The Plant Cell, Vol. 3, 11-22, January 1991 © 1991 American Society of Plant Physiologists

Ammonia-Regulated Expression of a Soybean Gene Encoding Cytosolic Glutamine Synthetase in Transgenic *Lotus corniculatus*

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A full-length cDNA clone encoding cytosolic glutamine synthetase (GS), expressed in roots and root nodules of soybean, was isolated by direct complementation of an *Escherichia coli gln A*$^-$ mutant. This sequence is induced in roots by the availability of ammonia. A 3.5-kilobase promoter fragment of a genomic clone (λGS15) corresponding to this cDNA was isolated and fused with a reporter [β-glucuronidase (GUS)] gene. The GS-GUS fusion was introduced into a legume (*Lotus corniculatus*) and a nonlegume (tobacco) plant by way of *Agrobacterium*-mediated transformations. This chimeric gene was found to be expressed in a root-specific manner in both tobacco and *L. corniculatus*, the expression being restricted to the growing root apices and the vascular bundles of the mature root. Treatment with ammonia increased the expression of this chimeric gene in the legume background (i.e., *L. corniculatus*); however, no induction was observed in tobacco roots. Histochemical localization of GUS activity in ammonia-treated transgenic *L. corniculatus* roots showed a uniform distribution across all cell types. These data suggest that the tissue specificity of the soybean cytosolic GS gene is conserved in both tobacco and *L. corniculatus*; however, in the latter case, this gene is ammonia inducible. Furthermore, the ammonia-enhanced GS gene expression in *L. corniculatus* is due to an increase in transcription. That this gene is directly regulated by externally supplied or symbiotically fixed nitrogen is also evident from the expression of GS-GUS in the infection zone, including the uninfected cells, and the inner cortex of transgenic *L. corniculatus* nodules, where a flux of ammonia is encountered by this tissue. The lack of expression of GS-GUS in the outer cortex of the nodules suggests that ammonia may not be able to diffuse outside the endodermis.

INTRODUCTION

Glutamine synthetase (GS; EC 6.3.1.2) is a key enzyme for the assimilation of ammonia. Several different isoforms of GS encoded by a small family of genes have been found in different parts of the plant (Hirel et al., 1987; Tingey et al., 1987; Forde et al., 1989; Edwards et al., 1990). These GS isoforms are located in different compartments of cells (cytosol or chloroplast) and in different tissues and organs of plants, meeting requirements of ammonia assimilation under various physiological conditions (Awonaike et al., 1981; Oaks and Hirel, 1985; Cullimore and Bennett, 1989). In leaves, ammonia produced primarily from photorespiration is assimilated by a chloroplastic form of GS, whereas the mineral nitrogen absorbed by plant roots is assimilated by the cytosolic root GS isoforms (Oaks and Hirel, 1985). A nonoverlapping pattern of expression of genes encoding these isoforms was revealed by their expression in transgenic plants (Edwards et al., 1990). The gene encoding chloroplastic GS (GS2) in pea is specifically expressed in photosynthetic cells of transgenic tobacco, whereas the gene for cytosolic GS (GS3A) is active in phloem elements of mature transgenic plants.

In legumes, root-specific and/or root nodule-specific GS is responsible for the assimilation of symbiotically fixed nitrogen. Nodule-specific GS isoforms have been identified in bean (Lara et al., 1983; Forde et al., 1989), alfalfa (Dunn et al., 1988), and lupin (Konieczny et al., 1988). In pea, however, it appears that the abundance of GS isoforms found in nodules is not unique to that organ (Tingey et al., 1987). In soybean, enhanced GS activity in nodules has been reported to be due to the synthesis of nodule-specific GS isoforms (Sengupta-Gopalan and Pitas, 1986). However, we have found that the increase in soybean nodule GS is apparently due to the NH_4^+-stimulated expression of GS isoforms in the root (Hirel et al., 1987). The bean *gln* γ gene is specifically expressed in infection zones of transgenic *Lotus corniculatus* nodules. The expression of

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gln β, however, was found in root as well as in the inner cortical and vascular tissue of the nodules, with little activity in the central infection zone. Thus, differential expression of GS genes suggests that GS isoforms synthesized in different cells or tissues of plant may perform different functions in nitrogen metabolism (Edwards et al., 1990).

The nitrogen metabolism is compartmentalized between the infected and uninfected cells in ureide-producing nodules (see Verma, 1989). Some of the enzymes involved in the early steps of nitrogen assimilation and ureide production (e.g., GS and xanthine dehydrogenase) appear to be located in both infected and uninfected cells (Verma et al., 1986; Branjeon et al., 1989; Newcomb et al., 1990); others, for example, uricase (nodulin-35), primarily occur in the uninfected cells (Nguyen et al., 1985; Kaneko and Newcomb, 1987; Stegink et al., 1987). In amide-producing nodules, the number of interstitial cells is much less than that in ureide-producing nodules (Stegink et al., 1987), suggesting that infected cells may transport amides directly.

The GS genes appear to be regulated in many different ways. Expression of some GS genes is influenced by light (Edwards and Coruzzi, 1989) and may be controlled by phytochrome (Sakamoto et al., 1990). Our earlier studies (Hirel et al., 1987) suggested that the expression of soybean cytosolic GS genes may be regulated by the availability of ammonia from external sources or from symbiotic nitrogen fixation. To understand the ammonia-induced expression of the GS genes, we isolated a soybean genomic clone corresponding to the ammonia-induced GS cDNA and used the promoter region of this gene fused with a reporter [β-glucuronidase (GUS)] gene. The chimeric GS-GUS gene was introduced into a nonlegume (tobacco) as well as into a legume (*L. corniculatus*) plant. We observed that soybean GS-GUS gene fusion is strongly expressed both in transgenic tobacco and in transgenic *L. corniculatus*, with activity being primarily localized in the root apices. However, the ammonia inducibility of soybean cytosolic GS only occurred in *L. corniculatus* and not in tobacco. These data suggest that some legume plants have brought the GS gene under the control of ammonia. A rapid flux of NH_4^+ occurs during symbiotic nitrogen fixation, as shown by induction of GS-GUS in the entire infection zone of the nodule including the uninfected cells and the inner cortex, whereas the outer cortex shows no GS-GUS activity.

