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High frequency transformation and regeneration of transgenic plants in the model legume *Lotus japonicus*

Jiri Stiller[1], Luca Martirani[2], Sunil Tuppale[1], Ru-Ju Chian[1], Maurizio Chiurazzi[2] and Peter M. Gresshoff[1,3]

[1] *Plant Molecular Genetics, Center for Legume Research, The University of Tennessee, 269 Ellington Plant Sciences Building, Knoxville, TN 37901–1071, USA*

[2] *International Institute of Genetics and Biophysics, Via Marconi 10, 80125 Naples, Italy*

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Abstract

The molecular analysis of plant genes involved in nodulation has been slowed by the inability to produce high numbers of transgenic legume lines. The high efficiency gene transfer and plant regeneration systems of the model legume *Lotus japonicus* is described. A collection of wild-type *A. rhizogenes* strains was tested for infectivity and the most virulent strains, 9402 and AR10, were selected for further use. Growth conditions for plantlets, induction of hairy roots and nodulation of composite plants were optimized for large-scale screening in Petri dishes. A cluster of 3–10 nodules was regularly formed on transgenic hairy roots 7–12 d after inoculation with the effective *Rhizobium loti* strain NZP2235. There were no apparent morphological differences between nodulation of hairy and wild-type roots. To test the applicability of the hairy root system for the trapping of symbiotic genes, transformation experiments with binary vectors possessing a *ß-glu*curonidase (*gus*, *uidA*) or a luciferase (*luc*) reporter driven by a cauliflower mosaic virus (CaMV) 35S promoter were performed. The frequency of cotransfer of a binary T-DNA with a root-inducing (Ri) T-DNA was 70%. Positive expression suggests that *gus* and *luc* trap vectors can be used for gene tagging in *L. japonicus*. To open the possibility of searching for mutant phenotypes, a regeneration system has been developed enabling the regeneration of large numbers of transgenic plants from hairy root cultures in about 5–6 months. At the same time, the *A. tumefaciens* hypocotyl transformation regeneration in *L. japonicus* has been improved. This new version provides fertile transgenic plants in about 4 months.

Key words: *Agrobacterium*, luciferase, nodulation, *Rhizobium*, symbiosis.

Introduction

Symbiotic interaction of leguminous plant species with bacteria of the (*Brady*)*Rhizobium* genera results in the invasive infection of plant roots and the establishment of a unique plant organ—the nodule. Inside this highly complex structure, and under nitrogen starvation, the microsymbiont converts atmospheric nitrogen to ammonia which is used by the legume in its nitrogen metabolism.

It is documented that mutual communication based on gene sets of both symbiotic partners controls nodule development and symbiotic functions. The signal molecules play a central role in these multiple cell-to-cell interactions (Long, 1996). Dozens of plant proteins, specifically expressed or up-regulated during nodulation, were identified in leguminous species. For simplicity, these proteins can be classified as early and late nodulins, depending upon the stage of nodule development when nodulin gene expression starts (Pawlowski and Bisseling, 1996). A limited number of nodulin genes have been cloned and sequenced (Kouchi and Hata, 1993; Wilson *et al.*, 1994). The establishment of hairy root transformation and nodulation procedures in several legumes, including, for example, *Lotus corniculatus*, *Vicia hirsuta* and soybean, has enabled the studies on regulation and function of nodulin genes in different genetic backgrounds (Cheon *et al.*, 1993; Quandt *et al.*, 1993; Laursen *et al.*, 1994).

Until now, most nodulin genes were isolated by differential screening or subtractive hybridization (Scheres

[3] To whom correspondence should be addressed. Fax: +1 423 974 2765. E-mail: jcrocket@utk.edu

et al., 1990; Cook *et al.*, 1995) and are usually expressed abundantly in nodules. The studies on chemically induced legume mutants and spontaneous nodulation, however, showed that, in addition to nodule 'housekeeping' genes, plant regulatory genes controlling nodulation processes exist (for review see Gresshoff, 1993). The identification of such genes by conventional genetic, biochemical and recombinant techniques is complicated and time-consuming.

An attractive alternative to the above procedures is the use of insertional mutagenesis (Berg and Howe, 1989). *Agrobacterium* T-DNA or maize transposable elements are mainly used as insertion mutagens (Dinesh-Kumar *et al.*, 1995; Lawrence *et al.*, 1995). An essential prerequisite for the application of insertional mutagenesis in molecular and genetic research of the legume/(*Brady*)*Rhizobium* symbiosis is the existence of a legume with high transformation ability. The elaboration of transformation regeneration protocols in *L. corniculatus* in the middle of the last decade represented the first breakthrough in this area (Stougaard *et al.*, 1986; Petit *et al.*, 1987). The hairy root transformation-nodulation system of *L. corniculatus* was regularly used for promoter analysis of nodulin genes (Hansen *et al.*, 1989; Szczyglowski *et al.*, 1994; Andersson *et al.*, 1997). The tetraploidy of *L. corniculatus*, however, excludes application of insertion mutagenesis or genetic map-based strategies in this species.

