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Transfer of C_4 Photosynthetic Characters through Hybridization of *Flaveria* Species[1]

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ABSTRACT

Transfer of C_4 photosynthetic traits was studied through hybridization of *Flaveria trinervia* (Spreng.) Mohr (C_4) and *Flaveria brownii* A.M. Powell (C_4-like) with *Flaveria linearis* Lag. (C_3-C_4) and the C_3 species *Flaveria pringlei* Gandoger (C_3). Fertility was low, based on irregular chromosome pairing and low pollen stainability, except in *F. brownii* × *F. linearis* which had bivalent pairing and 76% stainable pollen. Hybrids had apparent photosynthesis values of 71 to 148% of the midparental means, while the CO_2 compensation concentration was similar to the C_4 or C_4-like parent, except in hybrids having the C_3 species *F. pringlei* as a parent. Inhibition of apparent photosynthesis by O_2, and phospho*enol*pyruvate carboxylase and NADP-malic enzyme activities and subunit levels in the hybrids were closer to the C_3 or C_3-C_4 parent. The species *F. brownii* and *F. trinervia* were equal in their capacity to transfer reduced O_2 inhibition of AP and CO_2 compensation concentration values to hybrids with *F. linearis* (C_3-C_4), although hybrids with *F. trinervia* had higher PEPC activity. The O_2 inhibition of AP was correlated with the logarithm of activities of phospho*enol*pyruvate carboxylase ($r = -0.95$) and NADP-malic enzyme ($r = -0.87$). These results confirm that C_4 traits can be transferred by hybridization of C_3-C_4 and C_4 or C_4-like species, with a higher degree of C_4 photosynthesis than exists in C_3-C_4 species, and at least in *F. brownii* × *F. linearis*, fertile progeny are obtained.

The characters associated with C_4 photosynthesis provide these plants with a selective advantage in certain environments (22, 27, 28). The CO_2-concentrating mechanism of this pathway is believed to allow ribulose bisphosphate carboxylase/oxygenase to operate at V_{max} and reduce its inhibition by O_2, resulting in an increased photosynthetic capacity (22, 23). The evolutionary origin of C_4 photosynthesis appears to have been polyphyletic, having arisen several times within both monocots and dicots (26, 28). An individual genus usually contains only C_3 or fully evolved C_4 species. This has hindered investigations of the evolution and genetic control of C_4 characters, since hybrids between photosynthetic types usually are not possible, and the few that have been made reproduce poorly (6, 9, 14, 15).

Recently, however, the genus *Flaveria* (Asteraceae) has received considerable attention owing to the presence of C_3, C_3-C_4, and C_4 species (9, 14, 23, 26). Powell (26) suggests that C_4 photosynthesis evolved along two separate phyletic lines in *Flaveria*. One line includes the C_4-like species, *F. brownii* A.M. Powell (12), and C_3-C_4 intermediate species *F. linearis* Lag., *F. floridana* J.R. Johnston, and *F. oppositifolia* (DC.) Rydb. The other phyletic line contains eight C_4 species, and also C_3 species, including *F. pringlei* Gandoger. Variable levels of F_2 fertility have been reported by Powell (26) after crossing *Flaveria* species.

Since most C_3-C_4 *Flaveria* species fix CO_2 primarily by the C_3 cycle (23, 24), the potential for intrageneric hybrids between C_4 and C_3 or C_3-C_4 *Flaveria* species provides an opportunity to study the inheritance and genetic control of C_4 characters. Several interspecific hybrids have been made in *Flaveria*. Hybrids between *F. brownii* and *F. pringlei* had characteristics similar to *F. pringlei*, except that Γ[2] was somewhat lower than the C_3 parent and C_4 enzyme activities were somewhat higher (14, 15). Biochemical and CO_2 exchange characteristics of hybrids between C_4 and C_3-C_4 *Flaveria* species were intermediate to parental values except that Γ was close to the C_4 parental values (9, 11). In a previous study (9) we reported on CO_2 exchange, enzymic, anatomical, and carbon isotopic characteristics of hybrids between *F. trinervia* (Spreng.) C. Mohr (C_4) and either *F. floridana* (C_3-C_4), *F. linearis* (C_3-C_4), or *F. oppositifolia* (C_3-C_4). Hybridization was well documented in that study, but, as was the case for *F. brownii* × *F. pringlei* (15), the hybrids were infertile, preventing the production of an F_2 generation. The objective of this work was to expand the hybridization to other *Flaveria* species and to evaluate cytogenetics, CO_2, exchange, and C_4 enzymic characteristics of resulting hybrids.