RESULTS

Induction of Soybean Cytosolic GS Transcript by Ammonia in Soybean

Our earlier observations (Hirel et al., 1987) suggested that GS genes in soybean encoding a cytosolic enzyme in root and root nodules may be regulated by the availability of ammonia, either provided externally or from symbiotic nitrogen fixation. That this phenomenon was specific to soybean GS is shown in Figure 1. Whereas the GS transcript was elevated in NH_4^+-treated soybean roots (Figure 1A), no effect was observed in tobacco tissue (Figure 1B). Moreover, the ammonia-enhanced expression of GS was not due to the overall metabolic changes caused by the treatment of ammonia because the level of ATP-synthetase transcript in soybean (Figure 1C) in comparison to tobacco (Figure 1D) remained almost constant after ammonia treatment. These results confirmed that the level of cytosolic GS transcripts in soybean root is specifically regulated by the availability of ammonia (Hirel et al., 1987). However, this effect may be due to a direct increase in the rate of GS transcription or to an increase in the stability of GS transcripts in soybean root. To directly address this question, we isolated a full-length ammonia-induced GS

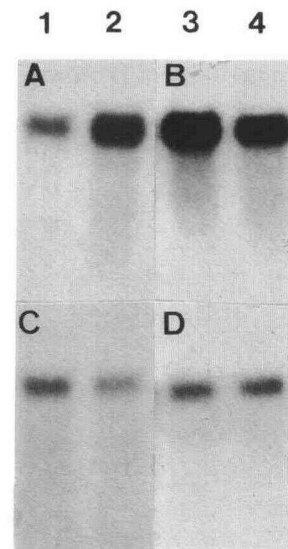

Figure 1. RNA Gel Blot Analysis Showing Ammonia Induction of GS Transcript in Soybean Root in Comparison to That in Tobacco.

(A) Soybean root poly(A)$^+$ RNA (3 μg) hybridized with 3′ end of ^{32}P-labeled soybean GS cDNA clone (pGS20). Lane 1, RNA from control (untreated) plants; lane 2, RNA from plants treated with 10 mM $(NH_4)_2SO_4$ for 12 hr before harvesting.

(B) Tobacco root poly(A)$^+$ RNA (3 μg) hybridized with tobacco cytosolic GS cDNA probe (Tingey and Coruzzi, 1987). Lane 3, RNA from control (untreated) plants; lane 4, RNA from ammonia-treated plants as in **(A)**.

(C) Soybean root poly(A)$^+$ RNA (3 μg) from control plants (lane 1) and ammonia-treated plants (lane 2) hybridized with β subunit of the mitochondrial ATP-synthetase (Boutry and Chua, 1985).

(D) Tobacco root poly(A)$^+$ RNA from control plants (lane 3) and ammonia-treated plants (lane 4) hybridized with the same probe as in **(C)**.

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   1  CCGTGAATCTCAGAATTCTCTAAAAGAGATCCTTTTCTGCTCTTTGAAGAAAGAAGGGTC    60
  61  TTTGCTTGATTTTGGAGATGTCTCTGCTCTCAGATCTCATCAACCTTAACCTCTCCGATA   120
                       M  S  L  L  S  D  L  I  N  L  N  L  S  D  T
 121  CCACCGAGAAGGTGATCGCAGAGTACATATGGATCGGTGGATCAGGAATGGACCTGAGGA   180
        T  E  K  V  I  A  E  Y  I  W  I  G  G  S  G  M  D  L  R  S
 181  GCAAAGCAAGGACTCTCCCAGGACCAGTTAGCGACCCTTCAGAGCTTCCCAAGTGGAACT   240
        K  A  R  T  L  P  G  P  V  S  D  P  S  E  L  P  K  W  N  Y
 241  ATGATGGTTCCAGCACAGGTCAAGCTCCTGGTGAAGACAGTGAAGCGATTTATACCCACA   300
        D  G  S  S  T  G  Q  A  P  G  E  D  S  E  A  I  Y  T  H  K
 301  AGCCATTTCAGGATCCATTCAGAAGGGGTAACAATATCTTGGTTATCTGTGATGCCTACA   360
        P  F  Q  D  P  F  R  R  G  N  N  I  L  V  I  C  D  A  Y  T
 361  CTCCTGCTGGAGAACCTATTCCCACTAACAAGAGGCACGCTGCTGCCAAGGTTTTCAGCC   420
        P  A  G  E  P  I  P  T  N  K  R  H  A  A  A  K  V  F  S  H
 421  ATCCTGATGTTGTTGCTGAAGTGCCATGGTACGGTATTGAACAAGAATACACCTTGTTGC   480
        P  D  V  V  A  E  V  P  W  Y  G  I  E  Q  E  Y  T  L  L  Q
 481  AGAAAGATATCCAATGGCCTCTTGGGTGGCCTGTTGGTGGTTTCCCTGGACCTCAGGGTC   540
        K  D  I  Q  W  P  L  G  W  P  V  G  G  F  P  G  P  Q  G  P
 541  CATACTACTGTGGTGTTGGCGCTGACAAGGCTTTTGGCCGTGACATTGTTGACGCACACT   600
        Y  Y  C  G  V  G  A  D  K  A  F  G  R  D  I  V  D  A  H  Y
 601  ACAAAGCCTGTATTTATGCTGGCATCAACATCAGTGGAATTAATGGAGAAGTGATGCCCG   660
        K  A  C  I  Y  A  G  I  N  I  S  G  I  N  G  E  V  M  P  G
 661  GTCAGTGGGAATTCCAAGTTGGACCTTCAGTTGGAATCTCAGCTGGTGATGAGATTTGGG   720
        Q  W  E  F  Q  V  G  P  S  V  G  I  S  A  G  D  E  I  W  A
 721  CAGCTCGTTACATCTTGGAGAGGATCACTGAGATTGCTGGTGTGGTGGTTTCCTTTGACC   780
        A  R  Y  I  L  E  R  I  T  E  I  A  G  V  V  V  S  F  D  P
 781  CCAAGCCAATTCCGGGTGATTGGAATGGTGCTGGTGCTCACACAAACTACAGCACCAAGT   840
        K  P  I  P  G  D  W  N  G  A  G  A  H  T  N  Y  S  T  K  S
 841  CCATGAGAGAAGATGGAGGCTATGAAGTGATCAAAGCAGCAATTGACAAGTTGGGGAAGA   900
        M  R  E  D  G  G  Y  E  V  I  K  A  A  I  D  K  L  G  K  K
 901  AGCACAAGGAGCACATTGCTGCTTATGGAGAAGGCAACGAACGTCGTTTGACAGGACGCC   960
        H  K  E  H  I  A  A  Y  G  E  G  N  E  R  R  L  T  G  R  H
 961  ACGAAACCGCTGACATCAACACCTTCTTATGGGGAGTTGCAAACCGTGGAGCTTCTGTTA  1020
        E  T  A  D  I  N  T  F  L  W  G  V  A  N  R  G  A  S  V  R
1021  GGGTTGGGAGAGACACAGAGAAAGCAGGGAAGGGATATTTTGAGGACAGAAGGCCTGCTT  1080
        V  G  R  D  T  E  K  A  G  K  G  Y  F  E  D  R  R  P  A  S
1081  CCAACATGGACCCATACGTGGTTACTTCCATGATTGCAGACACAACCATTCTGTGGAAGC  1140
        N  M  D  P  Y  V  V  T  S  M  I  A  D  T  T  I  L  W  K  P
1141  CATGAGCAAAACCTGCATGTTTTCTCCCTTTGGATGGAAAGGAACAGTTATGCTTTTCTT  1200
        *
1201  AGTAGGATTTGGTCTCTCTCTCTTTTTACCTTTTGATTGGTACTATGGTTGGTGCCTTGT  1260
1261  TGGTTGGTGCAACTAACTGGCAAGGGTTGTTCATTGTTTTCTTCTATTCCTTTCCCTCGT  1320
1321  TTTCCGATTGTTACAATGACAATAATTTAATGGTTATTATCAGTCTTGAAGGTTATTATC  1380
1381  AGTCTTGCAAGAAATGCTGATTGGAAGTATAATAATATATATGAAATTGTCATGTTCATT  1440
1441  GAGTAGGAAA                                                    1450
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Figure 2. Sequence of Soybean Cytosolic Ammonia-Induced GS cDNA Expressed in Root and Root Nodules.

