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Susceptibility of Common and Tepary Beans to *Agrobacterium* spp. Strains and Improvement of *Agrobacterium*-mediated Transformation Using Microprojectile Bombardment

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Abstract. **To develop an efficient protocol for *Agrobacterium*-mediated transformation of common bean (*Phaseolus vulgaris* L.) and tepary bean (*P. acutifolius* A. Gray), we have tested the susceptibility of six genotypes to eight *Agrobacterium tumefaciens* and two *A. rhizogenes* strains. The virulence of the *Agrobacterium* strains was shown to be genotype dependent. In general, the tumors observed on common bean cultivars were larger than those observed on tepary bean cultivars. The *A. tumefaciens* AT8196 and Ach5 strains and the *A. rhizogenes* 8196 strain induced the best responses in all genotypes tested. Polymerase chain reaction (PCR) analysis confirmed the presence of T-DNA in tumors derived from inoculation with three *A. tumefaciens* strains in common beans. Apical meristems of *P. vulgaris* cv. Jalo were bombarded with tungsten microprojectiles and then inoculated with an *A. tumefaciens* wild-type strain (Ach5). One month later, the explants showed a high frequency of tumor formation (50% to 70%). Similarly, when bombarded meristems were inoculated with an *A. tumefaciens* disarmed strain (LBA4404/p35SGUSINT), 44% of them showed substantial sectors of GUS activity, suggesting the expression of introduced gene. The bombardment/*Agrobacterium* system appears to be a promising method to stably transform bean through the regeneration of plants directly from transformed apical meristems.**

Agrobacterium tumefaciens and *A. rhizogenes* are soil bacteria that are the causative agents of the crown gall and hairy root plant diseases, respectively. These diseases are the result of a genetic parasitism by the bacterium on the plants. During infection, part of a large plasmid present in virulent strains is transferred to the plant genome where it is stably maintained (for review, see Hooykaas and Beijersbergen, 1994; Zupan and Zambryski, 1995). This transferred region (called T-DNA) contains several oncogenic genes that encode enzymes involved in phytohormone synthesis. These enzymes are expressed in the transformed plant cells, disturbing the cell division and resulting in formation of tumors at the infection site (Weiler and Schröder, 1987). Other T-DNA genes encode enzymes that synthesize opines, a group of conjugated amino acids and sugars that is specifically catabolized by the inciting bacterium strain (Dessaux et al., 1993). Thus, *Agrobacterium* strains are classified according to the opines that are synthesized by their T-DNAs (e.g., octopine, nopaline, agropine, etc.).

Successful transference of foreign genes to a plant (*Nicotiana tabacum*) was first described in 1985 using genetically manipulated strains of *Agrobacterium tumefaciens* (Horsch et al., 1985). *Agrobacterium* has since been used to transform numerous plant species and, except for monocots, has remained the method of choice (De Block, 1993; van Wordragen and Dons, 1992). Nevertheless, plant species have different susceptibility to agrobacteria, and successful transformation requires specific knowledge of *Agrobacterium*–host compatibility (Godwin et al., 1992).

In bean, previous works have reported the susceptibility of *Phaseolus vulgaris* and *P. coccineus* to different *A. tumefaciens* and *A. rhizogenes* strains (Franklin et al., 1993; Lewis and Bliss, 1994; Mariotti et al., 1989; McClean et al., 1991). However, the lack of efficient and reproducible regeneration and transformation protocols did not allow for the production of transgenic beans using the *Agrobacterium* co-cultivation strategy.

Russell et al. (1993) have described the stable transformation of *P. vulgaris* plants using an electrical particle acceleration device. The method involved the direct transformation of apical meristems from mature seeds. Nevertheless, the efficiency of this transformation protocol was extremely low, i.e., 0.03% germline transformed plants per bombarded meristem.

We have developed an efficient and reproducible method to induce multiple shoot formation from apical and axillary meristems of bean mature embryos using high levels of cytokinin. To achieve transgenic bean plants using the *Agrobacterium* system, we have evaluated the susceptibility of three Brazilian common bean cultivars (*Phaseolus vulgaris* L. cvs. Jalo, Costa Rica, and Carioca) and three tepary bean cultivars (*Phaseolus acutifolius* L. cvs. GL477, GL495, and GL114), to *A. tumefaciens* and *A. rhizogenes* wild-type strains. In a second step, we have tested the possibility to improve *Agrobacterium*-mediated transformation of bean by wounding embryo meristems with microprojectiles before infection with *Agrobacterium*.