MATERIALS AND METHODS

Species and Hybridization

Species were selected for hybridization from both phyletic lines of *Flaveria* proposed by Powell (26). The C_4-like species *F. brownii* and the C_3-C_4 *Flaveria* species are from the line possessing 5-6 phyllaries in the involucre and *F. trinervia* (C_4)

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[2] Abbreviations: Γ, CO_2 compensation concentration; PEP, phospho*enol*pyruvate; PEPC, phospho*enol*pyruvate carboxylase (EC 4.1.1.31); NADP-ME, NADP-malic enzyme (EC 1.1.1.40); PPDK, pyruvate, Pi dikinase (EC 2.7.9.1); AP, apparent photosynthesis.

and *F. pringlei* (C_3) are from the opposing 3-4 phyllary line. Hybrids were made using *F. trinervia* (ID No. 84-10) and *F. brownii* (85-250) as maternal parents. In the hybrid between these two species, *F. trinervia* was the maternal plant. The other species used were *F. pringlei* (85-207) and *F. linearis* (C_3C_4) (84-8). Procedures for emasculation and pollination of crosses involving *F. trinervia* as a maternal parent were the same as documented previously (9). When *F. brownii* was used as the maternal parent, emasculation consisted of the removal of all disc florets just prior to flowering. This left only the male sterile ray florets, which were then dusted with pollen from the appropriate male parent.

Cytogenetics

For both parents and hybrids, chromosome number was determined on root tips prefixed in a solution of cycloheximide (70 mg L^{-1}) and 8-hydroxyquiniline (270 mg L^{-1}), fixed in a 3:1 (v/v) mixture of ethanol and acetic acid and hydrolyzed in 5 N HCl for 5 min at room temperature, stained in Feulgen, and squashed in acetocarmine. The exception to this procedure was the use of the saturated solution of monobromonapthelene in the prefixation step with *F. pringlei* and its hybrids. Young flowers were fixed in Carnoy's solution (6:3:1 [v/v/v] ethanol:chloroform:acetic acid) and pollen mother cells were examined for meiosis after squashing anthers in acetocarmine. Pollen was collected at anthesis, and stained with a triple pollen stain of a mixture of acid fuchsin-malachite green-orange G (2) as an indication of viability.

CO_2 Exchange

Plants were grown from vegetative cuttings in 3-L pots filled with a 1:1:1 (by volume) mixture of soil, peat, and Perlite. They were fertilized three times weekly with Hoagland solution. In experiment 1 plants were grown in a growth chamber set at 30°C during the day (14 h) and 25°C at night (10 h). Irradiance was 430 μmol photons m^{-2} s^{-1} (400–700 nm). After 3 weeks of growth in the chamber, CO_2 exchange measurements were made. Plants for experiment 2 were grown from cuttings as in experiment 1, except they were grown in a greenhouse with maximum daytime temperatures of 30 to 35°C and nighttime temperatures of 20 to 25°C. During growth, supplemental lighting was provided for 8 h/d with multivapor lamps to provide at least 1.5 mmol photons m^{-2} s^{-1} at midday.

Plants were removed from the greenhouse or growth chamber, and CO_2 exchange measurements were made as described earlier (9) with the following modifications. A data acquisition and control system was used to monitor and maintain chamber CO_2 constant at 330 μmol mol^{-1} using a flow controller (Datametrics model A 25) to add CO_2 to the gas stream. AP was calculated from the flow rate and CO_2 concentration (approximately 10 mmol mol^{-1}) of the added gas. The leaf was maintained at 30°C, and dewpoint in the leaf chamber ranged from 17 to 22°C depending on transpiration. CO_2 exchange was measured at 2 mmol photons m^{-2} s^{-1} (400–700 nm).

The youngest fully expanded leaf on a stem was enclosed in the leaf chamber, and steady rates of CO_2 uptake were measured at 210 and 20 mmol O_2 mol^{-1}. Afterward the leaf was excised and the area determined. The opposite leaf on the stem was removed for enzyme assays. Measurements were made on leaves of three separate plants (vegetative clones) of each genotype in each experiment.

Γ was measured in experiment 1 using a syringe technique described earlier (9). Temperature in the 30-mL syringes was maintained at 30 ± 0.5°C and irradiance was 430 μmol photons m^{-2} s^{-1} (400–700 nm) inside the syringes. Whole leaves or leaf pieces were incubated for 20 min and a 20-mL sample of air was injected into the N_2 stream flowing through the sample cell of an IR analyzer.

Preparation of Crude Extracts

Leaf tissue (1.0 g fresh weight) was ground in 0.2 g sand and 2.0 mL grinding buffer at 4°C (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] and 10 μM PMSF [Sigma]). The crude extract was centrifuged at 12,000× *g* for 30 min at 4°C and the supernatant fluid was frozen at −80°C and later assayed for PEPC and NADP-ME activities.

Enzyme Activity

Activity of PEPC was determined by the method of Uedan and Sugiyama (31). 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^{-1} malate dehydrogenase (pigeon breast muscle, Sigma), and 5 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 extracts, $NaHCO_3$, 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} at 25°C. Three replicates, at two dilutions, were assayed for each sample. When appropriate, measurements were corrected for endogenous activity in the absence of PEP.

Activity of NADP-ME was assayed in crude extracts by the methods of Kanai and Edwards (18). The assay buffer contained 50 mM Tris (pH 8.0 at 22°C), 2.5 mM EDTA, 20 mM $MgCl_2$, 5 mM DTT, and 2.5 mM malate (Sigma) in a final volume of 1.0 mL. The reaction was initiated by the addition of 0.4 mM NADP and the activity was assayed by following the increase in A_{340} at 25°C. Reactions were run without $MgCl_2$ to determine endogenous NADP-malic dehydrogenase activity which was negligible in the direction of oxaloacetate formation.

