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Involvement of Ammonia in Maintenance of Cytosolic Glutamine Synthetase Activity in *Pisum sativum* Nodules

Norio Suganuma, Mayuko Watanabe, Takayuki Yamada, Takumi Izuhara, Koji Yamamoto, Maya Nishimura and Kanako Toriyama

Department of Life Science, Aichi University of Education, Kariya, Aichi, 448-8542 Japan

Regulatory mechanisms of glutamine synthetase (GS, EC 6.3.1.2) activity in pea (*Pisum sativum* L.) nodules were investigated by using the pea mutant E135 (*sym*13) that forms ineffective nodules. GS activity, and the levels of polypeptides and mRNA for cytosolic GS in nitrogen-fixing effective nodules increased during plant development while those in roots remained low. In the ineffective E135 nodules, GS activity and the level of polypeptides for GS were similar to those in effective nodules at the early stages of plant development, but later GS activity and level of polypeptides were lower than those found in roots although the level of the mRNA in the ineffective nodules did not decrease, and was much higher than that in roots. GS activity in the ineffective nodules at the later stages of plant development was enhanced by treatment with ammonia although the effect was transient. The treatment with ammonia also increased the level of polypeptides, but hardly affected the level of the mRNA for GS in ineffective nodules. These results suggest that the maintenance of cytosolic GS activity in pea nodules is regulated post-transcriptionally by the effectiveness of the nodules, and that ammonia produced by nitrogen fixation is involved in the maintenance of GS activity at a post-transcriptional level.

Key words: Ammonia — Glutamine synthetase (EC 6.3.1.2) — Ineffective nodules — Nitrogen fixation — *Pisum sativum* — Post-transcription.

The metabolism of carbon and nitrogen in host-plant cells of legume nodules is closely linked with the nitrogen-fixing activity of the rhizobia. Carbon metabolism yields compounds providing the energy and reductant required for nitrogenase. Ammonia derived from fixation is quickly assimilated into amides or ureides, for export to other plant organs. The activities of several enzymes in host-plant cells of nodules are higher than those in roots, and increase with nodule development (Rawsthorne et al. 1980, Tajima and Kouchi 1997). Furthermore, comparison of enzyme activities between effective nodules and ineffective nodules induced by bacterial or plant mutants showed that the activities of some enzymes, such as phospho*enol*pyruvate carboxylase (PEPC), glutamine synthetase (GS), glutamate synthase (GOGAT) and aspartate aminotransferase (AAT), are markedly lower in ineffective nodules (Werner et al. 1980, Vance and Johnson 1983, Egli et al. 1989, Häser et al. 1992, Suganuma and LaRue 1993, Suganuma et al. 1993, Romanov et al. 1995, 1998). These results indicate that the activities of these enzymes in host-plant cells are regulated by rhizobial nitrogen-fixing activity.

Vance and his collaborators have been studying the regulatory mechanisms for several enzymes involved in carbon and nitrogen metabolism by using an alfalfa (*Medicago sativa*) Fix^- mutant (Vance and Gantt 1992, Vance et al. 1994). They showed that expression of PEPC, GS, NADH-GOGAT and AAT are regulated differentially and that expression of the gene for NADH-GOGAT has a strict requirement for a factor(s) related to effective nodules. In contrast, induction of expression of PEPC, GS and AAT occurs independent of nitrogenase activity, but a post-transcriptional control by the effectiveness of the nodules is shown to be involved in the regulation of activities of PEPC, GS and AAT. Similar results with PEPC have been obtained using a pea (*Pisum sativum*) Fix^- mutant E135 (Suganuma et al. 1997). However, how nitrogenase activity in microsymbionts regulates the activities of these enzymes in the host-plant cells remains unclear.

