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Photosynthesis of F_1 Hybrids between C_4 and C_3-C_4 Species of *Flaveria*[1]

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ABSTRACT

Photosynthetic characteristics were studied in several F_1 hybrids between C_4 and C_3-C_4 species of *Flaveria*. Stable carbon isotope ratios, O_2 inhibition of apparent photosynthesis, and phosphoenolpyruvate carboxylase activities in the hybrids were similar to the means for the parents. Values of CO_2 compensation concentrations were nearer to those of the C_4 parent and apparent photosynthesis was below that of both parents, being only 60 and 74% of that of the lowest (C_3-C_4) parent in two experiments. Reductions of CO_2 compensation concentration and O_2 inhibition of apparent photosynthesis as well as increases in carbon isotope ratios and phosphoenolpyruvate carboxylase activities compared to values in C_3-C_4 species suggest transfer of a limited degree of C_4 photosynthesis to the F_1 hybrids. However, the lower apparent photosynthesis of the hybrids suggests that transfer of C_4 characteristics to non-C_4 species is detrimental unless characteristics associated with C_4 photosynthesis are fully developed. There was a highly significant negative correlation ($r = -0.90$) between CO_2 compensation concentration and the logarithm of phosphoenolpyruvate carboxylase activity in the parents and hybrids, suggesting involvement of this enzyme in controlling the CO_2 compensation concentration. Although bundle-sheath cells were more developed in leaves of hybrids than in C_3-C_4 parents, they appeared to contain lower quantities of organelles than those of the C_4 parent. Reduced quantities of organelles in bundle-sheath cells could indicate incomplete compartmentation of partial pathways of the C_4 cycle in the hybrids. This may mean that the reduction of CO_2 compensation and O_2 inhibition of apparent photosynthesis relative to the C_3-C_4 parents is less dependent on fully developed Kranz anatomy than is increased apparent photosynthesis.

To understand how C_4 photosynthesis evolved and how its associated characteristics are inherited it is important to study hybrids between photosynthetic types. C_3 and C_4 species have not been found which are closely enough related to produce highly fertile offspring. But species have been discovered in several genera which have photosynthetic and leaf anatomical characteristics intermediate between C_3 and C_4 species (C_3-C_4) (11, 20, 22). These intermediate characteristics may indicate closer phylogenetic relationships with C_4 species and greater success in hybridization. Because C_3-C_4 species in most cases fix CO_2 exclusively by the C_3 cycle (10, 11, 20, 29), hybrids between C_4 and C_3-C_4 species may be useful in understanding genetic control of characteristics associated with C_3 and C_4 photosynthesis.

Flaveria is a small genus containing C_3, C_4, and a substantial proportion of C_3-C_4 species (11, 20, 26). These C_3-C_4 species, like similar species in *Moricandia* and *Panicum*, have low Γ[2], photorespiration, and inhibition of AP by O_2 compared to C_3 species (11, 17, 20). Hybridization of C_3-C_4 and C_3 *Panicum* species produced F_1 plants with Γ, photorespiration, leaf anatomy, and O_2 inhibition of AP intermediate to those of the parents (7). In C_3-C_4 *Panicum* species CO_2 assimilation in air is apparently entirely by the C_3 cycle (10) so that neither parent exhibited C_4 photosynthesis. Hybridization of C_4 and C_3-C_4 species in *Flaveria* should, however, provide plants for study of genetic control of characters differing in C_3 and C_4 photosynthesis.

Hybrids between photosynthetic types have been made in *Flaveria*, but there has not been much description of their photosynthetic characteristics (8, 9, 12, 16, 26). This paper reports CO_2 exchange, enzymic, anatomical, and carbon isotopic characteristics of F_1 hybrids between C_3-C_4 and C_4 species of *Flaveria*.

MATERIALS AND METHODS

Plant Material and Hybridization. *Experiment 1.* Plants of *Flaveria linearis* Lag. (C_3-C_4) and *F. trinervia* (Spreng.) Mohr (C_4) were grown in a greenhouse from seeds supplied by Dr. Maurice Ku of Washington State University. They were established in 3-L pots filled with a 1:1:1 (by volume) mixture of soil, peat, and Perlite. When in full flower, cross-pollinations were made using emasculated maternal parents isolated in a growth

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[2] Abbreviations: Γ, CO_2 compensation concentration; AP, apparent photosynthesis; PEPC, phosphoenolpyruvate carboxylase; RuBPC, ribulose bisphosphate carboxylase; BSC, bundle sheath cell; c_a, CO_2 concentration external to the leaf; c_i, CO_2 concentration in intercellular spaces.

chamber with a 14/10-h light/dark cycle. *F. linearis* was emasculated daily by removal of all disk florets as they emerged from within the phyllaries, leaving only the male-sterile ray florets. Pollen collected from *F. trinervia* was dusted on the ray florets. Seed were dissected out of the synflorescences 3 weeks after the end of the pollination period. To make the reciprocal cross, *F. trinervia* was emasculated daily by removal of all heads containing emerging disk florets, conspicuous by their bulbous yellow tops. The emasculated flowers were dusted with pollen from *F. linearis*. Synflorescences were harvested 3 weeks after the last pollination, dried, and rubbed to separate seed. Viable seed were isolated on the basis of density (viable seed sank when placed in water). The numbering system for parents used in experiment 2 was not instituted when these hybrids were made, therefore numbers are not given to the parents used in this experiment.

Experiment 2. Five hybrids were made as described above between C_3-C_4 species and *F. trinervia*. They were (a) *F. trinervia* (84-1) × *F. linearis* (84-9), (b) *F. trinervia* (84-1) × *F. linearis* (85-1), (c) *F. trinervia* (84-10) × *F. linearis* (84-2), (d) *F. trinervia* (84-1) × *F. floridana* Johnston (85-7), and (e) *F. trinervia* (84-1) × *F. oppositifolia* (DC.) Rydb. (85-22). In each of these crosses *F. trinervia* was the maternal parent. *F. linearis* plants 84-2 and 84-9 and both *F. trinervia* plants trace back to seeds obtained from Dr. Maurice Ku. Plants of *F. linearis* (85-1), *F. floridana* (85-7), and *F. oppositifolia* (85-22) were collected in Florida by P. T. Evans and R. H. Brown.

