

Soybean (*Ibc3*), *Parasponia*, and *Trema* Hemoglobin Gene Promoters Retain Symbiotic and Nonsymbiotic Specificity in Transgenic Casuarinaceae: Implications for Hemoglobin Gene Evolution and Root Nodule Symbioses

Claudine Franche, Diaga Diouf, Laurent Laplaze, Florence Auguy, Thierry Frutz, Maryannick Rio, Emile Duhoux, and Didier Bogusz

Physiologie Cellulaire et Moléculaire des Arbres (ORSTOM/GeneTrop), 911 avenue Agropolis, BP 5045, 34032 Montpellier cédex 1, France

Accepted 25 May 1998.

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 (*Ibc3*), *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 pre-disposition 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).

Corresponding author: D. Bogusz; E-mail: bogusz@mpl.orstom.fr

In our laboratory, we recently developed genetic transformation techniques for two actinorhizal trees in the Casuarinaceae 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 *c3* (*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) *e35S-gus* showed GUS activity throughout the transgenic *A. verticillata* plants (Franche et al. 1997). In untransformed

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

Construct ^b	Shoot ^c	Root	Nodule
<i>lbc3-gus</i>	0.2 \pm 0.3	0.43 \pm 0.5	1.08 \pm 0.7
<i>P-gus</i>	1.3 \pm 0.3	0.7 \pm 0.2	12.7 \pm 0.2
<i>T-gus</i>	0.97 \pm 0.4	456.5 \pm 35	0.3 \pm 0.3
<i>e35S-gus</i>	214.7 \pm 29	293.8 \pm 52	1.6 \pm 0.2
Untransformed	0.26 \pm 0.2	0.15 \pm 0.07	0.01 \pm 0.0

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

^b *lbc3*, soybean hemoglobin gene promoter; *P*, *Parasponia andersonii* hemoglobin gene promoter; *T*, *Trema tomentosa* hemoglobin gene promoter; *e35S*, cauliflower mosaic virus (CaMV) promoter containing a duplication of the B domain of the 35S promoter.

^c Shoot tissue consists of reduced leaves around the stem segment.

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-

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 internodes (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, AAGAT 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 actinorhiza. 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, Andersson 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 (Soltis 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

