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Two Glutamine Synthetase Genes from *Phaseolus vulgaris* L. Display Contrasting Developmental and Spatial Patterns of Expression in Transgenic *Lotus corniculatus* Plants

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The *gln*-γ gene, which specifies the γ subunit of glutamine synthetase in *Phaseolus vulgaris* L., has been isolated and the regulatory properties of its promoter region analyzed in transgenic *Lotus corniculatus* plants. A 2-kilobase fragment from the 5′-flanking region of *gln*-γ conferred a strongly nodule-enhanced pattern of expression on the β-glucuronidase reporter gene. Parallel studies on the promoter of another glutamine synthetase gene (gln-β) showed that a 1.7-kilobase fragment directed 20-fold to 140-fold higher levels of β-glucuronidase expression in roots than in shoots. Histochemical localization of β-glucuronidase activity in nodules of the transgenic plants indicated that the chimeric *gln*-γ gene was expressed specifically in the rhizobially infected cells; expression of the *gln*-β construct was detected in both cortical and infected regions of young nodules, and became restricted to the vascular tissue as the nodule matured. We conclude that *gln*-β and *gln*-γ genes are differentially expressed both temporally and spatially in plant development and that the *cis*-acting regulatory elements responsible for conferring these contrasting expression patterns are located within a 2-kilobase region upstream of their coding sequences.

INTRODUCTION

Glutamine synthetase (GS; EC 6.3.1.2) is the major enzyme responsible in higher plants for the first step in the assimilation of ammonia (Miflin and Lea, 1980). Photosynthetic tissues of most plants have two GS isoenzymes, GS_1 and GS_2, located in the cytosol and the chloroplast, respectively (McNally et al., 1983). In addition, some legumes have a further cytosolic GS isoenzyme present only in the root nodules that develop from the symbiotic association with the soil bacterium, *Rhizobium* (Lara et al., 1983; Knight and Langston-Unkefer, 1988). The primary function of plant GS in nodules is the rapid assimilation of the ammonia excreted into the plant cytosol of infected cells by the nitrogen-fixing bacteroids (Atkins, 1987). In *Phaseolus vulgaris* there is a 16-fold increase in GS activity during nodule development, due entirely to the appearance of the nodule-specific isoenzyme, GS_{N1} (Lara et al., 1983); a second nodule isoenzyme, GS_{N2}, is indistinguishable from the root form (Cullimore et al., 1983).

Plant GS is octameric, and two-dimensional electrophoresis has shown that the cytosolic enzyme in *P. vulgaris* is composed of up to three isoelectric focusing variants of the 40-kD subunit (α, β, and γ), which occur in varying proportions in different organs of the plant (Lara et al., 1984). The α, β, and γ subunits are specified by three distinct but homologous genes (now designated *gln*-α, *gln*-β, and *gln*-γ, respectively), which are differentially expressed during plant development (Cullimore et al., 1984; Gebhardt et al., 1986; Bennett, Lightfoot, and Cullimore, 1989). One gene (*gln*-β) is expressed preferentially in roots, whereas another (*gln*-γ) is strongly induced during nodule development. Although initially thought to be nodule-specific, gln-γ mRNA has recently been detected, at lower levels, in stems, petioles, and green cotyledons (Bennett et al., 1989). Nodule-specific, or at least nodule-enhanced, expression of one or more GS genes appears to be widespread in the legumes, having also been reported in soybean (Sengupta-Gopalan and Pitas, 1986; Hirel et al., 1987), pea (Tingey et al., 1987), lupin (Konieczny et al., 1988), and alfalfa (Dunn et al., 1988).

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Studies relating the timing of GS induction to other events in *P. vulgaris* nodule development (Lara et al., 1983; Gebhardt et al., 1986; Padilla et al., 1987) suggest that *gln-γ* belongs to the group of genes that specify the "late nodulins," the name given to the nodule-specific proteins that first appear just before or during the onset of nitrogen fixation. Other well-characterized late nodulins include leghemoglobin, uricase II, and sucrose synthetase (for recent reviews see Verma et al., 1986 and Nap et al., 1987). Almost nothing is known about the direct or indirect signals that pass between the *Rhizobium* and the plant to activate the expression of this group of genes. Analysis of nuclear extracts has recently led to the identification of a nodule-specific factor that binds to two AT-rich sequences in the 5′-flanking region of a soybean leghemoglobin gene (Jensen et al., 1988), but a regulatory function has yet to be established for this interaction.

With the long-term aim of investigating the mechanism by which *gln-γ* and other GS genes are regulated in plant development, we now report the cloning of the *gln-γ* gene and the analysis in transgenic plants of its promoter, along with that of the *gln-β* gene isolated previously (Turton et al., 1988). The results show that a fragment of about 2 kb from the 5′-flanking region of each gene is able to confer the appropriate nodule-specific or root-enhanced expression on a bacterial β-glucuronidase gene (GUS; Jefferson et al., 1987), and histochemical localization of GUS activity in tissue sections reveals that the two genes display very different spatial patterns of expression in the developing nodule.

RESULTS

Gene Isolation and Analysis

Figure 1 shows the restriction maps of three GS clones isolated from a *P. vulgaris* genomic library. λPvGSN-56 and λPvGSN-57 hybridized at high stringency to the *gln-γ* GS cDNA clone, pcPvNGS-01 (Cullimore et al., 1984), and are identical except that the former has an additional 3.4 kb at one end. The third clone, λPvGSR-4, is quite distinct and contains the *gln-β* gene (Turton et al., 1988).

