The Plant Cell, Vol. 2, 633–641, July 1990 © 1990 American Society of Plant Physiologists

Nonlegume Hemoglobin Genes Retain Organ-Specific Expression in Heterologous Transgenic Plants

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Hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa* have been isolated [Landsmann et al. (1986). Nature 324, 166–168; Bogusz et al. (1988). Nature 331, 178–180]. The promoters of these genes have been linked to a β-glucuronidase reporter gene and introduced into both the nonlegume *Nicotiana tabacum* and the legume *Lotus corniculatus*. Both promoters directed root-specific expression in transgenic tobacco. When transgenic *Lotus* plants were nodulated by *Rhizobium loti*, both promoter constructs showed a high level of nodule-specific expression confined to the central bacteroid-containing portion of the nodule corresponding to the expression seen for the endogenous *Lotus* leghemoglobin gene. The *T. tomentosa* promoter was also expressed at a low level in the vascular tissue of the *Lotus* roots. The hemoglobin promoters from both nonlegumes, including the non-nodulating species, must contain conserved *cis*-acting DNA signals that are responsible for nodule-specific expression in legumes. We have identified sequence motifs postulated previously as the nodule-specific regulatory elements of the soybean leghemoglobin genes [Stougaard et al. (1987). EMBO J. 6, 3565–3569].

INTRODUCTION

Hemoglobin is present in the nitrogen-fixing nodules of both legumes and nonlegumes, where it transports oxygen to the nitrogen-fixing endophytes. The oxygen-binding characteristics of these hemoglobins ensure that there is an adequate supply of oxygen for bacterial respiration at the low concentrations of free oxygen that prevail in infected cells. Damage to the oxygen-sensitive nitrogenase enzyme present in the *Rhizobium* bacteroid or *Frankia* encapsulated within the nodule tissues (reviewed in Appleby, 1984) is avoided at these low oxygen concentrations.

Soybean contains a number of hemoglobins (leghemoglobins) and their corresponding gene sequences, one of which, *lbc*$_3$, has been studied extensively at the molecular level and shown to be expressed solely in the nodules induced by *Bradyrhizobium japonicum* (Marker et al., 1984). When the promoter of the *lbc*$_3$ gene is linked to a reporter gene and introduced into another legume, *Lotus corniculatus*, using *Agrobacterium*-mediated transformation, the chimeric gene, following nodulation by *Rhizobium loti*, is expressed specifically in the nodules of the transgenic plants (Stougaard et al., 1987b). Clearly, the regulatory signals necessary for nodule-specific expression are conserved between different legume species.

The nitrogen-fixing nonlegume *Parasponia andersonii* has a single hemoglobin gene (Landsmann et al., 1986) that is strongly expressed in *Parasponia* nodules induced by *Rhizobium* strain CP283 and is also expressed, at a much lower level, in the roots of non-nodulated plants (Bogusz et al., 1988). The phylogenetically related non-nodulating, non-nitrogen-fixing species *Trema tomentosa* also contains a single hemoglobin gene, and expression has been detected only in roots (Bogusz et al., 1988).

We have investigated whether the promoters of the hemoglobin genes of these two nonlegumes will function when introduced into the genomes of either a legume species, *L. corniculatus*, or a non-nodulating, nonlegume host, tobacco (*Nicotiana tabacum*), and whether the nodule- and/or root-specific pattern of expression of the hemoglobin genes is maintained in the heterologous hosts. We have constructed chimeric genes consisting of the *Parasponia* and *Trema* promoters linked to the β-glucuronidase (GUS) reporter gene and introduced them into both *Lotus* (using *Agrobacterium rhizogenes*) and *N. tabacum* (using *A. tumefaciens*), and analyzed their expression in different organs by histochemical and quantitative enzyme assays.

Both promoters showed high levels of expression in

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nodules of *Lotus*, with expression concentrated in the central region of the nodule, which contains the cells infected by the bacteria. The pattern of expression is similar to that of the endogenous *Lotus* leghemoglobin as well as to that of a GUS reporter gene driven by the soybean *lbc*$_3$ promoter. The chimeric genes also showed low levels of expression in the roots of transgenic *Lotus*. In transgenic tobacco plants both promoters showed preferential expression in roots, a pattern similar to that in nonnodulated *Trema* or *Parasponia* plants.