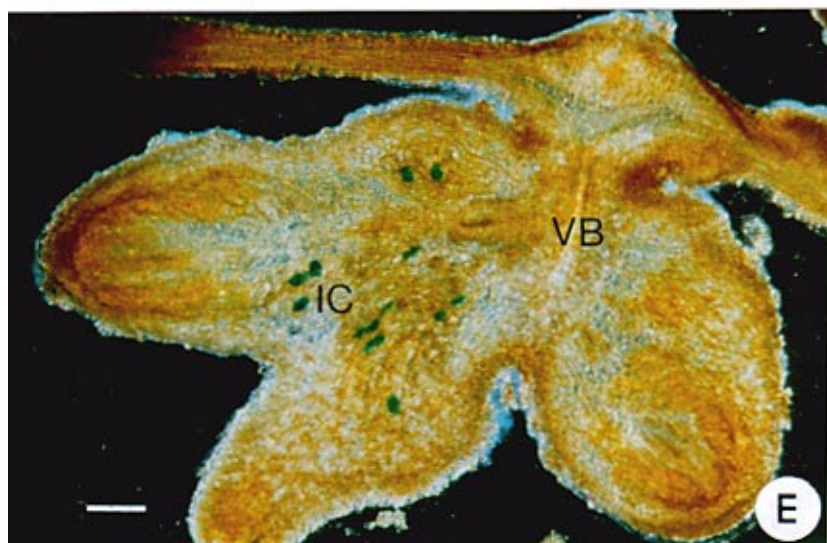
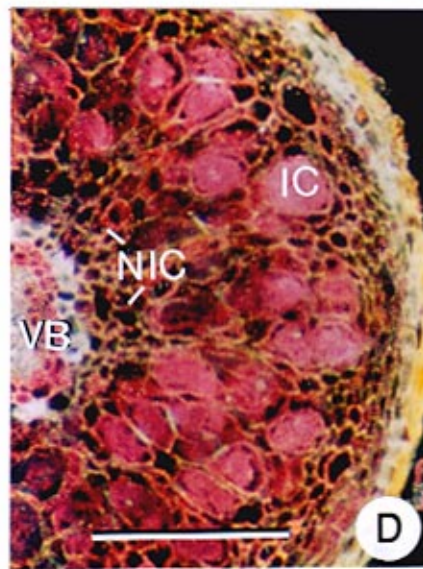
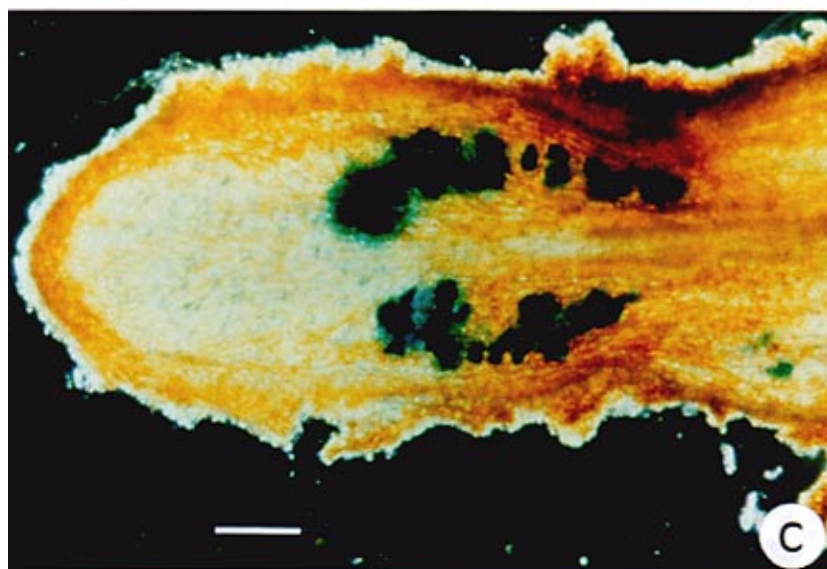
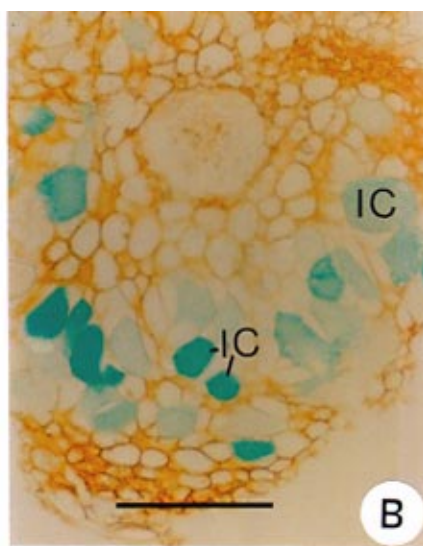
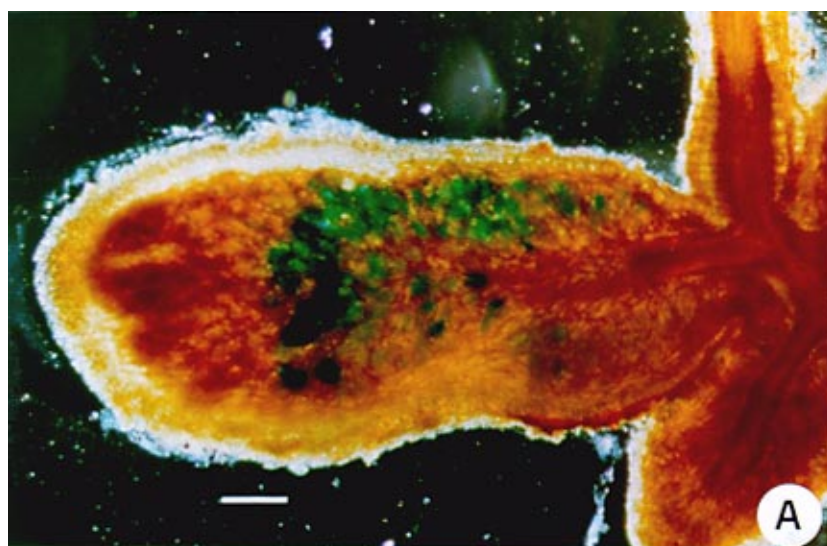




Fig. 1. A–F, Histochemical analysis of β -glucuronidase (GUS) activity of the chimeric plant hemoglobin *gus*-gene (A, B) *lbc3*, (C, D) *Parasponia andersonii*, and (E, F) *Trema tomentosa* of transgenic *Allocauarina verticillata* nodules. A, B, C, E, and F, Bright field micrographs. D, A dark-field micrograph in which GUS staining is visible as pink color. A, Longitudinal section (45 μ m thick) of mature nodule lobe. B, Detail of 3- μ m-thick cross section around vascular bundle. C, Longitudinal section (45 μ m thick) of mature nodule lobe. D, Detail of 3- μ m-thick cross section in region of *Frankia*-infected cells. E Longitudinal section (45 μ m thick) of three nodule lobes. F, Longitudinal hand-cut section of a nodule lobe. IC, *Frankia*-infected cells; NIC, noninfected cells; VB, vascular bundle. Bars = 200 μ m.