GS sequences in λPvGSN-56 and λPvGSN-57 were mapped by hybridization to the incomplete *gln-γ* cDNA clone, pcPvNGS-01, and to a full-length *gln-β* cDNA clone, pcGS-β1 (Figure 1). The location of the putative *gln-γ* gene was identified as a region in both clones that hybridized (at reduced stringency) to pcGS-β1 and which includes some sequences that hybridized (at high stringency) to pcPvNGS-01. Additional sequences hybridizing to pcGS-β1, which map several kilobases downstream of the putative *gln-γ* gene (Figure 1), appear to belong to a novel, fifth GS gene (designated *gln-ε*) that has no known gene product (B.G. Forde, J.E. Oliver, J.F. Turton, and A.P. Hopley, unpublished observations).

A 2.1-kb HindIII/BamHI fragment from λPvGSN-57, which was expected from the hybridization analysis to contain the 5′ end of the *gln-γ* gene, was subcloned and partly sequenced. Figure 2 shows 186 bp of the DNA sequence immediately preceding an HpaI site in the subclone (pN/HB57d), which has been aligned with the corresponding sequence from *gln-β* (Turton et al., 1988). The coding sequence in pN/HB57d begins at position 116 and is homologous but not identical to the equivalent sequences in the pcGS-α1 and pcGS-β1 cDNA clones (formerly pR-2 and pR-1, respectively; Gebhardt et al., 1986) and unrelated to the cDNA clone for the chloroplast subunit precursor, pre-δ (Lightfoot et al., 1988). Confirmation that the gene we have cloned is *gln-γ* has been obtained by comparison with the sequence of a full-length cDNA clone for the γ subunit (Bennett et al., 1989).

Primer extension analysis (not shown) indicates heterogeneity in the transcription start sites in *gln-γ*. The majority are clustered at nucleotides 43 to 49, with the most

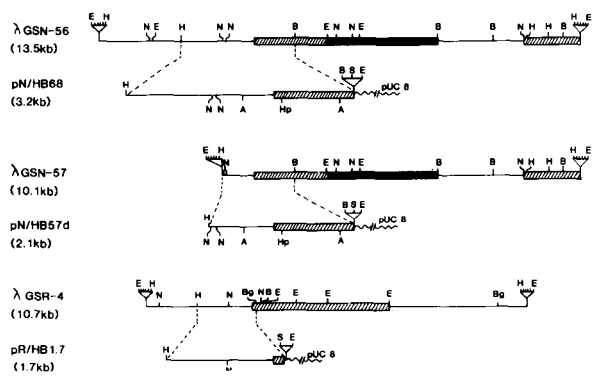

Figure 1. Restriction Maps of GS Genomic Clones and Subclones.

Three clones from a *P. vulgaris* genomic library in bacteriophage λ were mapped by single and double restriction digests and agarose gel electrophoresis. To locate transcribed GS sequences, DNA gel blots of the restriction digests were hybridized at high stringency to the 700-bp insert from pcPvNGS-01 (which represents only the 3′ half of the *gln-γ* mRNA; Cullimore et al., 1984) and at reduced stringency to the 1400-bp insert from pcGS-β1 (formerly pR-1; Gebhardt et al., 1986). The black rectangles indicate fragments that hybridized to both pcPvNGS-01 and pcGS-β1; the shaded rectangles indicate the sequences that hybridized only to pcGS-β1. Fragments from the 5′ ends of the *gln-γ* (λPvGSN-56 and -57) and gln-β (λPvGSR-4) genes were subcloned in pUC8 for more detailed mapping and sequencing. Figures in parentheses indicate the size of the cloned phage or plasmid inserts, E, H, N, B, Bg, S, A, and Hp refer to EcoRI, HindIII, NdeI, BamHI, BglII, SmaI, AccI, and HpaI, respectively.

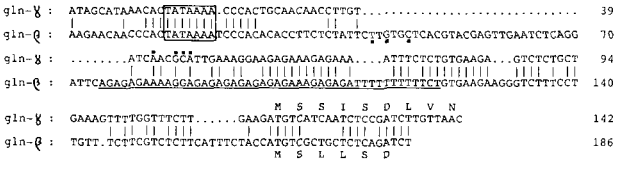

Figure 2. Partial Nucleotide Sequences of the Promoter Regions of *gln-β* and *gln-γ*.

The sequence of the 5′ end of the *gln-γ* gene was obtained by analysis of pN/HB57d and only the region immediately upstream and downstream of the translation initiation codon is shown. The 3′ end of the sequence is delimited by the HpaI site that was used to construct the *gln-γ* translational fusion (N/HH68-GUS; see Figure 3). The *gln-γ* sequence has been aligned with the corresponding part of the *gln-β* sequence (Turton et al., 1988), introducing gaps (indicated by dots) to optimize the number of matches (indicated by vertical lines). The amino-terminal sequences encoded by the two genes are presented using the single letter code. Putative TATA box sequences are boxed, and an extended homopurine-homopyrimidine sequence in the 5′-untranslated region of *gln-β*, which is partially conserved in *gln-γ*, is underlined. The major cap sites, as determined by primer extension, are indicated by dots above (*gln-γ*) or below (*gln-β*) the nucleotide sequences.

prominent bands being at nucleotides 43 and 46 to 48 (Figure 2), but there were also indications of several minor cap sites further upstream, the most distal being about 130 bp from the major cap sites. An additional weak initiation site, upstream of the major site, was also a reported feature of an alfalfa GS gene (Tischer et al., 1986). About 30 bp upstream of the first major cap site in both *gln-γ* and *gln-β* is a sequence (TATAAAA) that resembles the canonical TATA box sequence found 32 ± 7 nucleotides from the transcription initiation site of most plant genes (Joshi, 1987). Otherwise the noncoding sequences are poorly conserved, with perhaps the most striking common feature being an extended homopurine sequence followed by a shorter stretch of pyrimidines within both 5′-untranslated sequences.