RESULTS

The Promoters of *Parasponia* and *Trema* Hemoglobin Direct Root-Specific Expression in Tobacco

The *Parasponia*-GUS (P-GUS), *Trema*-GUS (T-GUS), and soybean-GUS (*lbc*$_3$-GUS) constructs were introduced into tobacco using *A. tumefaciens* transformation procedures. Roots and expanded leaves of six randomly chosen transformed tobacco plants growing in tissue culture were assayed for each construct; the data are displayed graphically in Figure 1. The *lbc*$_3$-GUS plants were initially screened by DNA gel blot hybridizations to identify those containing complete inserts. As seen in Figure 1, the P-GUS and T-GUS constructs gave comparable, high levels of GUS expression in roots [means of 301 and 289 specific activity units (picomoles of 4-methylumbelliferone produced per minute per microgram of soluble protein), respectively] but considerably less expression in leaves (means of 4.2 and 34.0 units, respectively). There was some variation in the level of GUS activity between individual transgenic plants, presumably the result of differences in the position of integration of the chimeric genes (Dean et al., 1988), but for any one plant expression was always higher in the roots. In P-GUS the ratio of root-to-leaf expression varied from 15 to 650 with a mean of 71. The T-GUS plants, on the other hand, had smaller ratios, varying from 1.5 to 33 in different plants with a mean of 6.5, largely due to the relative increase in levels of leaf expression. Therefore, of the two, P-GUS had the more tightly controlled pattern of organ-specific expression.

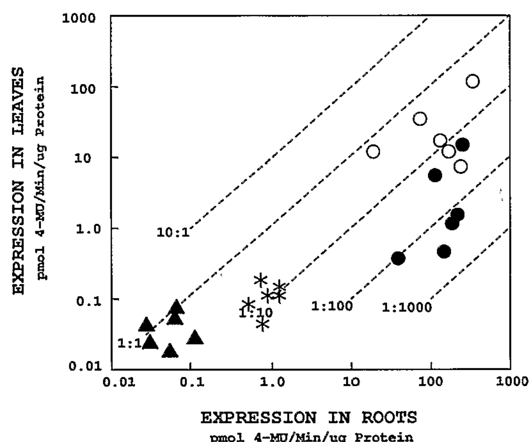

Figure 1. GUS Expression in the Roots and Leaves of Transgenic Tobacco Plants.

The specific activity of the β-glucuronidase enzyme was assayed in the roots and leaves of tobacco plants transformed with the P-GUS (●), T-GUS (○), and *lbc*$_3$-GUS (*) gene constructs or control plants without a chimeric GUS gene (▲) as described in Methods. Each point represents the root and leaf activity for a separate transformant for each of the gene constructs. The dotted lines represent the locations of common coordinates having leaf-to-root activity ratios of 10:1, 1:1, 1:10, 1:100, and 1:1000, respectively.

The *lbc*$_3$-GUS construct from soybean, consistent with previous reports of expression being restricted to nodules (Stougaard et al., 1987a, 1987b), gave very little expression in any part of the transgenic tobacco plants, although the levels of expression in the roots (mean of 1.4 specific activity units) were significantly above the background level observed in leaves (mean of 0.12 units) or in the roots or leaves of either transformants containing a promoterless GUS gene or nontransformed control tobaccos (all with activities less than 0.09 units). Figure 2 shows the distribution of P-GUS and T-GUS expression in tobacco roots and leaves as determined by histochemical staining for GUS activity. Figures 2A and 2B show that GUS activity was greatest in the meristematic region of the root tip in both the P-GUS and T-GUS plants. The root caps of both groups of plants were not stained. In those plants containing the P-GUS construct, GUS expression was also detected in differentiated tissues in the vascular cylinder (shown in Figure 2A), 2 mm to 3 mm proximal to the root tip. Further up the root, low-level staining was also observed in the cortex (not shown). T-GUS plants, on the other hand, showed little or no staining in the differentiated root tissues (Figure 2B) despite the intense staining in the root tips. In those T-GUS plants that expressed the highest levels of GUS activity in the leaves (not shown), this activity appeared to be confined to the vascular system in a manner similar to the GUS gene driven by the 35S promoter of cauliflower mosaic virus analyzed by Jefferson et al. (1987) but at much lower absolute levels. When tested in *N. plumbaginifolia* protoplasts, both nonlegume gene constructs were expressed at low but detectable levels relative to a 35S-GUS gene construct. The relative promoter strengths were 35S-GUS > P-GUS > T-GUS, a pattern not reflected in the root tissues of transgenic plants where T-GUS and P-GUS had similar mean values that were only slightly below 35S-GUS constructs (not shown).

The *lbc*$_3$-GUS construct had no detectable activity in tobacco protoplasts.