Recently, another member of the *Loteae*, a diploid *Lotus japonicus*, has been developed as a model legume for a determinate type of nodulation (Handberg and Stougaard, 1992; Jiang and Gresshoff, 1993, 1997; Handberg *et al.*, 1994). To investigate indeterminate nodulation, *Medicago truncatula* is widely used (Sagan *et al.*, 1995). The autogamous *L. japonicus* possesses all the characteristics necessary for molecular and genetic research: the existence of *A. tumefaciens* hypocotyl transformation and regeneration protocols, small genome size (0.5 pg), short generation time, good seed production, and fair crossing amenability. The establishment of a tagging system in *L. japonicus* by the use of a maize autonomous transposable element *Ac* (Thykjaer *et al.*, 1995), the construction of a skeletal linkage map based on molecular and morphological markers (Jiang and Gresshoff, 1997), and the construction of BAC and YAC clones (Pillai *et al.*, 1996) further favour this species, as does the existence of symbiotically altered plant mutants (J. Stougaard, F. de Bruijn, personal communication), the elucidation of nod-factor structure (H. Spaink, personal communication) and the availability of recombinant inbred lines from a cross of Gifu and Funakura (Jiang and Gresshoff, 1997).

Here the effective production of large numbers of transgenic plants derived via regeneration in *L. japonicus* is described. Furthermore, the suitability of this system for the tagging of symbiotic genes is discussed.

Materials and methods

Bacterial strains and plant material

Bacterial strains used are listed in Table 1. *Agrobacterium* strains were grown at 28 °C on LB medium (Handberg *et al.*, 1994) except 9402 (YEB medium; Vervliet *et al.*, 1975). *R. loti* NZP2235 was cultivated on YMB medium (Handberg *et al.*, 1994). Antibiotic concentrations were used as described in Van Haute *et al.* (1983). *L. japonicus* ecotype B-129–56 'Gifu' seeds were originally provided by J Stougaard, and were propagated in our Knoxville greenhouse.

Induction of hairy roots

Sterilized seeds (15 min in 3% H_2O_2 in 70% ethanol, five rinses with sterile water) were germinated on a wet filter paper pile in the dark, 24 °C for 2 d. Germinated seeds were transferred on the top of agar slopes in square Petri dishes (½ × B5 medium, ½ × B5 vitamins, no sucrose, pH 5.2, 1.2% Bacto-agar) with root tips dipped into the medium. This allows primary roots to grow into the medium and plantlets are anchored. The dishes were incubated in a growth chamber at vertical position (18/6 h day/night cycle, 20/19 °C temperature regime). Three days later plantlets with unfolded cotyledons were inoculated at the hypocotyl area with a syringe and 1–2 drops of *Agrobacterium* inoculum was applied at the wounds. The inoculum was prepared by washing the 2-d-old bacteria culture off the solid medium with sterile water (milky appearance). After inoculation plates with plantlets were kept overnight in the dark (20 °C) and then cultivated under the same conditions as above.

Nodulation of composite plants

Plantlets with 1–2 cm long hairy roots, and primary roots removed, were transfered on ½ × B5 agar slopes with ½ × B5 vitamins and 2–4 d later on B&D slopes (Broughton and Dilworth, 1971) supplied with 2–3 mM KNO_3 or 2 mM NH_4^+ (as $(NH_4)_2SO_4$) plus 1 mM KNO_3. Bacto-agar (1.4% and pH 6.8) were used in both media. Cultivation conditions were as above. Inoculation with *R. loti* NZP2235 was performed using 3-d-old culture diluted with YMB medium (1:1). Inoculum was applied by pipette directly on root tips.

Regeneration and transformation

All regeneration media are based on Gamborg's B5 medium (Gamborg, 1970) and contain B5 basal salts (Sigma, cat. no. G-5768), B5 vitamins (Sigma, cat. no. G-1019), 2% sucrose pH 5.5, and 0.6% agar (Sigma, cat. no. A-7921) if not otherwise specified. Media differ in hormone and antibiotic composition. Preparation of hormone and antibiotic stock solutions followed manufacturer instructions. Petri dishes and plant containers (Sigma, cat. no P1552) were used for cultivation of explants. Explants were subcultured every 7 d and cultivated in tissue culture chambers (Revco) with 16/8 h day/night cycle and 23/20 °C temperature regime. Axenic hairy root cultures were grown in the dark under the same temperature cycle.

