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Distribution of photorespiratory enzymes between bundle-sheath and mesophyll cells in leaves of the C_3–C_4 intermediate species *Moricandia arvensis* (L.) DC

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Abstract. In order to study the location of enzymes of photorespiration in leaves of the C_3–C_4 intermediate species *Moricandia arvensis* (L.). DC, protoplast fractions enriched in mesophyll or bundle-sheath cells have been prepared by a combination of mechanical and enzymic techniques. The activities of the mitochondrial enzymes fumarase (EC 4.2.1.2) and glycine decarboxylase (EC 2.1.2.10) were enriched by 3.0- and 7.5-fold, respectively, in the bundle-sheath relative to the mesophyll fraction. Enrichment of fumarase is consistent with the larger number of mitochondria in bundle-sheath cells relative to mesophyll cells. The greater enrichment of glycine decarboxylase indicates that the activity is considerably higher on a mitochondrial basis in bundle-sheath than in mesophyll cells. Serine hydroxymethyltransferase (EC 2.1.2.1) activity was enriched by 5.3-fold and glutamate-dependent glyoxylate-aminotransferase (EC 2.6.1.4) activity by 2.6-fold in the bundle-sheath relative to the mesophyll fraction. Activities of serine- and alanine-dependent glyoxylate aminotransferase (EC 2.6.1.45 and EC 2.6.1.4), glycollate oxidase (EC 1.1.3.1), hydroxypyruvate reductase (EC 1.1.1.81), glutamine synthetase (EC 6.3.1.2) and phosphoribulokinase (EC 2.7.1.19) were not significantly different in the two fractions. These data provide further independent evidence to complement earlier immunocytochemical studies of the distribution of photorespiratory enzymes in the leaves of this species, and indicate that while mesophyll cells of *M. arvensis* have the capacity to synthesize glycine during photorespiration, they have only a low capacity to metabolize it. We suggest that glycine produced by photorespiratory metabolism in the mesophyll is decarboxylated predominantly by the mitochondria in the bundle sheath.

Abbreviation: RuBP = ribulose 1,5-bisphosphate

Key words: C_3–C_4 intermediate plant – Glycine decarboxylation – *Moricandia* (photorespiration) – Photorespiratory enzymes (localization) – Protoplast isolation

Introduction

The aim of this work was to study the localization of reactions of the photorespiratory pathway in leaves of the C_3–C_4 intermediate species *Moricandia arvensis* (L.) DC. We have recently demonstrated using immunogold localization that glycine decarboxylase is largely confined to the mitochondria of the bundle-sheath cells of *M. arvensis* (Rawsthorne et al. 1988). The same technique demonstrated that enzymes which catalyse initial steps in the photorespiratory pathway, ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase and glycollate oxidase, are present in both mesophyll and bundle-sheath cells of *M. arvensis* (Rawsthorne et al. 1988). In leaves of the C_3 species *M. moricandioides* all three enzymes are found in all chloroplast-containing cells (Rawsthorne et al. 1988). We believe that the differential distribution of glycine decarboxylation in leaves is the reason for the low rate of apparent photorespiration in *M. arvensis* (Hunt et al. 1987; Rawsthorne et al. 1988) and in other C_3–C_4 intermediate species of *Moricandia, Panicum, Flaveria,* and *Mollugo* (Hylton et al. 1988).

Differential distribution of glycine decarboxylase between cell types has profound implications for the fate of ammonia which is released during the glycine-decarboxylation reaction, and the movement of metabolites involved in photorespiration between different cell types. To obtain more detailed information about the partitioning of the photorespiratory pathway between the mesophyll

and bundle-sheath cells, we have devised techniques for preparation of protoplast fractions enriched in either of the two cell types. Activities of photorespiratory enzymes were determined in these protoplast fractions. We have paid particular attention to the known difficulties of obtaining good recoveries of enzyme activity after preparation of protoplasts.

Material and methods

Chemicals. All chemicals were of the highest purity available commercially from BDH Chemicals or Sigma (London) Chemical Co., both of Poole, Dorset, UK. Radioisotopes were purchased from Amersham International, Amersham, Bucks., UK.

