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Expression of glutamine synthetase genes in roots and nodules of *Phaseolus vulgaris* following changes in the ammonium supply and infection with various *Rhizobium* mutants

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Abstract

In this paper we have examined whether the four glutamine synthetase (*gln*) genes, expressed in roots and nodules of *Phaseolus vulgaris* are substrate-inducible by ammonium. Manipulation of the ammonium pool in roots, through addition and removal of exogenous ammonium, did not elicit any changes in the abundances of the four mRNAs thus suggesting that the *gln* genes in roots of this legume are neither substrate-inducible by ammonium nor derepressed during nitrogen starvation. In nodules the effect of the ammonium supply on expression of the *gln* genes has been examined by growing nodules under argon/oxygen atmospheres, or with a number of Fix$^-$ *Rhizobium* mutants, and following addition of exogenous ammonium. The results of these experiments suggest that the expression of the *gln-γ* gene, which is strongly induced during nodule development, is primarily under a developmental control. However nitrogen fixation appears to have a quantitative effect on expression of *gln-γ* as the abundance of this mRNA is about 2 to 4-fold higher under nitrogen-fixing conditions. This effect could not be mimicked by addition of exogenous ammonium and moreover is not specific to the *gln-γ* gene as mRNA from a leghaemoglobin gene was similarly affected. Taken together these results have failed to find an effect of ammonium on specifically inducing the expression of glutamine synthetase genes in roots and nodules of *P. vulgaris*.

Introduction

Glutamine synthetase (GS, EC 6.3.1.2) catalyses the assimilation of ammonium into glutamine. This ammonium is produced by the reduction of the primary nitrogen sources of the plant (nitrate or, for legumes, dinitrogen) and is released from other metabolic pathways such as photorespiration, phenylpropanoid metabolism and catabolism of amino acids and ureides [30]. The enzyme therefore occupies a key position in the utilisation of the primary nitrogen sources and in maintaining the nitrogen economy of the plant.

In higher plants GS is encoded by a small nuclear gene family [see 13, 18]. One of the genes encodes a chloroplast located polypeptide that is

synthesized as a higher molecular weight precursor and post-translationally imported into the chloroplasts [40, 27] whereas several genes encode cytosolic polypeptides [20, 39, 40, 5]. These polypeptides then assemble into active, octameric isoenzymes in their respective subcellular compartments [4]. Measurements of mRNA abundance indicate that the different GS genes are differentially regulated during development in an organ-dependent manner [15, 20, 39, 40].

Recently it has been suggested that the genes encoding cytosolic GS in soybean are directly induced by the available ammonium [22]. This conclusion was based on the observations that the abundance of GS mRNA increased in soybean roots following addition of ammonium to nitrogen-starved plants and in nodules by growth in nitrogen-fixing conditions compared to nodules grown in an atmosphere of argon/oxygen or with a Fix^- mutant of *Rhizobium*. In addition, it has been shown in pea leaves that mRNA for the chloroplast located GS is more abundant in plants grown under photorespiratory conditions (which produce the major flux of ammonium in leaves) than in plants grown in conditions in which this pathway is suppressed [17]. These results may therefore suggest that GS, like nitrate reductase in higher plants [12, 14, 8] and *Chlorella* NADP-dependent glutamate dehydrogenase [3], may be a substrate-inducible enzyme.

In this study we set out to examine whether the GS genes in the legume *Phaseolus vulgaris* are also regulated by ammonium. This species possesses five GS genes, four of which (*gln-α*, *gln-β*, *gln-γ* and *gln-δ*) have been shown to be expressed and corresponding cDNA clones have been obtained and sequenced [15, 20, 27, 5]. The *gln-α*, *gln-β* and *gln-γ* genes encode the cytosolic GS polypeptides, α, β and γ, previously identified in plant tissue by Lara *et al.* [25], whereas the *gln-δ* gene encodes a precursor to the chloroplast-located GS [27]. An RNase protection technique has been set up to measure the abundances of each of these four GS mRNAs in plant tissue, in a specific and quantitative manner [5]. Using this technique we have investigated the role of ammonium in regulating the expression of these genes in roots and nodules of *P. vulgaris* and have used a number of *Rhizobium* mutants to study the developmental expression of the GS genes in nodules.

