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Communication

Induction of Glutamine Synthetase Activity in Nonnodulated Roots of *Glycine max, Phaseolus vulgaris,* and *Pisum sativum*[1]

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ABSTRACT

Nitrate or ammonium fertilization significantly increased glutamine synthetase (GS) activity in nonnodulated roots of French bean (*Phaseolus vulgaris*), soybean (*Glycine max*), and pea (*Pisum sativum*). Western analysis revealed substantial GS antibody-positive protein in root extracts that had minimal GS activity, indicating that an inactive form of GS may be present in nonfertilized plants.

GS[2] (EC 6.3.1.2) is a key enzyme in plant NH_4^+ metabolism, generating glutamine, ADP, and Pi from glutamate, NH_4^+, and ATP. Several GS isozymes have been identified in different plant tissues (4, 16, 19, 21). Considerable research effort has focused on GS in nodulated roots and it has been shown that nodule GS activity is high relative to root activity, and that nodule GS activity is the result of a pool of GS isozymes with at least one isozyme identical to that found in nonnodulated roots. Although some reports indicate that the increased GS activity in nodules is due to induction of one or more GS isozymes by NH_4^+ (7, 11, 21, 22), other research shows increased nodule GS prior to NH_4^+ production by nitrogenase, the source of nodule NH_4^+ (2, 3, 23). Cock et al. (3) found no differences in GS mRNA levels in nonnodulated roots of French bean grown under a wide range of N conditions. Vezina et al. (22) found NO_3^- induction of pea root plastid GS activity, and Groat and Vance (6) also found increases in root GS activity after NO_3^- or NH_4^+ treatment, using nodulated but symbiotically ineffective alfalfa plants.

Two problems that often arise when trying to analyze these contrasting reports are the use of different legume genera and complications due to nodule-root interactions. We used spectrophotometric assays combined with western analysis of crude root extracts to investigate induction of GS activity in nonnodulated pea (*Pisum sativum* L.), soybean (*Glycine max* L. Merr.), and French bean (*Phaseolus vulgaris* L.). Our results suggest that a significant portion of the inducible GS activity may be regulated posttranslationally.

MATERIALS AND METHODS

Plants were grown in sterile sand in a growth chamber maintained at 27°C day/16°C night with a 14-h photoperiod. Plants were watered daily until the day before harvest, when they were flooded for 5 min with 10 mM KCl, KNO_3, NH_4Cl, or NH_4NO_3 in phosphate buffer (10 mM, pH 7.0). This treatment was repeated on the day of harvest approximately 3 h before tissue collection.

Plants were harvested immediately prior to analysis. Root tissue was rinsed with water, and 1.0 g of coarsely chopped roots (the youngest tissue was taken) was ground in a chilled tissue grinder with 0.2 mL of grinding buffer, pH 7.0, (100 mM Tris-acetate, 1 mM EDTA, 0.5% Triton-X 100, 1 mM DTE, 5 mM benzamidine, 1 mM ϵ-amino-*N*-caproic acid, 2 mM PMSF, and 2 mM iodoacetamide). Samples were kept on ice at all times during processing.

For western analysis, the tissue slurry was diluted with 1.3 mL of grinding buffer supplemented with 2% SDS and centrifuged at 12,000*g*. The supernatant fractions were assayed for protein content (18), and 50 μg of protein per lane was loaded onto a polyacrylamide gel (9). Discontinuous gels were electrophoresed overnight at 7 mA and the proteins were electrotransferred onto Immobilon-P membrane (Millipore Corp., Bedford, MA) using standard procedures (Bio-Rad, Richmond, CA). The membrane was probed with *Phaseolus vulgaris* nodule GS antibody generously provided by Dr. P. Wong (Manhattan, KS), which cross-reacts with all legume GS proteins (16). Alkaline phosphatase-conjugated goat anti-rabbit antibody was used as a secondary antibody. GS antibody-specific protein bands were visualized by the alkaline phosphatase color reaction (12).

