Plant Physiol. (1988) 87, 867–873
0032-0889/88/87/0867/07/$01.00/0

Photosynthesis in *Flaveria brownii*, a C_4-Like Species

LEAF ANATOMY, CHARACTERISTICS OF CO_2 EXCHANGE, COMPARTMENTATION OF PHOTOSYNTHETIC ENZYMES, AND METABOLISM OF $^{14}CO_2$[1]

Received for publication November 25, 1987 and in revised form March 21, 1988

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ABSTRACT

Light microscopic examination of leaf cross-sections showed that *Flaveria brownii* A. M. Powell exhibits Kranz anatomy, in which distinct, chloroplast-containing bundle sheath cells are surrounded by two types of mesophyll cells. Smaller mesophyll cells containing many chloroplasts are arranged around the bundle sheath cells. Larger, spongy mesophyll cells, having fewer chloroplasts, are located between the smaller mesophyll cells and the epidermis. *F. brownii* has very low CO_2 compensation points at different O_2 levels, which is typical of C_4 plants, yet it does show about 4% inhibition of net photosynthesis by 21% O_2 at 30°C. Protoplasts of the three photosynthetic leaf cell types were isolated according to relative differences in their buoyant densities. On a chlorophyll basis, the activities of phosphoenolpyruvate carboxylase and pyruvate, Pi dikinase (carboxylation phase of C_4 pathway) were highest in the larger mesophyll protoplasts, intermediate in the smaller mesophyll protoplasts, and lowest, but still present, in the bundle sheath protoplasts. In contrast, activities of ribulose 1,5-bisphosphate carboxylase, other C_3 cycle enzymes, and NADP-malic enzyme showed a reverse gradation, although there were significant activities of these enzymes in mesophyll cells. As indicated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the banding pattern of certain polypeptides of the total soluble proteins from the three cell types also supported the distribution pattern obtained by activity assays of these enzymes. Analysis of initial ^{14}C products in whole leaves and extrapolation of pulse-labeling curves to zero time indicated that about 80% of the CO_2 is fixed into C_4 acids (malate and aspartate), whereas about 20% of the CO_2 directly enters the C_3 cycle. This is consistent with the high activity of enzymes for CO_2 fixation by the C_4 pathway and the substantial activity of enzymes of the C_3 cycle in the mesophyll cells. Therefore, *F. brownii* appears to have some capacity for C_3 photosynthesis in the mesophyll cells and should be considered a C_4-like species.

A diagnostic feature of C_4 photosynthesis is the fixation of atmospheric CO_2 into C_4 dicarboxylic acids in the leaf MC,[2] with rapid transport of C_4 acids to the BSC and subsequent decarboxylation and refixation of the CO_2 by the C_3 reductive pentose phosphate pathway (10). C_4 metabolism provides a spatial separation of the PEP carboxylation and C_4 acid decarboxylation reactions and enables the leaf to concentrate CO_2 at the site of Rubisco in the BSC. Among NADP-ME type dicots such as *Gomphrena celosioides* and *Flaveria trinervia*, PEPC and PPDK are considered to be restricted to the MC, while Rubisco and NADP-ME are localized in the BSC (4, 24, 26, 30); however, NADP-MDH occurs with substantial activity in both cell types (26, 31).

Flaveria brownii has previously been classified as an NADP-ME type C_4 dicot based on its having distinct Kranz anatomy (14, 15, 29), a low Γ (2, 14, 15), high C_4 enzyme activities (4, 15), and a high percentage of C_4 acids as initial photosynthetic products (3, 13). Recently, however, *in situ* immunofluorescent localization of PEPC and Rubisco in leaves of *F. brownii* indicated that there is no strict intercellular compartmentation of either protein (4, 30), as appreciable fluorescence was observed in both cell types with fluorescein isothiocyanate-labeled antibodies to both enzymes. Unfortunately, the immunofluorescent staining technique does not allow a quantitative determination of enzyme distribution, nor an evaluation of whether the enzyme is functional. Thus, these results do not fully indicate the significance of the incomplete compartmentation of the carboxylases.

In this study, we have isolated MP and BSP from leaves of *F. brownii* to examine the intercellular localization of the major photosynthetic enzymes by activity assays and by SDS-PAGE. The results demonstrate that not only the carboxylases, but also other photosynthetic enzymes lack strict intercellular compartmentation. Furthermore, $^{14}CO_2$ pulse studies suggest that the Rubisco in the MC is active in fixing atmospheric CO_2 through the C_3 cycle. This unusual enzyme distribution and photosynthetic metabolism is discussed with respect to the evolution of C_4 photosynthesis in the genus *Flaveria*.

MATERIALS AND METHODS

Reagents. Sumizyme (source of cellulase) was a gift from Dr. Ryuzi Kanai, Saitama University, Urawa, Japan. Pectolyase Y-23 (source of pectinase) was purchased from Seishin Pharmaceutical Co., Ltd., Noda, Chiba, Japan. Dextran (mol wt 35,000 to 50,000) was from U.S. Biochemical Corp., Cleveland, OH. All other reagents were obtained from Sigma, St. Louis, MO.

Plant Material. Plants of *Flaveria brownii* A. M. Powell (clone B6) were grown in a growth chamber under a 16-h photoperiod with a 25°C day/18°C night thermoperiod. Light was provided by a combination of fluorescent and incandescent lamps, giving a photosynthetic photon flux density of 400 $\mu E.m^{-2}.s^{-1}$ at plant

[1] This work was supported in part by a Washington State University summer research assistantship to S.-H.C. and by National Science Foundation Grant DMB-8506197.

