9)$$

where  $\text{CA}_{\text{leaf}}/F_{\text{in}}$  represents the mean number of hydration reactions for each  $\text{CO}_2$  molecule inside the leaf (Gillon and Yakir, 2000a). Leaf CA activity ( $\text{CA}_{\text{leaf}}$ ) is determined as the product of the CA hydration rate constant ( $k_{\text{CA}}$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$ ) and the mesophyll  $p\text{CO}_2$  ( $p_m$ ). The rate constant  $k_{\text{CA}}$  is calculated from in vitro measurements of CA activity in leaf extracts (see "Materials and Methods"). The gross influx of  $\text{CO}_2$  into a leaf ( $F_{\text{in}} = g_t p_a$ ) as well as  $p_m$  determine the residence time ( $\tau = p_m/F_{\text{in}}$ ) of  $\text{CO}_2$  within the leaf. The parameter  $g_t$  is defined as the total conductance of  $\text{CO}_2$

from the atmosphere to the site of  $\text{CO}_2\text{-H}_2\text{O}$  oxygen exchange (Gillon and Yakir, 2000a). The relationship of  $\text{CA}_{\text{leaf}}/F_{\text{in}}$  indicates that conditions that influence  $p_a$ ,  $p_m$ ,  $g_t$  or  $k_{\text{CA}}$  can alter the value of  $\theta$ .

## RESULTS

### Light Response and Isotopic Equilibrium

The online  $\Delta^{18}\text{O}$  values were determined by directly coupling a mass spectrometer to the outlet of a LI-6400 gas exchange system via a gas permeable silicone membrane (Cousins et al., 2006). This allowed the measurements of the  $\text{C}^{18}\text{OO}/\text{C}^{16}\text{OO}$  ratio of the  $\text{CO}_2$  in the air stream without prior purification of  $\text{CO}_2$ . We obtained a range of  $\Delta^{18}\text{O}$  values and rates of net  $\text{CO}_2$  assimilation by manipulating the light conditions that *F. bidentis* and tobacco (*Nicotiana tabacum*) leaves were exposed to during the gas exchange measurements (Table II). Leaf CA activity ( $\text{CA}_{\text{leaf}}$ ), determined as the product of the rate constant ( $k_{\text{CA}}$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$ ) and the mesophyll  $p\text{CO}_2$  ( $p_m$ ), increased as rates of net  $\text{CO}_2$  assimilation decreased under limiting light conditions because  $p_m$  increased (Table II). The  $k_{\text{CA}}$  was determined from leaf extracts using mass spectrometry to measure the rates of  $^{18}\text{O}_2$  exchange from labeled  $^{13}\text{C}^{18}\text{O}_2$  to  $\text{H}_2^{16}\text{O}$  (von Caemmerer et al., 2004; Cousins et al., 2006). The  $\text{CA}_{\text{leaf}}$  activity for tobacco was similar to earlier published values (Williams et al., 1996; Gillon and Yakir, 2000a), whereas  $\text{CA}_{\text{leaf}}$  in *Z. mays* was higher than previously reported by Gillon and Yakir (2001) but similar to a more recent publication (Affek et al., 2006). *F. bidentis* plants with reduced levels of Rubisco (caused by antisense RNA constructs

**Table II.** Gas exchange, CA activity,  $\Delta^{18}\text{O}$ , and isotopic equilibrium measurements

Net  $\text{CO}_2$  assimilation rate (A), the  $p\text{CO}_2$  at the site of  $\text{CO}_2\text{-H}_2\text{O}$  oxygen exchange in the mesophyll cytosol ( $p_m$ ), the ratio of mesophyll cytosolic to ambient  $\text{CO}_2$  partial pressure ( $p_m/p_a$ ), the residence time of  $\text{CO}_2$  in the aqueous phase within the leaf ( $\tau$ ), stomatal conductance ( $g_s$ ), leaf CA activity ( $\text{CA}_{\text{leaf}}$ ), online  $\Delta^{18}\text{O}$  discrimination, and proportion of chloroplast  $\text{CO}_2$  in isotopic equilibrium with chloroplast water ( $\theta$ ) determined from online  $\Delta^{18}\text{O}$  measurements (Eq. 7) and predicted from in vitro CA assays and gas exchange (Eq. 9) in *F. bidentis* and tobacco measured at various irradiances ( $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) and the *F. bidentis* with reduced levels of Rubisco (anti-SSU) and wild-type *Z. mays* measured at 2,000 ( $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ). Other conditions are as described in the legend to Figure 1.  $g_w = 10 \text{ mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$  for all plants except tobacco where  $g_w = 5 \text{ mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$ .  $n = 3$  to 5.

Plant	Irradiance $\mu\text{mol m}^{-2} \text{s}^{-1}$	A		$g_s$			$\text{CA}_{\text{leaf}}$	$\Delta^{18}\text{O}$	Measured	Predicted
		$\mu\text{mol m}^{-2} \text{s}^{-1}$	$p_m$	$p_m/p_a$	$\tau$	$\text{mol m}^{-2} \text{s}^{-1}$	$\mu\text{mol m}^{-2} \text{s}^{-1}$	‰	$\theta$	$\theta$
<i>F. bidentis</i> wild type	150	6.2 ± 0.2	410 ± 19	0.77 ± 0.03	11.5 ± 1.6	0.12 ± 0.03	2,855 ± 602	182 ± 38	1.06 ± 0.04	1.00 ± 0.01
	300	12.2 ± 0.2	363 ± 32	0.70 ± 0.06	7 ± 1	0.21 ± 0.07	2,558 ± 652	103 ± 29	0.89 ± 0.02	1.00 ± 0.01
	800	25.9 ± 0.9	283 ± 28	0.59 ± 0.06	3.8 ± 0.4	0.33 ± 0.09	2,000 ± 532	43 ± 10	0.65 ± 0.04	0.99 ± 0.01
	1,400	33.7 ± 1.3	252 ± 25	0.56 ± 0.05	2.8 ± 0.2	0.41 ± 0.07	1,844 ± 262	31 ± 5	0.54 ± 0.04	0.99 ± 0.01
	2,000	37.6 ± 1.2	234 ± 15	0.53 ± 0.03	2.6 ± 0.1	0.42 ± 0.04	1,868 ± 180	23 ± 2	0.45 ± 0.04	0.99 ± 0.01
Tobacco	300	15.9 ± 0.9	161 ± 14	0.45 ± 0.04	5.0 ± 0.3	0.18 ± 0.03	1,197 ± 194	37 ± 4	0.89 ± 0.08	0.99 ± 0.01
	500	21.1 ± 1.5	142 ± 13	0.41 ± 0.04	3.6 ± 0.2	0.25 ± 0.04	1,060 ± 182	29 ± 2	0.81 ± 0.10	0.99 ± 0.01
	800	24.7 ± 1.9	130 ± 11	0.39 ± 0.04	2.9 ± 0.2	0.30 ± 0.06	976 ± 183	23 ± 1	0.67 ± 0.04	0.99 ± 0.01
<i>F. bidentis</i> anti-SSU	2,000	23.6 ± 3.1	323 ± 5	0.68 ± 0.02	4.0 ± 0.7	0.40 ± 0.09	2,285 ± 231	51 ± 3	0.63 ± 0.02	0.99 ± 0.01
<i>Z. mays</i>	2,000	33.9 ± 1.5	86 ± 8	0.19 ± 0.02	1.9 ± 0.1	0.18 ± 0.01	296 ± 32	13 ± 1	0.62 ± 0.10	0.88 ± 0.03

targeted to the nuclear-encoded gene for the small subunit of Rubisco [SSU; anti-SSU plants]) had lower photosynthetic rates than wild-type plants and higher  $CA_{\text{leaf}}$  activity at similar light levels because of higher values of  $p_m$  (Table II).