Promoter Analysis in Transgenic Plants

Figure 3 shows the three chimeric gene fusions used to analyze and compare the promoter activities of *gln-β* and *gln-γ* genes. Two constructs were made with the *gln-γ* promoter, one a translational fusion with the GUS gene, the other a transcriptional fusion. The transcriptional fusion (N/BA68-GUS) contains about 2 kb of upstream sequence and includes only the first 53 bp of the 5′-untranslated region, so that the translation initiation codon is supplied by the first ATG codon of the GUS gene. The translational fusion (N/HH68-GUS) differs from N/BA68-GUS by having an additional 44 bp of *gln-γ* sequence, consisting of the remainder of the 5′-untranslated region and the first 24 bp of coding sequence. The *gln-β* construct (R/HB1.7-GUS) is also a translational fusion, comprising 1.7 kb of *gln-β* upstream sequence and the first 19 bp of coding sequence. In addition, a fusion between the "constitutive" cauliflower mosaic virus (CaMV) 35S promoter and the GUS gene, as constructed by Jefferson et al. (1987), has been used for comparison.

To assay expression of the chimeric genes in transgenic plants, we used an *Agrobacterium rhizogenes* transformation system similar to that developed for the study of soybean nodule-specific promoters (Stougaard et al., 1986; Petit et al., 1987), except that the binary vector, pBin19 (Bevan, 1984), was used instead of an integration vector. The four gene fusions, inserted at the polylinker between the left and right borders of pBin19 (Figure 3), were transferred to a wild-type strain of *A. rhizogenes* by triparental mating and the trans-conjugants were used to

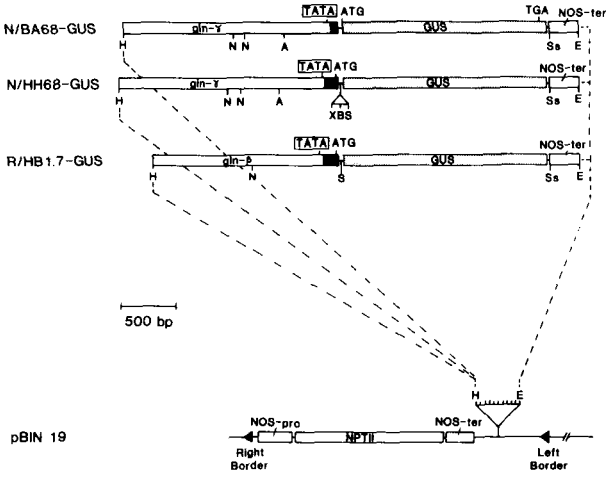

Figure 3. Chimeric Gene Fusions Used to Analyze GS Gene Expression in Transgenic Plants.

Gene fusions between the 5′-flanking regions of the GS genes and the coding sequence of the GUS gene were constructed as described in "Methods." N/BA68-GUS and N/HH68-GUS are transcriptional and translational fusions incorporating 2 kb of the *gln-γ* upstream sequence; R/HB1.7 is a translational fusion containing 1.7 kb of the *gln-β* upstream sequence. The GUS polypeptides encoded by the N/HH68-GUS and R/HB1.7-GUS fusions have amino-terminal extensions of 19 and 13 amino acids, respectively, (including sequences contributed by the pUC19 polylinker). The GS-GUS fusions were each transferred as a HindIII/EcoRI fragment to the polylinker of pBin19, adjacent to the neomycin phosphotransferase gene (NPTII); NOS-pro and NOS-ter are the promoter and terminator sequences from the nopaline synthetase gene (Bevan, 1984); X and Ss indicate XbaI and SstI sites; other abbreviations are as for Figure 1.

infect stems of *L. corniculatus* seedlings. Transgenic plants were regenerated from the hairy roots that developed from the infection sites and extracts of roots, shoots, and nodules were assayed for GUS activity and soluble protein content. Although there are cogent arguments against using protein as the denominator when comparing gene expression between tissues, because of differences between cell types in their protein contents (Jefferson et al., 1987), the alternative of using DNA as the denominator is similarly unsatisfactory owing to the differences in chromosome ploidy that are a common feature of differentiated plant cells (Deeley et al., 1957; Bryant, 1976), particularly in root nodules (Libbenga and Bogers, 1974), and the presence in nodules of large numbers of rhizobia, whose DNA could contribute significantly to the total DNA content.

Figure 4 presents the results obtained from the analysis of lines carrying each of the four GUS fusions. For each regenerated line, the data are plotted on a logarithmic scale as root activity versus shoot activity (Figure 4, A and B) and nodule activity versus root activity (Figure 4, C and D). In this way contrasting patterns of expression can be highlighted despite considerable differences between the overall levels of expression in lines with the same construct.

The distributions of GUS activity in the R/HB1.7-GUS and 35S-GUS lines are compared in Figure 4, A and C. Although the 35S promoter gave similar GUS activities in roots and shoots (root:shoot ratios from 1:1 to 3:1 in seven transformed lines), six of the seven lines carrying the R/HB1.7-GUS fusion had 20-fold to 50-fold higher activities in roots than in shoots and a seventh line had 140-fold higher root activity. There was no significant difference between the 35S-GUS and R/HB1.7-GUS lines in the distribution of GUS activity between the nodules and roots: both constructs gave nodule activities that were 20% to 140% of the corresponding root activity. The only exception was the R/HB1.7-GUS line that had the exceptionally high ratio of root to shoot activity in which nodule activity was approximately 5% of root activity. There was no evidence with either construct that the distribution of GUS activity in the plant was affected by the overall level of gene expression, as is evident from the tendency of each set of points in Figure 4, A and C, to lie along a straight line, parallel to the lines of constant ratio that are indicated in the figure.

