Transgenic Root Nodules of *Vicia hirsuta*: A Fast and Efficient System for the Study of Gene Expression in Indeterminate-Type Nodules

Hans-Joachim Quandt, Alfred Pühler and Inge Broer

Lehrstuhl für Genetik, Fakultät für Biologie, Universität Bielefeld, D-33501 Bielefeld, Germany
Received 24 March 1993. Accepted 25 August 1993.

The *Agrobacterium rhizogenes*-mediated transformation of *Vicia hirsuta* resulted in the induction of transgenic 'hairy roots' on wounded epicotyls of young seedlings. As soon as the 'hairy roots' had developed enough, the original wild-type roots were excised and composite plants were thus established that consisted of transgenic roots on untransformed shoots. The proportion of roots that proved by their β-glucuronidase (GUS) expression to be transgenic reached 88%. The inoculation of composite plants with *Rhizobium leguminosarum* bv. *viciae* led to the induction of transgenic indeterminate-type root nodules in less than 20 days after the *V. hirsuta* seedlings had been infected with *A. rhizogenes*. The structure and the function of the transgenic nodules were comparable to that of wild-type nodules. To illustrate the applicability of the system, the spatial and temporal expression pattern of an intron-containing p*35S-gus*A-int gene fusion was monitored during root and nodule development.

Additional keywords: symbiosis, vascular bundle-specific expression.

The symbiosis with soil bacteria of the genera *Azorhizobium*, *Bradyrhizobium,* and *Rhizobium* allows leguminous plants to utilize atmospheric, molecular nitrogen resources by the induction of morphologically defined organs, termed nodules, on the legume root. The legume-*Rhizobium* interaction is highly specific. The species of a defined legume genus can be nodulated by a certain *Rhizobium* species only (Vincent *et al.* 1980).

Plant genes that are preferentially or exclusively expressed in nodules (nodulin genes) have been isolated via nodule-specific cDNA cloning (Delauney and Verma 1988; Sanchez *et al.* 1991). However, at present little is known about the biochemical function, the localization of activity within the nodule or the regulation of the majority of nodulin genes. The establishment of symbiotic plant mutants via plant transformation and regeneration technologies in concert with the introduction of chimeric nodulin-reporter genes can help to elucidate the role of these symbiotic plant genes.

The most widely employed transformation procedure is the *Agrobacterium*-mediated gene transfer. Systems exist only for *Medicago sativa* (De Bruijn *et al.* 1989, 1990), *M. varia* (Brears *et al.* 1991; Deak *et al.* 1986), *Trifolium repens* (Diaz *et al.* 1989; Jorgensen *et al.* 1988), and *Lotus corniculatus* (Hansen *et al.* 1989; Petit *et al.* 1987; Stougaard *et al.* 1986). Nodules of *Medicago* and *Trifolium* belong to the indeterminate-type, while *L. corniculatus* forms determinate-type nodules. Transgenic *M. sativa* and *M. varia* plants result from the use of disarmed *A. tumefaciens* vector systems (Bevan and Goldsbrough 1988; Hoekema *et al.* 1983; Tepfer 1990; van Wordrangen and Dons 1992) followed by a complex regeneration protocol. Transgenic *L. corniculatus* plants are regenerated from *A. rhizogenes*-induced hairy roots cotransformed with the chimeric nodulin reporter gene fusion (Forde *et al.* 1989; Jorgensen *et al.* 1991; Miao *et al.* 1991; Stougaard *et al.* 1986; Stougaard *et al.* 1987a, 1987b, 1987c; Szabados *et al.* 1990). Although the regeneration of this plant species occurs spontaneously, it takes more than 5 mo for the transgenic root nodules to develop. Since this proved too time consuming to allow the routine examination of large amounts of independent transformants, a rapid technique was developed for *L. corniculatus* as well as *T. repens*. This procedure relies on the direct nodulation of transgenic roots that are induced by *A. rhizogenes* on the hypocotyl of seedlings (Jorgensen *et al.* 1988; Diaz *et al.* 1989, Hansen *et al.* 1989; Stougaard *et al.* 1990). Using this 'composite plant' approach, the expression of chimeric nodulin reporter genes can be analyzed in transgenic *L. corniculatus* root nodules within approximately 2 mo. Since each transgenic root represents a single transformation event, a great number of different transformants can be tested. This may minimize the influence of position effects on the average expression pattern.

A comprehensive system to analyze the major aspects of the legume-*Rhizobium* symbiosis would consist of three elements: 1) a collection of nodulin clones; 2) a fast and reliable transformation protocol for the establishment of great numbers of transgenic root nodules; and 3) a large collection of well-defined rhizobial mutants to elucidate the bacterial signals that regulate the activity of nodulin genes. All three components should belong to the same legume-*Rhizobium* association in order to obtain reliable and representative results.

A large collection of symbiotic mutants of *R. leguminosarum* bv. *viciae* exists, the microsymbiont of the *Vicia-Rhizobium* interaction. Recently, we established the other two components essential for the study of the *Vicia* sp.-*R. l.* bv. *viciae* symbiosis. That is a nodule-specific cDNA library of *Vicia* sp. (Perlick and Pühler 1993) and a fast and efficient transformation system for the generation of transgenic root nodules of *Vicia* sp. The transformation system for *V. hirsuta* is presented in this paper.

MPMI Vol. 6, No. 6, 1993, pp. 699-706

RESULTS

Construction of composite *Vicia hirsuta* plants which consist of transgenic roots on wild-type shoots.

The susceptibility of 2-day-old *V. hirsuta* seedlings to different *A. rhizogenes* strains was tested. Almost all *A. rhizogenes* strains induced 'hairy roots' on wounded epicotyls. Irrespective of the strain, the first visible roots usually appeared on the 7th day postinfection. Roots emerged from the wounds on more than 90% of the seedlings 14 days after beginning of the experiment. However, the intensity of the plants' responses was different. Bacteria carrying a pRi15834-type plasmid (AR15834, AR12, and C58C1:pRi15834) induced a strong response characterized by numerous roots growing from the wound. Bacteria bearing a pA4-type plasmid (ARqua1 and C58C1:pRiA4-24B) induced only one to three roots per wound (Fig. 1). Additionally, the 'hairy root' phenotype of the pRi15834-induced roots was more pronounced. Roots developed only occasionally from uninfected epicotyl wounds. Composite plants which had 'hairy roots' induced by the *A. rhizogenes* strain ARqua1 appeared most similar to wild-type plants as far as the shoot/root proportion was concerned. Consequently, composite plants which had 'hairy roots' induced by ARqua1 or its derivatives were chosen to develop the transformation system of *V. hirsuta* (Fig. 1).