To determine if any extracts contained PEPC or NADP-ME inhibitors, samples of *F. trinervia* or *F. brownii* were assayed after dilution with buffer or equivalent dilution with extract from each parent and hybrid sample. A decrease in enzyme activity, compared to *F. trinervia* or *F. brownii* plus assay buffer, would have suggested the presence of inhibitors in the other parents or hybrids ("Results"). Activities of PEPC and NADP-ME were compared among species by the General Linear Model test of SAS (30).

Chl Analysis

Chl measurements were made on cross-sectional segments of tissue from each leaf sampled for enzyme activity and protein concentration. The fresh tissue was cut cross-wise into small strips (1–2 × 10 mm), and extracted twice in 1.0 mL *N,N*-dimethylformamide overnight at room temperature (25). Extracts were stored at 4°C in the dark until assayed. The amount of Chl (total) was determined according to Inskeep and Bloom (17).

Protein Analysis

Protein concentration of each sample was determined in the centrifuged crude extract used for enzyme activity measurements by the method of Bradford (8) with BSA as a standard.

Protein Separation

Soluble proteins from each centrifuged crude extract were precipitated by the addition of 100 to 500 volumes of −20°C acetone. Samples (5 μg protein/lane) and mol wt standards (Pharmacia, 14,000–94,000) were prepared and electrophoresed in 12.5% acrylamide gels according to Laemmli (21). Gels were silver-stained according to Blum *et al.* (7).

Electrophoretic Transfer of Polypeptides and Immunoblotting

Samples (75 μg protein/lane) were electrophoresed as described above. The polypeptides were transferred to nitrocellulose filters and the subunits corresponding to PEPC and NADP-ME were visualized by probing each filter with polyclonal antibody against either PEPC (provided by S. Sun, University of Hawaii) or NADP-ME (provided by T. Nelson, Yale University) as previously described (10). No bands below either PEPC or NADP-ME were observed in these immunoblots, indicating that the samples were relatively undegraded.

RESULTS

Morphology

Flaveria trinervia possesses broad, subovate leaves with serrate margins and glomerule-like, axillary, and sessile capitulescences, while *F. linearis* and *F. brownii* both possess narrow, linear leaves and flat-topped corymbose, elongated terminal capitulescences (26). The *F. trinervia* × *F. linearis* and *F. trinervia* × *F. brownii* hybrids were intermediate in leaf shape and possessed terminal, but reduced capitulescences. The *F. brownii* × *F. linearis* hybrids were morphologically very similar to both parents. *Flaveria pringlei* had wide, ovate leaves with smooth, entire margins and terminal, corymbose panicles (26). All hybrids involving *F. pringlei* possessed morphology similar to *F. pringlei*. The *F. trinervia* × *F. pringlei* hybrid has never flowered and has poor growth and vigor.

Cytogenetics

F. pringlei is tetraploid ($2n=4x=72$), in contrast to the other parental species which are diploid ($2n=2x=36$) (Table I). Powell (26) also documented a tetraploid form of *F. pringlei*. The diploid number is certainly high, but no accessions with lower numbers were found by Powell in a fairly complete phylogenetic study of the genus. Anderson (3) describes an accession of *Flaveria campestris* for which $2n=18$, but concludes that it is a spontaneous polyhaploid rather than an ancestral diploid.

The *F. brownii* × *F. linearis* hybrid was a diploid ($2n=2x=36$) and possessed regular bivalent pairing and high pollen stainability (Table I). This plant has also been found to produce viable seed, and an F_2 population has been generated from it (16). The *F. trinervia* × *F. linearis* and the *F. trinervia* × *F. brownii* hybrids were diploids ($2n=2x=36$), but possessed low pollen stainability and several unpaired chromosomes (Table I). Although some pairing does exist in these hybrids, it is difficult to predict if the pairing results from partial homology between the *F. linearis* or *F. brownii* genomes and the F. trinervia genome or simply autosyndetic pairing within each parental genome.

F. pringlei was a fertile tetraploid with primarily bivalent pairing, but it also possessed a high degree of multivalent associations (Table I). Hybrids between it and *F. trinervia* and *F. brownii* were triploid ($2n=3x=54$). The *F. brownii* × *F. pringlei* hybrid showed a high degree of unpaired chromosomes, but averaged 13 paired chromosomes per cell. However, its fertility was low. The *F. trinervia* × *F. pringlei* hybrid has never flowered, therefore meiotic analyses were not performed on this plant.

CO_2 Exchange

In experiment 1 (chamber-grown plants), AP did not vary greatly among plants, and hybridization had only minimal effects on AP (Table II). In experiment 2 (greenhouse-grown plants), AP was higher for some plants than in experiment 1, probably because of the higher irradiance during growth. Increases in AP were especially apparent in *F. trinervia* and hybrids *F. brownii* × *F. linearis* and *F. brownii* × *F. pringlei*. The hybrid *F. trinervia* × *F. brownii* had AP similar to the midparent mean, whereas *F. brownii* × *F. linearis* and *F. brownii* × *F. pringlei* had AP 48 and 26% higher than the midparent means, respectively. The *F. trinervia* × *F. linearis* hybrids did not have AP lower than the *F. linearis* parent, in contrast to the same hybrid reported earlier (9).

