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Carbon-isotope discrimination by leaves of *Flaveria* species exhibiting different amounts of C_3- and C_4-cycle co-function

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Abstract. Carbon-isotope ratios were examined as $\delta^{13}C$ values in several C_3, C_4, and C_3–C_4 *Flaveria* species, and compared to predicted $\delta^{13}C$ values generated from theoretical models. The measured $\delta^{13}C$ values were within 4‰ of those predicted from the models. The models were used to identify factors that contribute to C_3-like $\delta^{13}C$ values in C_3–C_4 species that exhibit considerable C_4-cycle activity. Two of the factors contributing to C_3-like $\delta^{13}C$ values are high CO_2 leakiness from the C_4 pathway and pi/pa values that were higher than C_4 congeners. A marked break occurred in the relationship between the percentage of atmospheric CO_2 assimilated through the C_4 cycle and the $\delta^{13}C$ value. Below 50% C_4-cycle assimilation there was no significant relationship between the variables, but above 50% the $\delta^{13}C$ values became less negative. These results demonstrate that the level of C_4-cycle expression can increase from 0 to 50% with little integration of carbon transfer from the C_4 to the C_3 cycle. As expression increases above 50%, however, increased integration of C_3- and C_4-cycle co-function occurs.

Key words: C_3–C_4 intermediate plants – Carbon isotope discrimination (ratio, theory) – *Flaveria* – Photosynthesis (C_3, C_4, C_3–C_4).

Introduction

Species that exhibit characteristics of both the C_3 and C_4 photosynthetic pathways have been the subject of considerable recent research (see reviews by Monson et al. 1984; Holaday and Chollet 1984; Edwards and Ku 1988). Such species have importance in applied disciplines, in that they might contribute knowledge towards breeding efforts to introduce C_4 traits into otherwise C_3 plants, and more basic disciplines, in that they might contribute knowledge to the paths taken during the evolution of C_4 photosynthesis. In addition to C_3 and C_4 species, the genus *Flaveria* (Asteraceae) contains many species that exhibit anatomical and physiological traits characteristic of both the C_3 and C_4 syndromes (Ku et al. 1983; Holaday et al. 1984; Edwards and Ku 1988). Several of these C_3–C_4 species assimilate atmospheric CO_2 through both the C_3 and C_4 photosynthetic pathways (Rumpho et al. 1984; Bassüner et al. 1984; Monson et al. 1986). In a number of these species, C_4 photosynthesis can be responsible for up to 50% of the atmospheric CO_2 assimilation.

Despite the biochemical evidence for considerable C_4-cycle function in many of the *Flaveria* species, previous measurements of carbon-isotope values are suggestive of little contribution of C_4 photosynthesis to growth (Smith and Turner 1975; Powell 1978; Smith and Powell 1984). The presence of C_3-like carbon-isotope ratios in these otherwise intermediate plants has resulted in a paradox, since differences in the levels of atmospheric CO_2 assimilation through the C_3 or C_4 pathways should be detectable as differences in the level of discrimination against $^{13}CO_2$. The assimilation of atmospheric CO_2 through the C_3 cycle will result in greater discrimination against ^{13}C and a more negative $\delta^{13}C$ value, relative to CO_2 assimilation through the C_4 cycle (O'Leary 1981). Thus, in C_3 plants, $\delta^{13}C$ values between −25 and −30‰ are typically measured, whereas in C_4 plants the values are typically between −10 and −16‰. In plants that exhibit a balance of C_3- and C_4-cycle co-func-

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Abbreviations and symbols: RuBP carboxylase = ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39); PEP carboxylase = phosphoenolpyruvate carboxylase (EC 4.1.1.31); pa = atmospheric CO_2 partial pressure; pi = intercellular CO_2 partial pressure; δ = isotope ratio; ϕ = quantum yield for CO_2 uptake

tion in assimilating atmospheric CO_2, such as several of the *Flaveria* species, the $\delta^{13}C$ value should be intermediate between the C_3 and C_4 extremes.