(A) Hairy roots: To decontaminate, seedlings with induced hairy roots were shaken overnight in ½ × B5, 1% sucrose, 0.01% Tween20 and 500 $\mu g\ ml^{-1}$ cefotaxime sodium salt (Gemini Bio-Products, USA, cat. no. 400–115). After removal of shoots and primary roots decontaminated seedlings were grown on solid, hormone-free B5 medium (1.2% agar, 2% sucrose, 300 $\mu g\ ml^{-1}$ cefotaxime) in the dark. Two weeks later newly-grown hairy roots were cut off and transferred on fresh medium to establish axenic hairy root cultures. One centimetre long segments cut off from axenic hairy root cultures were used

as a starting material for regeneration. Shoot induction medium: 0.5 $\mu g\ ml^{-1}$ BA (Sigma, cat. no. B-9395), 0.05 $\mu g\ ml^{-1}$ NAA (Sigma, cat. no. N-0640), 10 mM NH_4^+ as $(NH_4)_2SO_4$; 150 $\mu g\ ml^{-1}$ cefotaxime added the first 3 weeks. Shoot elongation medium: 0.5 $\mu g\ ml^{-1}$ BA, 0.025 $\mu g\ ml^{-1}$ NAA. Root induction medium: ½ × B5 salts, ½ × B5 vitamins, 1% sucrose, 1 $\mu g\ ml^{-1}$ NAA. Root elongation medium: the same as root induction medium, but without NAA.

(B) Hypocotyls: Seeds sterilized as above were germinated 7 d in darkness under a 23/20 °C (16/8 h) temperature regime. Transformation was performed essentially the same way as described in Handberg *et al.* (1994) except that hypocotyls were cut into 2–3 pieces and were not cut longitudinally. Cocultivation medium: $^1/_{10}$ × B5 without sucrose, $^1/_{10}$ × B5 vitamins, 5 mM MES pH 5.2, 0.5 $\mu g\ ml^{-1}$ BA, 0.05 $\mu g\ ml^{-1}$ NAA. Regeneration medium: 0.5 $\mu g\ ml^{-1}$ BA, 0.05 $\mu g\ ml^{-1}$ NAA, 10 mM NH_4^+ as $(NH_4)_2SO_4$, 300 $\mu g\ ml^{-1}$ cefotaxime. Selection medium: as regeneration medium with 5 $\mu g\ ml^{-1}$ geneticin base (Sigma, cat. no. G-1279), 300 $\mu g\ ml^{-1}$ cefotaxime. Shoot induction medium: as regeneration medium except that 5 $\mu g\ ml^{-1}$ geneticin base was added only during the first 2 weeks, 150 $\mu g\ ml^{-1}$ cefotaxime. Shoot elongation medium: 0.2 $\mu g\ ml^{-1}$ BA, 150 $\mu g\ ml^{-1}$ cefotaxime. Root induction and root elongation media were the same as in the hairy root regeneration protocol except that half concentration of NAA (0.5 $\mu g\ ml^{-1}$) was used in root induction medium.

DNA constructs

A 2.3 kb *Bam*HI-*Xho*I fragment from the pMC81 (Chiurazzi and Signer, unpublished results) including the p35S-CODA-CAMV3' cassette (Perera *et al.*, 1993) was ligated into the pAR5 (Hansen *et al.*, 1989) to obtain the pAR5MCCODA. Homologous recombination of the pAR5MCCODA with the Ri T-DNA of MSU440 strain was performed as in Hansen *et al.* (1989). The resulting transconjugants were verified by Southern DNA analysis.

PCR analysis

Plant genomic DNA was isolated as described in Ausubel *et al.* (1987). The primer pairs used were: *rol*B1, 5'GCAATCTA TAGCCGTGAC3' and *rol*B2, 5'GGTTCCTCCGTGCGG CTG3'; *gus*5, 5'CCATCGCAGCGTAATGCTCT3' and *gus*6, 5'GCCGACAGCAGCAGTTTCAT3'; *vir*G1, 5'CGATGACG ATGTCGCTATGC3' and *vir*G2, 5'CAGCACCTCTTGCAG TCTTG3'. Amplification by *Taq* DNA polymerase was performed in 2.5 mM $MgCl_2$. Cycling conditions were 1 min at 95 °C, 1 min at 55 °C, 1 min at 72 °C for 30 cycles, followed by 5 min extension at 72 °C.

Reporter assays

An Hamamatsu photo-counting system (VIM camera, ARGUS-50 processor) was used for *in vivo* detection of luciferase activity. D-luciferin sodium salt was purchased from Molecular Probes (USA). Histochemical staining for GUS activity was performed as described (Jefferson, 1987).