[2] Abbreviations: MC (MP), mesophyll cells (protoplasts); BSC (BSP), bundle sheath cells (protoplasts); PEP (PEPC), phosphoenolpyruvate (carboxylase); Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; NADP-ME, NADP-malic enzyme; PPDK, pyruvate,Pi dikinase; NADP-MDH, NADP-malate dehydrogenase; Γ, photosynthetic CO_2 compensation point; LMP, protoplasts of larger, spongy mesophyll cells; SMP, protoplasts of smaller mesophyll cells; 3-PGA, 3-phosphoglycerate; Ru5P, ribulose 5-phosphate; $t_{½}$, half-time.

height. Plants were propagated vegetatively from cuttings and were watered with a dilute nutrient solution twice a week. Young expanded leaves from the third node were used throughout all experiments.

Leaf Anatomy. Leaf samples (approximately 4 mm^2) were dissected midway between the leaf margin and midrib, fixed at 4°C with 4% (v/v) glutaraldehyde in 50 mM phosphate buffer, pH 7.2, and embedded in "L.R. White" resin according to the supplier's instructions (Polysciences, Inc.). Sections were cut to 1-μm thickness and stained with either methylene blue plus safranin for organelles or periodic acid-Schiff reagent for insoluble carbohydrate.

CO_2 Exchange Measurement. Γ was determined at various O_2 levels in a closed plexiglass chamber. Gas samples were withdrawn from the chamber and injected into an IR gas analyzer (32). Photosynthesis rates under different O_2 concentrations were measured with an open IR gas analysis system (19). CO_2 exchange measurements were conducted with a photosynthetic photon flux density of 1000 to 1200 $\mu E.m^{-2}.s^{-1}$ and at 30 ± 0.5°C.

Protoplast Isolation and Purification. Fresh leaf material was sliced into segments (0.5 × 5 to 15 mm) and suspended in 10 volumes of digestion medium containing 2% (w/v) Sumizyme cellulase, 0.1% (w/v) Pectolyase, 0.5 M sucrose, 20 mM Mes (pH 5.5), 10 mM Na ascorbate, 1 mM $CaCl_2$, and 0.5% BSA. Leaf segments were incubated at 30°C for 2.5 h, with an irradiance of about 100 $\mu E.m^{-2}.s^{-1}$.

Following incubation, leaf digests were filtered through a 211-μm nylon net, and the remaining segments were washed gently with sucrose medium containing 0.5 M sucrose, 5 mM Hepes (pH 7.0), 1 mM $CaCl_2$, and 0.2% BSA and were refiltered. Filtrates were combined and subsequently passed through an 80-μm net and finally through a 61-μm net. Aliquots (3 ml) of the filtrate were poured into 16 × 125-mm glass tubes; these were overlaid first with 1 ml of sorbitol medium (0.5 M sorbitol, 5 mM Hepes [pH 7.0], 1 mM $CaCl_2$, and 0.2% BSA) and then with 1 ml of Hepes medium (0.25 M Hepes [pH 7.0], 1 mM $CaCl_2$, and 0.2% BSA). After centrifugation at 200*g* for 50 s, four fractions of protoplasts were obtained which had either floated to solution interfaces or had pelleted at the bottom of the tube. Fraction I, partitioning at the Hepes/sorbitol upper interface, was the source of LMP; fraction II, partitioning at the sorbitol/sucrose lower interface, and fraction III in the sucrose medium, were the sources of SMP; while fraction IV, the pellet, was the source of BSP.

Fraction I contained purified LMP (with an average diameter of 56 μm). The protoplasts were collected with a Pasteur pipette and resuspended in 2 to 3 volumes of sucrose medium. After sitting on ice for about 1.5 h, the LMP floated to the top of the solution and formed a concentrated protoplast layer. The solution below the protoplasts was removed.

Fraction II contained mostly SMP (with an average diameter of 24 μm) plus some LMP and BSP. SMP were further purified in 16 × 125-mm glass tubes by adding 2-ml aliquots of solution in the following order: 15% dextran medium (sucrose medium plus 15% [w/v] dextran), sucrose medium, fraction II protoplast suspension (collected from the original sorbitol/sucrose interface), and finally Hepes medium. Following centrifugation at 200*g* for 1 min, SMP were collected from the sorbitol/sucrose interface. Protoplasts appearing in other interfaces were discarded.

Fraction III protoplasts were those remaining in the sucrose medium after the initial centrifugation (200*g*, 50 s). This fraction, which contained about equal amounts of SMP and BSP, was used as an additional source of SMP. Dextran powder was added to this fraction, giving a final concentration of 15% (w/v). In 16 × 125-mm glass tubes, aliquots (3 ml) of the dextran-supplemented fraction III suspension were sequentially overlaid with 2 ml each of 10.7% dextran medium (sucrose medium plus 10.7% [w/v] dextran), sucrose medium, and sorbitol medium. After centrifugation at 300*g* for 10 min, pure SMP were collected from the upper interface between the sorbitol and sucrose layers, while protoplasts in other parts of the gradient were discarded. These purified SMP were then combined with those purified from fraction II.