GS (EC 6.3.1.2) plays a primary role in the assimilation of fixed nitrogen in combination with NADH-GOGAT (Ohyama and Kumazawa 1980). There are two classes of GS isoenzymes that are located in the cytosol (GS1) and chloroplast (GS2) (Lam et al. 1996). The cytosolic GS gene family has been well characterized in legumes, and a nodule-specific or nodule-enhanced GS has been isolated (Gebhardt et al. 1986, Hirel et al. 1987, Tingey et al. 1987, 1988, Bennett et al. 1989, Grant et al. 1989, Walker and Coruzzi 1989, Boron and Legocki 1993, Roche et al. 1993, Stanford et al. 1993, Temple et al. 1995). Analyses of expression of their GS genes have shown that expression of GS genes in soybean (*Glycine max*) or *Phaseolus vulgaris* nodules is regulated by symbiotically fixed nitrogen (Hirel et al. 1987, Cock et al. 1990, Miao et al. 1991). However, relatively little attention has been paid to post-transcriptional regulation of GS. Recently, Temple

Abbreviations: AAT, aspartate aminotransferase; DTT, dithiothreitol; GOGAT, glutamate synthase; GS, glutamine synthetase; PEPC, phospho*enol*pyruvate carboxylase.

et al. (1996) have presented evidence showing that turnover of GS holoprotein is involved in the regulation of GS activity in soybean nodules.

Pea contains five distinct GS polypeptides, which are expressed at different relative levels in leaves, roots and nodules (Tingey et al. 1987). Leaves contain five GS polypeptides, 44 kDa, 38 kDa and three 37 kDa having different charges; the 44-kDa GS polypeptide, which is localized to the chloroplast stroma, is predominant. In roots, two GS polypeptides, 37 kDa and 38 kDa, the 38-kDa GS polypeptide of which is predominant, and three 37-kDa GS polypeptides increase in concentration after *Rhizobium* infection. Furthermore, four cDNA clones have been isolated for GS: two nearly identical GS genes, GS341 and GS132, which encode the 37-kDa cytosolic GS polypeptide, the second type of cytosolic GS gene, GS299, encoding 38-kDa polypeptide, and GS197, which encodes a putative precursor to the mature chloroplast GS polypeptide (Tingey et al. 1987, 1988). Walker and Coruzzi (1989) showed that both mRNAs for GS341 and GS132 accumulate to wild-type levels in ineffective nodules induced on the E135 pea mutant. However, in the E135 nodules, GS activity is much lower than that in effective nodules (Suganuma and LaRue 1993, Suganuma et al. 1993). These results suggest that the activity of GS in pea nodules is regulated post-transcriptionally by the effectiveness of the nodules.

In this study, we compared GS activity and the levels of polypeptides and mRNA for cytosolic GS among effective nodules, ineffective nodules induced on the E135 and roots, and examined the effects of exogenous ammonia in order to elucidate the regulatory mechanisms of GS activity. We found that GS activity and the level of polypeptides for GS, but not the level of mRNA for GS, were greatly reduced in ineffective E135 nodules compared with those in effective nodules, and that those in ineffective nodules were enhanced by exogenous ammonia. This suggests that the ammonia derived by nitrogen fixation is involved in the regulation of cytosolic GS activity at a post-transcriptional level.

Materials and Methods

Plant materials—The seeds of the parental pea (*Pisum sativum* L. cv. Sparkle) and the Fix$^-$ mutant E135 derived from it (Kneen et al. 1990) were surface-sterilized and inoculated with *Rhizobium leguminosarum* bv. *viciae* strain 128C53. The plants were grown in vermiculite with a nitrogen-free nutrient solution in a greenhouse under natural daylight conditions, as described previously (Suganuma et al. 1993). The mutant nodules lack acetylene reduction activity throughout plant development (Suganuma et al. 1993, 1998). Freshly harvested nodules and roots from Sparkle plants and nodules from E135 plants were used for preparation of protein extracts. Some of them were frozen in liquid nitrogen and stored at −80°C until use for isolation of RNA.

For treatment with ammonia, 5-week-old plants were subirrigated with a solution of 10 mM potassium phosphate buffer (pH 7.0) that contained appropriate concentrations of ammonium sulfate or urea. The control plants were subirrigated with the buffer alone.