Results

The establishment of hairy root nodulation

The virulence of different *A. rhizogenes* strains (Table 1) was tested on *L. japonicus* 3–10-d-old seedlings. It was possible to induce hairy roots with all strains although with different frequency (Table 2; Plate 1A). The most virulent strains, namely 9402, A4RSII and AR10, belong to the agropine type of *Agrobacterium*. No observation was made of any substantial differences in the frequency of hairy root induction between plantlets of different age. Approximately 3 d post-infection wounded hypocotyl areas became swollen and 2–3 d later, small callus started to grow from the wounds. In most strains the first hairy roots appeared about 14 d post-infection. The only exception was strain 9402 in which hairy root induction appeared 2–3 d earlier. In all strains 1–3 cm long hairy roots developed in 3–4 weeks. Based on virulence and hairy root phenotype, it was decided to use two strains in future experiments; 9402 and AR10. These strains provided satisfactory frequencies of hairy root induction, but differed in virulence, number of induced roots, and root phenotype. Strain 9402 usually induced 3–5 hairy roots per seedling and roots showed a more pronounced hairy root phenotype than AR10. In about 10–20% of seedlings inoculated with strain 9402, primary shoots and roots did not grow although a cluster of hairy roots grew up from the infection sites. On the contrary, hairy roots induced with strain AR10 showed similar morphology to wild-type roots and 1–3 roots usually grew up from the inoculation site. The influence of different inocula on the frequency of hairy root induction was also tested. Although different cultivation techniques were tried [solid versus liquid medium; washed versus unwashed bacterial pellet; liquid carriers (water, YEB, LB, B5 media) and cell titres], no major differences were observed. Finally, the simplest procedure was used, which provided satisfactory results; 2-d-old bacteria grown on solid plates were washed off by sterile water until the bacterial suspension had a milky appearance. The growth of seedlings was adversely affected by sealing Petri dishes with Parafilm™, and by higher temperatures (23–26 °C), which lowered frequency of hairy root induction.

Nodulation experiments were performed on seedlings with 1–2 cm long hairy roots and primary (i.e. untransformed) roots cut off. Composite plants were transferred on ½ × B5 agar slopes (½ × B5 vitamins) to promote seedling growth (lower nodulation frequency was obtained when this step was omitted) and 2–4 d later on B&D slopes supplied with 2–3 mM KNO_3 or combination of 2 mM NH_4^+ (as $(NH_4)_2SO_4$) with 1 mM KNO_3. *R. loti* NZP2235 inoculation was done by applying a drop of inoculum (2-d-old culture, 1:1 dilution in YMB) on newly-grown root tips. Excess of inoculum was removed by pipette. First 'bumps' could be seen by 6 d and first pink nodules by 10–12 d. Clusters of 3–10 nodules regularly developed on roots at inoculation spots or below them (Plate 1B). Approximately 12 d post-inoculation new green shoots started to grow from abaxial buds. There were no apparent morphological differences between wild-type and hairy root nodulation. Several

Table 1. *Bacterial strains used in this study*

Strain (plasmid)	Name	Characteristics	Origin
Agrobacterium ssp.			
15834 (pRi15834a, b, c)	15834	Wild type	Nester EG (UW, USA)
C58C1 (pRi15834b)	AR10	His^-, Rif^r	Hansen *et al.* (1989)
C58C1 (pRi15834b, p35S::*gus*)	AR10GUS	His^-, Rif^r, Km^r	This study
C58C1 (pRi15834b, p35S::*luc*)	AR10LUC	His^-, Rif^r, Km^r	This study
A4 (pRiA4a, b, c)	A4RSII	Rif^r, Sp^r	Pellegrineschi *et al.* (1994)
MSU440 (pRiA4b)	MSU440		Sonti *et al.* (1995)
MSU440 (pRiA4b, p35S::*gus*)		Km^r	This study
R1000 (pRiA4b)	ARqual	Sm^r	Quandt *et al.* (1993)
1855 (pRi1855)	LBA9402	Rif^r	Pellegrineschi *et al.* (1994)
1855 (pRi1855, p35S::*gus*)	9402GUS	Rif^r, Km^r	This study
1855 (pRi1855, p35S::*luc*)	9402LUC	Rif^r, Km^r	This study
K599	K599	Wild type	Savka *et al.* (1990)
8196	8196	Wild type	Savka *et al.* (1990)
LBA4404 (pAL4404, p35S..*gus*)		Km^r	This study
LBA4404 (pAL4404, p35S::*luc*)		Km^r	This study
Rhizobium loti			
NZP2235			de Bruijn F (MSU, USA)

parameters seems to be critical for hairy root nodulation in Petri dishes: Petri dishes should not be sealed; inoculation should be performed on vigorously growing seedlings; and cultivation temperature should be about 20 °C.