Fraction IV protoplasts, containing about 65% BSP (with an average diameter of 37 μm) and 35% SMP, were those pelleted after the initial centrifugation. After removing the first three fractions, the pellet was first gently dispersed in 3 ml of 15% dextran medium, then overlaid with 2 ml each of the 10.7% dextran medium, sucrose medium, and finally the sorbitol medium. These were centrifuged at 300*g* for 10 min. BSP were collected from the interface between the sucrose and 10.7% dextran media. This preparation was still contaminated with about 15% SMP. To further purify this BSP fraction, dextran powder was added to the fraction, giving a final concentration of 13.5% (w/v). Aliquots (3 ml) of the dextran-supplemented BSP fraction were overlaid sequentially with 2 ml each of the 10.7% dextran, sucrose, and sorbitol media, followed by centrifugation at 300*g* for 10 min to obtain purified BSP from the sucrose and 10.7% dextran interface. Based on microscopic examination and cell counts of several LMP, SMP and BSP preparations, cross-contamination was 2% or less.

Enzyme Extraction and Assays. For the whole leaf extract, leaves were harvested under light after 4 to 6 h into the photoperiod, powdered with a mortar and pestle in liquid N_2, and ground with 5 volumes of 100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTE, 0.1 mM EDTA, 2.5 mM Na pyruvate, 0.5% (w/v) BSA, 0.05% (v/v) Triton X-100, and 2% (w/v) insoluble PVP. The homogenate was filtered through one layer of Miracloth and centrifuged at 15,000*g* for 1 min. For the protoplast extracts, intact protoplasts were first illuminated by an incandescent lamp with a photosynthetic photon flux density of 800 $\mu E.m^{-2}.s^{-1}$ for 1 min (long enough to activate photosynthetic enzymes such as NADP-MDH), and then osmotically shocked in a breaking medium containing 50 mM Hepes (pH 7.5), 10 mM DTE, 2.5 mM Na pyruvate, 1.5 mM K_2HPO_4, 0.5% BSA, and 1% soluble PVP. Chloroplasts were quickly ruptured by two cycles of freezing and thawing and were spun at 15,000*g* for 1 min. The clarified supernatant fluids from both the whole leaf and protoplast extracts were immediately used for enzyme assays. All enzyme assays were conducted at 30°C. PPDK (EC 2.6.1.2) was assayed as described by Edwards *et al.* (9); PEPC (EC 4.1.1.32) was determined after Uedan and Sugiyama (35); and NADP-ME (EC 1.1.1.40) and NADP-MDH (EC 1.1.1.82) were assayed according to Kanai and Edwards (16). The NADP-MDH of the whole leaf extract was fully activated by N_2 and DTE (24). Rubisco (EC 4.1.1.39) was measured radiometrically using $H^{14}CO_3^-$ (21). NADP-triose phosphate dehydrogenase (EC 1.2.1.13) and 3-PGA kinase (EC 2.7.2.3) were assayed as described by Latzko and Gibbs (20). Ru5P kinase (EC 2.7.1.19) was measured as described by Kobza and Edwards (17).

Chl Determination. Chl contents and *a*/*b* ratios were measured according to Wintermans and De Mots (36).

$^{14}CO_2$ labeling and ^{14}C Product Identification. $^{14}CO_2$ labeling experiments were conducted with excised, whole leaves. Conditions and procedures of such experiments followed those of Moore *et al.* (24). Extraction and analysis of metabolites were conducted as described by Rumpho *et al.* (32). Recovery of ^{14}C radioactivity from the plates was essentially 100%. Glycerate was counted with 3-PGA.

Gel Electrophresis. Polypeptide patterns of whole leaves and protoplasts were analyzed by SDS-PAGE. Soluble proteins from whole leaves were extracted according to Wittenbach (37). For

electrophoretic analysis of protoplast proteins, BSA was omitted from all of the media used in protoplast purification. While BSA may protect protoplasts from breakage during isolation, its elimination from the media did not appear to influence the final purity of the various protoplast types. The protein content of whole leaf and protoplast extracts was determined by the Bradford method (6). To prepare protein samples for SDS-PAGE, extracts were mixed with an equal volume of SDS-extraction medium containing 50 mM Tris (pH 7.3), 2% (w/v) SDS, 10% (v/v) glycerol, and 10% (v/v) 2-mercaptoethanol and were then immersed in boiling water for 2 min. SDS-PAGE was performed on a vertical slab gel (0.75-mm thickness) containing a 7.5 to 15% (w/v) linear gradient of polyacrylamide stabilized by a 5 to 17% linear sucrose gradient (resolving gel) (7). The resolving gel was overlaid with a 6% (w/v) polyacrylamide stacking gel. Electrophoresis was conducted at room temperature with a constant current of 20 mA for 2 h. The gel was stained for 2 h in a solution containing 0.1% (w/v) Coomassie brilliant blue R-250, 40% (v/v) methanol, and 10% (v/v) glacial acetic acid. Molecular weight marker proteins were run simultaneously using a premixed solution. The marker proteins used and their mol wt were: rabbit muscle phosphorylase *b* (97,400), human transferrin (80,000), BSA (68,000), ovalbumin (43,000), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (20,000), and α-lactalbumin (14,200).

RESULTS

Leaf Anatomy. Light microscopy showed that *F. brownii* exhibits a Kranz anatomy somewhat characteristic of C_4 dicotyledonous plants. Vascular tissues are surrounded by well developed BSC that contain numerous centripetally arranged chloroplasts (Fig. 1A) (14, 15). However, there are two MC types that can be differentiated by size and organelle content. Smaller MC surround the BSC and contain many chloroplasts. Larger, spongy MC are located between the smaller MC and epidermal cells and have fewer chloroplasts. The arrangement of smaller MC is more compact than that of the larger MC; in the latter case, there are large intercellular air spaces. This interesting leaf anatomy can also be seen in the micrographs from separate studies (14, 15) (although it was not discussed). Starch grains are more prevalent in the chloroplasts of the BSC, but they also occur in the two MC types (Fig. 1B, as indicated by periodic acid-Schiff staining of leaf cross-sections).

