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Immunocytolocalization of Glutamine Synthetase in Green Leaves and Cotyledons of *Lycopersicon esculentum*[1]

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ABSTRACT

Glutamine synthetase was localized in leaves and cotyledons of young tomato (*Lycopersicon esculentum* Mill.) plants using immunogold techniques coupled to transmission electron microscopy. The enzyme occurs only in chloroplasts and is most probably a stroma constituent.

Glutamine synthetase exists in two isoforms (GS_1[2] and GS_2) in the leaf cells of some higher plants (13, 15), which have previously been compared and studied by immunological methods (1, 9).

The intracellular localization of GS isoforms was initially investigated by classical subcellular fractionation in aqueous and nonaqueous media. GS_1 was specifically associated with the cytosolic fraction, whereas GS_2 was present in the chloroplast fraction (7, 8, 14, 18).

Specific antibodies prepared against GS_2 from spinach leaves (10) were used to study the intracellular localization of GS in sections of spinach leaves by indirect immunofluorescence. These experiments confirmed that spinach GS_2 is specifically localized in the chloroplast.

Recently, a detailed cellular and subcellular localization of GS in cyanobacteria was carried out (3, 12) by immunogold techniques (6) coupled to transmission electron microscopy. The enzyme was present in all cell types with no specific label associated with subcellular inclusions. Green leaves of tomato plants contain only one GS isoenzyme (15), which has been purified to homogeneity (5). The kinetics and heat stability of this enzyme suggest it to be chloroplastic in nature (4, 5, 15). However, this enzyme has not been localized at the subcellular level.

In this report, we present for the first time the application of immunogold techniques to study the localization of GS_2 in the leaves and cotyledons of a higher plant (*Lycopersicon esculentum*).

MATERIALS AND METHODS

Plant Material. Young leaves were collected from small, 15 cm, tomato plants (*Lycopersicon esculentum*, cv Hellfrucht frühstamm), which were approximately 30 d old. Cotyledons were collected from smaller, 5 to 7 cm plants, some 15 d old.

Electron Microscopic Techniques. *Plastic Embedding.* Leaves and cotyledons were cut in small pieces and fixed in 3% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3) for 3 h followed by dehydration in ethanol (50–80–100%). Samples were embedded in L.R. White resin (London Resin Co. Ltd., UK) which was then polymerized at 60°C. Ultrathin sections (0.05 μm) were cut with a Sorvall ultramicrotome MT2-B and placed on Formvar-coated copper grids.

Immunogold staining. The samples were washed twice for 10 min with 0.1 M sodium phosphate buffer (pH 7.3) containing glycine (0.75 mg/mL) and 0.1% BSA. They were then incubated for 45 min with immunoaffinity purified antibodies against GS_2 (30 μg/mL) diluted with phosphate buffer; washed three times in phosphate buffer containing 0.1% BSA; and incubated for 30 min in goat anti-rabbit IgG conjugated with 15 nm gold particles (GAR 15, Janssen Pharmaceutica, Beerse, Belgium), diluted 1:30 (v/v) in phosphate buffer containing 0.1% BSA. The samples were washed three times in phosphate buffer containing 0.1% BSA, followed by three washings with phosphate buffer and double-distilled water.

The control sections were prepared by the same procedure used for the experimental sections but nonimmune serum was used instead of antibodies against GS_2.

Immunogold labeled sections were stained with 2% uranyl acetate (pH 4) for 20 min and lead citrate for 8 min.

Cryosections. Samples for cryosectioning were fixed by the same procedure used for plastic embedding, then were washed twice for 10 min with phosphate buffer; rinsed in diluted sodium borohydride for 30 min; washed twice for 10 min with phosphate buffer; and finally immersed in 0.8 M sucrose for 14 h.

Ultrathin cryosections were cut at −100°C with the Sorvall ultramicrotome MT2-B equipped with a LTC-2 cryoattachment. The sections were placed on Formvar carbon-coated grids which were ionized prior to use.

The staining procedure was the same as for the plastic sections except after gold-labeling the samples were postfixed for 5 min with 2% glutaraldehyde in phosphate buffer, then stained with uranyl acetate (pH 7) for 20 min and embedded in a thin layer of 1% methyl cellulose plus 0.02% uranyl acetate (pH 4) for 10 min.

Tansmission electron microscopy was performed with a JEOL 1200 electron microscope (Jeol Ltd., Tokyo, Japan) for visual inspection and micrography of the sections.

