Plant Science, 65 (1989) 33-38
Elsevier Scientific Publishers Ireland Ltd.

MULTIPLE FORMS OF GLUTAMINE SYNTHETASE IN NODULES OF TROPICAL LEGUMES INOCULATED WITH *BRADYRHIZOBIUM SPP.* AND *RHIZOBIUM FREDII*

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(Received January 2nd, 1989)
(Revision received May 30th, 1989)
(Accepted June 12th, 1989)

The profiles of glutamine synthetase (GS) activity in the plant fraction of nodule extracts from tropical legumes, inoculated with either fast- or slow-growing rhizobia, were analyzed by non-denaturing polyacrylamide gel electrophoresis. The electrophoretic mobility of nodule GS was plant-dependent but was virtually unaffected by the type of *Rhizobium* present in the nodules. Only *Macroptilium atropurpureum (DC.)* Urb. nodule extracts displayed two distinct GS activity bands similar in electrophoretic mobility to those previously reported in *Phaseolus vulgaris* L. nodules. Nodule extracts of *Vigna unguiculata (L.)* Walp. exhibited one major and one minor band of GS activity. In contrast, nodule extracts of *Glycine max (L.)* Merr. and *Sesbaniaa rostrata* Brem displayed a single zone of GS activity. These experiments indicate that multiple GS activity bands in root nodules are not restricted to *P. vulgaris* and *P. lunatus* L. but are probably of widespread occurrence in legumes.

Key words: glutamine synthetase; nodules; legumes; *Rhizobium fredii;; Bradyrhizobium japonicum*

Introduction

In higher plants, glutamine synthetase (GS, EC 6.3.1.2), in conjunction with glutamate synthase (GOGAT, EC 1.4.1.14), plays a major role in the assimilation of ammonia [1]. Dinitrogen fixation by rhizobia in the root nodules of legumes generates ammonia which is also processed by the GS/GOGAT pathway after its excretion into the plant cytosol [2-6].

Several isoforms of GS occur in various plant organs (leaves, roots, nodules) [7-11]. In *Phaseolus vulgaris* root nodules, two cytosolic isozymes were found [7,19]. One was nodule specific (GS.) and was synthesized during the course of nodule development; the other (GS_{n2}) was virtually identical to the root form of GS (GS_r) [7,12]. GS_{n1} and GS_{n2} were consistently detected in the nodules of 62 cultivars of P. *vulgaris* by non-denaturing PAGE [9]. The electrophoretic mobility (EM) of the GS_{n1} band depended on the plant cultivar and on the development stage of the nodule [9].

In nodule extracts of *Cajanus cajan, Cicer arietinum, Glycine max, Lupinus angustifolius, Vicia faba,* and *Vigna unguiculata* either a single peak or a single band of GS activity was detected by either ion exchange chromatography or by native gel electrophoresis [3,12,13].

In this study, nodule extracts of the tropical legumes *Macroptilium atropurpureum, V. unguiculata,* and *G. max,* inoculated on separate occasions with *Bradyrhizobium sp., B. japonicum,* and *Rhizobium fredii,* were analyzed by native PAGE for differences in GS activity profiles. The patterns of GS activity in the stem and root nodules of the tropical leguminous tree *Sesbania rostrata,* inoculated with *Azorhizobium sesbaniae,* were also examined.

Abbreviations: EM, electrophoretic mobility; GOGAT, glutamate synthase; GS, glutamine synthetase; GS., nodule form of GS; GS, root form of GS; PAGE, polyacrylamide gel electrophoresis; cv., cultivar; bv., biovar.

Materials and Methods

Plant material and inoculation

Seeds of V. *unguiculata (L.)* Walp. and of P. *vulgaris* L. were purchased from Burpee Seed

0168-9452/89/$03.50

Co., Clinton, IA; seeds of *Glycine max (L.)* Merr. cvs. Peking and Williams were provided by Dr. H. Keyser and Dr. B.B. Bohlool respectively; seeds of M. *atropurpurem (DC.)* Urb. and of S. *rostrata* Brem were provided by Dr. B.B. Bohlool. The seeds were surfaced-sterilized either in 1 : 1 (v/v) Clorox for 20 min. (soybean), or in concentrated H_2S0_4 for 15 min *(Macroptilium; Sesbania),* or in 30% H_20_2 for 20 min *(Phaseo*lus). The seeds were germinated for 2 days on filter paper and planted in 6-inch pots containing a 3 : 1(v/v) sterilized mixture of vermiculite and perlite [14]. They were inoculated with a culture of the appropriate rhizobium (10^{10} cells/ pot) grown in yeast-extract-mannitol broth [15]. *G. max cv.* Peking, V. *unguiculata cvs.* California and Purple Hull, and M. *atropurpureum* seeds were inoculated with *Bradyrhizobium sp.* strains TAL 420 and TAL 163, *B. japonicum* USDA 110, and *R. fredii* HH003. P. *vulgaris* cvs. Blue Lake 274 and Green Crop were inoculated with *R. leguminosarum* biovar phaseoli Viking 1. S. *rostrata* seeds were inoculated with A. *sesbaniae ORS* 571. *Sesbania* stems were inoculated with the same strain after 28 days by using a cotton tip dipped in a broth culture (10^8 cells/ml). The pot surface was covered with a layer of silicone-coated sand to protect against contamination by airborne rhizobia. The pots were irrigated three times a week alternately with water and N-free Hoagland nutrient solution [16]. The plants were grown in a growth chamber at 25 °C with 16-h days (220 µE m^{-2} s^{-1} light) and 8-h nights.

