Plant Physiol. (1988) 88, 532–536
0032-0889/88/88/0532/05/$01.00/0

Communication

Inheritance of C_4 Enzymes Associated with Carbon Fixation in *Flaveria* Species[1]

Received for publication February 10, 1988 and in revised form May 31, 1988

RANDALL G. CAMERON[2] AND CAROLE L. BASSETT*
Department of Agronomy, University of Georgia, Athens, Georgia 30602 (R.G.C.), *and U. S. Department of Agriculture, Agricultural Research Service, P.O. Box 5677, Athens, Georgia 30613* (C.L.B.)

ABSTRACT

Activities and subunit levels of three C_4 enzymes were determined for F_1 hybrids between C_4 and C_3-C_4 *Flaveria* species. For phosphoenolpyruvate carboxylase and pyruvate orthophosphate, dikinase, enzyme amounts in the hybrids were close to the mid-parent means. However, activity and subunit levels of NADP-malic enzyme were approximately one-half the mid-parent mean.

A selective advantage has been observed for C_4 over C_3 plants in a variety of habitats (22). One reason for this greater fitness is the higher level of AP[3] in C_4 species believed to result from the CO_2 concentrating mechanism in these plants (18, 19). This mechanism relies on the enhanced activities and compartmentation of enzymes associated with the C_4 pathway which include PEPC, PPDK, and NADP-ME (5a, 12).

The discovery of C_3, C_4, and C_3-C_4 intermediate species in the genus *Flaveria* (Asteraceae) provides an opportunity to hybridize species which can produce potentially fertile F_1 offspring (6, 21). Characterization of hybrids between C_3 and C_4 species (12, 21) and between C_3-C_4 and C_4 species (6, 8) has demonstrated that a number of C_4 traits can be transferred. For example, increased PEPC activity (6, 7, 12), reduced CO_2 compensation concentration (6, 7, 12), decreased O_2 inhibition of AP (6, 7), and increased ratios of $^{13}C/^{12}C$ (6) have all been observed for some of the hybrids. However, in all cases examined to date, AP has been equal to or less than that of the C_3-C_4 or C_3 parent (6, 7). Thus it is clear that transferring sufficient C_4 characters to C_3 or C_3-C_4 species to increase yield by maximizing photosynthesis requires a more complete understanding of the inheritance and regulation of the enzymes associated with this pathway.

The present study follows the inheritance of PEPC, PPDK, and NADP-ME in hybrids between *Flaveria trinervia* (Spreng.) C. Mohr (C_4) and the intermediate (C_3-C_4) species, *Flaveria floridana* J. R. Johnston, *Flaveria linearis* Lag., and *Flaveria oppositifolia* (O.C.) Rydb. The activities of two of these enzymes were measured to assess transfer of the genes encoding them in the F_1 hybrids; in addition, subunits of all three enzymes were identified by immunoblotting using antibodies against the maize and pearl millet enzymes, and subunit amounts were estimated from SDS-polyacrylamide gels. The results are interpreted in the light of what is currently known about the regulation of expression of enzymes associated with carbon fixation.

MATERIALS AND METHODS

Preparation of Crude Extracts. All plant material was kindly provided by Dr. Joe Bouton (Agronomy Department, University of Georgia). *Flaveria trinervia* and *Flaveria linearis* were grown from seed obtained from M.S.B. Ku; *Flaveria oppositifolia* and *Flaveria floridana* were collected in Florida by H. Brown and P. Evans. Replicas of each species were vegetatively propagated and grown in a glass greenhouse under natural photoperiod conditions (Athens, GA, summer 1986). Samples were harvested under full sunlight. Leaf tissue from the third node below the growing apex was extracted as previously described (6), and the supernatants were stored at −80°C before assaying for PEPC and NADP-ME activity. Extracts were prepared from different branches of the same individual weekly for three consecutive weeks.

Enzyme Activity: Activity of PEPC [EC 4.1.1.31] in crude extracts was assayed as previously described (7). Three replicas at two dilutions were assayed for each sample. Measurements were corrected for endogenous activity in the absence of substrate, when appropriate.

Activity of NADP-ME [EC 1.1.1.40] was assayed in crude extracts by the method of Kanai and Edwards (15). The reaction was initiated by the addition of 0.4 mM NADP and the activity was determined by following the increase in A_{340}. Reactions were run without $MgCl_2$ to correct for endogenous NADP-MDH activity.