Hairy root regeneration

In preliminary experiments, it was found that the direct use of nodulated hairy roots for regeneration experiments was hampered by *Agrobacterium* contamination even with high levels of cefotaxime (500 $\mu g\ ml^{-1}$). To eliminate this problem axenic hairy root cultures were established. First, seedlings with nodulated hairy roots were shaken 1–2 d in liquid ½ × B5 medium supplied with 1% sucrose, 0.01% Tween20 and 500 $\mu g\ ml^{-1}$ cefotaxime to eliminate bacteria. Seedlings were then transferred on to solid hormone-free medium, 2% sucrose with 300 $\mu g\ ml^{-1}$ cefotaxime and incubated in the dark. In 2–3 weeks new hairy root cultures were started from newly-grown roots. Typically, 3–5 cm long pieces of roots including root tips were used as a starting material. About 90% of hairy root lines grew on hormone-free solid or in liquid medium vigorously and often showing disturbed geotropism (Plate 1C). The use of liquid root culture was very convenient if a large mass of roots needed to be produced. In experiments with wild-type roots no or very limited growth of roots was observed on hormone-free medium. There were no apparent differences in growth characteristics of hairy root lines induced with different *A. rhizogenes* strains. In 2–3 weeks the amount of root mass on plates was sufficient to start regeneration experiments. As an initial material, 1 cm long segments excised from root portions of different age were used. In the first experiments, the original *L. japonicus* hypocotyl regeneration protocol (Handberg and Stougaard, 1992; Handberg *et al.*, 1994) on hairy root lines induced with different *A. rhizogenes* strains (Table 2) was tested. On the callus medium, 100% callus formation was obtained in 2–3 weeks with all root lines. However, no shoot induction was observed even after 4 months of cultivation on shoot induction medium. As a next step, media were tested with different combinations of hormones. Finally, leaf and shoot induction were obtained with the combination of 0.5 $\mu g\ ml^{-1}$ 6-benzylaminopurine (BA), 0.05 $\mu g\ ml^{-1}$ 1-naphthaleneacetic acid (NAA) (Golds *et al.*, 1991) and 10 mM NH_4^+ (Fig. 1; Plate 1E, F). On this regeneration medium the ends of segments became swollen and callogenesis started in 2 weeks. In 2–4 months the calli became dark green, and leaf and shoot primordia developed. Shoot elongation was achieved on the medium with half concentration of NAA and no ammonium (0.5 $\mu g\ ml^{-1}$ BA, 0.025 $\mu g\ ml^{-1}$ NAA). Table 3 summarizes the frequency of regeneration in different hairy root lines. It was possible to regenerate seedlings from hairy root lines induced with all tested *Agrobacterium* strains. The regeneration frequency ranged from 70% to 90%. Approximately 10% of the segments grew as root teratoma-like tissues. In com-

Plate 1. Hairy root (A–F) and hypocotyl (G) transformation regeneration in *L. japonicus*. (A) Hairy root induction. Plantlets grown on agar slopes in Petri dishes (½ × B5 medium) were inoculated with *A. rhizogenes* strain AR10 at the hypocotyl. Transgenic hairy roots start to grow from the wound sites (3 weeks post-infection). (B) Hairy root nodulation. Plantlets with induced hairy roots and decapitated primary roots were transferred on B&D medium (2 mM KNO_3) and inoculated with *R. loti* strain NZP2235. The cluster of nodules has developed (7 d post-inoculation). (C) The axenic hairy root culture derived from AR10 hairy root. (D) The image of nodulation of CaMV 35S::*luc* hairy root (8 d post-inoculation, 3 min data collection). (E) Shoot regeneration from AR10 hairy root. (F) Root elongation on shoots regenerated from AR10 hairy root. (G) Selection of transgenic calli using the hypocotyl transformation. GUS-positive, geneticin-resistant calli start to grow from hypocotyl segments after 2 weeks on selection medium (GUS assay performed). *A. tumefaciens* strain LBA4404 with a 35S::GUS-INT binary vector was used in this experiment.