Nodule and root harvest

Nodules from 28-day-old plants were removed, kept on ice until frozen in liquid nitrogen, and stored at - 70 °C as previously described [9]. Uninoculated roots were harvested after 14 days using the same procedure. At harvest time, S. *rostrata* root and stem nodules were, respectively, 56 and 28 days old.

Preparation of nodule and root extracts

To obtain that GS present in the plant cytosol, nodule crude extracts were prepared, as in [9], by grinding 1 g of nodules in an ice-chilled mortar with 2 ml of 0.05 M Tris (hydroxymethyl aminomethane)-HCI buffer at pH 7.5 containing 1.0 mM dithiothreitol. The extract was filtered through eight layers of gauze, centrifuged at 27000 x *g* to remove plant debris and bacteroids, frozen in liquid nitrogen, and stored at - 70 °C.

A crude extract of uninoculated roots (40 g roots/80 ml Tris buffer, was obtained as in [9]. The root GS was partially purified by treating the extract with 1% (w/v) protamine sulfate followed by centrifugation at 27000 x *g* at 4 °C for 20 min. The supernatant fluid was fractionated with solid $(NH_4)_2SO_4$ (35-55% saturation). The precipitate was separated from the fluid by centrifugation as before and dissolved in 1 ml of 'running buffer' (containing 10 mM Tris-HCI buffer (pH 7.8), 5 mM Na-glutamate, 10 mM $MgSO_4$, and 10% (v/v) glycerol) [7]. The root extract was then dialyzed against "running buffer."

Non-denaturing PAGE

Discontinuous native gels [1.5 mm x 14 cm x 12 cm separating gel (7.5%) and a 4-cm stacking gel] were prepared as in [9]. Electrophoresis was carried out at 4°C and at 8 W for 5.5 h. The samples were prepared as in [9] and approximately 120 µg of protein (quantified by Bradford's procedure [17]) was loaded/well.

Determination of GS activity bands

The transferase assay [18] was used to detect GS activity zones on native gels.

Results

All of the legumes were effectively nodulated (red nodules, green plants) by the fast and slow-growing strains of *Rhizobium* used. Figure 1 compares the profiles of the electrophoretic mobility (EM) of nodule GS from herbaceous legumes, inoculated with either *R. fredii* (fast-growing strain), *Bradyrhizobium sp.* or *B. japonicum* (slow-growing strains), with the GS profiles of nodule extracts from P. vulg*aris* Green Crop used as a reference. The nodules GS from each legume displayed distinc-

