Soybean (lbc3), Parasponia, and Trema Hemoglobin Gene Promoters Retain Symbiotic and Nonsymbiotic Specificity in Transgenic Casuarinaceae: Implications for Hemoglobin Gene Evolution and Root Nodule Symbioses

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The purpose of this study was to compare the control of expression of legume and nonlegume hemoglobin genes. We used the Casuarina glauca and Allocasuarina verticillata transformation system to examine the properties of the soybean (lbc3), Parasponia andersonii, and Trema tomentosa hemoglobin gene promoters in actinorhizal plants. Expression of the hemoglobin promoters gus genes was examined by fluorometric and histochemical assays. The fluorometric assays in various organs showed that the soybean and P. andersonii promoters were most active in nodules whereas the T. tomentosa promoter gave a very high activity in roots. The histochemical study showed that Gus activity directed by the soybean and the P. andersonii gus chimeric genes appeared mainly confined to the infected cells of the C. glauca and A. verticillata nodules. The T. tomentosa hemoglobin promoter was primarily expressed in the root’s cortex and vascular tissue. The results indicate that the soybean, P. andersonii, and T. tomentosa hemoglobin promoters retain their cell-specific expression in transgenic members of the Casuarinaceae, suggesting a close relationship between legume, Ulmaceae member, and actinorhizal hemoglobin genes. The conservation of the mechanism for nodule-specific expression of soybean, P. andersonii, and C. glauca and A. verticillata hemoglobin genes is discussed in view of recent molecular phylogenetic data that suggest a single origin for the predisposition to form root nodule symbioses.

Additional keywords: actinorhiza, Frankia, nitrogen fixation.

Plant hemoglobins were first identified and characterized in the nitrogen-fixing root nodules that develop through the symbiotic association of Rhizobia spp. bacteria with leguminous plants. More recently, hemoglobins have also been found in nodules of nonlegumes, such as Parasponia andersonii nodulated by Bradyrhizobium spp. (Appleby et al. 1983; Landsmann et al. 1986) and Casuarina glauca, an actinorhizal plant that undergoes symbiosis with the actinomycete Frankia (Fleming et al. 1987; Jacobsen-Lyon et al. 1995). The role of hemoglobin in symbiosis is to supply oxygen to the bacterial respiration chain while preserving the activity of the oxygen-intolerant nitrogenase enzyme complex (Appleby 1984). Besides the symbiotic hemoglobin genes that are expressed specifically in nitrogen-fixing nodules, a second type of hemoglobin gene has been isolated in nodulating and non-nodulating plants. A nonsymbiotic hemoglobin gene was first recorded in Trema tomentosa, a non-nodulating relative of P. andersonii (Bogusz et al. 1988). Since then, nonsymbiotic hemoglobin genes have been identified in cereals such as barley, wheat, maize, and rice (Taylor et al. 1994) and in Arabidopsis thaliana (Trevaskis et al. 1997). These genes are expressed in different tissues, such as roots, stems, and seeds. The presence of nonsymbiotic hemoglobin genes in symbiotic plants has also been reported in soybean (Andersson et al. 1996) and in the actinorhizal tree C. glauca (Christensen et al. 1991; Jacobsen-Lyon et al. 1995). In contrast to their symbiotic counterparts, the nonsymbiotic hemoglobin genes of soybean and C. glauca were expressed in various nonsymbiotic tissues and the level of expression in nodules was very low, compared with that of hemoglobin symbiotic genes (Andersson et al. 1996; Jacobsen-Lyon et al. 1995). P. andersonii possesses a single hemoglobin gene that shows expression in both nodules and non-nodulated roots, suggesting symbiotic and nonsymbiotic roles for P. andersonii hemoglobin (Landsmann et al. 1986, 1988; Bogusz et al. 1988). The function of these nonsymbiotic hemoglobins is still unknown. It has been suggested that hemoglobin could act as a sensor of oxygen tension or as an oxygen carrier (Appleby et al. 1988).

