

Glutamine Synthetase in Spinach Leaves¹

IMMUNOLOGICAL STUDIES AND IMMUNOCYTOCHEMICAL LOCALIZATION

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ABSTRACT

By polyacrylamide gel electrophoresis, DEAE Sephacel, and hydroxyapatite chromatography, one form of glutamine synthetase has been identified in spinach (*Spinacia oleracea* L. cv. Monstrueux de Viroflay) leaves. It is localized only inside the chloroplast. The enzyme was purified to homogeneity and specific antibodies against the protein were raised by immunization of rabbits. The intracellular localization of glutamine synthetase in spinach leaves was studied by indirect immunofluorescence microscopy on thin-sectioned spinach leaves. It has been demonstrated that the enzyme is specifically associated with the chloroplasts of parenchymatous cells.

In several plants, two isoforms of GS³ (L-glutamate: ADP, EC 6.3.1.2) have been identified in leaves. In rice (5, 8) and in barley (14, 15) one, named GS₁ is located in the cytosol, the other, called GS₂ is located in the chloroplast (7, 9, 14). In this study, we have investigated the presence of isoforms of GS in green spinach leaves, by means of classical intracellular localization and chromatographic technique. To investigate further, we have purified to homogeneity GS from spinach leaves and immunized rabbits against the enzyme. Specific antibodies were obtained and an immunocytochemical experiment was then employed to investigate the intracellular localization of the enzyme of thin-sectioned spinach leaves.

MATERIALS AND METHODS

Materials. Acrylamide and bisacrylamide were purchased from Serva (Heidelberg). DEAE Sephacel was purchased from Pharmacia (Uppsala) and hydroxyapatite was from Bio-Rad Laboratories (Richmond, CA). All fine chemicals were from Sigma. Isothiocyanate-labeled sheep antirabbit immunoglobulins were purchased from Pasteur Institute (Paris).

Plant Material. Spinach (*Spinacia oleracea* L. cv. Monstrueux de Viroflay) leaves were purchased at a local market for chloroplast isolation and enzyme purification. Immunocytochemical and chromatographic investigations were performed on 4-week-old

spinach leaves previously grown in a controlled environment chamber. Plants were watered daily with a complete Hoagland solution (11). Air temperature was 22°C during the day (14 h) and 18°C in the dark. The relative humidity was 70% and the light intensity 0.2 w/cm².

DEAE Sephacel and Hydroxyapatite Column Chromatography. All operations were carried out at 4°C. Extraction and purification of spinach leaf GS were conducted using the following procedure: 20 g fresh leaves were used in each experiment and ground in 50 ml 25 mM Tris-HCl (pH 7.6), containing 1 mM MgCl₂, 1 mM DTT, and 10 mM 2-mercaptoethanol (4-fold, 1 min) in a Waring Blendor with 15-s stop every 1 min. The brei was filtered through two layers of cheesecloth and centrifuged at 40,000g for 30 min. The soluble proteins were then directly fixed on DEAE Sephacel on a hydroxyapatite column (1 × 10 cm) and eluted as described previously (8).

Isolation of Chloroplasts on a Percoll Layer. Isolation of spinach chloroplasts was conducted by the method of Mills and Joy (17). Fresh leaves (200 g) were cut into small pieces and homogenized in 800 ml ice-cold medium (330 mM sorbitol; 50 mM Tricine KOH [pH = 7.9], 2 mM EDTA, 1 mM MgCl₂, and 0.1% BSA) for 5 s in a Waring Blendor at full speed. The brei was filtered on four layers of cheesecloth and 120 ml were placed in 200-ml centrifuge tubes. The brei was underlayered with 60 ml of a Percoll Medium (40% [v/v] Percoll; 330 mM sorbitol; 50 mM Tricine KOH [pH 7.9], and 0.1% BSA). Chloroplasts were pelleted by centrifugation at 2,500g for 1 min in a Martin-Christ centrifuge equipped with a swinging rotor. The supernatant was discarded and the pellet resuspended in 50 ml 25 mM Tris-HCl (pH 7.6), containing 1 mM MgCl₂ and 10 mM DTT. The suspension was finally homogenized with a "Polytron mixer" for 5 s and centrifuged 15 min at 20,000g. The supernatant was directly fixed on a DEAE Sephacel or hydroxyapatite column (1 × 10 cm) and the proteins eluted as previously described (8).