Growth of Plants. In both experiments plants were vegetatively propagated and three pots of each parent and hybrid were established. A 1:1:1 mixture of soil, peat, and Perlite was used in 3-L pots. Plants were fertilized three times weeky with Hoagland solution. They were grown in a greenhouse during late spring and summer. In experiment 1, plants were grown during May and June 1985 and measurements were made during the period from June 18 to June 27. Plants for experiment 2 were grown during May, June, and July 1985 and measurements were made from July 15 to August 1. Maximum daily temperatures during growth of the plants were 32 to 36°C and night temperatures dropped to approximately 20 to 22°C. Solar radiation was supplemented by multivapor lamps because the greenhouse was covered with shade fabric to reduce radiation for growth of other plants. Maximum daily photosynthetic photon flux density was at least 1.5 mmol quanta m^{-2} s^{-1}.

Gas Exchange Measurements. Measurements of AP and transpiration were made in acrylic plastic chambers 13 cm long, 7 cm wide, and 7.5 cm deep. The chambers had a removable top which was held in place by bolts and wing nuts. Soft closed-cell plastic gaskets provided an effective seal when leaves were enclosed. The chamber was separated into two compartments by an acrylic plastic partition which had a 2.5 cm diameter hole in each end. The upper compartment was 1 cm deep and the lower was 6.2 cm deep. A fan and a cooling coil were installed in the bottom compartment. Air was circulated through the coil, through the hole in the opposite end of the partition, and past the leaf enclosed in the upper compartment. A fine wire thermocouple was pressed against the lower side of the enclosed leaf. Four similar chambers were arranged in a circle under a 1500 W multivapor lamp and a switching system was used to monitor chambers for both exhaust gas and temperature for sequential 5-min periods. Thus, four leaves could be measured in one run.

Gases supplied to the chamber were from cylinders containing mixtures prepared in advance. In each set of measurements leaves were exposed to the following gas mixtures: experiment 1: (a) 325 μl L^{-1} CO_2, 210 ml L^{-1} O_2; (b) 331 μl L^{-1} CO_2, 20 ml L^{-1} O_2; and (c) 3 μl L^{-1} CO_2, 210 ml L^{-1} O_2; Experiment 2: (a) 340 μl L^{-1} CO_2, 210 ml L^{-1} O_2; (b) 345 μl L^{-1} CO_2, 20 ml L^{-1} O_2; and (c) 5 μl L^{-1} CO_2, 210 ml L^{-1} O_2. In each case the balance of the mixture was N_2. Gases were humidified to a dewpoint of 11 to 13°C (constant to within 0.3°C for a given run). Transpiration raised the dewpoint in the chamber to 18 to 24°C. Water vapor was measured with a chilled mirror dewpoint hygrometer, and CO_2 differential in intake and exhaust gas with an IR gas analyzer. Water vapor and CO_2 exchange were calculated from flow rates and concentration differentials in intake and exhaust air. Since air was well stirred in the leaf chamber, c_a was taken as the CO_2 concentration in exhaust air. Intercellular CO_2 concentration, (c_i) was calculated from the equation, $c_i = c_a - AP/g_l$, where g_l is leaf conductance to CO_2 (stomatal and boundary layer). Leaf temperature was maintained at 30°C and incident irradiance was 2 mmol quanta m^{-2} s^{-1} (400–700 nm).

In experiment 1, the youngest fully expanded leaves of the four *Flaveria* plants were monitored simultaneously and measurements were made over a 2- to 3-h period beginning at noon. Replicate measurements were made on separate plants on three different days. On a separate day the same measurements were made on alfalfa (*Medicago sativa* L) leaves. In experiment 2, 12 plants were included and only four leaves could be measured each day. A scheme was set up so that in each run, each hybrid would be paired with at least one of its parents and both parents would be in the same run as their progeny at least once. One replication of measurements was made per week for 3 consecutive weeks.

After measurements of AP and transpiration were finished, Γ was measured on other leaves of each plant similar in age and position. Measurements were made at 30°C and an irradiance of 2 mmol quanta m^{-2} s^{-1} (400–700 nm) by enclosing leaves in a water-jacketed acrylic plastic chamber (10 cm long × 2 cm wide × 1 cm deep) and adjusting the mixture of two gases (one containing zero CO_2 and the other 116 μl L^{-1} CO_2; both with 210 ml L^{-1} O_2, balance N_2) fed into the chamber until the IR gas analyzer indicated no net CO_2 exchange in the chamber. This was done by calibrating the IR gas analyzer for 0 to 100 μl L^{-1} CO_2 and, with N_2 passing through the reference cell, alternately passing gas from the intake and exhaust of the leaf chamber through the sample cell until adjustment of the mixture produced similar readings on the analyzer.

PEPC Analysis. We chose to measure PEPC activity since, of the whole suite of enzymes commonly compared in C_3 and C_4 plants, it was expected that PEPC was likely to exhibit the most difference among genotypes and to be most closely related to CO_2 exchange characteristics such as Γ (2, 14). For PEPC activity measurements (Tables I and II), leaves opposite to those used for AP were selected and analyzed on the day following AP measurements.

Preparation of Crude Extracts. Leaf tissue was ground in washed, ignited sand and grinding buffer at a ratio of 1.0 g fresh weight leaf:0.2 g sand:2.0 ml buffer for 2 min at 4°C. The grinding buffer contained 100 mM Tris (pH 8.0) at 22°C, 20 mM $MgCl_2$, 5 mM DTT, 20 mg ml^{-1} PVP-40 (Sigma), 0.15 ml ml^{-1} glycerol, 50 μM leupeptin (Sigma), 7 mM diethyldithiocarbamic acid (Sigma), and 10 μM phenylmethylsulfonyl fluoride (Sigma). The crude extract was centrifuged at 40,000*g* for 15 min at 4°C and the supernatant was assayed for PEPC activity.