Materials and methods

Plant material

Root material from non-inoculated plants of *Phaseolus vulgaris* L. cv. Tendergreen was obtained from plants grown in perlite under the growth room conditions previously described [27]. The plants were watered with a nutrient solution lacking a nitrogen source or containing 1 mM $(NH_4)_2SO_4$, 1 mM $Ca(NO_3)_2$ or 1 mM NH_2CONH_2 [9]. For the experiments described in Figs. 1 and 2, plants grown for 10 days in perlite were suspended (with their cotyledons removed) in trays containing 5 l of nutrient solution and

Table 1. Strains of *R. leguminosarum* bv. *phaseoli.*

Strain	Characteristics	Nodule morphology	Reference
CE3	Str^r Nod^+ Fix^+ (wild-type)	large, pink nodules	Noel *et al.* [32]
CE108	Str^r Km^r Nod^+ Fix^-	large, pink nodules	Noel *et al.* [32]
CE116	Str^r Km^r Trp^- Ndv^{slow} $Fix^{+/-}$	medium, white nodules	Noel *et al.* [32]
CE123	Str^r Km^r Noi^+ Fix^-	small, nodule-like swellings	K.D. Noel, unpublished
CE106	Str^r Km^r Pur^- Inf^- Noi^+ Fix^-	small, nodule-like swellings	VandenBosch *et al.* [43]
4292	Rif^r Nod^+ Fix^+ (wild-type)	large, pink nodules	Johnston *et al.* (1982)*
F51	Rif^r Km^r NifA1	large, pink nodules	F.K.L. Hawkins and A.W.B. Johnston, unpublished

CE108, CE116, CE123 and CE106 are Tn5 derivatives of CE3. F51 is a Tn5 derivative of 4292.
Ndv = nodule development, Noi = nodule initiation, Inf = infection threads. *J Gen Microbiol 128: 85–93.

grown for a further 3 days in liquid culture. The nutrient solution was then changed as indicated and the whole root systems were harvested at the appropriate times, washed 3 times in distilled water, frozen in liquid nitrogen and stored at -80 °C. The nutrient solution either lacked a nitrogen source [9] or was supplemented with 5 mM $(NH_4)_2SO_4$.

Strains of *R. leguminosarum* bv. *phaseoli*, used to nodulate plants, are described in Table 1. Nodulated plants, with their root systems enclosed in 80% Ar/20% O_2 and 80% N_2/20% O_2 atmospheres, were grown essentially as described by Atkins *et al.* [2] except that we used 2.5 l pots containing Leca (porous mineral granules; see [6]) as a support for the root systems of six plants per pot. The plants were initially germinated in perlite or vermiculite for several days and then transferred to the growth pots. The seedlings were then inoculated with *Rhizobium* strain 4292 (Day 0) and about four days later the pots were sealed with silicone rubber glue and the appropriate gas mixture was passed through the pots at a flow rate of about 250 ml/min. The gas composition in the pots was checked periodically by gas chromatography and was generally less than 0.5% N_2 in the Ar/O_2 pots. In some experiments 10 mM $(NH_4)_2SO_4$ containing nutrient solution was sprayed over the root systems by introduction of the degassed solution through a gas-tight seal; the ammonium concentration was diluted to about 10 mM by the nutrient solution already present in the pots. In the experiments described in Fig. 5 which used mutant Fix$^-$ *Rhizobium* strains, the plants were grown in sterilised open pots containing perlite and were watered with sterilised nutrient solution. Nodules were then harvested 21 or 22 days after inoculation, but it should be noted that the nodule-like swellings formed with mutants CE106 and CE123 were about 50% contaminated with attached roots. The nodule samples were checked for nitrogenase activity as described below. The nutrient solutions used for growing nodules lacked a nitrogen source and was either the Long Ashton medium [21] for the experiments in Figs. 3 and 5 or the solution described by Chaillou *et al.* [9] for the experiment in Fig. 4. The experiment described in Fig. 4 was carried out in growth rooms as described by Chen and Cullimore [10] whereas experiments in Figs. 3 and 5 were carried out in a greenhouse with the day-length increased to 14 h by artificial lighting.

RNase protection assay of mRNA abundance

An RNase protection method [23] was used to assay for specific mRNAs. Isolation of total RNA and synthesis of probes specific to *gln-α*, *gln-β*, *gln-γ*, *gln-δ* and Lhb-1 mRNA have been described previously [5]. Note that the probes to the four GS genes are of different sizes and are prepared to different parts of the mRNA. They can, therefore, be used in the same hybridization without competing with each other as they hybridize to the GS mRNAs. Two different sized probes have been used for the *gln-β* gene; a 309 nucleotide probe in Figs. 1 to 4 and a 166 nucleotide probe in Fig. 5. For each of the GS probes, the fragment protected by its homologous mRNA is always of a defined size (between 4 and 42 nucleotides shorter than the synthesised probe) and it is this defined fragment that is quantified as described below. Each probe has been shown to be absolutely specific for assaying its homologous mRNA under the conditions used. No signal of the defined size is produced with the other GS mRNAs [5]. Following RNase protection assays, hybridized probe was quantified by scanning autoradiographs with a Joyce-Loebl Chromoscan 3 densitometer. RNase protection of calibration curves of (m)RNA synthesized *in vitro* from the four GS cDNA clones allowed quantification of GS mRNA abundances in the plant RNA samples [5]. Abundance of the Lhb-1 mRNA was estimated in relation to the abundance in fixing nodules, which has been experimentally determined to be about 100-fold higher than *gln-γ* mRNA.

