Plant Physiol. (1991) 96, 518–528
0032-0889/91/96/0518/11/$01.00/0

Received for publication September 9, 1990
Accepted January 29, 1991

Photosynthetic and Photorespiratory Characteristics of *Flaveria* Species[1]

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ABSTRACT

The genus *Flaveria* shows evidence of evolution in the mechanism of photosynthesis as its 21 species include C_3, C_3-C_4, C_4-like, and C_4 plants. In this study, several physiological and biochemical parameters of photosynthesis and photorespiration were measured in 18 *Flaveria* species representing all the photosynthetic types. The 10 species classified as C_3-C_4 intermediates showed an inverse continuum in level of photorespiration and development of the C_4 syndrome. This ranges from *F. sonorensis* with relatively high apparent photorespiration and lacking C_4 photosynthesis to *F. Among the intermediates, the photosynthetic CO_2* compensation points at 30°C and 1150 micromoles quanta per square meter per second varied from 9 to 29 microbars. The values for the three C_4-like species varied from 3 to 6 microbars, similar to those measured for the C_4 species. The activities of the photorespiratory enzymes glycolate oxidase, hydroxypyruvate reductase, and serine hydroxymethyltransferase decreased progressively from C_3 to C_3-C_4 to C_4-like and C_4 species. On the other hand, most intermediates had higher levels of phosphoenolpyruvate carboxylase and NADP-malic enzyme than C_3 species, but generally lower activities compared to C_4-like and C_4 species. The levels of these C_4 enzymes are correlated with the degree of C_4 photosynthesis, based on the initial products of photosynthesis. Another indication of development of the C_4 syndrome in C_3-C_4 *Flaveria* species was their intermediate chlorophyll *a*/*b* ratios. The chlorophyll *a*/*b* ratios of the various *Flaveria* species are highly correlated with the degree of C_4 photosynthesis suggesting that the photochemical machinery is progressively altered during evolution in order to meet the specific energy requirements for operating the C_4 pathway. In the progression from C_3 to C_4 species in *Flaveria*, the CO_2 compensation point decreased more rapidly than did the decrease in O_2 inhibition of photosynthesis or the increase in the degree of C_4 photosynthesis. These results suggest that the reduction in photorespiration during evolution occurred initially by refixation of photorespired CO_2 and prior to substantive reduction in O_2 inhibition and development of the C_4 syndrome. However, further reduction in O_2 inhibition in some intermediates and C_4-like species is considered primarily due to the development of the C_4 syndrome. Thus, the evolution of C_3-C_4 intermediate photosynthesis likely occurred in response to environmental conditions which limit the intercellular CO_2 concentration first via refixation of photorespired CO_2, followed by development of the C_4 syndrome.

[1] Supported in part by National Science Foundation grants DMB-8512521 and DCB-8816322.

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A unique feature of the small genus *Flaveria* is that it contains many C_3-C_4 intermediate species in addition to C_3 and C_4 species (12). It has been speculated that some of these species may be in the process of evolution towards C_4 photosynthesis (28). So far, 7 of the 21 species in the genus have been classified as C_3-C_4 intermediates (12). Compared to C_3 plants, the C_3-C_4 intermediates all exhibit a reduced level of photorespiration and a more differentiated Kranz-like leaf anatomy. However, it is not clear if all the intermediates in the genus possess a similar photosynthetic mechanism. That is, the underlying biochemistry of photosynthetic intermediacy may be different among the C_3-C_4 *Flaveria* species. In addition, several recent studies have shown that *F. brownii*, a species previously classified as a C_4 plant, is actually an advanced, C_4-like intermediate (4, 9, 10, 14, 23). Furthermore, a study on enzyme compartmentation and initial photosynthetic products with five C_4 *Flaveria* species suggested that three of them could be classified as C_4-like species (25). These results showed there may be a continuous gradation in both the physiology and biochemistry of photosynthesis among the *Flaveria* species. This gradation should allow a critical evaluation of the relationship between development of C_4 biochemistry and physiology of photosynthesis. In this study we examined a number of photosynthetic and photorespiratory characteristics of 18 *Flaveria* species, and the relationships between these parameters were evaluated. The possible implications of the variation of photosynthetic characteristics among these species relative to the evolution of C_4 photosynthesis are discussed. We also report on the finding of three new intermediate species in this group of plants.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Eighteen identified *Flaveria* species were used in this study: *F. angustifolia* (Cav.) Pers. (C_3-C_4), *F. anomala* B. Robinson (C_3-C_4), *F. australasica* Hook (C_4), *F. bidentis* (L.) Kuntze (C_4), *F. brownii* A. M. Powell (C_4-like), *F. chloraefolia* A. Gray (C_3-C_4), *F. cronquistii* A. M. Powell (C_3), *F. floridana* J. R. Johnston (C_3-C_4), *F. linearis* Lag. (C_3-C_4), *F. oppositifolia* (DC.) Rydb. (C_3-C_4), *F. palmeri* J. R. Johnston (C_4-like), *F. pringlei* Gandoger (C_3), *F. pubescens* Rydb (C_3-C_4), *F. ramosissima* Klatt (C_3-C_4), *F. robusta* Rose (C_3), *F. sonorensis* A. M. Powell (C_3-C_4), *F. trinervia* (Spreng.) C. Mohr (C_4), and *F. vaginata* B. L. Robinson and Greenman (C_4-like). Two genotypes of *F. oppositifolia* were used in this study. One of them was collected by L. J. Mets and was keyed by A. J. Gilmartin (former systematicist, Washington State Univer-

sity). This plant differs in many morphological characters (*e.g.* leaf size, pubescence, and pigmentation) from that provided by R. H. Brown. The designations of photosynthetic groups in parentheses are based on results of the present and recent studies, which include examination of leaf anatomy, Γ[3], sensitivity of photosynthesis to O_2, enzyme compartmentation, and initial photosynthetic products (1, 3, 6, 9, 16, 20, 22, 25, 29).

Plants were grown from either seeds or vegetative cuttings in a compost:sand:perlite mixture (2:1:1, by volume) in 4 L plastic pots, and cultivated in a greenhouse under natural light. The maximum PPFD in the greenhouse on a clear day during the summer months was about 1750 μmol quanta m^{-2} s^{-1}. The temperature was controlled at 25 ± 1°C day and 18 ± 0.5°C night, and the RH was maintained at 60 ± 5%. Plants were fertilized twice a week with commercial fertilizer and supplemented with micronutrients. Young, fully expanded leaves (usually the third or fourth leaves from the apex) from plants grown during the summer months (June to September) were used for various experiments.

Photosynthetic CO_2 Compensation Point

Two methods were employed to estimate Γ in intact leaves. In the first method, Γ was measured in a closed, plexiglass leaf chamber (volume, 300 mL) using an Anarad IRGA in a differential mode. The technique for determining CO_2 concentration in the leaf cuvette was essentially as described by Atkins and Pate (2). A gas sample of 3 mL was withdrawn from the leaf chamber through a rubber septum at 5 min intervals with a gas tight glass syringe and was immediately injected into the N_2 gas flowing to the sample tube of an IRGA. Γ was measured at varying light, temperature, and O_2 levels. Leaf temperature was maintained during measurements with a jacket of circulating water. The PPFD inside the cuvette, provided from a 1000 W sodium vapor lamp (Lucalux, General Electric), was about 1150 μmol m^{-2} s^{-1}. Lower light intensities were obtained by inserting layers of cheesecloth between the light source and the cuvette. Light intensities were measured using a Lambda Li-85 quantum sensor, inserted into the cuvette. Different O_2 levels were generated by mixing gases from pure O_2 and N_2 tanks using precision flow meters. The leaf cuvette was flushed with the desired gas mixture at 1.5 L min^{-1} for 2 min before it was completely sealed. After 25 to 35 min, the first sample was taken for analysis. At least three consistent measurements were obtained for each condition before the value was recorded.

