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Communication

Detection of a Cytosolic Glutamine Synthetase in Leaves of *Nicotiana tabacum* L. by Immunocytochemical Methods[1]

Helena Carvalho*, Susana Pereira, Claudio Sunkel, and Roberto Salema

Centro de Citologia Experimental (Instituto Nacional de Investigação Científica) (H.C., S.P., C.S., R.S.), and Instituto de Botânica (S.P., R.S.), Universidade do Porto, 4100-Porto, Portugal

ABSTRACT

Two glutamine synthetase (GS) polypeptides (44 and 39 kD) were immunodetected on western blots of leaf extracts from tobacco (*Nicotiana tabacum* L.), a plant that has been reported to contain only chloroplast GS in the leaves. By immunocytochemical methods, we confirmed the localization of GS in the cytosol of cells in the vascular tissue and in the chloroplasts of mesophyll cells.

In plants, ammonia is assimilated into organic form, primarily via the GS[2]-GOGAT cycle (8). In green leaves of most plant species, GS exists in both the cytosol (GS1) and chloroplasts (GS2) (8). There are considerable species-specific differences in the ratio of these two isoforms. It has been reported that members of the Leguminosae, namely *Phaseolus vulgaris* L. and *Pisum sativum* L., contain both GS1 and GS2 activities in leaves, whereas members of the Solanaceae, including tobacco (*Nicotiana tabacum* L.), contain only GS2 activity in mature leaves (7).

Promoters for chloroplastic and cytosolic GS from *P. sativum* direct a cell-specific expression of reporter gene GUS (β-glucuronidase) in transgenic tobacco (4). The promoter for chloroplastic GS2 directs GUS expression within photosynthetic cells, whereas the promoter for a cytosolic GS directs expression of GUS in phloem cells.

Recently, we reported the occurrence of GS in the chloroplasts of the mesophyll cells and in the cytoplasm of phloem companion cells in the Solanaceae *Solanum tuberosum* L. by immunogold EM, suggesting a differential localization of GS1 and GS2 in distinct leaf tissues (9).

In the present study, we examined the GS isoform content in the leaves of *N. tabacum*, and we report the detection and immunolocalization of the chloroplastic GS and also of a cytosolic isoform that is localized in the vascular tissue.

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[2] Abbreviations: GS, glutamine synthetase; GOGAT, glutamate synthase; GUS, β-glucuronidase reporter gene.

MATERIALS AND METHODS

Plant Material

Tobacco seeds (*Nicotiana tabacum* L. cv Havana 425) were sown in soil, and plants were grown for 3 months in a growth chamber at a daylength of 16 h (200 μmol/m^2·s) and a temperature of 16/22°C.

Antibodies

Polyclonal antibodies against GS from *Phaseolus vulgaris* root nodules were produced in rabbits. These antibodies cross-react with GS protein from other higher plants (3).

Leaf Extracts

Whole expanding leaves (including midribs) and excised midribs were ground in a mortar and pestle in the presence of quartz sand and extraction buffer containing 25 mM Tris-HCl (pH 8), 9 mM $MgCl_2$, 5 mM DTT, 10% glycerol, 0.05% Triton X-100, and 5% insoluble PVP. The homogenate was filtered through four layers of gauze, and the filtrate was centrifuged at 20,000*g* for 30 min. The supernatant was used for SDS-PAGE analysis.

SDS-PAGE and Immunoblotting

The soluble proteins were separated by SDS-PAGE according to Laemmli (6) and electroblotted onto nitrocellulose filter paper. The blots were incubated with primary anti-GS antibody, and peptides were detected with secondary peroxidase-conjugated goat anti-rabbit immunoglobulin G.

Western Tissue Printing

Tissue prints were obtained by pressing freshly cut sections of expanding leaves (similar to those used for leaf extracts) on a nitrocellulose membrane. Antibody staining for GS on the tissue print was performed as described for immunoblots of SDS polyacrylamide gels, except that endogenous peroxidases were first blocked by treatment of tissue prints with 1% periodic acid for 30 min. Anti-GS antibody was omitted in the control prints. Antigen on the tissue prints was localized with a Nikon photomicroscope.

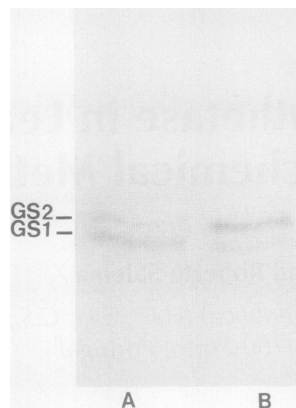

Figure 1. Relative abundance of GS polypeptides on western blots of leaf midrib (A) and whole leaf (B) extracts. The molecular masses of GS polypetides are 44 and 39 kD for GS2 and GS1, respectively.

