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Leaf Anatomy of C_3-C_4 Species as Related to Evolution of C_4 Photosynthesis[1]

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ABSTRACT

This study was undertaken to examine the degree of Kranz anatomy development in the species intermediate to C_3 and C_4 types (C_3-C_4) in *Panicum, Neurachne, Flaveria,* and *Moricandia*. In each genus, C_3 and/or C_4 species were used for comparison. Leaf transections from each species were examined by light and transmission electron microscopy. The percentages of leaf photosynthetic cell profiles partitioned to bundle sheaths were higher in C_4 than in C_3 species, while C_3-C_4 species tended to be in between. However, percentages for C_3-C_4 species in *Moricandia* and some C_3-C_4 *Flaveria* species were not greater than C_3. When expressed on a cell profile area basis, C_3-C_4 species partitioned more photosynthetic tissue to bundle sheaths than C_3 species in *Moricandia*, but not in *Flaveria. Neurachne minor* S. T. Blake (C_3-C_4) partitioned a very small portion of cell profile area to the inner bundle sheaths (5%) compared to *Neurachne munroi* F. Muell (C_4) (21%). The percentage of organelles partitioned to bundle sheaths was much greater in C_3-C_4 than in C_3 species. The average C_3 percentages for mitochondria plus peroxisomes were 19, 8, and 19.5% for *Neurachne, Flaveria,* and *Moricandia,* respectively, compared to 41, 29, and 46.5% for the C_3-C_4 species. The CO_2 compensation concentration was negatively related to the partitioning of tissue to bundle sheaths and to the percentage of organelles in bundle sheaths. It is concluded that all of the C_3-C_4 species examined have developed some degree of Kranz anatomy and that this altered anatomy is involved in their reduced apparent photorespiration.

Plants with C_4 photosynthesis have a specialized leaf anatomy which is requisite for their CO_2 metabolism and which effectively eliminates photorespiration and increases the capacity for CO_2 assimilation (3, 9). This specialized anatomy is characterized by a well-developed vascular bundle sheath with BSC[2] containing large quantities of organelles (4). The leaf mesophyll of C_4 species is also radially arranged around the bundle sheath and MC are usually located no more than one cell distant from BSC (12). Thus, close spacing of veins and radial arrangement of MC provides maximum contact between MC and BSC, facilitating transport between cell types.

Evolution of C_4 species undoubtedly involved steps in which anatomical characteristics were between those of C_3 and C_4 species. Species intermediate to C_3 and C_4 types have been described in seven genera (8). Although C_3-C_4 species have been characterized mainly by CO_2 exchange and biochemical analyses, they also have anatomical characteristics between those of C_3 and C_4 (5, 7, 14, 15, 17, 20, 21). It is not clear how much development of Kranz anatomy is required to reduce Γ and O_2 inhibition as observed in C_3-C_4 species, but it is certain that well developed Kranz anatomy is necessary for C_4 species.

Although anatomical observations have been made on most species reported to be C_3-C_4 (5, 7, 13–17, 21), quantitative assessment of Kranz anatomy has not been reported except in *Panicum* (7). This report describes a quantitative study of the leaf anatomy of several C_3-C_4 species in different taxa to relate anatomical characteristics to the physiological and biochemical traits reported for these species. The main emphasis is on development of the vascular bundle sheath.

MATERIALS AND METHODS

Plant Selection and Culture

Sixteen species listed in Table I, representing four genera, were selected. *Panicum milioides* was included for comparison with C_3-C_4 species in the other genera, since it and other C_3-C_4 and C_3 species in *Panicum* were examined quantitatively for ultrastructure earlier (7).

The plants were grown from seedlings, except for *Neurachne munroi* and *N. tenuifolia*, which were propagated from vegetative tillers. All plants were grown in a potting mixture containing peat, soil, and perlite in pots ranging in size from 1 to 3 L. Each species was replicated twice. They were fertilized with nutrient solution twice weekly. The plants were grown under fluorescent lighting in a growth cabinet set for a 14-h light/10-h dark period. The temperature was 33 ± 1°C during the light period and 24 ± 1°C in the dark. PPFD was maintained at 500 μmol quanta m^{-2} s^{-1} near the top of the plants during the photoperiod. RH was approximately 50% during the day and 80 to 95% at night.