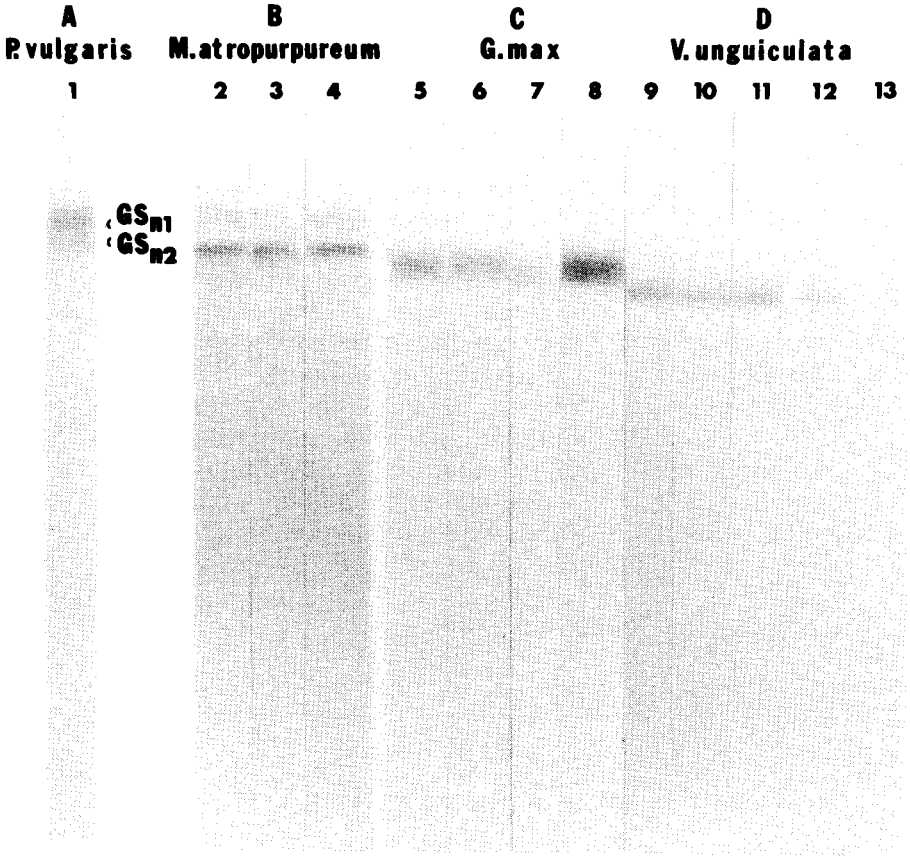

Fig. 1. Native polyacrylamide gel of nodule crude extracts showing glutamine synthetase (GS) activity bands (approximately 120 μg of protein was loaded into each well). GS_{n1} is the nodule-specific form and GS_{n2} is the band similar to GS_r in *P. vulgaris*. A. *P. vulgaris* cv. Green Crop inoculated with *R. Leguminosarum* bv. phaseoli Viking 1 (lane 1). B. *M. atropurpureum* inoculated with *R. fredii* HH003 (lane 2), *B. japonicum* 110 (lane 3), and *Bradyrhizobium* sp. TAL 420 (lane 4). C. *G. max* cvs. Peking (lane 5, 6) and Williams (lane 7, 8) inoculated with *R. fredii* HH003 (lanes 5, 7) and *B. japonicum* 110 (lanes 6, 8). D. *V. unguiculata* cvs. California (lanes 9, 10) and Purple Hull (lanes 11, 12, 13) inoculated with *B. japonicum* 110 (lanes 9, 12), *Bradyrhizobium* sp. TAL 163 (lanes 10, 13), and *R. fredii* HH003 (lane 11). All GS activity bands were located in the upper 2.5 cm of the gel.

tive patterns of electrophoretic mobility. In *M. atropurpureum* two distinct GS bands (GS_{n1} and GS_{n2}), similar in electrophoretic mobility to those previously reported in *P. vulgaris [7,9,19],* were detected. Nodule extracts of *V. unguiculata* cvs. California and Purple Hull showed identical GS_n profiles which consisted of one major GS activity band of high EM and of one minor band of slower EM. The latter was barely detectable in nodule extracts of cv. Purple Hull housing *Bradyrhizobium sp. TAL 163.* In contrast, in both *G. max* cultivars, one single band of nodule GS activity was observed. The EM of nodule GS in the various legumes was virtually unaffected by the type of *Rhizobium* strain present in the nodules.

The GS activity in root extracts was much lower than in nodule extracts and was detectable only after partial purification and concentration (approx. *30* times) of the protein

fraction with solid $(NH_4)_2SO_4$ GS, activity was demonstrated in all of the root extracts examined; those of *M. atropurpureum* were omitted due to the scarcity of root material available.

A comparison of the EM of GS in nodules and roots (Fig. 2) showed that the distinctive feature of all root extracts was the presence of a single band of GS activity (GS_r). The EM of this GS, band was as high or slightly higher than that of the lower GS activity band in nodule extracts of *V. unguiculata.* In G. max the GS_r band was thinner than the single GS band found in the nodules of Peking and Williams cultivars.

Both stem and root nodule extracts of *S. rostrata* showed a single zone of GS activity (Fig. 3). Not enough root material was available to permit a successful extraction and concentration from non-nodulated roots.

Discussion

The results indicated that the EM of nodule GS activity zones was virtually unaffected by the type of microsymbiont housed in the nodules (Fig. 1). Since the GS investigated here is of plant origin, the regulation of the GS isoform production is under plant control and would more likely depend on the amount of nitrogen fixed than on the type of rhizobium. In contrast, the profiles of nodule GS activity varied greatly with the legume (Fig. 1).