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Table 1. Wild type *Agrobacterium* spp. strains used.

Name	Species	Opine	Biovar	Origin	References
AT2553	*A. tumefaciens*	Nopaline	1	Common bean	L.M.G. Barros, personal communication
AT8196	*A. tumefaciens*	Nopaline	1	Poplar	L. Jouanin, personal communication
T37	*A. tumefaciens*	Nopaline	1	Walnut	Sciaky et al., 1978
82.139	*A. tumefaciens*	Nopaline	2	Wild cherry	Michel et al., 1990
Bo542	*A. tumefaciens*	Agropine	1	Dahlia	Sciaky et al., 1978
Ach5	*A. tumefaciens*	Octopine	1	Cherry	Lin and Kado, 1977
R10	*A. tumefaciens*	Octopine	1	Unknown	Petit et al., 1983
15955	*A. tumefaciens*	Octopine	1	Tomato	Sciaky et al., 1978
A4	*A. rhizogenes*	Agropine	2	Rose	Moore et al., 1979
8196	*A. rhizogenes*	Mannopine	2	Unknown	Petit et al., 1983

Material and Methods

Agrobacterium strains. Eight wild-type strains of *A. tumefaciens* and two of *A. rhizogenes* were used in susceptibility tests (Table 1). These strains belong to different opine and biovar groups. Before inoculation, they were grown at 28 ± 2 °C for 48 h on MYA-agar plates (Tepfer and Casse-Delbart, 1987). The *Agrobacterium* culture in each plate was then suspended in 2 mL of distilled sterile water.

In the first bombardment assays, the wild-type *A. tumefaciens* octopine strain Ach5 (Table 1) was used to easily identify the transformation occurrence. Further assays were performed with the disarmed strain LBA4404/p35SGUSINT, a disarmed derivative of Ach5 (Ooms et al., 1982) harboring the binary vector p35SGUSINT (Vancanneyt et al., 1990). This vector contains the *gus-intron* and *neo* genes between the T-DNA borders.

For bombardment assays, the Ach5 and the LBA4404/p35SGUSINT strains were incubated in MYA liquid medium at 28 ± 2 °C on a shaker at 100 rpm. Cells were grown to a density of ≈10^8 cells/mL (O.D. $_{600\ nm}$ = 1.0). Bacteria were pelleted by centrifugation and suspended in liquid MS medium (Murashige and Skoog, 1962) to a final concentration of 10^9 cells/mL.

Plant inoculation with wild-type strains. Three current important Brazilian common bean (*Phaseolus vulgaris* L.) cultivars, 'Jalo', 'Costa Rica', and 'Carioca', and three tepary bean (*P. acutifolius*) cultivars, 'GL477', 'GL495', and 'GL114', were used for inoculation assays. The seeds were germinated in a greenhouse with 60% relative humidity and a temperature of 26 ± 2 °C.

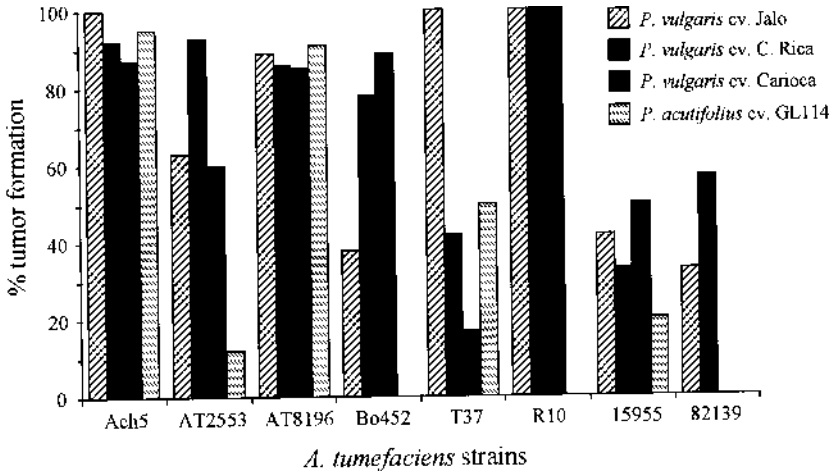
Plants were infected 14 days after germination by wounding twice in different parts of the plant (hypocotyl, epicotyl, petiole, and leaf) with a trident forceps first dipped into the bacterial suspension. Fifteen to 20 plants of each bean cultivar were inoculated with each *Agrobacterium* strain (Table 1). The symptoms were evaluated ≈1 month after inoculation, analyzing the number and size of tumors or roots formed. The controls received the same treatment, but without bacteria.