Anatomy

Leaves were harvested 0.5 to 1 h after the start of the light period. A section was taken from the midlamina region of youngest fully expanded leaves and cut into small pieces in fixative. Specimens were taken from each of the three plants

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[2] Abbreviations: BSC, vascular bundle sheath cells; MC, mesophyll cells; C_3-C_4, species intermediate to C_3 and C_4 types; Γ, CO_2 compensation concentration.

of each species, except *N. munroi, N. tenuifolia,* and *F. linearis,* in which case two specimens were taken from one plant and one from another plant. The pieces from the three replicate leaves were fixed separately in glutaraldehyde (30 mL L^{-1}) in 50 mM phosphate buffer (pH 6.8) at 4°C for 2 h and postfixed in OsO_4 (10 g L^{-1}) at room temperature for 2 h. In the case of *N. minor* and *N. munroi*, the OsO_4 fixation was reduced to 30 min to prevent BSC from staining too heavily. The leaf pieces were embedded in LR white embedding medium under vacuum. Transverse sections about 2-μm thick were cut and stained with toluidine blue for light microscopy. Silver or gold colored transverse sections were cut for electron microscopy. After staining with uranyl acetate and poststaining with lead citrate, sections were viewed and photographed with a Hitachi 500 or Hitachi 600 transmission electron microscope at 75 kV.

Whole cell profiles were photographed in the electron microscope at magnifications of ×1500 or greater. In some cases, cells were too large to photograph at ×1500, and portions of the cell were photographed and a collage prepared of the whole cell profile. In those species for which a distinctive palisade parenchyma was observed, separate photographs were taken of palisade and spongy MC. In *N. minor* and *N. munroi*, cells of both inner and outer bundle sheaths were photographed. At least three, and in some cases as many as 10, cell profiles of each type were photographed for each of the three leaves sampled.

Photographs, 20 × 25 cm (minimum magnification ×3600), were prepared and organelles were counted with the aid of a ×4.5 magnifying lens in the case of mitochondria and peroxisomes. In some photographs, mitochondria and peroxisomes could not be differentiated and so all counts for these organelles are combined. Cell profile areas were measured on the photographs with an electronic planimeter digitizer.

Thick sections were photographed at magnifications ranging from ×100 to ×400 and profiles of BSC and MC were counted. In all *Flaveria* species except *F. brownii,* palisade and spongy MC were counted separately. The percentage of cell profile numbers in the bundle sheaths were calculated from light micrographs. The percentage of total cell profile area in the bundle sheaths was calculated from cell profile numbers in light micrographs and cell profile areas from electron micrographs. The proportion of organelles in BSC was calculated from the number per cell profile and the ratio of BSC to MC profiles determined from light micrographs.

CO_2 Compensation Concentration

To confirm the photosynthetic type of each species, Γ was measured in 50-mL glass syringes using a method described earlier (6). Leaf sections were incubated in the syringes in the same growth chamber in which plants were grown. The distance of the syringe from the lamps was adjusted so that irradiance in the syringe was 500 μmol quanta m^{-2} s^{-1}, and the temperature in the syringe was set at 30°C by adjusting the temperature of the growth chamber. Four replicates of each species were measured.

Data Analysis

Analysis of variance was performed on data from all measurements using a completely randomized design. Species means were compared using Fisher's protected least significant difference (22).

RESULTS

General Anatomical Observations

Characteristics for most of the species studied here have been given in a general way earlier (7, 13–17, 21), and only a few qualitative observations with possible physiological implications will be made. In the two dicotyledonous genera, there was little obvious development of Kranz anatomy in the C_3-C_4 species compared to C_3 when observed in the light microscope (Fig. 1, A, D, E, and F). Although BSC of C_3 species appeared essentially devoid of organelles (Fig, 1, D and E), some development occurred in the C_3-C_4 species represented by *F. anomala* (Fig. 1A) and *M. spinosa* (Fig. 1F). In C_3 and C_3-C_4 species of *Flaveria* a distinct palisade layer was obvious, but it was much less distinct in *Moricandia.* Kranz anatomy was well developed in *F. brownii* (Fig. 1C) and *F. trinervia* (Fig. 1B) as has been documented earlier (1, 16, 21, 29). *F. trinervia* possessed a palisade layer, although not as deep nor as distinct as in the C_3 and C_3-C_4 *Flaveria* species. The palisade in *F. brownii* was almost absent (Fig. 1C).

Panicum milioides had well developed BSC (Fig. 1G) as did *N. minor* (Fig. 1J); however, BSC were much larger than MC in the former but smaller than MC in the latter. *N. tenuifolia* possessed BSC that appeared almost empty at the magnification in Figure 1I. The chlorophyllous BSC in *N. minor* (Fig. 1J)were surrounded by an outer sheath which appeared to have few organelles, and in *N. munroi* (Fig. 1K) the sheaths were similar to *N. minor*, except the outer sheath is less distinct and incomplete for many veins.