Nine of the ten lines carrying the *gln-γ* translational fusion (N/HH68-GUS), and two of the three lines carrying the *gln-γ* transcriptional fusion (N/BA68-GUS) had very low levels of GUS activity in roots and shoots and from 180-fold to 10,000-fold higher activities in nodules (Figure 4, B and D). The two lines with unusually high root and shoot activities also had enhanced activities in nodules, but only by 12-fold in one line and 25-fold in the other. The explanation for the unusually high levels of expression in non-nodule tissues of these two lines is not known.

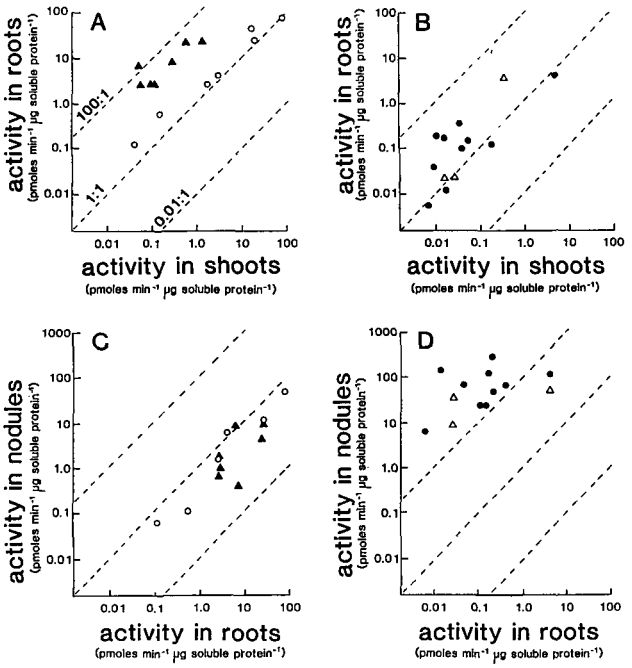

Figure 4. GUS Activities in Roots, Shoots, and Nodules of Transgenic Plants.

The specific activity of GUS was assayed in tissue extracts from *L. corniculatus* plants carrying the R/HB1.7-GUS (▲), 35S-GUS (○), N/BA68-GUS (△) or N/HH68-GUS (●) fusions, as described in "Methods." For each transformed line, root activity has been plotted against shoot activity, **(A)** and **(B)**, and nodule activity against root activity, **(C)** and **(D)**. Each point indicates the results obtained from a single transformed line and represents the average of at least two independent measurements. The broken lines have been drawn to indicate the location of coordinates having a root:shoot or nodule: root ratio of 100:1, 1:1, or 0.01:1.

Some of the variability that we observe in the organ-to-organ distribution of GUS activity in different lines carrying the same construct may be attributable to differences in the severity of the hairy root phenotype (Ooms et al., 1986; Hänisch ten Cate et al., 1988). However, variability in overall level of expression between lines with the same construct is a well-established phenomenon in transgenic plants and seems to be unrelated to the number of copies of the introduced gene (recently reviewed by Dean et al., 1988). This type of variability, which can be up to 100-fold, is generally attributed to poorly understood "position effects," which appear to operate even over distances greater than 2 kb (Dean et al., 1988). The exceptional variability observed in the present studies (1000-fold in the 35S-GUS lines) may be due in part to the sensitivity of the fluorometric GUS assay, which increases the range over which gene expression is detectable, but may also be a consequence of the particular binary vector system used for plant transformation. pBin19 was developed for use

with disarmed strains of *A. tumefaciens* (Bevan, 1984), and transformed plant cell lines are normally selected for resistance to kanamycin, a phenotype that is conferred by the *nptII* gene located within the pBin19 T-DNA and next to the site of integration of the chimeric gene fusion. In the binary system used here, the only selection was for root development from the site of infection, and this phenotype is under the control of the pRi TL-DNA genes, which, being located on a separate plasmid, are likely to be integrated into the plant genome independently of the pBin19 T-DNA. Thus, in our experiments, there was no selection against those transformed cell lines in which the chimeric gene was integrated at a particularly unfavorable chromosomal location, with the result that we may have been observing the full range of possible position effects.

Histochemical Localization of GUS Activity

Figure 5 shows the results obtained when nodules from the different transgenic lines were stained for GUS activity. In lines carrying either of the *gln-γ* constructs, staining in both young and mature nodules was restricted to the central core (Figure 5, A to C), the region that contains the rhizobially infected cells (Vance et al., 1982). At higher magnifications it could be seen that the most intense staining in lines with the N/BA68-GUS fusion was localized to the infected cells, and that the interstitial noninfected cells appeared to have little or no GUS activity (Figure 5, H and I).

In lines with the *gln-β* construct, the distribution of GUS activity was quite different and changed significantly as the nodule matured (Figure 5, D to F). Staining in the newly emerged white nodule (Figure 5D) was widely and evenly dispersed (cf. Figure 5A), whereas in an intermediate-sized pink nodule, staining could be seen in both the vascular tissue and the inner layers of the nodule cortex and only faintly in the central infected zone (Figure 5E). In the mature nodule only the vascular traces became stained (Figure 5F). Similar studies with the 35S-GUS lines revealed GUS distribution patterns that were not significantly different from those observed with the *gln-β* promoter, as illustrated by comparison between Figure 5E and 5G. The intense staining seen here in the vicinity of the vascular tissue of both root and nodule in the 35S-GUS lines is consistent with the histochemical observations of Jefferson et al. (1987) on stem sections of tobacco plants carrying the same 35S construct.

