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Enzymic and Photosynthetic Characteristics of Reciprocal F_1 Hybrids of *Flaveria pringlei* (C_3) and *Flaveria brownii* (C_4-Like Species)[1]

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A. Scott Holaday*, R. Harold Brown, J. Michael Bartlett, Elizabeth A. Sandlin, and Raymond C. Jackson
Department of Biological Sciences, Texas Tech University, Lubbock, Texas 79409 (A.S.H., J.M.B., E.A.S., R.C.J.); *and Department of Agronomy, University of Georgia, Athens, Georgia 30602* (R.H.B.)

ABSTRACT

The activities of key C_4 enzymes in gel-filtered, whole-leaf extracts and the photosynthetic characteristics for reciprocal F_1 hybrids of *Flaveria pringlei* (C_3) and *F. brownii* (C_4-like species) were measured to determine whether any inherited C_4-photosynthetic traits are responsible for their reduced CO_2 compensation concentration values (AS Holaday, S Talkmitt, ME Doohan Plant Sci 41: 31–39). The activities of phosphoenolpyruvate carboxylase, pyruvate, orthophosphate dikinase, and NADP-malic enzyme (ME) for the reciprocal hybrids are only about 7 to 17% of those for *F. brownii*, but are three- to fivefold greater than the activities for *F. pringlei*. The low activities of these enzymes in the hybrids appear to be the result of a partial dominance of *F. pringlei* genes over certain *F. brownii* genes. However, no such dominance occurs with respect to the expression of genes for NADP-malate dehydrogenase, which is as active in the hybrids as in *F. brownii*. In contrast to the situation with the enzymes above, cytoplasmic factors appear to determine the inheritance of NAD-ME. The NAD-ME activity in each hybrid is comparable to that in the respective maternal parent. Pulse-chase $^{14}CO_2$ incorporation analyses at ambient CO_2 levels indicate that the hybrids initially assimilate 7 to 9% of the total assimilated CO_2 into C_4 acids as compared to 3.5% for *F. pringlei*. In the hybrids, the percentage of ^{14}C in malate decreases from an average of 6.5 to 2.1% after a 60-second chase in $^{12}CO_2$/air. However, this apparent C_4-cycle activity is too limited or inefficient to substantially alter CO_2 exchange from that in *F. pringlei*, since the values of net photosynthesis and O_2 inhibition of photosynthesis are similar for the hybrids and *F. pringlei*. Also, the ratio of the internal to the external CO_2 concentration and the initial slopes of the plot of CO_2 concentration versus net photosynthesis are essentially the same for the hybrids and *F. pringlei*. At 45 micromoles CO_2 per mole and 0.21 mole O_2 per mole, the hybrids assimilate nearly fivefold more CO_2 into C_4 acids than does *F. pringlei*. Some turnover of the malate pool occurs in the hybrids, but the labelling of the photorespiratory metabolites, glycine and serine, is the same in these plants as it is in *F. pringlei*. Thus, although limited C_4-acid metabolism may operate in the hybrids, we conclude that it is not effective in altering O_2 inhibition of CO_2 assimilation. The ability of the hybrids to assimilate more CO_2 via phosphoenolpyruvate carboxylase at low levels of CO_2 than does *F. pringlei* may result in an increased rate of reassimilation of photorespiratory CO_2 and CO_2 compensation concentrations below that of their C_3 parent. If the hybrids do possess a limited C_4 cycle, it must operate intracellularly. They are not likely to have inherited an intercellular compartmentation of C_4 enzymes, since *F. brownii* has incomplete compartmentation of key C_3 and C_4 enzymes.

Flaveria is a small genus in Asteraceae which contains C_3, C_4, and C_3-C_4 intermediate species (2, 19, 25). Several interspecific hybrids have been made both within and between photosynthetic types (1, 4, 9, 15, 25, 28), but results from only one $C_3 \times C_4$ hybrid have been reported (15) (the 'C_3' species used in Ref. 28 is actually a C_3-C_4 intermediate species [18, 19]). Although this F_1 hybrid between *Flaveria pringlei* (C_3) and *Flaveria brownii* (now considered a C_4-like species [20]) exhibits Γ[2] values at 0.21 mol $O_2 \cdot mol^{-1}$ (28–33 μmol $CO_2 \cdot mol^{-1}$) that are between those values of the parents, they are somewhat closer to values measured for *F. pringlei* (46–64 $\mu mol \cdot mol^{-1}$) than to those for *F. brownii* (6–7). Also, even though the activities of PEP carboxylase, PPDK, and NADP-ME in whole-leaf extracts from the hybrid plants are two- to fivefold greater than in *F. pringlei*, they are only 7 to 10% of activities determined for *F. brownii*. Even the morphological features of the hybrid are close to those of *F. pringlei*. These weak expressions of most C_4 parental traits in this $C_3 \times C_4$ hybrid might be explained by cytoplasmic factors controlling the inheritance of some traits. Or it is possible that when genes of both parents are present, those of *F. pringlei* are preferentially expressed and exhibit partial dominance over the genes of *F. brownii*. However, the possibility of cytoplasmic influence cannot be evaluated from information in the initial report, since reciprocal hybrids were not studied.