Immunogold Transmission EM

Small leaf pieces were fixed for 2 h in 4% *p*-formaldehyde, 0.2% glutaraldehyde, 1% sucrose, and 0.05% $CaCl_2$ in 0.1 M Pipes buffer, pH 7.2, and then dehydrated in ethanol and embedded in LR White resin. Immunogold labeling of GS in ultrathin sections was performed with anti-rabbit immunoglobulin G gold conjugate (15 nm) essentially as described for experiments with *Solanum tuberosum* L. (9). Control sections were prepared as experimental sections except that primary antibody was replaced with rabbit nonimmune serum (immunoglobulin G, 20 $\mu g/cm^3$).

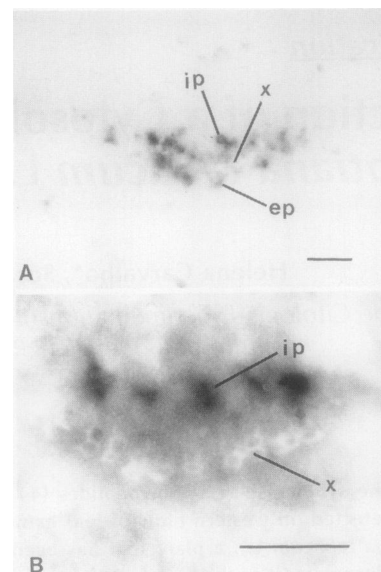

Figure 2. Localization of GS on tissue prints of *N. tabacum* leaves. A, Tissue print of leaf cross-section. B, Detail of a leaf midrib cross-section. Immunolabeling is localized in the phloem, particularly in the internal phloem. ep, External phloem; ip, internal phloem; x, xylem. Bar, 150 μm.

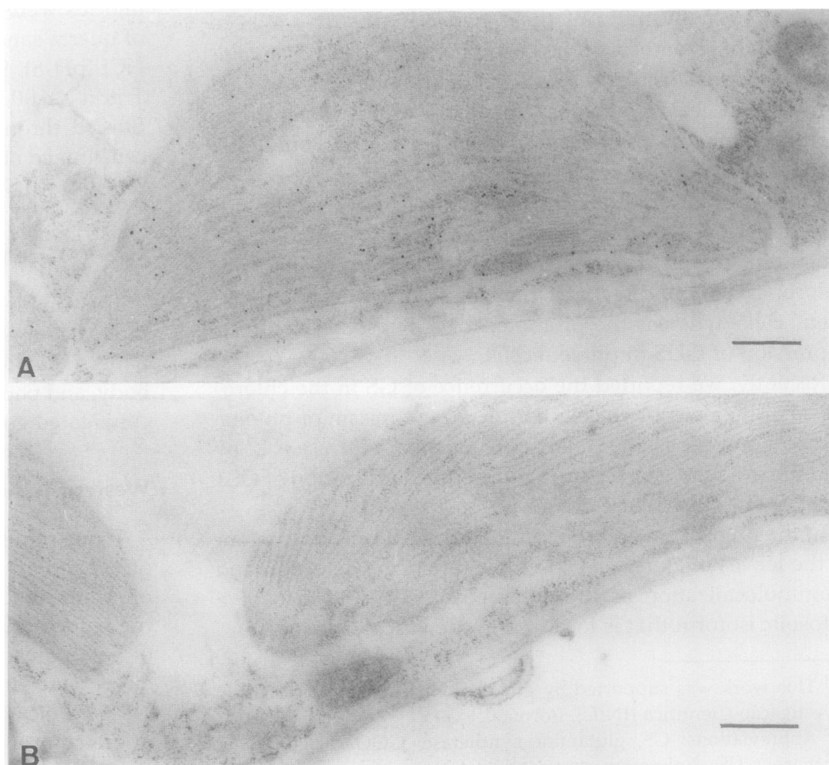

Figure 3. A, Section of mesophyll cell after immunogold staining for GS showing significant labeling limited to the chloroplasts. B, Nonimmunolabeled control section of mesophyll cell. Bar, 0.5 μm.