Immunological Methods. The methods of obtaining the immune serum and performing the immunotitration curve, double immunodiffusion and immunoelectrophoresis experiments were described previously (1). The immunotitration curve showed a similar pattern to the one previously reported (1). The monospe-

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[2] Abbreviations: GS_1, cytosolic glutamine synthetase; GS_2, chloroplastic glutamine synthetase; Fd-GOGAT, ferredoxin dependent glutamate synthase.

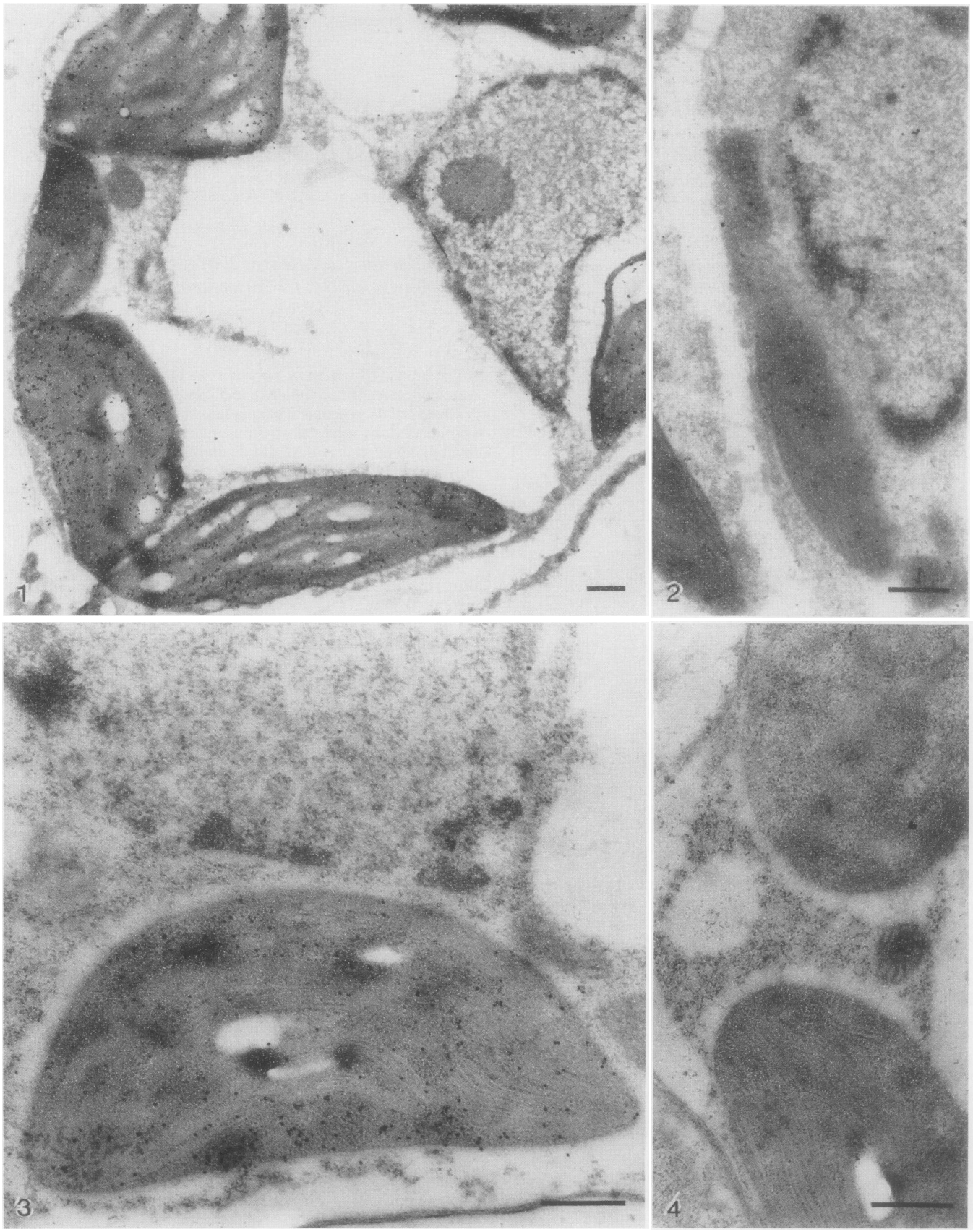

FIG. 1. Low magnification micrograph of a cryosection through the mesophyll of a tomato leaf, incubated with antibodies against GS. Gold label is abundantly present on the chloroplasts. There is negligible labeling of nucleus, cytoplasm, mitochondria, vacuole and cell wall. Bar = 500 nm.

FIG. 2. Nonimmune control cryosection showing profiles of chloroplasts, cell wall, cytoplasm, mitochondrion and nucleus. Bar = 500 nm.