The nodule GS activity profiles of *M. atropurpureum* were similar to those reported for

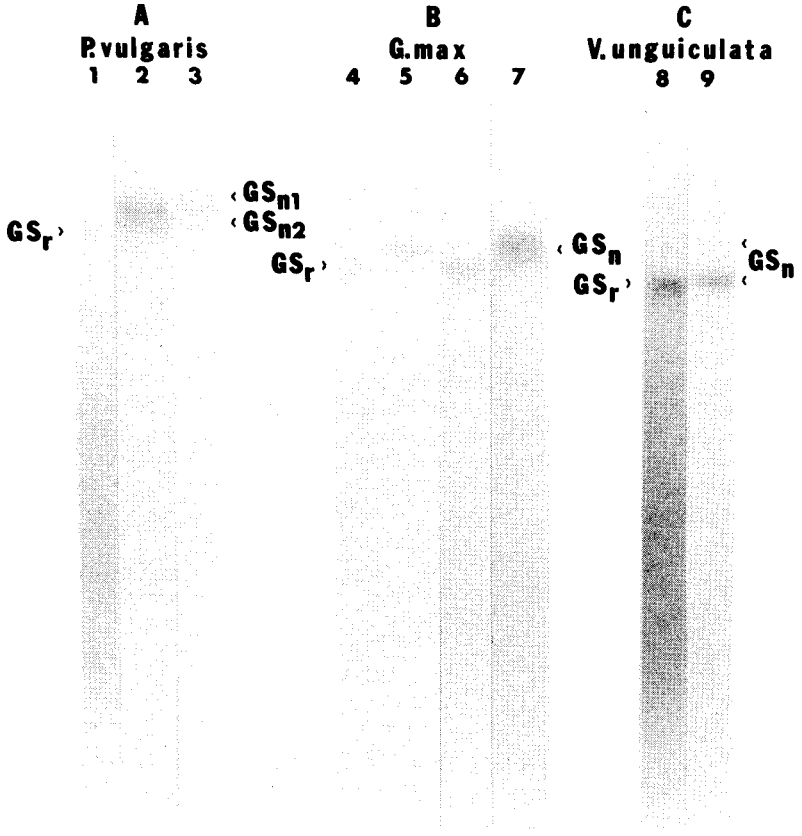

Fig. 2. Comparison of GS activity bands, on native polyacrylamide gel, of nodule crude extracts and partially purified root extracts. A. *P. vulgaris* cv. Blue Lake 274 roots (lane 1), and nodules housing *R. leguminosarum* bv. *phaseoli* Viking 1 (lane 2); cv. Green Crop nodules housing Viking 1 (lane 3). B. *G. max* cvs. Peking and Williams roots (lanes 4, 6), and nodules housing *R. fredii* HH003 (lanes 5, 7). C. *V. unguiculata* cv. California roots (lane 8) and nodule housing *R. fredii* HH003 (lane 9).

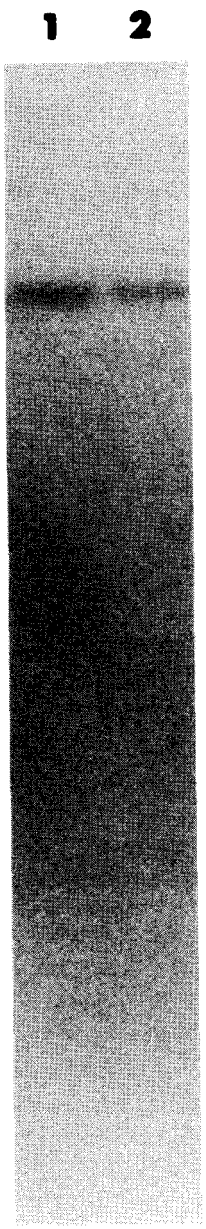

Fig. 3. Native gel electrophoresis of *S. rostrata* stem and root nodules housing *A. sesbaniae* ORS 571. The distance traveled by the GS activity bands of stem (lane 1) and root (lane 2) nodule extracts was 1.8 cm.

some *P. vulgaris* cultivars whose GS_{n1} displayed a slow EM [9].

In *G. max*, both nodule and root extracts produced only one band of GS activity (Figs. 1 and 2) as reported previously [3,12].

In this study, 2 bands of nodule GS activity (GS.) were detected in both cultivars of *V. unguiculata* (Fig. 1). Since the upper band is a minor one, it may not have been detectable by ion exchange chromatography in previous studies [12]. Also, the EM of this upper band could increase during nodule development, as seen in P. vulgaris [9]. This would cause the fusion of the upper with the lower band of GS activity and the detection of a single form of GS in the nodules of *V. unguiculata.* A time-course experiment is needed to verify this hypothesis.

These results suggest that multiple GS activities, previously observed in *P. vulgaris* and *P. lunatus* [7,9,19], also exist in the plant cytosol fraction of root nodules of other legumes such as *M. atropurpureum* and *V. unguiculata*

Acknowledgements

This research was funded in part by Cooperative Agreement (DAN-4177-A-00-6035-00) to the NifTAL Project (Paia, Hawaii) from the Agency for International Development.

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