Enzyme Activity. Activity of PEPC [EC 4.1.1.31] in crude extracts was assayed by the method of Uedan and Sugiyama (30). The assay buffer contained 100 mM Tris (pH 8.0) at 22°C, 10 mM $MgCl_2$, 5 mM DTT, 0.2 mM NADH (Sigma), 10 mM $NaHCO_3$, 7.8 units/ml malic dehydrogenase (pigeon breast muscle, Sigma), and 5.0 mM PEP in a final volume of 1.0 ml. Stock solutions of NADH, $NaHCO_3$, and PEP were prepared fresh before each assay. Aliquots of extract, $NaHCO_3$ and NADH were added to the assay mixture just prior to spectrophotometric analysis. The reaction was initiated by the addition of PEP, and PEPC activity was assayed by following the decrease in A_{340}. When appropriate, measurements were corrected for endogenous

activity in the absence of substrate. To determine if any extracts contained PEPC inhibitors, samples of *F. trinervia* were assayed for PEPC activity after dilution with an equal volume of assay buffer or undiluted extract from each C_3-C_4 and hybrid sample. A decrease in enzyme activity compared to *F. trinervia* plus assay buffer would have suggested the presence of PEPC inhibitors in the C_3-C_4 or hybrid samples. No inhibition was observed.

Chl Analysis. Chl measurements were made on 50 mg fresh weight of midleaf tissue from each leaf sampled for PEPC activity. The tissue was cut into small strips (1–2 × 10 mm), and extracted twice in 1.0 ml *N,N*-dimethylformamide overnight at room temperature (21). Extracts were stored at 4°C in the dark until assayed. The amount of Chl (total) was determined according to Inskeep and Bloom (13).

Protein Separation. Soluble proteins from the C_3-C_4 and C_4 parents and their F_1 hybrids were precipitated by the addition of TCA (0.125 g ml^{-1}, final concentration) to extracts prepared for enzyme assays. After centrifugation the pellet was washed once in acetone. Samples, mol wt standards (BioRad, 14,000–93,000 mol wt) and partially purified maize PEPC (Sigma) were prepared and electrophoresed according to Laemmli (18). Gels were stained using the $AgNO_3$ procedure of Oakley *et al.* (24).

Carbon Isotope Analysis. The same leaves from each parent and hybrid used for AP measurements were dried and ground to a fine powder. Ten mg of each sample were loaded into VYCOR ampulae with 1 g of CuO, 1 g of copper, and a small piece of silver foil and then sealed under vacuum. Ampulae were heated at 800°C for 3 h. After being cooled to room temperature they were opened *in vacuo* and the CO_2 was cryogenically purified and its $^{13}C/^{12}C$ ratio determined mass spectrometrically. Isotope ratios are expressed as $\delta^{13}C$ values, where

$$\delta^{13}C = \left[\frac{R \text{ sample}}{R \text{ standard}} - 1\right] \times 10^3‰$$

and R is the $^{13}C/^{12}C$ ratio. The standard used was Peedee belemnite (PDB) carbonate. The precision of isotopic analysis was ±0.2‰.

RESULTS

Apparent Photosynthesis and Γ. AP of the C_3-C_4 species was lower than that of *F. trinervia*, being only 63% as high for *F. linearis* in experiment 1 and averaging 83% as high for the three species in experiment 2 (Tables I and II). The inhibition of AP by 210 ml L^{-1} O_2 was about 20% in the C_3-C_4 species and near zero in *F. trinervia*. The mean O_2 inhibition of AP in F_1 hybrids (12.8%) was similar to the mean for the parents (11.1%). In *F. linearis* 84-9, there was a negative inhibition or a stimulation of about 15% by 210 ml L^{-1} O_2. This O_2 stimulation of AP has been observed repeatedly and its dependence on irradiance and CO_2 concentration has been described (6). For the F_1 hybrids, O_2 inhibition was about midway between the value for the parents (except *F. linearis* 84-9). Although in *F. linearis* 84-9 AP was stimulated by air levels of O_2, in the 84-1 × 84-9 hybrid AP was inhibited 12%, similar to the other hybrids. In alfalfa, the O_2 inhibition of AP was typical of that for C_3 species. Whereas the C_3-C_4 species evolved small amounts of CO_2 in the light (≈0.5 μmol m^{-2} s^{-1}), typical of reports for other C_3-C_4 species (7, 22), neither the hybrids nor the C_4 parent lost appreciable amounts (data not shown). In contrast, alfalfa lost an amount equivalent to 18% of AP at 210 ml L^{-1} O_2 (experiment 1). The Γ-values for the C_3-C_4 species ranged from 14 to 21 μl L^{-1}, but ranged only from 2.8 to 5.0 μl L^{-1} in the C_4 parents and from 4.6 to 10 μl L^{-1} in the F_1 hybrids. Values of Γ were higher in experiment 2 than in experiment 1. Alfalfa had a Γ of 55 ± 1 in both experiments, typical of C_3 plants.

PEPC Activity. Activity of PEPC in both experiments was over 10-fold higher in the C_4 than the C_3-C_4 species (Tables I and II). Although PEPC in the C_3-C_4 plants in experiment 2 ranged from 59 to 188 μmol mg Chl^{-1} h^{-1} the differences were not associated with species since the extreme values were observed in two accessions of *F. linearis*. The F_1 hybrids were intermediate between the parents in PEPC activity, possessing on average only 36% as much as the C_4 parent in experiment 1, and 49% as much in experiment 2. The hybrids in experiment 2 had activities ranging from 323 to 937 μmol mg Chl^{-1} h^{-1}, only one of which (84-1 × 85-7) approached the PEPC activities of the C_4 parent. Alfalfa had PEPC activity only one-half as high as the C_3-C_4 *Flaveria* species in experiment 1 but only slightly less in experiment 2.