Γ was also determined at 30°C, 21% O_2, and 1150 μmol m^{-2} s^{-1} PPFD by measuring photosynthesis (CO_2 assimilation) in response to low CO_2 concentrations (0–100 μbars for C_3 and C_3-C_4 species and 0–50 μbars for C_4-like and C_4 species) and extrapolating the initial CO_2 response curve through the *x*-axis. In most cases, the two methods gave very similar values, but the extrapolation method tended to give more consistent results.

[3] Abbreviations: Γ, photosynthetic CO_2 compensation point; GO, glycolate oxidase; HPR, hydroxypyruvate reductase; SHMT, serine hydroxymethyltransferase; NADP-ME, NADP-malic enzyme; PEPC, phospho*enol*pyruvate carboxylase; PPDK, pyruvate, Pi dikinase.

Photosynthesis-CO_2 Assimilation

CO_2 and H_2O vapor exchanges of intact leaves were measured with an open IRGA system as previously described (20). The cuvette was 18.5 cm (L) × 4.5 cm (W) × 5.5 cm (H). Usually one or two attached leaves, depending on the leaf size, were sealed into the cuvette. Unless otherwise stated, the leaf temperature was maintained at 30 ± 0.5°C using a peltier-cooled heat exchanger. The temperature dependence of photosynthesis was determined from 15 to 45°C, starting with the lowest temperature and progressing to the highest temperature. The leaf-to-air water-vapor deficit was controlled by regulating the moisture content at 15 to 25 mmol H_2O mol^{-1} air by passing incoming air through a condenser. The PPFD inside the leaf chamber, provided by a 1000 W multivapor lamp and filtered though 20 cm of water, was 1650 μmol m^{-2} s^{-1}. Various gas mixtures were generated by mixing gases from cylinders containing 1% CO_2 in N_2, and CO_2-free air or 2% O_2 in N_2 using two Wosthoff mixing pumps. The CO_2 concentration in the atmosphere around the leaf was carefully maintained at 325 ± 5 μbars by regulating the flow rate of incoming air. Oxygen inhibition of photosynthesis was measured at 30°C, 325 μbars CO_2 and 1650 μmol m^{-2} s^{-1} PPFD by starting with 21% O_2, then switching to 2% O_2. The rates of photosynthesis were measured after a steady state was reached.

Enzyme Extraction and Assay

About 0.5 to 1 g of illuminated leaf tissue was harvested in the middle of the day, frozen in liquid nitrogen, and quickly ground into fine powder using a mortar and pestle. Five volumes of extraction medium were added and grinding was continued until complete maceration was achieved. The extraction medium contained 50 mM Hepes-KOH (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 10 mM dithiothreitol, 25% (w/v) insoluble PVP, 12.5% (v/v) glycerol along with 10 μM leupeptin and 1 mM PMSF. The crude extract was passed through one layer of Miracloth and aliquots were taken for Chl and protein determination. The filtrate was centrifuged at 15,000*g* for 20 min in a cold room, and the supernatant was rapidly desalted by passing through a small Sephadex G-25 column (0.8 cm in diameter, 5 cm in length). The column was preequilibrated with the extraction medium without PVP and centrifuged at 1,400*g* for 2 min. About 0.4 mL of the crude extract was applied to the column and desalted by centrifugation at 1,400*g* for 2 min at room temperature (13). The eluate obtained by this technique was not diluted and the protein recovery was usually over 90%. The enzyme extract was kept on ice until assay.

Activities of photosynthetic and photorespiratory enzymes were assayed within 30 min of extraction and the assays were performed at 30°C. The amount of enzyme added to the assay medium (0.4–1.0 mL) was 10 to 50 μL, equivalent to 1 to 5 μg Chl of initial extract. GO was measured spectrophotometrically by following the formation of glyoxylate-phenylhydrazone at 324 nm (27). HPR was assayed by monitoring the utilization of NADH at 340 nm (36). SHMT was assayed using [^{14}C]serine following the method of Taylor and Weissbach (31). The assay mixture (0.4 mL) contained 75 mM K-

phosphate (pH 7.4), 0.25 mM pyridoxal-phosphate, 2 mM tetrahydrofolate, and 10 mM 2-mercaptoethanol. The reaction was initiated with addition of 5 mM serine, including 0.1 μCi 3-^{14}C-serine, and stopped after 15 min by addition of 0.3 mL 1 M Na-acetate (pH 4.5). The samples were then processed according to Taylor and Weissbach (31) and the radioactivity originating from methylenetetrahydrofolate counted. PEPC and NADP-ME were assayed spectrophotometrically at 340 nm following the formation of reduced pyridine nucleotide (18, 32).

Chl concentration and Chl *a*/*b* ratio were determined according to Wintermans and De Mots (35) after extraction in 96% ethanol. Soluble protein was assayed by the method of Bradford (5) with BSA as standard.

SDS-PAGE

Electrophoretic analysis of leaf soluble protein was conducted in a 7.5 to 15% linear gradient polyacrylamide gel containing 0.1% SDS, stabilized by a 5 to 17% linear sucrose gradient as previously described (11).

RESULTS AND DISCUSSION

Classification of Photosynthetic Types

Among the 18 *Flaveria* species investigated in this study (Table I), several have not been critically examined for classification into photosynthetic types since Powell's study in 1978 (28). Based on Γ and sensitivity of photosynthesis to O_2 (Table I), *F. robusta* was shown to be a C_3 plant. Like *F. cronquistii* and *F. pringlei,* it has a non-Kranz type leaf anatomy and exhibits a high Γ (62.1 μbars at 30°C and 1150 μmol m^{-2} s^{-1} PPFD) and a high sensitivity of photosynthesis to O_2 inhibition (35.0% inhibition by 21% O_2 at 30°C, 325 μbars CO_2, and 1650 μmol m^{-2} s^{-1} PPFD). In addition to the seven C_3-C_4 intermediates previously reported in the genus *Flaveria* (12) this study identified *F. angustifolia, F. oppositifolia* (Mets), and *F. sonorensis* as new C_3-C_4 intermediates. Similar to other intermediates, these three species have reduced Γ (20.5–29.6 μbars) and reduced O_2 inhibition of photosynthesis (21.0–26.8%), relative to C_3 plants. These species also possess a Kranz-like leaf anatomy with numerous organelles located in a centripetal position (J Constable, unpublished results), similar to that of *F. chloraefolia, F. floridana,* and *F. linearis* (16). *F. oppositifolia* (Brown), also a C_3-C_4 intermediate (6), had a higher Γ but a lower O_2 inhibition of photosynthesis than *F. oppositifolia* (Mets). Since the two plants have very different morphology and physiology of photosynthesis they may be two different species. Further clarification on identification is needed. *F. vaginata* and *F. palmeri,* previously classified as C_4 plants by Powell (28), are now considered C_4-like species (Table I; 24). Although they have a low Γ (3.0–4.7 μbars) and a typical Kranz leaf anatomy

Table I. *Photosynthetic CO_2 Compensation Points, CO_2 Assimilation, and O_2 Inhibition of CO_2 Assimilation in Various Flaveria Species*

