TECHNICAL ADVANCE

***Agrobacterium rhizogenes* Transformation of the *Phaseolus* spp.: A Tool for Functional Genomics**

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Submitted 24 March 2006. Accepted 7 July 2006.

A fast, reproducible, and efficient transformation procedure employing *Agrobacterium rhizogenes* was developed for *Phaseolus vulgaris* L. wild accessions, landraces, and cultivars and for three other species belonging to the genus *Phaseolus*: *P. coccineus*, *P. lunatus*, and *P. acutifolius*. Induced hairy roots are robust and grow quickly. The transformation frequency is between 75 and 90% based on the 35-S promoter-driven green fluorescent protein and β -glucuronidase expression reporter constructs. When inoculated with *Rhizobium tropici*, transgenic roots induce normal determinate nodules that fix nitrogen as efficiently as inoculated standard roots. The *A. rhizogenes*-induced hairy root transformation in the genus *Phaseolus* sets the foundation for functional genomics programs focused on root physiology, root metabolism, and root–microbe interactions.

Additional keywords: common bean, T-DNA, transgenic nodules.

Grain legumes are one of the most valuable sources of dietary proteins for both humans and domestic animals. *Phaseolus vulgaris* (common bean) is a basic staple food providing more than 70% of the dietary protein for poor people in Latin America and Eastern Africa; it is half of the grain legume consumed by humans worldwide (Broughton et al. 2003). The yields of common beans are low, and the quality of seed proteins is suboptimal. Selection over millennia for stable rather than high yield is the most likely explanation for these characteristics. This problem can be addressed by modern genetic techniques, provided that plant transformation is routine.

Production of genetically modified crops requires efficient techniques for DNA integration into the genome and plant regeneration methods. Plant transformation is a prerequisite for the molecular and physiological analysis of gene function. Many cultivated grain legumes are recalcitrant to current transformation technologies, limiting the possibilities for functional genetic studies. In particular, the genus *Phaseolus* has been extremely difficult to regenerate in vitro, creating a bottleneck for an efficient plant transformation protocol. Particle bombardment-mediated systems have been developed to obtain

transgenic plants of *P. vulgaris*; however, these are labor intensive and the transformation frequencies still have to be improved (Aragão et al. 2002; Christou 1997). Only one successful method using *Agrobacterium tumefaciens* for the production of transgenic plants in *P. acutifolius* has been reported for a few accessions and commercial cultivars (Zambre et al. 2005).

In addition to forming symbiosis with mycorrhizal fungi, many legume plants, when interacting with bacteria collectively known as Rhizobia, respond by forming nitrogen-fixing root nodules. A precise exchange of molecular signals between rhizobia and the host plant over space and time is essential for the development of an effective root nodule. Rhizobia enter through root hairs and infect root cortical cells. Once there, they differentiate into bacteroids before the nitrogen-fixation process begins. During initial developmental changes, plant early-nodulin genes (Oldroyd et al. 2005) are expressed and, at later stages, during nodule formation and functioning, a set of nodule-enhanced and nodule-specific genes (Mylona et al. 1995) also are induced.

Legume genomics has been focused primarily on the improvement of genetic resources and information on *Lotus japonicus* and *Medicago truncatula*. These two species are forage crops and have been adopted as legume model plants based on their small genome and plant size, diploid state, and number of seed produced, among other desirable traits (Udvardi et al. 2005). *Glycine max* (soybean) is a crop for which numerous genomic studies have been initiated because of its worldwide economic importance (Stacey et al. 2006). Recently, hundreds of differentially expressed gene transcripts were identified by microarray analysis during the early and late symbiotic interaction stages between the model legume *M. truncatula* and *Sinorhizobium meliloti* (El Yahyaoui et al. 2004). From these studies, it has been inferred that multiple potential plant regulators are part of the symbiotic program in addition to genes related to bacterial infection, nodule formation and functioning, defense response, and signal-transduction proteins (El Yahyaoui et al. 2004; Lohar et al. 2006). Currently, *G. max*, *L. japonicus*, and *M. truncatula* are the focus of genome sequencing and functional genomics programs (Udvardi et al. 2005). Recently, the Cross-legume Advances Through Genomics (CATG) Conference convened to take advantage of current research tools and technologies to develop robust genomic resources in reference legumes, including common bean (Gepts et al. 2005). In this regard, Ramirez and associates (2005) have established an

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initial platform for functional genomics of common bean by the identification of almost 8,000 unique genes assembled from 20,000 expressed sequence tags (ESTs) obtained from various organs, including nitrogen-fixing nodules. Common bean has a similar or slightly larger genome compared with other legume model plants (approximately 600 MBp/haploid genome) (Gepts et al. 2005). The rationale to sequence the genome would be stronger if the transformation was routine, permitting high-throughput functional genomics.

