Genetic transformation of bean callus via *Agrobacterium*-mediated DNA transfer

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Summary: Callus cultures were induced from hypocotyl of young bean seedlings. Callus developed and maintenaned on B5 medium supplemented with 2mg/l 2,4-D and 1mg/l kinetin. The results demonstrate that *A. tumefacins*-mediated transformation is a convenient method to obtain transient gene expression in callus of bean. The results have shown that the bean callus co-cultivated with *A. tumefaciens* can be transformed to get herbicide Finale (glufosinate-ammonium) resistant GUS positive tissues. Southern blot analysis of transformed calli showed integration of *gusA* marker gene carried by a binary vector. Transformed calli were selected on herbicide containing media. Data of molecular analysis (Southern blotting) confirmed the insertion of *gusA* gene in the genome of herbicide resistant calli with *bar* gene. There are three evidences that calli are stable transformants: (1) herbicide resistance, (2) GUS activity which is indicative since the coding region containing an intron, (3) the results of Southern hybridization technique.

Key words: *Phaseolus vulgaris*, bean, *Agrobacterium*, transformation, glufosinate-ammonium, GUS

Abbreviations: B5 = *Gamborg* et al. (1968), MS = *Murashige & Skoog* (1962), 2,4-D = 2,4- dichlorophenoxyacetic acid, KIN = kinetin, *bar* = phosphinothricin acetyl transferase gene, *gusA* = β-glucuronidase gene

Introduction

Common bean is a vegetable crop, a member of the genus *Phaseolus*, in the family *Fabaceae*. Most of the bean production is confined to *P. vulgaris*. Dry common beans are the major source of protein and the most important food legume in the developing world for millions of people. Moreover, *P. vulgaris* is nitrogen fixing, has a short life cycle to improve nitrogen status of soil.

Despite being an important pulse crop, its production has not been improved significantly. Consequently, there is considerable interest in the introduction of useful traits into common bean by breeding and genetic engineering. Classical breeding has met limited success. The other alternative approach is transferring desirable genes from other sources, but the main hindrance to transformation is its recalcitrance like most of the legumes. The genetic transformation of bean has been difficult and challenging till now. The members of genus *Phaseolus* have been shown to be susceptible to *A. tumefaciens* and transgenic callus lines have been generated.

The use of cell culture and genetic engineering is viewed as a logical approach to improve legume crops (*Morginski & Kartha* 1985, *Hildebrandt* et al. 1986 and *Mok* et al. 1986). Gene transfer techniques will have a great impact on legumes. In vitro techniques are important for the production of useful targets for gene delivery systems. In vitro culture, organogenezis and embriogemesis as well as callus induction from different parts of bean have been reported (*Eissa* et al., 1998a, 1998b, 1999; *Gamborg*, 1968, *Gémesné* et al., 1995; *Mohamed* et al., 1993; *Mok* et al., 1986, *Morginski* et al., 1985). The particle delivery system have beeen used successfully for bean tissue transformation (*Eissa* et al., 2000b, 2001a, 2001b, 2003). Some research groups report on chimaeric gene transfers to grain legume using the leaf disc method with *A. tumefaciens*, in *Vigna unguiculata* (*Garcia* et al. 1986). Transformed calli could be obtained. *McClean* et al. (1991) were able to introduce genes into beans using the *Agrobacterium* system. Preliminary data regarding the capability of *Phaseolus* to establish a compatible reaction with *Agrobacterium* were reported by *Allavena* (1985) and *McClean* et al. (1988) and for the introduction of reporter genes by *Eissa* et al., (2000a).

The objective of this study was to produce stable transgenic bean callus with *A. tumefaciens* containing phosphinothricin acetyl transferase gene (*bar*) and β-glucuronidase gene (*gusA*).

Material and method

Plant material

The bean cultivars, Fönix and Maxidor were evaluated in this study. Seeds were obtained from the breeder (Prof. *I.*

Velich). All the experiments were carried out and kept at the Department of Genetics and Plant Breeding, Faculty of Horticultural Sciences, Szent István University in Budapest, Hungary.