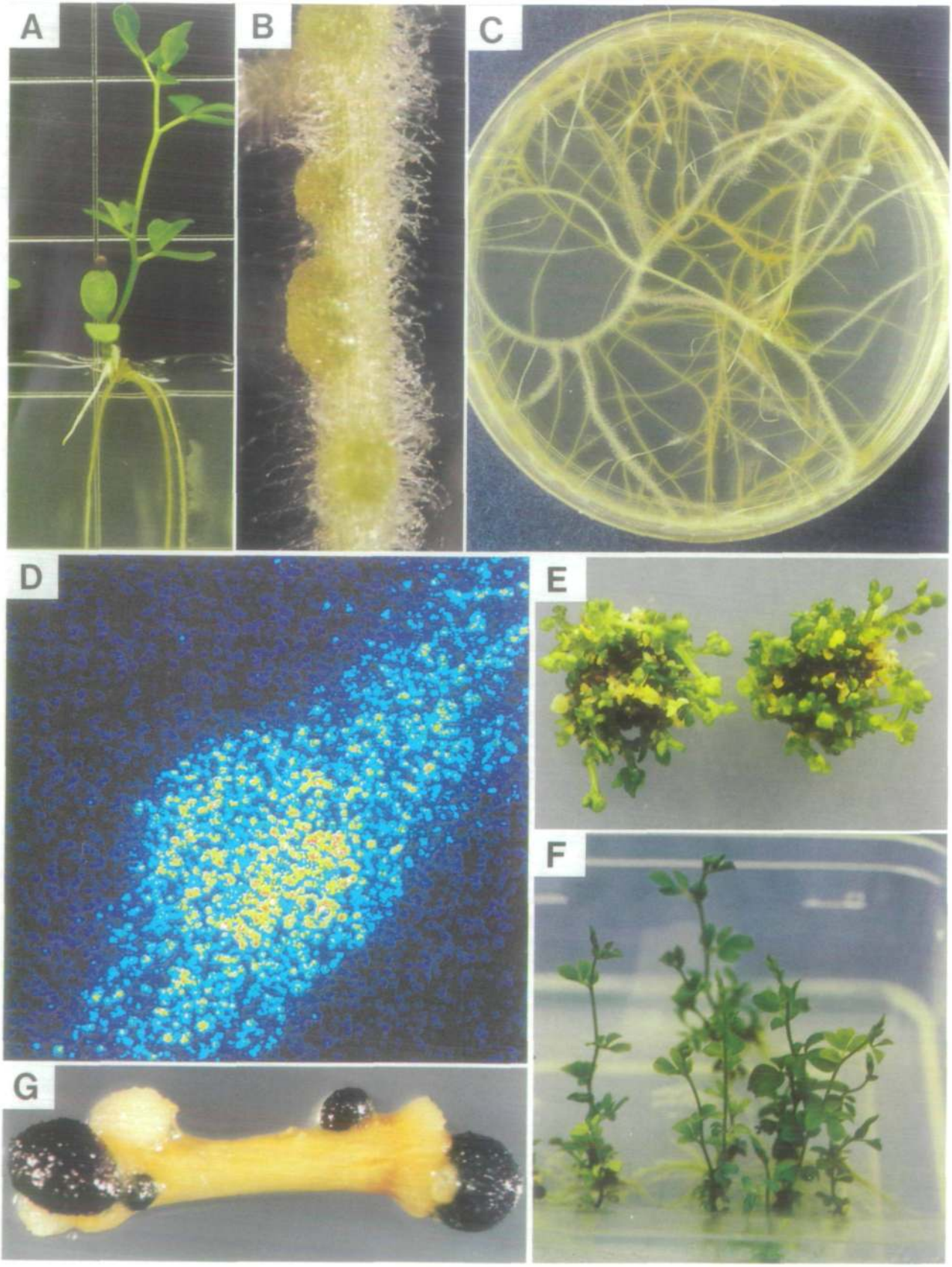

Table 2. *The infectivity of* A. rhizogenes *strains in* L. japonicus

Strain	Hairy root induction (% of plants)[a]
9402	90–100
A4RSII	80–90
15834	70–90
AR10	50–90
ARqual	50
8196	20
K599	10

[a]100–150 plants tested per strain.

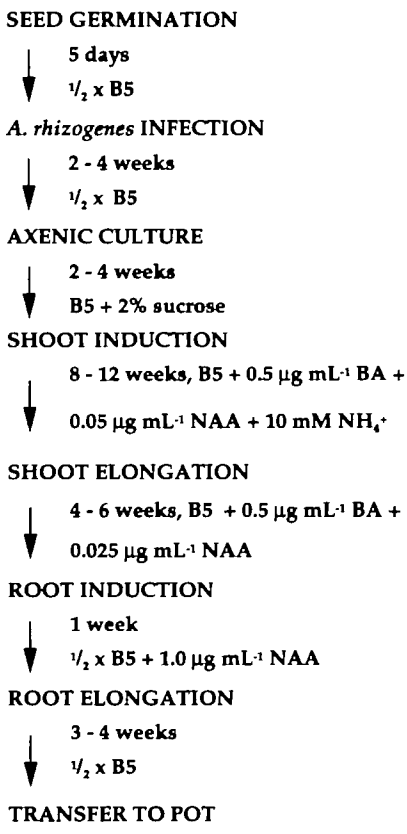

Fig. 1. The flowchart for hairy root transformation regeneration in *L. japonicus*.

Table 3. *The regeneration potential of hairy roots induced with different* A. rhizogenes *strains*

Strain	Number of tested lines	Number of regenerating lines (%)
LBA9402, 9402GUS, 9402LUC	16	15 (94)
AR10, AR10GUS, AR10LUC	36	29 (81)
15834	2	1
A4RSII	2	1
8196	2	1

parison to the standard hypocotyl regeneration protocol the induced shoots grew slowly and had shorter internodes. Root induction on shoots was performed on 0.5–1 cm long shoot cuttings using twice the NAA (1 $\mu g\,ml^{-1}$). The rooting efficiency was 90–100%. Alternatively, 95% root induction was obtained on half-strength MS hormone-free agar slopes. The transfer of plants into soil and their growth conditions were as in Handberg *et al.* (1994). Transgenic plants flowered and set seeds. Hairy root symptoms (Tepfer, 1984), such as wrinkled leaves and shortened internodes, were observed in several lines (detailed biometric analysis was not performed). The transgenic character of hairy roots was confirmed by several procedures: hairy root cultures grew on hormone-free medium contrary to wild-type roots; non-geotropic patterns of growth; PCR analysis for the presence of *rol* and *gus* genes (Fig. 3); detection of selection and reporter gene activities (Plate 1D) and absence of *Agrobacterium* chromosomal sequences (*vir*G, Fig. 3).