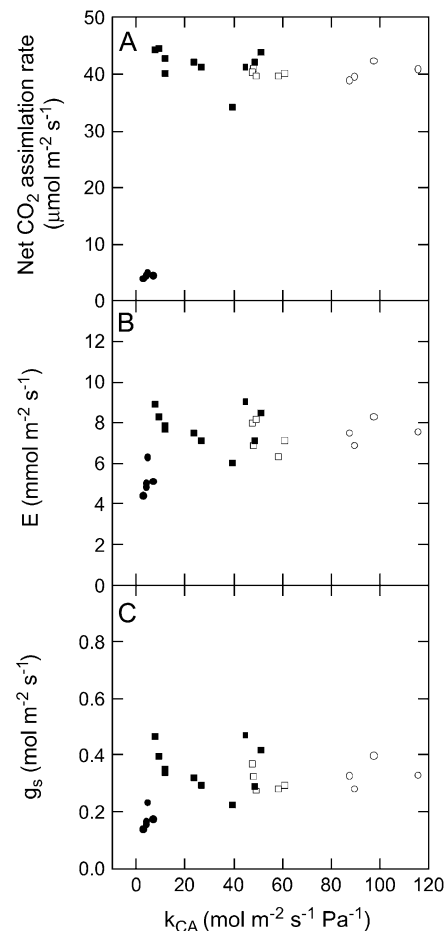
In both wild-type *F. bidentis* and tobacco the predicted isotopic equilibrium ( $\theta$ ) determined from the in vitro CA assays (Eq. 9) did not vary with irradiance, whereas  $\theta$  determined from the measured  $\Delta^{18}\text{O}$  (Eq. 7) decreased dramatically with increasing irradiance (Table II). The residence time ( $\tau = p_m/F_{\text{in}}$ ) of  $\text{CO}_2$  within the leaf increased in both *F. bidentis* and tobacco at the low irradiances (Table II). This was due to an increase in  $p_m$  as well as a decrease in  $F_{\text{in}}$  caused by a reduction in  $g_s$  (Table II). In both species, the values of  $\theta$  predicted from in vitro CA assays (Eq. 9) were substantially higher than the values measured from  $\Delta^{18}\text{O}$  (Eq. 7), except at the low light levels where the predicted and measured values of  $\theta$  started to converge. In *F. bidentis* we used the low light measurement, when isotopic equilibrium is expected to be complete (i.e.  $\theta = 1$ ), to estimate an internal conductance to the diffusion of  $\text{CO}_2$  between the intercellular air space and the site of  $\text{CO}_2\text{-H}_2\text{O}$  oxygen exchange ( $g_w$ ) of  $10 \text{ mol m}^{-2} \text{ s}^{-1} \text{ Pa}^{-1}$  (method described by Gillon and Yakir, 2000a). The value of  $g_w$  for tobacco ( $5 \text{ mol m}^{-2} \text{ s}^{-1} \text{ Pa}^{-1}$ ) was determined from  $\Delta^{13}\text{C}$  measurements as described by von Caemmerer and Evans (1991). Both the predicted  $\theta$  values from in vitro CA assays (Eq. 9) and the measured values determined from  $\Delta^{18}\text{O}$  (Eq. 7) in *Z. mays* were lower than in wild-type *F. bidentis* plants at similar light levels (Table II). The *F. bidentis* anti-SSU plants with reduced levels of Rubisco had higher online determined values of  $\theta$  and  $\tau$  than wild-type *F. bidentis* at similar light conditions because of higher values of  $p_m$  (Table II).

### Gas Exchange and CA Activity

We obtained plants with a range of CA activity from the progeny of *F. bidentis* plants containing the anti-sense RNA constructs targeted toward the putative cytosolic CA (anti-CA plants, see Fig. 1). As previously reported, decreases in CA activity in *F. bidentis* had little effect on net  $\text{CO}_2$  assimilation until CA levels were less than 20% of wild type (Fig. 1A; see also von Caemmerer et al., 2004; Cousins et al., 2006). The transpiration rate ( $E$ ) and stomatal conductance ( $g_s$ ) recorded after approximately 1.5 h in the leaf chamber under steady-state conditions were similar in plants with a large range of leaf CA activities (Fig. 1, B and C). However, both  $E$  and  $g_s$  were lower (5.1 versus 7.5 for  $E$  and 0.36 versus 0.17 for  $g_s$ ) in the anti-CA plants with dramatically reduced CA activity and net  $\text{CO}_2$  assimilation rates compared to wild-type plants (Fig. 1).

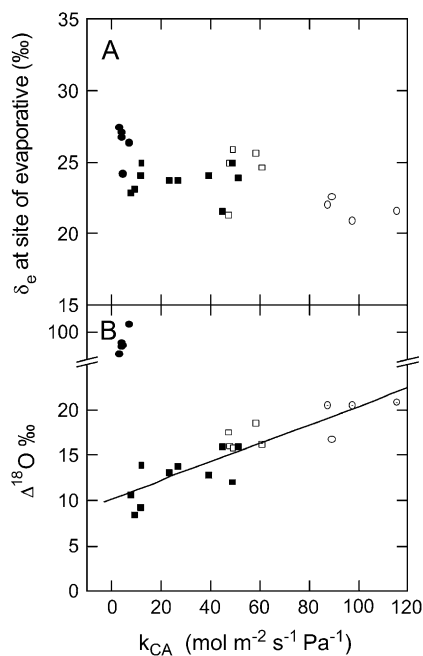
### $\delta^{18}\text{O}$ of Water at the Site of Evaporation

The oxygen isotope composition of water at the evaporative site in the leaves ( $\delta_e$ ) determined from the model of Craig and Gordon (1965) and further devel-



**Figure 1.** Net  $\text{CO}_2$  assimilation rate, transpiration rate ( $E$ ), and stomatal conductance ( $g_s$ ) as a function of the rate constant of leaf CA ( $k_{\text{CA}}$   $\text{mol m}^{-2} \text{ s}^{-1} \text{ Pa}^{-1}$ ). Each point represents a measurement made on a different plant grown in a glass house at ambient  $\text{CO}_2$  or in a growth cabinet at 0.96 kPa  $\text{CO}_2$ : wild-type plants grown at ambient  $\text{CO}_2$  ( $\square$ ); anti-CA plants grown at ambient  $\text{CO}_2$  ( $\blacksquare$ ); wild type grown at 0.96 kPa  $\text{CO}_2$  ( $\circ$ ); and anti-CA plants grown at 0.96 kPa  $\text{CO}_2$  ( $\bullet$ ). Gas exchange measurements were made at an irradiance of  $2,000 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ , leaf temperature of  $30^\circ\text{C}$  and an inlet  $\text{CO}_2$  concentration of 52 Pa in 90.5 kPa of  $\text{N}_2$  and 4.8 kPa of  $\text{O}_2$  gas mixture.