FIG. 3. Part of a mesophyll cell in a plastic section incubated with antibodies against GS. The specific label is limited to the chloroplasts. The nucleus (upper part of field) and the cytoplasm are free of label. Bar = 500 nm.

FIG. 4. Nonimmune control plastic section of a mesophyll cell. Bar = 500 nm.

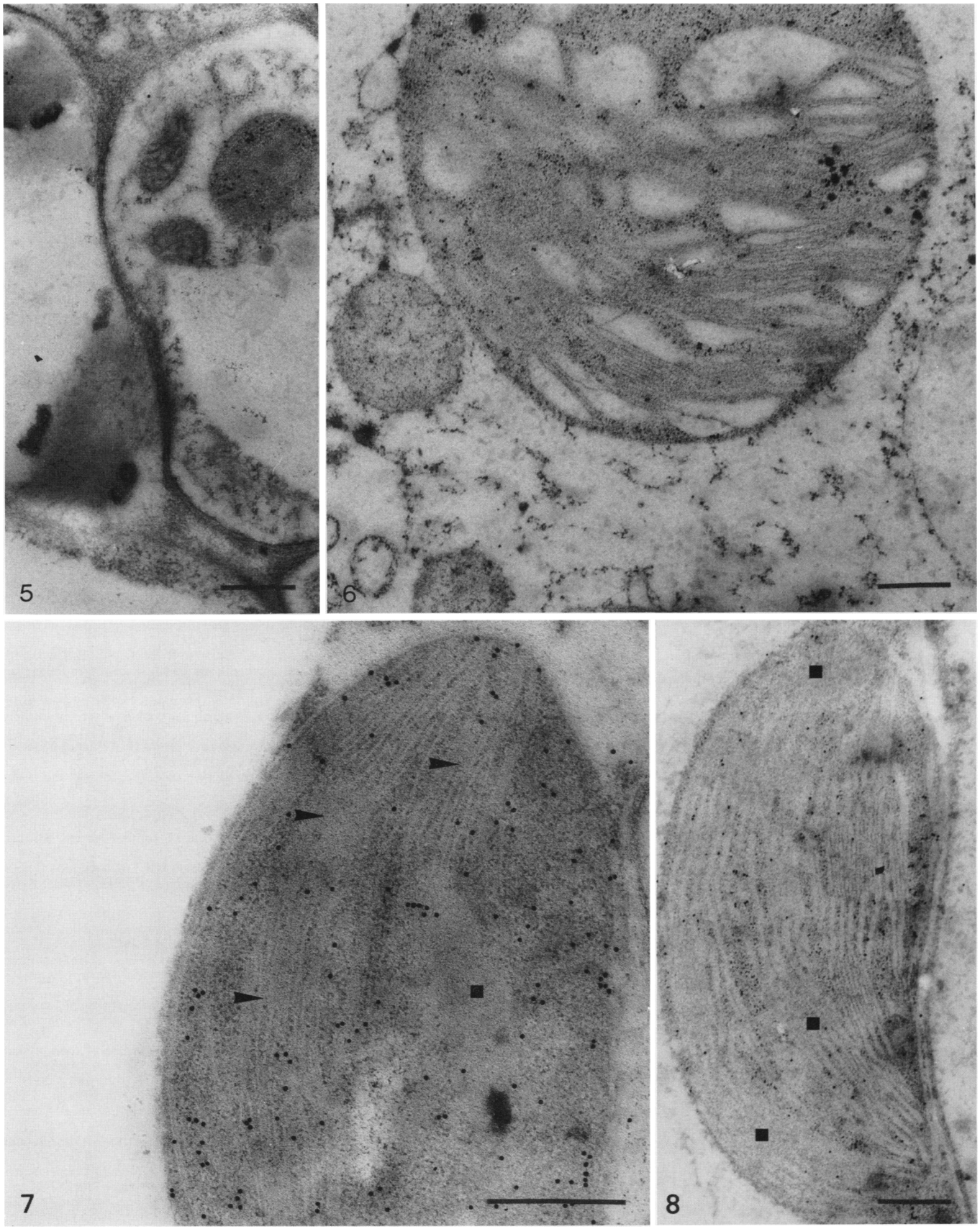

FIG. 5. Section through a xylem parenchyma cell (right side of picture). The plastid clearly contains glutamine synthetase. The left part of the picture represents a section through a xylem vessel with secondary wall thickenings. Bar = 500 nm.

FIG. 6. Plastic section through a senescent cotyledon, labeled for glutamine synthetase. The structure of the cytoplasm and the plastid is deteriorated. The stroma of the plastid is particularly well marked with colloidal gold particles. Bar = 500 nm.