To verify the root transformation via cotransfer and expression of the *gus*A-int reporter gene, the *A. rhizogenes* strain ARqua1 was equipped with the binary plant vector p*35S-gus*A-int, and the new strain was termed ARqua2 (Table 1).

Two weeks after infection with *A. rhizogenes* ARqua2, the roots that had formed at the wound site of *V. hirsuta* seedlings were well-developed (1–5 cm long). The substrate 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc) was used for the histochemical analysis of transgenic roots. The blue staining of the roots indicated the expression of the GUS enzyme (Fig. 2). Two weeks after the infection with ARqua2, between 54 and 71% of the roots tested showed histochemically detectable GUS activity resulting from the expression of the intron-containing *gus*A-int reporter gene under the control of the CaMV promoter. Five weeks after ARqua2 infection, the proportion of GUS-positive roots tended to be higher than the proportion measured 2 wk after ARqua2 infection. Up to 88% of these 5-wk-old roots showed histochemically detectable GUS enzyme activity. Every measurement included five independent experiments with at least 40 individual roots monitored in every experiment. *V. hirsuta* wild-type roots as well as hairy roots induced by the *A. rhizogenes* strain ARqua1 did not show any β-glucuronidase background activity. The same result was obtained when the substrate of the β-glucuronidase enzyme (X-Gluc) was added to logarithmically growing suspension cultures of *A. rhizogenes* ARqua1 and ARqua2.

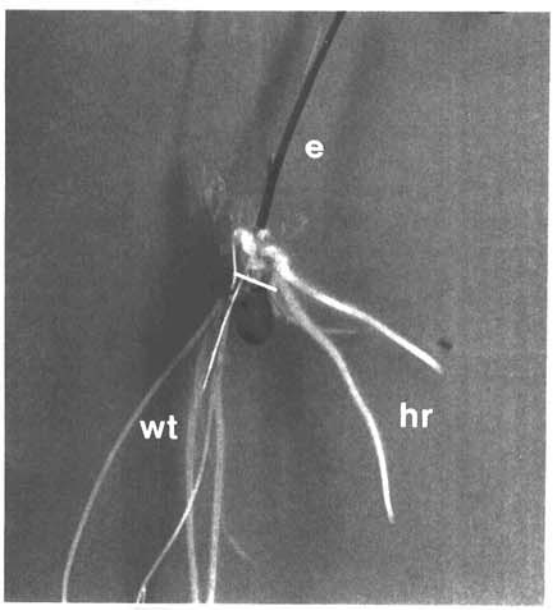

Fig. 1. The induction of transgenic 'hairy roots' on a *Vicia hirsuta* plant. The epicotyls of *V. hirsuta* seedlings were wounded and the wound sites infected with *Agrobacterium rhizogenes* ARqua1 or ARqua2. Twelve to fourteen days postinfection, the 'hairy roots' emerging from the wound sites were developed enough to monitor the GUS gene expression as well as to excise the original wild-type roots to result in composite plants consisting of transgenic roots on untransformed wild-type shoots. The bar indicates the site where the epicotyl was cut. Abbreviations: e, epicotyl; hr, transgenic 'hairy roots'; wt, wild-type roots.

The spatial localization of the p*35S-gus*A-int gene activity in transgenic *V. hirsuta* roots.

The differential blue staining of the transgenic *V. hirsuta* roots induced by the *A. rhizogenes* strain ARqua2 was analyzed after their incubation in X-Gluc. Figure 2A depicts the GUS activity in a branched root system transformed with the p*35S-gus*A-int construct. The younger the lateral roots are the more intensive is the GUS staining. Figure 2B shows a photograph of a stained apex of a young transgenic root. GUS activity is mainly observed in the two differentiation zones of the root meristem and the central vascular cylinder. Figure 2C depicts the differential GUS activity in the central vascular tissue distal to the root apex. The blue precipitate is only located in the cells of the phloem, while the solid star-shaped xylem remains unstained. The GUS activity of the root cortex usually was too faint as to be detected in a semithin cross section of a transgenic root. This overall GUS activity pattern was consistent in all roots independently induced by the

Table 1. Bacterial strains and plasmid used in this study

Strain name	Relevant characteristics	Origin or source
A. rhizogenes		
15834	pRi15834, wild-type	Schiemann *et al.* 1989
C58C1	pRi15834, Rif^r	Hansen *et al.* 1989
AR12	pRi12, Rif^r, Km^r p*35S-gus*A inserted into TL-DNA of strain C58C1:pRi15834	Hansen *et al.* 1989
C58C1	pRiA4-24B, Sm^r	Schiemann *et al.* 1989
R1000	Derivative of strain A4T	White *et al.* 1985
ARqua1	Sm^r-derivative of R1000	This work
ARqua2	ARqua1 containing p*35S-gus*A-int, Sm^r, Km^r	This work
R. leguminosarum bv. *viciae*		
VH5e	Wild type	B. Kosier, unpublished
VH5eSm^r	Sm^R-derivative of VH5e	This work
The plasmid		
p*35S-gus*A-*int*	pBIN19 derivative carrying an intron-containing *gus*A gene fused to the *35S* promoter, Km^r	Vancanneyt *et al.* 1990

A. rhizogenes strain ARqua2. However, the intensity of the GUS activity varied considerably between the individual roots.

Transgenic roots of composite *V. hirsuta* plants can be nodulated.