Data are means of three to four replicates ± SD

Species	CO_2 Compensation Point[a]	CO_2 Assimilation[b]		O_2 Inhibition[c]
	μbar	μmol m^{-2} s^{-1}	μmol mg^{-1} Chl h^{-1}	%
C_3				
F. cronquistii	60.4 ± 1.7	16.8 ± 0.9	136 ± 10	30.2 ± 0.7
F. pringlei	62.0 ± 0.3	20.9 ± 0.8	198 ± 23	32.3 ± 1.0
F. robusta	62.1 ± 1.0	21.2 ± 0.8	184 ± 14	35.0 ± 1.5
C_3-C_4				
F. angustifolia	24.1 ± 0.4	24.4 ± 0.3	255 ± 16	26.8 ± 0.2
F. anomala	15.5 ± 0.7	13.9 ± 0.3	131 ± 6	22.4 ± 1.7
F. chloraefolia	29.0 ± 2.0	14.2 ± 0.3	116 ± 13	25.7 ± 1.5
F. floridana	9.5 ± 2.0	16.1 ± 1.6	117 ± 10	25.3 ± 0.6
F. linearis	27.0 ± 1.7	17.0 ± 1.7	202 ± 16	25.9 ± 0.9
F. oppositifolia (Brown)	15.0 ± 0.2	25.5 ± 1.9	168 ± 12	28.3 ± 0.3
F. oppositifolia (Mets)	22.3 ± 0.8	20.9 ± 0.8	158 ± 16	21.0 ± 2.5
F. pubescens	21.3 ± 1.2	19.4 ± 0.4	190 ± 12	23.7 ± 1.2
F. ramosissima	9.0 ± 1.7	22.6 ± 0.9	263 ± 12	19.2 ± 0.5
F. sonorensis	29.6 ± 1.0	20.2 ± 0.3	155 ± 7	27.3 ± 0.6
C_4-like				
F. brownii	6.0 ± 1.3	25.2 ± 0.6	205 ± 8	11.4 ± 0.5
F. palmeri	4.7 ± 0.3	29.7 ± 0.3	290 ± 24	4.1 ± 0.9
F. vaginata	3.0 ± 1.2	27.2 ± 2.1	278 ± 19	7.1 ± 0.3
C_4				
F. australasica	5.1 ± 0.4	31.9 ± 0.8	333 ± 22	−1.7[d] ± 0.5
F. bidentis	3.2 ± 0.3	32.4 ± 0.5	200 ± 11	−1.2 ± 0.4
F. trinervia	3.5 ± 0.4	32.0 ± 0.4	322 ± 29	1.5 ± 0.4

[a] Determined at 30°C, 21% O_2, and 1150 μmol quanta m^{-2} s^{-1}. [b] Measured at 30°C, 21% O_2, and 1650 μmol quanta m^{-2} s^{-1}. [c] Expressed as percentage of reduction relative to rate measured at 2% O_2. [d] Negative values represent stimulation of photosynthesis by O_2.

(J Constable, unpublished results), like C_4 plants, they still show some O_2 inhibition of photosynthesis (4.1–7.1%). Together with *F. brownii,* these C_4-like species have high levels of C_4 enzymes but commonly lack a strict compartmentation of key photosynthetic enzymes (*e.g.* Rubisco) between mesophyll and bundle sheath cells, as compared with their C_4 counterparts (9, 14, 25). The presence of some Rubisco in mesophyll cells can account for the small amount of O_2 inhibition of photosynthesis. The three C_4 species *F. australasica, F. bidentis,* and *F. trinervia* all have low Γ and exhibit very little O_2 inhibition. In fact, *F. australasica* and *F. bidentis* showed some stimulation of photosynthesis by 21% O_2.

Photorespiratory Characteristics

CO_2 Compensation Point

Comparisons were made of the Γ of several representative *Flaveria* species measured under different environmental conditions. Γ is a physiological trait that distinguishes C_3 from C_4 plants and can be used as one criterion to identify C_3-C_4 intermediate species. Γ is defined as the ambient CO_2 concentration at which CO_2 uptake through photosynthesis is balanced by photorespiration and to a small extent by respiration and, therefore, Γ is a good indicator of photorespiratory loss of CO_2 from the leaf. The response of Γ to increasing light intensity is shown in Figure 1A. The C_4 plant *F. trinervia* exhibited a typical Γ that changed little with PPFD above about 250 μmol quanta m^{-2} s^{-1}. The two C_3 species had the highest Γ, but both showed substantial decreases in Γ with increasing PPFD. The two C_3-C_4 species had intermediate Γ, and their Γ decreased markedly with increasing light intensity. However, *F. sonorensis* had much higher Γ than those of *F. floridana.* The decrease in Γ with increasing light intensity has been previously observed in other C_3-C_4 intermediates and is greater than in C_3 species (7, 15). However, the present data show that this phenomenon occurs in C_3 species as well and, therefore, cannot be regarded a distinct characteristic of C_3-C_4 intermediates. The reason for this decrease in Γ with increasing light has not been determined, although it may reflect a greater degree of refixation of photorespired CO_2 during photosynthesis at higher PPFD. It would not appear linked to dark-type mitochondrial respiration, since under 2% O_2 (where mitochondrial respiration occurs with little photorespiration) there is not a decrease in Γ with increasing PPFD (15).

In the C_3 species *F. pringlei* and *F. cronquistii,* Γ increased almost linearly with increasing O_2 concentration (Fig. 1B), typical of C_3, while Γ remained low in *F. trinervia* as O_2 concentration increased up to 50%, typical of C_4. The increase in O_2 concentration increases the ratio of soluble O_2/CO_2 in the leaves, which in turn promotes Rubisco oxygenase activity over carboxylase activity in C_3 plants. The C_4 pathway of photosynthesis is thought to concentrate CO_2 and raise the ratio of soluble CO_2/O_2 at the site of Rubisco, thus suppressing the oxygenase activity. The C_4 machinery appears to be very effective in preventing photorespiration and does not allow apparent photorespiration to take place even under 50 to 60% O_2. Earlier studies (9, 16) also reported that Γ of the C_4-like species *F. brownii* is low and is insensitive to increases in O_2

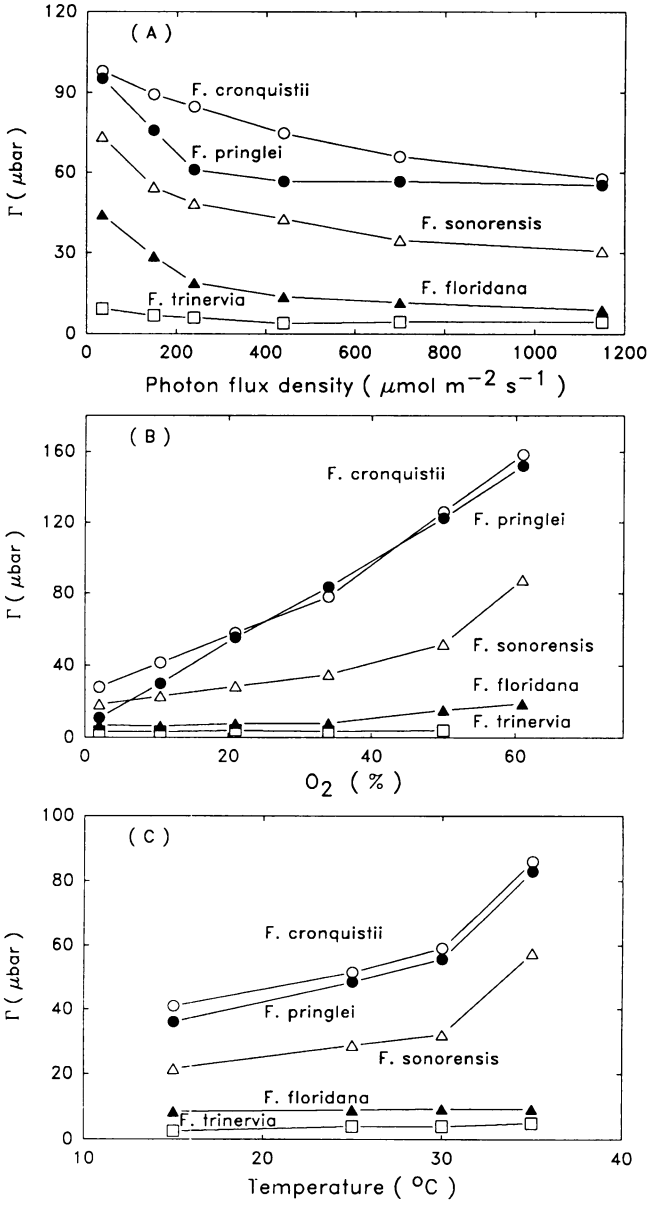

Figure 1. Photosynthetic CO_2 compensation point as a function of light intensity (A), O_2 level (B), and temperature (C) for several *Flaveria* species, representing different photosynthetic types. The measurement conditions were 30°C, 21% O_2, and 1150 μmol quanta m^{-2} s^{-1}, when they were not varied. Duplicate measurements were performed for each species in the various experiments, but only one set of data is presented. For most cases, the differences from the two replicates were within 5 to 10%.

up to 50%. Compared with the C_3 species, Γ for the intermediates *F. sonorensis* and *F. floridana* were less sensitive to changes in O_2 concentration, especially when O_2 concentration was below 50%. These intermediate species possessed a biphasic-like response of Γ to changes in O_2 concentration, although Γ was much lower in *F. floridana* than in *F. sonorensis*. The biphasic response of Γ to O_2 has been observed in other intermediate species belonging to several different genera (12), and may reflect the relative efficiency of the biochemical machinery (*e.g.* the ability to refix photorespired CO_2 or to perform C_4 photosynthesis) of an intermediate species in reducing its photorespiration. Both *F. cronquistii* and *F. sonorensis* had high Γ at low O_2 concentrations (Fig. 1B). This may be caused by a significant contribution of respiratory CO_2 to Γ in these species.