Polymerase chain reaction (PCR) analysis of tumors. Total genomic DNA was extracted from tumors and leaves of common bean according to the method described by Dellaporta et al. (1983). These tumors were derived from inoculation with 82139, Ach5, and R10 strains of *A. tumefaciens*. DNA from leaves of noninfected plants was used as a negative control. The total DNA from *A. tumefaciens* 82139 and Ach5 strains (positive controls) was isolated as described by Dhaese et al. (1979). DNA from plants and *Agrobacterium* was suspended in deionized water to a final concentration of 5 ng·μL^{-1}.

For each PCR, the reaction mixture consisted of 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 160 μM of each dNTP, 0.2 μM of each primer, 2 units of Taq-polymerase (GIBCO BRL, Grand Island, N.Y.), and 10 ng of extracted DNA. Sterile water was added to give a total volume of 25 μL. Two primers of 20 bp were used for the detection of the *nos* gene from pTi82139 (primer 1: 5′ TGGTCTACCTGCTCTTGAAC 3′; primer 2: 5′ TCGTCAGTGAACTAGCCAAC 3′). This primer pair amplified a 529-bp fragment in the *nos* gene coding sequence (Drevet et al., 1994). Two primers of 21 bp were used for the detection of the tryptophan 2-mono-oxygenase (*tms1*) gene from pTiAch5 (primer 1: 5′ CCTCTCCTTGATAACCAGTGC 3′; primer 2: 5′ CATTCTCGTACAGCGGCATTG 3′). This primer pair amplified a 493-bp fragment in the *tms1* gene coding sequence (Gielen et al., 1984). The mixture was overlaid with mineral oil and each PCR reaction was submitted to an initial denaturation for 5 min at 95 °C, followed by 40 cycles (95 °C/1.5 min; 60 °C/1.5 min and 73 °C/2.5 min) and a final step for 7 min at 73 °C. The reactions were performed in a PTC-100 Thermal Cycler (MJ Research, Watertown, Mass.). The amplification products were electrophoresed in a 1% agarose gel, stained with ethidium bromide and visualized at ultraviolet light.

Explant preparation for bombardment. Mature seeds of common bean (*P. vulgaris* cv. Jalo) were sterilized in 70% (v/v) ethanol for 1 min followed by 20 min in 1% (v/v) sodium hypochlorite. Nonwrinkled seeds were then rinsed three times and soaked overnight in sterile deionized water. Apical meristems were exposed by removing cotyledons and primary leaves as described by Aragão et al. (1993). Subsequently, the root tips were also excised. The embryonic axes were placed on a petri dish containing a thin layer of 20% (w/v) sterile gum xanthan (G1253; Sigma, St. Louis), to immobilize the apical meristem for bombardment. The explants

Fig. 1. The effect of *A. tumefaciens* strains on common bean (cv. Jalo, Costa Rica, and Carioca) and on tepary bean (cv. GL114), expressed as the percentage of plants giving tumors per inoculated plants, 30 days after inoculation. The 82.139 strain was not assayed on the Carioca and GL114 cultivars.



were positioned in a circle (2.5 cm in diameter) in the center of the dish (four explants per dish), with the apical region directed upward.

Bombardment. Tungsten M10 particles (0.2 to 1.5 μm in diameter, Sylvania, Towanda, Pa.) were sterilized in 70% (v/v) ethanol and suspended in 50% glycerol at a final concentration of 400 $\mu g \cdot \mu L^{-1}$. For bombardment, 2.5 μL of particle suspension were used (1 mg/shot). The helium-driven particle bombardment device used was essentially as described by Finer et al. (1992). The tissue was placed at a distance of 9 cm from the screen containing the particle suspension. The exposed meristems were subjected to a bombardment using a helium pressure of 80 or 100 psi, under partial vacuum of 27 inches Hg.

Postbombardment inoculation. Immediately after bombardment, a droplet (≈1 μL) of the bacterial suspension (10^9 cells/mL) was laid directly onto the bombarded meristems. Controls were inoculated with the bacteria, but were not bombarded. The bombarded-inoculated meristems were maintained on gum xanthan for 24 h, transferred to MS medium and incubated at 26 ± 2 °C under continuous light (40 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Two days after bombardment, the explants were transferred to MS medium supplemented with 500 $mg \cdot L^{-1}$ cefotaxime and 250 $mg \cdot L^{-1}$ ampicillin to avoid bacterial contamination.