Determination of Enzyme Activity and Protein Measurements. Proteins were determined by the Scopes method (23). GS was assayed by using the biosynthetic reaction based on a γ -glutamyl hydroxamate formation as described in a previous study (8). One unit of the activity represents 1 μ mol γ -glutamyl hydroxamate formed/min = 16.67 nkat. Visualization of enzyme activity in polyacrylamide gels and Coomassie Blue staining were conducted as previously described (8).

Preparation of Antibodies Against Chloroplastic GS from Spinach Leaves. All operations were carried out at 4°C. GS was extracted from spinach leaf chloroplasts. Spinach leaves (4 kg) were ground in a total volume of 8 L sucrose medium (3) (200 g/400 ml approximately for one grinding) in a Waring Blendor for 3 × 5 s. The homogenate was filtered through three layers of cheesecloth and centrifuged for 5 s at 2,500g in a Beckman J 21 C centrifuge equipped with a JA 14 rotor. Each pellet of chloroplasts corresponding to one grinding was resuspended in 50 ml 10 mM

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³ GS, glutamine synthetase; GS₁, cytosolic glutamine synthetase; GS₂, chloroplastic glutamine synthetase.

Tris-HCl (pH 7.6), containing 1 mM MgCl₂ and 10 mM DTT and treated with a "Polytron" at maximum speed for 15 s. Broken chloroplasts were centrifuged for 30 min at 40,000g and the supernatant was directly fixed on a DEAE Sephacel column (2.5 × 25 cm) equilibrated in the previous buffer. Elution was performed with a linear NaCl gradient (0–400 mM). Fractions exhibiting GS activity were pooled and fixed on a hydroxyapatite column (10 × 2.5 cm) equilibrated with 100 mM K-phosphate (pH 7) containing 1 mM DTT. The column was rinsed with 100 ml 100 mM K-phosphate (pH 7) containing 1 mM DTT. Elution of proteins was achieved by using a linear gradient of K-phosphate (pH 7) (100–300 mM) containing 1 mM DTT. The fractions exhibiting enzyme activity were precipitated with solid ammonium sulfate at 80% of the saturation and filtered on a Sephadex G25 column equilibrated in 10 mM Tris-HCl (pH 7.6) containing 1 mM MgCl₂ and 1 mM DTT. The partially purified extract was then submitted to preparative polyacrylamide gel electrophoresis in a 5% acrylamide cylindrical gel (2.5 × 6 cm). After the run, the Pi produced by hydrolysis of ATP by the enzyme was localized in the gel by precipitation with 200 mM CaCl₂ as described in a previous paper (8).

The gel was then frozen (20 min, –20°C) and a 2-mm band containing the enzyme was cut with a razor blade and homogenized with a Potter-Elvehjem homogenizer in 5 ml 10 mM Tris-HCl (pH 7.6) containing 1 mM MgCl₂ and NaCl 0.9%. This enzyme preparation was used for primary injection to the rabbits. For booster injections, the enzyme was extracted from the gel by electrophoresis and recovered in an elution chamber covered by a dialysis membrane.

Immunization Procedure. Three rabbits (Fauve de Bourgogne) 2.5 kg each were immunized by four subcutaneous injections. One injection (500 μl emulsion) was composed of 250 μl polyacrylamide gel suspension containing 170 μg pure GS and 250 μl complete Freund's adjuvant. Three intravenous booster injections of antigen extracted from the polyacrylamide gel (500 μl extract each containing 220 μg protein) were given 1 month later at 3-d intervals. The rabbits were bled from the ear a week after the last injection. The blood was kept for 12 h at 4°C. Serum immunoglobulins precipitated by ammonium sulfate (33% saturation) were collected by centrifugation and dissolved in the minimal volume of 150 mM borate-NaOH (pH 8.14) containing 0.9% NaCl and dialyzed overnight against a large excess of the same buffer. The preparation was kept in 500-μl aliquots and frozen at –20°C until further use.