Analytical procedures

Ammonium was assayed in 0.5 g of fresh root material extracted in a pestle and mortar with 1 ml

of 0.1 M HCl. The homogenate was centrifuged in a microfuge for 4 min and the supernatant assayed for ammonium by the method of McCullough [28]. The Western blot was carried out exactly as described in Bennett *et al.* [5] with constant amounts of 100 μg protein loaded in each track. Nitrogenase activity was measured either in isolated nodules or in nodulated root systems using the acetylene reduction assay [24].

Results

Effect of exogenous addition of ammonium or nitrogen starvation on abundances of GS mRNAs in roots

Plants grown for 10 days in vermiculite in the absence of an externally applied nitrogen source were set up in aerated, liquid, nitrogen-free culture. At this time their cotyledons were removed and the plants were then nitrogen-starved for a further 3 days. Figure 1A shows that when ammonium was added (to 10 mM) the ammonium level in the root tissue increased transiently 26-fold to about 5 μmol/g fresh weight within 4 h. The abundances of the four GS mRNAs were then measured in samples of total RNA isolated from roots of these ammonium-supplemented plants and from control plants, using an RNase protection technique (Fig. 1B). Densitometry scanning of this data showed that the steady state abundances of each of the four GS mRNAs varied by less than 30% in this experiment and there was no evidence for an increased abundance of any of the GS mRNAs following addition of ammonium. This experiment was then repeated over a longer time-course and again a transitory rise in the level of ammonium in the root tissue was observed although by 48 h the level had decreased to less than 1 μmol/g fresh weight (data not shown). Figure 2A shows the abundance of the *gln-α* and *gln-β* mRNAs (which constitute over 98% of the root GS mRNA) during this experiment (data for *gln-γ* and *gln-δ* are not shown). Again there were no significant changes in the abundance of the GS mRNAs following the ammonium addition; note that part of one of the control samples was lost in this experiment.

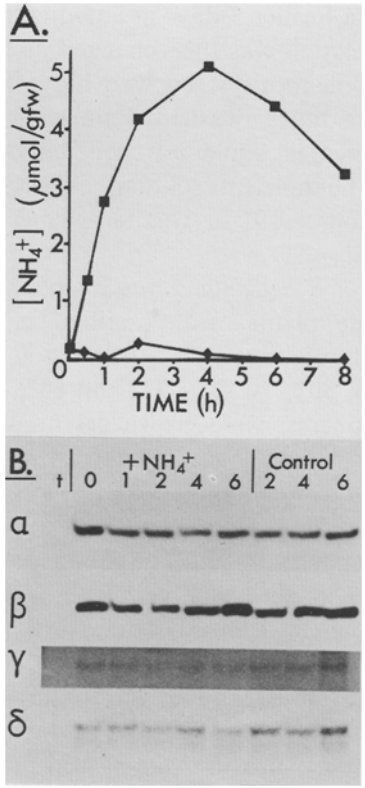

Fig. 1. Effect of exogenously applied ammonium on tissue ammonium levels and the abundances of the four GS mRNAs, in roots previously starved of nitrogen. 10 mM ammonium was added exogenously to plants grown in nitrogen-free culture and roots were harvested in these ammonium-supplemented plants (+ NH_4^+) and in the nitrogen-starved plants (control) for up to 8 h following the addition. A. Tissue ammonium levels following ammonium addition (■) and in control plants (◆). B. Abundances of the four GS mRNAs. These were measured using an RNase protection assay in a yeast tRNA control (t) and in total RNA samples isolated from the root samples. The autoradiographs were exposed for different times but by reference to standard curves the abundances of the *gln-α*, *gln-β*, *gln-γ* and *gln-δ* GS mRNAs were assessed to be approximately 6.0, 20.0, 0.1 and 0.4 pg/μg total RNA respectively.