This clone represents the full length of pGS20 (Hirel et al., 1987) and was obtained by complementation of an *E. coli* (A318) *gln A*$^-$ strain (Sundaresan et al., 1983). The consensus sequence (TCAGATCTCATCAA) of GS used as probe for defining the 5′ end of the coding region in the genomic clones is underlined.

cDNA (pGS20) and a corresponding genomic clone (λGS15) to analyze further the effect of ammonia on GS gene expression in both transgenic legume (*L. corniculatus*) and nonlegume (tobacco) plants.

Isolation and Analysis of Ammonia-Inducible Soybean Cytosolic GS cDNA

Analysis of the previously isolated cDNA clones encoding GS in soybean nodules (Hirel et al., 1987) showed that they were not full-length sequences. To get a full-length GS cDNA clone encoding soybean cytosolic GS, we took a genetic complementation approach (Delauney and Verma, 1990a). An *Escherichia coli* GS mutant (A318) (Sundaresan et al., 1983), *gln A*$^-$, was used as the host for complementation experiments. Transformation of A318 with 100 ng of soybean nodule expression library in phagemid (Delauney and Verma, 1990b) gave several colonies complemented for glutamine. One of the full-length cDNA clones thus isolated corresponded to pGS20, which is regulated by the availability of ammonia (Hirel et al., 1987). The sequence in Figure 2 revealed an open reading frame of 1068 nucleotides encoding a GS polypeptide of 38.8 kD. Comparison of the predicted amino acid sequence of this soybean cytosolic GS with other plant GS genes (Gebhardt et al., 1986; Tischer et al., 1986; Tingey et al., 1987; Snustad et al., 1988) showed an extensive (>85%) similarity at the amino acid sequence level (data not shown). However, the amino acid sequence of GS20 showed only 23% identity with the *E. coli* GS, suggesting that these highly conserved amino acids between plants and bacteria may be essential for GS function. The plant GSs function effectively in *E. coli* despite such limited homology, and allow complementation of glutamine auxotrophy (DasSarma et al., 1986; Snustad et al., 1988).

Tissue-Specific Expression of the Soybean Cytosolic GS Gene in Transgenic *L. corniculatus* and Tobacco

Several genes encoding cytosolic forms of GS enzyme were isolated from a soybean genomic library using pGS20 as a probe. Figure 3A shows one of the genomic clones (λGS15) of soybean GS containing the coding region, which spans about 4 kb. The 5′ region was located by using a synthetic oligonucleotide probe corresponding to a consensus coding sequence (TCAGATCTTATCAA) at the 5′ end (9 bp downstream from the ATG initiation codon) of the GS genes of pea (Tingey et al., 1987) and bean (Gebhardt et al., 1986). In the soybean GS gene, T was found to be substituted by C (Figure 2). The approximate location of the 3′ end of the coding region was determined using the GS 3′ end fragment from pGS20 as a probe. The sequence of λGS15 at the junction between the translation start site and the TATA box (Figure 3A) was found to have few differences as compared with GS20. A single base pair change in the 5′ end of pGS20 resulted in the loss of the BglII site present in the genomic clone λGS15. The sequences of pGS20 and λGS15 were identical at the 3′-noncoding region. These data suggest that these two GS sequences may be allelic, both being regulated by ammonia (Hirel et al., 1987; see below). The transcription start site of GS (shown in Figure 3B) was determined by the S1 protection assay using an appropriate 5′ fragment as described previously (Wong and Verma, 1985).

To determine the tissue specificity of expression of GS15, a transcription fusion (Figure 3B) was made in a

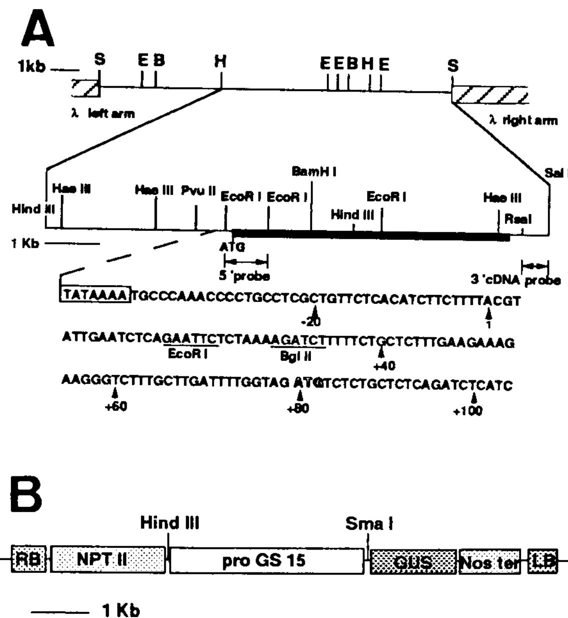

Figure 3. Structure of an Ammonia-Induced GS Gene of Soybean and Construction of a Chimeric Gene.

(A) Restriction map of a soybean GS genomic clone (λGS15) which corresponds to cDNA clone pGS20. DNA sequence of the 5′ region and the position of the BglII site, upstream of the initiation codon, used for making the transcription fusion with the GUS gene.