Plant material and growth conditions. Plants of *Moricandia arvensis* (L.) DC were grown in a 1:1 mixture of John Innes No. 1 compost and grit in a glasshouse. Day and night temperatures were maintained at 20° C and 16° C, respectively. Between October and April, plants were provided with supplementary illumination (300 μmol quanta photosynthetically active radiation (PAR)$\cdot m^{-2} \cdot s^{-1}$) from high-pressure sodium lamps for 14 h per day during the photoperiod. Plants were fed twice-weekly with a 1.0 $g \cdot l^{-1}$ solution of Solinure (2:1:1, N:P:K, respectively; Fisons, Ipswich, UK). All leaves used in these studies were 75–100% fully expanded.

Preparation of protoplast fractions. Protoplast preparation was based on the protocol of Edwards et al. (1978). The lower epidermis was peeled from leaves immediately after removal from plants and the leaves were floated peeled-side-down on ice-cold sorbitol wash medium (SWM) containing 0.6 M sorbitol, 20 mM 3-(N-morpholino)propanesulphonic acid (Mops), 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$ and adjusted to pH 7.0. When sufficient leaves had been collected, one of two separate procedures was applied. To produce a protoplast fraction enriched in mesophyll cells the SWM was replaced by a digestion medium (medium A) containing 2.0% cellulase (Onozuka R-10), 0.5% pectinase (Macerozyme R-10; both from Yakult Honsha Co., Minatoku, Tokyo, Japan), 0.6 M sorbitol, 20 mM 2-(N-morpholino)ethanesulfonic acid (Mes), 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 1.0% (w/v) bovine serum albumin (BSA) and adjusted to pH 5.8, and the leaves were incubated for 15 min at 28° C. The medium A was then removed and replaced by ice-cold SWM. Protoplasts were released by gentle swirling and the suspension was filtered through a 200-μm nylon mesh and centrifuged at 350 $\cdot g$ for 5 min in 15-ml glass tubes. All manipulations with protoplasts were carried out at 0–4° C. The supernatant was decanted and the pellet resuspended carefully in 5 ml of a sucrose resuspending medium (SRM) which was identical to SWM except that 0.6 M sucrose replaced the sorbitol. Two milliliters of a 1:4 (v/v) mixture of SWM and SRM and then 2 ml of SWM were layered over the SRM and the gradient centrifuged at 500 $\cdot g$ for 5 min. Protoplasts at the upper interface were removed with a Pasteur pipette and resuspended in 10 ml of SWM. This was designated the mesophyll (MSC) cell fraction.

To produce a protoplast fraction which contained a mixture of mesophyll and bundle-sheath cells, the leaves used to prepare the MSC fraction were incubated in medium A for a further 30 min at 28° C. Protoplasts released by this second digestion were purified as described above and were designated the mixed (MIX) fraction.

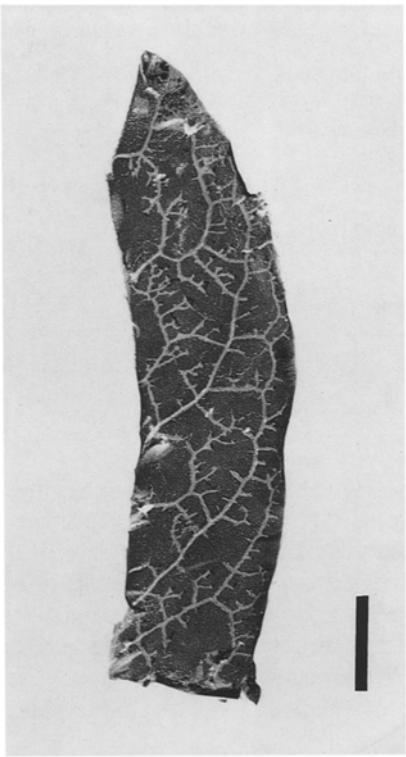

Fig. 1. A leaf of *Moricandia arvensis* after digestion with 0.01% cellulase and 0.5% pectinase for 45 min followed by mechanical removal of the vascular strands. The strands were used to produce a protoplast fraction enriched in bundle-sheath cells. ×1.25; bar = 1 cm

Protoplasts of bundle-sheath cells were produced by a procedure which combined mechanical manipulations and digestion of leaves with enzymes. Leaves were incubated for 45 min at 28° C in a digestion medium (medium B) which was identical to medium A except that the cellulase concentration was altered to 0.01% (w/v). Leaves were removed from the digestion medium and floated on ice-cold SWM. The vascular strands, including much of the fine-vein network (Fig. 1), were removed using fine forceps under a dissection microscope and were placed in ice-cold SWM. Once all the vascular strands had been collected the SWM was replaced with 5 ml digestion medium A and the tissue incubated at 28° C for 25 min. Cells which had been attached to the vascular strands were liberated as protoplasts by gentle agitation. The protoplasts were then purified as described above and the fraction designated bundle-sheath cells (BSC).