Figure 2B depicts the abundances of the *gln-α* and *gln-β* mRNAs in the reverse experiment where plants were grown continuously on 10 mM ammonium and then were transferred to nitrogen-free medium for up to 48 h. Over this period of nitrogen starvation the ammonium level in the root tissue declined from about 0.3 μmol/g fresh weight to barely detectable levels. Again, in this

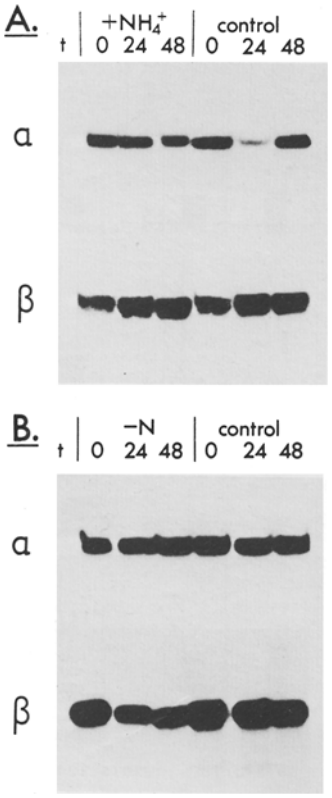

Fig. 2. Effect of changes in exogenous ammonium on the abundances of *gln-α* and *gln-β* mRNAs in roots. The abundance of the mRNAs were measured using an RNase protection assay in a yeast tRNA control (t) and in total RNA samples harvested 0, 24 and 48 h after the test plants were transferred to the new conditions. A. Effect of addition of 10 mM exogenous ammonium to plants previously grown in nitrogen-free culture. B. Effect of nitrogen-starvation on plants previously grown in the presence of 10 mM exogenous ammonium.

experiment, no significant changes in the abundances of any of the four GS mRNAs were observed (data for only *gln-α* and *gln-β* mRNAs, are shown).

Plants were also grown continuously in vermiculite either with no nitrogen source or with a 2 mM nitrogen source of either ammonium, nitrate or urea. No obvious differences were observed in the abundance of the four GS mRNAs in roots from 14-day-old plants grown under these different conditions (data not shown).

Effect of ammonium supply and nodule development on GS mRNA abundances in root nodules

In root nodules the main supply of ammonium is from dinitrogen fixation. In order to examine whether the ammonium supply affects the abundance of the four GS mRNAs, dinitrogen fixation was inhibited by growing the nodules either in an atmosphere lacking dinitrogen or with Fix^- mutants of *Rhizobium*.

In the first experiment plants inoculated with a Fix^+ strain of *Rhizobium* were grown in a nitrogen-free medium with their root systems enclosed in either a 80% Ar/20% O_2 (Ar/O_2) or, as a control, in a 80% N_2/20% O_2 (N_2/O_2) atmosphere. At day 21 following inoculation, the nodules grown under Ar/O_2 were found to have about 60% of the acetylene reduction activity of the control nodules, showing that nitrogenase was expressed under the Ar/O_2 conditions, but at

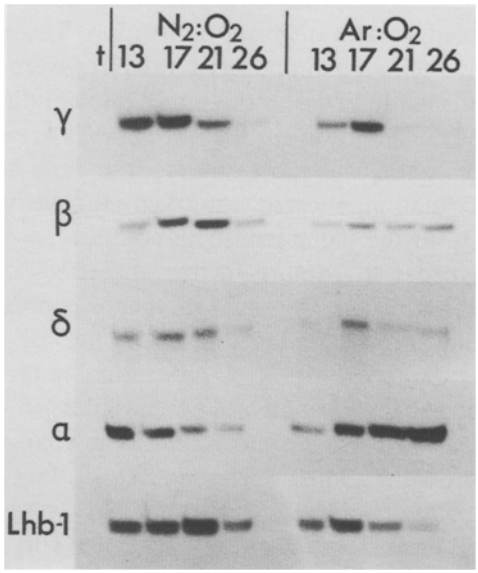

Fig. 3. Abundance of the four GS mRNAs, and Lhb-1 mRNA, in nodules grown under atmospheres of N_2/O_2 or Ar/O_2. The mRNA abundances were measured using an RNase protection technique in yeast tRNA (t) and in total RNA samples from nodules harvested 13, 17, 21 and 26 days after inoculation with *Rhizobium* Fix^+ strain 4292. The abundances of mRNA at day 13 under the N_2/O_2 atmosphere were assessed to be 13.0, 8.8, 1.1, 1.4 and approximately 1300 pg/μg total nodule RNA for the *gln-γ*, *gln-β*, *gln-δ*, *gln-α* and Lhb-1 mRNAs respectively.