For the spectrophotometric enzyme assays, the tissue slurry was diluted with 0.8 mL of grinding buffer and centrifuged for 15 min at 37,000*g*. The supernatant fraction was desalted with Sephadex G-50 and aliquots of this crude extract containing approximately 100 μg of protein were added to the assay buffer. The transferase and synthetase reaction mix-

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[2] Abbreviation: GS, glutamine synthetase.

tures (5) were adjusted to pH 6.7 and 6.5, respectively. These pH values differ from published values obtained from highly purified preparations (4), but were determined to be optimum with crude extracts. After incubation at 37°C, 1 mL of ferric chloride reagent (3.3% $FeCl_3$, 2% TCA in 1.25 M HCl) was added and absorbance measured at 508 nm. γ-Glutamyl monohydroxamate was used for the standard curve.

RESULTS

GS activity in pea, soybean, and French bean roots was induced by N treatment (Table I). Bush Blue Lake 74 and Tendergreen, two other cultivars of *P. vulgaris*, were also found to have NH_4^+-inducible GS activity (data not shown). The ratio of transferase to synthetase activity was consistently higher in KCl-treated plants than in N-treated plants in all three genera. In both *G. max* and *P. sativum*, the change in the transferase/synthetase ratio followed the same pattern when nutrient treatments changed. Apparent variations in the degree of induction of GS activity between the transferase and synthetase assays may reflect a differential sensitivity of the assays to different isoenzymes (6, 23).

The lack of relative differences between treatments in GS antibody-positive protein seen with the western analysis (Fig. 1) does not reflect the pattern seen with the spectrophotometric assays (Table I); there was a similar amount of GS antibody-positive protein in all extracts, including those that contained minimal GS activity. This phenomenon was observed repeatedly; in some cases, slightly less GS antibody-positive protein was observed in extracts from KCl-treated plants, but in all cases a significant amount of GS protein was observed in all treatments, including those that had very low GS activity. Specificity of the antibody was verified using starch activity gels; the only areas that bound the GS antibody were those where active GS protein was located (data not shown). Protein extracts isolated under highly denaturing conditions (pH 9.0, 3% SDS) and subjected to western analysis produced only one band per lane, further confirming the specificity of the antibody.

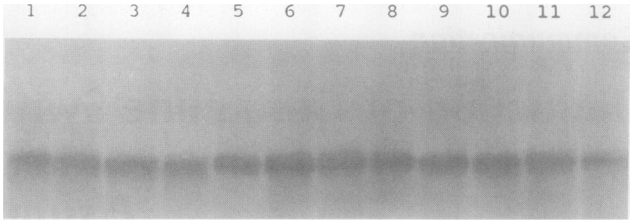

Figure 1. Western analysis of GS-positive protein in extracts of legume roots. Lanes 1–4, *P. sativum*; lanes 5–8, *G. max*; lanes 9–12, *P. vulgaris*. Treatments: lanes 1, 5, 9, NH_4NO_3; lanes 2, 6, 10, NH_4Cl; lanes 3, 7, 11, KNO_3; lanes 4, 8, 12, KCl. Fifty micrograms of protein were loaded per lane.

Numerous reports have documented toxic effects of NH_4^+ on plant growth, although buffered nutrient solutions can largely circumvent negative plant responses to NH_4^+ (see ref. 1). Furthermore, in experiments where plants were continuously cultured in unbuffered NH_4-based nutrient solutions, toxic responses such as browning and reduced root growth did not occur for several days. Our treatments were short flooding periods (5 min) with buffered solutions, and were initiated approximately 28 h before harvest. Negative responses to fertilization were not observed, and differences in leaf or root growth between nutrient treatments were not apparent at harvest.

DISCUSSION

Our results indicate that induction of root GS activity in *P. vulgaris*, *P. sativum*, and *G. max* occurs in the presence of NH_4^+ and NO_3^-. Furthermore, there is GS protein present in all root extracts, but it is apparently largely inactive in *P. vulgaris* and *P. sativum* roots not treated with N.

Table I. *GS Activity in Crude Extracts of Roots from P. sativum, G. max, and P. vulgaris*
Plants were harvested 14 days after planting.