To determine if any extracts contained PEPC or NADP-ME inhibitors, samples of *F. trinervia* were assayed after dilution with an equal volume of buffer or equivalent dilution of extract from each C_3-C_4 parent and hybrid sample. A decrease in enzyme activity compared to *F. trinervia* plus assay buffer alone would have suggested the presence of inhibitors in the other parents or hybrids. No decrease in activity was observed for the two enzymes using any sample tested.

Protein Analysis. Protein concentration of each sample was determined by the method of Bradford (5) using BSA as a standard.

Protein Separation. Soluble proteins from each sample were precipitated overnight at −20°C by the addition of 100 to 500 volumes of cold acetone to extracts prepared for enzyme assays. Samples (10 μg protein/lane) and mol wt standards (Sigma) were

[1] Supported by the University of Georgia Program in Biological Resources and Biotechnology.

[2] Present address: Citrus and Subtropical Products Laboratory, Winter Haven, FL 33883.

[3] Abbreviations: AP, apparent photosynthesis; PEPC, phosphoenolpyruvate carboxylase; PPDK, pyruvate Pi,dikinase; NADP-ME, NADP-malic enzyme.

prepared and electrophoresed in 12.5% acrylamide gels according to Laemmli (16). The gels were silver stained according to the procedure of Blum *et al.* (4).

Electrophoretic Transfer of Polypeptides. After SDS-PAGE, the polypeptides (75 μg/lane) were transferred to nitrocellulose filters (0.45 μm pore size, Schleicher & Schuell) in an LKB Trans Blot apparatus at 360 V·h. Transfer buffer contained 4% methanol, 0.625 mM Tris, 4.8 mM glycine, and 0.025% SDS. Transferred polypeptides were first visualized by staining with Ponceau S (Sigma), and their positions on the filters were marked. The filters were then blocked with 1% BSA (Sigma) in PBS containing 10 mM Na_3PO_4 and 140 mM NaCl.

Polypeptides of PEPC, PPDK, and NADP-ME were visualized in separate experiments by probing each filter with polyclonal antibody against either PEPC (provided by Dr. J. Rawson, SOHIO, against pearl millet), PPDK (provided by Dr. K. Aoyagi, Rockefeller University, against maize), or NADP-ME (provided by Dr. T. Nelson, Yale University, against maize). After incubating the filters in PBS + goat anti-rabbit alkaline phosphatase conjugate (Sigma), the blots were stained with 5-bromo-4-chloro-3-indolyl phosphate/*p*-nitro blue tetrazolium chloride for alkaline phosphatase activity (3). In subsequent experiments the filters were probed with a mixture of the three antibodies to visualize the polypeptides in a single extract, thereby minimizing loading and sample preparation artifacts. No bands below NADP-ME were observed in the immunoblots.

Densitometric Scans: After confirming the positions of each enzyme subunit from immunoblots, the silver stained gels were scanned with an LKB 2202 Ultroscan Laser Densitometer and the relative peak area corresponding to the appropriate enzyme subunit was calculated with the software provided (Gel Scan Program). The linearity of the scan data was confirmed by scanning a gel loaded with increasing amounts of partially purified maize PEPC (Sigma).

RESULTS AND DISCUSSION

Enzyme Activity. Activities of PEPC and NADP-ME were determined for two seedlings of *F. Trinervia*, three C_3-C_4 *Flaveria* species, and the F_1 progeny resulting from their crosses. Activity was computed on the basis of total soluble protein extracted. The activities of both enzymes were greatest in the two C_4 parents, being approximately 10- to 20-fold higher than in the C_3-C_4 individuals (Table I). The F_1 hybrids had levels of PEPC activity that were elevated with respect to the C_3-C_4 parents, but approximately one-half that of the C_4 parents. Similarly, NADP-ME activities in the hybrids were elevated compared to the C_3-C_4 parents; however, the levels were only about one-fourth those of the C_4 species. When compared to the estimated mid-parent mean which takes into account the contribution (though minimal) of the C_3-C_4 parent, PEPC activity in the hybrids averaged approximately 88%, whereas NADP-ME activity averaged only about 54%. Similar results were obtained when activity was computed on the basis of leaf fresh weight.