Extraction of soluble protein—Nodules and roots were homogenized, separately, with a mortar and pestle in 0.2 M potassium phosphate buffer (pH 7.5) that contained 0.3 M sucrose, 5 mM DTT, 1 mM $MgCl_2$ and 0.4 mM EDTA with 30% (w/w) Polyclar AT powder. The homogenate was centrifuged at 16,000 × *g* for 30 min. The resulting supernatant fluid was used for assay of GS activity and western blot analysis.

Protein was measured by a modified version of Lowry's procedure (Bensadoun and Weinstein 1976), with bovine serum albumin as the standard.

Assay of GS activity—The in vitro semi-synthetase activity of GS was determined by monitoring the formation of γ-glutamyl hydroxamate (Farnden and Robertson 1980). The reaction mixture containing 50 mM imidazole-HCl buffer (pH 7.0), 40 mM sodium glutamate, 4 mM ATP, 33 mM $MgSO_4$ and 33 mM hydroxylamine was incubated at 30°C for 15 min. Control assays minus hydroxylamine were included. The reaction was stopped by the addition of $FeCl_3$ reagent followed by centrifugation, and the absorbance of the supernatant was measured at 540 nm.

Cloning of GS cDNA clone—A λgt10 cDNA library constructed with poly(A)$^+$ RNA from 5-week-old Sparkle nodules (Suganuma et al. 1995) was probed with alfalfa GS cDNA (generously provided by Dr. C.P. Vance, Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN 55108, U.S.A.). One of the cDNA inserts obtained by plaque hybridization, which was 1.3 kb long, was subcloned into pBluescript II SK(+) (Stratagene, La Jolla, CA, U.S.A.) and the nucleotide sequence was partially determined by the method of Sanger et al. (1977) with a BcaBEST Dideoxy Sequencing Kit (TaKaRa, Kyoto, Japan). The nucleotide sequence determined, namely, 150 bp from the 5′ end or 80 bp from the 3′ end, was completely identical to that of GS341 isolated from nodulated roots of pea (Tingey et al. 1987, 1988). GS341 cDNA insert encodes a 37-kDa polypeptide for cytosolic GS, which is the most abundant in nodules. Thus the isolated cDNA insert was used for preparation of antiserum against GS, and for northern blot analysis.

Preparation of antiserum against recombinant GS—The isolated cDNA insert, which has *Eco*RI adaptors at both ends, was digested with *Bgl*II, with a restriction site located at position +15 from the start codon and then the predicted protein lacked 5 amino acid residues as compared with the complete protein. The digested cDNA fragment was ligated into the *Bgl*II and *Eco*RI cloning sites of pRSETC expression vector (Invitrogen, San Diego, CA, U.S.A.) and the construct was introduced into *E. coli* strain BL21(DE3).

The host cells carrying the construct were grown in 2 × YT medium with ampicillin at 37°C and the expression of histidine-tagged recombinant GS protein was induced by addition of IPTG to a final concentration of 1 mM. The cells collected by centrifugation were sonicated and centrifuged. The target protein was solubilized with 6 M urea from the resulting pellet and purified by using His·Bind Resin (Novagen, Madison, WI, U.S.A.). The protein obtained was further purified by SDS-PAGE and the corresponding band, with a molecular mass of 43 kDa, was excised from the gel stained with Coomassie brilliant blue. The bands ground in liquid nitrogen were injected into a rabbit with adjuvant and antiserum was raised by the standard method.

Western blot analysis—Soluble proteins from nodules and

roots were separated by SDS-PAGE on a 12.5% (w/v) polyacrylamide gel. After SDS-PAGE, the polypeptides were transferred electrophoretically to an Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.), and immunoreactive polypeptides with the antiserum raised against recombinant GS protein were visualized with a ProtoBlot Western Blot AP System (Promega, Madison, WI, U.S.A.) as described previously (Suganuma et al. 1993).

Northern blot analysis—Total RNA was isolated by phenol extraction and LiCl precipitation (Suganuma et al. 1995). Electrophoresis of total RNA on an agarose gel that contained formaldehyde and transfer to a Hybond-N membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) were performed by standard procedures (Sambrook et al. 1989). DNA probes were prepared from the isolated GS cDNA with ^{32}P-dCTP and a *Bca*BEST Labeling Kit (TaKaRa). Hybridization and washing were done as described previously (Suganuma et al. 1997).