oped by Farquhar and Lloyd (1993) ranged between  $18\text{‰}$  and  $27\text{‰}$  (Eq. 1). We waited a minimum of 1.5 h under steady conditions to measure  $\Delta^{18}\text{O}$ , and subsequently collected transpired water to calculate  $\delta_e$ . Under steady-state conditions the isotopic composition of transpired water vapor ( $\delta_t$ ) should be equal to the isotopic composition of source water ( $\delta_s$ , the water take up by the root system). The values of  $\delta_t$  and  $\delta_s$  were similar for plants grown under 1%  $\text{CO}_2$ ,  $-6.2 \pm 0.3\text{‰}$  and  $-5.1 \pm 0.4\text{‰}$ , respectively. However,  $\delta_t$  and  $\delta_s$  in the glass house-grown plants were  $-2.6 \pm 0.5\text{‰}$  and  $-5.3 \pm 0.3\text{‰}$ , respectively. Values of  $\delta_e$ , calculated from Equation 1 using values of  $\delta_t$ , were similar between anti-CA and wild-type plants grown under ambient  $\text{CO}_2$  in the glass house (Fig. 2). However,  $\delta_e$  in wild-type plants grown in 1%  $\text{CO}_2$  tended to be more depleted in  $^{18}\text{O}$ , whereas  $\delta_e$  in the anti-CA plants



**Figure 2.** The isotopic composition (‰) of the water at the site of evaporation ( $\delta_e$ ) and the oxygen isotope discrimination ( $\Delta^{18}\text{O}$ ) as a function of the rate constant of leaf CA ( $k_{\text{CA}}$  mol m<sup>-2</sup> s<sup>-1</sup> Pa<sup>-1</sup>). Values of  $\delta_e$  were calculated as described in the text using Equation 1 and source water ( $\delta_s$ ) was  $-5.3 \pm 0.3$ ‰. The line represents a linear regression ( $R^2 = 0.87$ ) for all data except the plants with high  $\Delta^{18}\text{O}$  values. Symbols and gas exchange conditions are as in Figure 1 and  $\delta$  values are expressed in reference to VSMOW.

grown under similar conditions was more enriched in  $^{18}\text{O}$  (Fig. 2). The difference in  $\delta_e$  between the high  $\text{CO}_2$ -grown wild-type and low anti-CA plants is attributed to the differences in  $E$  and  $g_s$ , which caused  $e_k$  (Eq. 2) to increase from  $30 \pm 0.1$ ‰ to  $31 \pm 0.1$ ‰ and the ratio of  $e_a/e_i$  (used in Eq. 1) to decrease from  $0.45 \pm 0.01$ ‰ to  $0.32 \pm 0.02$ ‰. These changes in  $E$  and  $g_s$  as well as the shift in  $p_m/p_a$  contribute to the large shift in  $\Delta^{18}\text{O}$  in the low anti-CA plants (see below).

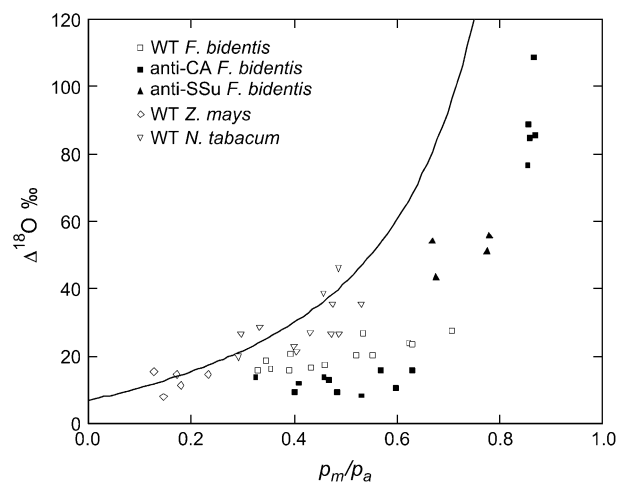
### Measured $\text{C}^{18}\text{OO}$ Discrimination and Isotopic Equilibrium

Although there was no detectable change in the net  $\text{CO}_2$  assimilation rate in anti-CA plants with greater than 20% of wild-type CA activity,  $\Delta^{18}\text{O}$  decreased with reduced CA activity (Fig. 2B). There was a strong relationship between  $\Delta^{18}\text{O}$  and CA activity, where  $\Delta^{18}\text{O}$  decreased with CA activity until low levels of CA dramatically altered the rates of net  $\text{CO}_2$  assimilation. Plants with low photosynthetic rates had  $\Delta^{18}\text{O}$  values markedly higher than the other anti-CA plants corresponding to the high  $p_m/p_a$  in these plants (Figs. 2B and 3).

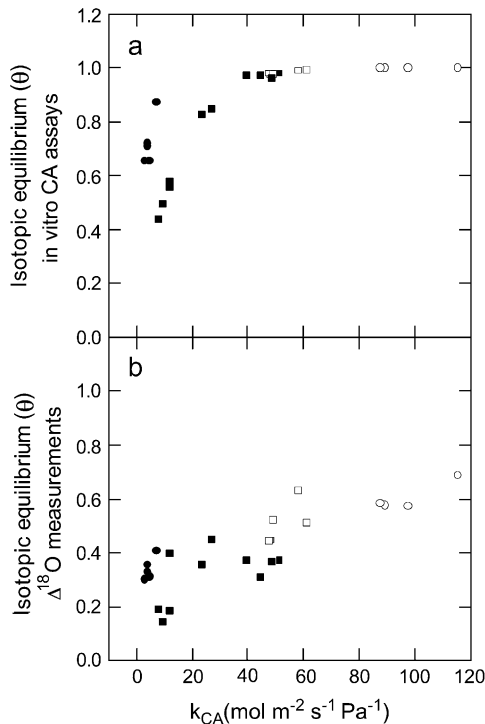
The values of  $\Delta^{18}\text{O}$  increased with  $p_m/p_a$  as predicted from Equation 4, but the measured values for *F. bidentis* were always less than those predicted at full isotopic equilibrium with an assumed constant  $\Delta_{\text{ea}}$  of 33.7‰ (Fig. 3). The value of  $\Delta_{\text{ea}}$  was taken as the average value

determined from all wild-type plants at high irradiance ( $2,000 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ). At similar  $p_m/p_a$  values the anti-CA plants, with wild-type rates of net  $\text{CO}_2$  assimilation, had lower  $\Delta^{18}\text{O}$  and were thus further away from the predicted values of  $\Delta^{18}\text{O}$ . The values of  $\Delta^{18}\text{O}$  and  $p_m/p_a$  were high in the anti-CA plants with extremely low CA activity compared to wild-type plants due to the lack of net  $\text{CO}_2$  assimilation (Fig. 3). However, the measured values of  $\Delta^{18}\text{O}$  were even further away than wild type from the predicted  $\Delta^{18}\text{O}$  values. *F. bidentis* plants with reduced amounts of Rubisco (anti-SSU plants) have a high  $p_m/p_a$ , due to the low rates of net  $\text{CO}_2$  assimilation, but wild-type levels of extractable CA activity. Their measured values of  $\Delta^{18}\text{O}$  are closer to the predicted values of  $\Delta^{18}\text{O}$ , compared to wild-type plants (Fig. 3). The values of  $\Delta^{18}\text{O}$  for both *Z. mays* and tobacco correspond to lower  $p_m/p_a$  and were closer to the predicted values of  $\Delta^{18}\text{O}$  compared to wild-type *F. bidentis* (Fig. 3).