Binary vectors and Agrobacterium

A. tumefaciens strain A281 was used for transformation experiments, contains the binary plasmid pRGG *bar* H1. Agrobacteria were obtained from Agricultural Biotechnology Center, Gödöllő, Hungary. In the strain, *gusA* contains an intron in the coding region to ensure that the observed GUS activity occurring in the plant cells and not due to the endogenous *Agrobacterium* cells. Bacterial cultures were grown in Luria-Bertani (LB)-agar medium containing 50mg/l kanamycin at 28 °C overnight.

Callus induction and transformation

Seed sterilization and germination methods: Bean seeds were selected and sterilized as by *Eissa* (2003). Sterilized seeds were incubated on agarized hormone-free (*Murashige & Skoog* 1962) (MS) medium.

Explant preparation: Hypocotyl segments (5–7 mm in thickness), shoot apex, primary leaf with petiole, leaf discs 8 mm in diameter and roots from 7–10 days old aseptically grown bean seedlings were used as explants.

Callus initiation and culture: The procedure and conditions of callus induction were followed described *Eissa* (2003).

Finale sensitivity test: Before co-cultivation with *Agrobacterium*, Fönix and Maxidor bean calli were tested for the herbicide glufosinat ammonium (Finale) (AgrEvo GmbH, Berlin) sensitivity. Calli were cultured on B5 medium containing Finale in concentrations ranged from 0.5 to 5mg/l (0.5, 1, 2, 3, 4 and 5mg/l). This experiment was repeated 4 times and 12 replicates/one were used.

Culture medium and growth of bacteria: *Agrobacterium* strain A281, harboring the helper plasmid pRGG bar H1 was grown on solid LB-medium at 28 °C with antibiotic kanamycin 50mg/l. Plasmid pRGG *bar* H1 contains the phosphinothricin acetyl transferase gene (*bar*) as selectable marker and â-glucuronidase gene (*gusA*) as reporter.

Agrobacterium sensitivity test to augmentin: *Agrobacterium* was grown on solid LB-medium containing 300, 400, 500, 600, 700, 800, 900 and 1000mg/l augmentin at 28 °C. This experiment was repeated two times in order to define the concentration that was effective to kill the Agrobacteria.

Co-cultivation with Agrobacterium and transformation:, Bacteria were grown overnight in solid LB-medium, for transformation. Freshly streaked bacteria were resuspended in sterile distilled water. The fresh calli were infected by 1–2ml of bacterial suspension and co-cultivated with the bacteria for three days at 24–26 °C under light on agar-B5 medium (pH 5). Addition of 375–500mg/l augmentin or 200mg/l Claforan (Cefotaximum)(Hoechst) to B5 medium controlled bacterial growth. The callus was transferred two times at three day intervals to fresh B5 medium containing 375–500mg/l augmentin or 200mg/l Claforan (pH 5.7) for three days. Transformed calli were selected on 2–5mg/l Finale. Calli were then placed on B5 medium containing 200mg/l claforan+2mg/l Finale. Calli cultures were considered to be free of Agrobacteria if exudates from crushed cells did not exhibit bacterial growth in LB liquid after 7 days. The Finale concentration was then gradually increased over an eight month period to 5mg/l.

Callus proliferation and genetic selection: Calli inoculated with *Agrobacterium* were maintained for three months. Secondary callus (E1 generation) was removed from the original inoculated callus (E0) at three months after inoculation. To screen for transformants, callus (E2-10 generation) of each subclone were transferred to the B5 medium containing 2–5mg/l Finale over an 8 month period and 3-4 weeks transferred according to a 3–4 week schedule.

Transformation tests

Selection test: Calli derived from 3mg/l Finale-selected medium were cultured on solidified medium containing 4mg/l Finale and later on B5 medium containing 5mg/l Finale to test their ability to form an actively growing callus in the presence of the herbicide. It was necessary to keep the callus in direct contact with the selective medium to develop transformed callus.

Histochemical staining test: Putative transformed calli were determined and evaluated by a histochemical assay for the presence of GUS activity (checked by staining a small portion of callus with X-Gluc) according to the method of *Jefferson* 1987). Small portions of calli were incubated overnight in a thermostat at 37 °C in a staining buffer. Next morning, (β-glucuronidase) was measured histologically using X-Gluc substrate.