A. rhizogenes is a soil bacterium responsible for development of hairy root disease on a range of dicotyledonous plants (Tepfer 1990). Infection at the wounding sites by *A. rhizogenes* results in the transfer, integration, and expression of T-DNA (TR-DNA and TL-DNA in agropine strains) from the root-inducing (Ri) plasmid into the plant cells (Grant et al. 1991). The expression of this integrated T-DNA in plant cells induces transformed roots and synthesizes certain low molecular weight carbon compounds called opines (Petit et al. 1983). There are four opine-type Ri plasmids in *A. rhizogenes*, including agropine, mannopine, cucumopine, and mikimopine (Savka et al. 1990). *A. rhizogenes* strain K599 (NCPB2659) used in this work is a cucumopine-type strain (Savka et al. 1990) that has only one T-DNA fragment in the Ri plasmid (Combard et al. 1987).

A. rhizogenes-mediated root transformation has been described for a number of legumes: *L. corniculatus* (Petit et al. 1987), *Trifolium repens*, (Díaz et al. 1989), *T. pratense* (Díaz et al. 2000), *Vigna aconitifolia* (Lee et al. 1993), *G. max* (Cheon et al. 1993), *Vicia hirsuta* (Quandt et al. 1993), *L. japonicus* (Stiller et al. 1997), *M. truncatula* (Boisson-Dernier et al. 2001), and *Sesbania rostrata* (Van de Velde et al. 2003). Legume composite plants with a transformed root but untransformed shoot can be nodulated by rhizobia and colonized by mycorrhizal fungi (Oldroyd et al. 2005). Provided that root transformation is efficient, such composite plants are an alternative to generating stable transgenic lines.

We document a simple, fast, and reproducible protocol using *A. rhizogenes* to produce hairy roots on several wild accessions, landraces, and cultivars of *P. vulgaris* and three other species within *Phaseolus*. The induction of transgenic roots in the genus *Phaseolus* offers a new strategy for overexpressing or suppressing endogenous genes, and the method can be scaled up readily to perform functional genomics focused on root biology and root-microbe interactions.

RESULTS

A. rhizogenes-mediated root transformation in the genus *Phaseolus*.

Induction of *A. rhizogenes*-mediated root transformation in *Phaseolus* spp. was modified from a protocol originally developed for *G. max* using *A. rhizogenes* strain K599 (Bond and Gresshoff 1993). Several common bean and *Phaseolus* landraces, wild accessions, and cultivars were highly susceptible to the *A. rhizogenes* K599 strain and produced hairy roots. Briefly, for hairy root induction, bean seedlings were infected with *A. rhizogenes* K599 by wounding the cotyledonary nodes with a syringe (Fig. 1A). Later, sprouts were transferred to a tray covered with a plastic lid (Fig. 1B). Over 5 to 8 days, a globular tumor developed on the stem at the wound site in *Phaseolus* plants. (Fig. 2A, a to g). Visible hairy roots emerged from the tumor as early as 1 week after infection (Fig. 2B, a to c). At 3 weeks postinfection, between 85 and 100% plants had abundant hairy roots (Fig. 2B, b; Table 1). Conversely, none of the plants injected with water produced tumors or roots at the wounded sites (data not shown). Hairy roots are distinct from normal roots because they have altered geotropic behavior and a high degree of branching (Odegaard et al. 1997; Tepfer 1987). The efficiency to induce hairy roots of different *A. rhizogenes* strains was evaluated in several *Phaseolus* spp. Strain K599 (cucumopine) was the most efficient at inducing hairy roots. High transformation efficiency rates (75 to 90% frequency) were reproducibly found in three independent experiments with different *P. vulgaris* wild accessions, landraces, and cultivars analyzed (Table 1). Strains A4 and A15834 (agropine type) induced hairy roots at 75 to 90% and 40 to 50% frequencies only in vaquita and peruano landraces, respectively (data not shown). No significant results were obtained with the other cucumopine and mannopine *A. rhizogenes* strains tested (data not shown).

Analysis of β -glucuronidase and green fluorescent protein expression in hairy roots.

To demonstrate gene transfer, β -glucuronidase (GUS) and green fluorescent protein (GFP) expression were monitored (see Materials and Methods). Hairy roots in all *Phaseolus* spp. tested expressed both reporter genes (Fig. 3). For example, GUS staining was confirmed in hairy roots induced by *A. rhizogenes* K599 harboring the binary vector p35SGFPGUS + ([GFP])-intron [catalase]-*uidA* gene fusions) (Fig. 3B to E);

Fig. 1. *Agrobacterium rhizogenes* infection of bean plantlets. **A**, Five-day-old bean plantlets were infected with 10 to 15 μ l of an *A. rhizogenes* fresh culture by wounding into the cotyledonary nodes, as shown. **B**, After infection, plantlets were kept in a plastic tray covered with a transparent lid, as described in Materials and Methods.

conversely, no GUS expression was detected in *A. rhizogenes* K599-transformed roots (Fig. 3A). Intron removal in the nucleus is a prerequisite for GUS activity to be expressed (Vancanneyt et al. 1990). Based on the high transformation frequencies found (Table 1) and the GUS and GFP expression results (Fig. 3E and F, respectively), we conclude that the expression of the reporter genes in hairy roots reflects nuclear integration events.