In a recent study, Peisker (1985) attempted to make quantitative predictions of what the $\delta^{13}C$ values should be in C_3–C_4 species. His results demonstrated that C_3-like $\delta^{13}C$ values would result if the C_4 cycle was rate-limited by factors other than phosphoenolpyruvate (PEP)-carboxylase activity, for example PEP-regeneration rate. One implication of Peisker's study is that although PEP-carboxylase activities in C_3–C_4 species are measurably higher than in C_3 species, they do not accurately reflect C_4-cycle assimilation of CO_2. In essence, according to Peisker's model the C_3-like $\delta^{13}C$ values in otherwise C_3–C_4 species were attributed to a low C_4-cycle activity. However, as mentioned above, recent studies with several C_3–C_4 *Flaveria* species have demonstrated considerable potential for C_4-cycle assimilation of atmospheric CO_2 (Monson et al. 1986). Thus, an enigma still exists as to why C_3-like $\delta^{13}C$ values occur in plants with such a large fraction of C_4-cycle CO_2 assimilation. In this study, we have compared measured $\delta^{13}C$ values with those predicted from theoretical models in order to identify factors which may be responsible for the discrepency. Such an approach has previously been used to explain why $\delta^{13}C$ values in fully-expressed C_4 species are more negative than those predicted solely from biochemical and biophysical fractionation processes (Farquhar 1983). Our analysis showed that an inefficient transfer of CO_2 from the C_4 to the C_3 cycle, following the decarboxylation of C_4-acids, could result in C_3-like $\delta^{13}C$ values in the C_3–C_4 *Flaveria* species.

Material and methods

Plant material. Plants of all species, except *Flaveria brownii* A.M. Powell and *F. floridana* Johnson, were established from seeds obtained from Dr. A.M. Powell (Sul Ross State University, Alpine, Tex., USA). The seeds were originally collected from field populations in Mexico (see Powell 1978). Plants of *F. brownii* and *F. floridana* were established from seeds collected by Dr. L.J. Mets (University of Chicago, Chicago, Ill., USA) from Texas and Florida, respectively. Following establishment the plants were maintained in greenhouse or growth-chamber culture, each species being propagated from branch cuttings.

For one group of plants grown in a growth chamber, intensive studies of leaf $\delta^{13}C$ values and biochemical traits were conducted (data reported in Table 1). These plants were grown in a controlled-temperature regime of 27° C day/22° C night. The 14-h light period was produced by a combination of fluorescent and incandescent lamps. The photosynthetic photon fluence rate (400–700 nm) at plant height was between 650 and 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$. In calculating the predicted $\delta^{13}C$ values reported in Table 1, measurements of the quantum yield for CO_2 uptake were required (see *Theory* section). Measurements of the quantum yield were conducted on established cuttings of the same plants used in the growth-chamber studies, although in this case the plants were grown in a greenhouse in Boulder, Colo. Care was taken to match the greenhouse growth conditions to those used in the growth chamber in the following ways: the quantum-yield measurements were conducted between late-May and early-August, 1985, when photoperiods ranged between 14 and 15 h, greenhouse temperatures were maintained at 25–30° C during the day and 18–23° C during the night, and the photon fluence rate was maintained between 700–900 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (400–700 nm) at midday on clear days by partially shading the plants.

We assumed a value of −7.5‰ for the $\delta^{13}C$ of the air in the growth chamber. This is the value typically assumed for clean ambient air (Keeling et al. 1979). As a check of this value, we calculated the $\delta^{13}C$ value of the growth chamber air to be −7.4‰ using the measured value of −29.4‰ for the C_3 plant *F. cronquistii* and equation (12) from Farquhar et al. (1982). *Flaveria cronquistii* exhibits a small amount of atmospheric CO_2 assimilation through PEP carboxylase (Monson et al. 1986, Table 1). However, this small amount of C_4-cycle activity would only introduce an error of +1.3% into the calculation of the $\delta^{13}C$ value for the growth-chamber air (compare the predicted and actual $\delta^{13}C$ values for *F. cronquistii* in Table 1).