Results

Activity, protein and mRNA for GS in ineffective nodules—The activity of GS in effective nodules and ineffective E135 nodules during plant development has been studied previously (Suganuma et al. 1993). In the present study, we also determined the activity in roots for comparison. GS activity in ineffective nodules from 3-week-old E135 plants was similar to that in effective nodules from Sparkle plants, and both had activity higher than that in 3-week-old Sparkle roots (Fig. 1). However, GS activity in ineffective nodules decreased gradually and became lower than that in roots after 6 weeks while the GS activity in effective nodules increased and that in roots remained low during plant development.

Antiserum raised against histidine-tagged recombinant GS protein immunoprecipitated GS activity in nodule crude extracts up to 56% of the initial activity with increasing amount of the antiserum (data not shown), indicating that the antibody against GS was certainly obtained by using the recombinant GS protein. Western blot analyses using the antiserum prepared revealed that the antiserum recognized a band corresponding to a molecular mass of approximately 39 kDa in both nodules and 40 kDa in roots, which were nearly identical to the molecular mass (39,295) of the deduced amino acid sequence from isolated GS cDNA. Changes in levels of immunologically detectable polypeptides for GS in effective nodules, ineffective nodules and roots during development paralleled GS activities shown in Figure 1 (Fig. 2). The level of GS polypeptides in ineffective nodules declined during plant growth, and the band corresponding to GS was undetectable at 6 and 7 weeks. Besides the 39 kDa polypeptide, two bands corresponding to polypeptides of approximately 27 kDa and 25 kDa were also detected in effective nodules at the later stages, but very weakly in ineffective nodules and in roots. These two polypeptides may represent proteolytic products of the 39 kDa polypeptide.

A single band estimated to be approximately 1.4 kb in

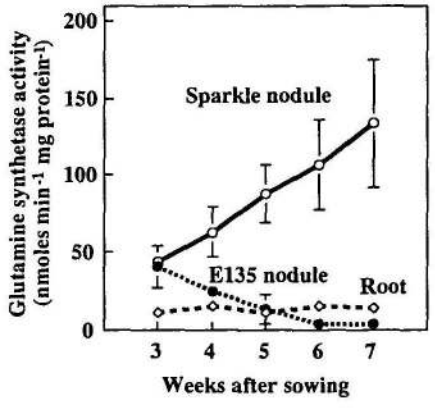

Fig. 1 In vitro activity of GS in effective Sparkle nodules, ineffective E135 nodules, and roots during plant development. Samples from Sparkle roots were prepared with tissue from which nodules had been harvested. All values are means of results of three determinations and bars represent standard errors.

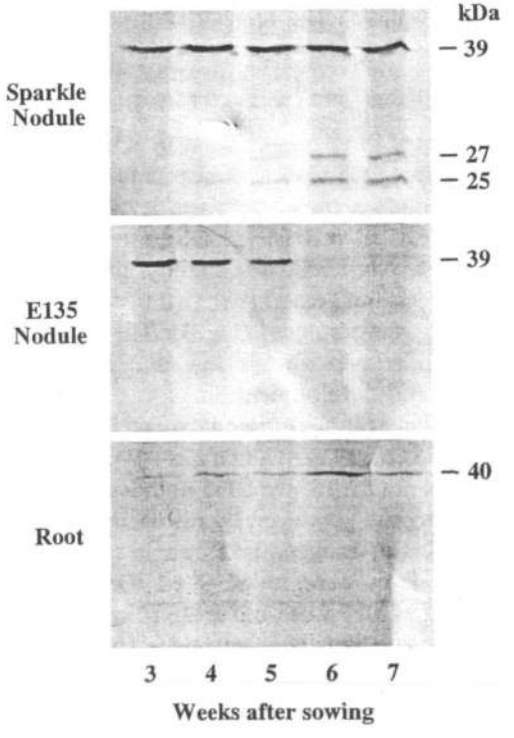

Fig. 2 Immunodetection of GS polypeptides in effective Sparkle nodules, ineffective E135 nodules, and roots during plant development. Samples from Sparkle roots were prepared with tissue from which nodules had been harvested. Twenty μg of soluble protein from each source were subjected to SDS-PAGE on a 12.5% (w/v) polyacrylamide gel. The GS polypeptide was detected by western blotting with an antiserum raised against recombinant pea GS protein.