P-GUS and T-GUS Are Highly Expressed in *Lotus* Nodules

The P-GUS, T-GUS, and *lbc*$_3$-GUS chimeric genes were introduced into *Lotus* using *A. rhizogenes*. Nodules were produced on the hairy roots by inoculation with *R. loti.* GUS activities were quantified fluorometrically in individual roots and their associated nodules; the data are displayed in Figure 3. Each root system represents a separate transformation event. Between four and 11 mature pink nodules were assayed for each construct. Although there was considerable variation among different transformants, all three constructs showed high levels of expression in *Lotus* nodules, the soybean promoter being the most active and the *Trema* promoter the least active. On average, the level of GUS activity in the nodules increased by threefold between T-GUS (245 specific activity units) and P-GUS (791 units) or twofold between P-GUS and *lbc*$_3$-GUS (1312 units).

When transgenic *Lotus* nodules were stained for GUS activity, expression in the mature nodules was confined to the central core of tissue, corresponding to the bacteroid-containing cells, which also express the endogenous *Lotus* leghemoglobin. Most T-GUS nodules showed relatively uniform expression throughout the core (Figure 2C), whereas P-GUS expression seemed to be more intense at the periphery of the central core, probably just internal to the vascular tissue that surrounds the bacteroid-containing cells (Figure 2D). This difference in the staining pattern may simply reflect absolute levels of GUS expression because some T-GUS nodules, which expressed lower levels of GUS, showed a similar differential between the nodule vascular tissue and the inner bacteroid-containing cells (Figure 2F, larger nodule). GUS activity was absent from the cortical cells surrounding the nodule (Figures 2C and 2D) for both constructs. Not all apparently mature nodules on any particular transformed root were positive for GUS expression. It is unclear whether these nodules were somehow disturbed in nodule development, but they did appear normal at a gross structural level. The *lbc*$_3$-GUS nodules were not analyzed in any great detail, but they, too, showed GUS staining only in the central core of the nodule and not in the cortical cells or the non-nodulated part of the root.

The time of expression of the nonlegume hemoglobin promoters in the transgenic legume nodules appeared to correlate with nodule size and, therefore, to the developmental stage of the nodule (Figure 2F and results not shown). We have not been able to determine precisely whether GUS expression and *Lotus* leghemoglobin synthesis are initiated simultaneously, but the highest levels of staining occurred in large nodules that had already turned pink because of leghemoglobin accumulation.

P-GUS and T-GUS Show Low Levels of Expression in *Lotus* Roots

Fluorometric GUS assays of transformed *Lotus* roots, both nodulated and non-nodulated, showed a low level of expression of both the P-GUS and T-GUS chimeric genes (Figure 3). The expression of the T-GUS construct (37.2 specific activity units) was considerably higher than that of the P-GUS gene (9.6 units), whereas the *lbc*$_3$-GUS gene was poorly expressed (1.8 units). There was a decreasing mean ratio of nodule-to-root expression from *lbc*$_3$-GUS (727) to P-GUS (82) to T-GUS (6.6), primarily because of the relative increase in expression in the non-nodulated parts of the roots.

The distribution of GUS expression in transgenic *Lotus* roots was determined histochemically. In contrast to tobacco roots, P-GUS expression was not detected at high levels in the root tip. There was a low level of staining that was difficult to localize but was probably associated with the vascular tissues. The T-GUS construct was expressed at slightly higher levels in the transgenic *Lotus* roots but low compared with its expression in tobacco. Staining was generally confined to the vascular tissues of mature roots (Figure 2C). In general the root tips were not stained; occasionally, newly emerged roots expressed the gene but in the vascular region and not the meristem (Figure 2E). No staining was detected in the transgenic roots containing the *lbc*$_3$-GUS gene (not shown).

DISCUSSION

We have demonstrated that the promoters of the hemoglobin genes of the two nonlegumes *Parasponia* and *Trema* function at elevated levels in the transgenic nodules of the legume *Lotus*. The soybean *lbc*$_3$ promoter, as expected from previous studies with a chimeric chloramphenicol acetyltransferase gene construction (Stougaard et al., 1987b), also showed an extremely high level of expression in transgenic *Lotus* nodules with only low levels of expression in the non-nodulated roots. The general spatial and temporal distribution of GUS activity, determined histochemically, directed by the two nonlegume hemoglobin-GUS chimeric genes within the *Lotus* nodule appeared to parallel both that of endogenous *Lotus* leghemoglobin and the introduced soybean hemoglobin-GUS gene. This finding indicates that nodule-specific regulatory sequences are sufficiently conserved between nonlegumes and legumes to control expression of the nonlegume promoters in transgenic legume plants.