Hypocotyl transformation and regeneration

Although the standard hypocotyl transformation regeneration in *L. japonicus* is reliable and provided high numbers of transgenic calli, it still took 6–9 months to obtain transgenic plants in soil. Once the hairy root regeneration medium was defined, it was decided to test this medium in hypocotyl transformation to shorten the protocol. In initial experiments, hypocotyl segments from 7-d-old seedlings (germinated under light) were cultivated on the 'hairy root' medium. First leaf primordia were already seen by 1–2 weeks which was approximately 1 month earlier than in the original protocol. As a next step, transformation and regeneration experiments with an *A. tumefaciens* strain LBA4404 (pAL4404) possessing a binary vector pBINLUC (a 35S::*luc* fusion construct; Creissen *et al.*, 1995) or a p35SGUS-INT (a 35S::*gus*; Vancanneyt *et al.*, 1990), both with a neomycin phosphotransferase gene (nptII) as a selectable marker, were started. The outcome of six independent experiments was an improved hypocotyl transformation protocol presented in Fig. 2. The antibiotic geneticin (G418) was superior as a selection agent to kanamycin. About 70–90% of segments provided transgenic calli. Typically, 1–3 transgenic calli grew up from segments in 2–4 weeks (Plate 1G). In comparison to the original protocol this improved version has several advantages: (i) it provided transgenic plants more than 1 month earlier, it used long hypocotyls from seedlings germinated in the dark, which were not cut longitudinally (less laborious, 2–3 segments cut from one hypocotyl; first used by L Schauser); (ii) it did not use 2,4-D (potential mutagen) in the regeneration protocol; (iii) transgenic calli were compact, easily separable from hypocotyl segments; (iv) 60–90% transgenic calli regenerated into shoots.

Expression analysis of gus, luc *and* codA *markers in hairy roots*

To test the applicability of this hairy root short-cut system and binary vector strategy for insertional mutagenesis in

SEED GERMINATION
↓ 7 days
in dark
HYPOCOTYL COCULTIVATION
↓ 7 days, $^1/_{10}$ x B5 +
0.5 μg mL^{-1} BA + 0.05 μg mL^{-1} NAA
REGENERATION MEDIUM
↓ 5 days, B5 + 0.5 μg mL^{-1} BA +
0.05 μg mL^{-1} NAA + 10 mM NH_4^+
SELECTION MEDIUM
↓ 2 - 5 weeks, repeat +
5 μg mL^{-1} G418
SHOOT INDUCTION
↓ 3 - 6 weeks
repeat
SHOOT ELONGATION
↓ 4 - 6 weeks
B5 + 0.2 μg mL^{-1} BA
ROOT INDUCTION
↓ 1 week
$^1/_2$ x B5 + 0.5 μg mL^{-1} NAA
ROOT ELONGATION
↓ 3 - 4 weeks
$^1/_2$ x B5
TRANSFER TO POT

Fig. 2. The flowchart for hypocotyl transformation regeneration in *L. japonicus*.

L. japonicus, experiments with binary 35S::*gus* and 35S::*luc* constructs (see vectors in hypocotyl transformation) were performed. The expression (Plate 1D) and PCR analysis (Fig. 3) of hairy roots induced with strains 9402, MSU440 and AR10 possessing these constructs showed that cotransfer frequency of a binary T-DNA with a Ri T-DNA was 70%. Expression patterns of *gus* and *luc* reporters driven by 35S promoter did not differ from those in other legume species (primary expression in root tip meristem and vascular tissues, data not shown). The *luc* reporter was found as a very suitable reporter for non-destructive detection of hairy roots with integrated binary T-DNA, and seemed to be a good reporter candidate for large-scale trapping experiments in *L. japonicus*. Detection of LUC activity was simple and enabled the analysis of reporter gene expression at different developmental stages in a dozen plants at the same time. In addition, rescue of LUC positive roots is less complicated in comparison to GUS roots.

A negative selection marker, cytosine deaminase (*codA*; Perera *et al.*, 1993; Stougaard, 1993), was introduced into the Ri T-DNA of *A. rhizogenes* strain MSU440 and induced hairy roots were tested for negative selection.