With increasing temperature, Γ increased in the C_3 species, reflecting an increase in photorespiration (Fig. 1C). The increase in Γ was more pronounced above 30°C. In the C_4 species *F. trinervia,* Γ was low throughout the temperature range, indicating low photorespiration due to the CO_2 concentrating mechanism, and refixation of photorespired CO_2. The C_4-like species *F. brownii* also had a temperature response of Γ similar to that of *F. trinervia* (data not shown). Two very different patterns were observed in Γ *versus* temperature with the two intermediates, with *F. floridana* resembling the C_4 species, and *F. sonorensis* resembling the C_3 species.

The variation in Γ for the C_3-C_4 intermediate species *F. floridana* and *F. sonorensis* under different environmental conditions may be due to differences in the degree and relative efficiency of C_4 photosynthesis. *F. floridana* has a functional C_4 cycle and is capable of fixing up to 50% of the atmospheric CO_2 via the cycle (22, 24). However, based on low fixation of atmospheric CO_2 into C_4 acids, *F. sonorensis* appears to have no functional C_4 cycle (24). Intermediates which reduce photorespiration by refixation of photorespired CO_2 in bundle sheath cells with little or no C_4 photosynthesis, would have a limited capacity for refixation. As the rate of production of CO_2 via photorespiration increases with increasing temperature or O_2 concentration, the refixation potential in the bundle sheath approaches saturation and there will be a continuous increase in Γ as demonstrated in the model of von Caemmerer (33). Therefore, intermediates which have some C_4 photosynthesis will have a lower Γ by concentrating CO_2 and limiting the production of glycolate.

The photosynthetic CO_2 compensation points of 18 *Flaveria* species, measured at 21% O_2, 30°C, and 1150 μmol quanta m^{-2} s^{-1}, are shown in Table I. Among the C_3-C_4 intermediate species, there was a large variation in Γ ranging from 9.0 to 29.6 μbars: *F. ramosissima, F. floridana, F. anomala* tend to have a lower Γ while *F. angustifolia, F. chloraefolia, F. linearis, F. oppositifolia* (Mets), *F. pubescens,* and *F. sonorensis* have a higher Γ. The C_4-like species *F. brownii, F. palmeri,* and *F. vaginata* all have a low Γ approaching that of C_4 *Flaveria* species. Differences in the ability to refix photorespired CO_2 and to perform C_4 photosynthesis are factors which could result in variation in Γ among the C_3-C_4 species. A common feature of intermediates may be the ability to refix photorespired CO_2 in the bundle sheath cells. The model of von Caemmerer (33) shows how variations in the partitioning of glycine decarboxylase (a mitochondrial enzyme which catalyzes the release of photorespiratory CO_2) between mesophyll and bundle sheath cells, the fraction of Rubisco in bundle sheath cells, and the leakiness of bundle sheath cells to CO_2 will influence Γ. In addition, intermediates possessing a degree of C_4 photosynthesis can have a lower Γ if the C_4 cycle results in an increased concentration of CO_2 as a substrate for Rubisco.

Photorespiratory Enzymes

The activities of photorespiratory enzymes have not been examined in intermediate species, relative to those in C_3 and C_4 species of *Flaveria*. Three enzymes of the glycolate pathway, GO (located in peroxisomes), SHMT (located in mitochondria), and HPR (located in peroxisomes), were assayed in representative species of the different photosynthetic types of *Flaveria*. The results showed a similar pattern for the three enzymes, with the C_3 species having the highest activity, followed by C_3-C_4 intermediates, then C_4-like species, and last the C_4 species which had the lowest activity (Fig. 2). Among the three photorespiratory enzymes, GO showed the highest correlation (linear correlation coefficient $r = 0.85$) with Γ (Fig. 2D). Plants having the C_4 cycle produce little glycolate via photorespiration, which is consistent with the lower activities of glycolate pathway enzymes. The large variation in activity of these enzymes among the intermediates (Fig. 2), which in some cases was as high as in C_3 species, may be related to differences in the degree of C_4 photosynthesis and glycolate production. That is, the reduction of the expression of photorespiratory enzymes in these species may be a biochemical adaptation to the decreased capacity for glycolate formation during the evolution of C_4 photosynthesis.

O_2 Inhibition of Photosynthesis

O_2 inhibition of photosynthesis (CO_2 assimilation rate at 21% compared to 2%), another qualitative measure of photorespiration, was also determined in the various *Flaveria* species. Compared to the C_3 species, all the intermediate species were less sensitive to O_2 inhibition of photosynthesis at atmospheric levels of CO_2 (Table I). Since the measurements were performed at the same ambient CO_2 concentration, differences in stomatal response among species may also influence the O_2 sensitivity due to variation in intercellular CO_2 concentration. However, the difference between C_3 and C_3-C_4 intermediate species was not accounted for by differences in intercellular CO_2 concentration. At 30°C, 325 μbars CO_2 and a light intensity of 1150 μmol m^{-2} s^{-1}, the intercellular CO_2 concentrations varied from 265 to 270 μbars for the C_3 species, 270 to 280 μbars for the C_3-C_4 species, 240 to 255 μbars for the C_4-like species, and 190 to 220 μbars for the C_4 species. For most intermediates, the percentage of inhibition of photosynthesis by 21% O_2 ranged from 20 to 28%. However, the percentage of inhibition of photosynthesis by O_2 was only 5 to 10% in the C_4-like species. Under the same conditions, photosynthesis of the C_3 species was inhibited by over 30%, whereas there was little effect of O_2 on photosynthesis in the C_4 *Flaveria* plants. At 21% O_2, there was a slight stimulation of photosynthesis in *F. australasica* (1.7%) and *F. bidentis* (1.2%) (Table I), presumably due to the Mehler reaction providing additional ATP for the C_4 pathway.