O_2 inhibition of AP in *F. trinervia* was near zero, typical of C_4 species, whereas *F. brownii* exhibited values of 10 and 4% in experiment 1 and 2, respectively, values somewhat higher than most C_4 plants (Table II). *F. pringlei* had values of 26 ± 4 and 28 ± 1%, just slightly below those reported for most C_3 species. Hybrids between either *F. trinervia* or *F. brownii* and *F. linearis* or *F. pringlei* exhibited O_2 inhibition of AP as high or higher than the mean of the parents, ranging from 100% of the midparent mean in *F. brownii* × *F. linearis* in experiment 1 to 169% for *F. brownii* × *F. pringlei* in experiment 2. In fact, for *F. brownii* × *F. pringlei* the O_2 inhibition of AP was similar to that of *F. pringlei* in both experiments.

In experiment 1, Γ values determined at low irradiance were 6 μmol mol^{-1} or less for *F. trinervia*, *F. brownii*, and hybrids between these species and *F. linearis* (Table II). *F. linearis* had a Γ of 40 ± 4 μmol mol^{-1}. This higher than

Table I. *Chromosome Number and Meiotic Behavior and Pollen Stainability of Flaveria Species and Their Hybrids*

Plants	Somatic Chromosome No. (2n)	Metaphase I Chromosome Pairing[a]				Stainable Pollen
		I	II	III	IV	
		avg. no. cell^{-1}				%
Parents						
F. trinervia (C_4)	36	1.4	17.0	0	0.1	99
F. brownii (C_4-like)	36	0	18.0	0	0	96
F. linearis (C_3-C_4)	36	0	18.0	0	0	95
F. pringlei (C_3)	72	1.9	19.0	0.4	7.5	98
Hybrids						
F. trinervia ×						
F. brownii	36	10.0	13.0	0	0	6
F. linearis	36	12.9	9.8	1.1	0.1	2
F. pringlei[b]	54					
F. brownii ×						
F. linearis	36	0	18.0	0	0	76
F. pringlei[c]	54	21.6	13.1	1.6	0.1	11

[a] Notations I, II, III, and IV indicate the univalent, bivalent, trivalent, and quadrivalent chromosome associations, respectively. [b] *F. trinervia* × *F. pringlei* hybrid grew poorly and never flowered, thus preventing any meiotic and pollen stainability observations. [c] The *F. brownii* × *F. pringlei* hybrid possessed undeveloped anthers. Therefore the meiotic analysis and pollen stainability were determined on the reciprocal hybrid (*F. pringlei* × *F. brownii*).

Table II. *Apparent Photosynthesis (AP), O_2 Inhibition of AP, and Γ for Flaveria species and hybrids*

Values in parentheses are the percentage of the calculated midparent means. AP was measured at 30°C, 330 μmol mol^{-1} CO_2, 210 mmol mol^{-1} O_2, and 2 mmol photons m^{-2} s^{-1}. Γ was measured at 30°C, 210 mmol mol^{-1} O_2, and 430 μmol photons m^{-2} s^{-1}.

Plants	AP		O_2 inhibition[a]		Γ
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1
	μmol m^{-2} s^{-1}		%		μmol mol^{-1}
Parents					
F. trinervia	23	36	−0.6	2	4
F. brownii	27	25	10	4	6
F. linearis	17	21	24	22	40
F. pringlei	22	21	26	28	68
Hybrids					
F. trinervia ×					
F. brownii	27 (108)	30 (98)	3	7	3
F. linearis	20 (100)	21 (74)	13 (111)	17 (142)	4
F. pringlei	16 (71)	ND[b]	17 (134)	ND	69
F. brownii ×					
F. linearis	19 (86)	34 (148)	17 (100)	16 (123)	5
F. pringlei	22 (90)	29 (126)	28 (156)	27 (169)	56
LSD (0.05)	11	6	10	3	7

[a] Inhibition of AP at 210 mmol mol^{-1} O_2 expressed as a percentage of AP at 20 mmol mol^{-1} O_2. [b] Not determined.

previously reported value (9) probably resulted from the low irradiance under which plants were both grown and measured (13). Hybrids involving *F. pringlei* exhibited Γ values much higher than midparent means and in *F. trinervia* × *F. pringlei* Γ was similar to the C_3 parent value.

Enzyme Activity

Although growth irradiance was higher in experiment 2 than in experiment 1, PEPC activities were not consistently different (Table III). Activities in *F. trinervia* and its hybrids were higher in experiment 2 than experiment 1, while in *F. brownii* and its hybrids activity decreased. Activity in *F. trinervia* and *F. pringlei* behaved similarly to *F. brownii*, *i.e.* a decrease in experiment 2 compared to experiment 1. On the other hand, NADP-ME activity was consistently higher in experiment 2 than in experiment 1. Soluble leaf protein also was higher in experiment 2, except for *F. brownii* and *F. pringlei*, as indicated by the protein/Chl ratios in Table III.