Using Equation 9 and in vitro CA assays to predict  $\theta$  indicated that wild-type plants should be at complete isotopic equilibrium with water at the site of oxygen exchange (Fig. 4A). This is in contradiction to the relatively low  $\theta$  values determined from measurements of  $\Delta^{18}\text{O}$  and Equation 7 (Fig. 4B), highlighting the discrepancy in  $\theta$  determined from either Equation 7 or 9. The values of  $\theta$  predicted from in vitro CA assays (Eq. 9) were substantially higher in both the wild-type and anti-CA *F.*



**Figure 3.** Oxygen isotope discrimination ( $\Delta^{18}\text{O}$ ) as a function of the ratio of mesophyll cytosolic to ambient  $\text{CO}_2$  partial pressure ( $p_m/p_a$ ). Where  $p_m$  was calculated with  $g_w = 10 \text{ mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$  for the  $\text{C}_4$  plants and  $5 \text{ mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$  for tobacco. The line represents the theoretical relationship of  $\Delta^{18}\text{O}$  and  $p_m/p_a$  at full isotopic equilibrium where  $a = 7.7$ ‰ and  $\Delta_{\text{ea}} = 33.7$ ‰ (Eq. 4) and the  $\text{CO}_2$  supplied to the leaf had a  $\delta^{18}\text{O}$  of 24‰ relative to VSMOW. Each point represents a measurement made on a different plant grown under ambient conditions in a glass house or at 0.96 kPa  $\text{CO}_2$  in growth chambers. Measurement conditions are as in Table II and Figure 1. Each point represents a measurement made on a different plant or different light level: wild-type *F. bidentis* ( $\square$ ); anti-CA *F. bidentis* ( $\blacksquare$ ); anti-SSU *F. bidentis* ( $\blacktriangle$ ); wild-type *Z. mays* ( $\diamond$ ); and wild-type tobacco ( $\triangle$ ). Plants grown in ambient and 0.96 kPa  $\text{CO}_2$  were grouped together in this figure.



**Figure 4.** The extent of isotopic equilibrium ( $\theta$ ) as a function of the rate constant of leaf CA ( $k_{CA}$  mol m<sup>-2</sup> s<sup>-1</sup> Pa<sup>-1</sup>) in wild-type and anti-CA *F. bidentis* plants. The predicted values of  $\theta$  were determined from in vitro CA assays using Equation 9 (A) and the measured values of  $\theta$  were determined from  $\Delta^{18}\text{O}$  using Equation 7 (B). Symbols and gas exchange conditions are as in Figure 1. Calculations were made with  $g_w = 10$  mol m<sup>-2</sup> s<sup>-1</sup> Pa<sup>-1</sup>.

*bidentis* plants than the values measured from  $\Delta^{18}\text{O}$  (Eq. 7), as shown in Figure 4 and Table II. Both methods of determining  $\theta$  indicated a decrease in  $\theta$  as the amount of CA decreased in the leaves of anti-CA plants (Fig. 4), although values estimated from in vitro CA assays were more sensitive to changes in CA (Fig. 4A).

#### The Water at the Site of Exchange, Internal CO<sub>2</sub> Conductance, and the Extent of Isotopic Equilibrium

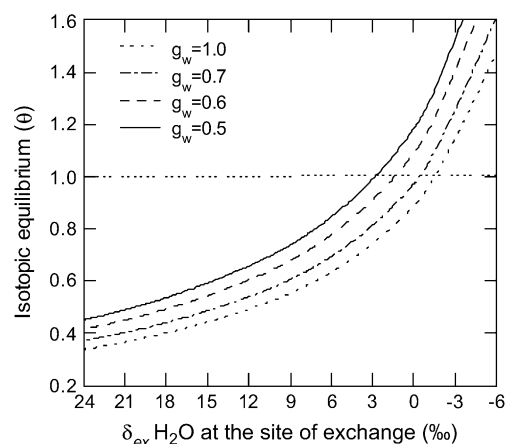
The value of  $\theta$  calculated from the online isotope measurements (Eq. 7) is influenced not only by  $\Delta^{18}\text{O}$  but also by the partial pressure of CO<sub>2</sub> and the isotopic signature of leaf water at the site of CO<sub>2</sub>-H<sub>2</sub>O oxygen exchange,  $p_m$  and  $\delta_{ex}$ , respectively. The Craig-Gordon model describes the water enrichment at the site of evaporation ( $\delta_e$ ), but does not provide information on the isotopic gradient of water within a leaf. It is generally assumed that  $\delta_e$  is equal to  $\delta_{ex}$  but theoretically  $\delta_{ex}$  can vary between  $\delta_e$  ( $\delta_e = 18\text{‰}$  to  $25\text{‰}$ ) and source water ( $\delta_s = -5.3 \pm 0.3\text{‰}$ ). Figure 5 shows that modeled changes in  $\delta_{ex}$  shifts  $\theta$  from around 0.4 to 1.4, demonstrating that  $\delta_{ex}$  is crucial for calculating  $\theta$  when using Equation 7. The model is also used to vary  $g_w$  which is used to estimate  $\varepsilon$  in Equation 4, to see how  $g_w$  influences the value of  $\theta$  estimated from  $\Delta^{18}\text{O}$  measurements (Fig. 5).

## DISCUSSION

### The Influence of CA on C<sup>18</sup>O Discrimination during C<sub>4</sub> Photosynthesis

We measured  $\Delta^{18}\text{O}$  concurrently with gas exchange in transgenic *F. bidentis* with a range of leaf CA activity due to an antisense construct targeted to a putative cytosolic CA to explore the role CA plays in  $\Delta^{18}\text{O}$  in a C<sub>4</sub> species. These plants have been previously described by von Caemmerer et al. (2004) and Cousins et al. (2006). Our measurements show that even small reductions in CA activity resulted in decreased  $\Delta^{18}\text{O}$  (Fig. 2) and reductions in the extent of isotopic equilibrium,  $\theta$ , estimated from  $\Delta^{18}\text{O}$  (Fig. 4). However, these plants showed no differences in the rate of net CO<sub>2</sub> assimilation or stomatal conductance (Fig. 1). It has been reported that low levels of leaf CA, particularly in C<sub>4</sub> monocots, limits the extent of  $\theta$  (Gillon and Yakir, 2000a, 2000b, 2001). The limited amount of CA activity in these leaves is thought to be insufficient to fully equilibrate the exchange of oxygen between CO<sub>2</sub> and water. However, the C<sub>4</sub> dicot *F. bidentis* has high levels of leaf CA, compared to these C<sub>4</sub> monocots, with levels comparable to many C<sub>3</sub> plants (Table II; Gillon and Yakir, 2000a, 2000b, 2001; von Caemmerer et al., 2004; Cousins et al., 2006). It was therefore intriguing to measure low  $\Delta^{18}\text{O}$  and  $\theta$  values in wild-type *F. bidentis* and observe the responsiveness of  $\Delta^{18}\text{O}$  to small reductions in CA activity.