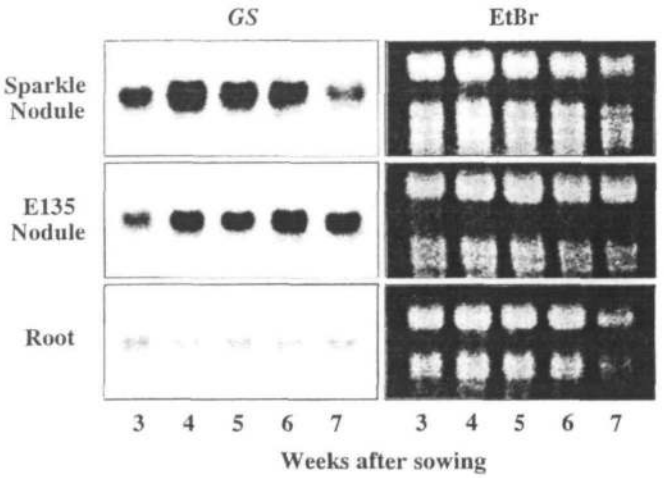

Fig. 3 Steady-state expression analysis of GS mRNA in effective Sparkle nodules, ineffective E135 nodules, and roots during plant development. Samples from Sparkle roots were prepared with tissue from which nodules had been harvested. Ten μg of total RNA from each source were subjected to electrophoresis on a 1.25% (w/v) agarose gel that contained formaldehyde. The blotted bands of RNA were probed with the full-length sequence of GS341 cDNA that had been radiolabeled with [^{32}P]dCTP. The rRNA stained with ethidium bromide (EtBr) are shown as controls.

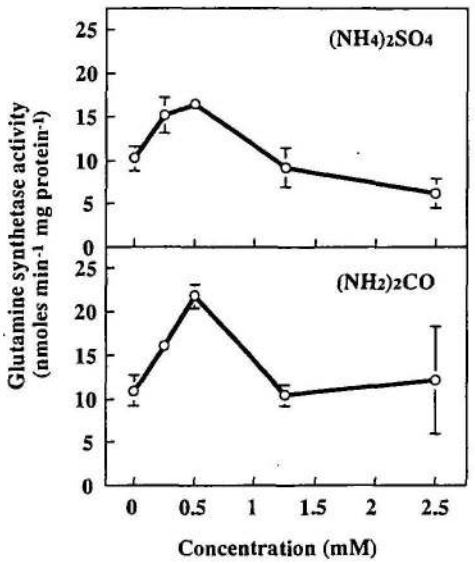

Fig. 4 Effects of ammonia on in vitro activity of GS in ineffective E135 nodules. A 10 mM potassium phosphate buffer (pH 7.0) containing appropriate concentrations of ammonium sulfate or urea was supplied to 5-week-old E135 plants. After 2 d of treatment, nodules were harvested and their in vitro activities of GS were determined. All values are means of results of three determinations and bars represent standard errors.

size was detected in all three tissues by northern blot analyses using isolated GS cDNA clone as a probe. Contrary to the results regarding the activity and level of polypeptides for GS, the level of mRNA for GS in ineffective nodules during plant development was slightly lower than that in effective nodules, but greater than that in roots even in later stages of plant development (Fig. 3). The level of mRNA for GS in roots was always low and almost constant throughout plant development.