Lysis of protoplast fractions. For assays of all enzymes except glycine decarboxylase and RuBP carboxylase the protoplast fractions were centrifuged at 350 $\cdot g$ for 5 min and the pelleted protoplasts were lysed by resuspension in 50 mM Mops, 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 5 mM dithiothreitol (DTT) and 0.1% (w/v) BSA, adjusted to pH 7.0 (extraction medium A). Complete breakage of organelles was achieved by three cycles of freezing to liquid-nitrogen temperature and thawing to 4° C. Where fractions were assayed for serine hydroxymethyltransferase and glutamine synthetase the lysates were immediately supplemented with pyridoxal phosphate (to 200 μM) or $MgCl_2$ (to 20 mM),

respectively. For assays of glycine decarboxylase, the extraction medium contained in addition 0.3 M sucrose, and protoplasts were lysed by repeated pipetting action through a fine-bore tip using an automatic pipette. For assays of RuBP carboxylase, protoplast fractions were lysed in a medium containing 200 mM N,N-bis(2-hydroxyethyl)glycine (Bicine), 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 10 mM DTT, adjusted to pH 8.0.

Recovery of enzyme activity following leaf incubation on digestion medium. Method I. The lower epidermis of a leaf was peeled away and the leaf divided along the midrib. One-half of the leaf was floated on digestion medium A for 70 min before washing gently with SWM. Leaf pieces were ground in an all-glass homogenizer at 0–4° C in extraction medium A containing 0.15 g polyvinylpolypyrrolidone (PVPP) per g fresh weight of leaf tissue. For assays of RuBP carboxylase the leaves were homogenized in the medium described above for protoplast lysis containing 0.15 g PVPP per g fresh weight of leaf tissue. The other half of the leaf was chopped finely and homogenized as described above. Homogenates were centrifuged at 4° C for 10 min at 12000·*g* and the supernatant fraction desalted on a Sephadex G-25 column (Rawsthorne et al. 1988). Extracts made for measurement of RuBP carboxylase were not desalted. Activities of enzymes were calculated on a chlorophyll basis and the values before and after incubation were compared.

Method II. Three leaf halves were chopped and homogenized as described for method I. The opposite halves of the same leaves were used to prepare protoplasts as described for the MSC fraction except that the digestion time was extended to 70 min. This led to almost complete liberation of chlorophyll from the leaf and at least 50% of this chlorophyll was present in the purified protoplast fraction. Enzyme activities were measured in the leaf homogenate and in the protoplast fraction after lysis.

Recovery of enzyme activity following protoplast lysis. The activities of enzymes were determined in separate lysates of MSC and BSC fractions and in a lysate containing known quantities of the same MSC and BSC fractions mixed before lysis. The MSC and BSC fractions were prepared as described above.

Measurement of enzyme activities. All assays were done at 30° C and procedures and reaction mixtures were as follows:

Glycine decarboxylase (EC 2.1.2.10) (Walton and Woolhouse 1986) except that the assay medium contained 0.3 M sucrose as osmoticum and 5 mM DTT.

Serine hydroxymethyltransferase (EC 2.1.2.1) (after Taylor and Weissbach 1965). Assays contained 50 mM Bicine pH 8.5, 10 mM 2-mercaptoethanol, 5 mM [3-^{14}C]serine (3.7 GBq·mol^{-1}), 1 mM tetrahydropteroyl-monoglutamate (tetrahydrofolate) and 0.25 mM pyridoxal phosphate.

Fumarase (EC. 4.2.1.2) (Hill and Bradshaw 1969).