The value of  $\theta$  is related to the mean number of hydration reactions a CO<sub>2</sub> molecule experiences inside a leaf. This in turn is the product of residence time ( $\tau = p_m/F_{in}$ ) and the hydration constant of leaf CA,  $k_{CA}$  (as described in the theory section). We therefore also experimentally thought to increase  $\theta$  by using transgenic *F. bidentis* with reduced Rubisco activity. This increased  $\tau$ ,  $\Delta^{18}\text{O}$ , and the measured  $\theta$  values because



**Figure 5.** The predicted isotopic equilibrium ( $\theta$ ) determined with various isotopic compositions of water at the site of CO<sub>2</sub>-H<sub>2</sub>O oxygen exchange ( $\delta_{ex}$ ). Calculations were made with  $g_w$  values ranging from 10 to 5 mol m<sup>-2</sup> s<sup>-1</sup> Pa<sup>-1</sup>.  $\theta$  was calculated using Equation 7 where  $\Delta_{ea}$  was determined using Equation 5 and substituting  $\delta_e$  with various values of  $\delta_{ex}$ .  $\Delta_{ca}$  was calculated from parameters taken from the high light wild-type *F. bidentis* measurements in Table II.  $\delta_{ex}$  values are presented relative to VSMOW.

reduced assimilation rates are not matched by reductions in stomatal conductance, thus leading to an increased  $p_m$  and  $p_m/p_a$  (Table II; Fig. 3). This further suggests that CA activity is not saturating for isotopic equilibrium in wild-type *F. bidentis* plants.

#### CA Activity as a Predictor of Isotopic Equilibrium

In both *Z. mays* and *F. bidentis* the value of  $\theta$  determined from  $\Delta^{18}\text{O}$  measurements was considerably less than that estimated from in vitro measurements of CA activity that predicted  $\theta$  values close to one for all species (Table II; Fig. 4). As previously reported and shown in Table II,  $\theta$  in the  $\text{C}_3$  plant tobacco determined from  $\Delta^{18}\text{O}$  measurements was higher than in  $\text{C}_4$  plants (Williams et al., 1996; Gillon and Yakir, 2000a, 2000b). As noted above, the extent of  $\theta$  is in part determined by the CA activity associated with the  $\text{CO}_2\text{-H}_2\text{O}$  oxygen exchange, but knowing the isotopic signature of the water at the site of exchange ( $\delta_{\text{ex}}$ ), as well as knowing the  $p\text{CO}_2$  at the site of exchange, are also both important factors (see below).

The discrepancy between our estimates of  $\theta$  in *F. bidentis* from online measurements and the in vitro CA activity (Fig. 4) could be due to the fact that measurements of total leaf CA activity are not representative of the CA activity associated with the  $\text{CO}_2\text{-H}_2\text{O}$  oxygen exchange that influences  $\Delta^{18}\text{O}$ . Multiple forms of CA have been reported from leaf tissue of both  $\text{C}_3$  and  $\text{C}_4$  species (Badger and Price, 1994; Badger, 2003). In  $\text{C}_4$  plants the majority of CA is located within the mesophyll cytoplasm (Ku and Edwards, 1975; Burnell and Hatch, 1988; Hatch and Burnell, 1990) and in *F. bidentis* approximately only 5% of the total leaf CA activity is specifically located within the BSC cells (Ludwig et al., 1998). In  $\text{C}_3$  plants the chloroplasts, which contain the majority of the leaf's CA, are appressed against the cell walls adjacent to the intercellular air space, thus positioning CA in close proximity to the water at the site of evaporation. Under such conditions it is likely that the isotopic signature of water at the site of evaporation ( $\delta_e$ ) is similar to  $\delta_{\text{ex}}$  and this may explain why in tobacco we found quite close agreement between  $\theta$  estimated from  $\Delta^{18}\text{O}$  and in vitro CA activity (Gillon and Yakir, 2000a, 2000b). However, in  $\text{C}_4$  plants, because the CA activity is distributed throughout the mesophyll cytoplasm, the majority of CA may not be positioned adjacent to the intercellular air spaces and the site of evaporation. This suggests that whole leaf in vitro CA assays likely overestimate the amount of CA important for determining  $\Delta^{18}\text{O}$  in  $\text{C}_4$  plants. The different localization of CA activity in  $\text{C}_3$  and  $\text{C}_4$  species may be in part responsible for the different relationship between in vitro CA activity and  $\Delta^{18}\text{O}$  in *F. bidentis* and tobacco.

#### The Water Isotopic Signature and the Internal Conductance to $\text{CO}_2$

The Craig-Gordon model (Eq. 1) is used for predicting the isotopic composition of water at the site of

evaporation,  $\delta_e$ , but there are a number of studies showing that there can be a large isotopic gradient within a leaf between the water supplied via the vascular tissue and water at the site of evaporation (Farquhar and Gan, 2003; Barbour and Farquhar, 2004; Barbour et al., 2004). As shown in Figure 5, hypothetically varying the isotopic signature of the water at the site of exchange ( $\delta_{\text{ex}}$ ) has a large influence on the calculated values of  $\theta$ . The values of  $\delta_{\text{ex}}$  presented in Figure 5 are a reasonable isotopic gradient to consider when  $\delta_e$  in the *F. bidentis* plants varied between 19‰ and 25‰ and source water was  $-5.3 \pm 0.3$ ‰. Due to the fact that the majority of CA occurs within the mesophyll cytoplasm in  $\text{C}_4$  plants it is possible that the  $\text{CO}_2\text{-H}_2\text{O}$  oxygen exchange occurs in a range of  $\delta_{\text{ex}}$  values. Indeed the average  $\delta_{\text{ex}}$  values may be closer to the isotopic composition of source water (Fig. 5). Thus in  $\text{C}_4$  plants water at the site of evaporation may not be the only water that influences  $\Delta^{18}\text{O}$ .

The difference between the measured and predicted values of  $\Delta^{18}\text{O}$  is also due to uncertainty in determining the  $p\text{CO}_2$  within the mesophyll cytoplasm ( $p_m$ ) that is used to calculate  $\varepsilon$  in Equation 4. Knowing the internal conductance to  $\text{CO}_2$  ( $g_w$ ) is necessary to accurately calculate values of  $p_m$ ; however, in  $\text{C}_4$  plants it is difficult to determine  $g_w$  with the traditional  $\Delta^{13}\text{C}$  measurements because of the low  $\Delta^{13}\text{C}$  values (Evans and von Caemmerer, 1996). We have used  $\Delta^{18}\text{O}$ , as described by Gillon and Yakir (2000a), under low light conditions when  $\theta = 1$  would be expected to estimate values of  $g_w$  in *F. bidentis* (Table II). However, Figure 5 shows that dramatically varying  $g_w$  has only a small influence on the calculated  $\theta$  values and although  $g_w$  is important for estimating  $p_m$ , uncertainty in this value would not be sufficient to raise the measured  $\theta$  values to the values predicted by the in vitro CA assays.