When either *F. trinervia* or *F. brownii* was crossed with the

Table III. *Activity of PEPC and NADP-ME from Flaveria Parents and Hybrids*

Plants	Experiment	Protein/Chl	PEPC	NADP-ME
		$\mu g\ \mu g^{-1}$	$\mu mol\ h^{-1}\ mg^{-1}$ protein	
Parents				
F. trinervia	1	4.8	41.8	34.1
	2	5.8	79.9	83.0
F. brownii	1	6.3	23.3	61.3
	2	5.0	13.5	85.7
F. linearis	1	5.1	3.5	5.1
	2	7.2	2.3	8.2
F. pringlei	1	5.6	1.5	0.1
	2	5.4	0.9	0.3
Hybrids				
F. trinervia ×	1	3.6	25.1 (77)[a]	34.0 (71)
F. brownii	2	5.7	52.6 (113)	90.3 (107)
F. trinervia ×	1	4.0	14.4 (64)	5.3 (27)
F. linearis	2	5.9	23.3 (57)	25.0 (55)
F. trinervia ×	1	2.7	7.1 (33)	2.6 (15)
F. pringlei	2	ND[b]	ND	ND
F. brownii ×	1	3.2	4.6 (34)	9.6 (29)
F. linearis	2	5.3	2.8 (35)	16.8 (36)
F. brownii ×	1	4.4	2.9 (24)	2.1 (7)
F. pringlei	2	5.6	2.0 (27)	2.9 (7)
LSD (0.05)	1		11.2[c]	11.8
			3.6[d]	1.9
	2		22.6	28.4
			5.6	6.2

[a] Numbers in parentheses are the percentages of the mid-parent means. [b] Not determined. [c] LSD for all means. [d] LSD for means excluding *F. trinervia*, *F. brownii* and their F_1 hybrid.

C_3 species *F. pringlei*, enzyme activities were between those of the parents although closer to the C_3. On the other hand, when the same species were crossed with *F. linearis* the results were not consistent. When *F. trinervia* was used, PEPC activity in the hybrid was several-fold greater than in *F. linearis* (also ref. 9), but when *F. brownii* was involved PEPC activity in the hybrid was nearly as low as in *F. linearis*. The hybrid *F. brownii* × *F. linearis* was significantly higher in NADP-ME activity than *F. linearis* in experiment 1, while the *F. trinervia* × *F. linearis* hybrid values were nearly identical. In experiment 2, however, both hybrids had higher NADP-ME activity than the C_3-C_4 parent; with *F. trinervia* activity was 3.1-times higher and with *F. brownii* 2.1-times higher than in *F. linearis*.

Electrophoresis and Immunoblotting of Soluble Proteins

Figure 1 shows representative soluble polypeptide profiles extracted from all parents and hybrids. The most densely stained PEPC bands are present in *F. trinervia* and *F. brownii*. Immunostaining of polypeptides transferred to nitrocellulose consistently indicated one band corresponding to PEPC in *F. trinervia* (100 kD) and two PEPC bands in *F. brownii*, *F. linearis*, and all hybrids except where *F. pringlei* was a parent (Fig. 2A) (10). Although no PEPC band is detectable in the extracts from *F. pringlei* in Figure 2A, previous immunoblots have shown a single faint band of approximately the same size as in *F. trinervia* (RG Cameron, CL Bassett, unpublished data). In the hybrid *F. brownii* × *F. pringlei*, no band corresponding to PEPC was visible (Fig. 2A, lane 9); however, a band corresponding to PEPC was observed in *F. trinervia* × *F. pringlei*, although at a somewhat reduced level compared to the *F. trinervia* parent.

No silver-stained band corresponding to PPDK could be detected in *F. pringlei*, while all other samples (except *F. trinervia* × *F. brownii*) have a single band in the PPDK position (10), although apparently at a slightly lower mol wt in *F. brownii*.

Two NADP-ME bands are present in *F. trinervia* (10), a heavily stained band at approximately 64 kD and lightly stained band at 67 kD. Only one band is present in *F. linearis*, at approximately 66 kD (10). *F. brownii* has a heavily stained NADP-ME band at the same position as in *F. linearis*, slightly higher in mol wt than the heavily stained band in *F. trinervia*, although NADP-ME activity was nearly identical in both species (Fig. 1). This band was readily apparent in the extracts from *F. brownii* on immunoblots (Fig. 2B, lane 1), but was completely absent in extracts from *F. pringlei* (Fig. 2B, lane 2). No bands were detectable in hybrids having *F. pringlei* as one of the parents. On the other hand, a faint band corresponding to NADP-ME was observed from extracts of the *F. brownii* × *F. linearis* hybrid (Fig. 2B, lane 5).

DISCUSSION

The hybrid nature of the F_1 plants was confirmed in several ways. In all cases, except *Flaveria trinervia* × *Flaveria brownii*, PEPC and NADP-ME activities did not approach that of the female parent, strongly indicating that the plants were not selfs. Polypeptide bands in Figure 1 indicate the same. For crosses other than *F. brownii* × *F. linearis* and *F. brownii* × *F. pringlei*, morphology of the parents and F_1 plants was distinct enough for reliable verification. In *F. brownii* × *F. pringlei* and *F. trinervia* × *F. pringlei* the triploid chromosome number (Table I) showed the progeny to be hybrids. For *F. brownii* × *F. linearis*, whose parents are morphologically similar, the low enzyme activity (similar to *F. linearis*) as well as intermediate O_2 inhibition indicated hybridization. Successful hybridization among these species indicates fairly close phylogenetic relationships even between species considered by Powell (26) to be almost as divergent as any in the genus.