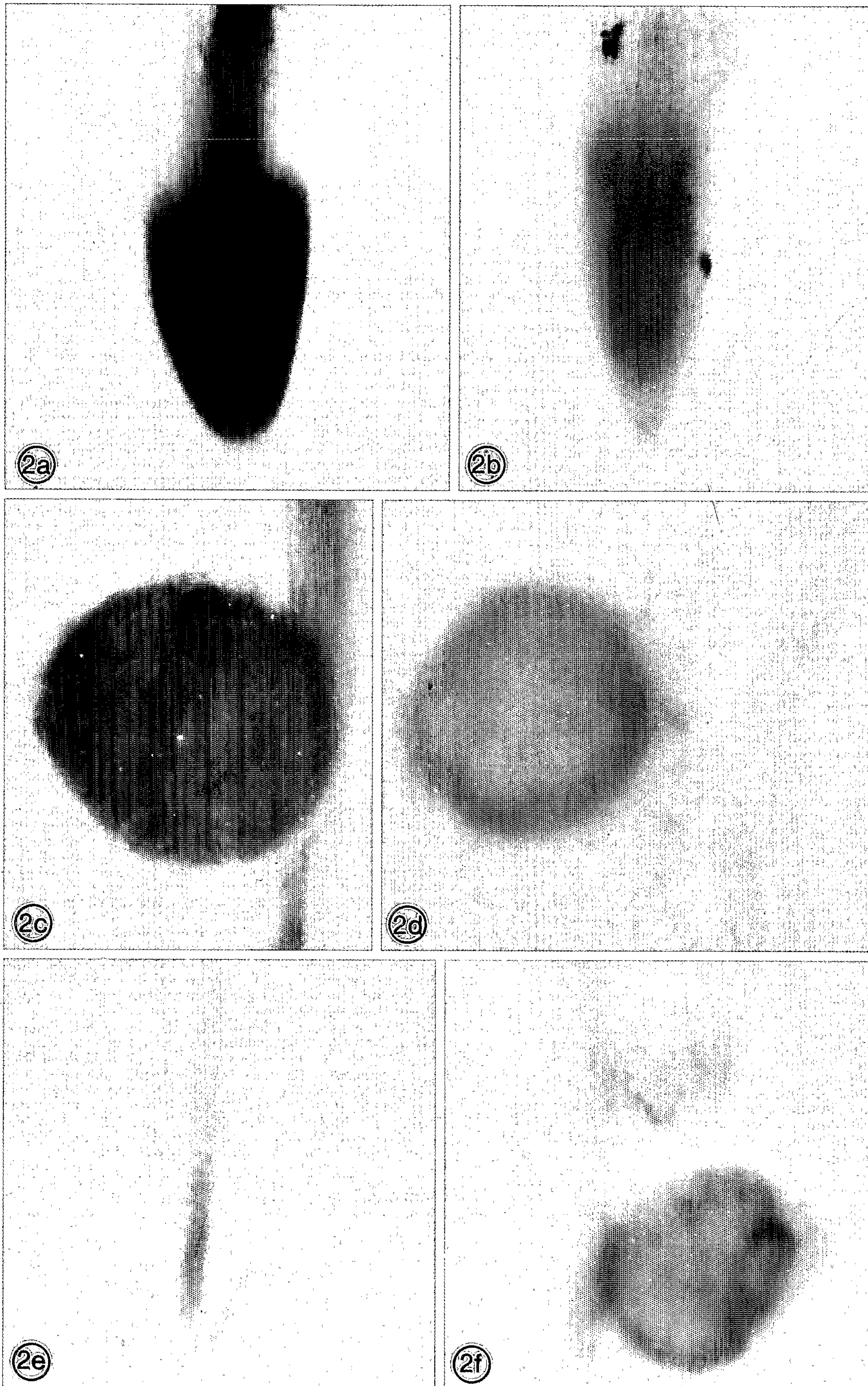

Figure 2. Histochemical Localization of GUS Gene Expression in Transgenic Plants.