#### The Influence of Light on CA Activity and Isotopic Equilibrium

The increase in  $\text{CA}_{\text{leaf}}$  activity at low photosynthetic rates is attributed to the increase in substrate availability for CA due to the lack of photosynthetic  $\text{CO}_2$  drawdown at the low irradiances. The increase in  $p_m$  causes  $\text{CA}_{\text{leaf}}$  to increase, assuming  $k_{\text{CA}}$  remains constant with changing light conditions, because CA activity is generally limited by  $\text{CO}_2$ . Additionally, at low light the residence time of  $\text{CO}_2$  ( $\tau = p_m/F_{\text{in}}$ ) is greater as  $p_m$  increases and the gross flux of  $\text{CO}_2$  into the leaf ( $F_{\text{in}} = g_t p_a$ ) decreases due to the reduction in  $g_t$  caused by the drop in  $g_s$  (Table II). Therefore, the number of hydration reactions per  $\text{CO}_2$  increases at low light as demonstrated by  $\text{CA}_{\text{leaf}}/F_{\text{in}}$  in Equation 9. It is unknown if CA activity in  $\text{C}_4$  leaves is modulated with changes in irradiances, but potentially the redox statues of mesophyll chloroplast could regulate CA activity (Lee et al., 2004). Under high light conditions, stomatal conductance ( $g_s$ ) in *F. bidentis* and tobacco is high compared to *Z. mays* ( $0.42 \pm 0.04$ ,  $0.30 \pm 0.06$ , and  $0.18 \pm 0.01$ , respectively; see Table II). In *Z. mays* the



low  $g_s$  causes the  $\Delta^{18}\text{O}$  to be largely influenced by the stomatal fractionation whereas in *F. bidentis* and tobacco the higher values of  $g_s$  allow for the greater potential influence of CA on  $\Delta^{18}\text{O}$  (Fig. 4). Therefore, in *Z. mays* the low  $g_s$  influences the values of  $\theta$  more so than in *F. bidentis* or tobacco.

Using Equation 9,  $C_4$  plants with less CA (e.g. grasses) would be predicted to have a much larger shift in  $\theta$  due to the changing light conditions. This is in contrast to *F. bidentis* where the high  $CA_{\text{leaf}}$  activity predicted that  $\theta$  would be close to 1 under all light conditions (Table II). The variation in  $\theta$  under different light conditions may have important implications for predicting the influence of plant communities on the  $\delta^{18}\text{O}$  of atmospheric  $\text{CO}_2$ .

## CONCLUSION

The amount of CA activity in a leaf plays an important role in determining  $C^{18}\text{O}$  discrimination during  $C_4$  photosynthesis. We have shown that  $\Delta^{18}\text{O}$  and the extent of  $\text{CO}_2\text{-H}_2\text{O}$  isotopic equilibrium ( $\theta$ ) in *F. bidentis* leaves, which contain high levels of in vitro CA activity relative to rates of net  $\text{CO}_2$  assimilation, is sensitive to small changes in CA activity even when rates of net  $\text{CO}_2$  assimilation are unaffected. We conclude that the cytoplasmic localization of the majority of CA in  $C_4$  species has important implications for predicting  $\Delta^{18}\text{O}$  and the estimation of  $\theta$  under different environmental conditions. It appears that in vitro CA activity on whole leaf extract of *F. bidentis* may not represent the CA activity associated with the  $\text{CO}_2\text{-H}_2\text{O}$  oxygen exchange and therefore may not be a good predictor of  $\theta$ . The ability to accurately predict  $\theta$  in  $C_4$  species will be limited by the uncertainties in CA activity involved in the oxygen exchange and knowing the isotopic composition of water at the site of exchange.

## MATERIALS AND METHODS

### Growth Conditions

*Flaveria bidentis* plants were previously transformed with antisense RNA constructs targeted to either the nuclear-encoded gene for the SSU (anti-SSU plants) or a putative cytosolic CA (anti-CA plants; Furbank et al., 1996; von Caemmerer et al., 1997b, 2004). The segregating T1 generations of anti-CA primary transformants with photosynthetic rates similar to wild type were grown during the summer months in a glass house under natural light conditions (27°C day and 18°C night temperatures). The wild-type tobacco (*Nicotiana tabacum*) plants were also grown in a glass house under similar conditions. Anti-CA (segregating T2 generation from primary transformants 15, 12, and 8) and anti-SSU plants (segregating T2 generation from primary transformant 136-13) with low photosynthetic capacities and wild-type plants were grown under 0.96 kPa of  $\text{CO}_2$  in a controlled environment growth cabinet at an irradiance of 400  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  at plant height and air temperature of 27°C during the day and 18°C at night with a day length of 14 h. *Zea mays* plants were grown in similar growth cabinet conditions at ambient  $\text{CO}_2$  concentrations. All 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 plus trace elements: B, Cu, Fe, Mn, Mo, and Zn; Scotts Australia Pty) and watered daily.

### Gas Exchange Measurements

The uppermost fully expanded leaves were placed into the leaf chamber of the LI-6400 (LI-COR) and equilibrated under measurement conditions for a

minimum of 1.5 h (Cousins et al., 2006). Air entering the leaf chamber was prepared by using mass flow controllers (MKS Instruments) to obtain a gas mix of 90.5 kPa dry  $\text{N}_2$  and 4.8 kPa  $\text{O}_2$  (Cousins et al., 2006). A portion of the nitrogen/oxygen air was used to zero the mass spectrometer to correct for  $\text{N}_2\text{O}$  and other contaminants contributing to the 44 and 46 peaks. Pure  $\text{CO}_2$  ( $\delta^{18}\text{O} = 24\text{‰}$  VSMOW) was added to the remaining air stream to obtain a  $\text{CO}_2$  partial pressure of approximately 52 Pa. Low oxygen (4.8 kPa) was used to minimize contamination of the 46 (mass-to-charge ratio) signal caused by the interaction of  $\text{O}_2$  and  $\text{N}_2$  to produce  $\text{NO}_2$  with the mass spectrometer source.

The gas mixtures were fed to the inlet of the LI-6400 console and a flow rate of 200  $\mu\text{mol s}^{-1}$  was maintained over the leaf. The remaining air stream was vented or used to determine the isotopic composition of air entering the leaf chamber (Cousins et al., 2006). 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 spec and the match valve simultaneously. Gas exchange parameters were determined by the LI-6400 and  $p\text{CO}_2$  leaving the chamber was subsequently corrected for the dilution of  $\text{CO}_2$  by water vapor (von Caemmerer and Farquhar, 1981).

### Online 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 that was housed in a temperature-controlled cuvette. The masses (mass-to-charge ratio) 44 and 46 were monitored continuously and the oxygen isotope discrimination during  $\text{CO}_2$  exchange,  $\Delta^{18}\text{O}$ , was calculated from the ratio of mass 46 to 44 in the reference air, determined before and after each sample measurement, entering the chamber ( $R_s$ ), and the composition of the sample air leaving the leaf chamber ( $R_o$ ) as (Evans et al., 1986)

$$\Delta = \frac{-\xi(R_s/R_o - 1)}{1 + \xi(R_s/R_o - 1)} \quad (10)$$

where  $\xi = p_e/(p_e - p_o)$ , and  $p_e$  and  $p_o$  are the  $p\text{CO}_2$  of dry air entering and leaving the leaf chamber, respectively. A summary of the symbols used in the text are listed in Table I. Zero values for the 44 and 46 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 6% of the 46 peak.