lower levels (see also [1], [22]). By day 26, plants grown in the Ar/O_2 regime were chlorotic (a symptom of nitrogen deficiency) suggesting that these plants were not fixing nitrogen. Moreover their nodules were smaller than the control nodules and had a green coloration indicating that they were senescing. Figure 3 shows the abundances of mRNA from the four GS genes and also from a leghaemoglobin (Lhb-1 [5]) gene in nodules at different stages of development, under the two conditions. The mRNA from the *gln-γ* gene, which is barely detectable in roots (Fig. 1B) and increases about 150- to 750-fold during normal nodulation [5] had reached a high abundance in nodules grown under both gaseous regimes by days 13 and 17. However the abundance of this mRNA was about 2-fold lower under the Ar/O_2 conditions and showed a more pronounced decline as the nodules senesced. The Lhb-1 gene, which shows nodule-specific expression (see [5]), was also expressed under the Ar/O_2 conditions and, like *gln-γ*, the abundance of its mRNA declined at later stages of nodulation. The abundance of the *gln-β* and *gln-δ* mRNAs were generally slightly lower in the non-fixing conditions. Surprisingly however, the *gln-α* mRNA, which under N_2/O_2 was most abundant at early stages of nodulation, showed a marked increase under Ar/O_2, in the older senescing nodules.

In order to explore further the effect of the ammonium supply on GS gene expression, nodules grown under the Ar/O_2 regime were either switched to N_2/O_2 or ammonium was added exogenously. In addition nodules grown on N_2/O_2 were switched to Ar/O_2. The changes to the growth regimes were made at 15 days after *Rhizobium* inoculation and nodules were harvested just before the switches (day 15) and 1 and 4 days later (days 16 and 19). The abundances of the GS and Lhb-1 mRNAs in these nodules are shown in Fig. 4A. With regard to *gln-γ*, *gln-β*, and Lhb-1 mRNAs, the following observations were made:

1) In nodules grown under Ar/O_2, all three mRNAs were at a lower abundance than in control nodules, in agreement with the previous experiment;

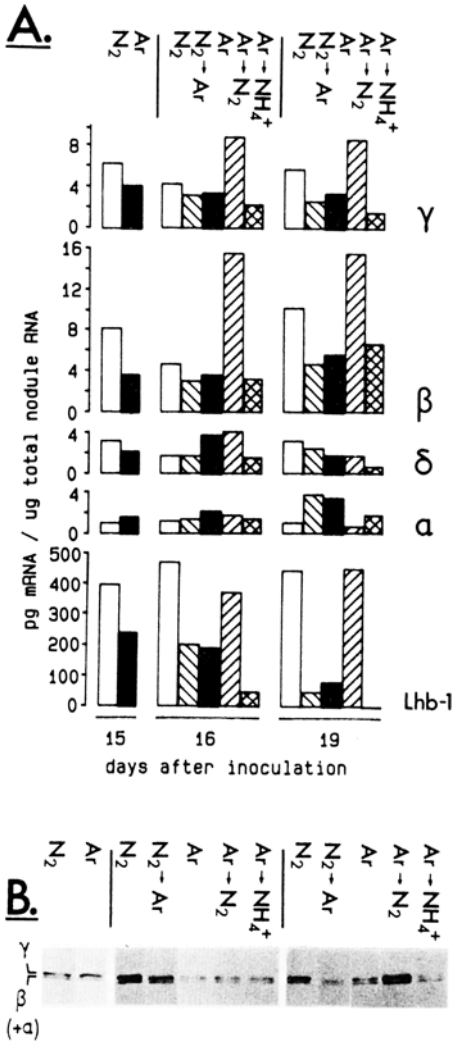

Fig. 4. Effect of changes in the ammonium supply on GS mRNAs and polypeptides in root nodules grown under Ar/O_2 and N_2/O_2 atmospheres. Nodules were grown for 15 days following inoculation with *Rhizobium* Fix^+ strain 4292 under an atmosphere of either N_2/O_2 or Ar/O_2. At this time, some of the nodules were harvested and the remaining plants were either allowed to grow further under the same regime or were treated as follows: the gas flow was either switched from Ar/O_2 to N_2/O_2 or *vice versa* or 20 mM ammonium was added exogenously to the nodulated root systems of Ar/O_2 grown plants. Samples of nodules grown under these various conditions were then harvested at day 16 and day 19. A. Abundances of the four GS and Lhb-1 mRNAs, quantified by an RNase protection technique. B. Western blot of GS polypeptides. Note that the β and α polypeptides cannot be separated, but β is generally much more abundant than α in nodules.

2) All increased in abundance within 1 day of switching from Ar/O_2 to N_2/O_2 and these increased abundances were sustained at 4 days;
3) Addition of ammonium to Ar/O_2-grown nodules did not elicit an increase in abundance of the three mRNAs and, in fact, caused a substantial decline in the *gln-γ* and Lhb-1 mRNAs by 4 days (it was noticeable that nodules treated with ammonium were green after 4 days and showed very little acetylene reduction activity);
4) Nodules that were transferred from N_2/O_2 to Ar/O_2 showed a decline in the abundance of these three mRNAs within 1 day and this was more pronounced at 4 days.