Meristems inoculated with the wild-type strain Ach5 were scored for tumor formation 4 weeks after inoculation. Tumors developed on the apical region of the embryonic axis were then excised and cultured on MS medium without antibiotics or growth regulators.

Meristems inoculated with the disarmed strain LBA4404/p35SGUSINT were analyzed 1 week after inoculation for *gus* gene expression, using the histochemical assay (Jefferson et al., 1987).

Results and Discussion

Genotype effect. The percentage of tumor formation in common beans (cultivars Jalo, Costa Rica, and Carioca) inoculated with different *A. tumefaciens* strains is shown in Fig. 1. The three common bean cultivars showed different degrees of susceptibility to each *Agrobacterium* strain. However, all of the *A. tumefaciens* strains tested were virulent in those cultivars. Initiation of tumors started at 8 to 10 days after inoculation, resulting in large tumors (Fig. 2a). The tumor size varied widely according to the tested strain. Only tumors larger than 5 mm were considered in this study.

Common bean cultivars also showed high susceptibility to both *A. rhizogenes* tested (Fig. 3), except to the A4 strain that did not infect 'Jalo'. The infecting ability of strain A4 in 'Carioca' is illustrated in Fig. 2b, showing the typical hairy root phenotype at the inoculation site. However, these roots grew very slowly.

In contrast, the inoculated tepary bean cultivars were not very susceptible to *A. tumefaciens* infection, showing very small tumors, ranging from 2 to 5 mm (Fig. 2c). The tepary bean cultivar GL114 gave the best quantitative and qualitative responses, especially with the Ach5 and AT8196 strains (Fig. 1). The others tepary cultivars (GL477 and GL495) were weakly susceptible, showing a tumor formation frequency lower than 20% (data not shown).

Agrobacterium rhizogenes displayed a low virulence on tepary bean cultivars, usually forming a small tumescence on the inoculation site. Only the GL495 cultivar shown to be susceptible to *A. rhizogenes* infection (Fig. 3). The other tepary beans cultivars (GL477 and GL114) were not susceptible to the infection by A4 and 8196 *A. rhizogenes* strains (data not shown).

Agrobacterium tumefaciens strains effect. All the *Agrobacterium tumefaciens* strains tested demonstrated different degrees of virulence and tumor sizes in the same bean cultivar (Fig. 1). For example, in common bean cultivars strains AT8196 (nopaline type) and Ach5 (octopine type) induced large tumors very quickly. However, the R10 strain (octopine type) was the most virulent, since the infectivity in all tested cultivars was 100%. These results indicated that the most efficient *Agrobacterium* strain for use as a vector to introduce important genes into common beans is the nopaline strain AT8196 or the octopine strains Ach5 or R10. The results also suggest that the *Agrobacterium*–bean interaction is opine and biovar independent.