### Collection and $^{18}\text{O}$ Isotopic Measurements of Water Vapor

A line connected directly to the exhaust port of the LI-6400 was used to cryogenically trap transpired water in a modified glass collection line submerged in an ethanol/dry ice bath. Water vapor was collected for 45 min (netting 50–100  $\mu\text{L}$  of water depending on transpiration rates) and allowed to warm to room temperature in a sealed collection tube. The tube was subsequently centrifuged and placed into an ice water bath for 30 min. The liquid water was removed from the tube and stored at 4°C in screw-top sample vials (Alltech) until measured. To measure the  $\delta^{18}\text{O}$  of the collected water, 10 mL head space vials (Alltech) with crimped tops containing butyl septa (Alltech) were flushed with 1.9 kPa  $\text{CO}_2$  (in a  $\text{N}_2$  background) at 2 L  $\text{min}^{-1}$  for 3 min and allowing the pressure inside the vials to remain at atmospheric levels. Water samples (25  $\mu\text{L}$ ) were injected through the septa with a gas-tight syringe (SGE) and the  $\text{CO}_2\text{-H}_2\text{O}$  was allowed to equilibrate for 48 h on a shaker at room temperature (Fessenden et al., 2002).

Prior to the isotopic measurements the vials were placed for a minimum of 2.5 h on a temperature block set a 25°C. The  $\text{CO}_2$  samples were analyzed by injecting 200  $\mu\text{L}$  of the headspace gas into a 500  $\mu\text{L}$   $\text{N}_2$  purged gas-tight temperature-controlled cuvette containing a Teflon gas permeable membrane linked to a mass spectrometer (micromass ISOPRIME, Micromass). Masses 44 and 46 were monitored continuously and the zero values determined before and after the sample measurements were subtracted from the values prior to determining the mass ratios. The zero values were typically 3% to 4% of the 44 and 46 peak. Two standard laboratory waters were measured during each measurement to calibrate our measured values against known standards. Our standard waters ( $S1 = -6.44$  and  $S2 = -22.83$ ) were calibrated by the Stable Isotope Facilities in the Earth Environment Group within the Research School of Earth Sciences at The Australian National University.

The measured  $\delta^{18}\text{O}$  value ( $\delta_m$ ) was corrected for the contribution of oxygen from the  $\text{CO}_2$  used for equilibration and normalized against VSMOW as outlined in Scrimgeour (1995). The dilution corrected value was obtained by

$$\delta_c = \delta_m + (\delta_m - \delta_g)\alpha/k \quad (11)$$

where  $\delta_g$  is the signal of the equilibrating gas,  $\alpha$  is the fractionation factor for  $^{18}\text{O}$  between water and  $\text{CO}_2$  (41.1‰ at 25°C), and  $k$  is the ratio of oxygen atoms in the water to the oxygen atoms in the  $\text{CO}_2$  ( $k = 78$ ). The  $\delta_c$  values were then normalized to VSMOW using the two standard waters (−6.44‰ and −22.83‰) and the following equation:

$$\delta^{18}\text{O}_{\text{VSMOW}}^{\text{SAM}} = (\delta_c^{\text{SAM}} - \delta_c^{\text{S1}})(\delta_c^{\text{S2}} - \delta_c^{\text{S1}}) / (\delta_c^{\text{S2}} - \delta_c^{\text{S1}}) + \delta_c^{\text{S1}} \quad (12)$$

where the superscripts SAM, S1, and S2 refer to the sample in question and the reference waters 1 and 2, respectively. The subscripts c and VSMOW refer to the dilution corrected values and those calibrate against VSMOW, respectively. The precision of analyses, based on the repeated measurements of gas samples sealed in vials was 0.1‰ (1 SD,  $n = 8$ ).

## CA Activity Measurements

CA activity was measured on leaf extracts using mass spectrometry to measure the rates of  $^{18}\text{O}_2$  exchange from labeled  $^{13}\text{C}^{18}\text{O}_2$  to  $\text{H}_2^{16}\text{O}$  (Badger and Price, 1989; von Caemmerer et al., 2004; Cousins et al., 2006). 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  $^{18}\text{O}$  loss over the uncatalyzed rate and the nonenzymatic first order rate constant was applied (pH 7.4 appropriate for the mesophyll cytosol). The CA activity was reported as a first order rate constant  $k_{\text{CA}}$  ( $\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$ ) and  $k_{\text{CA}P_m}$  gives the in vivo CA activity at that particular cytosolic  $p\text{CO}_2$ . 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.