FIG. 7. High magnification micrograph of a chloroplast of plastic embedded tissue, labeled for GS. Note the absence of gold spheres from grana stacks sectioned perpendicularly (▶) and obliquely (■) to the membrane surface. Bar = 500 nm.

FIG. 8. Section through a chloroplast of plastic embedded tissue, labeled for GS, showing areas of obliquely cut grana stacks (■). Colloidal gold is virtually absent from these sites. Bar = 500 nm.

cificity of the antiserum was tested by double immunodiffusion and immunoelectrophoresis. A single band of immunoprecipitate that confirmed the monospecificity was observed after both methods. The antibodies were purified by immunoaffinity before they were used for localization experiments.

Antiserum Purification by Immunoaffinity Chromatography. Homogeneous GS_2 (1.9 mg) was coupled to 3 g (dry weight) of CNBr-activated Sepharose 4B (Sigma Chemical Company). Antibodies to GS_2 were purified by circulating aliquots (3 ml) of immune serum within the column for 2 h (30 mL/h flux). The nonspecifically linked protein was eluted with 25 mM Mops-Tris buffer (pH 7.5) containing 1 M NaCl; the antibodies against GS_2 were eluted with 0.1 M Gly-HCl buffer (pH 2.5). The 2 mL fractions were immediately neutralized with crystalline Tris base.

RESULTS

In leaf mesophyll cells, the cloroplatic localization of GS_2 was evident even at low magnifications of cryosections (Fig. 1). Significant label density occurred only on the profiles of the plastids. The few gold grains present on nucleus, mitochondria, cytoplasm, cell wall, and vacuole can also be found in sections treated with nonimmune serum (Fig. 2) and are therefore negligible.

Plastic embedding and sectioning gave similar results as that of cryosections, but the resolution of the micrographs was, in general, better and the level of unspecific labeling was less (Fig. 3). Further, control sections reacted with nonimmune serum showed negligible labeling (Fig. 4).

The distributions observed in the epidermis and xylem parenchyma cells were similar to that in the mesophyll cells. The former, while not specifically photosynthetic tissues, had small or rudimentary plastids. Nevertheless, these plastids contained detectable amounts of GS_2, as can be seen in the micrograph of a xylem parenchyma cell (Fig. 5).

In the cotyledon, GS_2 was found typically in the plastids. This location was also found in healthy green tissue (results not shown) as well as in older, senescent organs in which the plastids were already partly disorganised (Fig. 6).

The gold label was always distributed over the entire section of the chloroplast. It was present not only in the typical stroma regions, but also in the vicinity of thylakoids. The presence of gold grains on the profiles of typical grana, however, was very rare, as can be seen on the micrographs with higher magnifications (Fig. 7). In some chloroplasts, the membrane stacks were sectioned obliquely and, consequently, they exposed large areas of grana membrane, free of interlacing stroma, to the antibody solution during labeling. Virtually no colloidal-gold grains could be found on these parts of the chloroplast sections (Fig. 8).

DISCUSSION

The present work was designed to immuno-localize glutamine synthetase in leaves and cotyledons of young tomato plants using both cryosections and plastic embedded sections. With both approaches, GS_2 was found to be a chloroplastic protein.

The localization of GS in chloroplasts reported in this work correlates well with recent findings. Very recently, nitrate reductase and Fd-GOGAT were localized by immunoelectron microscopy in plastids of seed plants (2, 11). In both cases, as in the present work, the plastids were the only site where the enzyme was detected.

For Fd-GOGAT the authors established that the enzyme is not a membrane protein but a stromal protein (2). In this present work, we used the same approach and gave special attention to obliquely sectioned grana stacks. The conclusion was that it is unlikely that GS can be an intrinsic part of the thylakoidal membrane at the same time that it occurs in the stroma. Indeed, when large areas of grana membrane were exposed on the surface of the section, little or no label adhered to these sites (Fig. 8). The occasional presence of gold spheres on, or close to, the thylakoid profiles could then be due to GS sitting in the neighboring stroma or on the surface of non stacked thylakoids.

The chloroplastic localization of tomato leaf GS_2 and Fd-GOGAT (2) indicates that all the ammonia arising from nitrite reduction must be assimilated in this organelle. In this case it is also obvious that the photorespiratory evolved ammonia must be reassimilated by the sequential action of GS_2 and the Fd-GOGAT in the chloroplast. The resulting glutamate will sustain the reassimilation of the photorespiratory ammonia (16) as well as the glyoxylate amination (17).

The unequivocal chloroplastic localization of both GS and Fd-GOGAT in tomato leaf cells suggests that it is now important to determine not only the levels of several amino acids and metabolites in the chloroplast compartment, but also their fluctuations in the transition from light to darkness.

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