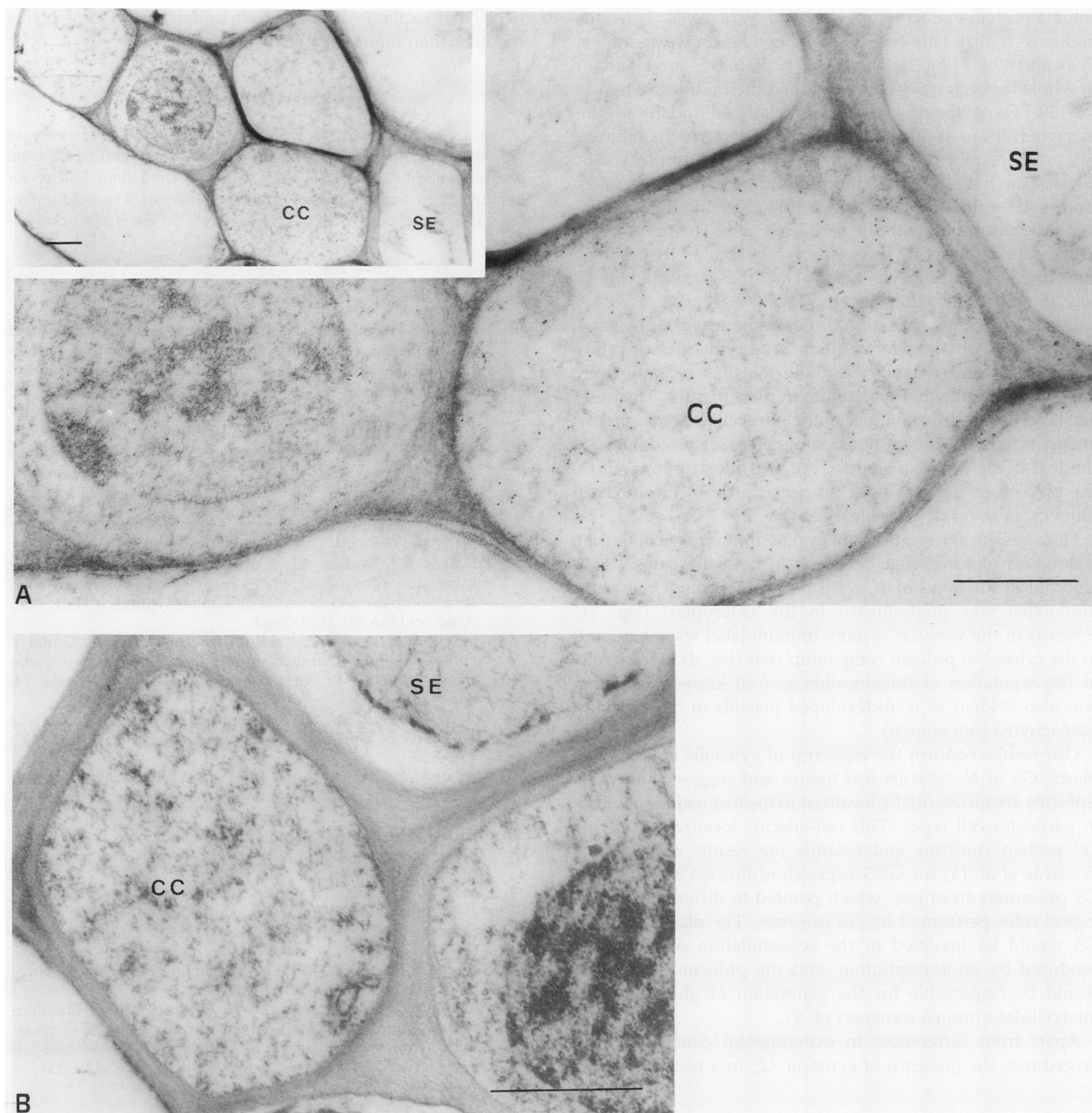

Figure 4. A, Antiglutamine synthetase-treated sections of leaf phloem cells. Labeling is abundant in the cytoplasm of companion cell and is absent from the sieve element and other phloem parenchyma cells. Low magnification inset shows the localization of labeled cell in the phloem bundle. B, Nonimmunolabeled control section, without labeling. CC, Companion cell; SE, sieve element. Bar, 1 μm.

RESULTS AND DISCUSSION

Higher plant GS is encoded by a number of genes that direct the synthesis of multiple GS polypeptides, which assemble into octameric isoenzymes (2). The subunits of the chloroplastic and cytosolic isoforms have M_r values of around 45,000 and 40,000, respectively, and can be resolved by SDS-PAGE (1). Peptides of similar sizes have been assigned to chloroplast and cytosolic GS isoforms in *Nicotiana* species (5, 10, 11). However, only the polypeptide corresponding to GS2 has been detected in the leaves of these species, although in *N. plumbaginifolia*, some mRNA encoding the cytosolic GS polypeptide might be present in leaves (10).