Immunofluorescence Microscope Experiment. Pieces of spinach leaves were fixed 3 h with 2% paraformaldehyde in 25 mM Na-phosphate (pH 7.3) containing 400 mM sucrose; then they were dehydrated in graded ethanol from 10 to 100% and embedded in araldit 502. Sections (1-μm thick) were mounted on glass plates and processed for immunocytochemistry. The indirect immunofluorescence technique was employed as described by Coons and Kaplan (4). First the resin was removed according to the method of Mayor et al. (16). Sections were incubated for 60 h at 4°C in different dilutions of rabbit antiserum. After 30 min of washing in 10 mM Na-phosphate (pH 7.3) containing 140 mM NaCl, sections were incubated 45 min at room temperature in fluorescein isothiocyanate-labeled sheep antirabbit immunoglobulins and cuts were rinsed again for three periods of 10 min in 10 mM Na-phosphate (pH 7.3) containing 140 mM NaCl. To conceal tissue autofluorescence, the sections were transferred for 10 min in 100 mM veronal buffer (pH 7.3) containing 0.01% Evans blue, rinsed and mounted in a mixture containing two volumes of glycerol and one volume of 10 mM Na-phosphate (pH 7.3) containing 140 mM NaCl. Slides were observed at 490 nm with a Leitz fluorescence photomicroscope using the reflected light system of Ploem and photographs taken in 1-min exposures (Kodak Ektachrome 200).

RESULTS

Chromatographic Properties of GS from Spinach Leaves. The purification of a crude extract of green spinach leaf shows the existence of only one peak of GS activity eluted at a concentration of 250 mM in NaCl from a DEAE Sephacel column (Fig. 1) and at 200 mM in K-phosphate from a hydroxyapatite column (Fig. 2). Similar elution patterns are obtained when a stroma fraction of isolated chloroplasts are chromatographed on both types of column (Figs. 1 and 2).

Results obtained with 5% polyacrylamide gel electrophoresis

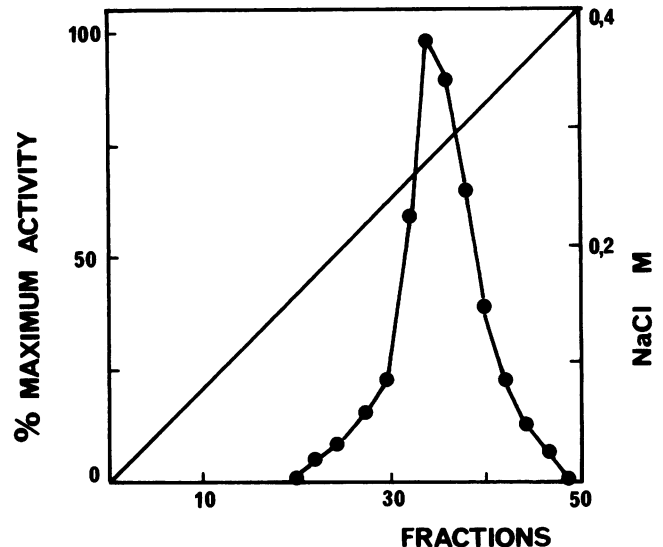


FIG. 1. Elution pattern of GS of a spinach leaf extract or chloroplast extract from a DEAE Sephacel column. Elution of proteins was performed by progressively mixing 100 ml 10 mM Tris-HCl (pH 7.6), containing 1 mM MgCl₂ and 1 mM DTT with 100 ml of the same 0.4 M buffer in NaCl. Four-ml fractions were collected and 200-μl aliquots were assayed for biosynthetic potency with hydroxylamine. Maximum activities for GS were 10 units in both experiments.