The hybrid between *F. pringlei* and *F. brownii* is the first reported successful hybridization between a C_3 and a C_4-species since the production of a $C_4 \times C_3$ *Atriplex* F_1 hybrid (5, 6, 23). Although some anatomical and biochemical characteristics of this *Atriplex* hybrid are similar to those features of C_3-C_4 intermediate species, its photosynthetic CO_2 exchange is reported to be considerably less than its C_3 parent. The substantial reduction in Γ relative to the values for the C_3 parent, the presence of leaf bundle-sheath cells with some centripetally arranged chloroplasts, and the substantial activities of key C_4 enzymes for *F. pringlei* $\times$ *F. brownii* plants are also similar to those characteristics of some C_3-C_4 intermediate *Flaveria* species and suggest that this *Flaveria* hybrid might be considered as an artificially produced C_3-C_4 intermediate (15). However, no comparative photosynthetic data have been reported for this hybrid.

We have been interested in studying physiological and biochemical features of the *Flaveria* hybrid for comparison with those features of naturally occurring intermediate species to see

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[2] Abbreviations: Γ, CO_2 compensation concentration; AP, apparent photosynthesis; c_a, c_i, ambient and intercellular CO_2 concentrations, respectively; MDH, malate dehydrogenase; ME, malic enzyme; pCO_2, partial pressure of CO_2; PEP, phosphoenolpyruvate; PPDK, pyruvate, orthophosphate dikinase; RuBP, ribulose 1,5-bisphosphate.

if these plants have common or different mechanisms for the reduction of Γ. Limited C_4 photosynthesis apparently occurs in at least some C_3-C_4 intermediate *Flaveria* species and may be patially responsible for their intermediate CO_2 exchange characteristics (19, 27). Considering the activities of PEP carboxylase, PPDK, and NADP-ME in *F. pringlei* × *F. brownii* plants, a limited C_4 photosynthetic pathway might also operate in them. However, the initial report of this hybrid (15) does not present the critical CO_2 exchange and biochemical data we require to test this hypothesis. Therefore, in the present study we have examined various biochemical and physiological characteristics of reciprocal hybrids between *F. pringlei* and *F. brownii* to evaluate the apparent dominance of the expression of *F. pringlei* traits over those of *F. brownii* and to determine whether these hybrids are capable of limited C_4 photosynthesis.

MATERIALS AND METHODS

Plant Material. *Flaveria pringlei* Gandoger, *F. brownii* A.M. Powell, and their reciprocal F_1 hybrids were grown in heated greenhouses at Texas Tech and the University of Georgia under a 14 h photoperiod. Sylvania 400 W metal halide lamps, providing a radiant flux density of 1200 to 1600 μmol quanta$\cdot$m$^{-2}\cdot$s^{-1} (400–700 nm), maintained this photoperiod when the natural daylength was less than 14 h. The seeds of *F. pringlei* and *F. brownii* were generously supplied by Dr. A. M. Powell, Sul Ross State University, Alpine, TX.

All hybridizations were performed in a growth chamber as described by Holaday *et al.* (15). The chromosome numbers of the F_1 hybrids were determined from floral material undergoing meiosis (17) and were found to be the same as those of their parents ($2n = 36$).

Enzymic Analyses. Whole-leaf extracts were prepared using young, fully expanded leaves previously exposed to full sunlight. The homogenization procedures and the analyses of the gel-filtered extracts for PEP carboxylase, PPDK, NADP-ME, NAD-ME, and NADP-MDH activities were performed according to the methods of Holaday *et al.* (15). Three to four different sexually reproduced plants were sampled for the activity of each enzyme.

CO_2 Exchange Measurements. Young, fully expanded leaves from vegetative plants were used to measure CO_2 exchange. The techniques were essentially the same as described previously (4). All measurements were made at 2000 μmol quanta$\cdot$m$^{-2}\cdot$s^{-1} and 30°C. Two experiments were conducted for measuring AP. In the first experiment, comparisons were made between AP at 0.02 and at 0.21 mol $O_2\cdot$mol^{-1} in order to determine O_2 inhibition. The CO_2 concentration surrounding the leaf was maintained between 300 and 320 μmol$\cdot$mol^{-1}. Three sets of measurements were made for each hybrid, along with three for *F. brownii* and six for *F. pringlei*. In the second experiment, the response of AP to CO_2 concentration was measured at 0.02 and 0.21 mol $O_2\cdot$mol^{-1} for three leaves each of *F. brownii*, *F. pringlei*, and each of the reciprocal F_1 hybrid plants. Measurements were made using five values of pCO_2 (5, 75, 140, 225, 340 μmol$\cdot$mol^{-1}) entering the leaf chamber. Intercellular CO_2 concentrations were calculated as described earlier (4). The initial slope of the AP response to c_i was calculated from the three lowest c_i values, all of which were less than 145 μmol$\cdot$mol^{-1}. Since results of measurements at air levels of CO_2 in the second experiment were similar to those in the first experiment, results for the two experiments were combined. Two separate *F. pringlei* × *F. brownii* hybrids and three reciprocal hybrids were used in these experiments.

Values of Γ were determined on one each of the reciprocal F_1 hybrids along with the parents. Measurements were made in triplicate using an open gas analysis system as described earlier (4).

$^{14}CO_2$ Incorporation Studies. Leaves were exposed to $^{14}CO_2$ in air at either 315 or 45 μmol $CO_2\cdot$mol^{-1}. Exposures were made in a cylindrical, 0.3-L Plexiglas chamber at 28 ± 2°C and 1500 μmol quanta$\cdot$m$^{-2}\cdot$s^{-1}. At the low pCO_2, the chamber was flushed with CO_2-free air for 3 min before the introduction of the $^{14}CO_2$. After 5 or 10 s of photosynthesis, either the leaf was plunged into liquid N_2 or the stoppered end of the chamber was removed and the chamber flushed with 296 or 45 μmol $^{12}CO_2\cdot$mol^{-1}/air at 7 L$\cdot$min^{-1} for 20, 60, or 180 s before plunging the leaf into liquid N_2. Duplicate experiments were performed for each time point using leaves from two different, sexually reproduced plants.