Electrophoresis of Soluble Proteins. Figure 1 shows representative profiles of polypeptides extracted from the C_3-C_4 and C_4 parents and their hybrids (*F. linearis* 84-10 [lane 1], *F. trinervia* 84-2 [lane 3], and their F_1 hybrid 84-2 × 84-10 [lane 2]). A densely stained band (approximately 100 kD) corresponding to PEPC was observed in extracts from *F. trinervia*, but considerably reduced in *F. linearis* extracts by either Coomassie brilliant blue or silver staining. The relative amount of PEPC in all F_1 hybrids examined was intermediate between that of the parents. Furthermore, in a reciprocal cross between *F. trinervia* and *F. linearis* (Table I) no difference in the relative amounts of PEPC between the two hybrids was evident (data not shown).

Carbon Isotope Ratios. The $\delta^{13}C$ values of the F_1 hybrids were about midway between those observed for the parental types in both experiments. The $\delta^{13}C$ values in experiment 2 were higher than experiment 1 possibly due to differences in ^{13}C content of the CO_2 in the greenhouse during the two growth periods.

Leaf Anatomy. Leaf cross-sections of *F. trinervia* exhibited the

Table I. *Apparent Photosynthesis, O_2 Inhibition of AP, c_i, Γ, PEPC Activity and $\delta^{13}C$ Values for F. trinervia, F. linearis, Their Reciprocal F_1 Hybrids and Alfalfa in Experiment 1*

Genotype	AP[a]	O_2 inhibition[a]	c_i[b]	Γ[c]	PEPC Activity	$\delta^{13}C$
	μmol m^{-2} s^{-1}	*%*	*%*	*μl L^{-1}*	*μmol mg chl^{-1} h^{-1}*	*‰*
F. trinervia (C_4)	39 ± 3	−0.4 ± 2.6	24	2.8 ± 0.5	3362 ± 307	−20.4 ± 0.2
F. linearis (C_3-C_4)	24 ± 3	21 ± 1	73	14 ± 2	280 ± 65	−33.5 ± 1.6
F_1 (*F. trinervia* × *F. linearis*)	15 ± 1	12 ± 1	73	4.7 ± 1.2	1304 ± 335	−27.9 ± 1.3
F_1 (*F. linearis* × *F. trinervia*)	14 ± 1	17 ± 0.5	78	4.5 ± 0.9	1142 ± 187	−28.4 ± 0.5
Alfalfa (C_3)	13 ± 3	31 ± 0.5	67	55 ± 1	150 ± 79	−30.4[d]

[a] Determined at 30°C, 2 mmol quanta and at c_a from 290 to 305 μl L^{-1}. O_2 inhibition is the reduction of AP at 210 ml L^{-1} O_2 compared to 20 ml L^{-1} O_2, expressed as a % of AP at 20 ml L^{-1} O_2. [b] c_i expressed as a percentage of c_a. [c] Determined at 30°C, 2 mmol quanta m^{-2} s^{-1} and 210 ml L^{-1} O_2. [d] Only one composite sample analyzed.

Table II. *Apparent Photosynthesis, O_2 Inhibition of AP, c_i, Γ, PEPC Activity and δ^{13} Values for F. trinervia and Three C_3-C_4 Species and F_1 Hybrids between the Photosynthetic Types, and Γ and PEPC Activity for Alfalfa in Experiment 2*

Genotype	AP[a]	O_2 inhibition[a]	c_i[b]	Γ[a]	PEPC Activity	$\delta^{13}C$
	$\mu mol\ m^{-2}\ s^{-1}$	%	%	$\mu l\ L^{-1}$	$\mu mol\ mg\ chl^{-1}\ h^{-1}$	‰
F. trinervia 84-1 (C_4)	30 ± 3	0.1 ± 2	45	5.2 ± 0.0	1313 ± 79	−16.8 ± 0.2
F. trinervia 84-10 (C_4)	27 ± 4	2.1 ± 0.2	55	5.2 ± 0.1	1125 ± 162	−16.7 ± 0.3
F. linearis 84-2 (C_3-C_4)	24 ± 4	24 ± 0.1	67	14 ± 2	59 ± 12	−31.3 ± 0.6
F. linearis 84-9 (C_3-C_4)	19 ± 3	−15 ± 5	63	21 ± 3	70 ± 3	−30.3 ± 1.1
F. linearis 85-1 (C_3-C_4)	22 ± 3	22 ± 2	70	20 ± 1	188 ± 26	−31.2 ± 0.7
F. floridana 85-7 (C_3-C_4)	24 ± 2	21 ± 1	72	16 ± 1	122 ± 11	−30.6 ± 0.6
F. oppositifolia 85-22 (C_3-C_4)	25 ± 2	20 ± 3	76	17 ± 0.4	54 ± 16	−32.2 ± 0.3
84-10 × 84-2	16 ± 1	12 ± 3	65	9.3 ± 0.9	323 ± 32	−25.2 ± 1.0
84-1 × 84-9	20 ± 1	12 ± 3	68	7.4 ± 1.8	659 ± 43	−23.3 ± 1.1
84-1 × 85-1	16 ± 5	14 ± 2	76	8.1 ± 0.6	616 ± 50	−25.3 ± 0.6
84-1 × 85-7	17 ± 4	13 ± 1	75	6.9 ± 0.1	937 ± 172	−24.8 ± 2.0
84-1 × 85-22	17 ± 3	10 ± 0.2	72	10 ± 1	473 ± 75	−24.6 ± 2.2
Alfalfa (C_3)	[c]	[c]	[c]	55 ± 1	72 ± 1	[c]
Means						
C_4	28	1	50	5	1219	−16.7
C_3-C_4	23	22[d]	70	18	99	−31.1
C_4 × C_3-C_4	17	12	71	8	602	−24.6

[a] Measurement conditions the same as for Table I. [b] c_i is expressed as a % of c_a. [c] Not determined. [d] This mean does not include data for *F. linearis* (84-9).

typical Kranz anatomy of C_4 species (Fig. 2A). The C_3-C_4 species examined had very little development of BSC and no conspicuous concentration of organelles in them as illustrated for *F. linearis* 84-2 (Fig. 2C). In some accessions of the C_3-C_4 species a well developed palisade parenchyma occurred; in others it was less apparent. The leaf anatomy of F_1 hybrids was between those of the parents, with considerable development of BSC but with low apparent organelle concentrations in BSC (Fig. 2B).