Experimental methods. The combustion and analytical methods used to determine leaf-$\delta^{13}C$ values were as described in Gurevitch et al. (1986). Carbon-isotope ratios were obtained for the youngest fully expanded leaves (third or fourth node from the apex) of non-flowering plants. The $^{14}CO_2$ pulse-$^{12}CO_2$ chase studies, that were used to establish the relative activities of the C_3 and C_4 cycles, and the gas-exchange methods used in the quantum-yield and intercellular (pi) and ambient (pa) CO_2 partial pressure measurements, are described in detail in a previous paper (Monson et al. 1986). Measurements of pi/pa were determined at a photosynthetic photon fluence rate of 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$, leaf temperature of 30° C, and leaf-to-air water-vapor concentration gradient of 12–15 $mmol \cdot mol^{-1}$.

Theory

Predictions of the $\delta^{13}C$ values for eight *Flaveria* species were generated using previous biochemical and physiological measurements (Monson et al. 1986, 1987), and previously described models of carbon-isotope discrimination resulting from diffusive and biochemical fractionation (Farquhar et al. 1982; Farquhar 1983). Knowing the proportion of atmospheric CO_2 assimilated by the C_3 and C_4 pathways, we calculated the carbon-isotope ratios of the C_3–C_4 species using the following expression:

$$\delta^{13}C_{tot} = (\delta^{13}C_{c4} \cdot c + \delta^{13}C_{c3} \cdot d) \quad (1)$$

where $\delta^{13}C_{tot}$ is the predicted $\delta^{13}C$ value for total carbon assimilated in C_3–C_4 plants, c represents the proportion of atmospheric CO_2 assimilated through the C_4 cycle, d represents the proportion of atmospheric CO_2 assimilated through the C_3 cycle, $\delta^{13}C_{c4}$ represents the predicted $\delta^{13}C$ value for carbon assimilated through the C_4 pathway,

and $\delta^{13}C_{c3}$ is the predicted $\delta^{13}C$ value for carbon assimilated through the C_3 pathway.

The values for $\delta^{13}C_{c4}$ and $\delta^{13}C_{c3}$ were determined according to Eq. (11) in Farquhar (1983) and Eq. (12) in Farquhar et al. (1982), respectively. The only correction was that we used a value of 29‰ as the fractionation caused by ribulose-1,5-bisphosphate (RuBP) carboxylase (see Roeske and O'Leary 1984). Determination of $\delta^{13}C_{c4}$ requires knowledge of the leakiness (ϕ) which is the proportion of CO_2 that is initially assimilated by PEP carboxylase, but not reassimilated by RuBP carboxylase following decarboxylation of the C_4 acids. In this study ϕ was determined from the C_4 quantum yield for C_4 uptake (q_4) using the expression for ϕ_2 in Farquhar (1983). In those species that exhibit atmospheric CO_2 assimilation simultaneously through both the C_3 and C_4 pathways, the C_4 quantum yield was calculated from the total quantum yield ($C_3 + C_4$) using the following expression:

$$q_{tot} = (c \cdot q_4) + (d \cdot q_3) \quad (2)$$

where q_{tot} is the total quantum yield, q_3 represents the C_3 quantum yield, and q_4 represents the C_4 quantum yield. Assuming the C_3 quantum yield to be 0.052 mol $CO_2 \cdot mol^{-1}$ quanta absorbed at 25–30° C (determined as the mean of over 30 species, see Ehleringer and Björkman 1977; Monson et al. 1982; Ehleringer and Pearcy 1983), Eq. (2) can be rearranged to:

$$q_4 = (q_{tot} - d \cdot 0.052)/c \quad (3)$$

In all species, except *F. trinervia*, the values for *d* and *c* were determined from 8-s $^{14}CO_2$-pulse experiments (Monson et al. 1986). Ideally, the values for *c* and *d* should be determined from pulse experiments of varying duration, that can be used to extrapolate to a pulse-time equal to zero. To date, the latter type of measurement has only been conducted with two C_3–C_4 species. *F. pubescens* (Bassüner et al. 1984) and *F. ramosissima* (Rumpho et al. 1984), and one C_4 species, *F. trinervia* (Rumpho et al. 1984). The studies with the C_3–C_4 species revealed little change in the proportion of the ^{14}C recovered from C_3 and C_4 products between 0 and 10 s, demonstrating that the use of the previous 8-s pulse data (Monson et al. 1986) for the *c* and *d* values is reasonable in the C_3–C_4 species. In the C_4 species *F. trinervia*, data from $^{14}CO_2$-pulse experiments of varying durations revealed that approximately 100% of atmospheric CO_2 is assimilated through the C_4 pathway at a time equal to 0. Therefore, we have used 1.0 for *c* in the calculations of predicted $\delta^{13}C$ for this species.

In theory, it is an oversimplification to treat the C_3 and C_4 pathways as isotopically separate, since previous observations have shown RuBP carboxylase and PEP carboxylase to be in both mesophyll and bundle-sheath cells in some C_3–C_4 *Flaveria* species (Bauwe 1984; Reed and Chollet 1985). Thus, both carboxylases are drawing upon the same intercellular pool of CO_2, and the potential exists for carbon-isotope discrimination by one to influence discrimination by the other. Such an influence will occur because the $\delta^{13}C$ value of the intercellular CO_2 pool will be adjusted to some value different from that expected in fully expressed C_3 and C_4 plants. In essence, such an adjustment can be considered as an extra discrimination factor since it acts to alter the $^{13}C/^{12}C$ ratio of the intercellular CO_2, just as with other discrimination. We examined the magnitude of this effect using equations described in O'Leary (1981) to calculate the $\delta^{13}C$ value of the intercellular CO_2 pool in C_3–C_4 species. Since the discrimination caused by the carboxylases is characterized by different signs (RuBP carboxylase discriminates against ^{13}C, whereas the combined effect of equilibration between CO_2 and HCO_3^- and discrimination by PEP carboxylase favors the assimilation of ^{13}C), to some extent the discrimination by one compensates for the discrimination by the other. For example, in C_3–C_4 species with nearly equal amounts of atmospheric carbon assimilated through the C_3 and C_4 cycles, we estimate that the presence of RuBP carboxylase in the mesophyll cells causes carbon assimilated by PEP carboxylase to be enriched in ^{13}C by approx. 9‰, compared to fully expressed C_4 plants. Conversely, the presence of PEP carboxylase in the mesophyll cells, causes carbon assimilated by RuBP carboxylase to be enriched in ^{12}C by approx. 6‰, compared to fully expressed C_3 plants. When the $\delta^{13}C_{tot}$ is calculated for such C_3–C_4 species, these differences translate into an error of only 1–2‰ by not considering the effect of discrimination by one carboxylase on discrimination by the other. We have not presented this analysis in a formal manner, since it should only be taken as a first approximation. More elegant modelling efforts should be conducted to accurately describe the effects of co-discrimination by the decarboxylases. Nonetheless, at first consideration the effect appears to be small.

Results

In one set of measurements the proportion of CO_2 assimilated through the C_3 and C_4 cycles was mea-

Table 1. Actual and predicted $\delta^{13}C$ values for growth-chamber-grown plants and the parameters used to calculate the predicted $\delta^{13}C$ values (see *Theory* section for calculation procedure) in nine *Flaveria* species