The extraction and identification of labeled compounds was performed essentially as described by Holaday and Chollet (14). However, besides the ethanol/water extractions, three extractions with 5% (v/v) HCOOH in water were employed instead of just one water extraction.

Protoplast Preparation and Isolation. The mesophyll and bundle-sheath protoplasts were prepared from *F. brownii* leaves by a method similar to that described for other dicotyledonous C_4 species (21). Leaf slices, which were 1 mm wide and lacked midribs, were first washed with the resuspension medium (0.5 M sorbitol, 10 mM Hepes [pH 7.0], 10 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1% [w/v] BSA) to be used during the protoplast isolation and then digested at 30°C. The protoplasts were released in a medium at pH 6.0 containing 0.5 M sorbitol, 20 mM MES, 10 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2% (w/v) BSA, 0.5% Macerase pectinase, and 2.0% (w/v) Cellulysin cellulase (Behring Diagnostics). During the digestion, the leaf slices were illuminated at 250 μmol quanta$\cdot$m$^{-2}\cdot$s^{-1}.

After 3 h of digestion, the protoplast suspension was removed with a Pasteur pipette and placed over 2 ml of a 5.0% (w/v) Ficoll/0.5 M sucrose solution in a centrifuge tube. The suspension was centrifuged at 100*g* for 1.5 min, and the protoplasts were removed from a layer on top of the Ficoll solution. This protoplast suspension was diluted with two volumes of the resuspension medium and then placed onto 10 ml of a 4.0% (w/v) Ficoll/0.5 M sucrose solution. Centrifugation at 300*g* for 3 min removed most cell debris and cloroplasts from the protoplast suspension remaining on top of the Ficoll solution. These protoplasts were removed, diluted with two volumes of resuspension medium, and layered onto a sucrose separation medium at pH 7.0 containing 0.6 M sucrose, 10 mM Hepes, 10 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1% (w/v) BSA. Two ml of the 5.0% Ficoll solution were included at the bottom of the centrifuge tube. Centrifugation at 300*g* for 5 min pelleted most of the bundle-sheath protoplasts onto the Ficoll solution, while the mesophyll protoplasts remained on top of the sucrose solution. The chloroplasts from ruptured protoplasts pelleted with the bundle-sheath protoplasts. This fraction was diluted with resuspension medium, and the chloroplasts were removed by layering the fraction onto a 4.0% Ficoll solution and centrifuging at 300*g* for 3 min.

The purity of each fraction was determined by counting 75 to 100 protoplasts and determining the percentage of each type present at ×43 magnification. The bundle-sheath protoplasts were distinguished from the mesophyll protoplasts by their egg-like shape and their cluster of chloroplasts at one end. More than 95% of the protoplasts in the mesophyll fraction were mesophyll protoplasts, and greater than 90% of the protoplasts of the bundle-sheath preparation were bundle-sheath protoplasts.

Both fractions were diluted threefold with resuspension medium and centrifuged at 300*g* for 3 min. The pellets were suspended in the appropriate breaking medium for enzymic analysis. These suspensions were either processed for immediate analysis or frozen in liquid N_2 until they could be analyzed the next day. If the extracts were to be frozen, no Triton X-100 was included until they were thawed and homogenized.

The breaking media were the same as the homogenization

solutions for whole-leaf enzymic analyses (15). For all but RuBP carboxylase, Triton X-100 at a concentration of 0.1% (v/v) was included. Homogenization was performed in a glass homogenizer at 0°C.

Only the extracts to be analyzed for PPDK activity were not frozen in liquid N_2 before homogenization. This enzyme was activated by allowing the extracts to incubate under N_2 at room temperature for 1 h prior to the assay. The extracts to be analyzed for RuBP carboxylase activity were incubated for 20 min at room temperature with 10 mM $NaHCO_3$ present in the breaking medium to activate the enzyme. All enzyme assays were performed as described by Holaday *et al.* (15).

RESULTS AND DISCUSSION

Enzymic Data. We obtained the enzymic activities in the initial report (15) from *F. pringlei* × *F. brownii* plants growing in a greenhouse during the winter and spring months without high-intensity supplemental lighting. Subsequent analyses of plants growing in the summer or in the winter under a long photoperiod sustained by a radiant flux density greater than 1200 μmol quanta·m^{-2}·s^{-1} (400–700 nm) show that the activities of PEP carboxylase and PPDK are greater than those in the initial report (Table I) (15). The largest relative increase in the PEP carboxylase activity as a result of the long period of high light intensity occurs for the F_1 hybrids. However, the largest relative increase in PPDK activity occurs for *F. brownii*. Monson *et al.* (20) mention that the greatest expression of C_4 photosynthesis in *F. brownii* as measured by CO_2 exchange or carbon isotope ratios occurs under summer conditions. Therefore, C_4 photosynthetic activity may be controlled to some extent by its PPDK activity. The longer periods of high light intensity have little effect on the activities of the other enzymes. Thus, some of the activities in Table I are similar to those in the initial report (15), but all of the values are representative of the activities in extracts from plants growing under high light intensities and a long photoperiod.