Twelve to fourteen days after the infection with *A. rhizogenes* ARqua2, the roots emerging from the wound site of *V. hirsuta* seedlings were sufficiently developed (1–5 cm long) to remove the original wild-type root system without killing the plant (Fig. 1). The composite plants were transferred to fresh nitrogen-free nodulation medium and inoculated with the *R. l.* bv. *viciae* strain VH5eSm^r. Four days after the inoculation the first nodules were visible on the transgenic root system. Eight days after the inoculation, the first nodules showed a pink color, indicating the onset of leghemoglobin expression and nitrogen fixation. Two weeks after the inoculation, almost all composite plants had developed the first pink nodules. These observations correspond to those of the wild-type plants. Additionally, at all developmental stages the shape of transgenic and wild-type nodules was comparable. Young nodules had a globular shape, whereas older ones became cylindrical. Three weeks after the inoculation, more than 90% of all composite plants had developed root nodules. At that point the majority of composite plants had developed between two and five nodules. This nodulation profile was comparable

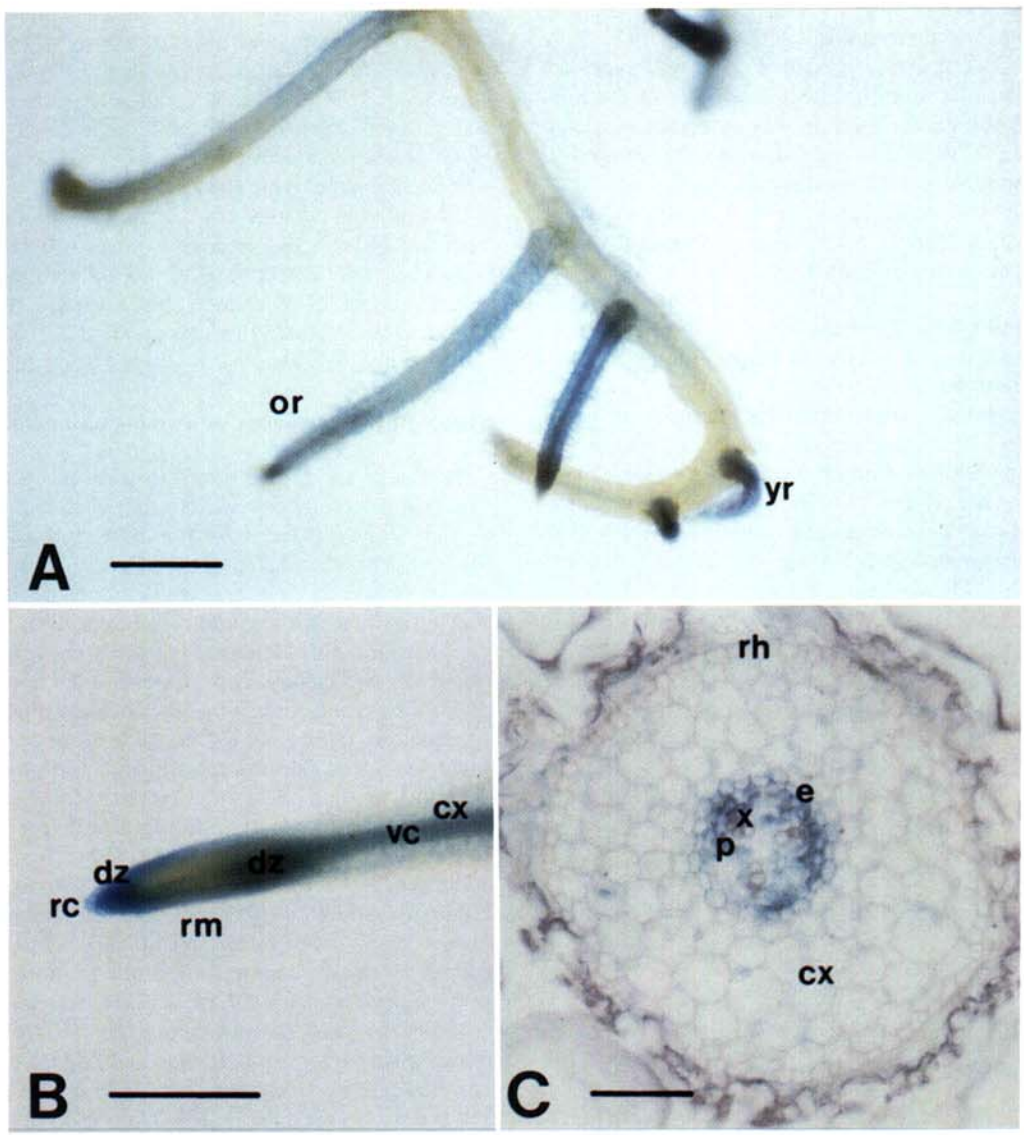

Fig. 2. The histochemical analysis of GUS activity in transgenic *Vicia hirsuta* roots transformed with the p*35S-gus*A-int construct. Whole roots or root sections were treated with X-Gluc and microphotographed with a binocular (A and B) or with a microscope (C). The blue precipitate indicates GUS activity. **A,** Localization of the GUS activity in a branched root system. Intense GUS staining in young lateral roots and less GUS staining in older lateral roots is clearly visible. Bar represents 1 mm. **B,** Localization of GUS activity in the apex of a young root. Strong GUS activity in the two differentiation zones of the root meristem and the central vascular cylinder and less activity in the root cap, the root meristem and the root cortex can be observed. Bar represents 1 mm. **C,** Micrograph of a semithin cross-section of a transgenic root. Strong GUS activity in the phloem of the central vascular cylinder is visible. Bar represents 100 µm. Abbreviations: cx, root cortex; dz, differentiation zone of root meristem; e, endodermis; or, old lateral root; p, phloem; rc, root cap; rh, rhizodermis; rm, root meristem; vc, central vascular cylinder; x, xylem; yr, young lateral root.

to that found on wild-type plants. Therefore, no influence of the 'hairy root' character of the transgenic root on the nodule formation frequency could be observed.