The degree of organelle development in BSC was greatly different in C_3, C_3-C_4, and C_4 species when viewed at electron microscope magnifications (Fig. 2), as indicated by the quantitative analysis later. Another feature which differed among species is the arrangement of chloroplasts and mitochondria in BSC. For the C_3 species in *Moricandia* and *Flaveria*, few organelles lined the centripetal wall (Fig. 2, A and E). For the C_3-C_4 and C_4 species (except *N. minor* and *N. munroi*), organelles were concentrated along the centripetal wall, with mitochondria lying adjacent to the wall and chloroplasts tending to overlay the mitochondria (Fig. 2). This tendency was very strong in *Moricandia* (see *M. spinosa*, Fig. 2F, and *M. arvensis* [17]), but less in *P. miliaceum* (Fig. 2G) in which mitochondria were also interspersed among chloroplasts. In *N. minor* (Fig. 3) and *N. munroi*, BSC were more nearly filled with organelles and mitochondria were distributed among chloroplasts (see also ref. 14).

Quantitative Aspects

Within a given genus, the percentage of photosynthetic cell profile numbers occurring in the bundle sheath was lower in C_3 than in C_4 species, and the C_3-C_4 species tended to be in

Table I. *Percentage of Photosynthetic Cell Profiles in the Bundle Sheaths and Percentage of Leaf Chloroplasts and Mitochondria plus Peroxisomes in BSC for Species of Panicum, Neurachne, Flaveria, and Moricandia*

Species	Type	Cell Profiles in Bundle Sheath		Organelles in BSC	
		No.	Area	Chloroplasts	Mitochondria + Peroxisomes
				%	
P. milioides Nees ex Trin.	C_3-C_4	12	31	25	52
P. miliaceum L.	C_4	23	28	29	74
N. minor S.T. Blake[a]	C_3-C_4	19	5	13	41
N. munroi F. Muell[a]	C_4	23	21	34	37
N. tenuifolia S.T. Blake	C_3	11	13	9	19
F. anomala B.L. Robinson	C_3-C_4	18	17	16	29
F. floridana J.R. Johnston	C_3-C_4	14	10	14	27
F. linearis Lag.	C_3-C_4	14	7	15	25
F. oppositifolia (DC.) Rydb	C_3-C_4	18	11	18	35
F. brownii Powell	C_4-like	28	17	53	64
F. trinervia (Spreng.) C. Mohr	C_4	22	20	28	30
F. pringlei Gandoger	C_3	10	9	6	8
M. arvensis (L.) DC.	C_3-C_4	15	17	20	46
M. spinosa Pomel	C_3-C_4	20	14	20	47
M. foleyii Batt.	C_3	14	8	8	21
M. moricandioides (Boiss.) Heywood	C_3	13	6	11	18
LSD (0.05)		5	5	10	12

[a] Outer bundle sheath cells included as mesophyll cells.

between (Table I). The cell profiles in bundle sheaths of C_4 species were about 23% of the total, and for the C_4-like species, *F. brownii*, the value was 28%. *Neurachne minor* also had a similar value (19%). Among the other C_3-C_4 species, only *F. anomala, F. oppositifolia,* and *M. spinosa* partitioned a significantly higher percentage of cell profiles into bundle sheath than their C_3 relatives. *Panicum milioides* had a percentage (12%) similar to the C_3 species in the other genera examined and identical to a value calculated for this species from data of Brown *et al.* (7).

Enlargement of BSC relative to MC would also tend to increase investment of photosynthetic tissue in the bundle sheath of C_4 and C_3-C_4 species. There was, however, no consistent trend for BSC to be larger than MC so that similar or lower percentages of total cell profile area occurred in bundle sheaths compared to the cell number (Table I). The notable exception was P. *milioides* which had 31% of its cell profile area in the bundle sheath, but only 12% of cell numbers, because BSC profiles were much larger (503 μm^2) than MC profiles (146 μm^2) (see Fig. 1G). In *N. minor* the trend was opposite: BSC profile areas (inner sheath) averaged 57 μm^2 and MC 358 μm^2 (Fig. 1J). The percentage of cell profile area in bundle sheaths was higher in C_3-C_4 than C_3 species in *Moricandia,* but in *Flaveria* this was true only for *F. anomala. N. minor* had a lower percentage of cell profile area in bundle sheaths than *N. tenuifolia*, but this is because of the inclusion of outer sheaths with MC in *N. minor.*

In addition to the greater partitioning of cells to bundle sheaths, concentrations of organelles, especially mitochondria plus peroxisomes, were generally higher in BSC of C_4 and C_3-C_4 than in C_3 species (Table II). The mitochondrial plus peroxisomal concentration in BSC of C_3-C_4 *Moricandia* species was twice, or more, than that in the related C_3 species. The largest concentration of organelles was in the small, inner BSC of *N. minor* where chloroplasts and mitochondria plus peroxisomes numbered 77 and 280 $\mu m^{-2} \times 10^{-3}$ cell profile area, respectively. The BSC/MC concentration ratios were also high for this species, being 3.1 and 20.0 for chloroplasts and mitochondria plus peroxisomes, respectively (Table II). An exception to the high concentration of organelles in BSC of C_4 plants was the chloroplast value of 18 $\mu m^{-2} \times 10^{-3}$ recorded for *P. miliaceum,* a concentration similar to that of C_3 *Neurachne* and *Flaveria* species and also similar to that in MC of *P. miliaceum.* On the other hand, mitochondria plus peroxisomes were present in BSC of *P. miliaceum* in the second highest concentration of any species examined and 8.4 times the concentration in MC. The trend for concentration of organelles in BSC of C_3-C_4 species was least evident for chloroplasts in *Moricandia* and mitochondria plus peroxisomes in *Flaveria,* where actual concentrations and the BSC/MC concentration ratio were not significantly higher in some C_3-C_4 than C_3 species.