Genomic DNA extraction: Total genomic DNA was isolated from transgenic and non-transgenic control callus with QIAGEN Dneasy Plant System Mini Kit (*Qiagen, GmbH* 2000). DNA manipulations including enzymatic cleavage, large-scale plasmid preparation and genomic DNA blot were performed following standard protocols (*Maniatis* et al. 1982). Using modifications recommended by the enzyme manufacturers. For Southern blot analysis, 10 µg of DNA samples were digested with *XhoI* and *XbaI* restriction enzymes and separated on 0.8% agarose gels, blotted on nylon membrane and 1.8 Kb long GUS coding region fragment marked radioactively was used as a probe.

Statistical analysis

The difference among different genotypes and concentrations Finale were calculated using analysis of

variance and the levels of significance were assessed by Duncan's test using the *SPSS program* (1999).

Results and discussion

Callus induction and maintenance: Using the method described we obtained rapidly growing callus cultures of bean to transform bean cells for transient or stable gene expression by using *Agrobacterium*-mediated transformation.

Effectiveness of Finale on the growth of control callus: The finale sensitivity of callus growth was studied to investigate the suitability of finale-resistance as a selectable marker for callus transformation. Phenotypically, we observed that 0.5mg/l Finale caused almost total inhibition the growing of callus in the two cultivars, they grew phenotypically brown and died. After repeating this experiment 4 times with 12 replicates per each treatment, the statistical analysis showed that this concentration was effective to kill the control callus and it was enough to differentiate the transgenic callus with *bar* gene from non-transgenic. This is the minimum concentration that should be necessary to select transformed callus *(Figure 1)*.

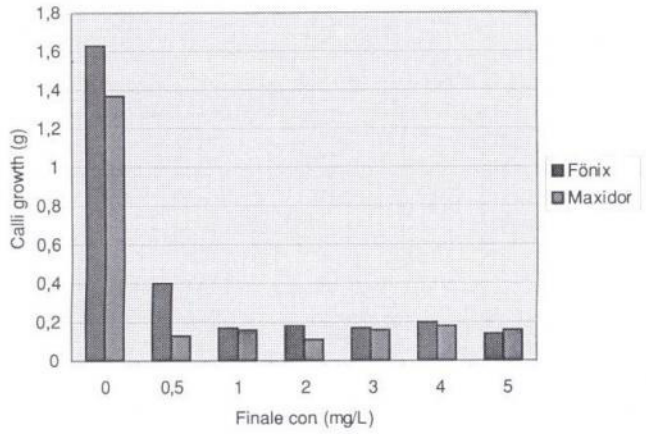

Figure 1 Effect of different concentrations of Finale on the growth of Fönix and Maxidor calli cultures growth on B5 medium

Effectiveness of augmentin on the growth of Agrobacteria: *Agrobacterium* strain 281, harboring plasmid pRGG *bar* H1 was grown on LB-agar medium supplemented with 300, 400, 500, 600, 700, 800, 900 and 1000mg/l augmentin under normal conditions. This experiment was repeated two times to define the minimum concentration that was effective to kill the Agrobacteria. We found that 300mg/l augmentin was enough to kill the Agrobacteria after repeated the same experiment. So, we used higher concentration 375mg/l to kill the Agrobacteria.

Transformation

Selection: A first transformation screening system exploited the ability of the enzyme phosphinothricin acetyltransferase (PAT, encoded by the *bar* gene) to detoxify the herbicide Finale. Calli were screened for bar gene expression by selecting for growth on high concentration 5mg/l finale containing medium. At first most of the non-transformed calli died within several weeks on low concentration 0.5, 1 or 2mg/l Finale. However, several-transformed calli remained white and healthy on high concentration 5mg/l Finale. The herbicide Finale was added to the media at various concentrations 2, 3, 4, 4.5 and 5mg/l respectively, and their effects on growth and color over a period of 6 months and 3–4 week transfer schedule to screen for transformant calli. Finale; 2, 3, 4 and 4.5mg/l allowed growth for about eight weeks. We found that at all of these concentrations of Finale the transformed calli survived and grew while control died. Resistance of the selected calli was verified by their ability to grow on a selective medium. Our initial experiments focused on the demonstration and optimization of transient expression of Finale resistance and *gusA* genes (*bar* and *gusA*) in callus cultures using the expression plasmid A281 pRGG. Using the optimal conditions established for transient gene expression, a series of subculture steps directed towards the recovery of stable transformant calli were conducted.