DISCUSSION

Confirmation of hybridization in the group of plants described here was made from several characteristics. All flowers that were pollinated had been previously emasculated. Upon examination, most characteristics of the F_1 hybrids were between those of the parents. The capitulescences of the C_3-C_4 species which we used are flat-topped corymbose terminal panicles, while those of *F. trinervia* are glomerule-like, axillary, and sessile (26). The hybrids had mostly terminal panicles, but much reduced compared to the C_3-C_4 species. Leaves in the hybrids were more elongate and narrow than in *F. trinervia* but shorter and wider than in the C_3-C_4 parents. For O_2 inhibition of AP, Γ, PEPC activity, and $\delta^{13}C$ values, the F_1 hybrids were intermediate and showed no overlap of values with those of the parents, except for O_2 inhibition in 84-1 × 84-9, which had a value higher than both parents (Table II). The F_1 hybrids also had low pollen stainability compared to fully stainable pollen in both parents (data not presented). All of these characteristics taken together leave no doubt that the hybrids were authentic.

Values of O_2 inhibition of AP, carbon isotope ratios and PEPC subunits and activity roughly midway between those of the parents, and of Γ closer to the C_4 parent, indicate a transfer of characteristics to the F_1 hybrids, perhaps sufficient to support a degree of C_4 photosynthesis. Cheng *et al.* (9) also reported O_2 inhibition of AP to be 10 to 15% in hybrids between C_4 and C_3-C_4 species in *Flaveria* compared to 21% inhibition in the C_3-C_4 parent. These results suggest a greater degree of C_4 photosynthesis than in the C_4 × C_3 F_1 hybrid in *Atriplex* (2, 3). In the *Atriplex* hybrid, although $\delta^{13}C$ values were between those of the parents (25), Γ was ony slightly lower than in the C_3 parent and O_2 inhibition of AP was equal to or higher than in the C_3 parent (3). In a hybrid between the C_4 species *F. brownii* and the C_3 species *F. pringlei*, Holaday et al. (12) found that although Γ of the F_1 hybrid (31 $\mu l\ L^{-1}$) was similar to the midparent mean (29.5 $\mu l\ L^{-1}$), enzyme activities of the hybrid approached those of one parent or the other. The activity of PEPC, for example, was 33 and 1449 $\mu mol\ mg^{-1}$ Chl h^{-1}, respectively, in the C_3 and C_4 parents, but only 121 $\mu mol\ mg^{-1}$ Chl h^{-1} in the hybrid. In contrast, PEPC activities of F_1 hybrids between C_4 and C_3-C_4 *Flaveria* species in our study were clearly intermediate to those of the parents, with the exception of 84-1 × 85-7 which approached that of the C_4 parent (Table II). The relative levels of PEPC subunits in soluble protein extracts from these plants were also intermediate to the parents (Fig. 1) (RG Cameron, CL Bassett, unpublished data).

Perhaps the greater transfer of C_4 traits in our *Flaveria* hybrids than in C_3 × C_4 hybrids of *Atriplex* (3, 25) or *Flaveria* (12) is due to the use of C_3-C_4 parents in which some features were already different from C_3. Ku *et al.* (16) reported that *F. linearis* exhibited higher activities of PEPC and pyruvate-P_i-dikinase than the C_3-C_4 species *Panicum milioides* and other C_3-C_4 species in *Flaveria* show even more C_4 tendencies than *F. linearis* (11, 17, 20). But as in the *Atriplex* hybrids, partial incorporation of C_4 characteristics did not confer an advantage in AP, the rate being (equal in the 84-1 × 84-9 hybrid) lower than in both parents (Tables I and II). In experiments 1 and 2 average AP of the hybrids was only 60 and 74% of the C_3-C_4 parents, respectively. In the *Atriplex* F_1 hybrid AP was only 55% of the C_3 parent (3). It is not clear why incorporation of C_4 traits has a detrimental effect on AP. Interspecific hybridization *per se* probably does not cause the reduction, since in three hybrids between C_3, and C_3-C_4 *Panicum* species, which apparently possess no C_4 photosynthesis, AP was as high or higher than the C_3 parent (7).

It may be that a mixture of C_3 and C_4 cycle enzymes in the mesophyll of hybrids is less efficient than the C_3 cycle alone. Cheng *et al.* (8) found that although there was a tendency for enzymes associated with mesophyll and BSC in C_4 plants to concentrate in those cell types in C_3-C_4 × C_4 *Flaveria* hybrids, compartmentation was far from complete. Incomplete compartmentation may result in a futile cycle in which a portion of the CO_2 is fixed by PEPC, C_4 acids are decarboxylated, and resulting CO_2 is refixed by PEPC or RuBPC. Such cycling without compartmentation of appropriate portions of the cycle and the asso-