The regulation of both symbiotic and nonsymbiotic hemoglobin gene expression has been studied with transgenic legumes. An examination of the expression of the chimeric hemoglobin promoter-reporter gene has shown that organ-specific expression is mediated by cis-acting elements (Stougaard et al. 1987, 1990; Szabados et al. 1990; Bogusz et al. 1990; Jacobsen-Lyon et al. 1995; Andersson et al. 1997). Furthermore, the study of the expression of various hemoglobin promoters in transgenic legume has contributed to better understanding of the origin of hemoglobin genes in plants (Appleby et al. 1988; Appleby 1992).
In our laboratory, we recently developed genetic transformation techniques for two actinorhizal trees in the Casuarinaeae family, C. glauca (Diouf et al. 1995) and Allocasuarina verticillata (Franche et al. 1997). To further investigate the evolution of plant hemoglobins, we introduced into C. glauca and A. verticillata chimeric genes consisting of the promoter region from the soybean e3 (lbc3), the P. andersonii, and the T. tomentosa hemoglobin genes linked to the coding region of the reporter gene gus (uidA) encoding β-glucuronidase (GUS).

We found that in transgenic nodules of C. glauca and A. verticillata, the soybean and P. andersonii hemoglobin promoters direct expression of the gus gene in Frankia-infected cells, thus indicating a conservation of cell-specific expression in the actinorhizal plants. We have also shown that the expression of the T. tomentosa hemoglobin gene promoter in C. glauca and A. verticillata nonsymbiotic tissue is essentially root specific. The different patterns of expression were compared with those of the endogenous soybean, P. andersonii, and T. tomentosa hemoglobin genes, and with the expression previously reported in the heterologous transgenic legume, Lotus corniculatus.

RESULTS

Quantitative GUS activity in roots, nodules, and shoots (stem/leaves) of transgenic A. verticillata.

GUS activity controlled by the hemoglobin promoters was determined in extracts of shoots (stems/leaves), roots, and nodules of transgenic A. verticillata. Since C. glauca and A. verticillata are characterized by distinctive foliage consisting of deciduous, long, needlelike, articulate branchlets with reduced, scalelike leaves organized in whorls, the GUS activity measured in shoots represents the activity in stems and leaves. These experiments were carried out on two to five plants regenerated from each of 20 independently transformed calli. Table 1 summarizes the results of GUS activity in transgenic A. verticillata plants. The variability observed between independent transformants can be explained by differences in transgene copy number, methylation, and/or integration site (for review see Finnegan and McElroy 1996). As expected, the control plants carrying cauliflower mosaic virus (CaMV) e3SS-gus showed GUS activity throughout the transgenic A. verticillata plants (Franche et al. 1997). In untransformed nodules, the GUS values were lower than in shoots, suggesting that the high content of phenolic compounds in A. verticillata nodules could reduce endogenous GUS activity (Serres et al. 1997).

The soybean lbc3-gus construct was not significantly expressed in the aerial part of the plants or in the non-nodulated roots. Although low GUS activity was observed in transformed lbc3-gus nodules, the values obtained were up to 100 times higher than those observed in nontransformed nodules. A similar pattern of GUS activity was observed in L. corniculatus transformed with the same lbc3-gus construct (Lauridsen et al. 1993).

The gus expression produced by the P. andersonii hemoglobin promoter in shoots and roots was slightly above the background of untransformed extracts. High reporter GUS activity was observed in nodules; the level of expression was about 10 times higher than the level detected with the lbc3-gus construct (Table 1).

The T. tomentosa gus construct gave a low level of GUS activity in shoots, whereas a high expression was observed in roots. In nodules, the measured reporter gene activity was up to 30 times higher than the level detected in nontransformed nodules (Table 1).

Expression of the soybean (lbc3) and P. andersonii hemoglobin promoters in transgenic C. glauca and A. verticillata nodules.

The GUS activity was further studied by examining sections of C. glauca and A. verticillata nodules. Similar results were obtained in the two systems.

In any given lobe of C. glauca or A. verticillata, Frankia is restricted to the cortical cells. The way Frankia invades cortical parenchyma cells growing acropetally toward the nodule lobe meristem creates a developmental gradient. Different zones have been characterized by both morphological and gene expression studies (Angulo Carmona 1974; Ribeiro et al. 1995; Gherbi et al. 1997), e.g., the apical meristematic zone at the apex (zone I), the infection zone (zone II) where Frankia infects some of the new cells derived from the meristem, the fixation zone (zone III) where active nitrogen fixation takes place, and the senescence zone (zone IV).