The reduced activities of NADP-ME were observed in the hybrids regardless of the C_3-C_4 parent. Diffusible inhibitors of enzyme activity produced in the C_3-C_4 parents could explain the reduction in enzyme activity observed in the hybrids. However, as the mixing experiments revealed no adverse effects on the activity of C_4 extracts *in vitro*, it is not likely that this explanation accounts for the observed reduction in activity in the F_1 hybrids. Another explanation is the possibility that the observed reduction in activity is maternally inherited. Current data concerning expression of NADP-ME (9) support the hypothesis that it is a nuclear encoded polypeptide and therefore should show no maternal effects. Also, *F. trinervia*, which expressed the highest activity, always served as the female parent in these experiments. A third possibility is that the amounts of the enzyme subunits themselves are reduced in the hybrid progeny. To assess this possibility, the soluble proteins extracted from these plants were separated by SDS-PAGE and identified by immunoblotting.

Electrophoresis and Immunoblotting of Soluble Proteins. Figure 1A shows representative polypeptide profiles obtained from extracts of the C_4 and C_3-C_4 parents and their hybrids. Bands corresponding to the individual subunits of PEPC, PPDK, and NADP-ME were identified from immunoblots using polyclonal antibodies to the enzymes isolated from other C_4 plants (Fig. 1C). Completeness of transfer was determined from Ponceau S stained filters prior to immunoblotting (Fig. 1B). Two PEPC bands were observed in extracts prepared from the C_3-C_4 parents and hybrids, but the larger band was not consistently observed in extracts from the two C_4 seedlings. The size of the larger band is estimated to be between 110 and 120 kD. The smaller band (*ca.* 100 kD) is similar in size to the PEPCs isolated from green and etiolated leaves of maize and pearl millet (20, 24). Two PEPC bands have previously been reported for *F. floridana* and *F. cronquistii* using antibodies raised against the maize enzyme (1). The results presented here were obtained using antisera prepared against PEPC purified from pearl millet, a C_4 grass (24).

Table I. *Enzyme Activity in Flaveria Parents and F_1 Hybrids*

Enzyme activity was measured in crude leaf extracts as described in "Materials and Methods." Standard errors of the mean are for $N = 3$ measurements.

Sample	Photosynthetic Type	*Flaveria* Species	PEPC	MPM[a]	NADP-ME	MPM
			μmol h^{-1} mg^{-1} protein	%	*μmol h^{-1} mg^{-1} protein*	%
84-1[b]	C_4	*F. trinervia*	26.9 ± 1.6	—[c]	32.9 ± 2.7	—
84-10	C_4	*F. trinervia*	32.7 ± 5.9	—	38.8 ± 3.1	—
85-7	C_3-C_4	*F. floridana*	3.2 ± 0.5	—	2.8 ± 0.3	—
85-22	C_3-C_4	*F. oppositifolia*	2.1 ± 0.2	—	5.5 ± 0.9	—
84-2	C_3-C_4	*F. linearis*	1.5 ± 0.1	—	3.3 ± 0.4	—
84-1 × 85-7			13.7 ± 2.6	91	10.1 ± 0.9	56
84-1 × 85-22			15.1 ± 2.1	104	10.7 ± 0.5	56
84-10 × 84-2			11.8 ± 1.6	69	10.7 ± 0.5	51
Average of F_1 hybrids			13.5	88	10.5	54

[a] Mid-parent means (MPM) are the averages between activities of each parent in the indicated cross. [b] Georgia identification number. [c] Not applicable.