The structure and the function of transgenic *V. hirsuta* root nodules was investigated. Individual nodules were sectioned by hand, and their zonal structure was evaluated. Figure 3 shows longitudinal hand-cut sections of a 3-wk-old wild-type nodule (A) and a transgenic nodule on roots induced by the *A. rhizogenes* strain ARqua2 (B). The zonal differentiation was similar in both types of nodules. The extension of the symbiotic zone is indicated by the spreading of the red leghemoglobin protein. The comparison of semithin sections of approximately 3-wk-old transgenic and wild-type root nodules showed in both types of organs the typical zonation of meristem, infection zone, symbiotic zone, and senescence zone. Finally, during all developmental steps of the nodules, the relative extent of the zones was comparable.

In addition to their structure, the function of wild-type and transgenic nodules was investigated. The activity of the *Rhizobium*-encoded nitrogenase enzyme was measured as acetylene reduction. The acetylene reduction rate of composite plants that had nodules on GUS-positive roots only, was comparable to that of wild-type plants. In both groups, 40 days after the inoculation with *R. l.* bv. *viciae*, individual plants produced 15–35 nmol ethylene plant^{-1} hr^{-1}.

The temporal and spatial localization of the p*35S-gus*A-int gene activity in transgenic *V. hirsuta* root nodules.

Composite *V. hirsuta* plants that had transgenic roots induced by ARqua2 were inoculated with *R. l.* bv. *viciae* VH5eSm^r and the nodules formed were investigated at different developmental stages. Accordingly, whole nodules, hand-cut sections as well as semithin vibratom sections of such nodules were treated with X-Gluc and the GUS activity was localized. No GUS activity was found in the undifferentiated, meristematic tissue irrespective of the developmental stage of the nodules (Fig. 4A and C). In transgenic nodules, the most intensive GUS activity was detected in the vascular bundles. Thus, especially in young transgenic nodules, the GUS activity pattern reflected the growth of the vascular system (Fig. 4A). In mature nodules, cells of the symbiotic zone also showed a strong response (Fig. 4D–F), while there was no activity in the infection zone (Fig. 4F). A more detailed analysis of the nodule vascular bundle revealed that the distribution of GUS activity in the different cell types paralleled that found in the central vascular cylinder of transgenic roots. That is, a strong response was detected in the cells of the phloem and no response occurred in xylem cells (Fig. 4G). Additionally, intense activity was detected in the cells of the nodule parenchyma embedding the vascular bundles (Fig. 4G). However, while the vascular bundle-specific GUS activity was the first activity to appear in young nodules, it was also the first to disappear in the basal parts of older, transgenic *V. hirsuta* root nodules. Irrespective of the developmental stage of the nodules, the GUS activity in the outer cortex remained low (Fig. 4C–F). The GUS staining of senescent nodule tissue was also faint.

In wild-type nodules of *V. hirsuta* and nodules formed on roots induced by the *A. rhizogenes* strain ARqua1, GUS enzyme activity was not detected. Thus, not only untransformed plant nodule tissues of *V. hirsuta*, but also *R. l.* bv. *viciae* bacteroids lack GUS activity during nodule formation and nodule function in *V. hirsuta*.

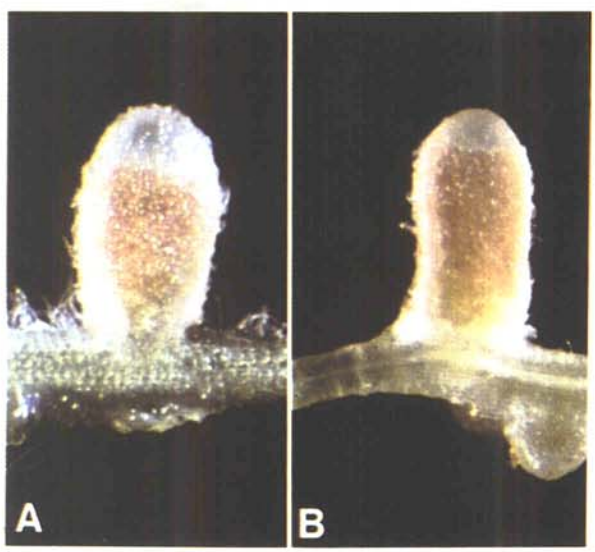

Figure 3. The comparison of the morphology of **A,** wild-type and **B,** transgenic *Vicia hirsuta* root nodules. Composite and wild-type *V. hirsuta* plants were inoculated with *Rhizobium leguminosarum* bv. *viciae*. Three-week-old nodules were bisected and the structure was evaluated under a binocular microscope. The structure of transgenic root nodules is identical to that of wild-type nodules.

DISCUSSION

In this paper, a new transformation protocol is presented that enables a fully developed, and nodulated, transgenic root system of the legume *V. hirsuta* to be established in less than 20 days. An integral step of the protocol is the *A. rhizogenes*-mediated induction of transgenic roots on young plantlets which substitute for the wild-type root system.

The usefulness of legumes in nodulation tests is determined mainly by two factors: 1) the time required for visible nodules to form on plants and 2) the ease of culturing great numbers of nodulated plants and individual transformants. Among the plant species of the pea cross-inoculation group (*Pisum* sp., *Lathyrus* sp., and *Vicia* sp.) nodulated by *R. l.* bv. *viciae*, it transpired that *V. hirsuta* fulfilled both prerequisites most extensively (Van Brussel *et al.* 1982). Compared to other *Vicia* species, young *V. hirsuta* plants are small and can be cultured easily in a petri dish, and the first visible nodules appear only 4 days after inoculation. These advantageous properties and the root induction rate of more than 90% support the development of the *V. hirsuta* transformation system.