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## LITERATURE CITED

- Affek HP, Kirsch MJ, Yakir D (2006) Effects of intraleaf variations in carbonic anhydrase activity and gas exchange on leaf (COO)-O-18 isoflux in *Zea mays*. *New Phytol* **169**: 321–329
- Badger M (2003) The roles of carbonic anhydrases in photosynthetic  $\text{CO}_2$  concentrating mechanism. *Photosynth Res* **77**: 83–94
- Badger M, Price GD (1994) The role of carbonic anhydrase in photosynthesis. *Annu Rev Plant Physiol Plant Mol Biol* **45**: 369–392
- Badger MR, Price GD (1989) Carbonic anhydrase activity associated with the cyanobacterium *Synechococcus* PCC7942. *Plant Physiol* **89**: 51–60
- Barbour MM, Farquhar GD (2004) Do pathways of water movement and leaf anatomical dimensions allow development of gradients in (H<sub>2</sub>O)-O-18 between veins and the sites of evaporation within leaves? *Plant Cell Environ* **27**: 107–121
- Barbour MM, Roden JS, Farquhar GD, Ehleringer JR (2004) Expressing leaf water and cellulose oxygen isotope ratios as enrichment above source water reveals evidence of a Pecllet effect. *Oecologia* **138**: 426–435
- Burnell JN, Hatch MD (1988) Low bundle sheath carbonic-anhydrase is apparently essential for effective C-4 pathway operation. *Plant Physiol* **86**: 1252–1256
- Cernusak LA, Farquhar GD, Wong SC, Stuart-Williams H (2004) Measurement and interpretation of the oxygen isotope composition of carbon dioxide respired by leaves in the dark. *Plant Physiol* **136**: 3350–3363
- Chitty JA, Furbank RT, Marshall JS, Chen ZH, Taylor WC (1994) Genetic transformation of the C-4 plant, *Flaveria-bidentis*. *Plant J* **6**: 949–956
- Cousins AB, Badger MR, von Caemmerer S (2006) Carbonic anhydrase and its influence on carbon isotope discrimination during C4 photosynthesis: insights from antisense RNA in *Flaveria bidentis*. *Plant Physiol* **141**: 232–242
- Craig H, Gordon LI (1965) Deuterium and oxygen-18 variations in the ocean and the marine atmosphere. In E Tongiorgi, ed, *Proceedings of a Conference on Stable Isotopes in Oceanographic Studies and Paleotemperatures*. Consiglio Nazionale delle Ricerche, Laboratorie Geologia Nuclear, Pisa, Italy, pp 9–130
- Evans JR, Sharkey TD, Berry JA, Farquhar GD (1986) Carbon isotope discrimination measured concurrently with gas-exchange to investigate  $\text{CO}_2$  diffusion in leaves of higher-plants. *Aust J Plant Physiol* **13**: 281–292
- Evans JR, vonCaemmerer S (1996) Carbon dioxide diffusion inside leaves. *Plant Physiol* **110**: 339–346
- Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. *Annu Rev Plant Physiol Plant Mol Biol* **40**: 503–537
- Farquhar GD, Gan KS (2003) On the progressive enrichment of the oxygen isotopic composition of water along a leaf. *Plant Cell Environ* **26**: 801–819
- Farquhar GD, Lloyd J (1993) Carbon and oxygen isotope effects in the exchange of carbon dioxide between terrestrial plants and the atmosphere. In JR Ehleringer, AE Hall, GD Farquhar, eds, *Stable Isotopes and Plant Carbon-Water Relations*. Academic Press, New York, pp 47–70
- Farquhar GD, Lloyd J, Taylor JA, Flanagan LB, Syvertsen JP, Hubick KT, Wong SC, Ehleringer JR (1993) Vegetation effects on the isotope composition of oxygen in the atmospheric  $\text{CO}_2$ . *Nature* **363**: 439–443
- Fessenden J, Cook C, Lott M, Ehleringer JR (2002) Rapid  $^{18}\text{O}$  analysis of small water and  $\text{CO}_2$  samples using a continuous-flow isotope ratio mass spectrometer. *Rapid Commun Mass Spectrom* **16**: 1257–1260
- Flanagan LB, Ehleringer JR (1998) Ecosystem-atmosphere  $\text{CO}_2$  exchange: interpreting signals of change using stable isotope ratios. *Trends Ecol Evol* **13**: 10–14
- Furbank RT, Chitty JA, Jenkins CLD, Taylor WC, Trevanion SJ, vonCaemmerer S, Ashton AR (1997) Genetic manipulation of key photosynthetic enzymes in the C-4 plant *Flaveria bidentis*. *Aust J Plant Physiol* **24**: 477–485
- Furbank RT, Chitty JA, von Caemmerer S, Jenkins CLD (1996) Antisense RNA inhibition of RbcS gene expression reduces rubisco level and photosynthesis in the C-4 plant *Flaveria bidentis*. *Plant Physiol* **111**: 725–734
- Gillon J, Yakir D (2001) Influence of carbonic anhydrase activity in terrestrial vegetation on the O-18 content of atmospheric  $\text{CO}_2$ . *Science* **291**: 2584–2587
- Gillon JS, Yakir D (2000a) Internal conductance to  $\text{CO}_2$  diffusion and (COO)-O-18 discrimination in C-3 leaves. *Plant Physiol* **123**: 201–213
- Gillon JS, Yakir D (2000b) Naturally low carbonic anhydrase activity in C-4 and C-3 plants limits discrimination against (COO)-O-18 during photosynthesis. *Plant Cell Environ* **23**: 903–915
- Harwood KG, Gillon JS, Griffiths H, Broadmeadow MSJ (1998) Diurnal variation of  $\Delta(\text{CO}_2)$ -C-13,  $\Delta(\text{COO})$ -O-18-O-16 and evaporative site enrichment of  $\delta(\text{H}_2\text{O})$ -O-18 in *Piper aduncum* under field conditions in Trinidad. *Plant Cell Environ* **21**: 269–283
- Hatch MD (1987) C-4 photosynthesis—a unique blend of modified biochemistry, anatomy and ultrastructure. *Biochim Biophys Acta* **895**: 81–106
- Hatch MD, Burnell JN (1990) Carbonic-anhydrase activity in leaves and its role in the first step of C-4 photosynthesis. *Plant Physiol* **93**: 825–828
- Kanai R, Edwards GE (1999) The biochemistry of  $\text{C}_4$  photosynthesis. In R Sage, R Monson, eds, *C<sub>4</sub> Plant Biology*. Academic Press, San Diego, pp 49–87
- Ku MSB, Edwards GE (1975) Photosynthesis in mesophyll protoplast and bundle sheath cells of various type of  $\text{C}_4$  plants. V. Enzymes of respiratory metabolism and energy utilizing enzymes of photosynthetic pathways. *Z Pflanzenphysiol* **77**: 16–32
- Lee K, Lee J, Kim Y, Bae D, Kang KY, Yoon SC, Lim D (2004) Defining the plant disulfide proteome. *Electrophoresis* **25**: 532–541
- Ludwig M, von Caemmerer S, Price GD, Badger MR, Furbank RT (1998) Expression of tobacco carbonic anhydrase in the  $\text{C}_4$  dicot *Flaveria bidentis* leads to increased leakiness of the bundle sheath and a defective  $\text{CO}_2$ -concentrating mechanism. *Plant Physiol* **117**: 1071–1081
- Mills G, Urey H (1940) The kinetics of isotopic exchange between carbon dioxide, bicarbonate ion, carbonate ion and water. *J Am Chem Soc* **62**: 1019–1026

- Scrimgeour CM** (1995) Measurement of plant and soil water isotope composition by direct equilibration methods. *J Hydrol* **172**: 261–274
- von Caemmerer S, Evans JR** (1991) Determination of the average partial pressure of CO<sub>2</sub> in chloroplast from leaves of several C<sub>3</sub> plants. *Aust J Plant Physiol* **18**: 287–305
- von Caemmerer S, Farquhar GD** (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* **153**: 376–387
- von Caemmerer S, Ludwig M, Millgate A, Farquhar GD, Price D, Badger M, Furbank RT** (1997a) Carbon isotope discrimination during C-4 photosynthesis: insights from transgenic plants. *Aust J Plant Physiol* **24**: 487–494
- von Caemmerer S, Millgate A, Farquhar GD, Furbank RT** (1997b) Reduction of ribulose-1,5-bisphosphate carboxylase/oxygenase by antisense RNA in the C<sub>4</sub> plant *Flaveria bidentis* leads to reduced assimilation rates and increased carbon isotope discrimination. *Plant Physiol* **113**: 469–477
- von Caemmerer S, Quinn V, Hancock NC, Price GD, Furbank RT, Ludwig M** (2004) Carbonic anhydrase and C<sub>4</sub> photosynthesis: a transgenic analysis. *Plant Cell Environ* **27**: 697–703
- Williams TG, Flanagan LB, Coleman JR** (1996) Photosynthetic gas exchange and discrimination against <sup>13</sup>CO<sub>2</sub> and C<sup>18</sup>O<sup>16</sup>O in tobacco plants modified by an antisense construct to have low chloroplastic carbonic anhydrase. *Plant Physiol* **112**: 319–326
- Yakir D, Sternberg LdL** (2000) The use of stable isotopes to study ecosystem gas exchange. *Oecologia* **123**: 297–311
- Yakir D, Wang X-F** (1996) Fluxes of CO<sub>2</sub> and water between terrestrial vegetation and the atmosphere estimated from isotope measurements. *Nature* **380**: 515–517