Γ and O_2 Inhibition. *F. brownii* possesses typical C_4 values for Γ, which are low and increase, but only very slightly, as the $[O_2]$ is raised (at 30°C, values of Γ are 0.9, 1.1, and 2.7 $\mu l.L^{-1}$ at 2, 21, and 51% O_2, respectively). Unlike most C_4 plants, which have O_2-insensitive photosynthesis (21% O_2 *versus* 2% O_2), *F. brownii* shows about 4% inhibition of net photosynthesis by 21% O_2 versus 2% O_2. A recent study with *F. brownii* also showed inhibition of net photosynthesis and quantum yield for CO_2 uptake by 21% O_2 (7 and 10%, respectively) (23).

Intercellular Localization of Photosynthetic Enzymes. Since the two MC types and BSC are very different in size and organelle content, we were able to isolate these cell types in the form of protoplasts according to differences in their buoyant densities (see "Materials and Methods"). BSP are much denser, apparently due to their large number of chloroplasts (Fig. 2C). Notably, the LMP are much bigger than the SMP (Fig. 2, A and B); they contain fewer chloroplasts and have a lower buoyant density.

The Chl *a/b* ratio of BSP is higher than those of both MP types in *F. brownii*, typical of NADP-ME type C_4 species (Table I). Selected photosynthetic enzymes were assayed in protoplast extracts. On a Chl basis, activities of the C_4 cycle enzymes PPDK and PEPC showed a gradation, being highest in LMP, intermediate in SMP, and lowest in BSP (Table I). Enzyme activity

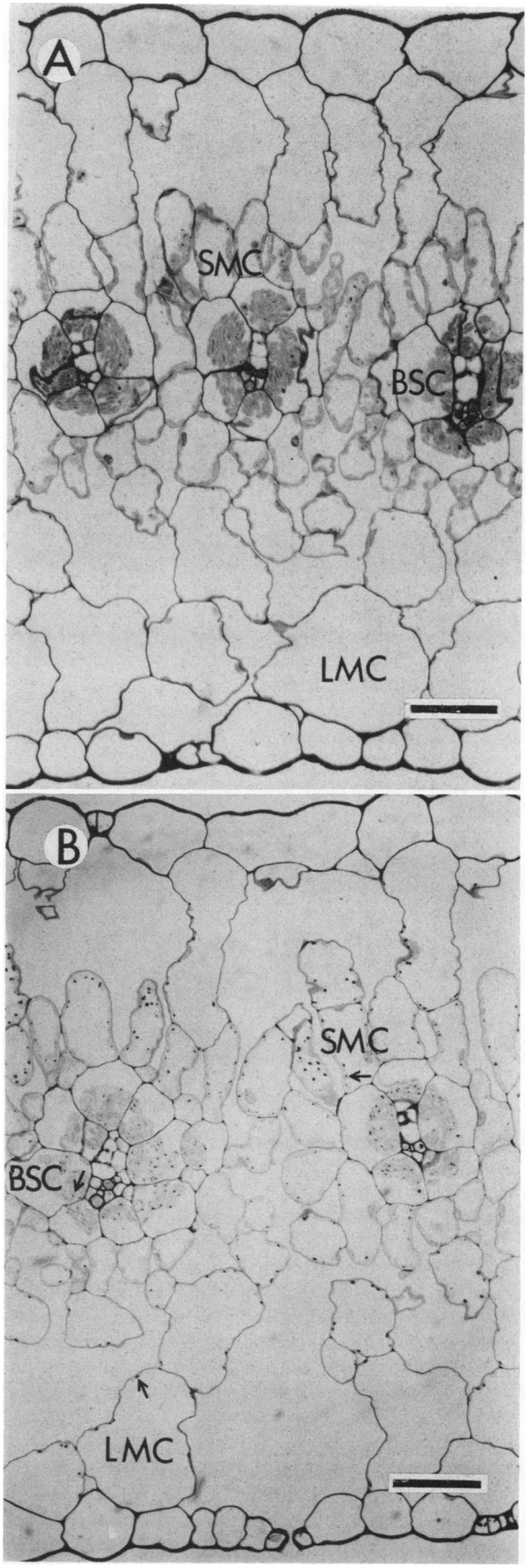

FIG. 1. Leaf cross-sections of *F. brownii*. A, General stain of organelles. LMC, larger, spongy mesophyll cells; SMC, smaller mesophyll cells; BSC, bundle sheath cells. B, Periodic acid-Schiff stain of starch grains. Arrows indicate starch grains. Bar represents 50 μm; × 257.