With the exception of NAD-ME, the activities of the selected C_4 enzymes are similar in the reciprocal F_1 hybrids of *F. pringlei* and *F. brownii* (Table I). Thus, cytoplasmic inheritance factors do not appear to be important in controlling the activities of these enzymes. Since activities of PEP carboxylase, PPDK, and NADP-ME in the reciprocal hybrids are only 7 to 17% of those in *F. brownii*, there appears to be a severe restriction of the expression of the *F. brownii* genes for these enzymes. However, the activities of these enzymes are still similar to those for several C_3-C_4 intermediate *Flaveria* species (4, 18) and even greater than the activities in extracts from *Moricandia* and *Panicum* intermediate species (13, 16, 22). On the other hand, the activity of NADP-MDH for the hybrids is comparable to that for *F. brownii* and is 10-fold greater than that for *F. pringlei*. The complete dominance of the *F. brownii* gene responsible for the activity of this enzyme in the reciprocal hybrids is a striking contrast to the dominance of *F. pringlei* genes for most other characteristics.

Contrary to the similarities between the reciprocal hybrids regarding the activities of the other C_4 enzymes, the activities of the mitochondrial decarboxylase, NAD-ME, in the reciprocal hybrids are not similar to each other, but rather are comparable to the activity for the respective maternal parent (Table I). If most of the mitochondria of each hybrid are inherited from the maternal parent, then our data would suggest a cytoplasmic influence on the inheritance of NAD-ME. In such a situation, the mitochondria could control the inheritance of the enzyme if they were the site of the synthesis of the enzyme. However, a recent study of varieties of *Panicum virgatum* (NAD-ME C_4 species) (29) suggests that the enzyme is nuclearly encoded, since the octoploid variety has 70% more NAD-ME activity on a leaf area basis than does the tetraploid. We cannot ascertain the site of the synthesis from our data. However, if NAD-ME synthesis is under nuclear control in *Flaveria* species, then our results would indicate that the *F. brownii* gene for the enzyme must be essentially fully expressed in *F. brownii* × *F. pringlei* with no hindrance to the enzyme's assembly in the mitochondria, and, in *F. pringlei* × *F. brownii*, some factors must either affect the expression of the *F. brownii* gene or affect the import and assembly of active NAD-ME in the mitochondria, which are most likely inherited from *F. pringlei*. The resolution of this problem requires studies on the site of synthesis of NAD-ME in *Flaveria* species and possibly on the ability of the mitochondria from one species to incorporate and assemble subunits of the enzyme synthesized by nuclear genes from another species.

CO_2 Exchange Analyses. The C_4 CO_2 exchange traits of *F. brownii* are weakly expressed (if at all) in the F_1 hybrids. The values for all of the CO_2 exchange parameters measured at 320 μmol CO_2·mol^{-1} are essentially the same for *F. pringlei* and the reciprocal F_1 hybrids (Table II). The values of AP and the O_2 inhibition of AP for the hybrids are not altered by the presence of *F. brownii* genes. Also, the initial slope of the CO_2 response curve at 0.21 mol O_2·mol^{-1} and values of c_i/c_a at air levels of O_2 and CO_2, both of which should be changed by the inheritance of C_4 photosynthesis, are essentially the same for the hybrid plants and their C_3 parent (Table II). Intermediate *Flaveria* species have rates of AP and c_i/c_a ratios that are similar to those values for C_3 species (4). However, they exhibit lower values of Γ than do the hybrids as well as a reduced O_2 inhibition of AP (4, 18). The only CO_2 exchange values of the hybrids which deviate from those of the C_3 parent are values of Γ (Table II) (15).

The Γ values in Table II for the hybrids determined with an open gas analysis system at 30°C are 5 to 10 μmol CO_2·mol^{-1} higher than those determined with sealed mylar bags at 25°C

Table I. *Activities of Key C_4 Enzymes in Whole-Leaf Extracts from F. Pringlei, F. Brownii, and Their Reciprocal F_1 Hybrids*

The assays were performed on the gel-filtered extracts at 30°C.

	F. pringlei	*F. brownii*	*F. pringlei* × *F. brownii*	*F. brownii* × *F. pringlei*
		μmol·mg Chl^{-1}·h^{-1}		
PEP carboxylase	40 ± 4[a]	1189 ± 85	183 ± 60	208 ± 10
PPDK	14 ± 5	542 ± 103	38 ± 8	44 ± 3
NADP-ME				
(Mg)	7 ± 1	1238 ± 121	68 ± 8	77 ± 14
(Mn)	11 ± 1	1090 ± 58	84 ± 4	97 ± 12
NAD-ME (Mn)	26 ± 3	73 ± 5	32 ± 7	61 ± 11
NADP-MDH	227 ± 28	2375 ± 89	2528 ± 268	2753 ± 132

[a] The values are the means, ± SD, of activities for extracts from three or four sexually reproduced plants.

Table II. *CO_2 Exchange Characteristics of F. Pringlei, F. Brownii, and Their Reciprocal F_1 Hybrids*

Measurements were made at 30°C, 320 μmol $CO_2 \cdot mol^{-1}$, 0.21 mol $O_2 \cdot mol^{-1}$, and 2000 μmol quanta $\cdot m^{-2} \cdot s^{-1}$ unless stated otherwise.