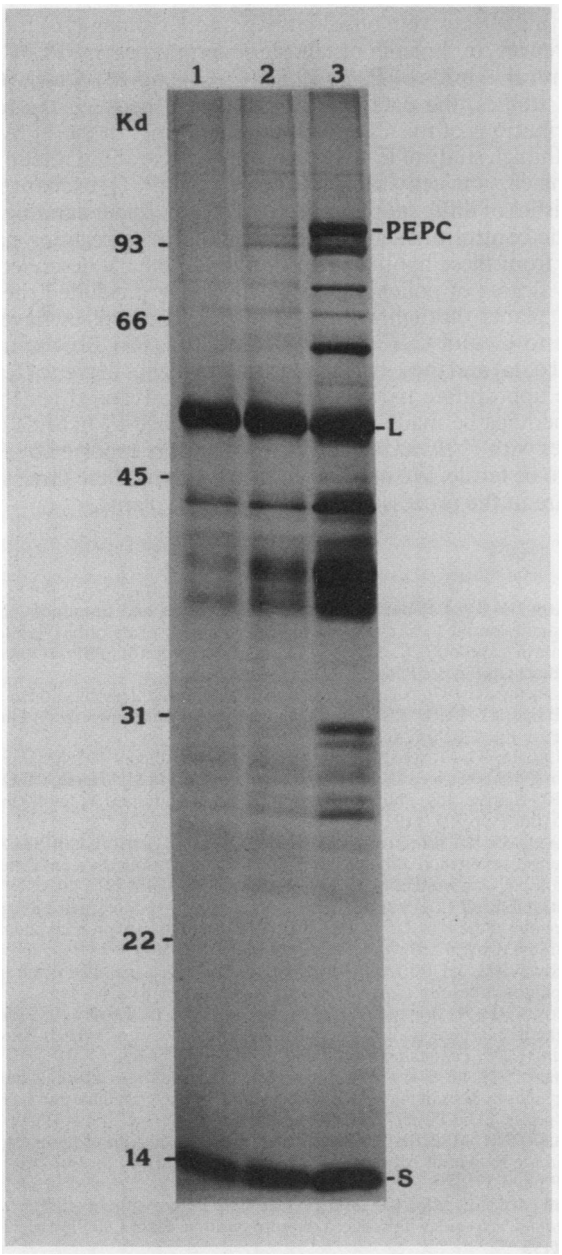

FIG. 1. SDS-PAGE of soluble proteins from *F. trinervia* 84-10, *F. linearis* 84-2 and their F_1 hybrid. Fifty μg of protein were loaded per well, and the polypeptides separated in a 12.5% polyacrylamide gel at 30 mamp for 5 h at room temperature. Individual polypeptides were visualized by silver staining. Lane 1, *F. linearis* (84-2); lane 2, F_1 hybrid (84-10 × 84-2); lane 3, *F. trinervia* (84-10). The migration of mol wt standards is indicated on the left and that of maize PEPC (Sigma) on the right. Identification of *Flaveria* PEPC was based on electroblotting and immunodetection (4, 15) with polyclonal rabbit antibody raised against *Pennisetum americanum* (C_4) PEPC (28). Large (L) and small (S) subunits of RuBPC were tentatively identified according to reported mol wt of about 56 and 14 kD, respectively (19).

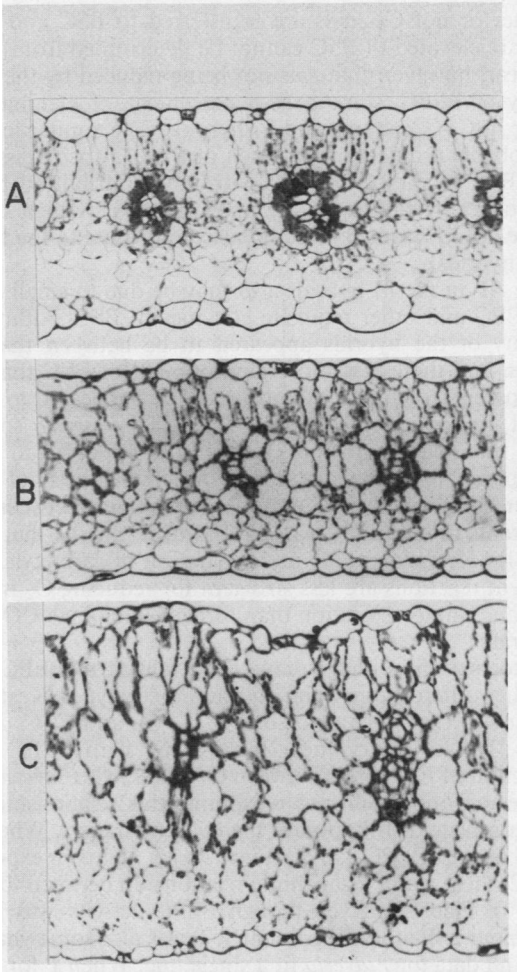

FIG. 2. Light micrographs of leaf cross-sections of (A) *F. trinervia* 84-10 (C_4), (C) *F. linearis* 84-2 (C_3-C_4), and (B) their F_1 hybrid 84-10 × 84-2. ×185.

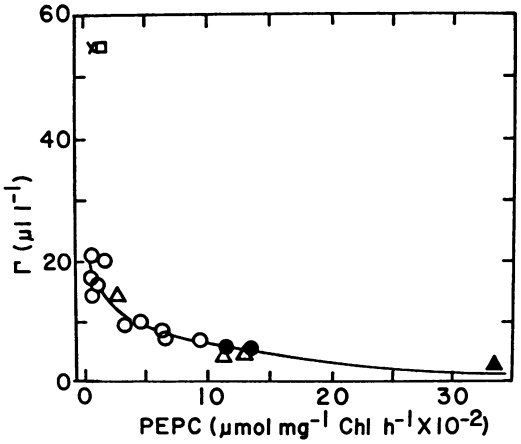

FIG. 3. Relationship between Γ and PEPC activity for C_4 and C_3-C_4 *Flaveria* species, their F_1 hybrids, and alfalfa. Experiment 1, triangles (except alfalfa—square); experiment 2, circles (except alfalfa—X). Solid symbols represent C_4 parents. The regression line (alfalfa omitted) was drawn from the equation, $\Gamma = 36.7 - 10.0 \log$ PEPC activity ($r = -0.90$).

ciated concentration of CO_2 in BSC would reduce the capacity for AP. In the *Atriplex* hybrids, however, a futile cycle is not indicated since a substantial proportion of fixed carbon appeared in C_4 acids (25–30%) and turnover of these C_4 acids was slow (25). A lack of complete compartmentation of carboxylases has been demonstrated by immunofluorescence techniques in *F. brownii* (1, 27), a species which exhibits rather typical C_4 characteristics (11, 17, 20). Thus, the presence of both carboxylase proteins in the mesophyll does not prevent C_4 photosynthesis.