Using the *gus*A transformation reporter gene located on a binary plant vector, on *V. hirsuta* and *L. corniculatus* up to 88 and 80%, respectively, of the *A. rhizogenes* induced roots showed a histochemically detectable GUS activity (Forde *et al.* 1989). This indicates that a high frequency of cotransformation of the vector and the pRi-born T-DNAs occurred (Stougaard *et al.* 1987a). The increase in the proportion of histochemically detectable, GUS-positive roots in older root systems of *V. hirsuta* in comparison with younger ones is probably due to an enhanced growth rate in further developed, composite *V. hirsuta* plants. Faster growing roots have

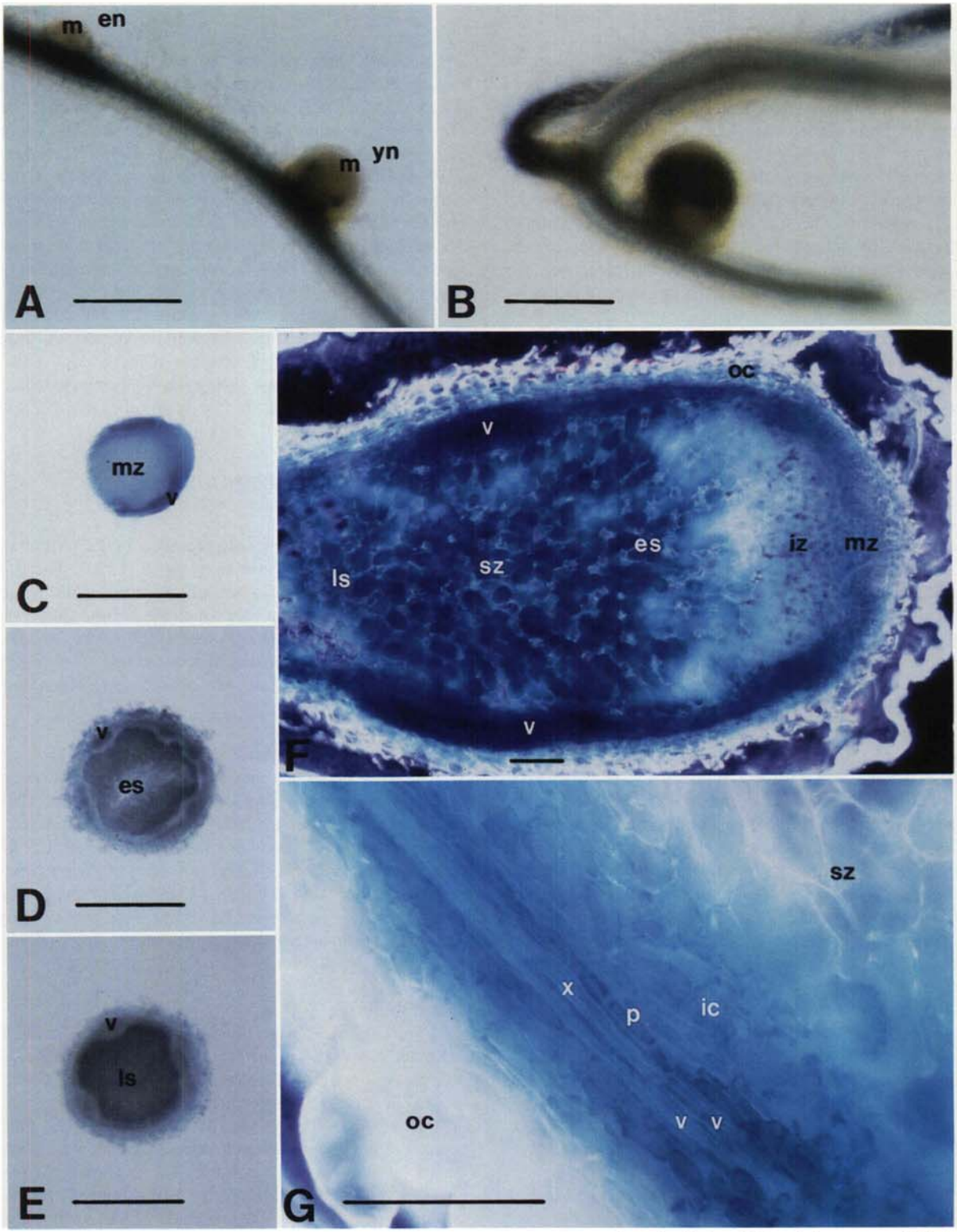

Figure 4. The histochemical analysis of GUS activity in transgenic *V. hirsuta* root nodules transformed with the p*35S-gus*A-int construct. Whole nodules or nodule sections were treated with X-Gluc and microphotographed either with a binocular (A-E) or with a microscope (F-G). The blue precipitate indicates GUS activity. The magnification bars represent 1 mm (A and B), 0.5 mm (C-E) and 100 μm (F and G). **A,** Localization of the GUS activity in emerging and young, pink nodules. **B,** Localization of GUS activity in an approximately 3-wk-old nodule. **C,** Cross section of the meristematic zone of an approximately 4-wk-old transgenic nodule. Intense GUS staining in the vascular tissue, no activity in the central meristematic tissue can be observed. **D,** Cross section of the early symbiotic zone of an approximately 4-wk-old transgenic nodule. GUS activity in the symbiotic tissue and in the vascular bundles is visible. **E,** Cross section of the late symbiotic zone of an approximately 4-wk-old transgenic nodule. GUS activity in the symbiotic zone and no activity in the vascular tissues are clearly visible. **F,** Darkfield micrograph of a longitudinal semithin section of an approximately 4-wk-old transgenic nodule. Intense GUS activity in the vascular and symbiotic tissues and less activity in the meristematic and infection zones can be observed. **G,** Micrograph of the longitudinal section of a nodule vascular bundle; higher power magnification of the same section as (F). Strong GUS activity in phloem cells is clearly visible. Abbreviations: en, emerging nodule; es, early symbiotic zone; ic, nodule parenchyma; iz, infection zone; ls, late symbiotic zone; m, meristem of young developing nodules; mz, meristematic zone; oc, outer cortex; p, phloem cell; v, vascular bundle; x, xylem cell; yn, young nodule.

more general gene activity and consequently, the p*35S-gus*A-int reporter gene might also be more active and result more often in a detectable signal. Thus, the real transformation frequency might be higher than that detectable using the GUS reporter.