	F. pringlei (9)[a]	*F. brownii* (6)	*F. pringlei* × *F. brownii* (6)	*F. brownii* × *F. pringlei* (12)
AP ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	17.5 ± 2.6	25.8 ± 3.1	21.1 ± 1.9	20.6 ± 3.2
O_2 Inhibition (%)[b]	28.0 ± 1.0	2.5 ± 7.4	26.8 ± 1.3	27.9 ± 2.8
c_i/c_a[c]	0.76 ± 0.05	0.52 ± 0.10	0.75 ± 0.06	0.76 ± 0.05
Initial slope of pCO_2 *versus* AP[d]	0.09 ± 0.02	0.22 ± 0.04	0.11 ± 0.01	0.09 ± 0.03
Γ(μmol $CO_2 \cdot mol^{-1}$)	47.7 ± 1.3	10.0 ± 0.8	40.0 ± 0.4	37.8 ± 0.3

[a] Numbers of leaves measured are shown in parentheses (experiments 1 and 2), except that the initial slopes of the CO_2 response curves of AP (experiment 2) and Γ-values are the means of values for three leaves of each genotype. [b] Inhibition of AP by 0.21 mol $O_2 \cdot mol^{-1}$ compared to 0.02 mol $O_2 \cdot mol^{-1}$. [c] Measured at 0.21 mol $O_2 \cdot mol^{-1}$ and c_a = 300 to 320 μmol $\cdot mol^{-1}$. [d] (μmol $CO_2 \cdot m^{-2} \cdot s^{-1}/\mu mol \cdot mol^{-1}$).

(15). In fact, these values are similar to those for the $C_4 \times C_3$ *Atriplex* hybrid (5), but are still 8 to 11 μmol $\cdot mol^{-1}$ lower than the values for *F. pringlei*. Why the two methods for determining Γ produce somewhat different results primarily just for the hybrids is not readily apparent. However, when considering all of the data for the *Flaveria* hybrids, they exhibit a slightly greater reduction in Γ than do the *Atriplex* hybrids relative to the values for their respective C_3 parent. Nevertheless, based on these CO_2 exchange results, if a C_4 cycle exists in the *Flaveria* hybrids, it must be very limited in activity.

The dominance of the CO_2 exchange by C_3 photosynthesis in the *Flaveria* hybrids is more reminiscent of the C_3-like CO_2 exchange of the *Atriplex* hybrid (5). However, AP for the *Flaveria* F_1 hybrids is equal to or slightly greater than AP for *F. pringlei*, whereas in *Atriplex*, AP of the F_1 plants is lower than that of either parent. The lack of such as reduction in AP in the *Flaveria* hybrids may be due to the dominance of *F. pringlei* genes, which may provide a more efficient, coordinated photosynthetic system than in the *Atriplex* hybrid. Also, the two parental photosynthetic systems of the *Flaveria* species may be more compatible due to a less developed intercellular compartmentation of enzymes in *F. brownii* as compared to other C_4 species (as will be discussed more fully later in the paper in Table V) (10).

$^{14}CO_2$ Incorporation. At 315 μmol $\cdot mol^{-1}$ $^{14}CO_2$ and 0.21 mol $\cdot mol^{-1}$ O_2, the hybrids initially incorporate into C_4 acids only 7 to 9% of the total $^{14}CO_2$ fixed (Table III). These findings are consistent with the CO_2 exchange results obtained at ambient CO_2 concentrations. Since *F. pringlei* only incorporates 3.5% of the total CO_2 into C_4 acids, the hybrids do have a slightly greater *in vivo* PEP carboxylase activity than does their C_3 parent. However, this activity is not nearly as great as reported to occur in certain C_3-C_4 intermediate *Flaveria* species, which can initially incorporate from 20 to 55% of the total assimilated CO_2 into malate and aspartate (19, 27). The *in vivo* activity in the *Atriplex* hybrid can also be quite high with approximately 28 to 30% incorporation into C_4 acids (23). Thus, the *in vivo* PEP carboxylase activity in air for our hybrid plants is more typical of that for *Moricandia* and *Panicum* intermediate species, which do not possess C_4 photosynthesis (14).

If a C_4 cycle does operate in the hybrids, then we should observe a fairly rapid decarboxylation of a major portion of the C_4 acid pool. After a 20-s chase period at 296 μmol $^{12}CO_2 \cdot mol^{-1}$, the percentage of label in the C_4 acids decreases slightly in the hybrids (Table III). Similarly, little change in the percentage of ^{14}C in the C_4 acids occurs in the *Atriplex* hybrid even after a 36-s chase period (23). However, the data which are used to support the proposal that a limited C_4 cycle operates in intermediate *Flaveria* species are from chase periods of 3 to 10 min (19, 27). For our hybrids during a 60-s chase, the percentage of the label in malate decreases from an average of 6.5 to 2% (Table III). Only a small change occurs for the percentage in aspartate. Therefore, the malate pool in the *Flaveria* hybrids does turn over at a reasonable rate, presumably as a result of decarboxylation. We believe that most of this putative decarboxylation occurs via NADP-ME in the chloroplasts, at least in *F. pringlei* × *F. brownii* plants, since it is the most active decarboxylase (Table I) and it appears to be localized in the chloroplasts of both *F. brownii* and *F. pringlei* (AS Holaday, unpublished data). In *F. brownii* × *F. pringlei*, in which more NAD-ME activity occurs (Table I), this mitochondrial enzyme may also be responsible for some malate decarboxylation. Decarboxylation via NADP-ME in the chloroplast would be more advantageous for the plant by favoring reassimilation of the CO_2 by RuBP carboxylase rather than by PEP carboxylase. However, in both reciprocal hybrids, the release of this CO_2 does not appear to substantially affect O_2 fixation by RuBP carboxylase/oxygenase as in C_4 species, since a high proportion of ^{14}C occurs in the photorespiratory metabolites, glycine and serine, during the chase periods (Table III), and O_2 inhibition of AP for the hybrids is the same as that for *F. pringlei* (Table II). This lack of an effect on O_2 fixation is probably due to a rather slow release of small quantities of CO_2 via decarboxylation.