Glycollate oxidase (EC 1.1.3.1) and *Glyoxylate aminotransferases* (EC 2.6.1.4, EC 2.6.1.45) (Walton and Woolhouse 1986).

Hydroxypyruvate reductase (EC 1.1.1.81) (Kohn and Utting 1982) except that the assay pH was 6.8.

Phosphoribulokinase (EC 2.7.1.19) and *RuBP carboxylase* (EC 4.1.1.39) (Smith et al. 1982).

Glutamine synthetase (EC 6.3.1.2) (O'Neal and Joy 1973) except that assays were at pH 7.9. The glutamate concentration was 160 mM and the diethylenetriamine pentaacetic acid was omitted.

Chlorophyll determination. Chlorophyll was determined as described by Arnon (1949).

Statistical analysis. The statistical significance of differences in enzyme activities between different protoplast preparations was assessed by one-way analysis of variance.

Results

Digestion with pectinase and 2.0% (w/v) cellulase of leaves with the lower epidermis removed resulted in release of mesophyll protoplasts from the abaxial surface only. Visual inspection of leaves showed that after 15 min of digestion, bundle-sheath cells were mostly intact. Protoplasts produced in this way were used as a mesophyll (MSC)-enriched fraction. Further digestion of the same leaves resulted in the release of bundle-sheath cells as well as mesophyll cells as protoplasts; these protoplasts were used as a mixed (MIX) fraction. Digestion of leaves with pectinase and 0.01% (w/v) cellulase did not release protoplasts but enabled vascular strands with bundle-sheath cells attached to be teased from the leaf (Fig. 1). Visual inspection of the strands indicated that contamination with mesophyll cells was low. Protoplasts produced by this method were used as a bundle-sheath (BSC)-enriched fraction.

Our measurements of enzyme activities are likely to be true reflections of their maximum catalytic activities in bundle-sheath and mesophyll cells in

Table 1. Recovery of initial enzyme activity in extracts from leaves of *M. arvensis* which had been incubated for 70 min on digestion medium A (see *Material and methods*). For each determination the lower epidermis of a single leaf was removed and the two leaf halves were assayed for the activity of an enzyme before and after incubation at 28° C, respectively. Assays after incubation were either made directly on leaf extracts (method I) or on lysates of protoplasts prepared from the digested leaves (method II)

Enzyme	Method	% recovery
Glycine decarboxylase	–	ND[a]
Serine hydroxymethyltransferase	I	108
Fumarase	I	88
Glycollate oxidase	I	90
Glyoxylate aminotransferase:		
Serine	I	83
Alanine	I	79
Glutamate	I	71
Hydroxypyruvate reductase	I	83
Glutamine synthetase	I	89
Phosphoribulokinase	I	71
	II	113
RuBP carboxylase	I	47
	II	43

[a] Not determined, see text

Table 2. Activities of enzymes in lysates of protoplast fractions enriched in mesophyll (MSC) or bundle-sheath (BSC) cells, or containing a mixture of both cell types (MIX). Each value is the mean from the number of separate protoplast preparations indicated. Where different numbers of replicate protoplast preparations were used in statistical analyses, these are given in parentheses. Recovery of the predicted activity from a lysate containing known proportions of MSC and BSC fractions mixed before lysis is also given, as is the ratio of mean enzyme activity in the BSC fraction to that in the MSC fraction

Enzyme	No. of preparations	Enzyme activity ($\mu mol \cdot min^{-1} \cdot (mg\ Chl)^{-1}$)			BSC / MSC	Recovery of activity (%)
		MSC	MIX	BSC		
Glycine decarboxylase	6	0.17	0.50[a]	1.27[a]	7.5	97
Fumarase	6	0.35	0.54	1.04[a]	3.0	100
Glycollate oxidase	3	1.72	1.67	1.72	1.0	97
Glyoxylate aminotransferase:						
Serine	5	1.85	1.90	1.52(6)	0.8	102
Alanine	9	3.94	4.92	5.69(6)	1.4	109
Glutamate	5	2.10	3.62	5.40[a](6)	2.6	99
Hydroxypyruvate reductase	5	16.0	13.6	16.6	1.0	89
Glutamine synthetase	5	1.94	2.20	2.98	1.5	105
Phosphoribulokinase	5	22.9	29.2	26.8	1.2	96