Changes in the abundance of the *gln-δ* mRNA were generally similar to those of the three mRNAs described above but its relatively low abundance made the quantitation more difficult. It was obvious however that the *gln-α* mRNA responded differently to the other four mRNAs and was of higher abundance in nodules either grown on Ar/O_2 or switched to this regime from N_2/O_2.

To see whether these changes in the GS mRNAs were affecting the amount of the different GS polypeptides, nodule extracts containing equal amounts of protein were subjected to western blotting. The two major nodule GS polypeptides, γ and β, can just be separated and detected in this way, although β co-runs with the small amount of α [4]. Fig. 4B shows that the abundances of these two polypeptides followed the abundances of the mRNAs; in the samples in which *gln-γ* and *gln-β* mRNAs were most abundant (nodules grown on N_2/O_2 or switched from Ar/O_2 to N_2/O_2) the two polypeptides were most prominent.

The conditions which lead to a strong induction of expression of *gln-γ* in nodules were examined further by growing nodules with a number of Fix^- mutants of *Rhizobium*. The characteristics of these mutants are shown in Table 1. In some cases the nodulated root systems were watered with a 20 mM ammonium solution for 3 days before harvesting. Figure 5 shows the abundances of the mRNAs from the *gln-γ*, *gln-β* and Lhb-1 genes in these different nodules. Mutants CE108

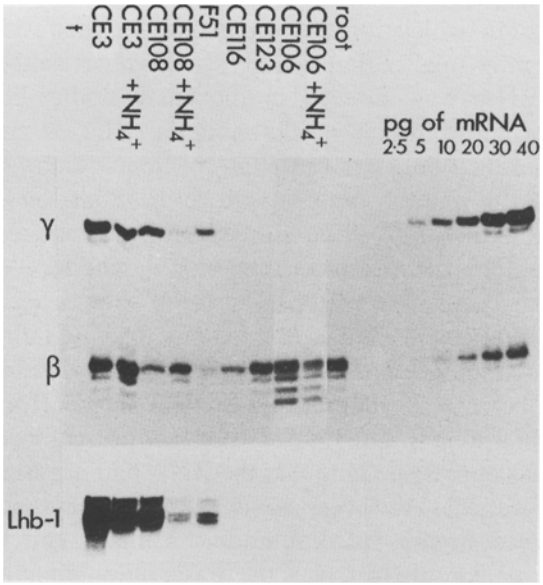

Fig. 5. Abundances of *gln-γ*, *gln-β* and Lhb-1 mRNAs in nodules produced with Fix^+ and Fix^- *Rhizobium* strains. Abundances of mRNAs were assayed by RNase protection in 3 μg samples of yeast tRNA (t), or total RNA isolated from nodules harvested 21 to 22 days after inoculation with either a Fix^+ *Rhizobium* strain (CE3) or with one of a number of Fix^- strains or total RNA from uninoculated roots. In some cases ($+NH_4^+$) 10 mM ammonium was applied exogenously to some of the nodulated root systems for 3 days prior to harvesting. Also shown in this figure are calibration curves of 2.5, 5, 10, 20, 30 and 40 pg of both *gln-γ* and *gln-β* (m)RNA synthesized *in vitro* from cDNA templates. Using these calibration curves, the abundances of mRNAs corresponding to *gln-γ*, *gln-β* and Lhb-1 in total RNA from 21-day-old nodules formed with *Rhizobium* strain CE3 were assessed to be 12.4, 17.1 and approximately 1300 pg/μg total RNA respectively.

and F51 (a *nif*A mutant) form nodules that are morphologically similar to the wild-type nodules (formed with CE3) but with no nitrogen-fixing activity. In these mutant nodules *gln-γ* expression was strongly induced compared to roots (Fig. 5) but the mRNA abundance was lower (about 2- and 4-fold for CE108 and F51 respectively) than in the fixing nodules (formed with CE3). Whereas addition of ammonium did not substantially affect the abundance of *gln-γ* mRNA in fixing nodules, it caused a decline in both *gln-γ* and Lhb-1, but not *gln-β*, mRNAs in the CE108 nodules. A just detectable induction of *gln-γ* and Lhb-1 mRNAs

(compared to roots) was observed with mutant CE116 which formed white nodules that were slightly smaller than wild-type. However neither mRNA was induced in the small nodule-like swellings formed with mutants CE123 and CE106. Moreover addition of ammonium to CE106 nodules did not lead to an induction of either the *gln-γ* or Lhb-1 mRNAs. It was noticeable that the relative changes in abundance of Lhb-1 and *gln-γ* mRNAs in the different nodule samples were similar, although in absolute terms the abundance of the Lhb-1 mRNA is estimated to be at least 100-fold higher than *gln-γ* mRNA (see also [5]). The *gln-β* mRNA was present in all the samples showing that the RNA had not been degraded. However, *gln-β* mRNA abundance varied in the different nodule samples and of particular interest was the observation that its abundance increased following addition of ammonium to CE108 nodules (this observation has been substantiated in a further experiment, data not shown).