An inherent problem to the 'composite plant' transformation method is the presence of the *Agrobacteria* after the transgenic roots have been induced. They could potentially harm the plant by subsequently infecting it, or suppress the plant's nodulation by the rhizobial bacteria. Moreover, the most crucial effect could be the activity of the promoter driving the reporter gene in the *A. rhizogenes* persisting in or on the transgenic roots. It has been shown in previous reports that constructs using the *35SCaMV* promoter employed in this study led to expression of the reporter gene not only in plants but also in *Agrobacteria* (Jefferson *et al.* 1987; Ohta *et al.* 1990; Vancanneyt *et al.* 1990). Other eukaryotic promoters are also known to be well active in *Agrobacteria* (Vancanneyt *et al.* 1990). Thus, composite *L. corniculatus* plants were decontaminated by submerging them for 4 days in antibiotic-containing medium (Hansen *et al.* 1989). Such an experimental step most probably damages the plants and thereby protracts the subsequent nodule induction by *Rhizobium*. In this work, the problem of contamination with bacterial GUS activity was circumvented by introducing an intron-containing *gus*A-int reporter gene (Vancanneyt *et al.* 1990). The *gus*A-int gene can only be expressed in eukaryotic tissues, since prokaryotes are devoid of the eukaryotic splicing apparatus. Thus, any signal in plant tissue must result from plant cell-specific expression. Composite *V. hirsuta* plants contaminated with *A. rhizogenes* were transferred to fresh nodulation medium and immediately inoculated with *R. l.* bv. *viciae*. The time period required until the first nodules became visible and the number of nodules in older plants were comparable to wild-type control plants, indicating that the *Agrobacteria* persisting on the transgenic roots did not interfere with the nodulation process. Further 'hairy root' induction in wounded plant organs due to experimental manipulations was rare, and no significant influence on the plant's general performance by the persisting *Agrobacteria* was detected. Thus, the decontamination of composite *V. hirsuta* plants prior to the inoculation with *R. l.* bv. *viciae* was not necessary.

In principle, it cannot be excluded that a disturbed hormone balance in the 'hairy root' may have an influence on nodule formation and gene expression in the transgenic nodule, especially when hormone-regulated transgenes are analyzed in 'hairy roots.' Nevertheless, according to our data, the nodule development, the morphology and the function of transgenic nodules seem to be comparable to the wild type.

The transformation system for *V. hirsuta* described in this paper is efficient and most suitable for the research into indeterminate nodule nodulin gene expression. In this work, the *gus*A-int gene under the control of the *35SRNA* promoter of the CaMV virus was chosen as a model transgene to demonstrate differential gene regulation in transgenic *V. hirsuta* roots and nodules. The *35SRNA* promoter is preferentially active in the vascular system (Harpster *et al.* 1988; Nagy *et al.* 1985; Odell *et al.* 1985; Sanders *et al.* 1987). Histochemical analyses of transgenic roots revealed that the expression is located mainly in the phloem and in the root tips (Benfey and Chua 1989; Benfey *et al.* 1989; Jefferson *et al.* 1987; Ohta *et al.* 1990). The *CaMV* promoter maintained this activity pattern in transgenic *V. hirsuta* roots. In previous reports, it has been demonstrated that the *CaMV* promoter maintained its vascular tissue-specific activity in determinate-type nodules of transgenic *L. corniculatus* plants (Forde *et al.* 1989; Szabados *et al.* 1990). The overall mechanism of the regulation of this viral promoter seems to be conserved in determinate-type and indeterminate-type nodules, since in transgenic *V. hirsuta* and *L. corniculatus* nodules, a strong expression of p*35S-gus*A genes has not only been detected in the vascular, but also in the symbiotic tissues (Szabados *et al.* 1990).

Since the GUS expression pattern in hairy roots and nodules is identical to that observed in organs of transgenic plants, the possibility of changes in expression patterns in composite plants is low. These results clearly indicate the suitability of this system for the comprehensive study of nodulin gene expression.

MATERIALS AND METHODS

The plant vector.

The p*35S-gus*A-int, kindly provided by L. Willmitzer, is a pBIN19 derivative (Vancanneyt *et al.* 1990). The *35S*RNA promoter of the cauliflower mosaic virus (CaMV) controls the *gus*A coding region into which the second intron (IV2) of the potato *ST-LS1* gene was introduced (Eckes *et al.* 1986). The binary plant vector was introduced into *A. rhizogenes* via the direct DNA transformation procedure (Höfgen and Willmitzer 1988).

Bacterial strains and media.

The bacterial strains are listed in Table 1. All *A. rhizogenes* strains were grown on YEB solid medium or in YEB liquid medium (Vervliet *et al.* 1975). *R. leguminosarum* bv. *viciae* was grown on TY solid medium or in liquid TY medium (Maniatis *et al.* 1982). Antibiotics were used as has been described before (Van Haute *et al.* 1983). The culture temperature was 28° C. Sm^r bacterial strains were generated by plating logarithmic suspension cultures on the appropriate media containing the antibiotic.

Plant material.

V. hirsuta seeds were obtained from John Chambers Ltd., London. Seeds were surface sterilized in concentrated sulphuric acid which was shaken in an Erlenmeyer flask for 20 min. Thereafter, they were rinsed six times in sterile water (modified according to Van Brussel *et al.* 1982). The seeds were swollen by a 4-hr incubation in sterile water, and then dried by removing all the water. The seeds were germinated in the same Erlenmeyer flask, by incubating the flask at 20° C in the dark, for 2 days. Pregerminated seeds were transferred, two individuals per plate, to 1.5% agar-solidified, nitrogen-free nodulation medium (Van Brussel *et al.* 1982) cast as slopes in petri dishes. The petri dishes (85 mm in diameter) were placed vertically in a 20° C growth cabinet which had a 16-hr photoperiod.

Generation of composite *V. hirsuta* plants.

Light-grown *V. hirsuta* seedlings having epicotyls of 1.5–2.0 cm in length (usually 2 days after the transfer into light, and 4 days after the sterilization) were infected with *A. rhizo-*

genes by stab inoculation. Accordingly, one side of the epicotyl of the seedling was stabbed three times proximal to the seed with the needle of an *Agrobacteria*-containing syringe. Finally, an approximately 5-µl drop of the *A. rhizogenes* suspension culture was placed on the wound area of the epicotyl. *A. rhizogenes* strains were freshly grown at 28° C overnight. Petri dishes containing infected plantlets were returned to the growth cabinet and incubation continued as described above. Fourteen days post *A. rhizogenes* infection the majority of the plants had developed transgenic 'hairy roots' and the primary wild-type roots including the seeds were exciced.

Nodulation of composite plants.