As shown in Figure 1A, the longitudinal section of mature A. verticillata nodules revealed that the lbc3-gus gene was active throughout the cortical cells that correspond to the Frankia-containing cells. Expression was at its highest in the early fixation zone III immediately adjacent to infection zone II. Although the C. glauca and A. verticillata nodule lobe has an indeterminate growth pattern, like indeterminate type legume nodules, the so-called interzone II-III (Vasse et al. 1990) between zone II and III has not been found. Toward the senescence zone IV, lbc3-gus expression progressively decreased. No activity was seen in the periderm, vascular bundle, or meristematic zone (Fig. 1A). The cell-specific expression of the lbc3-gus construct was studied in thin, transversal sections of transgenic nodules (Fig. 1B). GUS activity was localized in large Frankia-infected cells. No activity was seen in the uninfected cells, periderm, or vascular parenchyma (Fig. 1B). When transgenic lbc3 roots were stained for GUS activity, expression was not detected in lateral or primary roots (Fig. 2A) or in aerial parts (not shown). We concluded that, in mature C. glauca and A. verticillata nodules, the lbc3-gus con-

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Table 1. β-Glucuronidase (GUS) activity in different organs of transgenic Allocasuarina verticillata

<table>
<thead>
<tr>
<th>Construct</th>
<th>Shoot</th>
<th>Root</th>
<th>Nodule</th>
</tr>
</thead>
<tbody>
<tr>
<td>lbc3-gus</td>
<td>0.2 ± 0.3</td>
<td>0.43 ± 0.5</td>
<td>1.08 ± 0.7</td>
</tr>
<tr>
<td>P-gus</td>
<td>1.3 ± 0.3</td>
<td>0.72 ± 0.2</td>
<td>12.7 ± 0.2</td>
</tr>
<tr>
<td>T-gus</td>
<td>0.97 ± 0.4</td>
<td>456.5 ± 25</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>e3SS-gus</td>
<td>214.7 ± 29</td>
<td>293.8 ± 52</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Untransformed</td>
<td>0.26 ± 0.2</td>
<td>0.15 ± 0.07</td>
<td>0.01 ± 0.00</td>
</tr>
</tbody>
</table>

a GUS-specific activity was determined by fluorometric assays with tissue homogenates from at least 20 independent transgenic plants. Activity is expressed as μmol of MU (4-methyl umbelliferone) per min per mg of protein.

b Lbc3, soybean hemoglobin gene promoter; P, Pararosa anderssonii hemoglobin gene promoter; T, Trema tomentosa hemoglobin gene promoter; e3SS, cauliflower mosaic virus (CaMV) promoter containing a duplication of the B domain of the 3SS promoter.

c Shoot tissue consists of reduced leaves around the stem segment.
struct was expressed exclusively in Frankia-infected cells. This is similar to the localization of the endogenous C. glauca symbiotic hemoglobin mRNA (Gherbi et al. 1997) and protein (Goodchild and Miller 1997).

In a mature A. verticillata nodule lobe, the chimeric P. andersonii gene was highly active in the cortical, Frankia-infected part (Fig. 1C), and, occasionally, weaker GUS staining was observed in the vascular bundle (not shown). Analyses at high magnifications under the more sensitive dark-field conditions showed that expression was most prominent in mature Frankia-infected cells (Fig. 1D). GUS activity was also detected in Frankia-noninfected cells of the fixation zone and in the vascular bundle (Fig. 1D).

Expression of the T. tomentosa hemoglobin promoter in transgenic C. glauca and A. verticillata nodules.

As already mentioned for the lbc3 and P. andersonii-gus constructs, no difference in expression was observed between nodules developed on transgenic roots of C. glauca and A. verticillata. The T. tomentosa-gus expression was most frequently seen in the vascular bundle zone of the nodule lobe (Fig. 1F). A transverse section of the transgenic nodule lobe revealed a blue-stained vascular cylinder (not shown), and the central metaxylem appeared to be free of any blue precipitate (not shown). GUS activity was occasionally seen in a few Frankia-infected cells in the fixation zone without any expression in the vascular tissue of the nodule lobe (Fig. 1E).

Expression of the P. andersonii and T. tomentosa promoters in transgenic C. glauca and A. verticillata nonsymbiotic tissues.

The distribution of gus expression was determined histochemically in transgenic, non-nodulated C. glauca (not shown) and A. verticillata roots (Fig. 2), and in A. verticillata transgenic shoots (not shown). The gus expression observed in roots was comparable in the two transgenic plants.