DISCUSSION

Differential Expression of Two GS Genes in Roots, Shoots, and Nodules

The *gln-β* gene in *P. vulgaris* has been shown to be expressed preferentially in roots (Gebhardt et al., 1986), whereas expression of *gln-γ* is activated in the root nodules that develop from the symbiotic association with *Rhizobium* (Cullimore et al., 1984; Gebhardt et al., 1986; Cullimore and Bennett, 1988; Bennett et al., 1989). The isolation of genomic clones for *gln-γ* and *gln-β* (this paper and Turton et al., 1988) has enabled us to begin to investigate the factors that regulate their expression in plant development. Comparison of the DNA sequences at the 5′ ends of the two genes shows that the homology that is a feature of their coding sequences (Gebhardt et al., 1986) is only fragmentary in the immediate upstream region, and more extensive analysis (Turton et al., 1988, and unpublished observations) indicates that their 5′-flanking sequences have diverged considerably. In view of their very different patterns of expression, it seems likely that this sequence divergence at least partly reflects the acquisition by each gene of distinct *cis*-acting elements that confer responsiveness to different sites of regulatory signals. This hypothesis has been confirmed in the present study, which has demonstrated that chimeric gene fusions containing the 5′ flanking regions of *gln-β* or *gln-γ* are expressed in the appropriate root- or nodule-enhanced manner in transgenic plants.

In lines carrying the *gln-β* gene fusion, GUS activity in roots was 20-fold to 140-fold higher than in shoots, whereas the 35S promoter from CaMV produced only slightly higher activities in roots; the expression of both constructs was, on average, lower in nodules than in roots. These results compare well with the analysis of *gln-β* expression in *P. vulgaris*, which indicated that the abundance of its mRNA in roots is about 20-fold and twofold higher than in shoots and nodules, respectively (Gebhardt et al., 1986). The similarity between the 35S and *gln-β* promoters in their relative expression in roots and nodules suggests that the apparent partial repression of *gln-β* during nodule development is not due to any specific regulatory effect on this gene. Further dissection and functional analysis of the *gln-β* promoter region will be required to identify the regulatory sequences responsible for enhancing its expression in roots, or repressing it in shoots.

The majority of lines with the *gln-γ* gene fusions had low but significant GUS activities in roots and shoots, and high activities in nodules (at least 180-fold enhanced). Although *gln-γ* expression in *P. vulgaris* has been described as nodule-specific, there being no evidence of the mRNA in roots or leaves (Cullimore et al., 1984; Gebhardt et al., 1986), it is likely that the sensitivity of the techniques used would not have detected expression at levels several hundred times lower than in nodules. Thus the presence of GUS activity in the roots and shoots of the *gln-γ* lines probably reflects the sensitivity of the fluorometric GUS assay (Jefferson et al., 1987) rather than incorrect regulation of the gene in *L. corniculatus*. This conclusion is supported by results recently obtained with a sensitive RNase-protection technique which have established the