Chastain and Chollet (7, 8) note that labeling studies performed at a pCO_2 near Γ may be useful to ascertain the degree of limited C_4 photosynthesis present in a plant. They find that intermediate species which are thought to have little or no C_4 photosynthesis (*Moricandia arvensis*, *Panicum midioides* [14], and possibly *F. linearis* and *F. anomala* [19]) assimilate more CO_2 via PEP carboxylase at Γ than at ambient concentrations of CO_2 (7, 8, and personal communication). However, there is little turnover of the C_4 acids that they produce. This enhancement of the *in vivo* PEP carboxylase activity by low pCO_2 is proportionately less for *F. floridana* (7), which, like *F. ramosissima*, may possess a limited C_4 cycle (19, 27). Also, some turnover of the C_4 acids occurs at Γ for this species. In contrast, labeling patterns during $^{14}CO_2$-pulse and $^{12}CO_2$-chase experiments for certain C_3 and C_4 species are essentially unaltered by the low pCO_2 (7, 8).

At 45 μmol $CO_2 \cdot mol^{-1}$, the relative amount of CO_2 fixed via PEP carboxylase in the hybrids, increases to an average of 17% but does not change for *F. pringlei* (Table IV). Although the extractable PEP carboxylase activity for the hybrids is about fivefold less than the RuBP carboxylase activity (15), this increase in the *in vivo* activity of PEP carboxylase relative to that for RuBP carboxylase reflects its higher affinity for CO_2 (HCO_3^-). However, the *in vivo* activity is still limited by the amount of the

Table III. *Distribution of ^{14}C in Leaves of F. Pringlei, F. Brownii, and the Reciprocal F_1 Hybrids following 5 s of Photosynthesis in 315 μmol·mol^{-1} $^{14}CO_2$/Air or an Additional 20 or 60 s in 296 μmol·mol^{-1} $^{12}CO_2$/Air*

The incident photon flux density was 1500 μmol·m^{-2}·s^{-1}. The temperature was 28 ± 2°C. PGA, phosphoglyceric acid.

Metabolite	*F. pringlei*		*F. pringlei* × *F. brownii*			*F. brownii* × *F. pringlei*			*F. brownii*	
	5 s ^{14}C	20 s ^{12}C	5s ^{14}C	20 s ^{12}C	60 s ^{12}C	5 s ^{14}C	20 s ^{12}C	60 s ^{12}C	5 s ^{14}C	20 s ^{12}C
					% of ^{14}C fixed					
3-PGA	35.2[a]	20.7	33.2	14.3	9.4	33.8	16.3	7.0	9.6	17.7
Sugar phosphates	44.5	30.9	49.5	30.4	16.2	49.0	28.1	20.0	10.3	25.6
Malate	3.1	2.0	5.4	4.7	1.4	7.5	4.7	2.8	34.6	27.1
Aspartate	0.4	0.3	2.2	1.3	1.4	1.5	0.9	1.1	39.0	16.8
Glycine	3.3	18.0	1.7	21.4	23.5	0.7	15.6	23.9	0.9	6.5
Serine	0.2	4.3	0.1	4.2	10.7	0.1	5.3	13.5	3.7	0.7
Alanine	0.0	0.2	0.0	0.2	0.0	0.0	0.7	0.0	0.0	0.2
Neutrals	1.3	4.9	1.2	7.7	16.8	0.6	5.2	14.9	0.4	0.9
Unidentified	6.3	3.7	4.7	5.1	3.2	2.9	5.3	4.6	0.9	3.6
Water-insoluble	2.8	13.1	1.3	9.2	17.0	4.0	15.2	12.0	1.1	4.2
Total recovered	97.1	98.1	99.3	98.5	99.6	100.1	97.3	99.8	100.1	103.3

[a] The percentages are the means of data from two separate experiments with the exception of those for *F. brownii*, which are the results of one experiment.

Table IV. *Distribution of ^{14}C in Leaves of F. Pringlei, F. Brownii, and their Reciprocal F_1 Hybrids following 10 s of Photosynthesis in 45 μmol·mol^{-1} $^{14}CO_2$/Air or an Additional 60 or 180 s in 45 μmol·mol^{-1} $^{12}CO_2$/Air*

The incident photon flux density was 1500 μmol·m^{-2}·s^{-1}. The temperature was 28 ± 2°C. PGA, phosphoglyceric acid.