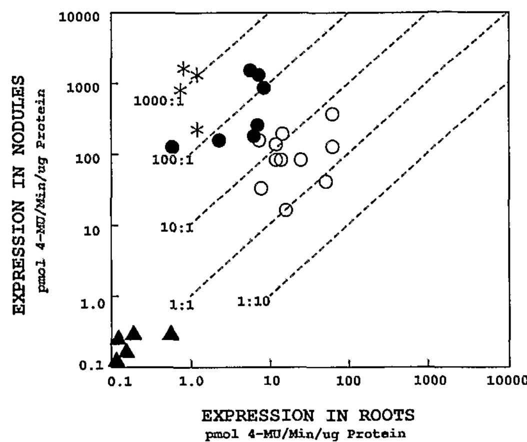

Figure 3. GUS Expression in the Roots and Nodules of Transgenic *Lotus* Plants.

The specific activity of β-glucuronidase enzyme was assayed in the hairy roots and their associated nodules from *Lotus* seedlings transformed with the P-GUS (●), T-GUS (O), and *lbc*$_3$-GUS (*) gene constructs or controls without a chimeric GUS gene (▲) and infected by *R. loti* as described in Methods. Each point represents the activity in a single mature pink nodule and a segment of non-nodulated root from the same root system of independent transformants. The dotted lines represent the locations of common coordinates having nodule-to-root activity ratios of 1000:1, 100:1, 10:1, 1:1, and 1:10, respectively.

Sandal et al. (1987) and Metz et al. (1988) have suggested that two consensus sequences, AAAGAT and CTCTTC, present in the so-called "organ-specific box" in the 5′ regions of all of the expressed hemoglobin and nodulin genes from soybean and other legume species could be the *cis*-regulatory sequences that determine high-level, nodule-specific expression. Deletion analysis of the soybean *lbc*$_3$ gene promoter has shown that the region responsible for nodule-specific expression contains copies of these sequences (Stougaard et al., 1987b). Figure 4 shows that these sequence motifs are also present in similar positions relative to the transcription start in the hemoglobin promoters of the *Parasponia* and *Trema* genes, but in *Trema* the CTCTTC subsequence is on the noncoding DNA strand. Furthermore, in *Trema* the AAAGAT motif is a poor match, but there is a perfect match further upstream in the same orientation (Figure 4). We are currently generating deletion derivatives of both the P-GUS and T-GUS chimeric genes to identify precisely the sequence elements responsible for the nodule-specific expression of these promoters in transgenic *Lotus* plants.

Both *Parasponia* and *Trema* are tropical or temperate trees and, hence, are phylogenetically distinct from the legumes. *Parasponia*, like the legumes and some other plant species scattered in families throughout the Angiospermae, can be nodulated by *Rhizobium* (or other symbiotic micro-organisms), but *Trema*, despite being in the same subfamily as *Parasponia*, does not form nodules either when inoculated in the wild or in the laboratory (Bogusz et al., 1988). Both species possess single hemoglobin genes that show considerable nucleic acid sequence and inferred amino acid sequence similarity (Bogusz et al., 1988) but that are very different from the hemoglobins found in legumes (only 40% identical at the amino acid level and very little at the nucleic acid level).

Expression of the two nonlegume hemoglobin proteins and their mRNAs has so far been detected at low levels in the roots of *Parasponia* and *Trema* plants (Bogusz et al., 1988; Landsmann et al., 1988) but not in their leaves, demonstrating organ-specific control in expression and suggesting that they may have some function independent of nodulation. When *Parasponia* roots are nodulated, the level of hemoglobin gene expression in the mature nodules is higher than root expression by several orders of magnitude (Landsmann et al., 1986). It has now been shown that the *Trema* hemoglobin gene has an expression potential similar to the *Parasponia* gene because it, too, can direct nodule expression when introduced into transgenic *Lotus* plants. The 10-fold lower level of nodule expression is probably a true difference in relative promoter strength but may also be due to the absence of upstream promoter elements because the *Trema* promoter used is about 350 bp shorter than the *Parasponia* promoter. How is it, then, that this gene from a species that never forms nodules has the capacity to be expressed at elevated levels in nodules? The answer presumably has implications for the

Figure 2. (continued).

Various root and nodule tissues from transgenic *L. corniculatus* or *N. tabacum* lines were stained for GUS enzyme activity as described in Methods and photographed by bright-field microscopy. The blue staining of otherwise cleared tissues identifies cells or tissues actively expressing the chimeric GUS genes.

(A) P-GUS construct. Transgenic tobacco root showing staining in the meristematic region and vascular bundle.

(B) T-GUS construct. Transgenic tobacco root showing staining in the meristematic tissues.

(C) T-GUS construct. Transgenic *Lotus* root and mature pink nodule showing staining throughout the central core of nodule tissue and in the vascular bundle of the root.

(D) P-GUS construct. Transgenic *Lotus* root and mature nodule showing staining in central core and nodule vascular tissue.