The tendency for both greater percentages of cell profile in bundle sheaths of C_3-C_4 and C_4 species and for greater concentration of organelles in BSC resulted in a higher percentage of leaf organelles partitioned to BSC (Table I). Partitioning of chloroplasts to BSC was marginally higher for C_3-C_4 than C_3 species in *Moricandia* and *Flaveria* and no greater in *Neu-*

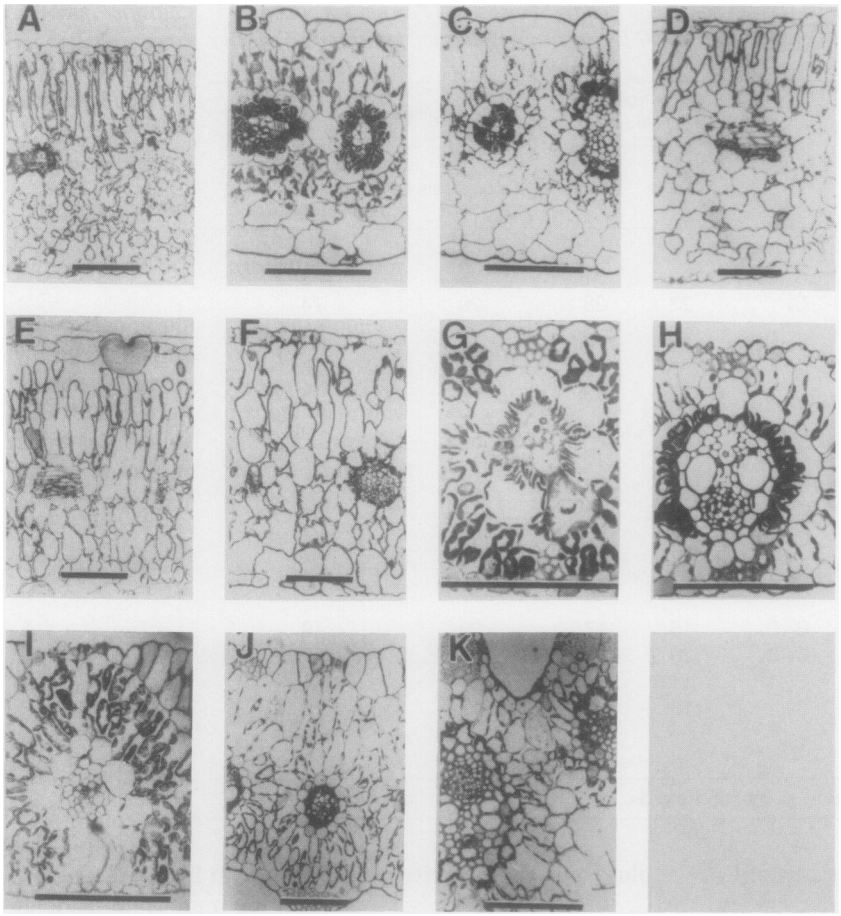

Figure 1. Light micrographs of leaf transverse sections of A, *F. anomala*; B, *F. trinervia*; C, *F. brownii*; D, *F. pringlei*; E, *M. foleyi*; F, *M. spinosa*; G, *P. milioides*; H, *P. miliaceum*; I, *N. tenuifolia*; J, *N. minor*; K, *N. munroi*. Bar = 100 μm.

rachne. However, for *P. milioides*, the C_4-like species *F. brownii*, and the three C_4 species, the percentage of chloroplasts in BSC was at least three times that in the C_3 species. The partitioning of mitochondria plus peroxisomes to BSC of C_3-C_4 and C_4 species was even greater than for chloroplasts. In each genus, the C_3-C_4 species partitioned significantly more mitochondria plus peroxisomes to BSC than in the C_3 species. In *P. miliaceum* and the C_4-like *F. brownii*, higher percentages of leaf mitochondria plus peroxisomes occurred in BSC than in their C_3-C_4 counterparts, but for *F. trinervia* and *N. munroi* the percentages were similar to those of the C_3-C_4 species.