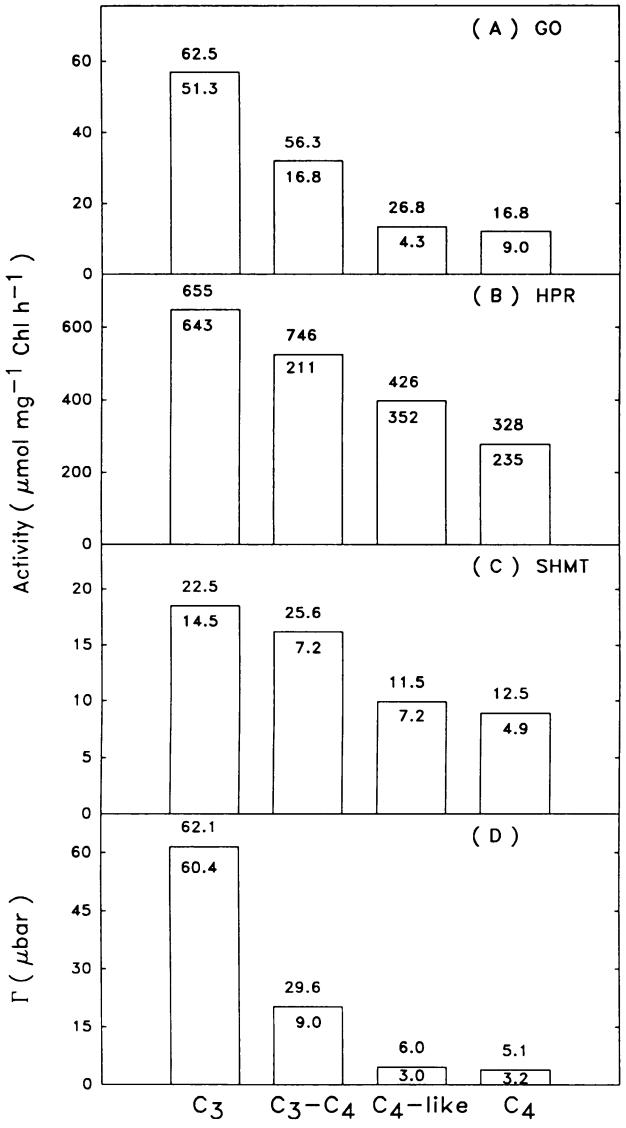

Figure 2. Averaged activities of three photorespiratory enzymes and the photosynthetic CO_2 compensation points for the various photosynthetic types of *Flaveria* species: glycolate oxidase (A), hydroxypyruvate reductase (B), serine hydroxymethyltransferase (C), and CO_2 compensation point (D). See Table I for the species included; data for individual species are not shown. Duplicate assays were performed for each enzyme and the mean values were used. The values presented in the figure represent the range of activity for each photosynthetic type.

A plot of O_2 inhibition of photosynthesis *versus* Γ for the various species was not linear (Fig. 3). Rather, as Γ decreased from C_3 to intermediates there was a limited decrease in O_2 inhibition of photosynthesis, until Γ was below 10 μbars. A simple interpretation of this relationship is that a major contributing factor for the initial reduction of Γ in the intermediates is refixation of photorespired CO_2 in the bundle sheath cells, with less contribution through a functional C_4 cycle. For example, an intermediate which has no C_4 cycle, with most of the Rubisco in mesophyll cells, but with glycine decarboxylase compartmentalized in bundle sheath cells, could have a low Γ due to refixation of photorespired CO_2. However, the Rubisco in the mesophyll cells would still undergo O_2 inhibition of photosynthesis as in C_3 plants during carbon assimilation. On the other hand, the C_4 cycle can effectively reduce O_2 inhibition of photosynthesis by concentrating CO_2 around Rubisco and intermediates having lower photorespiration may partition more Rubisco in bundle sheath cells. It has been shown that glycine decarboxylase, a key enzyme of the glycolate pathway, is almost exclusively localized in the bundle sheath mitochondria of C_3-C_4 intermediates (17, 26). This biochemical modification of glycolate metabolism appears to be common in all intermediates, with and without C_4 photosynthesis, and may be the very first step in improving the efficiency of C_3 photosynthesis. Consistent with this biochemical modification is the observation that C_3-C_4 intermediates partition more organelles (chloroplasts, mitochondria and peroxisomes) to bundle sheath cells than C_3 plants (6).

C_4 Characteristics

Initial Photosynthetic Products

The initial C_4 products of photosynthesis in the various *Flaveria* species was determined in earlier studies by measuring the amount of radioactive label appearing in the C_4-acids malate and aspartate after a short pulse with $^{14}CO_2$ (8, 9, 22, 24, 29). When the data of the percentage of ^{14}C initially incorporated into the C_4 acids from our earlier study (24) were compared with Γ for the various *Flaveria* species, there was a curvilinear relationship between the two (Fig. 4A). Lower Γ occurs in many intermediates, even in those with a relatively low capability for synthesis of malate and aspartate as the initial photosynthetic products via the C_4 pathway. This

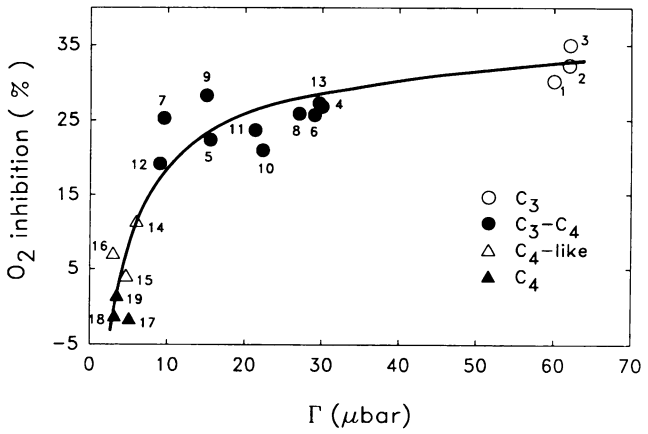

Figure 3. Relationship between photosynthetic CO_2 compensation point and inhibition of photosynthesis by 21% O_2 for various *Flaveria* species (data from Table I). 1, *F. cronquistii*; 2, *F. pringlei*; 3, *F. robusta*; 4, *F. angustifolia*; 5, *F. anomala*; 6, *F. chloraefolia*; 7, *F. floridana*; 8, *F. linearis*; 9, *F. oppositifolia* (Brown); 10, *F. oppositifolia* (Mets); 11, *F. pubescens*; 12, *F. ramosissima*; 13, *F. sonorensis*; 14, *F. brownii*; 15, *F. palmeri*; 16, *F. vaginata*; 17, *F. australasica*; 18, *F. bidentis*; 19, *F. trinervia*.

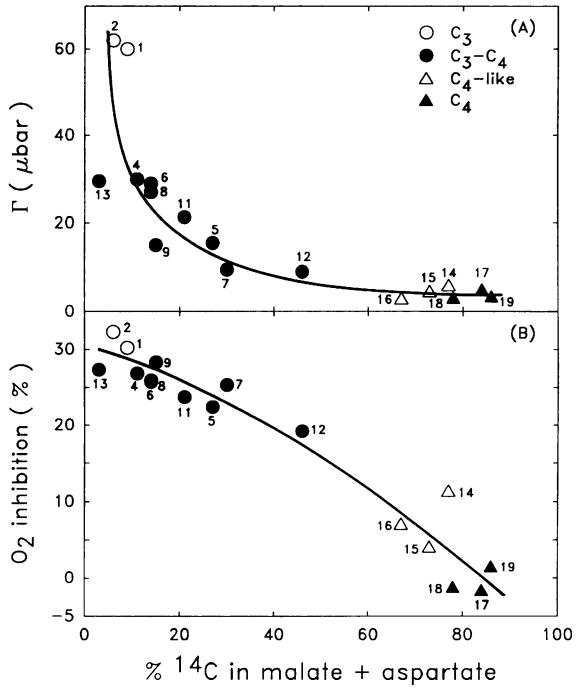

Figure 4. Relationships between the percentage of ^{14}C label incorporated into malate and aspartate following an 8 s pulse with $^{14}CO_2$ and photosynthetic CO_2 compensation point (A) and O_2 inhibition of photosynthesis (B) for various *Flaveria* species. The data of CO_2 compensation point and O_2 inhibition were from Table I and the data of the percentage of ^{14}C label initially incorporated into malate and aspartate were from an earlier study (24). See Figure 3 legend for the species included.

further supports the earlier suggestion that refixation of photorespired CO_2 is a major factor in the reduction of Γ in the intermediates (Fig. 3). On the other hand, the degree of C_4 photosynthesis based on initial products is highly correlated (linear correlation coefficient $r = 0.97$) with the decrease in O_2 inhibition of photosynthesis (Fig. 4B). Again, the results are consistent with the notion that development of the C_4 syndrome is more important than refixation of photorespiratory CO_2 in reducing O_2 inhibition, presumably by effectively concentrating CO_2 around Rubisco. The slower decrease in O_2 inhibition with increasing degree of C_4 photosynthesis in the intermediate and C_4-like species could be explained by a disproportional rather than a linear increase in CO_2 concentration in the leaf with increasing C_4 cycle activity. This relationship could be effected by lack of a strict cellular compartmentation of key photosynthetic enzymes in these species (3, 9, 16, 26), which would result in an inefficient operation of the C_4 pathway and allow more photorespiration to take place.