(E) T-GUS construct. Newly emerged *Lotus* root showing staining at the tip but restricted to the vascular bundle.

(F) T-GUS construct. Transgenic *Lotus* root and adjacent nodules showing the developmental increase in staining between a small white nodule and a larger intermediate pink nodule.

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Trema:AAAGTTTACTAAAGATTAAATCTTACTAAAAAGAAAACGAAAAAAA
      AAAAAAACCCAAGGAGATGGCTCTCCAGTACCCTGAAGAGTTACATCTTATGCTTATCCCCATTTTTTCCTATATATATACAG

Para: TAAAAAACCCAAAGATATGGCTCCCCAATACCCTGAAGAGTTACACACGATCCCCATTTTTTCTACTATATATACA

lbc3: AAGTTTTTGAAAAGATGATTGTCTCTTCACCATACCAATTGATCACCCTCCTCCAACAAGCCAAGAGAGACATAAG
           "ORGAN-SPECIFIC BOX"
                                   TTTTATTAGTTATTCTGATCACTCTTCAAGCCTTCTATATAAATAAG
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Figure 4. Conservation of Nodule-Specific Sequence Motifs between Legume and Nonlegume Hemoglobin Promoters.

The sequences at the 5′ end of the hemoglobin genes from *Trema* (Trema) and *Parasponia* (Para) are shown along with the upstream sequences from the legume hemoglobin *lbc$_3$* gene (lbc3). The sequences are aligned at the AAAGAT motif of the "organ-specific box" of the *lbc$_3$* gene (boxed) as identified by Stougaard et al. (1987b). Sequence motifs homologous to the soybean organ-specific sequences are in boldface type. The TATA boxes of all three promoters are underlined.

evolutionary origin of nodulation ability and nodule-specific gene expression among the Angiosperms.

P. andersonii and *T. tomentosa* are members of the widespread and taxonomically diverse (over 280 species) subfamily Celtidoideae of the family Ulmaceae (Hutchinson, 1967), which contains 12 genera. The ability to nodulate is restricted to the genus *Parasponia* (seven species) and is not seen in any of the other genera, including members of the morphologically similar genus *Trema* (55 species). Taxonomic evidence suggests, therefore, that a species ancestral to *Parasponia* gained the ability to nodulate independently of the legumes and subsequently was responsible for this property in the seven extant *Parasponia* species. The precise regulation of the temporal and spatial expression of the *Parasponia* hemoglobin promoter in the transgenic legume suggests that the legumes and nonlegumes both use the same regulatory mechanisms to control gene expression in the nodules and that this is reflected in the similarity of the DNA sequence motifs in the promoters of the hemoglobin genes. The expression of the *Trema* hemoglobin promoter gives some clue as to how these widely separated species arrived at the same regulatory process.

Our findings that both *Parasponia* and *Trema* hemoglobins are expressed in non-nodulated roots (Bogusz et al., 1988) originally suggested that hemoglobin may have a role in root metabolism that existed prior to its known oxygen transport role in symbiotic root nodules. The fact that the *Trema* promoter, normally expressed only in roots, can behave in a manner qualitatively similar to that of a nodule-expressed hemoglobin promoter in transgenic *Lotus* indicates that the DNA signals controlling nodule-enhanced expression must already be present in the root-expressed hemoglobin genes and, therefore, that a nodule-specific gene expression system may have evolved from a pre-existing root-specific expression system. The first primitive root nodules presumably contained the same rapidly dividing, highly active meristematic root tissues, possibly lateral root meristems, as those in the tobacco root tips where P-GUS and T-GUS are both highly expressed. Subsequent selection pressure for a more precise interaction between the plant and the symbiotic microorganism would have refined and enhanced the nodule specificity of hemoglobin expression. This could have been through changes in either the *cis*-acting DNA elements that regulate root expression or the *trans*-acting transcription factors that interact with these elements. These changes could have controlled precisely the temporal and spatial distribution of gene expression within the nodule, but in the nodulating nonlegumes, the original root function would need to have been maintained because these species possess only one hemoglobin gene that presumably carries out both root- and nodule-specific functions. The relationship between the root-specific and nodule-specific DNA signals in the two nonlegume promoters will eventually be determined by a functional analysis of the promoters in transgenic *Lotus* and tobacco.