CO_2 Compensation Concentration

The Γ values varied from 52 μL L^{-1} for *M. moricandioides* to zero for *F. trinervia* (Table II). There was no difference in Γ among the C_4 species and *N. minor* had a value of only 4 μL L^{-1}, which was significantly higher than only *F. trinervia*. The four groups of C_3-C_4 species differed with *Neurachne* < *Flaveria* < *Panicum* < *Moricandia*. The C_3 species in *Moricandia* had significantly higher Γ than those in *Flaveria* and *Neurachne*. Results from this experiment are similar to Γ values reported for these species earlier (6, 8, 14, 16, 21).

DISCUSSION

The increased partitioning of organelles to BSC in the C_3-C_4 species studied, compared to the C_3 species, indicates coevolution of leaf anatomy and reduced apparent photorespiration. Although some of the C_3-C_4 species, notably in *Flaveria* and *Moricandia*, do not have very well developed Kranz anatomy (Fig. 1) they all exhibit a tendency to partition more cells to the bundle sheath and to concentrate organelles in BSC. The tendency to partition organelles to the bundle sheath was not accomplished in a parallel way in the various C_3-C_4 species. The small BSC in *N. minor*, for example, resulted in only 5% of the total cell profile area being in the bundle sheath, but the high concentration of organelles in BSC compensated for their small size. In other C_3-C_4 species, increased partitioning of organelles in BSC compared to C_3 species resulted from both higher organelle concentrations and increased BSC size and/or number relative to MC.

It is likely that the partitioning of a substantial proportion of leaf organelles to BSC portends a different photosynthetic metabolism in C_3-C_4 than in C_3 species. Location of 30 to 50% of the leaf mitochondria plus peroxisomes in the BSC, which represent only 12 to 20% of the photosynthetic cells and 13 to 25% of the chloroplasts, suggests that this different metabolism strongly involves mitochondria and peroxisomes. It is not clear, however, whether the role of organelles in BSC is similar in the various C_3-C_4 species. Two schemes for reducing photorespiration in C_3-C_4 species have been postulated (8). In one, C_4 photosynthesis accounts for a portion of the CO_2 assimilated. Presumably, in such C_3-C_4 species there would be decarboxylation of C_4 acids in BSC and refixation