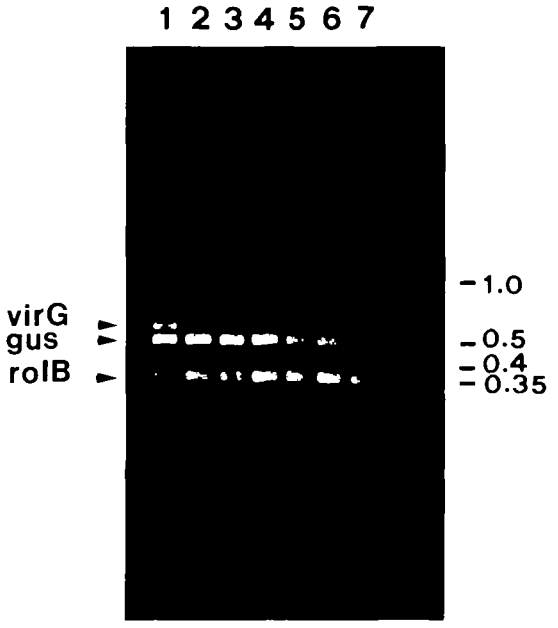

Fig. 3. PCR analysis of the MSU440 (pA4b, p35S::GUS-INT) hairy root lines for the presence of *rolB*, *virG* and *gus* genes (for primer sequences, DNA isolation and PCR conditions, see experimental procedures). Lane 1, *Agrobacterium* DNA; lanes 2–7, 6 independent hairy root lines. *rolB*, *virG* and *gus* amplified fragments are indicated by arrowheads. Molecular weights are indicated in kilobases.

The growth of hairy roots possessing the CaMV 35S::*cod*A construct was inhibited at the concentration of 500 μg ml^{-1} 5-fluoro-cytosine (5-FC) in 2 weeks (data not shown). It was assumed that double selection of transgenic progeny (5-FC plus selection marker on a binary T-DNA) could improve the probability of selecting segregants with only a binary T-DNA.

Discussion

The development of transformation and regeneration procedures in diploid legume species opened the possibility to isolate genes controlling the nodulation process by application of insertion mutagenesis approach.

The critical parameter of any insertional mutagenesis project is the feasibility with which the large number of plant lines with independent insertions of insertion elements can be produced. In *L. japonicus* insertion mutagenesis using T-DNA or transposable elements is currently pursued by several laboratories (Thykjaer *et al.*, 1995; J Webb, personal communication).

While the transposon strategy can be initiated with a limited number of transgenic lines, a system for large-scale production of transgenic lines is a prerequisite for T-DNA mutagenesis. The original *L. japonicus* transformation regeneration was based on hypocotyl transformation using a disarmed *Agrobacterium* system. This protocol enabled one to produce about 1000–2000 transgenic plants year^{-1} person^{-1}. However, this procedure

was still time consuming. It took 6–9 months to obtain fertile transgenic plants. Space for plant propagation and seed collection represented another obstacle in this procedure.

Agrobacterium rhizogenes hairy root short-cut systems were successfully applied for the molecular analyses of symbiotic genes in several legumes (Cheon *et al.*, 1993; Laursen *et al.*, 1994; Andersson *et al.*, 1997). The principal advantage of the hairy root system is that hundreds of independent transgenic roots can be produced in a short time and nodulation monitored directly on composite plantlets. In *L. japonicus* it was found that virulence of tested *A. rhizogenes* strains and nodulation data are comparable to those in *L. corniculatus* (Hansen *et al.*, 1989). Growth conditions for plantlets, induction of hairy roots and nodulation of composite plants for large-scale screening in Petri dishes, were optimized. Frequencies of 50–90% hairy root induction capable of normal nodulation in 7–12 d can be achieved using strains 9402 and AR10.

To assess suitability of using a binary vector strategy (Stougaard *et al.*, 1987) for trapping of symbiotic genes in *L. japonicus*, transformation experiments with a binary vector pBINLUC or a p35SGUS-INT were performed. The cotransfer frequency of 70% is in good accordance with the data available in other legumes (Stougaard *et al.*, 1987; Quandt *et al.*, 1993). The data on hairy root induction and normal nodulation suggest that composite plants of *L. japonicus* can be used as a tool for analyses of nodule-specific genes. Expression patterns of *luc* and *gus* genes showed that these genes can be considered as reporters for gene trapping in *L. japonicus*.

Regeneration of transgenic plants from hairy roots, which enabled plantlet regeneration from 70–90% of hairy root lines in 5–6 months, was described. The regenerated transgenic plants were fertile and developed seeds. Based on the hairy root regeneration medium, the simpler and shorter version of the hypocotyl transformation regeneration was developed. Using this system, seedlings prepared for the transfer into soil were obtained in 3.5–4.5 months.

One of the possible limitations of using composite plants as a tool for insertional mutagenesis is the oncogenic character of Ri T-DNA. The Ri T-DNA contains *rol* genes which are hypothesized to interfere with hormone signal transduction pathways in plant cells (Filippini *et al.*, 1996). It was found that *rol* gene expression does not interfere with nodulation in *L. japonicus* (cf. Beach and Gresshoff, 1988). Nevertheless, pleiotropic effects related to the *rol* genes expression in transgenic plants were reported (Schmülling *et al.*, 1988). Thus, phenotypes of tagged plant lines would be better analysed in a genetic background without *rol* genes. Segregation of the Ri T-DNA away from the binary T-DNA in the offspring of transgenic plants was recently reported (Otani *et al.*, 1996; Webb *et al.*, 1994). To assist segregation analysis, a negative selection marker, the *cod*A gene, into the Ri T-DNA of MSU440 strain was built. This marker enables efficient negative selection in *L. japonicus* and tobacco at the level of seedling germination (Stougaard, 1993).

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