Discussion

Effect of ammonium supply on GS expression in roots

Using samples of total RNA and a specific and quantitative detection technique, we have shown that the abundances of the mRNAs related to each of four GS genes expressed in roots of *P. vulgaris* are not affected by exogenous addition of ammonium to nitrogen-starved plants (Figs. 1B and 2A). This is despite a transient but substantial increase in the pools of ammonium within the root tissue (Fig. 1A). We therefore suggest that there is no evidence that GS genes in roots of *P. vulgaris* are induced, directly or indirectly, by the enzyme's substrate, ammonium. This result differs from that reported by Hirel *et al.* [22] who observed an increase in abundance of GS mRNA following ammonium addition to soybean roots. Further work is clearly required to investigate the differences between these results and to determine the extent to which plant species vary in the ammonium inducibility of their GS genes.

The expression of the GS genes in roots of *P. vulgaris* also appears not to change under conditions of nitrogen starvation (Fig. 2B). The regulatory controls on the expression of this enzyme in roots are therefore different to enteric bacteria [29, 38], cyanobacteria [34] and some green algae [41] which increase GS activity in the absence of fixed nitrogen.

The two major GS genes expressed in roots of *P. vulgaris* (*gln-β* and *gln-α*) have been shown to be under a developmental control during early root growth [35] and, as shown here, their expression is not then substantially influenced by the nitrogen supply. However, in these experiments we cannot rule out the possibility that there are localised changes in the expression of the GS genes in different parts of the root that are masked by analysing the whole root system. Moreover as we are using measurements of mRNA abundance as an indicator of expression, we may be failing to detect changes in GS gene expression that do not lead to changes in the abundance of the mRNAs.

It is interesting to note in these experiments, that plants previously starved of nitrogen and then presented with ammonium, showed a rapid but transient accumulation of ammonium in the root tissue to a maximum of about 5 mM within 4 hours. The ammonium concentration then started to decline and by 48 hours (and in plants grown continuously on ammonium) it was less than 1 mM. Similar results have also been observed by Breteler and Siegerist [7]. In our experiments we cannot distinguish between changes in ammonium accumulation occuring due to controls on uptake or changes in the capacity to assimilate the ammonium (perhaps related to the carbohydrate supply). Work on cereals however suggests that the rate of ammonium uptake is substantially affected by the nitrogen supply [26, 31].

Effect of ammonium supply on gln-γ expression in nodules

In nodules the effect of the ammonium supply on GS expression is more complicated than in roots. Ammonium is generated primarily from dinitrogen fixation in the *Rhizobium* bacteroids and is then excreted into the plant cytosol of the infected cells and assimilated by the plant GS (see [37]). In *P. vulgaris* the major GS isoenzymes in nodules are composed of the γ and, to a lesser extent, β polypeptides whereas the δ and α polypeptides make only minor contributions to the total GS activity [4]. Recently Forde *et al.* [19], using fusions of the 5'-upstream regions of GS genes with β-glucuronidase (GUS), have shown that *gln-γ* is expressed only in the rhizobially infected cells of the central zone of transgenic *Lotus corniculatus* nodules whereas the *gln-β* gene appears to be expressed initially throughout the nodule, but in older nodules its expression is largely confined to the nodule cortex. This partial separation of *gln-γ* and *gln-β* expression has been partly confirmed in *P. vulgaris* by nodule dissection [11]. It has previously been shown that the expression of the *gln-γ* gene is strongly induced during normal nodule development [20] before the onset of dinitrogen fixation [36]. However as its mRNA is detectable in roots, albeit at a 150- to 750-fold lower abundance, the increase during nodulation is more correctly considered as a strong enhancement of expression [5].

In this paper we have shown that when fixation is prevented by growing nodules either in an Ar/O_2 atmosphere (Fig. 3) or with certain Fix^- mutants that form nodules with a normal morphology (Fig. 5), the *gln-γ* gene is still strongly induced in expression compared to roots. These experiments, and the work of Padilla *et al.* [36], suggest that ammonium from dinitrogen fixation is not the primary signal for increasing the expression of this gene in nodules. However as the abundance of *gln-γ* mRNA in these non-fixing nodules is lower than in fixing nodules (Fig. 3 and 5) it appears that dinitrogen fixation (and the consequent production of ammonium) exerts a positive, quantitative effect on expression of the *gln-γ* gene. This is clearly seen by the substantial rise in *gln-γ* mRNA abundance that occurred when Ar/O_2 grown nodules were switched back to nitrogen-fixing conditions (Fig. 4). However this effect is not specific to *gln-γ* as the abundance of mRNA from a leghaemoglobin gene (Lhb-1), and also from *gln-β*, were affected in the same way (Fig. 4). We are therefore cautious in interpreting the results of such experiments to suggest that ammonium supplied from symbiotic nitrogen fixation induces GS genes in nodules as the effect is not specific to GS. Moreover, switching on dinitrogen fixation and hence the ammonium supply must initiate a myriad of changes in the metabolism of both the bacteroid and the plant part of the nodule and it is difficult to determine how directly ammonium is involved in eliciting the responses observed.