Tissue effect. There was a distinct plant response depend-

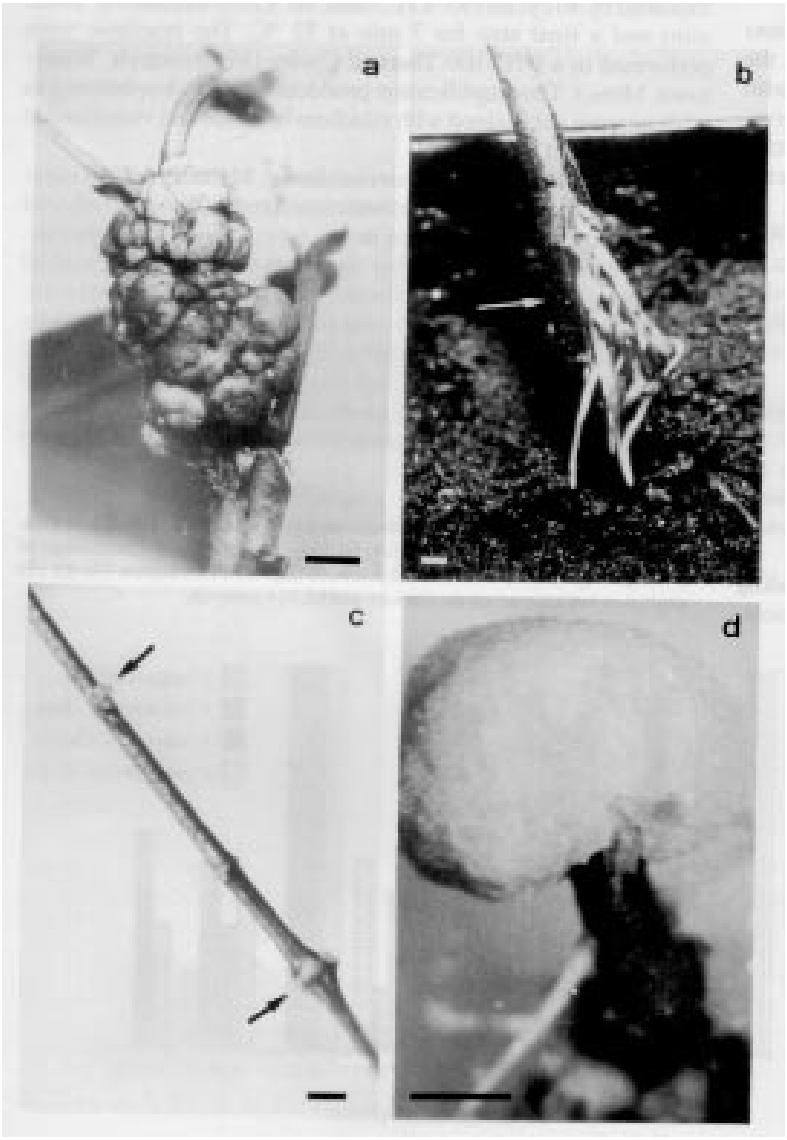


Fig. 2. (**a**) Tumor induced in *P. vulgaris* cv. Jalo, at epicotyl site, by *A. tumefaciens* R10 strain, 2 months after inoculation. (**b**) Hairy root induced in *P. vulgaris* cv. Carioca, at hypocotyl site, by *A. rhizogenes* A4 strain, 2 months after inoculation. (**c**) Tumor induced in *P. acutifolius* cv. GL114, at epicotyl site, by *A. tumefaciens* Ach5 strain, 2 months after inoculation. (**d**) Tumor formed on the apical meristem of the *P. vulgaris* cv. Jalo, that has been inoculated with Ach5 *A. tumefaciens* strain with prior bombardment (80 psi), 45 days after the treatment. Arrows indicate location of tumors or roots induced by *Agrobacterium* spp. inoculation. Bar = 1 cm.

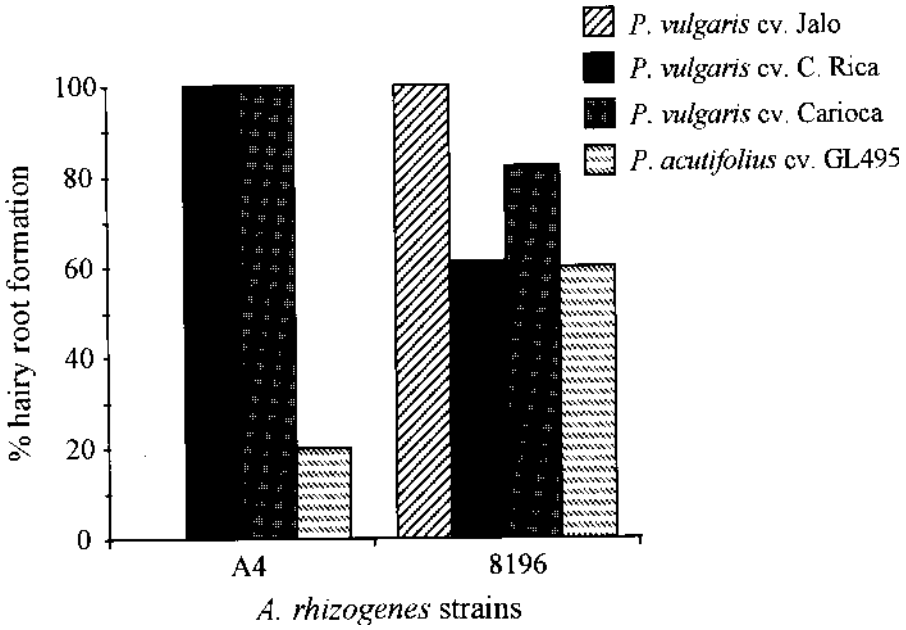


Fig. 3. The effect of *A. rhizogenes* strains on 'Jalo', 'Costa Rica', and 'Carioca' common bean and of 'GL495' tepary bean cultivars, expressed as the percentage of plants giving hairy roots per inoculated plants, 30 days after inoculation. The A4 strain was not assayed on 'Jalo'.