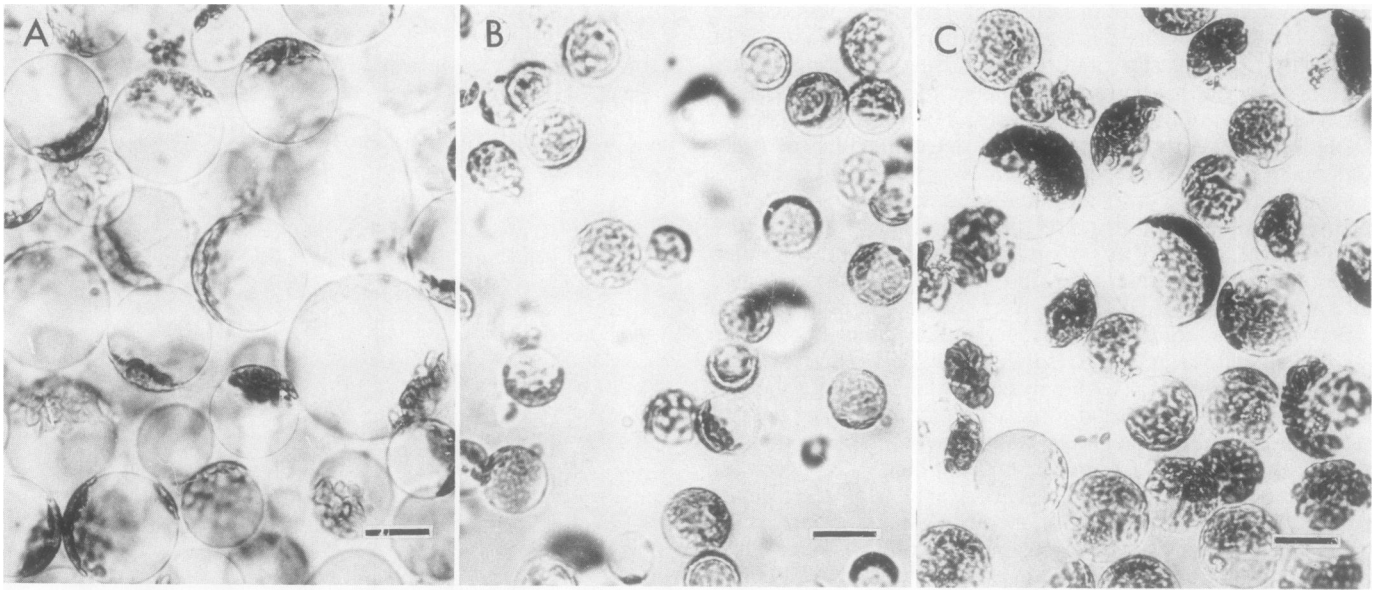

FIG. 2. Leaf protoplast types isolated from *F. brownii*. A, LMP. B, SMP. C, BSP. Bar represents 25 μm; × 484.

Table I. *Intercellular Localization of Photosynthetic Enzyme Activities in F. brownii*

The data presented are from one representative experiment; comparable results were obtained from several other experiments.

Chl *a/b* Ratio or Enzyme	WL[a]	Cell Type			Ratio	
		LMP	SMP	BSP	LMP/BSP	SMP/BSP
Chl *a/b* ratio	4.4	2.9	3.8	4.2		
		μmol·mg Chl^{-1}*·h*$^{-1}$				
PPDK	108	136	67	14	9.7	4.8
PEPC	553	908	463	79	11.5	5.9
NADP-MDH	980	424	309	298	1.4	1.0
NADP-ME	656	34	476	1120	0.03	0.4
Rubisco	305	118	341	420	0.3	0.8
3-PGA kinase	735	565	646	1024	0.6	0.6
NADP-triose phosphate dehydrogenase	1053	545	784	1050	0.5	0.7
Ru5P kinase	1494	288	377	850	0.3	0.4

[a] WL, whole leaf extract; LMP, larger mesophyll protoplast extract; SMP, smaller mesophyll protoplast extract; BSP, bundle sheath protoplast extract.

ratios between MP and BSP indicate that PPDK and PEPC are more abundant in MP. However, substantial activities of these enzymes are also present in BSP. Activities of NADP-MDH are similar in both SMP and BSP, which are lower than that in LMP (Table I). In contrast to the intercellular distribution of PPDK and PEPC, NADP-malic enzyme exhibited a reverse gradation in activity with the highest activity being in the BSP (Table I). Interestingly, substantial activities of the C_3 cycle enzymes Rubisco, 3-PGA kinase, NADP-triose phosphate dehydrogenase, and Ru5P kinase were found in both MP types as well as in the BSP (Table I, *e.g.* in the LMP, enzyme activities were 118, 565, 545, 288 μmol.mg Chl^{-1}.h^{-1}, respectively). These enzymes also showed the same gradation in activity among cell types as did NADP-malic enzyme. The presence of NADP-ME, Rubisco, and Ru5P kinase in the MC is unusual, since in C_4 species these enzymes are exclusively localized in the BSC (4, 18, 24, 31).

The polypeptide banding patterns of total soluble proteins of the three cell types and the whole leaf extract were examined by SDS-PAGE. Consistent with the enzyme activity data, PEPC (100 kD) and PPDK (95 kD) polypeptide bands (33) stained much more intensely in the LMP and SMP extracts than in the BSP extract (Fig. 3). In contrast, the polypeptide band of NADP-ME (68 kD) (33) was expressed predominantly in the BSP and SMP extracts, but showed very little staining in the LMP extract. The subunits of Rubisco (56 and 14 kD) (33) were found in all three cell types, with the majority being present in the BSP and SMP. These results further indicate that a considerable amount of Rubisco protein is present in the MC (also see Refs. 4 and 30), but it is uncertain whether the enzyme is actually functional in these cells *in vivo*. Since activities of 3-PGA kinase, NADP-triose phosphate dehydrogenase, and Ru5P kinase were also found in the MC (Table I), these cells may contain a fully functional C_3 cycle.