The legume-*Rhizobium* symbiosis may also have started with a single root-expressed hemoglobin gene like that in *Trema* and *Parasponia*. During the acquisition of nodulating ability, this hemoglobin gene could have been duplicated multiply (Brisson and Verma, 1982), allowing effective selection in some genes for high levels of purely nodule-specific expression without the necessity for preserving the root function in the same gene. The legume root-expressed and nodule-expressed genes may have diverged sufficiently so that they no longer cross-hybridized. As yet a root-expressed hemoglobin has not been identified in the legumes.

We previously suggested two possible functions for the root-expressed hemoglobins of nonlegumes (Appleby et al., 1988) as either sensors of oxygen tension in the root or oxygen transporters to actively dividing cells. Assuming that the nonlegume hemoglobin promoters behave in the same way in their native environment as they do in the nonlegume tobacco, the histological evidence presented here suggests that hemoglobin is not expressed throughout the root in *Parasponia* and *Trema* but is concentrated in a small area near the tip. We previously calculated (Appleby et al., 1988) the concentration of hemoglobin in *Parasponia* roots to be approximately 100 nM if averaged over the whole root volume, i.e., a value too low to function as an oxygen carrier. It now seems possible that the oxygen carrier function may be feasible because if the hemoglobin in the root were mainly at the tip, it would have a local concentration 100-fold or more greater than originally deduced. This could be sufficient for hemoglobin to function in the facilitation of diffusion of O_2 to the rapidly respiring cells in the vicinity of the root tip and in certain cells of the vascular bundle, provided that an oxygen concentration gradient sufficient for the oxygenation/deox-

ygenation of hemoglobin was established and maintained within the root tip. Further physiological work will be required to test this possibility.

METHODS

Plasmids and Constructions

The promoter fragments of *Parasponia* and *Trema* hemoglobin genes were obtained as follows. The *Parasponia* HpaI/EcoRI fragment from pL305 (Landsmann et al., 1986) was cloned in M13mp18 digested with SmaI and EcoRI. The *Trema* AccI/HindIII promoter fragment from pT16 (Bogusz et al., 1988) was filled in with Klenow fragment of DNA polymerase and cloned in M13mp18 digested with SmaI. A synthetic oligonucleotide 5′CTCTAGAGGATCCCTTCTGTGGGTTGAGG-3′ was used to generate a deletion terminating 3 bp upstream of the initiation codon (Eghtedarzadeh and Henikoff, 1986) for both the *Trema* and *Parasponia* clones in mp18.

The hemoglobin-GUS constructs are shown schematically in Figure 5 and were constructed as described below. The EcoRI/BamHI promoter fragment of the *Trema* plasmid was then cloned into EcoRI/BamHI-digested pGN100, a promoter expression cassette derived from pIBI21 (Jefferson et al., 1987) that carries the GUS reporter gene (*Esherichia coli uidA* gene) and the nopaline synthase (*nos*) termination and polyadenylation signals (J. Walker, unpublished data) (Figure 5). The BamHI promoter fragment of the *Parasponia* plasmid was cloned in pGN100 digested with BamHI (Figure 5). Finally, the pGN100 containing the *Parasponia* or the *Trema* promoter fragments were linearized with HindIII and ligated with the binary plant transformation vector pGA470 (An et al., 1985) previously digested with HindIII (Figure 5).

The soybean hemoglobin-GUS chimeric gene plasmid *lbc*$_3$-GUS-*NOS* (Figure 5) was kindly provided by Dr. Jens Stougaard. The 35S-GUS construct (not shown) containing the promoter of the 35S transcript of cauliflower mosaic virus is similar to that described in Jefferson et al. (1987) and was constructed by inserting the 430-bp BamHI 35S promoter fragment from p35SCAT (Walker et al., 1987) into pGN100. These last two plasmids were linearized with HindIII and inserted into the HindIII site of pGA470 (Figure 5).

Binary vectors were transferred into *Agrobacterium tumefaciens* strain LbA4404 (Hoekema et al., 1983) and *A. rhizogenes* strain A4 (White and Nester, 1980) by triparental mating as described in Ellis et al. (1987).

Plant Transformation

Leaf discs of *Nicotiana tabacum* (cv Wisconsin 38) were transformed with engineered *A. tumefaciens* cells using the leaf disc procedure as described in Ellis et al. (1987).

Chimeric *Lotus corniculatus* plants with normal, nontransgenic shoots but transformed hairy root systems were generated with engineered *A. rhizogenes* strains essentially by the procedures of Stougaard et al. (1987a) and Petit et al. (1987) and inoculated with *Rhizobium loti* (Stougaard et al., 1987a; Petit et al., 1987). After 2 weeks to 4 weeks plants were sacrificed and individual root systems dissected away. A single nodule from each root system was assayed for GUS activity by the fluorometric method to identify the transgenic roots and nodules, which were then analyzed further. Nontransformed roots often developed after the *Lotus* plants were transferred into the glasshouse, necessitating this preliminary screening.