The morphological and cytogenetic data indicate a closer relationship between *F. linearis* and *F. brownii* than between *F. linearis* and *F. trinervia*. *Flaveria brownii*, along with three C_3-C_4 *Flaveria* species, was placed by Powell in the 5-6 phyllary line of the genus while *F. trinervia* and *F. pringlei* are both in the 3-4 phyllary line (26). Data in this report further support the relationships suggested by Powell. It is difficult to predict the phylogenetic relationship of *F. pringlei* to the other species because its polyploidy complicates interpretation of the meiotic pairing of hybrids involving this species. However, it is likely that *F. brownii* is closely related to *F. linearis* and is therefore a more C_4-like form of the 5-6 phyllary line. The chromosome pairing exhibited in other hybrids indicates a more distant relationship between *F. brownii* and *F. linearis* of the 5-6 phyllary line and the C_4 annual species *F. trinervia* or the C_3 species *F. pringlei*. *Flaveria trinervia* and *F. pringlei* also are apparently too distantly related from one another to produce even partially stable offspring, even though both are in the 3-4 phyllary line.

The PEPC activity of F_1 hybrids was substantially higher

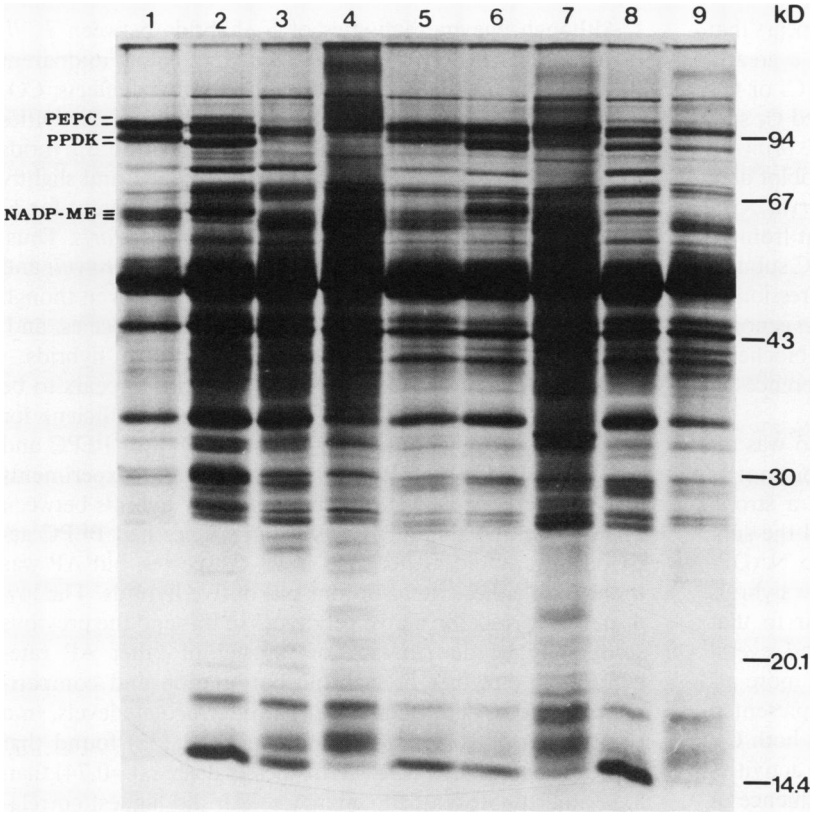

Figure 1. Representative silver-stained soluble polypeptide profiles of parents and hybrids. Plants were grown in the greenhouse in experiment 2, except *F. trinervia* × *F. pringlei* which was grown in the growth chamber in experiment 1. Lanes 1, *F. trinervia*; 2, *F. brownii*; 3, *F. linearis*; 4, *F. pringlei*; 5, *F. trinervia* × *F. linearis*; 6, *F. trinervia* × *F. brownii*; 7, *F. trinervia* × *F. pringlei*; 8, *F. brownii* × *F. linearis*; ; 9, *F. brownii* × *F. pringlei*.