Nodule development in *P. vulgaris* hairy roots.

Between 80 and 95% of the transformed hairy roots of *P. vulgaris* cv. Negro Jamapa expressing GFP (Fig. 4A) can form nodule primordia when inoculated with the *Rhizobium tropici* CIAT899 strain (Fig. 4B). These primordia developed into determinate nodules (Fig. 4C and D) that fix nitrogen. *R. tropici* CIAT899 was used because of its proven wide host nodulation range with Andean and Mesoamerican cultivars (Martínez-Romero et al. 1991). On average, more than 90% of *A. rhizogenes*-induced hairy roots (Table 1) had a similar number of nodules that developed with kinetics similar to those found in standard roots (data not shown). We found a range of 60 to 300 and 8 to 370 nodules in standard and transformed roots (nine plants each), respectively.

In addition, GFP expression was detected in fresh half-sectioned nodules (100 µm) (Fig. 4C and D) and also was observed in 3-µm sections of methacrylate-embedded transgenic nodules, visualized by laser scanning confocal microscopy (LSCM) (Fig. 4E and F). Vascular bundles in the nodule cortex, and infected and uninfected cells in the nodule central zone had the tissue organization typical of determinate common bean nodules (Fig. 4C to F). To compare the ultrastructure of *P. vulgaris* transformed (Fig. 5A, C, and E) and standard nodules (Fig. 5B, D, and F), 3-µm sections of methacrylate-embedded nodules were analyzed by low-vacuum scanning electron microscopy (LVSEM). Both transformed and standard nodules showed similar nodule morphology (Fig. 5A and B) and internal structure of infected and uninfected cells (Fig. 5C and D). Furthermore, infected cells appear to be filled with a similar number of symbiosomes, which exhibited the same elongated shape (Fig. 5E and F).

Nitrogen fixation in *P. vulgaris* hairy roots.

Nitrogenase activity in standard and transformed nodules was comparable in *P. vulgaris* cv. Negro Jamapa plants nodulated with *R. tropici*. Nitrogenase was assayed as acetylene reduction (Vessey 1994). Specific activity based on this reaction

Fig. 2. *Agrobacterium rhizogenes* K599-induced globular calli and hairy roots in diverse *Phaseolus* cultivars, landraces, and wild accessions of the genus *Phaseolus*. **A**, a to g, *Phaseolus vulgaris* and other *Phaseolus* spp. were injected in the cotyledonary nodes. Emergence of globular tumors in wounded regions are indicated with a white arrow. a, *P. vulgaris* cv. Negro Jamapa; b, *P. vulgaris* Peruano landrace; c, *P. vulgaris* “vaquita” landrace; and d, *P. vulgaris* wild accession from Morelos; e to g, Globular calli induced in three different *Phaseolus* spp.; e, *P. coccineus* Oaxaca landrace; f, *P. acutifolius* Chiapas landrace; g, *P. lunatus* cultivar from Peru. **B**, Composite *P. vulgaris* plants with hairy roots emerging from globular tumors formed at wounded sites. a, Flor de Mayo; b, cv. Negro Jamapa plant with a fully grown hairy root system, and c, Negro Querétaro 12, 20, and 8 days after inoculation, respectively.

Table 1. *Phaseolus* cultivars, landraces, and accessions transformed with *Agrobacterium rhizogenes* K599 strain with the binary vector p35SGFPGUS+

Region, species	Name	Type	Induced hairy roots/total plants ^a	Approximate transformed (GUS+)/ total hairy roots (%) ^b
Mesoamerican				
<i>Phaseolus vulgaris</i>	Negro Jamapa	Cultivar	49/50* €	95
	Negro Queretaro	Landrace	18/20* €	75
	Flor de Mayo	Landrace	20/20*	90
	Morelos	Wild accession	6/7 †	90
	DOR364	Cultivar	17/20*	75
<i>P. acutifolius</i>	Vaquita	Landrace	19/20* €	90
	Morelia	Wild accession	9/10 †	90
	Chiapas	Landrace	11/12 †	75
<i>P. coccineus</i>	Morelos	Wild accession	5/5 †	ND
	Oaxaca	Landrace	10/11 †	ND
Andean				
<i>P. vulgaris</i>	Peruano	Landrace	19/20*	85
<i>P. lunatus</i>	UNALM-1	Cultivar	6/7 †	75

^a Symbols: * = three different experimental trials, † = duplicated trials, and € = nitrogen fixation (acetylene reduction assay) was assayed in three individual inoculated whole hairy roots (three times). Nitrogen fixation activity was similar to standard inoculated roots.