Metabolite	*F. pringlei*			*F. pringlei* × *F. brownii*			*F. brownii* × *F. pringlei*		
	10 s ^{14}C	60 s ^{12}C	180 s ^{12}C	10 s ^{14}C	60 s ^{12}C	180 s ^{12}C	10 s ^{14}C	60 s ^{12}C	180 s ^{12}C
					% of ^{14}C fixed				
3-PGA	21.1[a]	7.8	5.4	23.1	6.0	4.6	19.3	5.6	3.0
Sugar phosphates	56.3	25.8	18.2	40.2	19.0	12.0	38.0	21.9	15.3
Malate	3.3	3.5	3.3	11.5	7.9	8.8	11.7	6.6	7.3
Aspartate	0.2	1.2	2.1	4.3	4.3	4.7	6.1	5.8	2.5
Glycine	8.2	15.0	11.2	7.3	19.3	19.1	9.8	21.4	12.9
Serine	1.2	28.8	28.8	1.0	20.2	23.9	1.1	20.0	26.7
Alanine	0.0	0.0	1.0	0.0	0.2	0.9	0.0	0.0	0.9
Neutrals	0.8	2.3	8.3	0.9	3.8	7.2	1.0	4.1	10.8
Unidentified	8.0	6.4	5.0	8.9	4.7	4.2	9.2	5.9	3.8
Water-insoluble	0.4	4.9	17.8	0.9	14.2	14.0	0.7	8.4	16.4
Total recovered	98.7	95.7	101.1	98.1	99.6	99.4	96.9	99.7	99.6

[a] The percentages are the means of data from two separate experiments.

protein present and the cell's capacity to produce PEP via PPDK, factors which are two- to fivefold greater in the hybrids than in *F. pringlei* (Table I) (15).

We would predict that the higher *in vivo* PEP carboxylase activity in the hybrids relative to that in *F. pringlei* at 45 μmol CO_2·mol^{-1} would produce a greater initial slope of AP versus pCO_2, but the initial slopes for the reciprocal hybrids are the same or only slightly greater than that for *F. pringlei* (Table II). Similarly, there is a report that *Panicum* intermediate species exhibit initial slopes of AP *versus* pCO_2 that are typical of those exhibited by C_3 species (3), yet low pCO_2 enhances their PEP carboxylation relative to RuBP carboxylation (8). It may be that these CO_2 exchange measurements are not sensitive enough to detect the small increase in the *in vivo* PEP carboxylase activity at low pCO_2 for these plants.

As at 296 μmol $^{12}CO_2$·mol^{-1}, a notable reduction in the percentage of label in malate occurs during 60-s and 180-s chase periods at 45 μmol $^{12}CO_2$·mol^{-1} for the hybrids, but no change occurs in the percentage in aspartate (Table IV). The hybrids appear to be capable of decarboxylating some of the malate pool at low pCO_2, but a portion of the malate may be unavailable for this process. The maintenance of 7 or 8% of the ^{14}C in malate during the chase periods could also be due to some refixation by PEP carboxylase of $^{14}CO_2$ from malate decarboxylation and photorespiration or could be due to the carboxylation of [^{14}C]PEP, as is thought to occur during long chase periods in intermediate species of *Faveria* (19). Clearly, as at ambient concentrations of CO_2, the amount of CO_2 produced by the apparent decarboxylation of malate is too small to significantly alter photorespiratory metabolism as in C_4 species, since the percentages of label in glycine and serine in the hybrids during the chase periods are not reduced relative to those in *F. pringlei* (Table IV).

Mesophyll and Bundle-Sheath Protoplast Study with *F. Brownii*. We have alluded to the fact that *F. brownii* lacks a strict intercellular segregation of C_3 and C_4 enzymes. This idea is based on recent immunofluorescence studies of the localization of PEP and RuBP carboxylases (26) and protoplast studies by Cheng *et al.* (10). Our concurrent protoplast experiments with *F. brownii* support the findings of these studies (Table V). Although most of the total protoplast PEP carboxylase activity, on a Chl basis, is present in the mesophyll protoplasts of this species (a mixture of large and small protoplasts), 17% is in the bundle-sheath protoplasts as opposed to less than 1% reportedly occurring in the bundle sheath of another C_4 dicot, *Atriplex spongiosa* (21).

Table V. *Activities of Key C_4 and C_3 Enzymes in Mesophyll and Bundle-Sheath Protoplasts and Whole Leaves of F. Brownii*

The assays were performed at 30°C. The whole leaf assays were performed on gel-filtered extracts.

	Mesophyll	Bundle Sheath	Whole Leaf
	$\mu mol \cdot mg\ Chl^{-1} \cdot h^{-1}$		
PEP carboxylase	1309[a]	266	1189
PPDK	276	212	469
NADP-ME	651	1195	1242
RuBP carboxylase	130	344	512

[a] The values are the means of data collected from three to five plants for each enzyme.

Some of the bundle-sheath activity may be due to the presence of a few contaminating small mesophyll protoplasts, but this contamination (less than 10%) cannot account for all of the activity in the bundle sheath preparations. In an analogous situation, even though no RuBP carboxylase can be detected in the mesophyll of other C_4 species (12, 24), in *F. brownii* we find that 27% of the total activity of this enzyme is in the mesophyll protoplast fraction (Table III). Thus, a complete separation of these two carboxylases does not occur in *F. brownii* leaves, and the RuBP carboxylase that we extract from the mesophyll is active *in vitro*. The higher percentage of $^{14}CO_2$ that *F. brownii* initially assimilates into 3-phosphogycerate and sugar phosphates than do C_4 species such as *F. trinervia* (10, 27) (Table III) and the measurable inhibition (as high as 9%) of photosynthesis by 0.21 mol $O_2 \cdot mol^{-1}$ (Table II) (20) indicate the extent of the *in vivo* activity of the mesophyll RuBP carboxylase and its ability to assimilate ambient CO_2 and O_2.