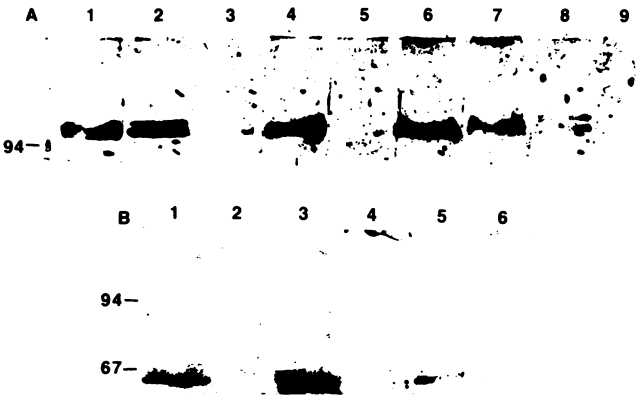

Figure 2. Immunoblot of soluble polypeptides from representative parents and hybrids. Plants were grown in the greenhouse as in experiment 2, except *F. trinervia* × *F. pringlei* which was grown in the growth chamber in experiment 1. The polypeptides were transferred to nitrocellulose, stained with Ponceau S to check for completeness of transfer, and bound with antibody as described in "Materials and Methods." Size markers are indicated on the left of the figure. A, Blots assayed with antiserum to PEPC: lanes 1, *F. trinervia*; 2, *F. brownii*; 3, *F. linearis*; 4, *F. trinervia* × *F. brownii*; 5, *F. pringlei*; 6, *F. trinervia* × *F. linearis*; 7, *F. trinervia* × *F. pringlei*; 8, *F. brownii* × *F. linearis*; 9, *F. brownii* × *F. pringlei*. B, Blots assayed with antiserum to NADP-,ME: lanes 1, *F. brownii*; 2, *F. pringlei*; 3, *F. trinervia* × *F. brownii*; 4, *F. trinervia* × *F. pringlei*; 5, *F. brownii* × *F linearis*; 6, *F. brownii* × *F. pringlei*.

than in C_3 or C_3-C_4 species when they were crossed with *F. trinervia*, but only marginally higher when *F. brownii* was used. Activity of PEPC was lower in *F. brownii* than *F. trinervia* in this and other work (4), and compartmentation of this enzyme is much less complete in *F. brownii* (12, 15, 29). Adams *et al.* (1) described two different molecular forms of PEPC in *Flaveria* based on peptide mapping. Both *F. floridana* (C_3-C_4) and *F. cronquistii* Powell (C_3) gave identical peptide maps that were different from that of *F. trinervia*. In support of these observations, the kinetic properties of PEPC from 10 *Flaveria* species were shown to be virtually identical for the C_3 and C_3-C_4 species examined (5). Interestingly, *F. brownii* PEPC showed hyperbolic kinetics typical of C_3 and C_3-C_4 species, but had a much higher K_m (PEP), although still below that of the C_4 species. Perhaps the differences in inheritance of PEPC activity observed in our study between *F. trinervia* and *F. brownii* relate to the structural and/or biochemical differences in forms of PEPC observed earlier (1, 5) or in the less complete compartmentation of this enzyme observed for *F. brownii* (12, 15, 29).

For both species, however, PEPC activity in hybrids with C_3-C_4 or C_3 species was below the midparent mean, ranging in the two experiments from 33 to 64% of midparent means in *F. trinervia* hybrids and 24 to 35% in hybrids with *F. brownii*. Although PEPC activity for *F. trinervia* hybrids with C_3-C_4 species reported earlier (9) was not always lower than midparent mean values, they were in five of the seven hybrids reported. In *Atriplex* hybrids PEPC was 50% of the midparent mean (6). Ku and Krishnan (19) found that allosteric and kinetic properties of PEPC from *F. brownii* × *F. floridana*

were closer to those of the C_3-C_4 parent. Thus, it appears that plants with a low level of C_4 photosynthesis exert a greater influence on inheritance of PEPC activity than do C_4 or C_4-like species and that the more completely developed C_4 species, *F. trinervia*, exerts a greater influence than *F. brownii*. This is especially true when comparing the immunoblot data which show that the hybrid derived from *F. trinervia* × *F. pringlei* has higher levels of PEPC compared to that from *F. brownii* × *F. pringlei*, which has no detectable PEPC subunit bands (Fig. 2A). This information suggests that expression of the enzyme is negatively influenced by the *F. pringlei* genome and that the lowered activity may not only be due to biochemical differences between the parents, but also to differences in expression at the polypeptide level.

Inheritance of NADP-ME activity by the F_1 also was affected by the choice of C_4 parent in experiment 1 but not as consistently as PEPC activity. *F. pringlei* exerted a strong negative influence on both NADP-ME activity and the subunit levels. In fact, from the immunoblot data, no NADP-ME subunit bands were detectable in either of the hybrids where *pringlei* was a parent. This result is similar to that observed previously in crosses between *F. trinervia* and several C_3-C_4 species showing that NADP-ME levels were more affected in the F_1 than PEPC (10). The additional data presented here with hybrids using *F. pringlei* indicate (a) that both C_3-C_4 and C_3 species exert a negative effect on enzyme activities and subunit levels in these hybrids, (b) that the influence of *F. pringlei* is much greater than that of *F. linearis*, and (c) that the influence lies at the level of expression and not simply at the level of biochemical differences among the enzymes.