[a] Activity in BSC or MIX fraction significantly greater ($P=0.01$) than that in the MSC fraction

the leaf for the following reasons. For each enzyme the pH and substrate concentrations used in the assay were optimized to give the maximum rate. The reaction rates were linear with time and were proportional to the amount of extract assayed. There was little or no loss of activity of most photorespiratory enzymes during incubation of leaf pieces on digestion medium (Table 1). The recoveries of the activities of the chloroplast enzymes phosphoribulokinase and RuBP carboxylase following incubation of leaf pieces for 70 min were 71% and 47%, respectively (Table 1). When incubations were carried out under an illumination of 100 μmol quanta $PAR \cdot m^{-2} \cdot s^{-1}$ the recovery of activity of RuBP carboxylase was increased to 60%. The recoveries of the activities of phosphoribulokinase and RuBP carboxylase following complete digestion of leaves to protoplasts (method II; *Material and methods*) were 113% and 43%, respectively (Table 1). Recoveries of enzyme activities using method II were not influenced by illumination during digestions. The distribution of RuBP-carboxylase activity between cell fractions was not determined because of the consistently poor recovery of initial activity during the digestion procedure. The stability of glycine-decarboxylase activity during incubations of leaves was not determined as the enzyme cannot be assayed in crude leaf homogenates because of the rupture of mitochondria and the resulting loss of activity (see Douce 1985). When protoplast fractions enriched in mesophyll or bundle-sheath cells were mixed together before lysis the activities in the lysed mixture of all the enzymes studied were within 89 to 109% of those predicted from measurements made on the separate fractions (Table 2). We are therefore confident that differences in enzyme activities between fractions are not the consequence of changes in activity occurring during protoplast isolation or of preferential activation/inactivation occurring during the lysis of different fractions.

The activities of glycine decarboxylase and fumarase were significantly and substantially greater (by 7.5- and 3.0-fold, respectively) in the BSC fraction than in the MSC fraction (Table 2). The activities of both enzymes in the MIX fraction were intermediate between those in the MSC and BSC fractions (Table 2). In three separate experiments the activity of serine hydroxymethyltransferase was consistently enriched in the BSC fraction relative to the MSC fraction (by an average of 5.3-fold), despite the differences in absolute activities between different batches of leaf material (Table 3). The variation in absolute activity is probably because the leaves used in experiments I and II for serine hydroxymethyltransferase were from plants growing in September while those for experiment III were from plants grown six months later (Table 3). Leaves used for measurements of each of the other enzymes were harvested over a four- to five-week period.

There were no significant differences in the activities of glycollate oxidase, hydroxypyruvate reductase, glutamine synthetase and phosphoribu-

Table 3. Activity of serine hydroxymethyltransferase in lysates of protoplast fractions prepared from three different batches of leaves of *M. arvensis*, and the ratio of enzyme activity in the BSC fraction to that in the MSC fraction. When known proportions of BSC and MSC fractions were mixed before lysis, 90% of the predicted activity was recovered in the lysed mixture

Expt.	Enzyme activity ($\mu mol \cdot min^{-1} \cdot (mg\ Chl)^{-1}$)			BSC / MSC
	MSC	MIX	BSC	
I	0.08	0.31	0.52	6.3
II	0.13	0.37	0.57	4.4
III	0.39	1.23	1.98	5.1

lokinase between different protoplast fractions (Table 2). Glyoxylate-aminotransferase activity was greatest with alanine and glutamate as amino donors in all fractions (Table 2). Whereas serine-dependent aminotransferase activity was approximately the same in all protoplast fractions, glutamate-dependent glyoxylate-aminotransferase activity in the BSC fraction was significantly greater (2.6-fold) than that in the MSC fraction.