^b GUS = β-glucuronidase and ND = not determined.

was $0.54 (\pm 0.18) \mu\text{M}$ ethylene per milligram of protein per hour in standard nodulated roots and $0.50 (\pm 0.25) \mu\text{M}$ ethylene per milligram of protein per hour in nodulated hairy roots. These results are the average of triplicate replicates with three whole roots in each individual assay ($n = 9$).

Polymerase chain reaction and Southern blot analysis.

Total DNA from hairy roots of *P. vulgaris* cv. Negro Jamapa, and Negro Queretaro and Flor de Mayo landraces, was isolated and digested with *EcoRI* and *EcoRV*, which cut one and four times, respectively, within the T-DNA (Fig. 6A). Restriction-digested DNA then was blotted and hybridized with the GFP gene as a probe. We found a 2,047-bp band when DNA was digested with *EcoRV*, which matches to the expected GFP gene-containing fragment size (Fig. 6A and B). A single hybridization band (approximately 10 kb) was obtained in *EcoRI*-digested DNA, suggesting a single T-DNA integration event (Fig. 6B). Standard roots used as a control do not show any band (data not shown). The presence of the T-DNA in total DNA from hairy roots was assessed by polymerase chain reaction (PCR) amplification of the *rolB* gene. Conversely, the *virD* gene was not found in transformed plant DNA samples, indicating that plant tissues were free of *A. rhizogenes* (Fig. 6C).

Fig. 3. β -Glucuronidase (GUS) and green fluorescent protein (GFP) expression in *Phaseolus vulgaris* transgenic roots and nodules; **A**, *Agrobacterium rhizogenes* K599 and **B**, *A. rhizogenes* K599 with p35SGFPGUS+ induced hairy roots showing none and approximately 85 to 90% GUS expression, respectively; **C**, **D**, and **E**, stereo-micrographs of **C**, transgenic nodules; **D**, roots with determinate nodules showing diverse levels of GUS expression; **E** and **F**, fresh nodule sectioned in half showing in **E**, GUS expression and **F**, GFP expression by laser scanning confocal microscopy.

DISCUSSION

The reliable hairy root transformation protocol in *Phaseolus* spp. described here provides a key tool that was previously missing. High-throughput functional genomics on root biology and root-microbe interactions is now possible in this genus. *P. vulgaris* has a relatively small genome size, and it is a diploid organism with many progeny (McClean et al. 2004).

The requirement for an additional model plant for this economically critical crop is highlighted by recent comparative gene analysis. Ramírez and associates (2005) found that, when 340 nodule-specific *M. truncatula* EST contigs identified by Fedorova and associates (2002) were compared by BLAST-N against 228 nodule-specific common bean EST sequences, only 25% of common bean ESTs had a homolog at $E < 10^{-12}$ in *M. truncatula*. Thus, in addition to a core of common plant nodulation genes, a substantial number (approximately 75 to date) of common bean-specific nodule genes are expressed. Furthermore, comparison of the 20 most abundant contigs in soybean nodules to those in *P. vulgaris* identified only leghemoglobins and sucrose synthase as shared, strengthening the

Fig. 4. Green fluorescent protein (GFP) visualization by laser scanning confocal microscopy in transgenic tissues of *Phaseolus vulgaris* cv. Negro Jamapa inoculated with *Rhizobium tropici* CIAT899. Single optical sections from **A**, root tip; **B**, transgenic nodule primordium; **C**, half-sectioned fresh transgenic nodule; **D**, merged images from the previous nodule section (GFP expression and dark field) showing the typical morphology of a determinate nodule; **E** and **F**, 3- μm methacrylate-embedded transgenic nodule sections treated for GFP expression visualization; **E**, $\times 20$ micrograph showing the periphery and central tissue of a transgenic nodule; **F**, $\times 40$ micrograph showing details from the nodule central zone (NCZ). Abbreviations: infected cells (IC), uninfected cells (UC), epidermis (E), nodule cortex (NC), vascular bundles (VB), root meristem (RM), and root central cylinder (RCC). Scale bars: A to D, 100 μm ; E, 50 μm ; and F, 20 μm .

rationale for a robust functional genomics platform in common bean (Ramírez et al. 2005). The gap to reaching this objective has been the lack of reproducible and speedy transformation protocols reflecting the recalcitrance of *P. vulgaris* to in vitro regeneration. *A. tumefaciens*-assisted transformation is a standard protocol successfully applied to generate transgenic legume plants of some *Phaseolus* spp., although the frequency of transformation for *P. vulgaris* has been very low (Zhang et al. 1997). Furthermore, there is only one report where *A. tumefaciens* was used to achieve expression of a *lea* gene that conferred abiotic tolerance in *P. vulgaris*. This very recent protocol is based on sonication and vacuum-assisted *A. tumefaciens*-mediated transformation of dry embryos attached to one cotyledon (Liu et al. 2005). Although this novel and cheap transformation method looks quite promising, the transformation efficiency (2 to 12%) must be improved.