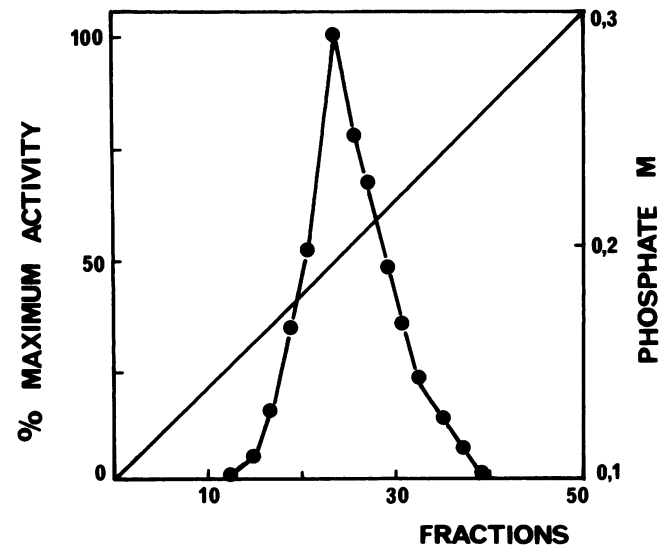


FIG. 2. Elution pattern of GS of a spinach leaf extract or chloroplast extract from a hydroxyapatite column. Elution of proteins was performed by progressively mixing 100 ml 100 mM K-phosphate (pH 7), with 100 ml 300 mM K-phosphate (pH 7). Four-ml fractions were collected and 200-μl aliquots were assayed for biosynthetic potency with hydroxylamine. Maximum activities for GS were 10 units in both experiments.

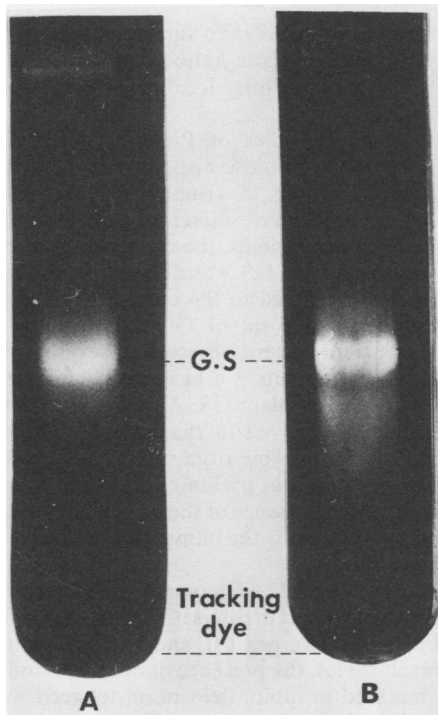


FIG. 3. Polyacrylamide gel electrophoresis of purified spinach leaf GS. Protein (50 μ g) was layered on the surface of 5% polyacrylamide gels (0.3 \times 5 cm). Electrophoresis was conducted at pH 8.3 in Tris-glycine (5 mamp/tube). (A), the band containing enzyme activity was visualized by adding the Fiske and Subba-Row reagent after 15 min incubation at 30°C with standard reaction mixture. A similar relative migration of the protein was obtained with the enzyme from a crude spinach leaves extract. (B), the gel was stained 1 h with Coomassie Brilliant Blue R, and destained overnight.

Table I. Purification of GS from Spinach Leaves

Purification Step	Total Protein	Total Units	Specific Activity	Purification
	mg	nkatal	nkatal/mg protein	-fold
Aqueous isolated chloroplasts	3,900	18,100	4.7	1
DEAE Sephacel	650	10,800	16.6	3.5
Hydroxyapatite	14	3,000	220	46
Preparative acrylamide gel electrophoresis	2	1,600	800	170

also indicated the presence of only one band of GS activity ($R_F = 0.5$) in the leaf and in the stroma fraction of isolated chloroplasts as shown in Figure 3A. In contrast with the results recently published for rice, where we gave evidence for the existence of two forms of GS in leaves (5, 8), this set of results indicates that only one GS is present in spinach leaves and it is located in the chloroplast.

Purification of GS from Spinach Leaf Chloroplasts. Table I summarizes the purification of GS after isolation of chloroplasts from spinach leaves. Two mg purified enzyme were obtained. The purity of the preparation was checked by acrylamide gel electrophoresis and Coomassie Blue staining. The band exhibiting the enzyme activity ($R_F = 0.5$) visualized by phosphate coloration corresponds to a single protein band as shown in Figure 3B.

Specificity of the Antibodies. Purity of the antiserum preparation was checked with crude extracts of spinach leaves using the Ouchterlony double diffusion test as shown in Figure 4. Only one