The P. andersonii-gus construct was expressed in root caps of the primary roots (Fig. 2C); staining was not seen in other parts of the primary root nor in lateral roots (Fig. 2B). A histochemical analysis of thin, longitudinal sections of the primary root tip showed staining in the outer part of the root cap (Fig. 2D). In P. andersonii, the endogenous hemoglobin gene is expressed at low level in roots (Bogusz et al. 1988; Landsmann et al. 1988).

The T. tomentosa-gus plants showed high levels of GUS activity in the primary roots (Fig. 2E). Staining was observed in the primary root tips but was not detected in the elongation region (Fig. 2F); newly emerging lateral roots expressed GUS activity in the region near the parent primary root (Fig. 2E). No staining was detected in the tip and the elongation region of the lateral roots containing the T. tomentosa-gus construct (Fig. 2E). The expression of the T. tomentosa hemoglobin gene was also observed in T. tomentosa roots (Bogusz et al. 1988). No staining was detected in transgenic A. verticillata shoots containing the soybean lbc3-gus construct (not shown).

Very diffuse staining was detected occasionally in leaves with the T. tomentosa-gus and the P. andersonii-gus construct that was located at the intermods (not shown). In T. tomentosa and P. andersonii, hemoglobin transcripts were not detected by Northern (RNA) blot analysis in leaves (Landsmann et al. 1988; Bogusz et al. 1988).

**DISCUSSION**

The soybean lbc3 5'-upstream region has been extensively studied (Stougaard et al. 1987, 1990). A deletion analysis of the lbc3 promoter identified cis-acting regulatory elements (OSE: organ-specific element) that determine high-level, nodule-specific expression. It has been suggested that two motifs, AAAGAT and CTCTTC, that are present in the OSE of all leg-hemoglobin genes and in the promoters of other legume nodule-specific genes, could constitute the regulatory sequences required for the nodule-specific expression (Sandal et al. 1987; Stougaard et al. 1987). In transgenic L. corniculatus, correct developmental regulation of the lbc3 promoter is maintained, suggesting the conservation of regulatory signals between different legume species. Also, Jacobsen-Lyon et al. (1995) have shown that the two motifs were also present in the promoter of the nonlegume C. glauca symbiotic hemoglobin gene, and that these elements were responsible for the nodule-specific expression in transgenic L. corniculatus plants. Our data indicate that the lbc3 promoter retains its cell-specific expression in transgenic actinorhizas. Altogether, heterologous expression analysis of the lbc3-gus construct indicated a conservation of the mechanisms that determine high levels of endophyte-infected cell expression in mature legume and actinorhizal nodules.

The pattern of expression of the P. andersonii-gus chimeric gene in transgenic C. glauca and A. verticillata nodules is comparable to that of the single P. andersonii hemoglobin gene in the P. andersonii nodule, which is expressed in both Rhizobium-infected cells and in uninfected cells (Trinick et al. 1989). Thus, it appears that P. andersonii, the only nonlegume nodulated by a Rhizobium strain, and actinorhizal plants have similar regulatory mechanisms that control cell-specific expression in nodules. Surprisingly, Anderson et al. (1997) found that the P. andersonii hemoglobin promoter directed the expression of the gus gene at a high level in uninfected cells of mature transgenic L. corniculatus nodules, whereas a low level of GUS staining was detected in Rhizobium-infected cells. This indicates that, unlike the lbc3 promoter, the P. andersonii hemoglobin promoter does not retain its cell-specific expression in transgenic legume nodules. It is interesting to note that both P. andersonii and C. glauca nodules are developmentally and structurally similar to lateral roots (Trinick 1979). Whereas legume nodules arise from the root cortex and exhibit peripheral vascular bundles, nonlegume nodules originate from pericycle cells, and their final structure resembles lateral roots with a single central vascular bundle. Furthermore, molecular phylogeny suggests that all root-nodule, nitrogen-fixing symbioses were contained in the same clade (Solís et al. 1995). In this analysis, legumes are grouped in a unique subclade, while representatives of the Ulmaceae family (P. andersonii/Rhizobium symbiosis) are found in another subclade together with intercellularly infected actinorhizal plants; the Casuarinaceae family belongs to a third subclade with intracellularly infected actinorhizal plants. Therefore, molecular phylogenetic data indicate that, although P. andersonii and legumes share the same symbiont, P. andersonii is not closer to legumes than to C. glauca and A. verticillata. Thus, it might be possible that legume and nonlegume symbioses have diverged to the point that nodule transcription factors of L. corniculatus do not recognize nodule-specific