$^{14}CO_2$ Labeling. To evaluate whether the C_3 pathway may be operating in the MC *in vivo*, one may estimate the partitioning of atmospheric CO_2 between the C_3 and C_4 cycles following a series of short exposures of leaves to $^{14}CO_2$ and extrapolation of the labeling curves to zero time. Such experiments indicate that at least 78% of the CO_2 is assimilated through the C_4 cycle, while about 20% directly enters the C_3 pathway (Fig. 4, malate + aspartate and PGA + sugar-phosphates, respectively). A similar carbon partitioning pattern of *F. brownii* was also observed by

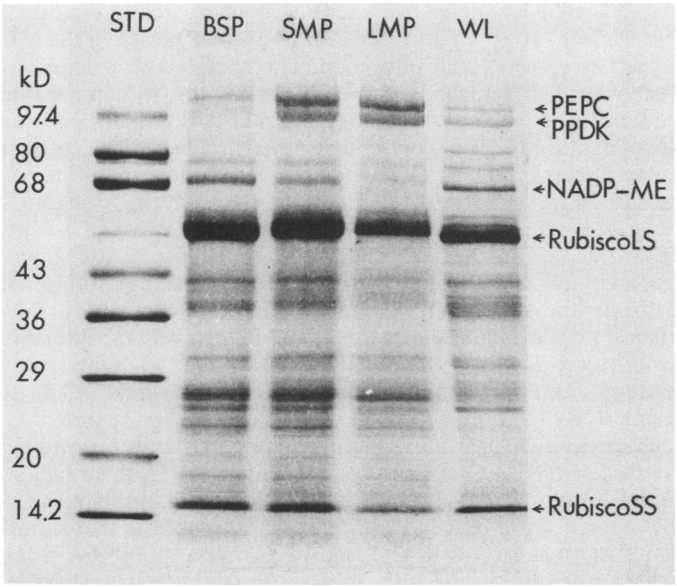

FIG. 3. SDS-PAGE analysis of leaf soluble proteins of *F. brownii* from different fractions. STD, standard proteins; BSP, bundle sheath protoplast extract; SMP, smaller mesophyll protoplast extract; LMP, larger mesophyll protoplast extract; WL, whole leaf extract. PEPC, subunit of PEPC; PPDK, subunit of PPDK; NADP-ME, subunit of NADP-ME; RubiscoLS, large subunit of Rubisco; RubiscoSS, small subunit of Rubisco. Twenty-five micrograms of protein were loaded for each sample. See "Materials and Methods" for molecule weight markers.

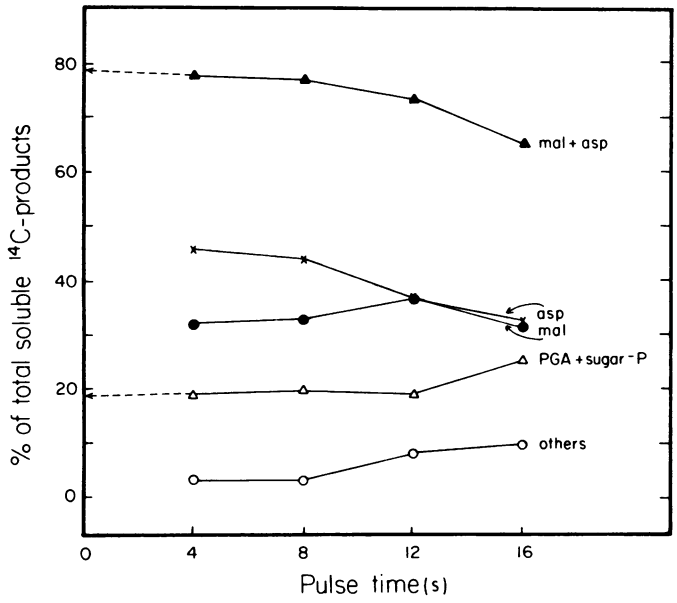

FIG. 4. Changes in percentage distribution of radioactivity among soluble ^{14}C products during continuous exposure to $^{14}CO_2$ (425 $\mu l.L^{-1}$) of leaves of *F. brownii*. The pulse experiments were performed at ~25°C and with an irradiance of ~1000 to 1200 $\mu E.m^{-2}.s^{-1}$. Dashed lines indicate extrapolation of labeling curves to zero time. Metabolite abbreviations are as follow: mal, malate; asp, aspartate; PGA, 3-phosphoglycerate; and sugar-P, sugar monophosphates plus sugar bisphosphates. Metabolites grouped as "others" include fumarate, citrate, glycolate, alanine, serine, and glycine.

Bassüner *et al.* (3). In contrast, in the C_4 species *F. trinervia*, the mature leaf assimilates CO_2 exclusively via the C_4 cycle (determined by the same technique) (32).

The metabolism of the initial photosynthetic products was further evaluated after an 8-s pulse in $^{14}CO_2$ and a subsequent chase in $^{12}CO_2$ for up to 5 min. Total label in the C_4 acids decreased relatively rapidly during the chase period, with the $t_{1/2}$ for apparent turnover of ^{14}C label equal to ~30 s (Fig. 5). This $t_{1/2}$ value is much shorter than those observed in C_3-C_4 intermediate *Flaveria* species ($t_{1/2}$ about 1 to 2.5 min) (22 and 32) but is significantly longer than that in the C_4 species *F. trinervia* ($t_{1/2}$ = 15 to 18 s) (25). Increased labeling of C_3 products (sugar-phosphates and sucrose) during the chase period provides direct evidence that C_4 acids are metabolized fairly rapidly to carbohydrates and are not themselves end products. While *F. brownii* does have a considerable capacity for C_4 photosynthesis and has little apparent whole leaf photorespiration (also see Refs. 2, 14, 15, and 23), the ^{14}C label in glycine plus serine increased gradually during the chase, and they became the major soluble photosynthetic products after 2.5 min.