Species	Photosynthetic pathway	c	d	q_{tot} (mol CO_2 · (mol quanta)$^{-1}$)	$\emptyset_2$	pi/pa	Predicted $\delta^{13}C$ (‰)	Actual $\delta^{13}C$ (‰)
F. cronquistii Powell	C_3	0.14	0.86	0.053	0.71	0.735	−28.1	−29.4
F. linearis Lag.	C_3–C_4	0.23	0.77	0.050	0.79	0.737	−28.0	−27.9
F. pubescens Rydb.	C_3–C_4	0.41	0.59	0.044	0.86	0.779	−28.0	−28.3
F. anomala Robinson	C_3–C_4	0.44	0.56	0.051	0.73	0.760	−26.1	−28.3
F. ramosissima Klatt	C_3–C_4	0.49	0.51	0.052	0.71	0.765	−25.4	−28.5
F. floridana Johnson	C_3–C_4	0.52	0.48	0.046	0.81	0.766	−26.2	−29.9
F. brownii A.M. Powell	C_3–C_4	0.65	0.35	0.052	0.73	0.482	−19.5	−17.4
F. palmeri Johnson	C_4	0.76	0.24	ND	ND	ND	ND	−16.5
F. trinervia (Spreng.) Mohr	C_4	1.00	0	0.051	0.72	0.441	−16.6	−14.3

Actual $\delta^{13}C$ values for *F. brownii* and *F. palmeri* were determined from greenhouse-grown plants, all others were from the same growth-chamber-grown plants used for determining c and d

c and d represent the percentages of atmospheric CO_2 assimilated through the C_4 and C_3 photosynthetic pathways, respectively. These data were taken from Monson et al. (1986)

q_{tot} represents the total quantum yield ($C_4 + C_3$), which were also taken from Monson et al. (1986)

$\emptyset_2$ represents the CO_2 leakiness values

pi/pa represents the ratio of intercellular (pi) to ambient (pa) CO_2 partial pressures

ND = not determined

sured directly using $^{14}CO_2$ pulse-$^{12}CO_2$ chase techniques and these proportions, along with measurements of the quantum yield for CO_2 uptake and pi/pa, were used to calculate the predicted $\delta^{13}C$ values for eight species (see Table 1). Leaves from the same plants were harvested and used for actual carbon-isotope measurements. Thus, direct comparisons could be made between predicted $\delta^{13}C$ values and actual $\delta^{13}C$ values. Calculations of CO_2 leakiness from the C_4 cycle ($\emptyset_2$) revealed relatively high values for all of the *Flaveria* species that we examined. Even for the fully expressed C_4 plant, *F. trinervia*, leakiness values were estimated to be 0.72. Values for pi/pa were much lower in the C_4 species, *F. trinervia*, and the C_3–C_4 species, *F. brownii*, relative to the C_3 species *F. cronquistii*. The other five C_3–C_4 species exhibited similar, or slightly higher, pi/pa values relative to *F. cronquistii*. In this study we have treated *F. cronquistii* as a C_3 species for purposes of classification in Table 1. However, previous pulse-chase studies have shown that, following an 8-s pulse with $^{14}CO_2$, approx. 14% of the assimilated ^{14}C can be recovered in C_4-acids (Monson et al. 1986). A considerable portion of the C_4-acid synthesis was accounted for by non-photosynthetic processes (Monson et al. 1986). Nonetheless, we have calculated a leakiness value for this C_4-cycle activity, and using it predicted a $\delta^{13}C$ value of −28.1‰. The latter value is only 1.3‰ less negative than the actual value of −29.4‰. However, the uncertain nature of C_4 assimilation in this species, and its influence on the $\delta^{13}C$ value, should be noted. The C_3–C_4 species were predicted to exhibit $\delta^{13}C$ values intermediate to the C_3 and C_4 plants. However, except for *F. brownii*, the values were closer to the C_3 extreme than the C_4 extreme. In this study we have classified *F. brownii* as a C_3–C_4 species based on previous reports of incomplete compartmentation of C_3- and C_4-cycle enzymes (Reed and Chollet 1985) and measurable oxygen inhibition of photosynthesis (Monson et al. 1987). The actual $\delta^{13}C$ values for C_3–C_4 species were within 4‰ of the predicted values.