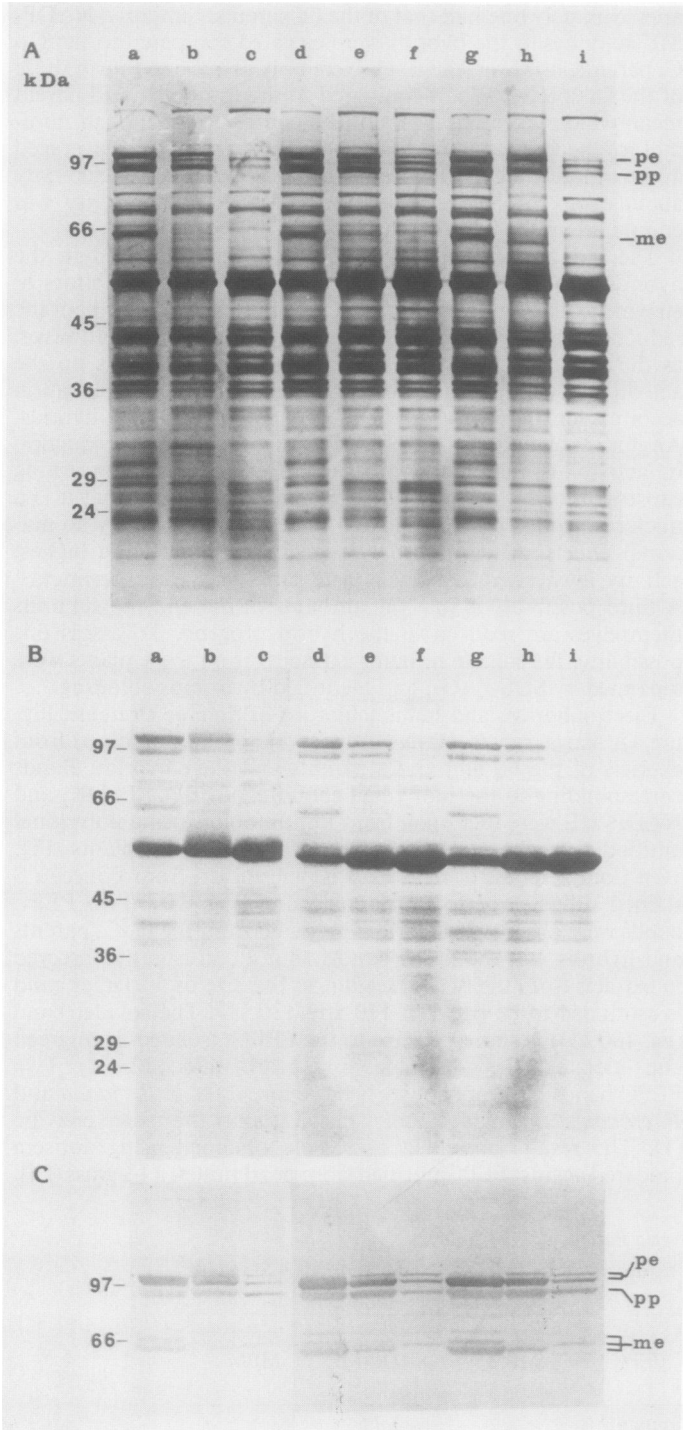

FIG. 1. Identification of PEPC, PPDK, and NADP-ME in C_4 and C_3-C_4 *Flaveria* species and their F_1 hybrids. Soluble extracts from fully expanded green leaves of *Flaveria* sp. and their F_1 hybrids were separated into their component polypeptides by SDS-PAGE according to Laemmli (16). The proteins were transferred to nitrocellulose filters and stained with Ponceau's reagent to monitor efficiency of transfer. Individual enzymes were identified by immunostaining using polyclonal antibodies raised against the maize (PPDK and NADP-ME) and pearl millet (PEPC) enzymes. Lane a, 84-10 (C_4); lane b, 84-10 × 84-2 (F_1 hybrid); lane c, 84-2 (C_3-C_4); lane d, 84-1 (C_4); lane e, 84-1 × 85-7 (F_1 hybrid); lane f, 85-7 (C_3-C_4); lane g, 84-1 (C_4); lane h, 84-1 × 85-22 (F_1 hybrid); lane i, 85-22 (C_3-C_4). A, Soluble proteins (10 μg/lane) separated by SDS-PAGE and stained with silver nitrate according to Blum *et al.* (4). B, Ponceau stained nitrocellulose filter after transfer of gel as in A loaded with 75 μg protein/lane. C, Immunoblot of filter in B. Molecular weight markers are on the left; pe, PEPC; pp, PPDK; me, NADP-ME.