(B) Diagram of the portion of the plasmid pBin GS-GUS containing the λGS15 promoter (HindIII/BglII fragment, 3.5 kb) and a reporter gene, GUS. The 3.5-kb HindIII/BglII fragment was first subcloned into HindIII/BamHI sites of pUC 19. This fragment was then ligated into HindIII/SmaI sites of pBI101.

binary vector (pBI101) using the 3.5-kb fragment (HindIII-BglII) of λGS15 and the GUS reporter gene (Jefferson et al., 1987). A legume (*L. corniculatus*) plant and a nonlegume (tobacco) plant were transformed with *Agrobacterium rhizogenes* and *A. tumefaciens*, respectively, with a GS-GUS construct. Table 1 shows that regenerated plants selected on kanamycin-containing media have GUS activity only in the root tissue, with no activity in the aerial parts in both transgenic tobacco and *L. corniculatus*. A very high level of GUS activity was also detected in nodules of transgenic *L. corniculatus*. As expected, the control plants carrying cauliflower mosaic virus (CaMV) 35S-GUS (pBI121) showed strong GUS activity throughout the transgenic tobacco and *L. corniculatus* plants (Table 1). Spatial localization of GS-GUS activity in roots of transgenic tobacco and *L. corniculatus* plants demonstrated that the activity in roots of tobacco (Verma et al., 1988) and *L. corniculatus* is localized primarily in the meristematic zone of the root, with some staining in the vascular bundles of mature root, as shown in Figure 4A. No GUS activity was observed in the elongation zone of the root in both transgenic tobacco (Verma et al., 1988) and *L. corniculatus* (Figure 4A). In contrast, the GUS activity driven by the CaMV-35S promoter was localized throughout the root of transgenic *L. corniculatus* and tobacco (data not shown). Closer examination of the GS-GUS activity in both transgenic tobacco and *L. corniculatus* showed that this activity was also localized at the apices of lateral roots, and that it can be detected at the site of emerging lateral roots and nodules (see below) before any visible morphological demarcation on the root surface. These results demonstrated that the soybean cytosolic GS gene retains its tissue specificity in both transgenic legume and nonlegume plants. Thus, the tissue-specific *cis*-acting regulatory elements carried by the soybean GS promoter can be correctly recognized by corresponding *trans*-acting factors present in both nonlegume (tobacco) and heterologous legume (*L. corniculatus*) plants.

Soybean GS Gene Is Regulated by Ammonia in Transgenic *L. corniculatus*

To directly demonstrate that ammonia-induced GS expression in soybean is controlled at the level of GS gene transcription, the GUS activity in roots of transgenic tobacco and *L. corniculatus* containing the GS-GUS gene fusions was measured fluorometrically and histochemically after treatment with different nitrogen sources. The GS-GUS activity in roots of transgenic tobacco did not respond to the application of external ammonia, as shown in Table 2 (see also Verma et al., 1988); however, treatment of the same transgenic *L. corniculatus* plant with 10 mM $(NH_4)_2SO_4$ resulted in enhanced GUS activity in roots (Table 2), suggesting that the soybean cytosolic GS gene promoter is regulated by the availability of ammonia in legume plants. Treatment with other kinds of reduced nitrogen had little or no effect on GS-GUS activity (Table 2). This again confirmed our previous hypothesis that the expression of the soybean GS genes in root and root nodules is directly regulated by ammonia (Hirel et al., 1987). Moreover, the GS-GUS activity was very high in the nodules of transgenic *L. corniculatus* (Table 1) because high concentrations of free ammonia may be available in functional nodules (see below). It has been demonstrated that the induction of cytosolic GS does not occur if the nodules are grown in an environment of argon or in nodules formed by ineffective strains of *Bradyrhizobium japonicum* (Hirel et al., 1987). Similar results are obtained in transgenic *Lotus* infected by a Fix^- strain of *Rhizobium loti* (data not shown). Thus, availability of ammonia from fixed nitrogen appears to be a main factor in enhancing GS gene expression in soybean nodules.

Histochemical localization of GS-GUS activity in ammonia-treated transgenic *L. corniculatus* roots showed that GUS activity is located uniformly in the entire root tissue (Figure 4B), whereas without ammonia treatment the GUS

Table 1. GUS Activity in Different Parts of Transgenic Tobacco and *L. corniculatus* Plants Carrying the Soybean GS Promoter Fused with the GUS Reporter Gene

		GUS Activity[a]			
Species	Construct	Stem	Leaf	Root	Nodule
Tobacco	GS-GUS	8 ± 7	6 ± 8	453 ± 140	–
	pBI121	4444 ± 1001	4403 ± 2173	3241 ± 1059	–
L. corniculatus	GS-GUS	12 ± 7	3 ± 3	564 ± 313	3917 ± 707
	pBI121	3621 ± 629	3467 ± 1100	2934 ± 262	2460 ± 726

Results are based on the mean of five independently transformed tobacco and *Lotus* plants except in the case of stem tissue, where the determinations were made on three samples only. As many as five separate assays of tissue derived from these independently transformed lines yielded the same pattern and variation in GUS activity, as shown above. Repeated analysis showed similar pattern and levels of activity.

[a] GUS activity was expressed in picomoles of the reaction product (methylumbelliferone) per minute per milligram of protein. Values are means ± SD. See Methods for details.

activity is mainly localized in root apices and vascular tissue (Figure 4A) of the same plant. This activity can be detected in the incipient and emerging lateral roots (see below). The ammonia-specific induction of GS gene expression in transgenic *L. corniculatus* takes 4 hr to 6 hr after ammonia application and reaches a maximum after about 12 hr, as shown in Figure 5. These results, along with the data in Figure 1, clearly demonstrate that the regulation of soybean cytosolic GS by ammonia occurs at the transcription level and is not due to the increased stability of GS transcripts. Furthermore, the ammonia-induced expression of GS appears to occur in legumes because the induction of soybean cytosolic GS expression by ammonia is only observed in soybean or in a transgenic legume (*L. cornicutus*) (Table 2, Figure 4, Figure 5), and not in a transgenic nonlegume (tobacco) plant (Table 2).

Expression of Soybean GS Gene in Root Nodules of Transgenic *L. corniculatus*

Subcellular localization of the GS-GUS activity in young nodules formed on transgenic *L. corniculatus* by *R. loti* showed that the GUS activity can be detected in emerging nodules (see also Forde et al., 1989) and incipient nodules before any morphological demarcation on the root surface, as shown in Figure 6B. The size and position of the zone having GUS activity and demarking nodule formation is clearly different from that in lateral root (cf. Figures 6A and 6B). In mature nodules, the infection zone, containing both infected and uninfected cells, and the cells in the inner cortex had most of the activity (Figure 6C). The strongest activity was found in the infected cells (Figures 6D and 6E). Because the uninfected cells have large vacuoles, the activity is localized only on the periphery of these cells. The outer cortex, including the suberized endodermis layer, showed no activity. Older nodules showed intense staining only in the outer part of the infection zone, the central tissue having less activity (data not shown). The latter could be due to the degeneration of the infection zone that usually begins at the center of the nodule. The activity was also present in the vascular bundles located in the inner cortex (Figures 6D and 6E). No GUS activity is detected in untransformed *Lotus* nodules and *R. loti* bacteroids do not have any GUS activity (see also Forde et al., 1989). *R. meliloti* also lacks GUS activity (Sharma and Signer, 1990). The entire infection zone, delimited by the endodermis (which is known to be impermeable to many gases), may encounter a flux of NH_4^+ that is diffused throughout this tissue, consequently inducing GS-GUS.