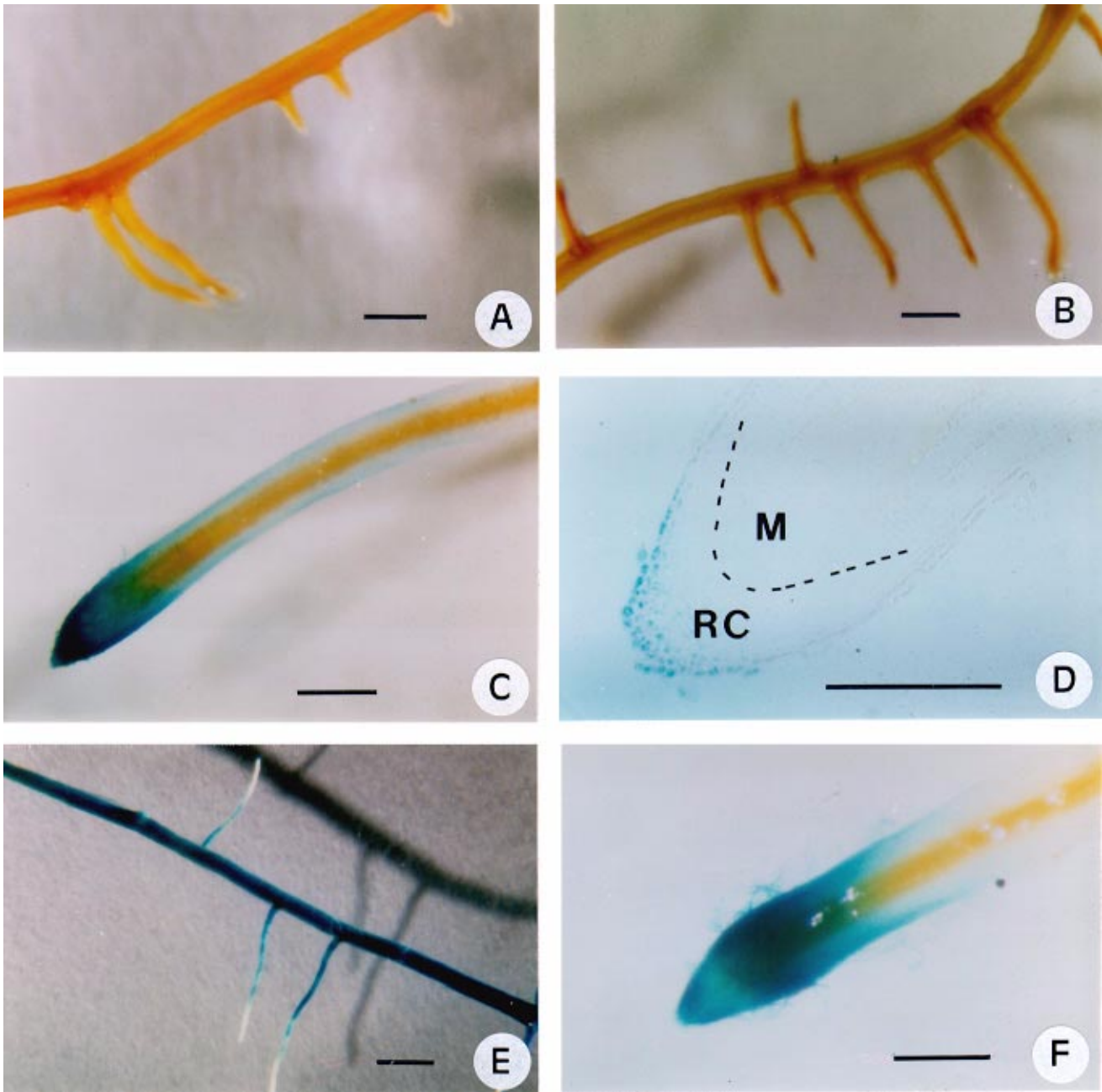


Fig. 2. A–F, Histochemical analysis of β -glucuronidase (GUS) activity of the chimeric plant hemoglobin *gus*-gene (A) *lbc3*, (B–D) *Parasponia andersonii*, and (E, F) *Trema tomentosa* of transgenic *Allocauarina verticillata* roots. A, B, Root fragments. C, Fragment of primary root tip section. D, Longitudinal section (3 μ m thick) of primary root tip. E, Root fragment showing intense GUS staining in primary root and proximal region of secondary root. F, Root tip fragment of primary root showing intense staining in meristematic region. M, meristem; RC, root cap. Bars = (A, B, C, F) 400 μ m, (D) 200 μ m, (E) 500 μ m.

DNA motifs of *P. andersonii* symbiotic genes. However, as reported previously, Jacobsen-Lyon et al. (1995) found that the symbiotic *cashb-sym* *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 *cashb-sym1* 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 (Rouhier 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 (Mylona et al. 1995; Mullin and Dobritsa 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).

Bacterial strains and plasmids.

The *Agrobacterium tumefaciens* strain C58C1(GV2260) (Vancanneyt et al. 1990) and *A. rhizogenes* A4RS (Jouanin et al. 1986) were used for transformation experiments. The soybean *lbc3-gus-NOS* chimeric gene plasmid, kindly provided

by K. Marker (Lauridsen et al. 1993), was digested with *Hind*III and inserted into the *Hind*III site of the binary vector pBin19 (Bevan 1984). The *P. andersonii* and *T. tomentosa* hemoglobin promoter *gus-NOS* constructs in the binary vector pGA470 were kindly provided by E. Dennis (Bogusz et al. 1990). CaMV 35S-*gus* gene fusion (pBI121) (Jefferson et al. 1987) was used as a control. Binary vectors were introduced in *A. tumefaciens* and *A. rhizogenes* by triparental mating (Koncz and Schell 1986) and individual colonies were tested by Southern analysis for the presence of the construct.

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 Thr (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 umbelliferone (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 7100; Labonord, Villeneuve d'Arcq, France). Two- to 3-µm-thick sections (Leica GRM 2055 microtome) were mounted on slides before observation by light microscopy.

ACKNOWLEDGMENTS

We would like to thank K. Pawlowski (Albrecht-V-Haller-Institut, Göttingen, Germany) for critical reading of the manuscript, S. Svistoonoff for help with cytological work, and L. Padiou for fluorometric analysis. The work was supported by ORSTOM (Institut Français pour le Développement en Coopération), by CIRAD (Centre de Coopération Internationale en Recherche Agronomique), and by European contract ERTBTS 3*-CT940978.