Taken together, the data indicate that within the genus *Flaveria* PEPC has been relatively conserved despite its divergence with respect to photosynthetic function. This observation also applies to the other two C_4 pathway enzymes examined (see below). Although the identity of the larger PEPC band is uncertain, the fact that antisera from two different sources recognize it argues against the possibility that it is an artifact related to the preparation or isolation of the polyclonal antisera. Furthermore this larger band has also been observed in immunoblots of green leaf extracts from pearl millet, *Sorghum*, and alfalfa (R Cameron, unpublished data), and from rice and wheat (17), again suggesting that it is not an artifact of sample preparation, nor is it confined to the genus *Flaveria*. Repeated attempts to visualize the larger band in green leaf extracts from *F. brownii* and maize were unsuccessful (data not shown), as has been reported by others (17). Two bands had previously been reported during purification of the maize enzyme on SDS-polyacrylamide gradient gels (11), but it is not known why immunoblotting fails to detect the larger band, especially since peptide mapping indicates that it is virtually identical to the smaller one (11).

Only one subunit band (*ca.* 95 kD) corresponding to PPDK was observed in all of the extracts examined. Previous studies have shown that PPDK isolated from C_3 and C_4 plants is composed of subunit polypeptides of similar weight (*ca.* 94 kD) and that there is good cross-reactivity between C_3 and C_4 plants (2). Hudspeth *et al.* (13) have estimated that there are at least two genes encoding PPDK in maize, although only one of them may be expressed.

Flaveria NADP-ME cross-reacted with antisera against maize NADP-ME, and two polypeptide bands were observed in extracts from C_4 plants. These bands differ only slightly in size (64 kD and 67 kD) and are similar to those reported for maize (9). From the evidence obtained in that study, the two subunits were hypothesized to arise from separate gene loci. However, since both polypeptides were synthesized as precursors to the *in vivo* form of the enzyme, as is consistent with current data on transport of nuclear encoded, chloroplastic enzymes, the relationship of these subunits to the presumed cytoplasmic and choroplastic isozymes isolated by Pupillo and Bossi (23) is unclear. Surprisingly, the same antisera identified only a single subunit band in C_3-C_4 extracts which was slightly different in size (66 kD) from the bands observed in the C_4 extracts. All three bands could be seen in the hybrid extracts, indicating that both sets of parental genes were transferred to the progeny.

Estimation of the Relative Levels of Enzyme Subunits in the Parents and Hybrids. Densitometric tracings of silver stained gels like the one shown in Figure 1A were used to estimate relative enzyme subunit levels identified from immunoblots (Fig. 1C). For PEPC and NADP-ME, only the bands corresponding to the major C_4 subunits (100 kD and 64 kD), respectively) were examined. As in the case of activity, amounts of NADP-ME in the hybrids averaged only 56% of the mid-parent means (Table II). PPDK averaged approximately 111% of the parental means and PEPC slightly less (88%), although for these two proteins percentages varied among the three hybrids.

There are two main criticisms associated with using stained one-dimensional gels to estimate subunit amounts. The first relates to differences in stainability between polypeptides which vary significantly in structure. Although there is biochemical evidence suggesting that isoforms of the enzymes examined here are different in C_3, C_4, and C_3-C_4 plants, there is also evidence indicating that considerable structural integrity has been maintained throughout their divergence (2, 14). As a result, differences

Table II. *Relative Subunit Levels of PEPC, PPDK, and NADP-ME in Parents and F_1 Hybrids*

Sample	PEPC 1[a]		PPDK		NADP-ME 1[a]	
	RI[b]	MPM[c]	RI	MPM	RI	MPM
		%		%		%
84-1	621	—[d]	545	—	643	—
84-10	445	—	236	—	412	—
85-7	56	—	60	—	ND[e]	—
85-22	45	—	84	—	ND	—
84-2	35	—	49	—	ND	—
84-1 × 85-7	350	103	377	124	184	57
84-1 × 85-22	296	89	266	85	204	63
84-10 × 84-2	173	72	178	125	98	47
Average of F_1 hybrids		88%		111%		56%

[a] PEPC 1 and NADP-ME 1: intensely stained subunit bands corresponding to polypeptides of 100 and 64 kD, respectively, as identified from immunoblots (Fig. 1C). The larger subunit bands of both enzymes did not stain intensely enough to give reliable scans. [b] RI, peak relative intensity. [c] MPM, mid-parent mean. [d] Not applicable. [e] ND, not detectable.

in stainability would probably not be significant when comparing isoforms which differ in expression by as much as 10- to 20-fold. Another potential difficulty is the possibility that on one-dimensional gels other polypeptides of similar size may contribute substantially to the amount of material detected in a given band. Preliminary separation of soluble polypeptides from *F. trinervia*, *F. linearis*, and their F_1 hybrid by two-dimensional gel electrophoresis indicates that few polypeptide spots migrating to positions of equivalent size to the three enzymes examined are detected by silver staining (R Cameron, unpublished data). Furthermore, the results obtained from the scans of silver stained gels are in close agreement with the results of the enzyme activity data for PEPC and NADP-ME. This suggests that the decrease in NADP-ME activity observed in the hybrids correlates with the reduced subunit levels estimated from the gels.