In order to obtain a broader perspective on how the $\delta^{13}C$ values of C_3–C_4 *Flaveria* species should vary as a function of the proportion of atmospheric CO_2 assimilated through the C_3 or C_4 pathways, theoretical calculations of $\delta^{13}C$ over a range of values for c and d were conducted. When CO_2 leakiness is zero, the $\delta^{13}C$ value is predicted to increase from −30.3‰ at 0% C_4 assimilation (100% C_3 assimilation), to −17.4‰ at 50% C_4 assimilation, to −4.6‰ at 100% C_4 assimilation (0% C_3 assimilation). If CO_2 leakiness is 0.75 (75% of the atmospheric CO_2 assimilated by the C_4 cycle is not reassimilated by the C_3 cycle following decarboxylation), the $\delta^{13}C$ value is predicted to only increase from −30.3‰ at 0% C_4 assimilation, to −27.0‰ at 50% C_4 assimilation, to −23.8‰ at 100% C_4 assimilation. The predictions were made assuming an average pi/pa of 0.75 which is typical of C_3–C_4 *Flaveria* secies (Table 1).

Table 2. $\delta^{13}C$ values for leaves of several *Flaveria* species grown in a greenhouse and sampled at various times during the year

Species	Genotype	Time of Collection	$\delta^{13}C$ (‰)
F. trinervia.	M2	March	−15.3
	K1	July	−14.5±0.1
F. cronquistii	K1	July	−28.0±0.3
F. brownii	MB6	March	−17.7
	MB6	July	−17.4±0.1
F. ramosissima	K1	September	−28.1
	K1	April	−28.3
	K1	July	−28.2±0.1
F. floridana	M1	April	−27.1
	M1	July	−28.3±0.1
F. pubescens	M1	March	−32.1
	K1	July	−28.8±0.2
F. linearis	M2	September	−29.6
	M1	March	−30.6
	M2	April	−25.3
	K1	July	−27.6±0.2

M-genotypes were obtained by Mets and grown at the University of Chicago; K-genotypes were obtained by Ku and grown at Washington State University or the University of Colorado

All values not followed by ± represent single measurements. Values followed by ± represent the mean ± SE of five replicate measurements

Measurements of $\delta^{13}C$ were conducted at various times during the year on seven *Flaveria* species grown in a greenhouse (Table 2). The purpose of these measurements was to provide a larger range of samples to assess whether the values reported in Table 1 were truly representative of the species. The $\delta^{13}C$ values of the greenhouse-grown plants were within ±3‰ of the growth-chamber-grown plants, with an exception being the March value for *F. pubescens*, which was 4.2‰ more negative than the growth-chamber value.

Discussion

Two of the principal factors underlying C_3-like $\delta^{13}C$ values in the C_3–C_4 *Flaveria* species appear to be the CO_2-leakiness value (ϕ_2) and the observed pi/pa value. As calculated here, leakiness may be overestimated (see Farquhar 1983). In essence, the leakiness calculations involve comparing actual to theoretical quantum yields (the greater the difference, the greater the calculated leakiness). The calculations do not adequately account for potential reductions in quantum yield due to energy-requiring non-photosynthetic processes, or the absorption of light by non-photosynthetic pigments and molecules. Additionally, the quantum yield is dependent on the wavelength distribution of absorbed light, being higher in red light (Evans 1987). Thus, the quantum-yield values reported here, which were measured in white light, may not reflect the true energetic demands of CO_2 assimilation through the C_3 and C_4 cycles. Finally, on theoretical grounds the leakiness values calculated from quantum yields measured at low light levels may differ from the values that exist at the higher light levels present during growth. For these reasons, the leakiness values and predicted $\delta^{13}C$ values reported in Table 1 should be viewed as estimates.