ing on the inoculated explant. Epicotyl tissues showed a high percentage of tumor and root formation in common and tepary beans (Figs. 1 and 3). These tumors were larger and more vigorous than those formed in the other inoculated tissues (Fig. 2 a and c). Hypocotyl has a variable response depending on the inoculated cultivar (Fig. 2b). In general, petioles and leaves showed a weak response to infection in common beans, and did not develop any symptom in tepary beans.

PCR analysis. The PCR analysis showed the presence of the bacterium T-DNA in tumors derived from inoculation with 82139, Ach5, and R10 strains on common bean plants (Fig. 4). A clear amplification signal of expected molecular weight (529 bp) was obtained with DNA from tumors induced by 82139 strain, corresponding to the *nos* gene from pTi82139. DNA from tumors induced by Ach5 strain also showed a fragment of 493 bp, corresponding to the *tms1* gene from pTiAch5. A weak amplification signal was obtained with DNA from tumors induced by the R10 strain (Fig. 4). This amplified fragment also showed an unexpected molecular weight (≈500 bp). Since the primers used were from pTiAch5 *tms1* gene, this result could be explained by a partial homology between the *tms1* gene from pTiAch5 and pTiR10. The *tms1* gene has been pointed as conserved between octopine strains, such as Ach5 and R10 (Weiler and Schröder, 1987). However, this homology was demonstrated by DNA hybridization assays and the complete nucleotide sequence of the *tms1* gene from pTiR10 has not yet been achieved.

The DNA from 82139 and Ach5 strains (positive controls) showed the expected amplification product (Fig. 4). No amplification was detected with the DNA from noninfected plants (negative control), using either *nos* or *tms1* primers.

Tumor formation after bombardment. Experiments were conducted to determine whether wounding tissues by microprojectile bombardment prior to *Agrobacterium* inoculation would improve the transformation efficiency. For that, bean meristems were subjected to bombardment with a pressure of 100 or 80 psi followed by *Agrobacterium* inoculation or inoculation with *Agrobacterium* without previous bombardment. For these assays, we have used the *A. tumefaciens* Ach5 strain that was revealed to be one of the most virulent strain on common bean plants. Tumors formed on all of the treatments, 1 month after inoculation, although differences in transformation efficiency were observed (Table 2). The tumors occurred exclusively on the apical region of the embryonic axis (Fig. 2d). Probably, these tumors originated from cells of the inoculated meristem region. Tumors did not form on the negative control treatment. Bombardment pretreatment clearly enhanced the percentage of tumor formation when compared with the inoculation without bombardment. The pressure of 80 psi was more efficient for inducing *Agrobacterium*-mediated transformation than a pressure of 100 psi (Table 2, Fig. 2d). About 30 days after the treatment, the tumors were transferred to MS medium, lacking growth regulators. These tumors grew rapidly, suggesting that they were transformed by the oncogenic T-DNA.

Further assays were made to confirm the stable expression of the introduced *gus* gene, after the bombardment/*Agrobacterium* treatment. The *Agrobacterium* inoculation was made using a disarmed strain harboring the *gus* gene between its T-DNA borders. The results showed similar responses to the assays using the wild-type strain (Table 3). The bombardment/*Agrobacterium* treatment is more efficient than the treatment with *Agrobacterium* without prior bombardment (Table 3). Meristems of both treatments exhibited substantial GUS-stained sectors, suggesting the stable expression of the foreign gene.

We have shown that the efficiency of *Agrobacterium*-mediated gene transfer to beans depends on plant genotype. The symptoms (crown gall or hairy root) formed on common bean cultivars were more expressive than those formed on tepary bean cultivars. The *A. tumefaciens* strains AT8196, Ach5, and R10 induced large tumors on Brazilian common beans. The *A. rhizogenes* strain 8196 produced hairy root on all genotypes tested. Thus, these results demonstrated the potential use of these strains as transformation vectors.