Confirmation of transformation (GUS assay): To verify the absence of *Agrobacterium*, transformed calli were transferred after four to five subcultures to an augmentin-free selection medium. Bacterial growth was not observed even after three more subcultures on augmentin-free medium. This indicates that Finale-resistant calli do not contain a significant number of residual *Agrobacterium*. Calli were assayed for GUS activity to confirm the expression of the *gusA* gene using the histochemical assay test. The *gusA* gene was also correctly expressed in callus cultures grown on a 4mg/l Finale-selecting medium, the typical blue color in the histochemical assay using the X-Gluc as substrate. We found that control callus showed no detectable GUS activity when stained with X-Gluc, while calli were grown on selective-medium stained blue. This suggests that the *gusA* gene expressed in bean cells. Control, non-transformed callus was not able to grow in the presence of Finale, neither showed any positive reaction in the *in vitro* assays *(not shown)*.

Similar results were obtained by *Mariotti* et al. (1989) in *P. vulgaris* L. & *P. coccineus* L., *Genga* et al. (1990) in *Phaseolus* species, *Becker* et al. (1994) in *P. vulgaris*, *Lewis* & *Bliss* (1994) in *P. vulgaris*, *Hinchee* et al. (1988) in soybean and *De Kathen* & *Jacobsen* (1990) in *Pisum sativum* L. Selection for phosphinotricin and assays for GUS activity indicated the expression of the phosphinotricin selectable marker gene (*bar*) and *gusA* reporter gene in the transformed bean calli. The callus transformation system described here can be used for quantifying the expression of a foreign gene integrated into bean cells.

Southern blot analysis: The genomic incorporation of *gusA* gene was proven by Southern hybridization carried out on genomic DNA from GUS expressing, Finale-resistant

calli that developed on a selective-medium. Southern hybridization analysis was conducted to verify the integration of the *gusA* gene within the bean genome. Border fragments from all transformed cell lines hybridized with a ^{32}P labeled *gusA* fragment. Genomic DNA from untransformed bean calli did not hybridize with the labeled *gusA* fragment. Southern hybridization analysis confirmed the stable integration of the *gusA* DNA into the bean genome. DNA volumes were loaded on gel after digesting with *XhoI* and *XbaI* restriction enzymes. From the total DNA extract 10–10 microgram per samples (from the wild type signed with K too), from plasmid DNA 100ng in P column. 1.8 Kb long *gusA* coding region fragment marked radioactively was used as a probe *(Figure 2)*.

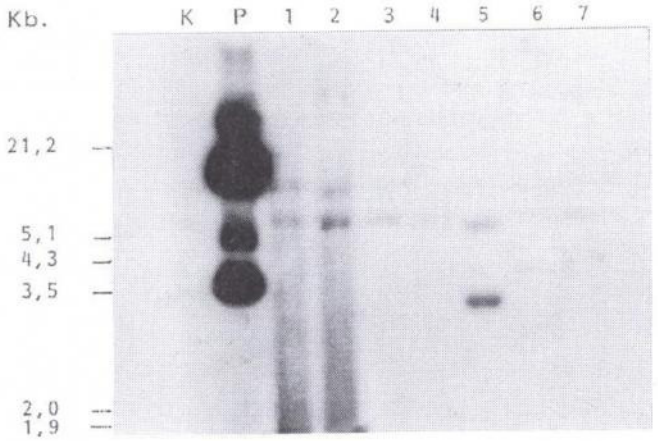

Figure 2 Southern hybridization of transgenic-calli with GUS activity

Notes: Lanes; **K**-DNA sample of a control callus; **P**-the plasmid used for the transformation (pRGG bar H1); **1, 2, 3, 4, 5, 6** and **7** – DNA samples of transgenic calli (transformed and selected on finale, GUS expression showing calli)

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