C_4 Enzymes

PEPC and NADP-ME, two key C_4 enzymes, were chosen for analysis in the various *Flaveria* species. The C_4 and C_4-like species had much higher activities of these enzymes than did the C_3 and intermediate species (Fig. 5). On average, the intermediates had higher activities of these enzymes than the C_3 species, but there was considerable variation in activities among the intermediates. In the intermediates *F. anomala, F. floridana,* and *F. ramosissima,* the activities of PEPC were about threefold higher than in the C_3 species (data for individual species were not shown). *F. anomala* and *F. ramosissima* also had four- to sixfold higher activity of NADP-ME than C_3 plants. *F. anomala, F. floridana,* and *F. ramosissima* are capable of fixing up to 50% of the atmospheric CO_2 through the C_4 pathway (8, 22, 24, 29) and exhibit the lowest Γ (9–15 μbars) among the C_3-C_4 intermediates. Thus, these three species appear to be intermediates both physiologically and biochemically with respect to C_4 photosynthesis. *F. anomala* and *F. ramosissima* also have distinct Kranz-like leaf anatomy resembling that of C_4 *Flaveria* species (20). However, the activities of PEPC and NADP-ME in other *Flaveria* intermediates like *F. angustifolia* and *F. sonorensis* were similar to those of C_3 plants, suggesting again that the conventional C_4 cycle has little or no role in reducing photorespiration in these species.

The higher activities of the two enzymes in some of the intermediate species (*e.g. F. anomala* and *F. ramosissima*) and the C_4-like species are due to higher amounts of the enzyme protein, as shown in the SDS-PAGE analysis of leaf soluble protein (Fig. 6). The amounts of PEPC (100 kD),

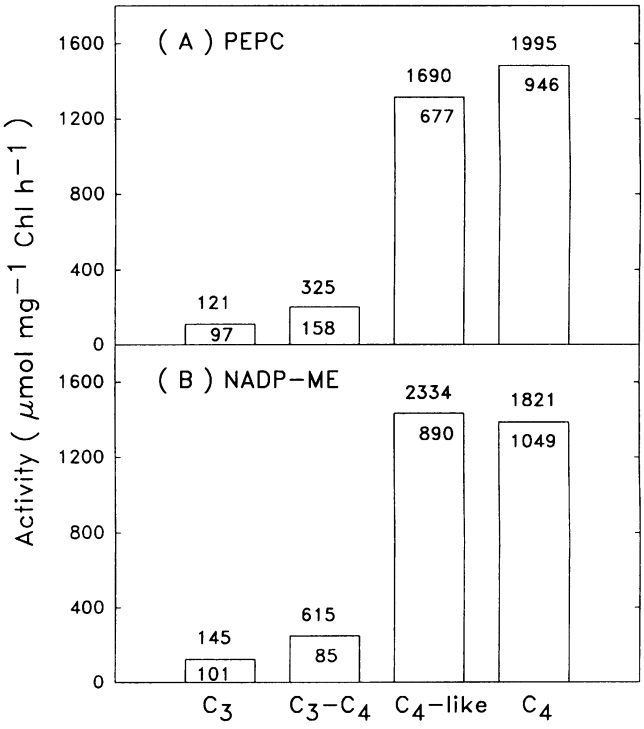

Figure 5. Averaged activities of PEP carboxylase (A) and NADP-malic enzyme (B) for the various photosynthetic types of *Flaveria* species. See Table I for the species included; data for individual species are not shown. Three to four replicates of assays were performed for each enzyme and the mean values were used. The values presented in the figure represent the range of activity for each photosynthetic type.

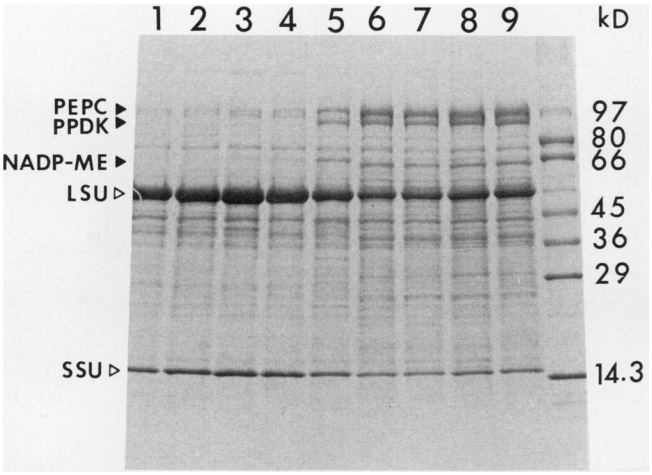

Figure 6. Electrophoretic analysis of leaf soluble protein extracted from several *Flaveria* species, representing different photosynthetic types. Polypeptides were resolved by SDS-PAGE in a 7.5 to 15% linear gradient polyacrylamide gel and stained with Coomassie brilliant blue R-250. All lanes contained 35 μg protein. 1, *F. cronquistii* (C_3); 2, *F. pringlei* (C_3); 3, *F. anamola* (C_3-C_4); 4, *F. ramosissima* (C_3-C_4); 5, *F. brownii* (C_4-like); 6, *F. vaginata* (C_4-like); 7, *F. australasica* (C_4); 8, *F. bidentis* (C_4); 9, *F. trinervia* (C_4). Arrows indicate polypeptides that increased (solid arrows) or decreased (open arrows) their expression from C_3 to intermediate to C_4 species. LSU, Rubisco large subunit; SSU, Rubisco small subunit. See Sheen and Bogorad (30) for the assignments of the various polypeptides. The molecular markers are indicated in kD. The leaf polypeptide composition for other C_3-C_4 *Flaveria* intermediates (not shown) were similar to those of the two C_3 species.

PPDK (95 kD), and NADP-ME (64 kD) polypeptides decreased progressively from C_4 to C_4-like to C_3-C_4 intermediate and C_3 species. In contrast, the amounts of Rubisco large (56 kD) and small (15 kD) subunits decreased progressively from C_3 and C_3-C_4 intermediate species to C_4-like and C_4 species, which is consistent with the results of an earlier study using different methods for quantification (34). Thus, during evolution of C_4 photosynthesis there is an inverse relationship between protein invested into C_4 *versus* C_3 cycle enzymes.

Overall, PEPC and NADP-ME activities, as expected, are positively correlated with the abilities of the species to assimilate atmospheric CO_2 into C_4 acids (linear correlation coefficient r = 0.85–0.90) and negatively correlated with the activities of the photorespiratory enzymes (linear correlation coefficient r = −0.55 to −0.70). Thus, these relationships further support the earlier suggestion that decreased expression of photorespiratory enzymes may be a biochemical adaptation to the development of the C_4 syndrome and the consequent reduction of glycolate formation. It is also interesting to note that none of the C_3-C_4 intermediates possess activities or protein levels of C_4 enzymes comparable to those of C_4-like or C_4 species (Figs. 5 and 6) and that among the intermediates the activities of C_4 enzymes tend to correlate with the development of Kranz anatomy (16, 20). These results suggest that development of Kranz anatomy is critical for and must precede the expression of C_4 biochemistry. Since C_4 photosynthesis requires extra energy to operate the pathway, a highly expressed C_4 biochemistry without a well developed Kranz anatomy and differential compartmentation would be energetically inferior due to futile cycling of the pathway and thus would not confer any ecological benefit.