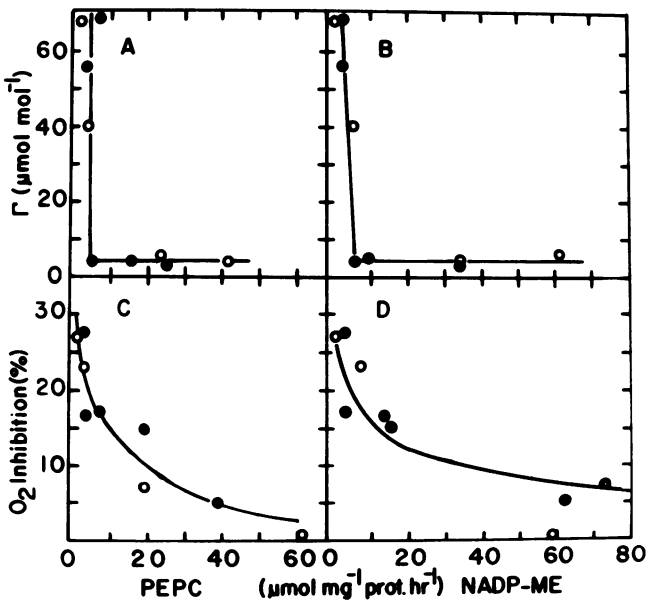

Figure 3. Dependence of Γ (A, B) and O_2 inhibition of AP (C, D) on activities of PEPC (A, C), and NADP-ME (B, D) for four *Flaveria* species (○) and five F_1 hybrids (●). Regressions fitted for O_2 inhibition of AP were (C) % O_2 inhibition = 29.6 ± 15.4 $\log_{10}$ PEPC ($r = -0.95$) and (D) % O_2 inhibition = 25.1-10.0 $\log_{10}$ NADP-ME ($r = -0.87$). Data points in A and B are from experiment 1 and data points in C and D are means for experiment 1 and 2, except for *F. trinervia* × *F. pringlei* which was not included in experiment 2.

Although enzyme activities of F_1 hybrids between *F. linearis* and *F. trinervia* or *F. brownii* were below midparent means and the latter two species had differential effects, CO_2 exchange characteristics did not follow the enzyme activities closely. Inhibition of AP by atmospheric O_2 in both hybrids was similar to midparent means in experiment 1 and slightly higher in experiment 2. Values of Γ were not different for *F. trinervia*, *F. brownii*, and their hybrids with *F. linearis*. Thus, for CO_2 exchange related to photorespiration, *F. brownii* and *F. trinervia* were similar in effect on F_1 hybrids, even though *F. trinervia* is a more completely developed C_4 species, and, at least for PEPC, transfers higher activity to its F_1 hybrids.

Enzyme activity in the parents and hybrids appears to be poorly correlated with AP. The correlation coefficient for these two parameters was only 0.57 ($P<0.01$) for PEPC and 0.52 ($P<0.01$) for NADP-ME for data from both experiments reported here. In earlier experiments (9) F_1 hybrids between *F. trinervia* and three C_3-C_4 *Flaveria* species had PEPC activities severalfold higher than in C_3-C_4 species, but AP was lower than either parent in four out of five hybrids. The low degree of correlation across genotypes in this and the previous study may be due to lack of transfer of other AP rate-controlling enzymes or lack of coordination and compartmentation of C_4 enzymes present at intermediate levels. In a comparison of 10 C_4 species, Usuda *et al.* (32) found that PEPC was correlated with AP to a lesser degree ($r=0.74$) than four other photosynthetic enzymes with the highest correlation observed between AP and PPDK ($r=0.94$).

Although increases in PEPC and NADP-ME activity in F_1 plants compared to C_3 or C_3-C_4 did not result in higher AP, Γ and O_2 inhibition of AP were reduced. When O_2 inhibition was regressed on $\log_{10}$ PEPC or NADP-ME activity, the correlation coefficient was −0.95 and −0.87, respectively (Fig. 3, C and D). This logarithmic relationship implies that small increases in C_4 photosynthesis, as may be indicated by the increase in PEPC or NADP-ME activity above the C_3 level, result in large reductions of O_2 inhibition. The change in Γ with increased PEPC or NADP-ME activity was even more dramatic, with Γ decreasing to C_4-like values at enzyme activities as low as those in C_3 or C_3-C_4 species (*cf. F. brownii* × *F. linearis* [Tables II and III; Fig. 3, A and B]). Large reductions in both Γ and O_2 inhibition of AP with little or no increase in both PEPC and NADP-ME may indicate that the activity of these enzymes is sufficient in the C_3 and C_3-C_4 species to reduce Γ and O_2 inhibition, but that some other C_4 enzyme may be limiting. Pyruvate,Pi dikinase activities, for example, are usually much lower than those for PEPC or NADP-ME in C_3-C_4 species (4, 20). On the other hand, low Γ in the C_4 or C_4-like × C_3-C_4 hybrids may result from more efficient recycling of photorespired CO_2 in bundle sheath cells.

Results presented here suggest that transfer of C_4 traits to C_3 or C_3-C_4 species is feasible. In all crosses, except *F. brownii* × *F. pringlei*, O_2 inhibition of AP was decreased relative to the non-C_4 parent and Γ was reduced in all hybrids except when *F. pringlei* was a parent. *F. brownii* appeared comparable to the better developed C_4 plant, *F. trinervia*, in transferring reduced O_2 inhibition of AP and Γ to its offspring, although inferior in transferring PEPC activity. *F. brownii* has the advantage of producing fertile hybrids with the C_3-C_4

species *F. linearis* (16). Additionally, AP of the hybrids was equal to, or greater than, values measured for the C_3 or C_3-C_4 parent (except for *F. trinervia* × *F. pringlei*). However, these results also indicate that for genetic transfer of C_4 photosynthesis a more complete understanding of the inheritance, regulation, and structure of the C_4 enzymes is required. Further studies with these species and their hybrids should provide answers to the mechanisms of inheritance as well as regulatory and structural differences between the C_3, C_3-C_4, and C_4 enzymes associated with C_4 photosynthesis.

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