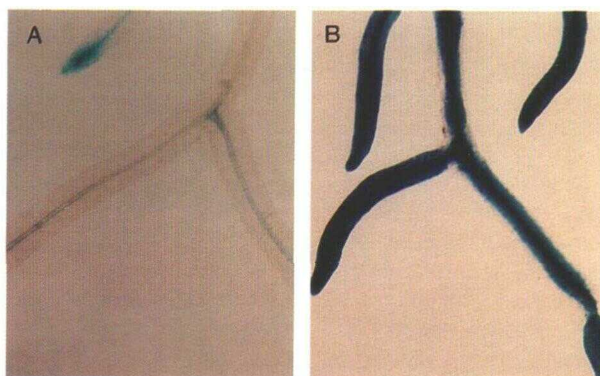

Figure 4. Root-Specific and Ammonia-Induced Expression of GS-GUS as Revealed by Histochemical Localization of GUS Activity in *L. corniculatus*.

(A) *L. corniculatus* roots from transgenic plants obtained after transformation with *A. rhizogenes* containing the GS-GUS construct shown in Figure 3.

(B) *L. corniculatus* roots treated with 10 mM $(NH_4)_2SO_4$ for 12 hr.

Table 2. NH_4^+ Induction of Soybean Glutamine Synthetase Gene as Determined by GS-GUS Expression in the Roots of Transgenic *L. corniculatus*

		GS Promoter Activity[a] (pmol mu[b]/mg protein/min[c])	
Construct	Treatment	*L. corniculatus*	Tobacco
GS-GUS	Untreated	564 ± 313	453 ± 140
	$(NH_4)_2SO_4$ (10 mM)	1868 ± 598	306 ± 163
	Asparagine (1 mM)	638 ± 97	ND[d]
	Glutamine (1 mM)	625 ± 89	ND
	KNO_3 (10 mM)	541 ± 180	ND
35S-GUS	Untreated	2934 ± 262	3241 ± 1059
	$(NH_4)_2SO_4$ (10 mM)	3125 ± 542	3315 ± 567

[a] Values are means ± SD. Increase in the elongation zone is much higher because these cells have very low (if any) GUS activity without ammonia treatment.
[b] mu = methylumbelliferone.
[c] Values are an average from three independent transgenic plants.
[d] ND, not determined.

The pattern of GS expression, however, may change depending upon the nature of the nodules, i.e., ureide or amide producers. Because *Lotus* is an amide-producing plant, it is possible that the expression of soybean GS (a ureide-producing plant) in a heterologous system may not mimic the native pattern.

DISCUSSION

Regulation of Soybean Cytosolic GS Genes by Ammonia in Soybean and Transgenic *L. corniculatus*

Primary assimilation of symbiotically fixed nitrogen is carried out by GS in the host cell cytoplasm, and in a number of legumes a nodule-specific form of GS has been detected (Lara et al., 1983; Sengupta-Gopalan and Pitas, 1986; Dunn et al., 1988; Konieczny et al., 1988; Forde et al., 1989). In soybean, on the other hand, it appears that there is no nodule-specific GS isoform (Hirel et al., 1987), and the root gene encoding cytosolic GS is regulated by the availability of ammonia produced through symbiosis with *Rhizobium*. That ammonia is specifically controlling the activity of the GS promoter in soybean is evident from the observation that externally supplied ammonia rapidly induces GS-GUS activity uniformly throughout the root. This induction, however, occurs only in a legume background because no increase in GS-GUS activity was observed in transgenic tobacco roots (Table 2). GS-GUS activity was also found to be very high in the infection zone of nodule tissue, suggesting that symbiotically fixed nitrogen is diffused rapidly as ammonia throughout the infection zone, inducing GS-GUS activity in both infected and uninfected cells. The specific activity of GS has been shown to be higher in the uninfected cells (Kouchi et al., 1988), although optically infected cells seem to have higher GS-GUS activity. It has recently been shown (Reynolds et al., 1990) that ammonia-fed *Trifolium* roots translocated assimilated nitrogen in a manner analogous to that of nodules.

In bean, GS genes appear to be developmentally controlled and are not responsive to externally supplied ammonia (Cock et al., 1990). The expression of one of the *Phaseolus* GS genes (*gln* γ) is restricted to infected cells, whereas the other GS gene (*gln* β) is preferentially expressed in vascular bundles in transgenic *L. corniculatus* nodules (Forde et al., 1989). The pea cytosolic GS gene is primarily expressed in the phloem tissue and, thus, helps in the intercellular transport of nitrogen (Edwards et al., 1990). The presence of GS-GUS activity in vascular bundles of transgenic *L. corniculatus* root and root nodules suggests that this tissue may participate in ammonia assimilation in amide-producing plants; however, the situa-

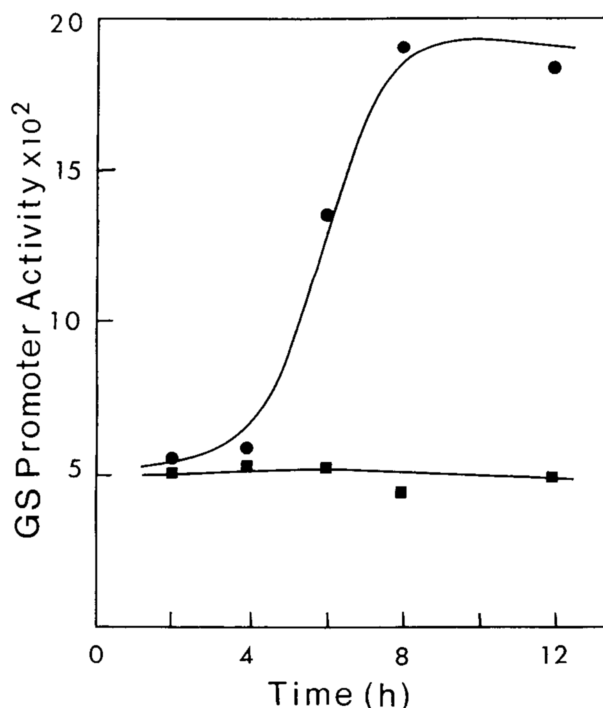

Figure 5. Time Course of Ammonia Induction of the Soybean Cytosolic GS Gene in Transgenic *L. corniculatus*.