Effects of exogenous ammonia on activity, protein and mRNA for GS in ineffective nodules—We postulated that low GS activity in ineffective E135 nodules might be due to a lack of ammonia produced by nitrogen fixation. Therefore, the effects of exogenous ammonia on GS activity in ineffective nodules were investigated. GS activity in ineffective nodules of 5-week-old E135 plants was enhanced by application of lower concentrations of ammonium sulfate or urea for 2 d; 0.5 mM ammonium sulfate or 0.5 mM urea was the most potent (Fig. 4). The enhancement of GS activity was also observed in roots but not in effective nodules (Fig. 5). In both E135 nodules and roots, urea was more potent than ammonium sulfate.

The enhancement of GS activity in ineffective nodules was transient and the GS activity returned to the initial level 2 d after treatment (Fig. 6). In effective nodules, GS activity was gradually decreased by prolonged treatment with urea. However, GS activity in roots increased at 1 d and the increased activity continued after treatment with urea.

Levels of polypeptides and mRNA for GS in nodules

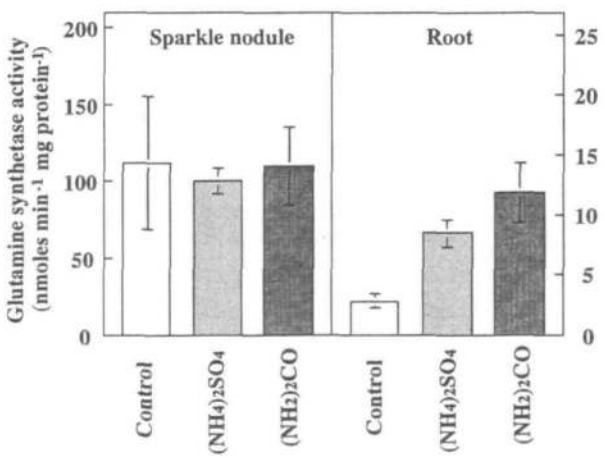

Fig. 5 Effects of ammonia on in vitro activity of GS in effective Sparkle nodules and roots. A 10 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM ammonium sulfate or urea was supplied to 5-week-old Sparkle plants. The control plants were supplied with the buffer alone. After 2 d of treatment, nodules and roots were harvested and their in vitro activities of GS were determined. All values are means of results of three determinations and bars represent standard errors.

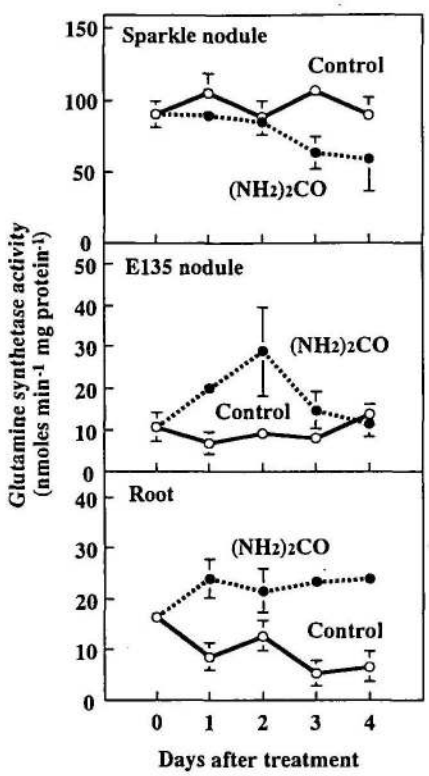

Fig. 6 In vitro activity of GS in effective Sparkle nodules, ineffective E135 nodules, and roots after treatment with ammonia. A 10 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM urea was supplied to 5-week-old Sparkle and E135 plants. The control plants were supplied with the buffer alone. Nodules and roots were harvested daily after treatment and their in vitro activities of GS were determined. All values are means of results of three determinations and bars represent standard errors.