A fast, reproducible, and efficient transformation procedure is crucial not only for gene function studies but also to allow crop improvement. In our hairy root induction protocol with the *A. rhizogenes* K599 strain, transformation frequencies up to 90% can be obtained for several *P. vulgaris* cultivars, landraces, and accessions and three other *Phaseolus* spp. (Table 1). The procedure is simple and has been successful when performed independently by five different laboratories (data not shown). Previous attempts to induce hairy roots with strains of

A. rhizogenes other than *A. rhizogenes* K599 were unsuccessful or gave poor results (McClellan et al. 1991). The susceptibility of bean plants to *Agrobacterium* strains varies greatly. Significant differences were observed between the transformation ability of different strains of *A. rhizogenes*. We tested four strains of *A. rhizogenes* to induce hairy roots on four species of *Phaseolus*, and the strain K599 (cucumopine type) was the most effective in inducing hairy roots. The mannopine-type (A8196), agropine-type (A4 and A1584), and cucumopine-type (A2659) strains of *A. rhizogenes* tested were poorly effective or completely ineffective in inducing hairy roots on common bean and other species. Hairy root formation also depended on the host plant genotype (data not shown).

A. rhizogenes-induced hairy roots in *P. vulgaris* produce determinate nodules that fix nitrogen as well as standard roots. The structure of common bean transgenic nodules was a major concern because Akasaka and associates (1998) reported that, in *Arachis hypogaea* L. (peanut), some nodules produced on transformed roots exhibited abnormal phenotypes. Although peanut generally has determinate nodules, enlargement of the nodule cortex region (NCR) in transformed nodules made them look like indeterminate nodules. Similar morphological alterations of *Agrobacterium rhizogenes*-mediated transformed root nodules in soybean previously were reported by Bond and Gresshoff (1993). In soybean, the altered anatomy probably re-

Fig. 5. Low vacuum scanning electron microscopy (LVSEM) analysis of *Phaseolus vulgaris* transgenic and standard nodule sections. The 3- μ m methacrylate-embedded nodule sections were treated to remove methacrylate, followed by gold coating the surface for scanning electron microscopy (SEM) analysis; **A** and **B**, whole nodule sections of **A**, transformed and **B**, standard nodules; **C**, transgenic infected and uninfected cells within the nodule at $\times 1,500$; **D**, infected and uninfected cells within the standard nodule at $\times 1,500$; **E** and **F**, infected cells filled with symbiosomes at $\times 5,000$ from **E**, transformed and **F**, standard infected cell. Abbreviations: infected cells (IC), uninfected cells (UC), central zone (CZ), nodule cortex (NC), and symbiosomes (S).

flects the abnormal presence of multiple active meristems in the NCR. More than one meristem causes such nodules to become elongated and branched, resembling indeterminate nodules of *Pisum sativum* and *M. truncatula*. The internal tissue

Fig. 6. A to C, Binary vector T-DNA, Southern blot, and polymerase chain reaction (PCR) analysis from hairy roots total DNA. **A**, Diagram of the T-DNA region from the binary vector p35SGFPGUS+ is shown; T-DNA is flanked with RB (right) and LB (left) borders. Unique *EcoRI* and three *EcoRV* restriction sites are indicated. The β -glucuronidase (GUS) gene with a catalase intron, the s65t variant green fluorescent protein (GFP) fused to a 35S promoter, and the hygromycin resistance gene (HPTII) fused to a *Cauliflower mosaic virus* promoter (CaMV35S) also are shown. **B**, Total DNA from hairy roots of *Phaseolus vulgaris*: Flor de Mayo (FM), Negro Queretaro (NQ), and Negro Jamapa (NJ) were digested with *EcoRI* and *EcoRV*. A DNA fragment carrying the *gfp* gene (750 bp) was 32 P labeled and used as a probe. DNA hybridization bands are indicated by arrows. MWM sizes are indicated to the left. **C**, DNA fragments from *rolB* (438 bp) and *virD* (423 bp) were PCR amplified using specific primers from the *Agrobacterium rhizogenes* A4 strain (see Materials and Methods). Amplified products from DNA isolated from standard and hairy roots of *P. vulgaris* cv. Negro Jamapa, *A. rhizogenes* A4; K599, and K599/p35SGFPGUS+ strains are shown; 1-kb ladder is shown to the right.

organization of nodule sections from transformed and standard *Phaseolus* nodules was analyzed by LSCM and LVSEM (Figs. 4E and F and Fig. 5, respectively). No obvious changes in the number of infected and uninfected cells and internal tissue organization of transformed (Fig. 5A, C, and E) and standard nodules (Fig. 5B, D, and F) were found. These data indicate that additional plant hormones produced in the *A. rhizogenes*-mediated transformation in *Phaseolus vulgaris* do not cause morphological alterations in transformed nodules.