Roots from transgenic *L. corniculatus* plants grown in vermiculite for several days without nitrogen were treated with 10 mM $(NH_4)_2SO_4$ for different time periods and root tissue was collected for the determination of GUS activity. GUS activity (pmol/min/mg of protein; see Table 1) in transgenic *L. corniculatus* without ammonia treatment (■) and with ammonia treatment (●) is shown.

tion may be different in ureide-producing tropical legumes, like soybean. In amide-producing legumes, glutamine is one of the final products of assimilated nitrogen and is translocated directly to the shoot; in the case of ureide-producing nodules, glutamine must be converted to ureide before translocation. Hence, the distribution of nitrogen assimilation enzymes in infected and uninfected cells may vary between the two types of nodules. Transfer of this gene to soybean or other tropical legumes such as *Vigna* (studies in progress) may help to answer this question.

Specificity for Ammonia Induction of Soybean GS Genes

The soybean GS15 gene retains its tissue specificity and is expressed in a similar spatial manner in both transgenic tobacco and *L. corniculatus* roots; however, the ammonia-enhanced expression is only observed in *Lotus*. This ammonia-enhanced expression of the GS gene is not due to a general effect of an enhanced level of reduced nitrogen because glutamine, asparagine, and nitrate did not increase the level of GS-GUS during the same period. The expression seems specific because the ATP-synthetase transcript is unaffected by the availability of ammonia. Therefore, these data suggest that the soybean GS promoter carries at least two *cis*-regulatory elements, one being root specific and the other, ammonia activated. The latter may require specific *trans*-acting factors present in legumes. Normally, ammonia is available from the assimilation of nitrate and nitrite, which occurs in the root apices (Emes and Fowler, 1979; Granato and Raper, 1989), where GS is also found to be localized (Figure 4A), thus converting ammonia into glutamine. This process, however, is relatively slow. Although the actual concentration of NH_4^+ is not known, a rapid flux of ammonia may occur from symbiotic nitrogen fixation in root nodules that must be metabolized to avoid any toxic effects. Thus, some of the GS genes appear to have come directly under the control of ammonia in certain legume plants. In germinating lettuce seeds, the cytosolic GS gene has been shown to be regulated by phytochrome (Sakamoto et al., 1990). Because there are several GS genes in plants, nitrogen metabolism may be fine-tuned depending upon the tissue and environment and the availability of carbon. The induction of GS by ammonia in soybean and transgenic *Lotus* also suggests that there are specific factors present in legumes that may interact with ammonia. The maize alcohol dehydrogenase gene has been shown to function only in the monocot background and is not inducible in tobacco (Ellis et al., 1987) unless upstream sequences from a constitutive promoter are added to this gene. We do not know whether the lack of inducibility of GS-GUS activity in tobacco roots is due to the lack of permeation of NH_4^+ through other parts of the root (except the apex) or to the lack of specific factors. Further promoter analysis with respect to the interactions of *trans*-acting factors may reveal these differences. The analysis of such factors may also shed light on the regulation of GS genes by ammonia in certain legume plants.

Soybean Cytosolic GS as an Indicator for Nitrogen Source/Sink in Root

The presence of GS in root apices, where nitrate reductase is also localized (Fentem et al., 1983; Weber et al., 1990), makes this tissue a source of reduced nitrogen. On the other hand, location of GS in incipient root primordia (Figure 6A) indicates that NH_4^+ may be mobilized to these cells from other parts of the root at this initial stage of lateral root development, where it is converted to glutamine by GS; thus, this tissue serves as an active sink for nitrogen. Once the apical meristem is fully developed and the lateral root has emerged from the main root, the apices of the lateral roots may act as a source of nitrogen. Similarly, the young emerging nodule (Figure 6B; Forde et al., 1989) is a sink for nitrogen, whereas the nitrogen-fixing nodule is a source. It has been shown recently that the nitrate reductase and GS are coordinately regulated in root (Weber et al., 1990). Thus, in both nodules and root apices, availability of reduced nitrogen may be controlling levels of GS supporting active nitrogen assimilation. The soybean GS promoter can, therefore, be used to monitor the effect of external nitrogen on the assimilation of symbiotically fixed nitrogen in legume roots.

Ammonia Permeability in the Infection Zone of Root Nodules and Possible Structural Barriers against Diffusion

Recent studies (Udvardi and Day, 1990) have shown that there is no specific carrier for NH_4^+ in root nodules, which should allow diffusion of this molecule throughout the tissue. The observation that GS-GUS is fully expressed in the inner cortex of the nodules of transgenic *Lotus*, whereas there is no activity in the outer cortex, suggests that the endodermis may act as a barrier to NH_4^+ diffusion. The GUS activity can be induced in the outer cortex by treating the nodule with ammonia (data not shown). Our studies suggest that NH_4^+ is rapidly diffused throughout the infection zone and the inner cortex, resulting in the induction of GS, and this activity is delimited by a single suberized cell layer (endodermis), which is known to be impermeable to many gases. It was first proposed by Tjepkema and Yocum (1974) that the endodermis is a barrier to oxygen, and this has been confirmed by recent in situ experiments (Hunt et al., 1988). Because the vascular bundles are also located within the inner cortex and the uninfected cells (including inner cortex) carry out terminal steps in ureide assimilation (Verma, 1989), the fixed nitrogen can be translocated easily from this region by