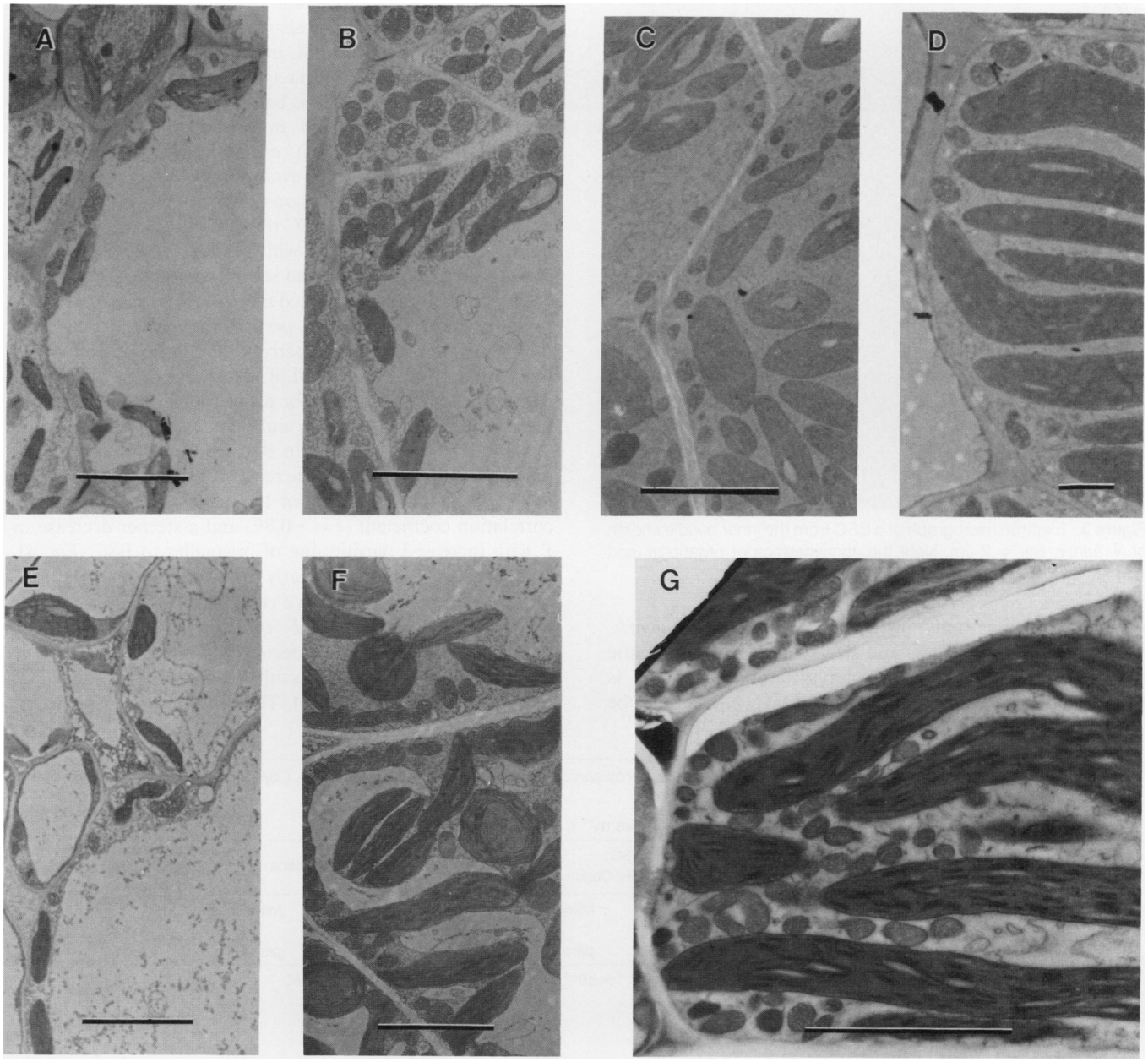

Figure 2. Electron micrographs of sections of BSC (with the centripetal wall to the left) and associated organelles of: A, *F. pringlei*; B, *F. anomala*; C, *F. brownii*; D, *F. trinervia*; E, *M. foleyi*; F, *M. spinosa*; G, *P. miliaceum*. Bar = 5 μm.

of CO_2 by the reductive pentose phosphate cycle. In addition, higher activities of phospho*enol*pyruvate carboxylase would be expected in MC of C_3-C_4 than in C_3 species or in BSC of the C_3-C_4 species. There is evidence that C_3-C_4 *Flaveria* species possess significant levels of C_4 photosynthesis (21, 23, 26, 27).

The second proposed scheme is that CO_2 evolved in photorespiration is refixed by ribulose bisphosphate carboxylase. This would probably require that CO_2 be released and refixed in a compartment separate (*i.e.* in BSC) from initial fixation of atmospheric CO_2. Although no such refixation cycle has been described, there is evidence that it exists (2, 6, 10, 18–20). The release of photorespired CO_2 in BSC might reduce apparent photorespiration for the following reasons. (a) Released CO_2 might be reassimilated by BSC chloroplasts which in some species are interposed between BSC mitochondria and MC (Fig 2). (b) BSC walls may be less permeable than MC walls, making photorespired CO_2 less likely to leak to MC or the atmosphere. (c) If CO_2 release in BSC is more rapid than refixation, CO_2 concentration may rise in BSC which may reduce the synthesis of glycolate and inhibition of carboxylation by BSC chloroplasts. This scheme has, until recently, been without support, but Hylton *et al.* (20) have shown by immunogold localization that glycine decarboxylase is highly restricted to BSC mitochondria in several C_3-C_4

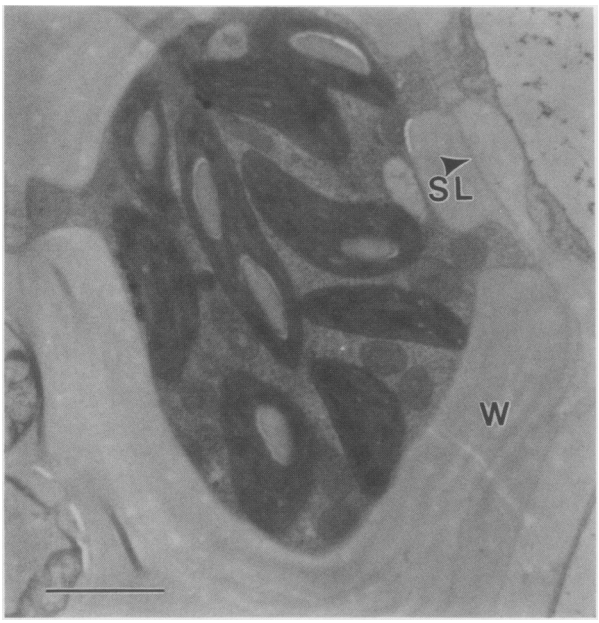

Figure 3. Electron micrograph of a BSC from the inner bundle sheath of *N. minor*. Bar = 2 μm. Note the concentration of organelles and lack of vacuole. SL, suberized lamella; W, cell wall.

species used in this study, including *P. milioides, F. linearis, F. floridana, M. arvensis,* and *M. spinosa.* This dramatic finding suggests that some photorespiratory metabolite is transported from MC to BSC and that CO_2 release may be restricted to BSC. The potential role of BSC mitochondria in reduced photorespiration is also supported by the high mitochondrial partitioning to BSC in the C_3-C_4 species in Table I. Partitioning of mitochondria to BSC is actually underestimated in Table I because it is based only on numbers, and at least for some of these species, mitochondria in BSC are much larger than in MC (20). That restriction of glycine decarboxylation to BSC may precede evolution of C_4 photosynthesis is suggested by the demonstration (20) that glycine decarboxylase is largely restricted to BSC mitochondria of C_3-C_4 species in *Moricandia* and *Panicum* which lack C_4 metabolism (8), as it is also in C_3-C_4 *Flaveria* and several C_4 species (20, 27, 28).