LITERATURE CITED

- Andersson, C. R., Jensen, E. O., Llewellyn, D. J., Dennis, E. S., and Peacock, W. J. 1996. A new hemoglobin gene from soybean: A role for hemoglobin in all plants. *Proc. Natl. Acad. Sci. USA* 93:5682-5687.
- Andersson, C. R., Llewellyn, D. J., Peacock, W. J., and Dennis, E. S. 1997. Cell-specific expression of the promoters of two nonlegume hemoglobin genes in transgenic legume, *Lotus corniculatus*. *Plant Physiol.* 113:45-57.
- Angulo Carmona, A. F. 1974. La formation des nodules fixateurs d'azote chez *Alnus glutinosa* (L.). *Acta Bot. Neerl.* 23:257-303.
- Appleby, C. A. 1984. Leghemoglobin and *Rhizobium* respiration. *Ann. Rev. Plant Physiol.* 35:443-478.
- Appleby, C. A. 1992. The origin and functions of haemoglobin in plants. *Sci. Progress (Oxford)* 76:365-398.
- Appleby, C. A., Bogusz, D., Dennis, E. S., and Peacock, W. J. 1988. A role for haemoglobin in all plant roots? *Plant Cell Environ.* 11:359-367.
- Appleby, C. A., Tjepkema, J. D., and Trinick, M. J. 1983. Hemoglobin in a nonleguminous plant, *Parasponia*. Possible genetic origin and function in nitrogen fixation. *Science* 220:951-953.
- Bevan, M. 1984. Binary *Agrobacterium* vectors for transformation. *Nucleic Acids Res.* 12:8711-8721.
- Bogusz, D., Appleby, C. A., Landsmann, J., Dennis, E. S., Trinick, M. J., and Peacock, W. J. 1988. Functioning hemoglobin genes in a non-nodulating plant. *Nature* 331:178-180.
- Bogusz, D., Llewellyn, D. J., Craig, S., Dennis, E. S., Appleby, C. A., and Peacock, W. J. 1990. Nonlegume hemoglobin genes retain organ-specific expression in heterologous transgenic plants. *Plant Cell* 2: 633-641.
- Christensen, T., Dennis, E. S., Peacock, W. J., Landsman, J., and Marker, K. A. 1991. Hemoglobin genes in non-legumes: Cloning and characterization of a *Casuarina glauca* hemoglobin gene. *Plant Mol. Biol.* 16:339-344.
- Diouf, D., Gherbi, H., Prin, Y., Franche, C., Duhoux, E., and Bogusz, D. 1995. Hairy root nodulation of *Casuarina glauca*: A system for the study of symbiotic gene expression in an actinorhizal tree. *Mol. Plant-Microbe Interact.* 8:532-537.
- Finnegan, J., and McElroy, D. 1996. Transgene stability. Pages 170-186 in: *Transgenic Plants: A Production System for Industrial and Pharmaceutical Proteins*. M. R. L. Owen and J. Pen, eds. Wiley & Sons, Chichester, UK.
- Fleming, A. I., Wittenberg, J. B., Wittenberg, B. A., Dudman, W. F., and Appleby, C. A. 1987. The purification, characterization and ligand-binding kinetics of hemoglobins from root nodules of the non-leguminous *Casuarina glauca*-*Frankia* symbiosis. *Biochem. Biophys. Acta* 911:209-220.
- Franche, C., Diouf, D., Le, Q. V., N'Diaye, A., Gherbi, H., Bogusz, D., Gobé, C., and Duhoux, E. 1997. Genetic transformation of the actinorhizal tree *Allocauarina verticillata* by *Agrobacterium tumefaciens*. *Plant J.* 11:897-904.
- Franche, C., Laplaze, L., Duhoux, E., and Bogusz, D. 1998. Actinorhizal symbioses: Recent advances in plant molecular and genetic transformation studies. *Crit. Rev. Plant Sci.* 17:1-28.
- Gherbi, H., Duhoux, E., Franche, C., Pawlowski, K., Nassar, A., Berry, A., and Bogusz, D. 1997. Cloning of a full-length symbiotic hemoglobin cDNA and *in situ* localization of the corresponding mRNA in *Casuarina glauca* root nodule. *Physiol. Plant.* 99:608-616.
- Girgis, M. G., Ishac, Y. Z., El-Haddad, M., Saleh, E. A., Diem, H. G., and Dommergues, Y. R. 1990. First report on isolation and culture of effective *Casuarina*-compatible strains of *Frankia* from Egypt. Pages 156-164 in: *Int. Casuarina Worksh.*, 2nd. M. H. El-Lakany, J. W. Turnbull, and J. L. Brewbaker, eds.
- Girgis, Z. M., and Schwencke, J. 1993. Differentiation of *Frankia* strains by their electrophoretic patterns of intracellular esterases and aminopeptidases. *J. Gen. Microbiol.* 139:2225-2232.
- Goodchild, D. J., and Miller, C. 1997. Immunogold localisation of hemoglobin in *Casuarina* root nodules. *Protoplasma* 198:130-134.
- Jacobsen-Lyon, K., Jensen, E. O., Jorgensen, J., Marker, K. A., Peacock, W. J., and Dennis, E. S. 1995. Symbiotic and non-symbiotic hemoglobin genes of *Casuarina glauca*. *Plant Cell* 7:213-222.
- Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. 1987. GUS fusion: β-Glucuronidase as a sensitive and versatile gene fusion marker in