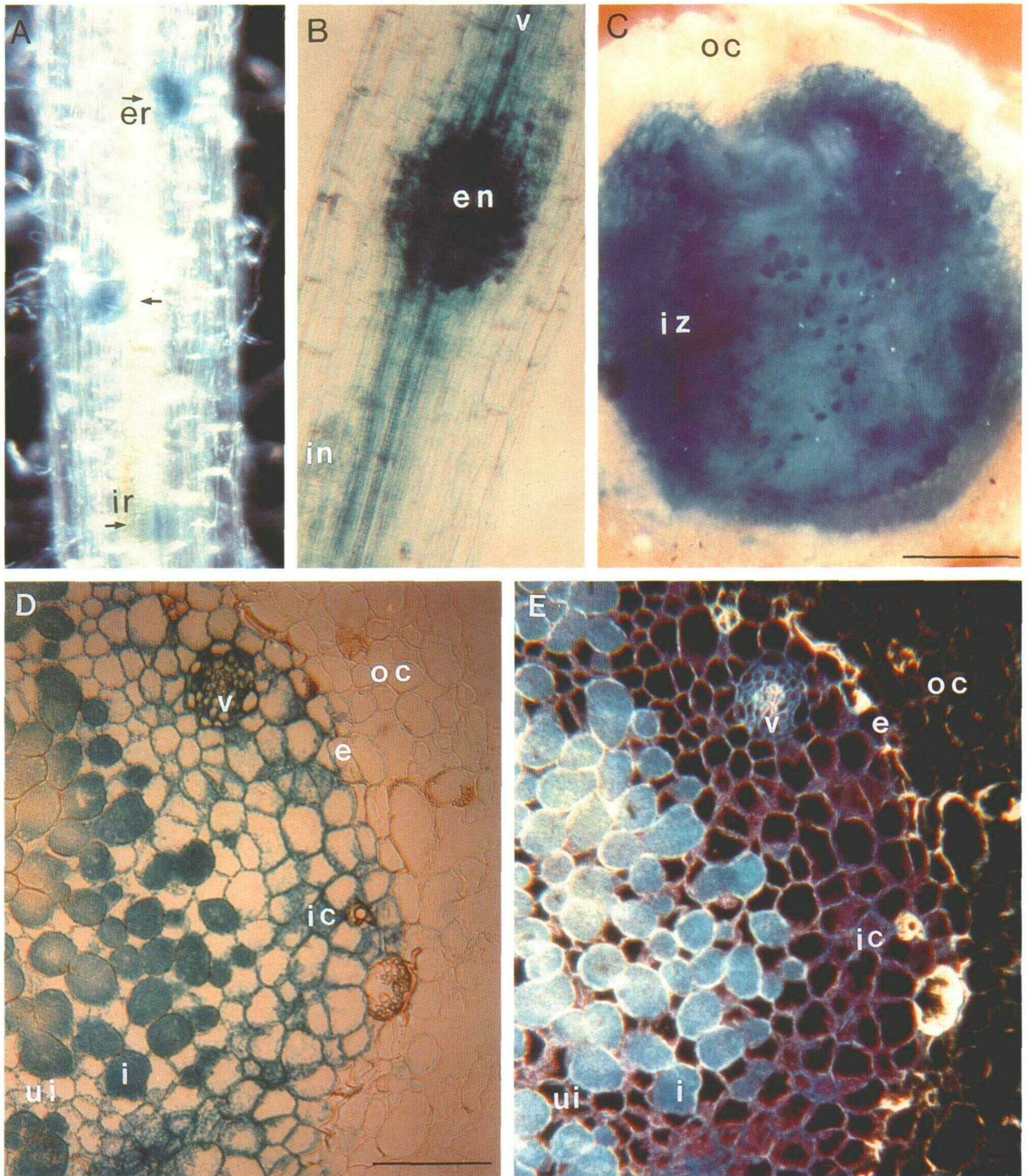

Figure 6. Expression of the Soybean Cytosolic GS-GUS Fusion in Transgenic *L. corniculatus* Root and Nodules.

GUS activity was localized by treating root and nodules with X-Gluc and processing them for light microscopy. e, endodermis; en, emerging nodules; er, emerging root; i, infected cells; ic, inner cortex; in, incipient nodules; ir, incipient root; iz, infection zone (including inner cortex); oc, outer cortex; ui, *uninfected cells*; v, *vascular bundle*.

way of the vascular tissue. Thus, either there is a clear diffusion barrier or a high rate of assimilation of ammonia in the infection zone that keeps NH_4^+ from reaching to the outer cortex.

The primary nitrogen assimilation products in temperate legumes (glutamine and asparagine) can be translocated directly from the infected cells to other parts of the plant through vascular tissue because amide-producing plants have fewer uninfected cells (Stegink et al., 1987). The localization of soybean GS-GUS activity in vascular tissue of transgenic *L. corniculatus* nodules (Figures 6D and 6E) also suggests the role of this tissue in assimilation of residual ammonia during the transport process (Edwards et al., 1990). In *Phaseolus*, which transports both ureide and amides, the *gln* γ gene is only expressed in the infected cells, as shown by the experiments in transgenic *L. corniculatus* (Forde et al., 1989). In the case of soybean, the terminal steps of ureide production take place primarily in the uninfected cells, whereas GS is localized in both cell types. This suggests that some of the intermediates of the nitrogen metabolism cross the infected and uninfected cells many times in ureide-producing nodules. This is consistent with the location of xanthine dehydrogenase in both infected and uninfected cells in cowpea (Newcomb et al., 1990). Alternatively, the uninfected cells can complete ureide production all the way from glutamine and translocate it, while the glutamine produced in the infected cell is utilized by subcellular processes of the infected cells and the nitrogen demand of the bacteroids. A detailed understanding of fine regulation of the GS genes by NH_4^+ in amide-producing and ureide-producing legumes may be necessary for enhancing the efficiency of assimilation of symbiotically fixed nitrogen.

METHODS

Plant Materials and Bacterial Strains

Tobacco (*Nicotiana tabacum* var Xanthi) and soybean (*Glycine max* var Prize) were grown in controlled environmental growth chambers at 26°C; *Lotus corniculatus* var Leo was grown at 22°C. The plants were watered once a day with nitrogen-free or nitrogen-containing half-strength complete Hoagland media.

Escherichia coli (XL1-Blue) from Stratagene was used as the host for constructing a soybean nodule cDNA library, as previously described (Delauney and Verma, 1990a, 1990b). The *E. coli* glutamine synthetase *gln* A^- mutant (A318) was used for glutamine auxotroph complementation. *Agrobacterium tumefaciens* (LBA 4404) containing appropriate GS-GUS constructs in pBIN (Bevan, 1984) was used for tobacco transformation. *A. rhizogenes* (A4), obtained from Dr. E. Nester, was used for *L. corniculatus* transformation. *Rhizobium loti* (USDA3471) was used for inoculating transgenic *L. corniculatus*.

RNA Gel Blot Analysis

Poly(A)$^+$ RNA isolation and RNA gel blot analysis were performed essentially as previously described (Hirel et al., 1987). The DNA probes were labeled with α-^{32}P-dCTP and used for hybridization. The hybridization was carried out at 65°C in 50 mM Tris-HCl, pH 7.6, 2% bovine serum albumin, 0.2% polyvinyl pyrrolidone, 0.2% Ficoll (40,000), 0.2% SDS, 0.1% sodium pyrophosphate, 6% NaCl, and 0.1 μg/mL denatured calf thymus DNA. Final washes were carried out in 0.1 × STE (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA), 0.1% SDS at 65°C, and filters were exposed to x-ray films at −80°C.

Isolation of Soybean Cytosolic GS cDNA Clone by Functionally Complementing an *E. coli gln* A^- Mutant

The complementation experiment was conducted as previously described (Delauney and Verma, 1990a). In short, the *E. coli* GS *gln* A^- auxotroph mutant (A318) was transformed with the total soybean phagemid library in λ*Zap*II (Stratagene) (Delauney and Verma, 1990b) by the method of Hanahan (1985). Transformants were selected on minimal A media (Ausubel et al., 1987) containing ampicillin (100 μg/mL) and isopropylthiogalactoside (1 mM). Plates were incubated at 37°C for about 24 hr. Plasmid DNA was isolated from rescued cells and used for restriction mapping (Maniatis et al., 1982) and DNA sequencing.