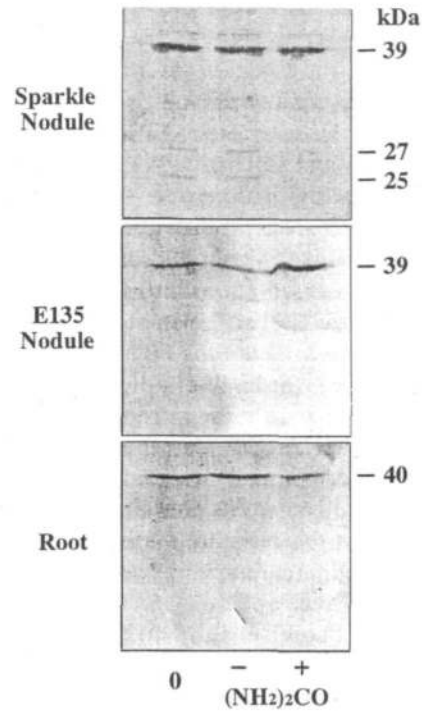

Fig. 7 Effects of ammonia on the level of GS polypeptides in effective Sparkle nodules, ineffective E135 nodules, and roots. A 10 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM urea was supplied to 5-week-old Sparkle and E135 plants. The control plants were supplied with the buffer alone. After 2 d of treatment, nodules and roots were harvested and the GS polypeptide was detected by western blot analysis as described in the legend of Figure 2.

and roots were examined in Sparkle and E135 plants treated with 0.5 mM urea for 2 d. The amount of polypeptides for GS in ineffective nodules was increased by urea although the urea hardly affected the amounts of GS polypeptides in roots and effective nodules (Fig. 7). On the contrary, the increase in the level of mRNA for GS by treatment with urea was not detected in ineffective nodules, nor in roots or effective nodules (Fig. 8).

Discussion

GS activity in ineffective E135 nodules was similar to that in effective nodules and higher than that in roots at the early stages of plant development (Fig. 1). However, in the later stages of nodule development, GS activity in ineffective nodules was lower than that in roots. This indicates that induction of GS activity in pea nodules occurs independent of nitrogenase activity, but that nodule effectiveness is required for maintenance of GS activity. With reduction in GS activity, the level of polypeptides for GS in

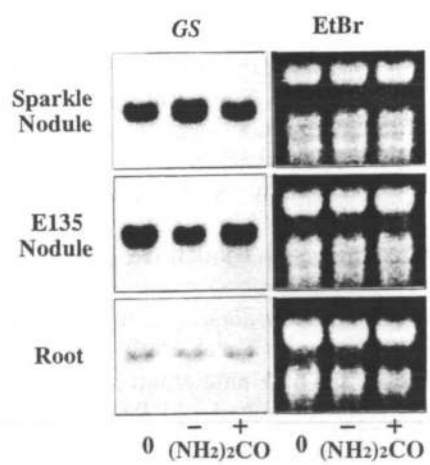

Fig. 8 Effects of ammonia on the steady-state level of GS mRNA in effective Sparkle nodules, ineffective E135 nodules, and roots. A 10 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM urea was supplied to 5-week-old Sparkle and E135 plants. The control plants were supplied with the buffer alone. After 2 d of treatment, nodules and roots were harvested and the GS mRNA was detected by northern blot analysis as described in the legend of Figure 3.

ineffective nodules decreased during plant development while the level of the mRNA was apparently higher than that in roots in the later stages of plant development (Fig. 2, 3). Our results concerning the level of mRNA for GS in ineffective nodules were consistent with those reported by Walker and Coruzzi (1989). From these results, it is suggested that the maintenance of GS activity in pea nodules is regulated post-transcriptionally by the effectiveness of the nodules. Vance and his collaborators have presented similar observations using an alfalfa mutant (Egli et al. 1989, Vance et al. 1994). However, in ineffective nodules induced on a *Vicia faba* Fix^- mutant, the activity of GS is not induced; the levels of polypeptides and mRNA for GS remain similar to those in roots during nodule development (Häser et al. 1992). In ineffective *V. faba* nodules, bacteria are occasionally released into nodule cells but differentiation into bacteroids does not occur (Häser et al. 1992). By contrast, bacteria are released into nodule cells and the bacteria differentiate into bacteroids in ineffective alfalfa nodules (Vance and Johnson 1983) and in ineffective E135 nodules (Kneen et al. 1990). Therefore, release of bacteria into nodule cells or differentiation into bacteroids may be required for induction of expression of GS in nodules.