Chl a/b Ratio

The amount of Chl per leaf area was about the same, on average, among the various photosynthetic types of *Flaveria* (Fig. 7). However, Chl *a/b* ratio increased progressively from C_3 to intermediate to C_4-like and C_4 species. Among the 18 *Flaveria* species, the C_3 species had the lowest Chl *a/b* ratios ranging from 2.49 to 2.85, while the C_4 species had the highest ratios ranging from 3.53 to 3.92. The C_3-C_4 intermediate species had a range of Chl *a/b* ratios from 2.77 to 3.39, but all except one of them were intermediate to those of C_3 and C_4 plants. Among the intermediates, *F. ramosissima* had the highest Chl *a/b* ratio. For the C_4-like species *F. brownii,* the Chl *a/b* ratio was similar to those of intermediate species (3.30), but *F. vaginata* and *F. palmeri* showed typical C_4 Chl *a/b* ratios (3.82 and 4.05, respectively). In C_3 plants, the light-harvesting Chl of PSI contains mostly Chl *a* and the light-harvesting Chl of PSII is thought to contain similar amounts of Chl *a* and *b*. NADP-ME type C_4 plants have high Chl *a/b* ratios in the bundle sheath chloroplasts, which probably reflects a low amount of light harvesting PSII Chl. This higher

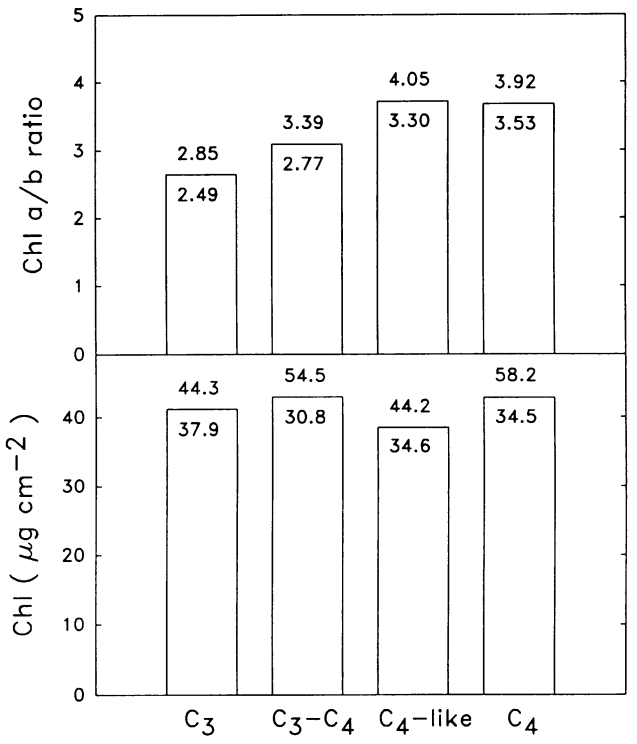

Figure 7. Averaged Chl *a/b* ratios (A) and contents (B) for the various photosynthetic types of *Flaveria* species. See Table I for the species included; data for individual species are not shown. Three to four replicates of assays were performed for each species and the mean values were used. The values presented in the figure represent the range for each photosynthetic type.

Chl *a/b* ratio in bundle sheath results in higher Chl *a/b* ratios for the whole leaf compared to C_3 species (19). The C_4 *Flaveria* species belong to the NADP-ME type (20). Thus, the C_3-C_4 *Flaveria* show some evidence of having the Chl composition which would be predicted of an intermediate. Interestingly, the Chl *a/b* ratios are highly correlated (linear correlation coefficient $r = 0.85$) with the degree of C_4 photosynthesis (percentage of ^{14}C initially assimilated into C_4 acids) in the various *Flaveria* species (Fig. 8). The result suggests that development of the C_4 syndrome (*e.g.* the leaf anatomy and biochemistry) and modification of PSII in the bundle sheath chloroplasts may have coevolved during the evolution of C_4 plants. Modification of the photochemical machinery during evolution of C_4 photosynthesis is apparently necessary in order to meet the specific energy requirements for operating the pathway.

Photosynthesis Rates

The influence of temperature on CO_2 assimilation was examined in several *Flaveria* species representing different photosynthetic types (Fig. 9). This shows that the optimum temperature for maximum rates of photosynthesis for each species varied from high to low in the following order: *F. australasica* (C_4), *F. vaginata* (C_4-like), *F. pubescens* (C_3-C_4), *F. floridana* (C_3-C_4), *F. sonorensis* (C_3-C_4), and *F. pringlei* (C_3). The C_4 species *F. australasica* had an optimal photosynthesis rate at 27 to 30°C, while *F. vaginata*, a C_4-like species, had an optimal photosynthesis rate at 25 to 27°C. The other C_4 *Flaveria* species exhibited a broad temperature optimum between 30 and 35°C (data not shown). The C_3-C_4 intermediate species *F. pubescens* had an optimum rate of photosynthesis at 25°C, compared to 20 to 25°C in the C_3 plant *F. pringlei*. It is also clear from Figure 9 that, at high temperatures, CO_2 assimilation rate decreased to a greater extent in the C_3 and C_3-C_4 intermediate species than in the C_4 and C_4-like species. The ratio of CO_2 assimilation rate at 25°C (near the maximum for most species) to the rate at 45°C increased from 1.5 for *F. australasica* to 1.8 for *F. vaginata*, 2.0 for *F. pubescens*, 2.2 for *F. floridana*, and 2.5 for *F. pringlei*. This can be ascribed to the susceptibility of C_3 and C_3-C_4 intermediate plants to reduced net CO_2 assimilation at higher temperatures due to elevated photorespiration (Fig. 1C). In C_4 plants, the lack of O_2 inhibition of PEPC and the higher internal CO_2 concentration allows relatively high rates of CO_2 assimilation even under the low levels of CO_2 which are expected at high temperatures or drought conditions.

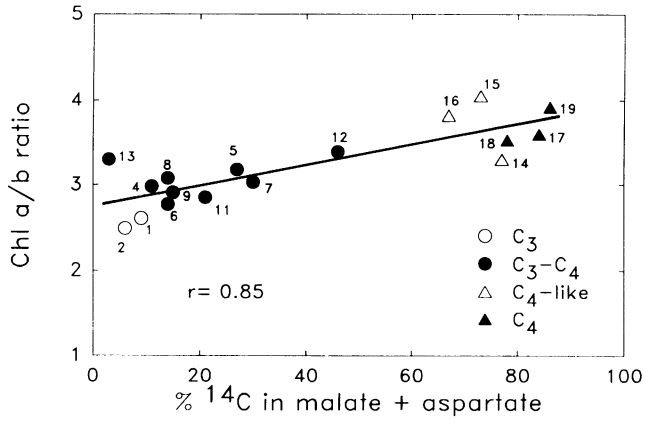

Figure 8. Relationship between Chl *a/b* ratio and the percentage of ^{14}C label incorporated into malate and aspartate following an 8 s pulse with $^{14}CO_2$ in various *Flaveria* species. The data of the percentage of ^{14}C label initially incorporated into malate + aspartate were from an earlier study (24). See Figure 3 legend for the species included.

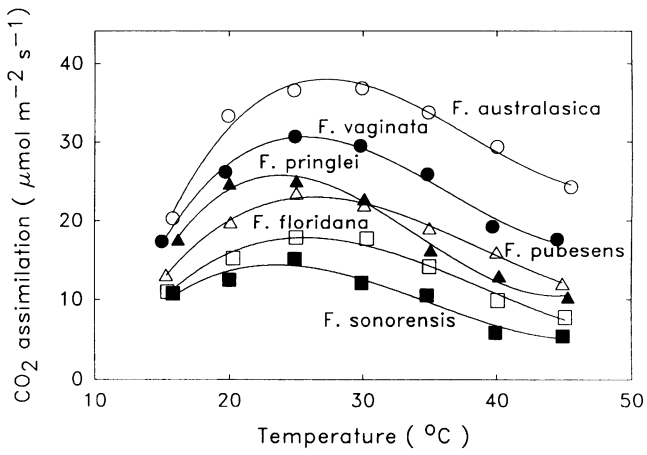

Figure 9. Rate of CO_2 assimilation as a function of leaf temperature in various *Flaveria* species, representing different photosynthetic types. The measurement conditions were 1650 μmol quanta m^{-2} s^{-1}, 21% O_2, and 325 ± 5 μbars CO_2. Duplicate measurements were conducted for each species, but only one set of data is presented. For all species, the temperature response curves from the two replicates were similar.