The intermediate values of $\delta^{13}C$ and O_2 inhibition of AP in these *Flaveria* hybrids (Tables I and II) indicate that some of the carbon fixed by PEPC is being assimilated and not released.

Whether or not C_4 acids are transferred to BSC and the CO_2 levels are elevated in BSC cannot be determined from the data. It is clear, however, that c_i is not being reduced by the elevated activity of PEPC, since c_i as a percentage of external CO_2 is similar in C_3-C_4 parents and F_1 hybrids. The similar levels of c_i are probably a reflection of much of the CO_2 being assimilated by RuBPC in mesophyll cells with any contribution by PEPC being offset by the lower AP in F_1 hybrids. A high degree of leakage of CO_2 from BSC could also contribute to the high c_i in the F_1 hybrids.

Low AP in the F_1 hybrids also may be due to small amounts of RuBPC and other requisite enzymes in BSC. Although leaf anatomy of the hybrids appeared to be between that of the parents, and there was more development of BSC than in the C_3-C_4 parents, organelle quantities in BSC appeared to be quite limited. In the hybrid between C_3 and C_4 *Flaveria* species reported by Holaday *et al.* (12), there also appeared to be weak development of Kranz anatomy. However, AP was not reported for that hybrid. Since the only parameters not between that of the parents in our study was AP and c_i, it may be that requirements for Kranz anatomy and separation of carboxylation and attendent reactions are less stringent for reducing O_2 inhibition of AP and photorespiration than for achieving high CO_2 assimilation rates.

Photorespiration and Γ have been related to PEPC activity and other differences among photosynthetic types. Kestler *et al.* (14) showed that in comparisons of C_3, C_4, and C_3-C_4 *Panicum* species, Γ had a curvilinear relationship with PEPC activity. Production of F_1 hybrids between C_4 and C_3-C_4 *Flaveria* species provides an opportunity to reexamine this relationship with a more complete distribution of the two parameters. When Γ was plotted against PEPC activity for *Flaveria* plants in experiments 1 and 2, there was a logarithmic relationship between the two as shown in Figure 3. Even though PEPC activity was lower in experiment 2 than in experiment 1, Γ was also somewhat higher so that all data for *Flaveria* fit a single line. When Γ for *Flaveria* was regressed on the logarithm of PEPC activity, the correlation coefficient was −0.90. The reasons for lower PEPC activity in experiment 2 are not known, but the corresponding higher Γ in experiment 2 and the high degree of correlation for all of the data indicate that the two parameters are closely linked in *Flaveria*. However, differences in Γ between C_3, and C_3-C_4 species do not appear to be related to levels of PEPC. Γ values for alfalfa lie well above the line relating this parameter to PEPC in *Flaveria* (Fig. 3). The fact that Γ is much higher in alfalfa than in C_3-C_4 *Flaveria* species that have nearly equal $\delta^{13}C$ values (Table I), suggests that fixation of CO_2 through PEPC may not be involved in reduced Γ of these C_3-C_4 species. A similar conclusion has been drawn by Edwards *et al.* (10) from $^{14}CO_2$ labelling studies and may be inferred from a report of similar $\delta^{13}C$ values for C_3 and C_3-C_4 *Panicum* species and their F_1 hybrids with Γ values ranging from about 8 to 60 $\mu l\ L^{-1}$ (29).

A role for PEPC in photosynthesis of the *Flaveria* hybrids is, however, suggested by the high activities of the enzyme, the low Γ, reduced O_2 inhibition, and intermediate $\delta^{13}C$ values. Although it is likely that the C_4 form of the enzyme predominates in the hybrids, the relationships between the existing forms is unknown. Nakamoto *et al.* (23) have shown that K_m (PEP)-values for PEPC isolated from *Flaveria cronquistii* (C_3) and *F. trinervia* (C_4) differ considerably, suggesting that the two enzymes are biochemically distinct. Furthermore, K_m values for *F. linearis* (C_3-C_4) PEPC were reported to be intermediate between those of the C_3 and C_4 species. Unfortunately, the genetic relationships between these enzymes have not been clearly established. In C_4 plants PEPC is considered to be the product of a nuclear gene (5), and the activity data and polypeptide profiles of hybrids from reciprocal crosses presented here support this hypothesis, since no apparent maternal effects were observed. Ku and Krishnan (16) found no differences in kinetic or allosteric properties of PEPC from reciprocal hybrids of *F. floridana* (C_3-C_4) and *F. brownii* (C_4). In these studies, the data do not distinguish between the relative contributions of the C_4 and C_3-C_4 isoenzymes in the F_1 hybrids.

Although study of F_1 hybrids is informative about relationships among enzyme activities, leaf anatomy, and CO_2 exchange characteristics of different photosynthetic types, understanding of the genetic control of such parameters requires segregating populations from these hybrids. The F_1 plants we have described have a low degree of pollen viability which may preclude generation of F_2 plants through selfing. However, backcrosses have been made to C_4 and C_3-C_4 plants (JH Bouton, RH Brown, unpublished data) and these backcrosses have a higher degree of fertility. More appropriate hybrids for producing advanced generations can perhaps be made using *F. brownii*, since F_1 hybrids of this species with *F. floridana* and *F. linearis* were reported by Powell (26) to be fertile. We have made hybrids using these three species and are in the process of evaluating the F_1 plants.