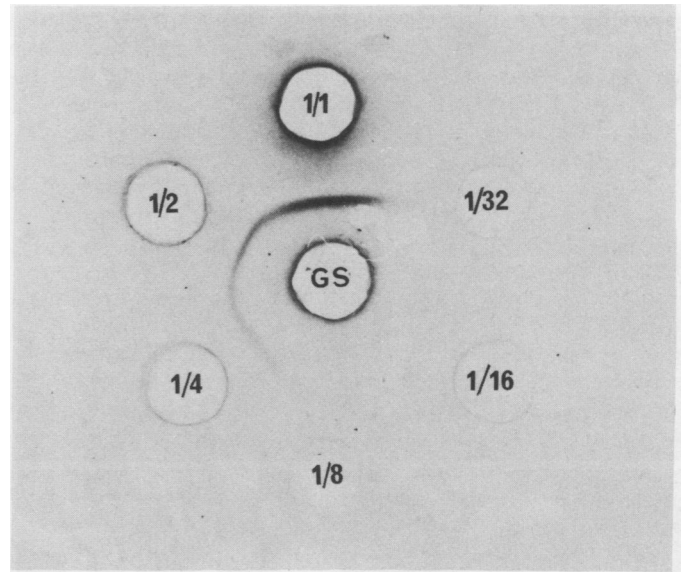


FIG. 4. Ouchterlony double diffusion test. Immunodiffusion was performed on agar plates (2%) according to the Ouchterlony technique. Homogenates of spinach leaf concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation of 80% saturation were prepared. Proteins were redissolved in a minimal amount of 50 mM borate NaOH (pH 8.14). The control well contained 10 μ l extract (0.5 units, 1 mg protein) and peripheric wells, dilutions of the antiserum (1 to 1:32). The plates were placed for 48 h at 4°C in a humid atmosphere and rinsed for 24 h in 0.9% NaCl solution. The plate was dried under filter paper at room temperature, stained 1 h with Coomassie Brilliant Blue R, and destained overnight.

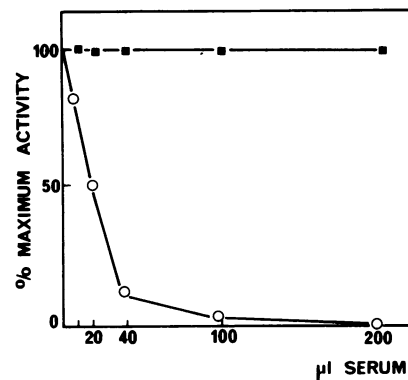


FIG. 5. Immunoprecipitation curve of GS from a crude spinach leaf extract. Constant amounts of GS activity (0.5 units) were incubated with increasing volumes of either antiserum or nonimmune serum. Samples were incubated for 1 h at 37°C, then overnight at 4°C, and finally centrifuged at 10,000g for 15 min. GS activity was assayed in the supernatant (○—○) serum anti-GS from spinach leaves. Control with non immune serum (■—■).

sharp precipitation band in the presence of antibodies was detected on the agar plates.

Behavior of total spinach leaf GS toward the antibodies was also studied by using an immunoprecipitation procedure. Figure 5 shows a titration curve in which constant amounts of GS were incubated with increasing volumes of antiserum. Units (0.5) of enzyme activity were entirely precipitated with 100 μ l antiserum.

Immunocytochemical Study of GS in Spinach Leaves. Immunofluorescence labeling of thin-sectioned spinach leaves treated with anti-GS antibodies followed by conjugation with fluorescein isothiocyanate-labeled sheep antirabbit immunoglobulins is presented in Figure 6. An intense fluorescent light was only exhibited