Composite *V. hirsuta* plants with transgenic 'hairy roots' on untransformed shoots (two individuals per plate) were transferred to fresh nitrogen-free nodulation medium (Van Brussel *et al.* 1982) slopes in petri dishes. Every plant was inoculated with 100 µl of an overnight grown *R. l.* bv. *viciae* suspension culture. Before the cultivation of the plants was continued in the growth cabinet, they were kept in the dark for 1 day. Acetylene reduction assays of nodulated plants were carried out 40 days after the inoculation with *R. l.* bv. *viciae* (Hardy *et al.* 1968).

Assays for GUS activity.

Histochemical staining of plant material was performed as described (Jefferson *et al.* 1987) with the following modifications. Intact or hand-cut sections of *V. hirsuta* roots and nodules were incubated, without prior fixation, in 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc), 50 mM sodium phosphate buffer (pH 7.0). The explants were subjected to vacuum infiltration for 10 min before incubation of them overnight at 37° C. Samples were washed at least three times with oxygen-enriched 50 mM sodium phosphate buffer (pH 7.0, 4° C), incubated under the same conditions overnight, cleared with 70% ethanol, incubated again overnight and photomicrographed using a binocular Wild M32 microscope. All experimental steps were carried out in Eppendorf tubes.

Semithin sections (30–100 µm) of fresh unfixed nodules were cut with an Oxford Vibratome sectioning device. Nodules were deposited in a layer of cyanoacrylate adhesive placed on a small wooden block, and the arrangement was fixed at the vibratome. Sectioning was carried out in 50 mM sodium phosphate buffer (pH 7.0) at room temperature. Sections were immediatly transferred to a thin layer of the X-Gluc substrate solution in a 10-ml glas vial and incubated overnight at 37° C. The sections were then mounted on microscope slides for observation and microphotography. Photographs were taken with an Olympus BH-2 microscope fitted with an Olympus OM-4 camera.

To stain the *A. rhizogenes* bacteria *in vivo*, the procedure described by Vancanneyt *et al.* (1990) was applied with the following modifications. Bacteria, freshly grown on YEB solid medium, were suspended in X-Gluc substrate solution (see above) to give a milky solution and incubated for 24 hr at 37° C.

NOTE ADDED IN PROOF

The expression of a *Vicia faba* leghemoglobin promoter fused to an intron-containing *gus*A coding region showed the expected pattern in transgenic root nodules of *V. hirsuta*.

ACKNOWLEDGMENTS

We wish to thank J. Schiemann, E. Nester, K. Marcker, and B. Kosier for the gift of bacterial strains, L. Willmitzer for the plant vector p*35S-gus*A-int, and K. Niehaus for helpful comments on the manuscript.

LITERATURE CITED

Benfey, P. N., and Chua, N.-H. 1989. Regulated genes in transgenic plants. Science 244:174-181.

Benfey, P. N., Ren, L., and Chua, N.-H. 1989. The CaMV 35S enhancer contains at least two domains which can confer different and developmental and tissue-specific expression patterns. EMBO J. 8:2195-2202.

Bevan, M., and Goldsbrough, A. 1988. Design and use of *Agrobacterium* transformation vectors. Genet. Eng. 10:123-140.

Brears, T., Walker, E. L., and Coruzzi, G. M. 1991. A promoter sequence involved in cell-specific expression of the pea glutamine synthetase *GS3A* gene in organs of transgenic tobacco and alfalfa. Plant J. 1:235-244.

De Bruijn, F. J., Felix, G., Grunenberg B., Hoffmann H. J., Metz, B., Ratet, P., Simons-Schreier, A., Szabados, L., Welters, P., and Schell, J. 1989. Regulation of plant genes specifically induced in nitrogen-fixing nodules: Role of *cis*-acting elements and *trans*-acting factors in leghemoglobin gene expression. Plant Mol. Biol. 13:319-325.

De Bruijn, F. J., Szabados, L., and Schell, J. 1990. Chimeric genes and transgenic plants are used to study the regulation of genes involved in symbiotic plant-microbe interactions (nodulin genes). Dev. Genet. 11:182-196.

Deak, M., Kiss, G. B., Koncz, C., and Dudits, D. 1986. Transformation of *Medicago* by *Agrobacterium* mediated gene transfer. Plant Cell Rep. 5:97-100.

Delauney, A. J., and Verma, D. P. S. 1988. Cloned nodulin genes for symbiotic nitrogen fixation. Plant. Mol. Biol. Rep. 6:279-285.

Diaz, C. L., Melchers, L. S., Hooykaas, P. J. J., Lugtenberg, B. J. J., and Kijne, J. W. 1989. Root lectin as a determinant of host-plant specificity in the *Rhizobium*-legume symbiosis. Nature 338:579-581.

Eckes, P., Rosahl, S., Schell, J., and Willmitzer, L. 1986. Isolation and characterization of a light-inducible, organ-specific gene from potato and the analysis of its expression after tagging and transfer into tobacco and potato sheets. Mol. Gen. Genet. 199:216-224.

Forde, B. G., Day, H. M., Turton, J. F., Wen-jun, S., Culimore, J. V., and Oliver, J. E. 1989. Two glutamine synthetase genes from *Phaseolus vulgaris* L. display contrasting developmental and spatial patterns of expression in transgenic *Lotus corniculatus* plants. Plant Cell 1:391-401.

Hansen, J., Jorgensen, J. E., Stougaard, J., and Marcker, K. 1989. Hairy roots—a short cut to transgenic root nodules. Plant Cell Rep. 8:12-15.

Hardy, R. W. F., Holsten, R. D., Jackson, E. K., and Burns, R. C. 1968. The acetylene-ethylene assay for N fixation: Laboratory and field evaluation. Plant Physiol. 43:1185-1207.

Harpster, M. H., Townsend, J. A., and Jones, J. D. G. 1988. Relative strength of the 35S cauliflower mosaic virus, 1′, 2′ and nopaline synthase promoters in transformed tobacco, sugarbeet and oilseed rape callus tissue. Mol. Gen. Genet. 212:182-190.

Höfgen, R., and Willmitzer, L. 1988. Storage of competent cells for Agrobacterium transformation. Nucleic Acids Res. 16:9877.