DNA motifs of *P. andersonii* symbiotic genes. However, as reported previously, Jacobsen-Lyon et al. (1995) found that the symbiotic *casbh-sym*1 *C. glauca* hemoglobin promoter retained its symbiont-infected, cell-specific expression in transgenic legumes. The conservation of the cell-specific expression of the *P. andersonii* hemoglobin promoter in *C. glauca* and *A. verticillata* nodules, the absence of cell-specific expression of the same promoter in *L. corniculatus*, together with the correct pattern of expression of the *casbh-sym*1 and *lbc3* promoters in transgenic *L. corniculatus*, suggest that the symbiotic function of the *P. andersonii* hemoglobin gene is more closely related to *C. glauca* and *A. verticillata* symbiotic genes than to the legume's. The behavior of the *P. andersonii* hemoglobin promoter in transgenic *C. glauca* and *A. verticillata* and *L. corniculatus* nodules could also be correlated to the unique feature of this hemoglobin gene with both a symbiotic and a nonsymbiotic function.

We found that the *T. tomentosa-gus* construct was expressed in transgenic *C. glauca* and *A. verticillata* nodules and that the cell-type specificity varied among individual transgenic nodules. Two different cell-type expressions were observed. Indeed, in most transgenic nodules, GUS staining was specifically seen in cells of the vascular bundle (Fig. 1F); in a few nodules there was GUS staining of the Frankia-infected cells (Fig. 1E). This suggests that, although some symbiotic expression was retained in *C. glauca* and *A. verticillata* nodules, the symbiotic regulatory sequences within the *T. tomentosa* promoter were imperfectly recognized by the transcription factors of the *C. glauca* and *A. verticillata* nodules that interact with the cis regulatory elements. Altogether, the sequence data analysis (Bogusz et al. 1988), our results, and previous reports from both Bogusz et al. (1990) and Andersson et al. (1997) suggest that the *T. tomentosa* hemoglobin promoter possesses all the basic elements for symbiotic regulation but that evolutionary change has not led to a precise control of cell expression within the nodule. As suggested by Andersson et al. (1997), it is possible that *T. tomentosa* might have been a nodulating species and lost its ability to form nodules.

The pattern of expression of the *P. andersonii-gus* chimeric gene in transgenic *C. glauca* and *A. verticillata* roots differs from that reported in transgenic *L. corniculatus* roots. Bogusz et al. (1990) detected a low level of staining in the vascular bundle whereas Andersson et al. (1997) did not observe any GUS activity. The root cap consists of living parenchyma cells derived from the apical meristems. These cells secrete a mucilage that coats root tips growing in the soil (Rougier 1981).

It has been suggested that the role of the *P. andersonii* hemoglobin in roots could be to facilitate oxygen diffusion in rapidly respiring cells (Appleby et al. 1988); thus, the expression of the *P. andersonii-gus* construct may be correlated with the high metabolic activity of the root cap cells.

In transgenic *L. corniculatus* roots, Andersson et al. (1997) reported that the expression of the *T. tomentosa-gus* construct was occasionally observed in the root caps or root meristem initial cells.

As already suggested above for the *P. andersonii* promoter, the *T. tomentosa* hemoglobin promoter seems to be more accurately recognized by *C. glauca* and *A. verticillata* than by legume transcription factors. Since *T. tomentosa* is a close relative of *P. andersonii*, this finding is in agreement with our previous suggestion that the Ulmaceae hemoglobin genes are more closely related to *C. glauca* and *A. verticillata* genes than to leghemoglobin genes.

On the basis of their protein sequence analysis, Trevaskis et al. (1997) reported that two classes of hemoglobin are present in plants. The nonsymbiotic *C. glauca*, legume, and *P. andersonii* and *T. tomentosa* hemoglobins belong to class 1, whereas the symbiotic *C. glauca* and legume hemoglobins belong to class 2.

Our results showed that symbiotic and nonsymbiotic functions of the *P. andersonii* and *T. tomentosa* hemoglobin gene promoters were maintained in transgenic *C. glauca* and *A. verticillata* nodules, whereas Andersson et al. (1997) reported that the same promoters were not correctly recognized in transgenic *L. corniculatus*. This suggests that *P. andersonii* and *T. tomentosa* hemoglobin genes share common regulatory mechanisms with symbiotic and nonsymbiotic *C. glauca* and *A. verticillata* hemoglobin genes. Thus, even if *P. andersonii*, *T. tomentosa*, and symbiotic *Casuarina* hemoglobin belong to different classes, the corresponding genes have acquired common regulatory mechanisms through evolutionary convergence.