Even less separation with respect to the cell types exists for NADP-ME and PPDK (Table V). Of the total protoplast activity of NADP-ME for *F. brownii*, only 65% is in the bundle-sheath fraction in comparison to 95 to 100% in other C_4 species (12, 21). A high decarboxylation potential must exist in the bundle sheath of C_4 species for the maintenance of a high pCO_2 to inhibit O_2 fixation and photorespiration (11). *Flaveria brownii* lacks sufficient NAD-ME or PEP carboxykinase activity for this purpose (Table I; AS Holaday, unpublished data), but the activity of NADP-ME in the bundle sheath is threefold greater than the RuBP carboxylase activity and should be sufficient to concentrate CO_2 there. Similarly, even though PPDK is essentially evenly distributed between the cell types, its *in vivo* activity in the mesophyll must be sufficient to maintain the C_4-like rates of CO_2 assimilation for *F. brownii* (Table II). However, a question arises as to the extent of malate decarboxylation in the mesophyll and whether the released CO_2 is reassimilated by PEP carboxylase or the small amount of RuBP carboxylase present.

The study by Cheng *et al.* (10) indicates that somewhat less PPDK activity occurs in the bundle sheath than we indicate here. Although we have no explanation for this difference in the data, we do not believe that our results are simply due to contaminating mesophyll protoplasts in the bundle-sheath preparation. The activity of this chloroplastic enzyme on a Chl basis in preparations containing only 60 to 70% bundle-sheath protoplasts is not substantially higher than in our purer preparations (not shown), probably because the bundle-sheath protoplasts contain considerably more chloroplasts than do the mesophyll protoplasts (15).

CONCLUSIONS

The enzymic data for the reciprocal F_1 hybrids of *F. pringlei* and *F. brownii* show that the low activity of several key C_4 enzymes in these plants is not due to cytoplasmic inheritance factors but rather to some partial dominance of C_3 genes over certain C_4 genes. A similar situation is reported for the inheritance of PEP carboxylase in a $C_4 \times C_3$ *Atriplex* F_1 hybrid (23), albeit not to the extent as with the *Flaveria* hybrids. If such dominance regularly occurs when certain genes of C_4 species are in the presence of genes from most C_3 species, it would greatly increase the difficulty in developing a highly active C_4 photosynthesis cycle in C_3 species. It is also important to note that cytoplasmic factors may influence the inheritance and development of active NAD-ME. Understanding these factors would be necessary if C_4 pathways based on this decarboxylase are to be developed in C_3 species.

Rates of AP for the *Atriplex* hybrid are considerably less than those for either parent, which indicates a considerable lack of compatability between its parents for this trait (5). In contrast, the rates of AP for our *Flaveria* hybrids are equal to or somewhat greater than those of their C_3 parent (Table II). Since *F. brownii* may be a recently evolved C_4-like plant which retains some C_3 characteristics, it may be more compatible with non-C_4 species when forming hybrids than would be true for more developed C_4 species. The lack of a complete development of C_4 characteristics in *F. brownii* may also explain why it exerts so little of its C_4 characteristics on its hybrid progeny when crossed with *F. pringlei*. However, hybrids between *F. brownii* and C_3-C_4 intermediate *Flaveria* species have values of Γ and O_2 inhibition of AP that are similar to those values for *F. trinervia* (C_4) $\times$ C_3-C_4 hybrids (RH Brown, J Bouton, unpublished data). One might expect that analyses of hybrids of *F. pringlei* and the classical C_4 species *F. trinervia* would resolve some of these uncertainties. We have formed these hybrids on two occasions, but we have found that they grow slowly and die before flowering. Thus, the parent species do not appear to be very compatible, and photosynthetic data from their hybrids may not be easily interpreted.

The *Flaveria* hybrids may be similar to several naturally occurring C_3-C_4 intermediate *Flaveria* species with respect to their activities of certain key C_4 enzymes (Table I), but unlike these species, they exhibit Γ values which are not greatly reduced relative to those for C_3 species (Table II) (15), and their photosynthesis is inhibited by 0.21 mol $O_2 \cdot mol^{-1}$ to the same degree as in C_3 species (Table II). CO_2 assimilation by the hybrids is most similar to that by *Moricandia* and *Panicum* intermediates (14), yet these intermediate species exhibit lower values of O_2 inhibition of photosynthesis, as do the intermediate *Flaveria* species. Contrary to the situation with the *Moricandia* and *Panicum* intermediates, our hybrids do appear to decarboxylate some malate, but we believe that this process does not influence their photorespiration substantially.

Because PEP carboxylase assimilates two- to fivefold more CO_2 in the hybrids than in *F. pringlei*, depending on the pCO_2 (Tables III and IV), and because a substantial portion of the resulting malate turns over in a relatively short time in the hybrids, we propose that they have inherited some C_4-acid metabolism. However, we stress that this metabolism is very limited in activity because of the low activity of key enzymes and is essentially ineffective in altering photosynthesis and the O_2 effects on photosynthesis from the situation in C_3 species. We conclude that the lower values of Γ for the hybrids between *F. pringlei* and *F. brownii* relative to the Γ values for their C_3 parent may be due largely to the existence in the hybrids of a greater capacity to reassimilate photorespired CO_2 via PEP carboxylase at low pCO_2 than occurs in *F. pringlei*. The higher *in vivo* PEP carboxylase activity in the hybrids may also be responsible for lowering Γ by increasing the direct assimilation of atmospheric CO_2 via this O_2-insensitive enzyme at low pCO_2 and 0.21 mol $O_2 \cdot mol^{-1}$.

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