We used anti-GS antibodies (3) to analyze total cellular

soluble protein extracts from leaves of *N. tabacum*. The antibodies recognize only two GS polypeptides on western blots of 44 and 39 kD (Fig. 1). The 44-kD peptide was predominant in whole leaf extracts (which included the midribs), whereas the 39-kD polypeptide was particularly abundant in the extracts from excised midribs (Fig. 1). These results indicate the presence of a cytosolic polypeptide (39 kD) in leaves of *N. tabacum* that is relatively more abundant in protein extracts from excised leaf midribs, suggesting that this polypeptide might be associated with the vascular tissue. The presence of a cytosolic isoform in the cytosol of vascular tissues was confirmed by immunocytochemical methods, which are more sensitive than biochemical methods.

Low-resolution localization of GS in the leaves of *N. tabacum* has been obtained by western tissue prints. Tissue printing is a fast and reliable technique that has recently been used to study enzyme localization in plant tissues. The antigen is accessible to antibodies in the nitrocellulose membrane and in its native form. Immunostaining was scattered through the leaf blade and leaf midrib, but was most prominent in the phloem in the leaf midribs, particularly in the internal phloem, as is visible in some cases (Fig. 2).

High-resolution localization by EM immunocytochemical techniques revealed that GS exists in both the cytosol and chloroplast in leaves of *N. tabacum*. In mesophyll cells, immunolabel was predominant in the chloroplast (Fig. 3), whereas in the vascular system, immunolabel was abundant in the cytosol of phloem companion cells (Fig. 4), whose role in the regulation of translocation is well known. Labeling was also evident in well-developed plastids in the vascular parenchyma (not shown).

Our results confirm the existence of cytosolic and chloroplastic GS in *N. tabacum* leaf tissues and suggest that these isoforms are differentially localized in distinct leaf tissues and in particular cell types. This cell-specific localization of the GS protein confirms and extends the results reported by Edwards et al. (4) for GUS expression directed by different GS promoters from pea, which pointed to different physiological roles performed by GS isoforms. The plastid-located GS would be involved in the reassimilation of ammonia produced by photorespiration, and the phloem-located GS would be responsible for the generation of glutamine for intercellular nitrogen transport (4, 9).

Apart from differences in experimental conditions and procedures, the presence of cytosolic GS in a restricted type of cell may explain why it has been undetected in some plants by other than immunocytochemical methods.

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LITERATURE CITED

1. **Cullimore JV, Bennett MJ** (1988) The molecular biology and biochemistry of plant glutamine synthetase from root nodules of *Phaseolus vulgaris* L. and other legumes. J Plant Physiol **132:** 387–393
2. **Cullimore JV, Gebhardt C, Saarelainen R, Miflin BJ, Idler KB, Baker RF** (1984) Glutamine synthetase of *Phaseolus vulgaris* L.: organ-specific expression of a multigene family. J Mol Appl Genet **2:** 589–599
3. **Cullimore JV, Miflin BJ** (1984) Immunological studies on glutamine synthetase using antisera raised to the two plant forms of the enzyme from *Phaseolus* root nodules. J Exp Bot **35:** 581–587
4. **Edwards JW, Walker EL, Coruzzi GM** (1990) Cell-specific expression in transgenic plants reveals non-overlapping roles for chloroplast and cytosolic glutamine synthetase. Proc Natl Acad Sci USA **87:** 3459–3463
5. **Hirel B, Weatherley C, Cretin C, Bergounioux C, Gadal P** (1984) Multiple subunit composition of chloroplastic glutamine synthetase of *Nicotiana tabacum* L. Plant Physiol **74:** 448–450
6. **Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685
7. **McNally SF, Hirel B, Gadal P, Mann AF, Stewart GR** (1983) Glutamine synthetases of higher plants. Plant Physiol **72:** 22–25
8. **Miflin BJ, Lea PJ** (1980) Ammonia assimilation. *In* BJ Miflin, ed, The Biochemistry of Plants, Vol 5. Academic Press, San Diego, pp 169–202
9. **Pereira S, Carvalho H, Sunkel C, Salema R** (1992) Immunocytolocalization of glutamine synthetase in mesophyll and phloem of leaves of *Solanum tuberosum* L. Protoplasma **167:** 66–73
10. **Tingey SV, Coruzzi GM** (1987) Glutamine synthetase of *Nicotiana plumbaginifolia*. Plant Physiol **84:** 366–373
11. **Yamaya T, Ishida H, Kamashi K, Ojima K** (1990) Immunochemical analysis of multiple subunit polypeptides of glutamine synthetase in methionine sulfoximine-sensitive and tolerant tobacco cell cultures. Plant Cell Physiol **31:** 325–331