- higher plants. *EMBO J.* 6:3901-3907.
- Jouanin, L., Tourneur, J., and Casse-Delbart, F. 1986. Restriction maps and homologies of three plasmids of *Agrobacterium rhizogenes* strain A4. *Plasmid* 6:124-134.
- Koncz, C., and Schell, J. 1986. The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol. Gen. Genet.* 204:383-396.
- Landsmann, J., Dennis, E. S., Higgins, T. J., Appleby, C. A., Kortt, A. A., and Peacock, W. J. 1986. Common evolutionary origin of legume and non-legume plant haemoglobins. *Nature* 324:166-168.
- Landsmann, J., Llewellyn, D., Dennis, E. S., and Peacock, W. J. 1988. Organ regulated expression of the *Parasponia andersonii* haemoglobin gene in transgenic tobacco plants. *Mol. Gen. Genet.* 214:68-73.
- Lauridsen, P., Franssen, H., Stougaard, J., Bisseling, T., and Marker, K. A. 1993. Conserved regulation of the soybean early nodulin ENOD2 gene promoter in determinate and indeterminate transgenic root nodules. *Plant J.* 3:483-492.
- Mullin, B. C., and Dobritsa, S. V. 1996. Molecular analysis of actinorhizal symbiotic systems: progress to date. *Plant Soil* 186:9-20.
- Mylona, P., Pawlowski, K., and Bisseling, T. 1995. Symbiotic nitrogen fixation. *Plant Cell* 7:869-885.
- Ribeiro, A., Akkermans, A. D. L., van Kammen, A., Bisseling, T., and Pawlowski, K. 1995. A nodule-specific gene encoding a subtilisin-like protease is expressed in early stages of actinorhizal nodule development. *Plant Cell* 7:785-794.
- Rougier, M. 1981. Secretory activity of the root cap. Pages 542-574 in: *Encyclopedia of Plant Physiology, New Series, Vol. 13B, Plant Carbohydrates II*. W. Tanner, and F. A. Loewus, eds. Springer-Verlag, Berlin.
- Sandal, N. N., Bojsen, K., and Marker, K. A. 1987. A small family of nodule-specific genes from soybean. *Nucleic Acids Res.* 15:1507-1519.
- Serres, B., McCown, B., and Zeldin, E. 1997. Detectable β -glucuronidase activity in transgenic cranberry is affected by endogenous inhibitors and plant development. *Plant Cell Rep.* 16:641-646.
- Soltis, D. E., Soltis, P. S., Morgan, D. R., Swensen, S. M., Mullin, B. C., Dowd, J. M., and Martin, P. G. 1995. Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms. *Proc. Natl. Acad. Sci. USA* 92:2647-2651.
- Stougaard, J., Jorgensen, J. E., Christensen, T., Kühle, A., and Marker, K. A. 1990. Interdependence and nodule specificity of *cis*-acting regulatory elements in the soybean leghemoglobin *lbc3* and N23 gene promoters. *Mol. Gen. Genet.* 220:353-360.
- Stougaard, J., Sandal, N. N., Gron, A., Kuhle, A., and Marker, K. A. 1987. 5' analysis of the soybean leghemoglobin *lbc3* gene: Regulatory elements required for promoter activity and organ specificity. *EMBO J.* 6:3565-3569.
- Szabados, L., Ratet, P., Grunenberg, B., and de Bruijn, R. J. 1990. Functional analysis of the *Sesbania rostrata* leghemoglobin *glb3* gene 5'-upstream region in transgenic *Lotus corniculatus* and *Nicotiana tabacum* plants. *Plant Cell* 2:973-986.
- Taylor, E. R., Nie, X. Z., MacGregor, A. W., and Hill, R. D. 1994. A cereal haemoglobin gene is expressed in seed and root tissues under anaerobic conditions. *Plant Mol. Biol.* 24:853-862.
- Trevaskis, B., Watts, R. A., Andersson, C. R., Llewellyn, D. J., Hargrove, M. S., Olson, J. S., Dennis, E. S., and Peacock, W. J. 1997. Two hemoglobin genes in *Arabidopsis thaliana*: The evolutionary origins of leghemoglobins. *Proc. Natl. Acad. Sci. USA* 94:12230-12234.
- Trinick, M. J. 1979. Structure of nitrogen-fixing nodules formed by *Rhizobium* on roots of *Parasponia andersonii*. *Can. J. Bot.* 25:565-578.
- Trinick, M. J., Goodchild, D. J., and Miller, C. 1989. Localization of bacteria and hemoglobin in root nodules of *Parasponia andersonii* containing both *Bradyrhizobium* strains and *Rhizobium leguminosarum* biovar *trifolii*. *Appl. Environ. Microbiol.* 55:2046-2055.
- Vancanneyt, G., Schmidt, R., O'Conner-Sanchez, A., Willmitzer, L., and Rocha-Sosa, M. 1990. Construction of an intron-containing marker gene: Splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium* mediated plant transformation. *Mol. Gen. Genet.* 220:245-250.
- Vasse, J., de Billy, F., Camut, S., and Truchet, G. 1990. Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. *J. Bacteriol.* 172:4295-4306.