DNA Sequence Analysis

DNA sequencing of the putative GS clones and the appropriate subclones of genomic DNA in pBluescript was carried out by the dideoxy sequencing method (Sanger et al., 1977). Crude prepared or CsCl-purified DNA was denatured by incubation in 0.2 N NaOH and 0.2 mM EDTA, and neutralized by 0.3 M sodium acetate, pH 5.0. The DNA was precipitated with 2.5 volumes of ethanol at −20°C for 15 min, pelleted in a microcentrifuge, resuspended in distilled water, and annealed with appropriate sequencing primers. Sequenase Version II (United States Biochemicals) was used for dideoxynucleotide-terminated chain polymerization. Different deletions of GS cDNA clones and synthetic nucleotide oligomer primers were used for sequencing the entire cDNA clone. The

Figure 6. (continued).

(A) Dark-field micrograph of GS-GUS activity in emerging and incipient lateral roots. Bar = 600 μm.
(B) Localization of GS-GUS activity in emerging and incipient root nodules. Bar = 600 μm.
(C) Cross-section of nodule exhibiting GUS activity in the infection zone and the lack of activity in the outer cortex. Bar = 200 μm.
(D) Light-field micrograph of a section of the transgenic nodule cortex and infection zone demonstrating the limit of GS-GUS activity. Bar = 200 μm.
(E) Dark-field micrographs of the nodule in **(D)**.

DNA sequence data was analyzed with the Wisconsin Genetics Group programs. Sequence data of a full-length soybean cytosolic cDNA have been submitted to the EMBL nucleotide sequence data bank.

Construction of the GS15 Promoter-GUS Gene Fusion

The junction between the coding region and the promoter of λGS15 was sequenced after appropriate subcloning. The promoter region of λGS15 (3.5 kb) located between the 5′ HindIII site and the BglII site in the 5′-noncoding region (see Figure 3) was first subcloned into the HindIII and BamHI sites of pUC 19. It was then transferred to pBI101 (Jefferson et al., 1987) using the HindIII and SmaI sites (Figure 3B). The resulting construct was introduced into tobacco and *L. corniculatus* by way of *Agrobacterium*-mediated transformations (see below). CaMV 35S-GUS gene fusion (pBI121) (Jefferson et al., 1987) was used as a control.

Transgenic Plants

The leaf disc transformation method was used for tobacco transformation (Horsch et al., 1984). The pBIN plasmid vectors with different constructs were directly introduced into *A. tumefaciens* (LBA 4404) by a modified freeze/thaw transformation procedure (An et al., 1988). The transformants, selected on kanamycin, were used to inoculate leaf discs of tobacco, and transformed callus and shoots were selected on MS (Murashige and Skoog, 1962) medium containing kanamycin (200 μg/mL). Transformed shoots were rooted on MS medium without any hormone. After rooting, the transgenic plants were transferred to soil and grown in controlled growth chambers. The primary transgenic plants were allowed to self-fertilize, and the mature seeds were collected and germinated on kanamycin-containing agar. The kanamycin-resistant seedlings were transferred to soil and grown at 26° C for further analysis.

Transgenic *L. corniculatus* plants were obtained using an *A. rhizogenes*-mediated transformation procedure (Petit et al., 1987). Surface-sterilized seeds of *L. corniculatus* were germinated and grown on MS medium without hormone. Five-day-old to 7-day-old plants were infected on the hypocotyls with *A. rhizogenes* containing the GS-GUS construct or a control plasmid, pBI121. Hairy roots were formed on infected stems 7 days to 14 days after inoculation. Transformed hairy roots were selected on half-strength MS medium containing kanamycin (100 μg/mL) and cefotaxim (200 μg/mL) in the dark at 26°C. After 2 weeks, fast-growing roots were transferred to shoot-inducing medium containing kanamycin and cefotaxim and incubated under continuous light at 26°C until shoots were formed. Shoots about 1 cm tall were transferred to half-strength MS medium, and rooted plants were propagated in soil in a growth chamber at 22°C. Several transgenic plants were generated from each independent transformant. The original transformed plants were inoculated with wild-type *Rhizobium loti* (USDA 3471) or a Fix^- mutant (PN1010, kindly provided by B. Scott, Massey University, New Zealand), and nodules were collected between 4 weeks to 8 weeks after inoculation. New plants were regenerated from cuttings.

The Fluorometric Assay

The fluorometric GUS assay was performed as described by Jefferson et al. (1987). Tissue extracts were made from different parts of transgenic and control plants treated with different nitrogen sources. Untreated tissue was obtained from the same plant before treatment. Tissue extracts were incubated with reaction buffer containing 1 mM 4-methylumbelliferyl glucuronide. The reaction was stopped after the appropriate incubation period, and the reaction product (4-methylumbelliferone) was measured using a fluorometer (DNA fluorometer model TKO 100, Hoefer Scientific Instrument). Protein concentration of tissue extracts was determined by the method of Bradford (1976).

Histochemical Localization of GUS

Histochemical localization of GUS activity was performed using 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-Gluc) as a chromogenic substrate (Jefferson et al., 1987). Freshly cut roots were directly incubated in 1 mM X-Gluc in 50 mM sodium phosphate buffer, pH 7.2, for 8 hr to 12 hr in a humidified chamber at 37°C. Samples were treated with 10% commercial bleach for 10 min and rinsed with distilled water before photomicrography. For histochemical localization of GUS in nodules, prestained nodules with X-Gluc were fixed in 2% glutaraldehyde/4% paraformaldehyde in 100 mM sodium cacodylate buffer, pH 7.2, overnight at 4°C, dehydrated in an ethanol series, and then gradually infiltrated with the methacrylate JB4 (Polysciences Inc., Warrington, PA). Tissue was left in 100% JB4 for at least 24 hr to ensure adequate penetration of resin, and was then embedded using catalyzed JB4 at room temperature. Sections 2 μm to 6 μm thick were cut on a rotary microtome using a dry glass knife. Section flattening on a drop of water was aided by the addition of ammonium hydroxide (0.01%) to the water. Sections were observed and photographed using bright-field and dark-field microscopy on a Zeiss microscope. No counterstain was applied.

ACKNOWLEDGMENTS

This work was supported in part with a grant from the National Science Foundation (DCB-8904101) and the National Sciences and Engineering Research Council of Canada to D.P.S.V. We would like to thank Frederick Ausubel for providing the *gln* A^- mutant of *E. coli*, Ashton Delauney for constructing the soybean expression library, C.P. Joshi for assistance with DNA sequence comparison, Kent Peters for comments on the manuscript, Pam Snyder for her excellent technical assistance, and Angela Kalb for help in preparing this manuscript.

Received July 11, 1990; accepted October 29, 1990.

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