Discussion

In all C_3–C_4 intermediate species the bundle-sheath cells of the leaf contain more mitochondria than the mesophyll cells (Edwards and Ku 1987). The threefold enrichment of the mitochondrial marker enzyme, fumarase, in our bundle-sheath-enriched protoplast fraction relative to our mesophyll-enriched fraction is consistent with this anatomical observation. Activities of the two mitochondrial enzymes of photorespiration, glycine decarboxylase and serine hydroxymethyltransferase (Douce 1985), were enriched in the bundle-sheath fraction to a considerably greater extent than the activity of fumarase. This indicates that the activity of these enzymes is considerably greater on a mitochondrial basis in the bundle sheath than in the mesophyll cells of the leaf. The activities of these two enzymes in the intact leaf cannot be estimated accurately since our two enriched protoplast fractions are certainly cross-contaminated and we know of no independent marker for either cell type that would allow us to quantify the cross-contamination. Contamination of the mesophyll fraction with bundle-sheath protoplasts will give higher activities of glycine decarboxylase and serine hydroxymethyltransferase in this fraction than in the mesophyll cells in vivo. Contamination of the bundle-sheath fraction with mesophyll protoplasts will give lower activities of these enzymes in this fraction than in bundle-sheath cells in vivo. The differences in activities of glycine decarboxylase and serine hydroxymethyltransferase between the two cell types in vivo are therefore considerably greater than the differences we found between the respective, enriched protoplast fractions.

The activity of serine hydroxymethyltransferase was enriched in the bundle-sheath fraction relative to the mesophyll fraction to a lesser extent than the activity of glycine decarboxylase (5.3- and 7.5-fold, respectively). There are plastidial, cytosolic and mitochondrial isoforms of serine hydroxymethyltransferase in leaves (see Cossins 1980), but only the mitochondrial isoform is involved in photorespiratory glycine decarboxylation (Douce 1985). Our value is the enrichment of total activity of the enzyme. The enrichment of the photorespiratory, mitochondrial isoform of the enzyme may well be greater than this value.

These results confirm and extend our previous observations on the intercellular location of the P-subunit of glycine decarboxylase, and of glycollate oxidase and RuBP carboxylase/oxygenase, in leaves of *M. arvensis* (Rawsthorne et al. 1988). Using immunogold localization we found that the P-subunit was detectable only in the mitochondria of the bundle-sheath cells. Our measurements of enzyme activity show that both of the enzymes involved in the mitochondrial decarboxylation of glycine, glycine decarboxylase and serine hydroxymethyltransferase, are highly enriched in the bundle sheath relative to the mesophyll cells.

None of the other enzymes of photorespiration that we assayed, apart from glutamate-dependent glyoxylate aminotransferase, were significantly enriched in either the mesophyll or the bundle-sheath fraction. For glutamate-dependent glyoxylate aminotransferase the enrichment in the bundle sheath was only 2.6-fold. Activities of glycollate oxidase and hydroxypyruvate reductase were also very similar in bundle-sheath- and mesophyll-enriched protoplast fractions from leaves of the C_3–C_4 intermediate species *Panicum milioides* (Ku et al. 1976). It appears that the only enzymes of the photorespiratory pathway that are markedly differentially expressed in the two cell types in leaves of *M. arvensis* are those involved in the decarboxylation of glycine, glycine decarboxylase and serine hydroxymethyltransferase.

It seems likely that the reactions of photorespiration occur in mesophyll cells as far as the production of glycine. The capacity of these cells to decarboxylate glycine is extremely limited and all or most of it moves to the bundle-sheath cells (see proposed scheme in Rawsthorne et al. 1988). Re-

lease of photorespiratory CO_2 almost exclusively from bundle-sheath mitochondria leads to an efficient recapture of CO_2 by the bundle-sheath chloroplasts and hence to the low apparent rate of photorespiration of this species (Hunt et al. 1987). Serine moves back from the bundle sheath to the mesophyll, completing the mesophyll photorespiratory cycle. This proposed scheme has profound implications for leaf nitrogen metabolism. First, release of ammonia during photorespiration will occur almost exclusively in the bundle-sheath mitochondria. Second, in order to maintain the nitrogen and carbon balance between the two cell types, a direct or indirect product of ammonia reassimilation must move from the bundle sheath to the mesophyll, and the carbon skeleton of this compound must move back to the bundle-sheath. The small enrichment of glutamate-dependent glyoxylate aminotransferase in the bundle-sheath fraction relative to the mesophyll may indirectly reflect the much higher requirement for ammonia assimilation in bundle-sheath cells. However, the activities of glutamine synthetase and other glyoxylate aminotransferase were not significantly enriched in either cell type. It appears that the predicted differences in nitrogen metabolism between the two cell types are not reflected in differential localization of the enzymes involved.

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