Hoekema, A., Hirsch, P. R., Hooykaas, P. J. J., and Schilperoort, R. A. 1983. A binary vector strategy based on the separation of the vir and T-region of the *Agrobacterium tumefaciens* plasmid. Nature 303:179-180.

Jefferson, R. A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. 5:387-405.

Jefferson, R. A., Kavanagh, T. A., and Bevan M. W. 1987. GUS fusions: β-Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901-3907.

Jorgensen, J. E., Stougaard, J., Marcker A., and Marcker, K. A. 1988. Root nodule specific regulation: Analysis of the soybean nodulin N23 gene promoter in heterologous symbiotic systems. Nucleic Acids Res. 16:39-50.

Jorgensen, J. E., Stougaard, J., and Marcker, K. A. 1991. A two-component nodule-specific enhancer in the soybean N23 gene promoter. Plant Cell 3:819-827.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Habor Laboratory Press, Cold Spring Habor, NY.

Miao, G. H., Hirel, B., Marsolier, M. C., Ridge, R. W., and Verma, D. P. S. 1991. Ammonia-regulated expression of a soybean gene encoding cytosolic glutamine synthetase in transgenic *Lotus corniculatus*. Plant Cell 3:11-22.

Nagy, F., Odell, J. T., Morelli, G., and Chua, N.-H. 1985. Relevance to agriculture in the eighties. Pages 227-336 in: Biotechnology in Plant Science. M. Zaitlin, P. Day, and A. Hollaender, eds. Academic Press, New York.

Odell, J. T., Nagy, F., and Chua, N.-H. 1985. Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313:810-812.

Ohta, S., Mita, S., Hattori, T., and Nakamura, K. 1990. Construction and expression in tobacco of a β-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. Plant Cell Physiol. 31:805-813.

Perlick, A., and Pühler, A. 1993. A survey of transcripts expressed specifically in root nodules of broadbean (*Vicia faba* L.). Plant Mol. Biol. 22:957-970.

Petit, A., Stougaard, J., Kühle, A., Marcker, K. A., and Tempé, J. 1987. Transformation and regeneration of the legume *L. corniculatus*: A system for molecular studies of symbotic nitrogen fixation. Mol. Gen. Genet. 207:245-250.

Sanchez, F., Padilla, J. E., Perez, H., and Lara, M. 1991. Control of nodulin genes in root-nodule development and metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42:507-528.

Sanders, P. R., Winter, J. A., Zarnason, A. R., Rogers, S. G., and Fraley, R. T. 1987. Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants. Nucleic Acids Res. 15:1543-1558.

Schiemann, J., and Eisenreich, G. 1989. Transformation of field bean (*Vicia faba* L.) cells: Expression of a chimeric gene in cultured hairy roots and root-derived cells. Biochem. Physiol. Pflanzen 185:135-140.

Stougaard Jensen, J., Marcker, K. A., Otten, L., and Schell, J. 1986. Nodule-specific expression of a chimeric soybean leghaemoglobin gene in transgenic *Lotus corniculatus*. Nature 321:669-674.

Stougaard, J., Abildsten, D., and Marcker, K. A. 1987a. *Agrobacterium rhizogenes* pRi TL-DNA segment as a gene vector system for transformation of plants. Mol. Gen. Genet. 207:251-255.

Stougaard, J., Sandal, N. N., Gron, A., Kühle, A., and Marcker, K. A. 1987b. 5′ Analysis of the soybean leghemoglobin *lbc*3 gene: Regulatory elements required for promoter activity and organ specificity. EMBO J. 6:3563-3569.

Stougaard, J., Peterson, T. E., and Marcker, K. A. 1987c. Expression of a complete soybean leghemoglobin gene in root nodules of transgenic *Lotus corniculatus*. Proc. Natl. Acad. Sci. USA 84:5754-5757.

Stougaard, J., Jorgensen, J. E., Christensen, T., Kühle, A., and Marcker, K. A. 1990. Interdependence and nodule specificity of *cis*-acting regulatory elements in the soybean *lbc*3 and N23 gene promoters. Mol. Gen. Genet. 220:353-360.

Szabados, L., Ratet, P., Grunenberg, B., de Bruijn, F. J. 1990. Functional analysis of the *Sesbania rostrata* leghemoglobin *glb*3 gene 5′-upstream region in transgenic *L. corniculatus* and *Nicotiana tabacum* plants. Plant Cell 2:973-986.

Tepfer, D. 1990. Genetic transformation using *Agrobacterium rhizogenes*. Physiol. Plant. 79:140-146.

Van Brussel, A. A. N., Tak, T., Wetselaar, A., Pees, E., and Wijffelman, C. A. 1982. Small leguminosae as test plants for nodulation of *Rhizobium leguminosarum* and other *rhizobia* and agrobacteria habouring a leguminosarum sym-plasmid. Plant Sci. Lett. 27:317-325.

Van Haute, E., Joos, H., Maes, M., Van Montagu, M., and Schell, J. 1983. Intergenic transfer and exchange recombination of restriction fragments cloned in pBR322: A noval strategy for reversed genetics of the Ti plasmids of *Agrobacterium tumefaciens*. EMBO J. 2:411-417.

Van Wordrangen, M. F., and Dons, H. J. M. 1992. *Agrobacterium tumefaciens*-mediated transformation of recalcitrant crops. Plant Mol. Biol. Rep. 10:12-36.

Vancanneyt, G., Schmidt, R., O'Connor-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.

Vervliet, G., Holsters, M., Teuchy H., Van Montagu, M., and Schell, J. 1975. Characterization of different plaque-forming and defective temperate phages in *Agrobacterium* strains. J. Gen. Virol. 26:33-48.

Vincent, J. M. 1980. Factors controlling the legume-*Rhizobium* symbiosis. Page 103 in: Nitrogen Fixation, Vol. 2. W. E. Newton, and W. H. Orme-Johnson, eds. University Park Press, Baltimore.

White, F. F., Taylor, B. H., Huffman, G. A., Gordon, M. P., and Nester, E. W. 1985. Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes*. J. Bacteriol. 164:33-44.