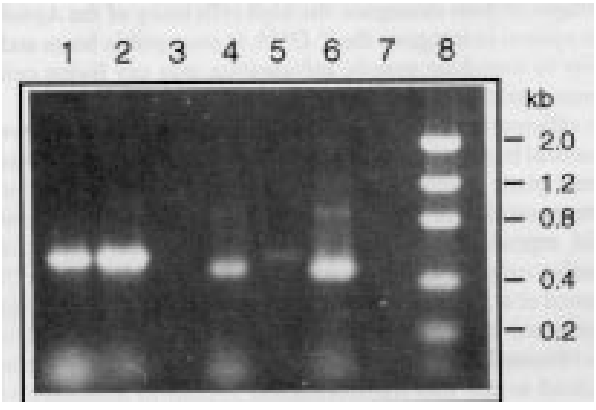


Fig. 4. PCR analysis from *Agrobacterium* and plant DNA. Lane 1: tumor derived from 82139 inoculation; lane 2: 82139 strain; lane 3: noninfected plant (negative control to the *nos* primers); lane 4: tumor derived from Ach5 inoculation; lane 5: tumor derived from R10 inoculation; lane 6: Ach5 strain; lane 7: noninfected plant (negative control to the *tms1* primers) and lane 8: Mass DNA ladder (GIBCO BRL). Lanes 1 and 2 amplified a 529-bp fragment (corresponding to the *nos* gene) and lanes 4 and 6 amplified a 493-bp fragment (corresponding to the *tms1* gene). The lane 5 amplified fragment of ≈500 bp.

Table 2. Percentage of tumor formation on the apical region of the embryonic axis of *Phaseolus vulgaris* cv. Jalo, 1 month after the treatment.

Treatment	Total no. treated meristems	Tumor formation (%)[z]
Nonbombarded	15	0
Only *Agrobacterium*[y]	30	31 ± 11
Bombardment with 100 psi	40	50 ± 8
Bombardment with 80 psi	40	70 ± 5

[z]Number of apical regions showing tumor formation per total number of treated meristems, in a total number of five experiments.
[y]*Agrobacterium tumefaciens* (Ach5) treatment without prior bombardment.

Table 3. Percentage of *Phaseolus vulgaris* cv. Jalo meristems expressing GUS, 1 week after the bombardment/*Agrobacterium* treatment. The meristems were bombarded using a pressure of 80 psi.

Treatment	Total no. treated meristems	GUS expression (%) [z]
Nonbombarded	27	0
Only bombardment[y]	27	0
Only *Agrobacterium* [x]	40	23 ± 7
Bombardment + *Agrobacterium*	59	44 ± 8

[z]Number of meristems expressing GUS gene per total number of treated meristems, in a total number of six experiments.
[y]Bombarded meristems without DNA or *Agrobacterium* treatment.
[x]*Agrobacterium* (LBA4404/p35SGUSINT) treatment without prior bombardment.

Our results also have confirmed that it is possible to use the bombardment/*Agrobacterium* system to transform apical meristems of bean. Bidney et al. (1992) showed that the wounding of tobacco leaves and sunflower apical meristems by microprojectile bombardment before inoculation of *Agrobacterium* increases, at least 100 times, the transformation frequency. Recently, May et al. (1995) obtained transgenic banana plants by *Agrobacterium*-mediated transformation of apical meristems that have been previously bombarded. The bombardment/*Agrobacterium* system also was used efficiently to transform zygotic embryos of *Eucalyptus globulus* (A. Boudet, Université Paul Sabatier, Toulouse/France, personal communication). The microwounds caused by the microprojectile bombardment can greatly enhance the frequency of *Agrobacterium*-mediated transformation in different target tissues. The bombardment/*Agrobacterium* method combines the advantages of both strategies: the high efficiency of the *Agrobacterium* system to integrate the T-DNA in susceptible hosts and the capacity to introduce genetic information into any living cell, of the microparticle bombardment system.

An efficient regeneration protocol is a prerequisite to develop a successful transformation system. Direct shoot organogenesis of different plant species could be induced by the cultivation of meristems on high level of cytokinin. This method of regeneration is rapid, reproducible, efficient and has been already reported for *Phaseolus vulgaris* and *P. acutifolius* (Malik and Saxena, 1992; Mohamed et al., 1992a, 1992b). Experiments are now in progress to regenerate bean plants directly from transgenic apical meristems obtained after the bombardment/*Agrobacterium* treatment. We intend to use this transformation system to introduce virus resistance genes as well genes to improve the nutritional quality of this legume (Aragão et al., 1992, 1995).

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