Despite the potential problems in calculating leakiness, estimates of $\delta^{13}C$ using the models described here are reasonably close to measured $\delta^{13}C$ values (Table 1; Farquhar 1983). The calculated values of ϕ_2 for the C_3–C_4 species are higher than those typically calculated for fully expressed C_4 species (e.g. Farquhar 1983). The utility of the leakiness values reported in Table 1 is that they provide evidence, in a relative sense, of inefficiency in the transfer of carbon from the C_4 pathway to the C_3 pathway in the C_3–C_4 species. The models used in this study demonstrate that such inefficiency, when combined with C_3-like pi/pa values, results in C_3-like $\delta^{13}C$ values in C_3–C_4 plants that assimilate up to 50% of their carbon through the C_4 pathway. Leakiness in the C_3–C_4 *Flaveria* species may be a consequence, in large part, of incomplete compartmentation of enzymes involved in the C_3 and C_4 cycles (Bauwe 1984; Reed and Chollet 1985), and futile cycling of CO_2 between carboxylation and decarboxylation events (see Discussion in Monson et al. 1986).

Through the models described earlier (see *Theory* section) and the leakiness values reported in Table 1, we calculated that, given equal pi/pa values, we should be able to detect differences of 3–4‰ in ^{13}C content when comparing values for fully expressed C_3 species and C_3–C_4 species that assimilate between 40 and 50% of their carbon through the C_4 pathway. We were not able to detect such a difference when comparing *F. conquistii* (C_3) with several C_3–C_4 species grown in growth-chamber and greenhouse environments. Two principal factors could have operated to ameliorate the anticipated differences between the C_3 and C_3–C_4 species. First, the predicted $\delta^{13}C$ values were generated from the biochemical and physiological traits measured at one instant during the lifetime of the leaf. There is some evidence that traits such as leakiness of the bundle-sheath tissue, and the relative proportions of C_3- and C_4-cycle activity can change as leaves mature in *F. trinervia* (Moore

et al. 1986). Young leaves tend to exhibit a greater ratio of C_3/C_4 activity than mature leaves. Given that the $\delta^{13}C$ value integrates the entire carbon-assimilation history of the leaves (including any carbon imported from other leaves or organs), there is potential for instantaneous predictions to deviate considerably from actual biomass values. If there exists a lower potential for C_4-cycle activity in young leaves of the C_3–C_4 species, the deviation between actual and predicted values in mature leaves would be such that the actual $\delta^{13}C$ values are closer to the C_3 value. Second, most of the C_3–C_4 *Flaveria* species exhibit slightly higher pi/pa values than *F. cronquistii* (Table 1). The higher pi/pa values in the C_3–C_4 species would influence the $\delta^{13}C$ values to become slightly more negative, once again bringing the C_3–C_4 values closer to the C_3 value.

The relationship between measured $\delta^{13}C$ values and ^{14}C-incorporation into C_4-acids (Table 1) may provide insight into the evolutionary relationship between the expression of the C_4 pathway and the level of integration of carbon transfer between the C_4 and C_3 cycles. In this study, we define a high level of integration as a greater amount of atmospheric CO_2 being assimilated by the C_4 cycle and efficiently transferred to the C_3 cycle following decarboxylation. In essence, a higher level of integration reflects a carbon-assimilation system that is less "open" (as defined in Berry and Troughton 1974). The less open system would result in less carbon-isotope discrimination by the C_3 cycle. In the C_3–C_4 *Flaveria* species, it is assumed that the $\delta^{13}C$ value is an index of biochemical integration, such that less negative values reflect more integration. The data presented in Table 1 demonstrate that among the *Flaveria* species an increase in expression of the C_4 cycle from 0% to 50% of atmospheric CO_2 fixation has occurred with no significant increase in integration between the C_4 and C_3 cycles. As the level of C_4-cycle expression increases above 50% there is a sharp increase in the level of integration. This increased integration is apparently associated with further development of Kranz leaf anatomy and an improved compartmentation of C_3- and C_4-cycle enzymes between mesophyll and bundle-sheath cells. Intuitively, it is reasonable to expect that there is an upper limit beyond which the evolution of increased activity of an inefficient, poorly integrated C_4 cycle becomes energetically too costly, relative to the benefit it might provide (e.g. reduction of photorespiration). In the C_3–C_4 *Flaveria* species this limit appears to be at the 50% expression point, above which improved compartmentation and co-function of the C_3 and C_4 cycles must occur before further increases in C_4 expression are possible.

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