The p35SGFPGUS+ binary vector used in the present protocol contains the GFP-intron (catalase)-*uidA* gene construct (Fig. 6A); both reporter gene activities are designed to be expressed only in plant tissues (C. Vickers, unpublished).

DNA prepared from hairy roots of different *P. vulgaris* cultivars was digested with different restriction enzymes, blotted, and hybridized with GFP as probe. One main hybridization signal (approximately 10 kb) was present in all transformed bean DNA samples analyzed with *EcoRI*. When plant DNA was digested with *EcoRV*, a 2.047-kb hybridization band corresponding to the expected size for the GFP gene was obtained. Similar results were observed in *P. acutifolius* wild accession (Morelia) and Peruano landrace (data not shown). No other fragments were detected, suggesting single-copy integration. Similar T-DNA single integration events have been reported in *Catharantus roseus* (Batra et al. 2004). Additionally, *rolB* PCR amplification of DNA isolated from hairy roots was used to verify the presence of the Ri T-DNA, and *virD* to monitor whether transformed plants were free of *A. rhizogenes* K599 (Fig. 6C).

In conclusion, no major differences were observed in the size, shape, nodule number, or color between nodules developing on transformed and standard plants. Nitrogen fixation also was equivalent for transformed and standard nodules.

Work in progress currently is focused on designing functional genomics tools for diverse root and nodule-specific genes employing miRNA, antisense, and cDNA-GFP fusions. The aim is to generate and select knockdown and gain-of-function mutant lines in transgenic common bean roots and nodules using high-throughput methods.

MATERIALS AND METHODS

Plant materials.

Mesoamerican *P. vulgaris* cv. Negro Jamapa, and landraces Negro Querétaro, Flor de Mayo, and Vaquita, were obtained from the local market. Cv. DOR364 (Davis et al. 1988) and other *Phaseolus* spp., landraces, and wild accessions, including *P. acutifolius*, landrace from Chiapas, México and a wild accession obtained from Morelia, México; and *P. coccineus* landrace from Oaxaca, México and a wild accession collected from Morelos, México, were obtained from the Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias collection. *P. vulgaris* Peruano, a South American landrace, was obtained from the local market. *P. lunatus* cv. UNALM-1, an Andean cultivar, was provided by E. Ormeño at Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México (UNAM), Cuernavaca.

A. rhizogenes strains and binary vector.

Cucumopine-type *A. rhizogenes* strain K599 (Bond and Gresshoff 1993) was used to infect cotyledon axes regions. *A. rhizogenes* K599 with p35SGFPGUS+ was grown in Luria-Bertani (LB) medium containing kanamycin (Km) at 50 μ g ml⁻¹. To get fresh cells, *Agrobacterium* was grown on LB plates containing Km and incubated overnight at 30°C. Cells were collected from these plates and diluted into 1 ml of sterile water in an Eppendorf tube. For control inoculations, a fresh

culture of *A. rhizogenes* K599 lacking the binary vector was grown in LB medium without antibiotics. A4 was grown in yeast extract-mannitol solid medium without antibiotic. All chemicals were from Sigma-Aldrich (St. Louis). Agar was from Difco Laboratories (Detroit).

The p35SGFPGUS+ binary vector (C. Vickers, *unpublished*) was constructed from a 1.82-kb fragment containing the 35S promoter (approximately 850 bp) and the GFP (approximately 720 bp)-*nos* terminator (approximately 250 bp) regions which were subcloned from p35SGFP as a *HindIII/EcoRI* fragment and subsequently ligated into pCambia1305.1 (GenBank AF354045) that was similarly digested with *HindIII* and *EcoRI*. This binary vector has a 35S promoter construct with two reporter genes: β -glucuronidase (*uidA*) fused to a catalase intron, followed by the GFP gene (p35SGFPGUS+).

Seed germination and infection with *A. rhizogenes*.

Legume seed were surface sterilized by an initial treatment with 100% ethanol for 1 min, rinsed with sterile water, treated with sodium hypochlorite for 5 min (except *P. lunatus*, which was treated for 2 min only), and followed by five rinses with sterile water. Bean seed were transferred to trays containing a wet paper towel; trays were covered with foil and held at 28°C until seed germination (approximately 3 days). Sprouts then were transferred to small plastic pots containing vermiculite, watered with B&D solution (Broughton and Dilworth 1971) supplemented with 8 mM KNO₃, and incubated in a growth chamber (light and dark for 16 and 8 h, respectively; temperature = 25 to 28°C). Five days after planting, plantlets with unfolded cotyledons were inoculated by injection directly into the cotyledonary nodes with a syringe delivering approximately 5 to 10 μ l of inoculum into the wound. To transform bean plantlets, the needle had to cross the vascular bundles (Fig. 1A). Each plant stem was wounded three times at different positions around the node. Typically, bean plants infected by *A. rhizogenes* started to show tumors approximately a week after inoculation. Later, sprouts were transferred to a tray and covered with a plastic lid (Fig 1B) and incubated in a growth chamber (light and dark for 16 and 8 h, respectively; temperature = 25 to 28°C).