The C_3-C_4 species partitioned more of their organelles (chloroplasts, mitochondria, and peroxisomes) to BSC than did their C_3 relatives, and the percentage of organelles in BSC was closely related to Γ in all of the species (Fig. 4). A single regression did not account for all of the variation in Γ. For the six species in *Moricandia* and *Panicum* the regression was Γ = 63 − 1.08% organelles in BSC ($r = -0.98$). For species in *Flaveria* and *Neurachne* the relationship was similar to that for *Panicum* and *Moricandia* but with a somewhat lower correlation coefficient ($r = -0.89$) and a steeper decrease in Γ with increased partitioning of organelles to BSC (Fig. 4). One species that deviated greatly from the relationship was *F. brownii,* which had a very low Γ, and much higher percentages of organelles in BSC than *F. trinervia* or *N. munroi.* The lower Γ in C_3-C_4 *Flaveria* species for a given percentage of organelles in BSC probably results at least in part from some C_4 cycle metabolism (23, 26). The same may be true for *N.*

Table II. *Concentration of Chloroplasts and Mitochondria plus Peroxisomes in Bundle Sheath Cells and CO_2 Compensation Concentration (Γ) of Species of Panicum, Neurachne, Flaveria, and Moricandia*

Γ was determined at 30°C, 0.21 L, O_2 L^{-1}, and 500 μmol quanta m^{-2} s^{-1} PAR.

Species	Type	BSC Organelle Conc.		Conc. Ratios		Γ
		Chloroplasts	Mitochondria + peroxisomes	Chloroplasts	Mitochondria + peroxisomes	
		No. $\mu m^{-2} \times 10^{-3}$		BSC/MC		$\mu L\ L^{-1}$
P. milioides	C_3-C_4	32	71	0.8	2.4	19
P. miliaceum	C_4	18	111	1.1	8.4	1
N. minor[a]	C_3-C_4	77	280	3.1	20.0	4
N. munroi[a]	C_4	60	60	2.8	4.9	1
N. tenuifolia	C_3	17	13	0.6	1.2	43
F. anomala	C_3-C_4	31	32	0.9	2.3	9
F. floridana	C_3-C_4	52	51	1.4	5.0	13
F. linearis	C_3-C_4	47	36	2.0	3.6	12
F. oppositifolia	C_3-C_4	30	35	1.4	3.6	14
F. brownii	C_4-like	60	36	4.2	7.9	2
F. trinervia	C_4	51	44	2.2	2.4	0
F. pringlei	C_3	15	19	0.5	1.0	43
M. arvensis	C_3-C_4	51	86	1.4	5.2	32
M. spinosa	C_3-C_4	34	48	1.6	6.0	25
M. foleyi	C_3	27	22	1.5	3.3	51
M. moricandioides	C_3	33	25	2.0	2.8	52
LSD (0.05)		34	34	1.3	5.2	4

[a] Organelle concentrations are for inner BSC. Concentration ratios are for inner BSC/MC.

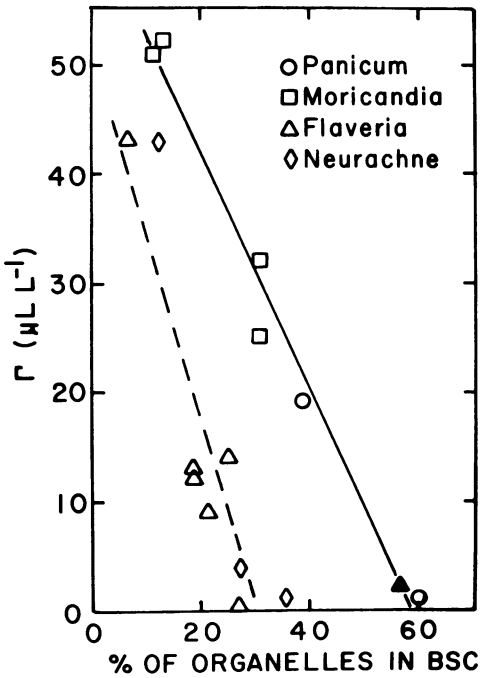

Figure 4. Relationship between the percentage of organelles (chloroplasts, mitochondria, and peroxisomes) in BSC and Γ for species differing in photosynthetic type in *Moricandia, Flaveria, Neurache,* and *Panicum.* The regression for *Panicum* and *Moricandia* (continuous line) is Γ = 63 − 1.08% organelles in BSC (r = −0.98) and for *Neurachne* and *Flaveria,* excluding *F. brownii* (▲) (dashed line) is Γ = 51 − 1.68% organelles in BSC (r = −0.89).

minor (11), but Moore and Edwards (25) found little ^{14}C assimilated into C_4 acids in this species, and its low Γ may also be due to impermeability of its BSC. Centrifugal walls of inner sheath cells of *N. minor* averaged 0.76 μm thick, compared to 0.14 μm (micrometers) for BSC of *P. milioides* (data not shown, but see Fig. 3). In addition, *N. minor* possesses a suberized layer in the centrifugal walls (Fig. 3).