The results presented here indicate that PEPC, PPDK, and NADP-ME are transferred to hybrids of C_4 and C_3-C_4 crosses. With respect to PEPC and PPDK the subunits appear to be expressed in F_1 hybrids at levels approximating what would be predicted for the transfer of a single, light-regulated C_4 gene, although we cannot from these data distinguish between the contributions of each parent. Studies on the transformation of light-regulated gene sequences in tobacco indicate that such sequences are under *cis* regulatory control (10). Since both PEPC and PPDK have been shown to be light-regulated at the molecular level in other C_4 plants (11, 13, 24, 25), it is likely that they would also be similarly regulated in *F. trinervia*. However, until we can assess the expression of PEPC and PPDK in the hybrids using specific gene probes or monoclonal antibodies which can distinguish between the various isoforms, it is impossible to know for certain whether the enzymes in the hybrids are predominantly of the C_4 or C_3-C_4 type.

NADP-ME in the *Flaveria* species is represented by three distinct subunit polypeptides differing in size. All three forms can be detected in hybrid progeny at roughly the ratio present in the individual parents (*i.e.* the lowest mol wt band is the most intensely stained in both the C_4 and hybrid extracts). The faint band corresponding to the C_3-C_4 form present in the hybrids shows no obvious increase in abundance compared to the intermediate parent. However, the predominant band (64 kD) in the hybrids is considerably reduced compared to the C_4 parent, being below the levels one would predict for the inheritance of a presumably single, light-regulated gene. Although other explanations may be considered, one likely interpretation of the data is that the regulation of this gene is under the control of a diffusible repressor in nuclei of the intermediate species. This would explain why mixing C_3-C_4 and C_4 extracts failed to effect any decrease in C_4 enzyme activity, whereas the hybrids which contain genomes from both parents have levels well below the mid-parent means. Furthermore, the reduced NADP-ME levels in the hybrids are not due to the loss of chromosomal material, as they contain the full complement of both parental genomes (J Bouton, personal communication) and express both parental NADP-MEs. If this hypothesis is correct and if reduced levels of NADP-ME contribute to the overall reduction in AP observed in the hybrids (6), it is possible that enhancing the expression of this enzyme would benefit apparent photosynthesis in the hybrids, especially since these hybrids have more fully developed Kranz anatomy than their C_3-C_4 parents (6). We are currently investigating the relationships and expression of the NADP-ME genes in *Flaveria* to test this hypothesis and provide answers to some of these questions.

Acknowledgments—The authors would like to thank Dr. J. R. Y. Rawson, Dr. K. Aoyagi, and Dr. T. Nelson for providing the antibodies used in this study. We would also like to thank Dr. J. Bouton and Dr. R. H. Brown (Agronomy Department, University of Georgia) for many helpful comments during the preparation of this manuscript.