Hairy root emergence and plant cultivation.

Twelve days after *A. rhizogenes* inoculation, plantlets exhibited numerous induced hairy roots per wound site. Primary root was removed from the plant by cutting approximately 1 cm below the cotyledon nodes. Plants with induced roots were repotted in fresh sterile vermiculite. Plants were watered with B&D, covered with a plastic lid, and grown in a growth chamber (light and dark for 16 and 8 h, respectively; temperature = 25 to 28°C). Lids were partially removed or plastic bags were perforated to facilitate gradual acclimation of transformed bean plants to the environment. Covers were removed 4 days after removing primary roots.

Nodulation of transgenic hairy roots.

R. tropici strain CIAT899 was used in all experiments. A fresh inoculum of CIAT899 was prepared by growing on PY liquid medium (Martínez-Romero et al. 1991) supplemented with CaCl₂ (at 7 mM final concentration), rifampicin at 50 μ g ml⁻¹, and nalidixic acid at 20 μ g/ml⁻¹ at 30°C to a cell density of 5 to 8 $\times 10^8$ ml⁻¹. Immediately after transferring *A. rhizogenes*-transformed bean plants into bigger pots with fresh vermiculite, each plant was inoculated by adding 1 ml of the *R. tropici* culture directly to the root. Plants were watered with B&D nitrogen-free solution. Nodulation was quantified 3 weeks after inoculation.

GUS and GFP expression.

To verify the number of transformed plants obtained by using this protocol, entire roots were tested. The expression of the GUS (*uidA*) gene was detected by histological assay described by Jefferson and associates (1987). Briefly, roots and hairy roots were incubated in sodium phosphate buffer (0.1 M) with GUS substrate for 12 h at 37°C. Enzymatic reaction was stopped by 70% ethanol. Pigments from tissues were removed by an acetone/methanol treatment (1:3). Tissues were analyzed by light stereomicroscopy after GUS staining. Additionally, transformation frequency was verified by GFP expression using confocal microscopy.

Biological nitrogen fixation.

Nitrogen fixation of standard and transgenic hairy roots (Table 1) was assayed using nitrogenase acetylene reduction (Vessey 1994). In brief, nodulated roots from fresh harvested plants were placed in a 160-ml vial closed with a serum cap. Immediately, 4 ml of air were withdrawn from the closed vial and replaced by acetylene gas. Ethylene production was assayed in a gas chromatograph.

Fixation, dehydration, embedding and sectioning of nodules.

Transgenic and standard roots and nodules were collected in stabilizing buffer (50 mM PIPES, 5 mM EGTA, and 2 mM MgSO₄, adjusted to pH 6.9 with KOH⁺) as described by Cyr (1991) and modified according to Collings (1998). Several modifications were introduced: a treatment in 100 μ M m-Maleimidobenzoyl-*N*-hydroxysuccinimide-ester (MBS) (Pierce, Rockford, IL, U.S.A.), and 0.1% Triton X-100 for 15 min followed by fixation in 4% paraformaldehyde for 30 min at room temperature, then washing three times with stabilizing buffer. The dehydration and embedding procedures described by Baskin and associates (1992) were modified as follows. First, 5 mM dithiothreitol was added to the ethanol series and to the methacrylate mixtures. The ethanol-methacrylate embedding series were 4:1, 3:1, 2:1, 1:1, 1:2, 1:3, and 1:4 (vol/vol) ethanol/methacrylate, followed by three changes of 100% methacrylate. The methacrylate mixture was 4:1 (vol/vol) butyl-methacrylate/methyl-methacrylate (Sigma Aldrich) with 0.375% (wt/vol) benzoin ethyl ether (Fluka Chemika, Buchs, Switzerland). Second, samples were deposited and oriented in gelatin capsules (Electron Microscopy Sciences, Fort Washington, PA, U.S.A.) with 100% methacrylate. Polymerization was performed in a digitally controlled temperature bath (VWR Scientific, Philadelphia) with antifreeze at -5°C, using long-wave UV (Blak-Ray long-wave UV lamp, Model B-100A; Electron Microscopy Sciences) placed 10 cm above the samples for 12 h. Embedded tissue was sectioned to 3 μ m in thickness using an ultramicrotome (Leica, Bensheim, Germany) and placed on top of small water drops in diagnostic multi-well slides (Erie Scientific Co., Portsmouth, NH, U.S.A.). Tissue was attached by heating for 2 min until the water evaporated. Methacrylate was removed with four changes of acetone (15 min each) and rehydrated with TBST buffer (0.15 M NaCl, 0.01 M Tris, pH 8.0, and 0.05% Tween 20). Samples were mounted using Citifluor (Ted Pella, Inc., Redding, CA, U.S.A.) and a coverslip for GFP observation under a confocal microscope.