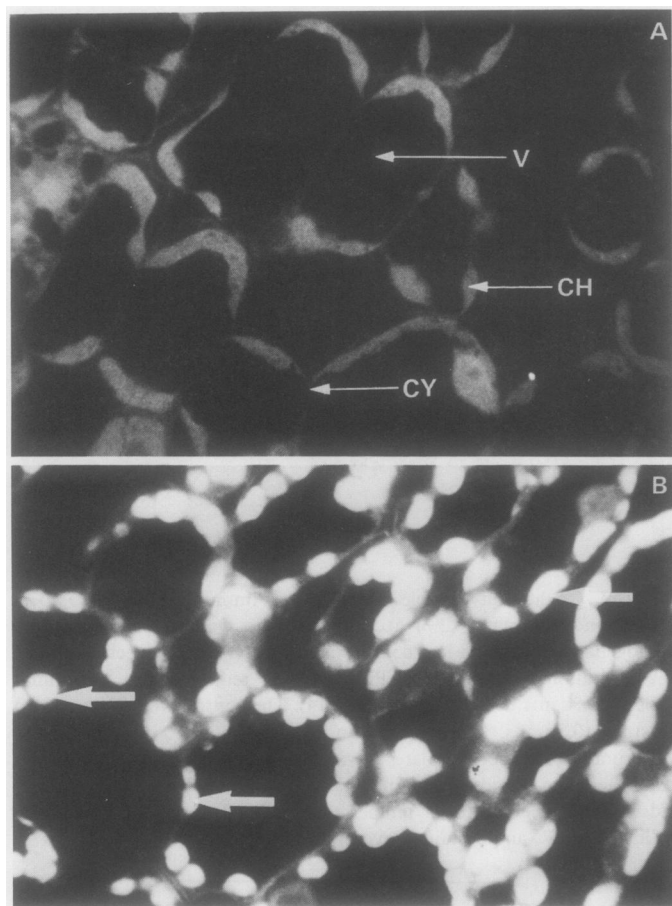


FIG. 6. Immunofluorescence localization of GS in spinach leaves. (A), control section treated by the preimmune serum (dilution 1:1,000). (B), specific anti-GS antibodies (dilution 1:1,000). Fluorescence location is indicated by large arrows. CH, chloroplasts; CY, cytoplasm; V, vacuole. Horizontal scale represents 10 μm .

in the chloroplasts of the chlorenchymatous cells. This result is in perfect agreement with our chromatographic investigation and clearly demonstrates the specific localization of GS in spinach chloroplasts.

DISCUSSION

Several years ago, it was admitted that GS in the leaves of higher plants is located in the chloroplasts (6, 20). In recent investigations, an increasing number of authors has demonstrated that in fact GS is present in two major molecular forms in the leaf (2, 8, 14). A chloroplastic (GS_2) and a cytosolic (GS_1) isoform of the enzyme have been characterized by their specific catalytic and regulatory properties in rice (8) and barley (15). According to Keys et al. (13) the cytosolic GS is likely to be involved in ammonia reassimilation during photorespiration. We have also suggested in a recent study that GS_1 could be responsible for glutamine synthesis in the dark (8, 12). Concerning GS_2 activity, strong evidence is in favor of its light control in the stroma by an increase of pH and Mg^{2+} concentration during illumination (8). It has been emphasized also that ATP in isolated chloroplasts is a limiting factor of GS in the dark (1, 18).

In the present study, we demonstrate, by using classical investigations based on isolation of chloroplasts in aqueous medium and identification of GS isoforms by chromatographic and electrophoretic methods, that in spinach the enzyme is located only in this organelle. It has the same chromatographic and electrophoretic properties as GS_2 , the chloroplastic GS isoform identified in

rice (8), pea (9), and barley (14). To further the investigation, we have decided to prepare specific antibodies against spinach leaf GS and to study the intracellular localization of the enzyme by immunochemistry.

Immunocytochemical studies of P-enolpyruvate carboxylase (21), β -amylase (19), and ribulose biphosphate carboxylase (22) have been successfully used to visualize the enzyme in thin-sectioned plant tissues. We have therefore examined, by indirect fluorescence-labeling experiments, the cellular distribution of GS in spinach leaves. Again, no GS was detected in the cytosol and the fluorescence was restricted to the chloroplasts. The presence of only a chloroplastic isoform of GS in spinach leaves is in contrast with the current pattern observed in several other higher plants. In parallel experiments, we have investigated the immunological behavior of other plant GS. A partial recognition of the leaf cytosolic GS was observed in rice and sorghum with antibodies raised against the enzyme from spinach chloroplast. These results were also confirmed in preliminary cytoimmunochemical experiments showing the presence of the enzyme in the chloroplast but also in the cytosol, by using the immunofluorescence technique (in preparation).

Moreover, in a recent study we have reported that in *Sorghum*, a C_4 plant, the cytosolic GS represents 67% of total enzyme activity in the leaf (10). In rice (8), pea (9), and barley (14) it was only 20%. As in spinach leaves, the presence of only one isoform of GS has also been reported in lupin, field bean, tobacco, and tomato (2). Physiological significance of these differences among different plant species is still unclear, but it seems valuable to consider that several higher plants have developed a specific regulatory mechanism of ammonia assimilation in the cytosol. It was predominant in *Sorghum*, low in rice, and lacking in spinach. Thus, it can be concluded that in spinach leaves, ammonia released from the cytosol or produced in the chloroplast is entirely dependent on a chloroplastic glutamine synthetase for incorporation.

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