To make comparisons on the rate of photosynthesis between the 18 *Flaveria* species, measurements were made at 30°C, 325 μbars CO_2, and 1150 μmol quanta m^{-2} s^{-1} (Table I). Although the temperature chosen (30°C) is lower than the optimum temperature for photosynthesis of C_4 *Flaveria* species it is near the optimum temperature for most of the other *Flaveria* species (Fig. 9). As expected, the C_4 species as a group had the highest averaged photosynthesis rate on the basis of either leaf area or Chl content (Fig. 10). In this group of plants, the CO_2 assimilation rates ranged from 31.9 to 32.4 μmol m^{-2} s^{-1} or from 200 to 333 μmol mg^{-1} Chl h^{-1} (Table I). The C_4-like species had an averaged rate (varied from 25.2–29.7 μmol m^{-2} s^{-1} or 205–290 μmol mg^{-1} Chl h^{-1}) slightly lower than the C_4 species, but higher than the intermediate and C_3 species. Although the intermediate species showed a large variation in CO_2 assimilation rate (13.9–25.5 μmol m^{-2} s^{-1} or 116–263 μmol mg^{-1} Chl h^{-1}) the averaged rate was similar to that of the C_3 species. Some of the intermediates, such as *F. angustifolia*, *F. oppositifolia* (Brown), and *F. ramosissima*, exhibited an intermediate rate, compared to C_3 and C_4 species whereas other intermediates like *F. anomala* and *F. chloraefolia* had much lower rates than the C_3 species. The higher rates in some of the intermediate species relative to their C_3 counter parts is apparently not related to whether they are capable of assimilating atmospheric CO_2 via the C_4

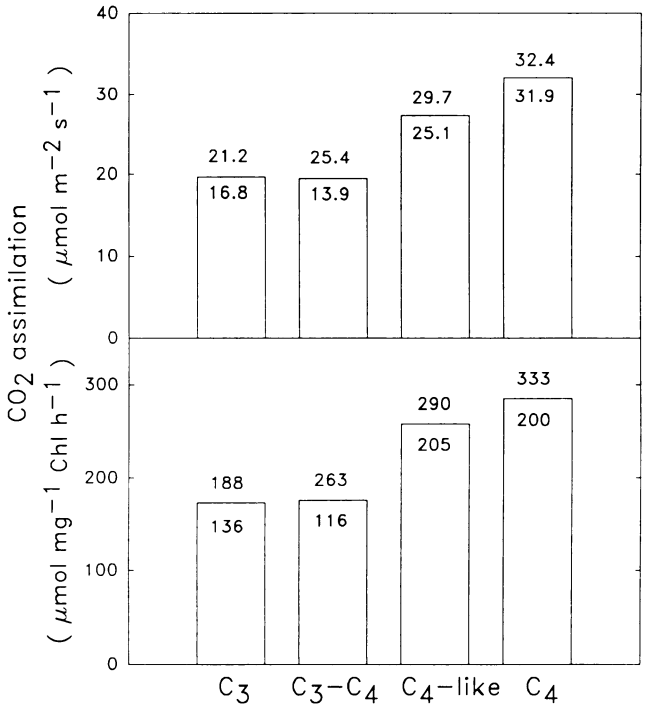

Figure 10. Averaged rates of CO_2 assimilation on a leaf area basis (A) and on a Chl basis (B) for the various photosynthetic types of *Flaveria* species (data from Table I). The values presented in the figure represent the range for each photosynthetic type.

pathway. Therefore, under optimum conditions the partial C_4 syndrome developed in the intermediates does not appear to confer any advantages over C_3 species in photosynthetic capacity. Only under environmental conditions where the internal CO_2 concentration may be reduced (*e.g.* drought or high temperatures) does the ability of these species to reduce photorespiration appear to be beneficial (20, 21).

CONCLUSION

We have examined various *Flaveria* species with respect to photorespiratory and C_4 cycle enzymes, leaf soluble protein profile, Chl *a*/*b* ratio, and gas exchange characteristics in this study and photosynthetic products in earlier studies (9, 22, 24, 25). Based on these photosynthetic characteristics, *Flaveria* species are divided into four photosynthetic groups: C_3, C_3-C_4 intermediate, C_4-like, and C_4 plants. The levels of photorespiratory enzymes and photorespiration (based on Γ and O_2 inhibition of photosynthesis) decreased, while Chl *a*/*b* ratio, C_4 cycle enzymes, the ability to fix atmospheric CO_2 via the C_4 pathway, and photosynthesis rate increased progressively from C_3 to C_4 *Flaveria* species. There is considerable variation in the physiology and biochemistry of photosynthesis and leaf anatomy among the intermediate and C_4-like *Flaveria* species. Several of the intermediate *Flaveria* species (*e.g. F. angustifolia, F. chloraefolia,* and *F. sonorensis*) apparently reduce photorespiration without a functional C_4 photosynthesis. On the other hand, intermediates such as *F. anamola, F. floridana,* and *F. ramosissima* are capable of assimilating atmospheric CO_2 through a limited, functional C_4 pathway. These plants exhibit a more advanced development of the C_4 syndrome (more distinct Kranz-like anatomy and elevated activities of C_4 enzymes) and tend to have a lower photorespiration and the associated O_2 inhibition of photosynthesis. The C_4-like species (*F. brownii, F. palmeri,* and *F. vaginata*) have typical Kranz anatomy, high activity of C_4 photosynthesis and low photorespiration, but still exhibit some sensitivity of photosynthesis to O_2, primarily due to lack of strict compartmentation of key photosynthetic enzymes. Under atmospheric and near-optimum temperature conditions, the C_4 and C_4-like *Flaveria* species have higher photosynthetic rates than their C_3 and C_3-C_4 counterparts. However, there is no consistent difference in photosynthesis rate between the C_3 and C_3-C_4 intermediate *Flaveria* species until CO_2 becomes limiting. Clearly, there is no evidence that the partial C_4 syndrome developed in the various intermediates confers any significant advantage in their capacity to assimilate CO_2 under optimum conditions.

When progressing from C_3 to C_4, the decrease in Γ among the 18 *Flaveria* species is much more rapid than that in O_2 inhibition of photosynthesis. The reduced O_2 sensitivity is highly correlated with the activities of key C_4 enzymes and the abilities of the species to assimilate atmospheric CO_2 via the C_4 pathway. These results suggest that reduction of photorespiration in the C_3-C_4 intermediates, as reflected in Γ, is mainly due to refixation of photorespired CO_2 rather than C_4 photosynthesis. Only when the C_4 syndrome is further developed such as those in the C_4-like species then is the O_2 sensitivity of photosynthesis largely reduced and the capacity of photosynthesis increased. The close relationship between Chl *a*/*b* ratio and the ability to fix atmospheric CO_2 via the C_4 pathway among the various *Flaveria* species also suggests that the photochemical machinery was progressively altered during evolution of C_4 photosynthesis to meet the specific energy requirements for operating the pathway. We conclude from these results that evolution of C_3-C_4 intermediate photosynthesis likely occurred in response to environmental conditions which limit the intercellular CO_2 concentration first via refixation of photorespired CO_2, followed by development of the C_4 syndrome.

ACKNOWLEDGMENTS

We are grateful to L. J. Mets, R. H. Brown, A. S. Holaday, and L. Bayraktaroglu for providing cuttings and Paul Peterson and E. Garcia for help in collecting seeds of several *Flaveria* species used in this study. The authors also wish to thank John Krall and Sandy Edwards for helpful comments on the manuscript.

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