In an attempt to assess the role of ammonium, ammonium was added exogenously to CE108 Fix^- nodules and to nodules grown under Ar/O_2 (Figs. 4 and 5). In both cases, ammonium addition did not increase the abundance of *gln-γ* mRNA and, in fact, caused a substantial decline (Fig. 4). However it is difficult to conclude from these experiments that ammonium arising from dinitrogen fixation is *not* directly, or indirectly, involved in eliciting *gln-γ* expression as ammonium added exogenously probably does not mimic the localised supply of ammonium from the bacteroids and moreover appeared to initiate the senescence of the nodule.

It is interesting to note that the abundance of *gln-β* mRNA responded differentially to *gln-γ* mRNA in these experiments (Figs. 4 and 5). There is evidence that the abundance of *gln-β* mRNA in CE108 nodules showed a relative increase over a period of several days, following addition of ammonium, suggesting that the *gln-β* gene may respond differently to the ammonium supply in nodules, compared to roots; this possibility is presently under further investigation. In contrast, the leghaemoglobin Lhb-1 gene, which is expressed in the same nodule cell type as *gln-γ* (the infected cells), showed a remarkable coordination of expression with *gln-γ* in both the Ar/O_2 experiments (Figs. 3 and 4) and in the experiment with

Fix$^-$ *Rhizobium* mutants (Fig. 5). However, there are significant differences in the expression of these genes as, in nodules, the Lhb-1 mRNA is about 100-fold more abundant than *gln-γ* mRNA and, in addition, the *gln-γ* gene is expressed in some organs that Lhb-1 is not [5].

Developmental control of gln-γ expression in nodules

In order to study the initial controls on the increased expression of *gln-γ* in nodules compared to roots, the expression of this gene was examined in nodules formed with a number of *Rhizobium* Fix$^-$ mutants blocked at different developmental stages (Fig. 5). Enhanced *gln-γ* expression did not occur in nodule-like swellings (formed with CE106 and CE123) that are devoid of infection threads and lack intra- and intercellular rhizobia [42]. A slightly increased abundance of *gln-γ* mRNA (compared to uninoculated roots) was observed in the medium-sized white nodules formed with CE116; these nodules have not been cytologically characterised but because they later turn pink and start to show low levels of nitrogenase activity [32], they probably contain intracellular rhizobia. In Fix$^-$ nodules (CE108 and F51) that appear morphologically similar to the wild type and, almost certainly, contain intracellular rhizobia, the *gln-γ* gene was expressed at fairly high levels. It thus appears that a certain stage of nodule development needs to be reached before increased expression of *gln-γ* is elicited. This stage of development clearly does not involve the development of nitrogen fixation activity (see [36] and Figs. 3 and 5) but it is not yet clear from the results presented here whether it requires penetration of the nodule cells by *Rhizobium* and the differentiation of the bacteroids.

Using a range of cytologically characterised nodules formed with *R. meliloti* mutants, Norris *et al.* [33] have shown that mRNA for a nodule-enhanced GS polypeptide is not present in lucerne nodules lacking elongate bacteroids but is present in Fix$^-$ nodules containing differentiated bacteroids. In addition, preliminary work by Dunn *et al.*[16] suggests that the expression of the nodule-enhanced GS gene is severely reduced in nodules formed with a rhizobial *ntr*A mutant (this gene is involved in regulating expression of nitrogen assimilatory genes in bacteria). It thus appears that expression of the nodule-enhanced plant GS in lucerne nodules may be correlated with the presence of differentiated bacteroids [33] and perhaps may be regulated by a symbiotic signal controlled by the *ntr*A gene [16]. In *P. vulgaris*, as the *gln-γ* gene is also expressed in certain organs of uninoculated plants, such as stems, petioles and germinating cotyledons [5], the presence of the symbiont is not a prerequisite for increased expression of *gln-γ* within the plant. In nodules our results suggest that the expression of *gln-γ* is primarily under a developmental control, which may be influenced by the bacteroids, and increased expression in nitrogen-fixing nodules may be considered as a secondary, quantitative response.

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