LITERATURE CITED

1. BAUWE H 1984 Photosynthetic enzyme activities and immunofluorescence studies on the localization of ribulose-1,5-bisphosphate carboxylase/oxygenase in leaves of C_3, C_4 and C_3-C_4 intermediate species of *Flaveria* (*Asteraccae*). Biochem Physiol Pflanzen 179: 253–268
2. BJÖRKMAN O 1976 Adaptive and genetic aspects of C_4 photosynthesis. *In* RH Burris, CC Black, eds, CO_2 Metabolism and Plant Productivity. University Park Press, Baltimore, pp 287–309
3. BJÖRKMAN O, M NOBS, R PEARCY, J BOYNTON, J BERRY 1971 Characteristics of hybrids between C_3 and C_4 species of *Atriplex*. *In* MD Hatch, CB Osmond, RO Slatyer, eds, Photosynthesis and Photorespiration. Wiley-Interscience, Sydney, pp 105–119
4. BLAKE MS, KA JOHNSTON, GJ RUSSELL-JONES, EC GOTSCHLICH 1984 A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots. Anal Biochem 136: 174–179
5. BROGLIE R, G CORUZZI, B KEITH, N-H CHUA 1984 Molecular biology of C_4 photosynthesis in *Zea mays*: Differential localization of proteins and mRNAs in the two leaf cell types. Plant Mol Biol 3: 431–444
6. BROWN RH, JH BOUTON, PT EVANS 1986 Oxygen stimulation of apparent photosynthesis in *Flaveria linearis*. Plant Physiol. 81: 212–215
7. BROWN RH, JH BOUTON, PT EVANS, HE MALTER, LL RIGSBY 1985 Photosynthesis, morphology, leaf anatomy, and cytogenetics of hybrids between C_3 and C_3/C_4 *Panicum* species. Plant Physiol 77: 653–658
8. CHENG S-H, MSB KU 1985 Intercellular localization of key enzymes of C_4 photosynthesis in the F_1 hybrids between C_3-C_4 intermediate and C_4 *Flaveria* species. Plant Physiol 77: S-90
9. CHENG S-H, MSB KU, LJ METS 1984 Further reduction of photorespiration in the F_1 hybrids between C_3-C_4 intermediate and C_4 *Flaveria* species. Plant Physiol 75: S-58
10. EDWARDS GE, MSB KU, MD HATCH 1982 Photosynthesis in *Panicum milioides*, a species with reduced photorespiration. Plant Cell Physiol 23: 1185–1195
11. HOLADAY AS, R CHOLLET 1984 Photosynthetic/photorespiratory characteristics of C_3-C_4 intermediate species. Photosynth Res 5: 307–323
12. HOLADAY AS, S TALKMITT, MS DOOHAN 1985 Anatomical and enzymic studies of leaves of a C_3XC_4 *Flaveria* F_1 hybrid exhibiting reduced photorespiration. Plant Sci 41: 31–39
13. INSKEEP WP, PR BLOOM 1985 Extinction coefficients of chlorophyll *a* and b in *N,N*-dimethylformamide. Plant Physiol 77: 483–485
14. KESTLER DP, BP MAYNE, TB RAY, LD GOLDSTEIN, RH BROWN, CC BLACK 1975 Biochemical components of the photosynthetic CO_2 compensation point of higher plants. Biochem Biophys Res Commun 66: 1439–1446
15. KNECHT DA, RL DIMOND 1984 Visualization of antigenetic proteins on western blots. Anal Biochem 136: 180–184
16. KU MSB, HB KRISHNAN 1984 Properties of PEP carboxylase from the F_1 hybrids between *Flaveria floridana* (C_3-C_4) and *Flaveria brownii* (C_4). Plant Physiol 75: S-188
17. KU MSB, RK MONSON, RO LITTLEJOHN JR, H NAKAMOTO, DB FISHER, GE EDWARDS 1983 Photosynthetic characteristics of C_3-C_4 Intermediate *Flaveria* species. I. Leaf anatomy, photosynthetic responses to O_2 and CO_2, and activities of key enzymes of the C_3 and C_4 pathways. Plant Physiol 71: 944–948
18. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227: 680–685
19. MCFADDEN BA 1980 A perspective of ribulose bisphosphate carboxylase/oxygenase, the key catalyst in photosynthesis and photorespiration. Acc Chem Res 13: 394–399
20. MONSON RD, GE EDWARDS, MSB KU 1984 C_3-C_4 intermediate photosynthesis

in plants. BioScience 34: 563–574
21. MORAN R, D PORATH 1980 Chlorophyll determination in intact tissues using *N,N*-dimethylformamide. Plant Physiol 65: 478–479
22. MORGAN JA, RH BROWN 1979 Photosynthesis in grass species differing in carbon dioxide fixation pathways. II. A search for species with intermediate gas exchange and anatomical characteristics. Plant Physiol 64: 257–262
23. NAKAMOTO H, MSB KU, GE EDWARDS 1983 Photosynthetic characteristics of C_3-C_4 intermediate *Flaveria* species II. Kinetic properties of phosphoenolpyruvate carboxylase from C_3, C_4 and C_3-C_4 intermediate species. Plant Cell Physiol 24: 1387–1393
24. OAKLEY BR, DR KIRSCH, NR MORRIS 1980 A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal Biochem 105: 361–363
25. PEARCY RW, O BJÖRKMAN 1971 Biochemical characteristics. Carnegie Inst Wash Year Book 69: 632–640
26. POWELL AM 1978 Systematics of *Flaveria* (*Flaveriinae-Asteraceae*). Ann Mo Bot Gard 65: 590–636
27. REED JE, R CHOLLET 1985 Immunofluorescent localization of phosphoenolpyruvate carboxylase and ribulose 1,5 bisphosphate carboxylase/oxygenase proteins in leaves of C_3, C_4 and C_3-C_4 intermediate *Flaveria* species. Planta 165: 439–445
28. RINEHART C 1984 Phosphoenolpyruvate carboxylase in *Pennisetum americanum* isolation, quantitation, light regulation and cell specificity of protein and mRNA. PhD dissertation. University of Georgia, Athens
29. STERNBERG DA SLL, MJ DENIRO, ME SLOAN, CC BLACK 1986 Compensation point and isotopic characteristics of C_3/C_4 intermediates and hybrids in *Panicum*. Plant Physiol 80: 242–245
30. UEDAN K, T SUGIYAMA 1976 Purification and characterization of phosphoenolpyruvate carboxylase from maize leaves. Plant Physiol 57: 906–910