DISCUSSION

Previously, *F. brownii* has been considered a typical C_4 dicot based on anatomical, physiological, and biochemical criteria (2, 3, 4, 13–15, 29). In agreement with other reports (14, 15, 29), our results show that it exhibits a very distinct C_4 Kranz anatomy based on development of BSC (Fig. 1A). However, it also contains starch grains in the MC as well as in the BSC (Fig. 1B), whereas typically in C_4 plants, starch is primarily found in BSC (10). The apparent whole leaf photorespiration, as indicated by Γ, is very low and insensitive to O_2, yet its photosynthesis is slightly inhibited by 21% O_2 (see "Results" and Refs. 2, 14, 15,

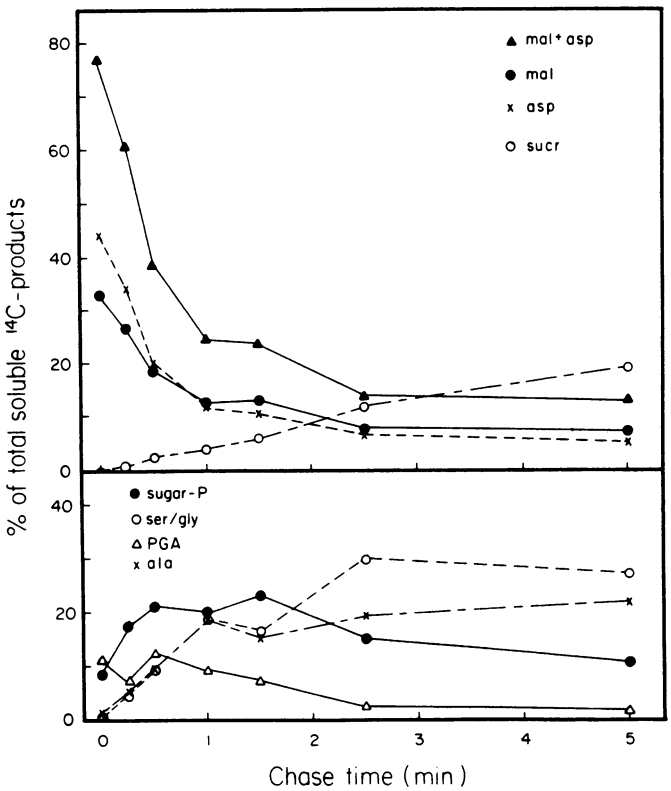

FIG. 5. Changes in percentage distribution of radioactivity among soluble ^{14}C products during a chase in $^{12}CO_2$ (340 $\mu l.L^{-1}$) after an 8-s pulse in $^{14}CO_2$ (425 $\mu l.L^{-1}$) in *F. brownii*. The pulse-chase experiments were performed at ~25°C and with an irradiance of ~1000 to 1200 $\mu E.m^{-2}.s^{-1}$. Metabolite abbreviations are as follows: mal, malate; asp, aspartate; sucr, sucrose; sugar-P, sugar monophosphates plus sugar bisphosphates; ser/gly, serine plus glycine; PGA, 3-phosphoglycerate; ala, alanine.

and 23). This relatively insensitive response of Γ to [O_2] was also observed by other investigators (2, 14), although the absolute Γ values were higher in their studies. O_2 appears to have less effect on apparent photorespiration (as measured from Γ) than on photosynthesis (as measured from O_2 inhibition of photosynthesis). This may be the result of a very effective refixation of photorespired CO_2 through the carboxylase(s), since substantial amounts of photorespiratory metabolites (glycine plus serine) were formed during photosynthesis (Fig. 5) (13).

An important feature of C_4 photosynthesis is the separation of photosynthetic functions between MC and BSC. *F. brownii*, which has been classified as a C_4 species in the past (2–4, 13–15, 29), in fact lacks strict intercellular compartmentation for carboxylases of initial CO_2 fixation (4, 30) and shows CO_2 gas exchange response atypical of C_4 plants (23). The results from Table I support and extend those obtained from localization studies using immunofluorescent staining of PEPC and Rubisco in this species (4, 30). Furthermore, in the present study we demonstrated that not only the carboxylases, but also other photosynthetic enzymes, have an atypical intercellular compartmentation compared to C_4 plants. Recently, a low amount of PPDK protein was also found in maize (an NADP-ME type monocot) leaf BSC, but it is not certain if the enzyme is active, since an immunoblot method was used to locate PPDK (1). The enzyme distribution of PEPC, PPDK, Rubisco, and NADP-ME contrasts markedly with that found in *G. celosioides*, another NADP-ME type C_4 dicot (31). Furthermore, *F. trinervia* (a typical C_4 species in the *Flaveria* genus) showed strict intercellular compartmentation of PEPC, Rubisco, and NADP-ME (4, 24, 26), although the distribution of NADP-MDH in different cell types is similar to that in *F. brownii*. In particular, in *F. brownii*, a gradation in activity is found in NADP-ME, Ru5P kinase, and Rubisco from BSP to SMP to LMP, whereas in C_4 plants these enzymes are exclusively located in BSC. It was uncertain whether the PEPC in BSC cells and the Rubisco in MC were catalytically functional in the previous studies with *F. brownii*, in which an immunofluorescence staining technique was employed (4, 30). Our activity assays of the carboxylases from the respective cell types demonstrated that these enzymes are, indeed, catalytically competent (Table I).