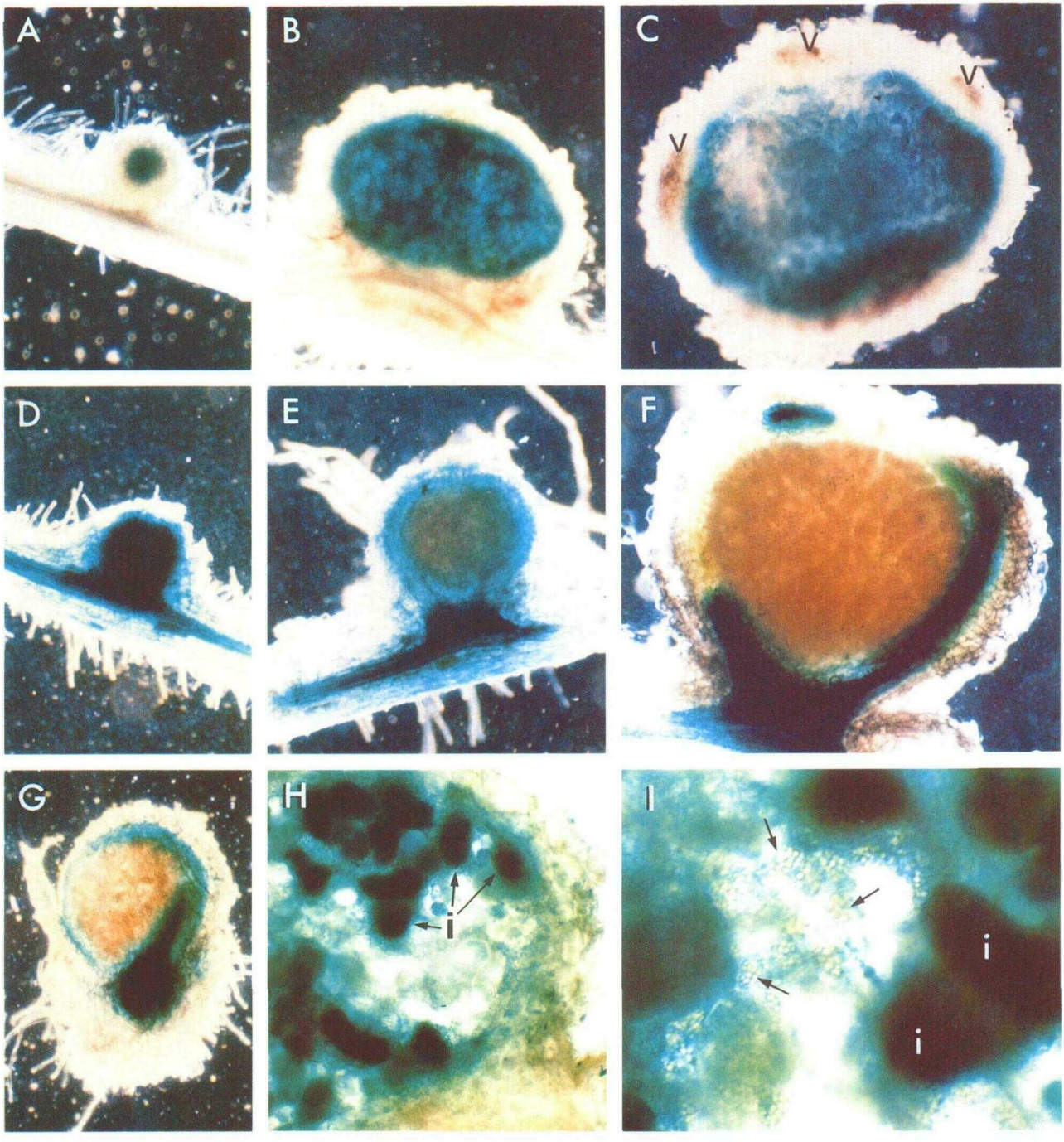

Figure 5. Histochemical Localization of GUS Activity in Nodules of Transgenic Plants.

Nodules of various ages from different transgenic *L. corniculatus* lines were stained as described in "Methods" and photographed by dark field, **(A)** to **(G)**, or bright field, **(H)** and **(I)**, microscopy: a blue precipitate indicates the location of GUS activity. The nomenclature used by Vance et al. (1982) has been adopted to describe the stages of nodule development.

(A) *gln-*γ construct (N/HH68-GUS); root and newly formed white nodule, stained intact (×40). Staining seen in central region of nodule only: no activity in nodule cortex or in root.

(B) *gln-*γ construct (N/BA68-GUS); root and intermediate pink nodule bisected longtitudinally (×40). Staining pattern as in **(A)**.

(C) *gln-*γ construct (N/BA68-GUS); equatorial section from large pink nodule (×40). Staining pattern as in **(A)** and **(B)**; the nodule vascular traces (v), and the cortical layer in which they are embedded, can be seen to be unstained.

(D) *gln-*β construct (R/HB1.7-GUS); root and newly formed white nodule bisected longtitudinally (×40). Staining in the vascular tissue of the root and in all but the outer cortical layers of the nodule.

(E) *gln-*β construct (R/HB1.7-GUS); root and small pink nodule bisected longtitudinally (×40). Staining most intense in the vascular tissue, but also present in the inner cortical layer of the nodule and, faintly, in the central infected zone.

presence of *gln-γ* mRNA in stems, petioles, and green cotyledons of *P. vulgaris* (Bennett et. al., 1989).

The finding that the *gln-γ* promoter is responsive to signals generated in the interaction between *L. corniculatus* and *R. loti* is in agreement with previous reports that promoters from two soybean nodule-specific genes, nodulin-23 and lbc_3, are able to confer the appropriate pattern of expression on the chloramphenicol acetyltransferase gene in *L. corniculatus* (Stougaard et al., 1986; Jørgensen et al., 1988). Deletion studies on the lbc_3 and nodulin-23 genes have delimited two positive regulatory elements in the 5′-flanking region of each, and located a 37-bp lbc_3 sequence that is required for nodule-specific expression (Stougaard et al., 1987; Jørgensen et al., 1988). Similar analysis of the *gln-γ* promoter is in progress to identify the *cis*-acting regulatory sequences and to establish whether the nodule-enhanced GS gene is under the control of the same set of factors as the other late nodulin genes.

Spatial Patterns of Gene Expression in Nodules

Histochemical analysis of the transgenic plants revealed a number of features of gene expression that were not apparent from analysis of tissue extracts. The most striking of these was the clear difference in the spatial distribution of GUS activity in the nodules of the *gln-β* and *gln-γ* lines. Whereas the *gln-γ* fusion was expressed only in the central infected zone of the nodule, the *gln-β* fusion was expressed in the vascular tissue and (in younger nodules) in the infected zone and the inner layers of the nodule cortex. The staining pattern seen with the *gln-β* lines closely resembled that generated by the "constitutive" CaMV 35S promoter, suggesting that the intense staining that is associated with the vascular traces may reflect higher overall transcriptional and translational activities in this region and/or a greater cell density (Vance et al., 1982).

Examination of stained nodule sections at higher magnifications indicated that *gln-γ* expression is confined to the infected cells of the central zone, with little evidence of GUS activity in the interspersed noninfected cells. This result is entirely consistent with the primary function of GS in nodules (Atkins, 1987), and the synthesis of high levels of the γ subunit in these cells can be seen as a symbiotic adaptation that facilitates rapid assimilation of the ammonia excreted by the bacterioids. The same cell-specific distribution has been reported for the leghemoglobins (Verma and Bal, 1976; Robertson et al., 1984; van de Wiel et al., 1988) and for a number of nodulins that are components of the peribacteroid membrane or the peribacteroid fluid (Fortin et al., 1985; Jacobs et al., 1987), but another late nodulin (uricase II or nodulin-35) is specific to the noninfected cells of ureide-producing legumes (Bergmann et al., 1983; Van den Bosch and Newcomb, 1986; Webb and Newcomb, 1987).

The results of the histochemical analysis may help to explain how the two nodule GS isoenzymes are generated in *P. vulgaris*. Analysis of the subunit composition of GS_{N1} has shown that it contains both γ and β, with γ being the major constituent (Lara et al., 1984). More recently, it has been shown that GS from *P. vulgaris* nodules can be resolved into at least seven bands by native polyacrylamide gel electrophoresis (Robert and Wong, 1986). This led to the hypothesis that GS_{N1} is composed of a mixture of up to eight different isoenzymes (i.e., γ_8 and $\gamma_7\beta_1$ to $\gamma_1\beta_7$) and that GS_{N2} is the β_8 form. To explain the over-representation of the β_8 form in the isoenzyme profile, the authors proposed that β has a greater tendency than γ to assemble into the octamer. However, an alternative explanation, which is supported by the histochemical data, is that, whereas some cells in the nodule synthesize both γ and β, others synthesize only β; thus a proportion of the β subunits in the nodule would have no access to γ subunits. Assuming that the expression of the *gln-β* and *gln-γ* genes in *P. vulgaris* is similar to that observed in *L. corniculatus*, we can hypothesize that the GS_{N1} isoenzymes are specific to the infected cells of the central core and that GS_{N2} is located predominantly outside the infected zone, in the region of the vascular traces and the inner cortical cell layers. It seems likely that some GS_{N2} is also present in the noninfected cells, although we have no experimental evidence to support this.