LITERATURE CITED

1. ADAMS CA, F LEUNG, SSM SUN 1986 Molecular properties of phosphoenolpyruvate carboxylases from C_3, C_3-C_4 intermediate, and C_4 *Flaveria* species. Planta 167: 218–225
2. AOYAGI K, JA BASSHAM 1984 Pyruvate orthophosphate dikinase of C_3 seeds and leaves as compared to the enzyme from maize. Plant Physiol 75: 387–392
3. BLAKE MS, KH JOHNSTON, GJ RUSSELL-JONES, EC GOTSCHLICH 1984 A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots. Anal Biochem 136: 175–179
4. BLUM H, H BEIER, HJ GROSS 1987 Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8: 93–99
5a. BOGLIE R, G CORUZZI, B KEITH, N-H CHUA 1984 Molecular biology of C_4 photosynthesis in *Zea mays*: differential localization of proteins and mRNAs in the two leaf cell types. Plant Mol Biol 3: 431–444
5. BRADFORD, MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
6. BROWN RH, CL BASSETT, RG CAMERON, PT EVANS, JH BOUTON, CC BLACK, LO'R STERNBERG, MJ DENIRO 1986 Photosynthesis of F_1 hybrids between C_4 and C_3-C_4 species of *Flaveria*. Plant Physiol 82: 211–217
7. CHENG S-H, MSB KU, LJ METS 1984 Further reduction of photorespiration in the F_1 hybrids between C_3-C_4 intermediate and C_4 *Flaveria* species. Plant Physiol 75: S-58
8. CHENG S-H, MSB KU 1985 Intercellular localization of key enzymes of C_4 photosynthesis in the F_1 hybrids between C_3-C_4 intermediate and C_4 *Flaveria* species. Plant Physiol 77: S-90
9. COLLINS PD, DR HAGUE 1983 Light-stimulated synthesis of NADP-malic enzyme in leaves of maize. J Biol Chem 258: 4012–4018
10. FLUHR R, C KUHLEMEIER, F NAGY, N-H CHUA 1986 Organ-specific and light-induced expression of plant genes. Science 232: 1106–1112
11. HAGUE DR, TL SIMS 1980 Evidence for light-stimulated synthesis of phosphoenolpyruvate carboxylase in leaves of maize. Plant Physiol 66: 505–509

12. HOLADAY AS, S TALKMITT, MS DOOHAN 1985 Anatomical and enzymic studies of leaves of a $C_3 \times C_4$ *Flaveria* F_1 hybrid exhibiting reduced photorespiration. Plant Sci 41: 31–39
13. HUDSPETH RL, CA GLACKIN, J BONNER, JW GRULA 1986 Genomic and cDNA clones for maize phosphoenolpyruvate carboxylase and pyruvate,orthophosphate dikinase: expression of different gene-family members in leaves and roots. Proc Natl Acad Sci USA 83: 2884–2888
14. IZUI K, S ISHIJIMA, Y YAMAGUCHI, F KATAGIRI, T MURATA, K SHIGESADA, T SUGIYAMA, H KATSUKI 1986 Cloning and sequence analysis of cDNA encoding active phosphoenolpyruvate carboxylase of the C_4-pathway from maize. Nucleic Acids Res 14: 1615–1628
15. KANAI R, G EDWARDS 1973 Separation of mesophyll protoplasts and bundle sheath cells from maize leaves for photosynthetic studies. Plant Physiol 51: 1133–1137
16. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
17. MATSUOKA M, S HATA 1987 Comparative studies of phosphoenolpyruvate carboxylase from C_3 and C_4 plants. Plant Physiol 85: 947–951
18. MONSON RK, PD MOORE, MSB KU, GE EDWARDS 1986 Co-function of C_3- and C_4-photosynthetic pathways in C_3, C_4 and C_3-C_4 intermediate *Flaveria* species. Planta 168: 493–502
19. MOORE PD 1982 Evolution of photosynthetic pathways in flowering plants. Nature 295: 647–648
20. NELSON T, MH HARPSTER, SP MAYFIELD, WC TAYLOR 1984 Light regulated gene expression during maize leaf development. J Cell Biol 98: 558–564
21. POWELL AM 1978 Systematics of *Flaveria* (Flaveriinae-Asteraceae). Ann MO Bot Gard 65: 590–636
22. PRENDERGAST HDV, PW HATTERSLEY, NE STONE, M LAZARIDES 1986 C_4 acid decarboxylation type in *Eragrostis* (Poaceae): patterns of variation in chloroplast position, ultrastructure and geographical distribution. Plant Cell Environ 9: 333–344
23. PUPILLO P, P BOSSI 1979 Two forms of NADP-dependent malic enzyme in expanding maize leaves. Planta 144: 283–289
24. RINEHART C 1980 Phosphoenolpyruvate carboxylase in *Pennisetum americanum*: isolation, quantitation, light regulation and cell specificity of protein and mRNA. PhD Thesis, University of Georgia, Athens
25. SIMS TL, DR HAGUE 1981 Light-stimulated increase of translatable mRNA for phosphoenolpyruvate carboxylase in leaves of maize. J Biol Chem 256: 8252–8255