We have also shown that the symbiotic and nonsymbiotic functions of the *P. andersonii* and the *T. tomentosa* hemoglobin genes are more specifically recognized in transgenic *C. glauca* and *A. verticillata* than in legume, although both legume and *P. andersonii* are nodulated by *Rhizobium*. This suggests that symbiotic microorganisms are not involved in the nodule-infected cell expression of hemoglobin genes and that the specializations of plant and endophyte symbiotic genes have evolved independently. As mentioned previously, Andersson et al. (1997) have determined the root and the nodule-specific DNA signals of the *P. andersonii* and *T. tomentosa* hemoglobin promoters in transgenic *L. corniculatus*; it would be interesting to make the same functional analysis in transgenic *C. glauca* and *A. verticillata* in order to establish whether the same DNA regulatory elements are recognized in legume and actinorhizal plants.

An increased number of nodule-specific and enhanced genes are being isolated in legumes and actinorhizal plants (Mylonas et al. 1995; Mullin and Dobrissa 1996; Franche et al. 1998). The prospect of introducing the promoters of these genes into heterologous symbiotic systems opens new avenues of study on the evolution of nodulation and symbiotic genes.

**MATERIALS AND METHODS**

**Plant materials.**

*C. glauca* seeds were kindly provided by H. H. El Lakani from the Desert Developmental Center (Cairo) and *A. verticillata* seeds collected in Australia were obtained from the Versepuy Company (Le Puy-en-Velay, France). For stable transformation, *C. glauca* and *A. verticillata* were propagated in tissue culture under sterile conditions at 26°C with a 16-h photoperiod (Diouf et al. 1995; Franche et al. 1997). The prospect of introducing the promoters of these genes into heterologous symbiotic systems opens new avenues of study on the evolution of nodulation and symbiotic genes.

**LITERATURE CITED**


**Plant transformation and nodulation by Frankia.**

Composite *C. glauca* plants, consisting of transgenic roots on untransformed shoots, were generated with engineered *A. rhizogenes* as described previously (Diouf et al. 1995). Transgenic *A. verticillata* was recovered through the transformation of mature zygotic embryos by engineered *A. tumefaciens*, as described previously (Franche et al. 1997). Transformed plants were characterized for stable integration by Southern and polymerase chain reaction analyses (Franche et al. 1997). Transgenic *C. glauca* and *A. verticillata* were grown in glass tubes containing a nutrient solution (Hoagland) or were transferred to soil in a glass house. Transgenic *C. glauca* was inoculated by the *Frankia* strain TR1 (Girgis et al. 1990) and *A. verticillata* by Allo2 (Girgis and Schwencke 1993).

**Fluorometric assay for GUS activity.**

Total protein (5 to 10 µg) from shoots (stem/leaves), roots, and nodules of transgenic and nontransformed control plants was used in the assay. The kinetic analysis of the GUS activity was performed with 1 mM methyl umbelliferyl glucuronide (MUG) as a substrate, as described by Jefferson et al. (1987). GUS activities were expressed as µmol of 4-methyl umbelliférole (4-MU) produced per mg of protein. The average activity for each construct was determined following the analysis of an average of 20 independent transformed plants.

**Histochemical localization of GUS activity.**

GUS activity was detected in small fragments of root and nodule samples essentially as described by Jefferson et al. (1987). Nontransformed plants were used as negative controls. Nodules that exhibited four to six lobes were analyzed. Samples were sliced into 45-µm-thick sections (VT 1000E vibratome; Leica, Heerbrugg, Switzerland). Tissue sections were viewed by a Leitz DMRB light microscope with bright- and dark-field optics. Alternatively, stained samples were post-fixed for 24 h in ethanol, acetic acid, formaldehyde (17, 2, 1) then dehydrated through graded ethanol solutions and embedded in resin (Histo Technik 7160; Labonor, Villeneuve d’Arq, France). Two- to 3-µm-thick sections (Leica GRM 2055 microscope) were mounted on slides before observation by light microscopy.

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Vol. 11, No. 9, 1998 / 893