Consistent with the enzyme distribution studies, the continuous $^{14}CO_2$ pulse experiment revealed that ~20% of the CO_2 is fixed directly by the C_3 cycle (Fig. 4, similar to that found in Ref. 3). These results support the idea that there is a fully functional C_3 cycle operating in the MC of *F. brownii*, but they do not preclude the possibility that this species may possess a rather leaky bundle sheath compartment, thus allowing external CO_2 to enter both MC and BSC. It is noteworthy, however, that a substantial amount of starch was found in the MC as well as in the BSC, which is an end product of C_3 photosynthesis (Fig. 1B). Based on microscopic observations (Fig. 1A, *i.e.* chloroplast numbers/leaf area in each cell type) and assuming an equal amount of Chl per chloroplast in each cell type, about 25% of the total whole leaf Chl is estimated to be distributed in MC (~7 and ~18% in LMC and SMC, respectively) and 75% in BSC. Therefore, when considering the Chl distribution and the Rubisco activities in cell types (Table I), the total activity of Rubisco in the MC would be approximately 20% of that in the whole leaf, which is consistent with ~20% direct entry of CO_2 into the C_3 cycle. Thus, it is likely that most of the direct fixation of atmospheric CO_2 into the C_3 pathway occurs in the MC.

While none of the photosynthetic enzymes examined are exclusively localized in either cell type, nevertheless, the enrichment in MC of enzymes of the carboxylation phase of the C_4 cycle and in BSC of Rubisco and an enzyme (NADP-ME) for decarboxylation is sufficient to support a high degree of C_4 photosynthesis. It is not surprising, though, that *F. brownii* exhibits a higher degree of O_2 sensitivity compared to typical C_4 species (see "Results" and Ref. 23), since the Rubisco in the MC would be subject to O_2 inhibition. The relatively slow apparent turnover of C_4 acids in *F. brownii* (Fig. 5) may, in part, be due to the presence of the two carboxylases and NADP-malic enzyme in both the MC and BSC compartments. This might cause a futile cycling of CO_2 fixation, and ultimately reduce the efficiency of carbon transfer from the C_4 cycle to the C_3 cycle. A larger C_4 acid pool size and/or a greater transfer resistance of C_4 acids from the MC to the BSC could also contribute to the slower apparent turnover of the C_4 acids in this species. Additional information is required to interpret these results critically. Furthermore, co-functioning of the C_3 and C_4 pathways presumably would allow Rubisco to incorporate part of the CO_2 in an "open system," resulting in a greater discrimination against $^{13}CO_2$. A leaf $\delta^{13}C$ value of −15.8‰ to −17.3‰ has been reported for this species, which is more negative compared with *F. trinervia* and other typical C_4 species (2, 34). Taking all of these factors into account, biochemically *F. brownii* could be considered a C_4-like species, *i.e.* a more advanced C_3-C_4 intermediate that assimilates the majority of its CO_2 by the C_4 cycle, yet shows some Rubisco activity in the MC. Thus, as noted by Hattersley (11), features such as Kranz anatomy and Γ cannot be used as exclusive criteria for classifying species as C_4 plants.

C_4 plants are thought to have evolved from C_3 plants (27, 29), but the evolutionary path is not yet known. Species exhibiting photosynthetic and photorespiratory features intermediate to C_3 and C_4 plants have been classified as C_3-C_4 species and interpreted as possible "evolutionary links" between C_3 and C_4 plants (29). Based on Γ and $\delta^{13}C$ values of intermediate species, Peisker (28) proposed a model for the evolution of C_4 photosynthesis that included nine hypothetical steps; the lack of Rubisco in the MC was considered as the final step before the completion of true C_4 photosynthesis. Ten intermediate species in the genus *Flaveria* have now been identified. Furthermore, among these 10 species, there is a gradation of intermediacy in the development of Kranz anatomy, reduced photorespiration, and C_4 biochemistry (see review in Ref. 8). *F. brownii* may represent an advanced stage of evolution toward C_4 photosynthesis: a well differentiated Kranz anatomy, low apparent photorespiration, and yet a kinetically intermediate PEPC (5) and a still incomplete compartmentation of photosynthetic enzymes. In fact, Powell had classified *F. brownii* as a more ancient perennial C_4 species in contrast to the more advanced annual C_4 species *F. trinervia* (29).

The NADP-ME type C_4 grass genus *Aristida* was found to be an unusual member of the Poaceae. It lacks a suberized lamella in the BSC such as occurs in other NADP-ME grasses, but it possesses double layers of BSC (namely outer and inner sheaths) (12). Using immunofluorescence staining, Rubisco protein was found in both the inner and outer sheaths but not in the MC. It was postulated that malate is transported from MC and decarboxylated in the inner sheaths (the true Kranz cells). It was suggested that part of the CO_2 may leak out of the inner sheaths due to the absence of a suberized lamella and may be refixed by Rubisco in the outer sheaths (12). When using an immunofluorescent probe to localize Rubisco in *F. brownii*, Bauwe (4) found an MC layer surrounding the BSC which contained a significant amount of Rubisco protein. It is apparent that this cell layer corresponds to the smaller MC of the present study, which have high amounts of both Rubisco activity (Table I) and subunits (Fig. 3). The smaller MC of *F. brownii* might be analogous to the outer sheath of *Aristida*. In *F. brownii*, PEPC in the smaller MC may also contribute to the refixation of CO_2 that leaks from BSC. The structural and biochemical relationships among the photosynthetic cell types of *F. brownii* may be advantageous in carbon assimilation prior to development of the full Kranz syndrome in the evolution of C_4 photosynthesis.

Acknowledgments—We are grateful for the technical assistance provided by Ms. A. J. Lansing (Washington State University, preparation of leaf anatomy) and Dr. V. R. Franceschi (Washington State University, preparation of photographs). We also thank Dr. L. J. Mets (University of Chicago) for giving us the cuttings of *F. brownii* and Mrs. S. Edwards for useful comments on the manuscript.

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