The specificity of *gln-γ* expression to the infected cells strongly suggests that the signals that activate the gene in nodules, or at least those that maintain its expression, are generated intracellularly when the infection thread penetrates the cell, or by some other event that follows on from infection (such as bacteroid release or ammonia production). In agreement with this, it has been found that the mRNA for the nodule-specific GS in alfalfa is not detectable in the nodules that are elicited by an Exo^- (exopolysaccharide minus) mutant of *R. meliloti*, which lack infection threads and intracellular bacteria (Dunn et al., 1988). Studies with other Fix^- mutants of *Rhizobium* or *Bradyrhizobium* (Lara et al., 1983; Sengupta-Gopalan and Pitas, 1986; Padilla et al., 1987; Dunn et al., 1988), with

Figure 5. (Continued).

(F) *gln-β* construct (R/HB1.7-GUS); longtitudinal slice of a large pink nodule (×40). Staining only in vascular tissue.
(G) CaMV 35S construct (35S-GUS); root and small pink nodule bisected transversely (×40). Staining mainly in vascular tissue but also in inner cortical layer of the nodule.
(H) *gln-γ* construct (N/BA68-GUS); section from intermediate pink nodule (×100). Staining restricted to the large infected cells **(I)**.
(I) *gln-γ* construct (N/BA68-GUS); higher power magnification of same section as **(H)** (×270). The noninfected cells (arrowed), which show very little staining, are identified by the large numbers of starch grains.

nodules formed in a nitrogen-free atmosphere (Atkins et al., 1984; Hirel et al., 1987), and analysis of the timing of appearance of the γ subunit (Padilla et al., 1987), indicate that, although ammonia production may be required for maximal induction of nodule GS, it is not essential for gene activation.

The resolution afforded by the histochemical techniques used in the present study does not allow us to relate the timing of appearance of GUS activity to the morphological changes taking place within the developing nodule. Other techniques, such as immuno-gold localization or in situ hybridization to mRNA, may be required to determine the precise stage at which the *gln-*γ gene is switched on in the infected cell. This information would be of considerable value in our attempts to elucidate the mechanism by which the interaction between the plant and bacterial cells leads to the coordinate activation of the set of plant genes that codes for the late nodulins.

METHODS

Plant Growth and Nodulation

Lotus corniculatus (Birdsfoot trefoil) cv Leo plants were grown in culture at 25°C, 50 $\mu E/m^2/sec$ on a 16-hr day. Seeds were surface-sterilized with Ca^{2+} hypochlorite and germinated axenically on MS agar (Murashige and Skoog, 1962) containing 20% sucrose (MS20). Nodulation was carried out by a modification of the method described by Rolfe et al. (1980). Stem segments with a single leaf node were taken from transformed plants and rooted on MS20 agar for 2 to 3 weeks until the roots were 1 to 2 cm long. The plantlets were transferred to a nitrogen-free nutrient agar slope (Fahraeus, 1957) in an 85-mm Petri dish (up to 4 plantlets per dish) and the roots were inoculated with a cell suspension of *Rhizobium loti* strain RCR3011. The dishes were kept in an upright position and stacked together in sets of three or more with the lower half of each set of plates wrapped in aluminum foil. Two days after the first rhizobial inoculation, the roots were re-inoculated. Nodules were first visible 1 to 2 weeks after inoculation and new nodules continued to appear for several weeks.

Nomenclature of cDNA Clones

Recent analysis has demonstrated that the two GS cDNA clones pR-1 and pR-2 (Gebhardt et al., 1986) encode the β and α subunits, respectively (Bennett et al., 1989). They have therefore been renamed pcGS-β1 and pcGS-α1, respectively.

Recombinant DNA Techniques

Unless otherwise stated, standard techniques were used (Maniatis et al., 1982). Frozen competent cells of *Escherichia coli* JM83 were prepared and transformed as described by Alexander et al. (1984). Nick translations were performed using a kit from Amersham International. Oligonucleotides were synthesized by phosphoramidite chemistry using a New Brunswick Biosearch 3810 DNA synthesizer.

Isolation of Genomic Clones and Sequence Analysis

To identify *gln-*γ clones, a genomic library of *Phaseolus vulgaris* (French bean) cv Tendergreen (MboI partial digest) in Charon 34 (kindly provided by Drs. M. Murray, J. Slightom, and T.C. Hall) was screened by plaque hybridization to the 700-bp insert from pcPvNGS-01 (Cullimore et al., 1984). The *gln-*β genomic clone, λPvGSR-4, was one of a number of clones in the same library that hybridized to a mixed probe consisting of the inserts from pcGS-β1 and pcGS-α1. The isolation of λPvGSR-4 has been reported briefly elsewhere, along with the sequence of a 1.1-kb segment of its 5′-flanking region (Turton et al., 1988). Partial sequence analysis of the 5′ end of one *gln-*γ clone, λPvGSN-57, was carried out by the dideoxy method (Sanger et al., 1977) using T4 polymerase-generated deletions of the 2.1-kb insert from pN/HB57d (see Figure 1) in M13mp18 and mp19 (Dale et al., 1985). Verification of the fusion boundaries in the GS-GUS constructs was obtained by double-stranded sequencing (Murphy and Kavanagh, 1988). The sequencing primer was a 17-mer (5′-GTTGGGGTTTCTACAGG-3′) complementary to the sense strand and terminating 8 bp downstream of the first nucleotide of the GUS coding sequence.