It would be expected that organelle development in BSC of C_3-C_4 species might reflect the C_4 type toward which it is evolving. Although it cannot be known with certainty which C_4 type may result from evolution of a given C_3-C_4 species, or even if a given species represents an intermediate evolutionary stage, consideration of the differences among C_3-C_4 species of the different genera may be fruitful. *Panicum milioides* is not very closely related to *P. miliaceum,* but centripetal location of organelles in BSC and the large mitochondria in BSC of *P. milioides* are similar to those of *P. miliaceum* and other NAD-malic enzyme C_4 species. Precursor species of NAD-malic enzyme types would be expected to have centripetal location of organelles and numerous large mitochondria in BSC.

Because mitochondria in BSC of NADP-malic enzyme and phosphoenolpyruvate carboxykinase type C_4 plants do not appear to be as directly involved in C_4 acid decarboxylation as in NAD-malic enzyme types (9), it might be expected that C_3-C_4 precursors of these C_4 types would invest fewer mitochondria in BSC. However, *F. brownii* had more of its mitochondria in BSC than any of the species examined, except *P. miliaceum* and *N. minor* had as many as any of the other C_3-C_4 species. *F. brownii* is considered to be a C_4-like C_3-C_4 intermediate based on anatomy, biochemistry, and CO_2 exchange (16, 23, 24, 26, 29). In both *Flaveria* and *Neurachne,* C_4 species are NADP-malic enzyme types (1, 8, 11, 16). Thus, high concentrations of mitochondria in BSC may be a condition which precedes full development of C_4 photosynthesis in all types. It is interesting, however, that the C_3-C_4 species in *Flaveria* and even *F. trinervia* partitioned fewer mitochondria plus peroxisomes to BSC than the C_3-C_4 species in *Panicum* or *Moricandia* (Table I).

Since functional BSC are required before compartmented metabolism of C_4 acids is possible, the first step in evolution of C_4 species may be increased metabolic activity of BSC, whether related to C_4 acid metabolism or not. The increased concentration of organelles, especially mitochondria plus peroxisomes, in BSC of the C_3-C_4 compared to C_3 species in this study suggests this may be the case. In *M. arvensis* and *P. milioides,* there is little evidence that C_4 metabolism occurs (8) and yet partitioning of these organelles to BSC in these species is at least as great as in the C_4 species *F. trinervia* and *N. munroi.* A secondary or concurrent change during evolution may be the investment of increased proportions of leaf tissue in BSC. One result of partitioning more tissue in the bundle sheath is reduced distance for transport of metabolites between cell types. This is apparently essential in C_4 photosynthesis, but may also be beneficial in other plants where BSC have specialized metabolism requiring interaction with MC. Based on total cell profile area (Table I), increased tissue investment has occurred in BSC of C_3-C_4 species in *Moricandia* and undoubtedly in *P. milioides* (7, 10), but not in C_3-C_4 *Neurachne* nor *Flaveria,* except *F. anomala.*

Once the anatomical and ultrastructural framework for BSC metabolism has been established, then modification of metabolism to accommodate C_4 photosynthesis may occur by acquisition or expression of genes for the necessary enzymes in the appropriate compartments. If decarboxylation of glycine occurs in BSC after transport of a precursor from MC in the C_3-C_4 species as suggested by data of Hylton *et al.* (20), and if subsequent products, perhaps serine, are returned to MC, then a carbon transport pathway may already be established in C_3-C_4 species for use by C_4 cycle metabolites. If some metabolite subsequent to serine is returned to MC then transport of amino groups must also occur to prevent N accumulation in BSC. Transfer of amino acids to maintain N balance is known to occur in C_4 species of the phospho*enol*pyruvate carboxykinase type (9).

This quantitative assessment of leaf anatomy in C_3-C_4 species suggests the importance of bundle sheath development in reduction of apparent photorespiration. The increased partitioning of organelles to BSC resulted from changes in both BSC/MC ratios and concentration of organelles in BSC. It is likely that in evolution of C_3-C_4 species metabolism in BSC occurred for some purpose beneficial to the plant, such as refixation of photorespired CO_2. The benefit of refixation in BSC is not clear at present, but it may also be that BSC development for a purpose other than C_4 acid metabolism preceded development of the C_4 syndrome.

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