Electroporation

N. plumbaginifolia protoplasts were prepared from suspension cultures and electroporated as described in Llewellyn et al. (1987).

GUS Assays

Histological Detection

Histological assays for GUS expression at the tissue level were done on non-nodulated roots or nodulated roots that had been shown to be transformed by the fluorometric assay, essentially as described by Finnegan et al. (1989). Whole organs were vacuum infiltrated in ice-cold 1% glutaraldehyde in 10 mM sodium phosphate, pH 7.1, and fixed on ice for 30 min to 60 min. After five changes in the same buffer, they were covered with substrate

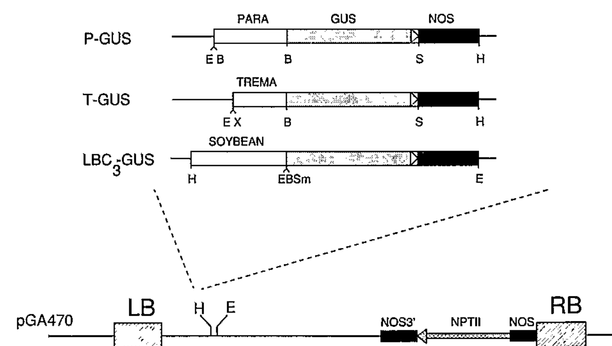

Figure 5. Chimeric Gene Fusions Used To Analyze Hemoglobin Gene Regulation in Transgenic Plants.

Gene fusions between the promoter segments of the *Parasponia* and *Trema* hemoglobin promoters and the β-glucuronidase reporter gene were constructed as described in Methods. P-GUS and T-GUS are transcriptional fusions incorporating 1 kb and 0.6 kb of upstream sequences of the *Parasponia* and *Trema* genes, respectively. The polyadenylation signals provided by a segment of the T-DNA encoded nopaline synthase gene (*NOS*) from *A. tumefaciens*. *lbc*$_3$-GUS is a transcriptional fusion between the 2-kb upstream sequences of the soybean *lbc*$_3$ leghemoglobin gene and the β-glucuronidase reporter cassette. All three constructs were cointegrated into the binary transformation vector pGA470 at their respective HindIII sites in such a way that they were between the left and right borders (LB and RB) of the T-DNA and adjacent to a selectable kanamycin resistance gene. Abbreviations are B, BamHI; E, EcoRI; H, HindIII; S, SacI; Sm, SmaI; X, XbaI; NPTII, neomycin phosphotransferase. Linear maps are not drawn to scale.

(10 mM sodium phosphate, pH 7.0, 1 mM X-glucuronide, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide) and incubated at 37°C for 12 hr to 24 hr in the dark. Roots were incubated whole or cut into 1 cm lengths, and larger (>3 mm diameter) nodules were bisected to facilitate entry of the substrate. After a brief buffer wash, tissues were dehydrated through a graded ethanol series to remove pigments and enhance the contrast of the blue reaction product.

For photography, cleared but blue tissues were either rehydrated or transferred to glycerol or immersion oil and recorded using either bright- or dark-field optics.

Fluorometric Assays

Quantitative determinations of GUS activity in various organs of transgenic plants were performed essentially as described by Jefferson et al. (1987) using 4-methylumbelliferyl glucuronide as the substrate. 4-Methylumbelliferone production was measured fluorometrically using a Hoeffer Mini Fluorimeter. Specific activities are expressed as picomoles of 4-methylumbelliferone produced per minute per microgram of total soluble protein. Protein levels in extracts were determined by the dye-binding assay of Spector (1978) using BSA as the standard.

Plant DNA Isolation and DNA Gel Blot Analysis

DNA was isolated from transgenic tobacco plants using the method of Taylor and Powell (1982) and analyzed for the presence of a complete soybean *lbc*$_3$-GUS-*NOS* chimeric gene by DNA gel blot hybridization as described in Dennis et al. (1984).

ACKNOWLEDGMENTS

We thank Jenny Thistleton and Celia Miller for their expert technical assistance and Kjeld Marker, Jens Stougaard, and Tove Christiensen for supplying the *lbc*$_3$-GUS construction, *Lotus* seeds, and helpful advice and protocols prior to publication. We also thank Jacques Tempe for useful discussions on *Lotus* transformation.

Received March 1, 1990; revised May 9, 1990.

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