Chimeric Gene Fusions

To construct the N/HH68-GUS fusion, a 3.2-kb fragment from λPvGSN-56 was first subcloned in pUC8 to give pN/HB68 (see Figure 1) and a 2-kb HindIII/HpaI fragment from this was further subcloned in pUC19 between the HindIII and HincII sites to give pN/HH68. This 2-kb fragment, which contains the 5′-flanking region of *gln-*γ and the first 24 bp of the coding region, was transferred (as a 2-kb HindIII/BamHI fragment) to the corresponding sites of the pUC19 polylinker upstream of the GUS-NOSter cassette from the pBin19 derivative, pBI101.1 (Jefferson et al., 1987). (To facilitate double-stranded sequencing of the fusion boundary, this fusion [and the two others below] was made with the GUS-NOSter cassette [2.1-kb HindIII/EcoRI fragment] in pUC8, rather than pBin19.)

In the absence of a convenient restriction site in the 5′-untranslated sequence of *gln-*γ, a transcriptional fusion (N/BA68-GUS) was constructed using one of the deletion mutants generated for sequence analysis. In this derivative of pN/HB57d, the *gln-*γ coding region had been removed along with the adjacent 20 bp of 5′-untranslated sequence. A 500-bp AccI/EcoRI fragment, containing the sequences next to the end point of the deletion, was used to replace the equivalent 550-bp AccI/EcoRI fragment in pN/HH68. The resultant clone (pN/BA68) differs from pN/HH68 only by the deletion of a 44-bp sequence that includes the *gln-*γ translation initiation codon. Finally, the 2-kb HindIII/EcoRI fragment from pN/BA68 (with the EcoRI site filled in using Klenow fragment of DNA polymerase I) was inserted between the HindIII and SmaI sites of the polylinker in the GUS-NOSter cassette from pBI101.1.

To construct the R/HB1.7-GUS fusion, a 1.7-kb internal HindIII/BglII fragment from λPvGSR-4, containing the 5′-flanking region of *gln-*β (Turton et al., 1988), was subcloned in pUC8 and transferred as an HindIII/SmaI fragment to the corresponding sites in

the polylinker of the GUS-NOSter cassette from pBI101.2 (Jefferson et al., 1987).

Plant Transformation and Regeneration

The pBin19 derivatives containing the GS-GUS fusions, and pBI121.1 (Jefferson et al., 1987) containing the CaMV-GUS fusion, were mobilized from *Escherichia coli* JM83 to *A. rhizogenes* LBA9402 by triparental mating (Ditta et al., 1980) using *E. coli* HB101 containing the helper plasmid pRK2013. Trans-conjugants were selected at 29°C on MM agar (Hooykaas, 1988) containing rifampicin (100 μg/mL) and kanamycin (50 μg/mL). The integrity of the chimeric genes was verified prior to plant transformation by analysis of restriction digests of total *A. rhizogenes* DNA, extracted as described by Ooms et al. (1981).

L. corniculatus seedlings were infected with *A. rhizogenes* at two sites on each stem by stab inoculation. After 2 to 4 weeks, roots that developed from the infection sites were excised and cultured for at least 2 weeks on MS20 agar containing 200 μg/ml cefataxime. When an *A. rhizogenes* strain carrying the 35S-GUS construct was used for the inoculation, 80% of the transformed roots had histochemically detectable GUS activity, indicating a high frequency of co-transformation with the vector and Ri T-DNAs as previously reported (Stougaard et al., 1987). To obtain shoot regeneration, 1-cm root segments were cultured on MS20 agar containing zeatin (1 μg/ml). After 4 to 8 weeks, shoots were excised and rooted on MS20 and a single shoot from each line was selected and propagated vegetatively.

Fluorometric Assay for GUS Activity

Plant material was harvested 3 to 5 weeks after inoculation with *Rhizobium*. Nodules were excised by first cutting the root immediately above and below the nodule, so that no residual nodule material would contaminate the root sample, and then detaching the nodules from their root segments. Nodules, de-nodulated root systems, and shoots from 1 to 3 plants were pooled, frozen in liquid N_2, and stored at −75°C. The tissue was ground in liquid N_2, using a pestle and mortar (roots and shoots), or glass rod and Eppendorf tube (nodules), and then in grinding buffer containing 50 mM Na^+ phosphate buffer, pH 7.0, 0.1 M NaCl, 1 mM Na EDTA, 0.1% Triton X-100, 10 mM β-mercaptoethanol. Extracts were assayed fluorometrically for GUS activity using 4-methylumbelliferyl glucuronide (Sigma) as substrate and grinding buffer as reaction buffer (Jefferson et al. 1987). Protein concentrations in the extracts were measured by the method of Bradford (1976) using a kit from Bio-Rad Laboratories.

Histochemical Staining

Intact or hand-cut sections of *L. corniculatus* roots and nodules were immersed, without prior fixation, in 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Research Organics, Inc.), 50 mM Na^+ phosphate buffer, pH 7.0, and subjected to vacuum infiltration for 1 min before incubation at 37°C overnight (Jefferson et al., 1987). An oxidation catalyst (0.5 mM K^+ ferricyanide and 0.5 mM K^+ ferrocyanide) was routinely included to avoid artefacts that could result from peroxidase activity or microaerobic conditions in the nodule sections (Pearse, 1972). No background staining activity was observed with nodules or roots from control *A. rhizogenes*-transformed plants, although prolonged incubation times (> 40 hr) sometimes produced a light blue coloration in the cortical region of the nodule.

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