Visualization GFP and GUS expression.

Whole fresh standard or transgenic roots first were stained for GUS activity (Jefferson et al. 1987) and then cross-linked for 15 min with MBS (100 μ M), fixed with 4% paraformaldehyde, and washed with methanol/acetone (1:3) to obtain a clear background. Nodules were bisected with a razor blade, mounted with Citifluor (Ted Pella, Inc.), and photographed un-

der a stereomicroscope. Subsequently, GFP was observed as described by Van Lent (2006). Briefly, a Zeiss LSM 510 Meta confocal microscope attached to an Axiovert 200 M was used. GFP excitation was obtained at 488 nm using an Argon laser and an HFT UV 488/543/633-nm dual dichroic excitation mirror with an LP 505-nm emission filter for detection. Simultaneously, autofluorescence was observed by exciting at 543 nm with an He/Ne laser, with the same dual dichroic excitation mirror and an LP 560-nm emission filter. Images were processed using Adobe Photoshop 7.0 software (Adobe Systems Inc., Mountain View, CA, U.S.A.)

LVSEM.

Methacrylate-embedded 3- μ m sections from transgenic and standard nodules were placed in folding copper grids. Embedded mixture was removed by washing with 100% acetone drop-lets for 3 min. The grids were opened and attached to carbon tape on top of an aluminum base, coating the surface with gold by sputtering (Denton Vacuum, Moorestown, NJ, U.S.A.) for SEM analysis in a Jeol JSM-5900LV low-vacuum scanning electron microscope.

PCR and Southern blot analysis.

Total genomic DNA was extracted from transformed roots using Versa Gene (Gentra Systems Inc., Minneapolis, MN, U.S.A.). Approximately 20 μ g of DNA from each sample was digested overnight with selected restriction enzymes. DNA fragments were separated by electrophoresis in 0.8% (wt/vol) agarose gel, transferred to a Hybond-N+ (Amersham Biosciences Little Chalfont, U.K.) nylon membrane, and cross-linked by UV light for 1 min. A GFP gene (approximately 750 bp) was labeled with [α - 32 P] as a probe for Southern hybridization. Filters were prehybridized in 1 mM EDTA, 0.5 M NaHPO₄, 1% bovine serum albumen, and 7% sodium dodecyl sulfate (SDS) buffer at 60°C and subsequently hybridized overnight with the GFP-labeled probe. After stringent washing (1 mM EDTA, 40 mM NaHPO₄, and 1% SDS at 60°C), filters were exposed in a Phosphor-Imager plate (Molecular Dynamics Storm, Sainte-Julie, Quebec, Canada).

The presence of Ri T-DNA in hairy roots was assessed by amplifying the *rolB* gene from total DNA obtained from hairy roots, and from *A. rhizogenes* K599 with and without p35SGFPUS+. As a positive control, 1.5 μ l of an overnight culture of *A. rhizogenes* strain A4 was added directly to the PCR reaction. Used primers were 5'-GCTCTTGCAGTGCTAGATT T-3' and 5'-GAAGGTGCAAGCTACCTCTC-3'. In order to confirm that plant tissues were free of *A. rhizogenes*, the *virD* gen was monitored with 5'-GCTCTTGCAGTGCTAGATT-3' and 5'-GAAGGTGCAAGCTACCTCTC-3' primers. PCR mixture consisted of 2 ng of plant DNA, 1.0 μ l of 10 $\times$ *Taq* buffer, 0.3 μ l of 50 mM MgCl₂, 0.25 μ l of dNTP, 1.0 unit of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.), and 0.25 μ l (10 pmol) of primer in a final volume of 10 μ l. For genomic DNA amplification, samples were heated to 94°C for 10 min, followed by 35 cycles at 94°C for 30 s, 61.1°C (*rolB* gene) or 46.4°C (*virD*) for 30 s, and 72°C for 1 min in iCycler thermal cycler (Biorad, Hercules, CA, U.S.A.). Amplified products were separated by electrophoresis on 1% agarose gels stained with ethidium bromide and visualized by fluorescence under UV light.

ACKNOWLEDGMENTS

Support for this research was provided by CONACyT 42562-Q and by Direccion General de Asuntos del Personal Académico IN-215805-2 grants. We thank C. Vickers for constructing the plasmid p35SGFPUS+; S. Silvente, A. Leija (CCG/UNAM), and I. Puente-Lee (FQ-USAI/UNAM)

for their technical assistance; and V. Walbot for critical reading of the manuscript.

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