The level of mRNA for GS in ineffective E135 nodules was slightly lower than that in effective nodules (Fig. 3). Walker and Coruzzi (1989) showed that mRNAs for nodule-enhanced forms of GS are accumulated in the ineffective nodules at the same level as in effective nodules, but that the accumulation of GS299, encoding GS polypeptide predominant in roots, is decreased in E135 nodules relative to wild-type nodules. In our study, northern blot analysis using the GS341 cDNA as a probe revealed the level of total transcripts for cytosolic GS polypeptides because the coding regions of cytosolic GS genes are homologous. Therefore, the lower abundance of mRNA detected in E135 nodules is likely to be attributed to the reduced level of mRNA for GS299. This suggests that expression of GS299 in pea nodules is regulated by the effectiveness of the nodules. The differential expression of GS genes in nodules has also been described in studies using soybean, *P. vulgaris* or *Medicago truncatula* (Padilla et al. 1987, Cock et al. 1990, Roche et al. 1993, Stanford et al. 1993, Temple et al. 1996).

Addition of 0.5 mM ammonium sulfate or urea enhanced GS activity in 5-week-old E135 nodules (Fig. 4), indicating that ammonia plays a role for supporting GS activity in nodules. Furthermore, the amount of polypeptides for GS in the ineffective nodules was also increased (Fig. 7), while no increase of mRNA for GS was detected after the treatment with ammonia (Fig. 8). By contrast, in nitrogen-fixing effective nodules, the GS activity, and levels of polypeptides and mRNA for GS were hardly affected by the treatment with ammonia (Fig. 5, 7, 8). In addition, GS activity in ineffective nodules of 3-week-old plants, which was similar to that in effective nodules, was not increased by the treatment with ammonia (data not shown). These results imply that exogenous ammonia applied to ineffective nodules replenished the lack of ammonia due to ineffective nitrogen fixation, and restored the polypeptides for GS, resulting in the enhancement of GS activity in ineffective nodules at the later stages of plant development. From these findings, we suggest that ammonia produced by nitrogen fixation is required for maintenance of GS activity in pea nodules at the level of post-transcription. On the other hand, the activity of GS in roots was also increased by ammonia (Fig. 5) and the increased activity continued during treatment with urea (Fig. 6), although no increase in polypeptides or mRNA for GS was detected (Fig. 7, 8). This may indicate that enhancement of GS activity in roots is induced by different controls from those observed in ineffective nodules.

The expression of GS genes or GS activity in legume roots has been shown to be increased by supplement with ammonium solution (Hirel et al. 1987, Hoelzle et al. 1992, Stanford et al. 1993). In the present study, we found that urea enhanced GS activity (Fig. 4). Since pea nodules have a significant urease activity (data not shown), urea may be able to supply ammonia moderately to nodules by avoiding any drastic changes in the physiological status of the nodules. However, it is unclear why the effect of urea on GS activity in ineffective nodules was transient (Fig. 6). The transient enhancement of GS activity in ineffective nodules was observed even when urea was renewed daily during treatment (data not shown). Therefore, the decline of GS activity in ineffective nodules observed 2 d after treatment with urea is not due to the exhaustion of ammonia applied to plants.

There is some evidence suggesting that cytosolic GS in roots or nodules is regulated post-translationally, such as turnover of protein or assembly of polypeptides (Hoelzle et al. 1992, Gao and Wong 1994, Temple et al. 1996). The present study shows that the maintenance of cytosolic GS activity in pea nodules is regulated post-transcriptionally, and that ammonia is required for maintenance of cytosolic GS activity at a post-transcriptional level. However, it remains to be determined how ammonia controls the amount of GS polypeptides in the nodules. Furthermore, GS activity in E135 nodules was not restored to the level in effective nodules, suggesting that another factor(s), besides ammonia, is involved in the maintenance of GS activity in pea nodules. Further study on in vitro and in vivo translation or turnover of GS protein is required for elucidation of molecular control mechanisms of GS activity in pea nodules.

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