Species and Enzyme Assay	Specific Activity[a]			
	KCl[b]	KNO_3	NH_4Cl	NH_4NO_3
P. sativum cv Sparkle				
Transferase	29.8 ± 8.8	95.5 ± 19.8	74.2 ± 9.3	94.2 ± 18.0
Synthetase	0.8 ± 0.2	3.1 ± 0.9	3.3 ± 0.4	4.7 ± 1.5
G. max cv Beeson 80				
Transferase	114.3 ± 16.0	149.9 ± 7.7	144.6 ± 13.9	157.3 ± 12.1
Synthetase	2.6 ± 0.4	3.7 ± 0.6	4.1 ± 0.6	6.1 ± 0.9
P. vulgaris cv Kentucky Wonder				
Transferase	9.4 ± 1.4	22.3 ± 3.2	52.8 ± 11.2	68.5 ± 18.1
Synthetase	0.2 ± 0.1	0.3 ± 0.1	1.1 ± 0.3	1.4 ± 0.4

[a] Enzyme activity expressed as μmol γ-glutamyl hydroxamate produced mg^{-1} protein h^{-1}. Values given are the mean and SE of four independent experiments. [b] Pots were flooded for 5 min daily for 2 d prior to harvest with a buffered 10-mM solution of the indicated salt.

Activation of an inactive GS protein has been observed in duckweed (*Lemna minor*) (15). These authors suggested that GS protein is maintained in an inactive form unless sufficient N and, importantly, C skeletons are available for glutamine synthesis. Thus, under conditions of low N and/or C availability, ATP is conserved and C remains available for other metabolic reactions.

Other studies can also be interpreted to provide support for this concept: Ratajczak et al. (14), working with lupine embryos (minus cotyledons), found increased levels of glutamate dehydrogenase and inhibition of GS activity if only NH_4^+ was supplied, but found increased GS and decreased glutamate dehydrogenase activities if sucrose was provided with NH_4^+. Sahulka and Lisa (17) incubated excised pea roots on NO_3^--containing media and found rapid decreases in GS activity unless sucrose was present. Finally, there are two reports of a strong correlation between energy charge and GS activity in sunflower root tissue (8, 24).

It is not clear whether this type of regulation applies to legume GS; however, the plants used in the current study were well illuminated and the cotyledons were not senescent at harvest, so they probably were not C limited. If the *Lemna* regulatory system was functioning, the presence of sufficient C presumably would have resulted in induction of GS activity in response to N fertilization, and this was observed.

Variations in technique may explain some differences between our results and those from other laboratories. Plant age influences GS activity: young plants express a high constitutive activity (10, 13), and differences between our results and those of Leon et al. (10) may be due to plant age. Some work has shown differences in GS activity in roots of different ages (16, 21). We did not compare activity of older versus younger root sections because at harvest our plants were only about 8 d past germination, so our more mature tissue would not have been significantly older than our younger tissue. Vezina and Langlois (22) documented increased GS activity in roots of NO_3^--treated peas but not in NH_4^+-treated plants. However, the plant culture conditions employed in their experiments utilized hydroponic conditions and nutrient solutions that were apparently not pH controlled. After 7 d of culture, root growth was significantly reduced in the NH_4^+-treated plants, indicating a toxic response and consequent reduced enzyme function; GS activity was lower in NH_4^+-treated plants than in the no-N controls. The apparent discrepancy between our results and those of Cock et al. (3) could be due to the presence of inactive GS protein in roots not treated with NH_4^+. These authors assayed plant material for mRNA coding for different GS isozymes but did not assay GS activity; it is possible that there is constitutive transcription and translation of GS mRNAs in *Phaseolus* roots, but that the enzyme is largely inactive unless NH_4^+ is present.

Further support for this suggestion comes from the same study, which showed that the tissue NH_4^+ level increased after roots were placed in an NH_4^+ solution, and then decreased after about 4 h. If GS activity were induced, a similar pattern of NH_4^+ accumulation and decrease would be expected. A different report from the same laboratory indicated a discrepancy between GS activity and the corresponding mRNA levels in developing *Phaseolus* cotyledons (20), similar to our results when comparing GS antibody-positive protein levels to activity.

Finally, results (not shown) from some preliminary experiments suggest that heat stress induces GS in *Phaseolus* and *Glycine* but not *Pisum,* and that drought stress reduces GS activity in *Phaseolus* and *Pisum* but not *Glycine*. Consequently, unintentional environmental stress to experimental plants may also be responsible for some differences in results between laboratories.

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