Production of transgenic bean callus via genetic transformation by DNA-coated tungsten particles

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Summary: Callus cultures were induced from hypocotyl of young bean seedlings. The B5 medium completed with 1mg/l KIN and 2mg/l 2,4-D proved the best. Callus developed and maintenaned on B5 medium supplemented with 1mg/l kinetin and 2mg/l 2,4-D. The B5 medium supplemented with 1mg/l KIN and 2mg/l 2,4-D induced much more callus than half strength MS medium supplemented with 0.5 or 0.75mg/l BA and 0.1mg/l NAA. The results demonstrate that Genebooster^{TM} is convenient method to obtain transient gene expression in callus of bean. The results have shown that the bean callus shot by Genebooster^{TM} can be transformed to get (kanamycin-resistant and stress mannitol-tolerant) calli. The presence of mannitol-dehydrogenase gene (*mtl*) was verified by PCR, showing the integration of *mtl* gene carried by two plasmids. Co-transformed calli were selected after bombardment on kanamycin, mannitol and (kanamycin+mannitol)-containing media. Data of molecular analysis (PCR) confirmed the insertion of *mtl* gene in the genome of mannitol-tolerant callus lines.

Key words: *Phaseolus vulgaris*, bean, Genebooster, transformation, stress, mannitol

Abbreviations: B5 = *Gamborg* et al. (1968) medium, MS = *Murashige & Skoog* (1962) medium, KIN = kinetin, 2,4-D = 2,4- dichlorophenoxyacetic acid, BA = 6-benzyladenine, NAA = naphtalene acetic acid, *mtl* = mannitol-dehydrogenase gene, PCR = polymerase chain reaction, npt II = neomycin phosphotransferase II gene

Introduction

Common bean (*Phaseolus vulgaris*) is the most cultivated species of the genus *Phaseolus*. 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. The main reasons are nutritional deficiencies and salt and drought intolerance. The insertion of genes encoding beneficial traits (such as disease resistance, yield improvement, and stress tolerance) into well-adapted crop plants is potentially of great agricultural importance.

Consequently, there is considerable interest in the introduction of agronomically 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 in order to increase its production. The main hindrance to transformation is that bean is recalcitrante like most of the legumes. Although attempts have been mad on in vitro culture and regeneration of bean (*Eissa* et al., 1998a, 1998b, 1999) the genetic transformation of bean has been difficult and challenging till now. 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). Common bean has been transformed using the biolistic DNA delivery system (*Aragao* et al. 1996, *Eissa* et al. 2000).

Our long term idea was to examine the activity of certain osmosis-protector genes and the effects of their gene products to drought stress in transgenic bean. We wanted to model it in bean. For that purpose we planned the transformation of bean with a gene responsible for the synthesis of osmosis -protecting chemicals (*Eissa* et al., 2001). The aim of this study was to produce transgenic bean callus with two genes, neomycin phosphotransferase II gene (*npt* II)+mannitol-dehydrogenase gene (*mtl*) by the direct gene bombardment system (Genebooster^{TM}) Here the succesfull transformation of bean callus and the selection of double resistant calli are reported

Material and method

Plant material: Two bean cultivars, Fönix and Maxidor were tested 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.

Seed sterilization and germination method: Bean seeds were selected based on a good and similar size. Seeds were surface sterilized by soaking first in 95% ethanol for 2 min, and then in 15% commercial bleach for 15 min and rinsing 4 times with sterile water. Sterilized seeds were incubated on agarized hormone-free (*Murashige & Skoog* 1962) (MS) medium. Uniform germination was achieved only with seeds which remained unwrinkled during sterilization.

Callus induction and maintenance

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: Six to ten from each type of explants were placed in 55×90-mm glass bottles on the appropriate medium (*Gamborg* et al. 1968) (B5) or MS to induce callus formation. The pH of the media was adjusted to 5.7 with KOH or NaOH before autoclaving. Media were supplemented with 20 or 30g/l sucrose and 7g/l agar. Media were dispensed into 55×90-mm bottle glasses and autoclaved at 120 °C for 20 min. Axenic seeds, explant cultures, calluses and transformed cells were incubated on the media at 24–26 °C under 16/8 hours light/dark photoperiod at white light in growth cabinet.

Leaf discs and roots were cultured horizontally, while shoot apices, primary leaves with petiole and hypocotyl segments were cultured horizontally or vertically on B5 medium. This medium contains macro and microelements, and supplemented with 1mg/l KIN and 2mg/l 2,4-D. Friable callus was produced within one week. After 7–10 days, the growing callus was transferred to fresh medium. After 3–4 weeks, callus was transferred to fresh medium and subcultured every 3–4 weeks for callus maintenance. Callus was maintained on fresh medium with the same composition and the same conditions by subculturing at four week intervals. Sometimes the explants were cultured on half strength MS medium with major salts and minor elements supplemented with B5 vitamins and 0.5 or 0.75mg/l BA and 0.1mg/l NAA. This experiment was repeated more than 10 times.

Callus sensitivity test

Before gene shooting, Fönix and Maxidor bean callus were tested for kanamycin and mannitol sensitivity according to *Eissa* et al., (2001) in two independent experiments using 12 replicates per each one. Calli were cultured on callus growing medium B5 containing kanamycin in concentrations ranged from 10 to 100 mg/l (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100mg/l). Mannitol in concentrations ranged from 0.2 to 2M/l (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2M/l). Calli were weighted by mg after 57 days from culture initial. Each experiment was repeated 3 times independently. Statistical analysis was made to define which dose was effective to kill the control callus to be able to differentiate later the transgenic from the non-transgenic cells.

Transformation

Plasmid DNA: Two plasmids were mixed in 1:1 ratio and used together for co-transformation process. Plasmid pFF19K, contains the *npt* II gene as selectable marker and plasmid pFF19-mtl contains the *mtl* gene. Plasmids were obtained from the Agricultural Biotechnology Center, Gödöllő, Hungary.

Gene shooting: For gene shooting the standard physical factors were used, as it were described by *Jenes* et al. (1996 and 1997) the appropriate pressure of N_2 gas, the shooting distance and the size of the tungsten particles used as microprojectiles. High-pressure nitrogen gas accelerated the macroprojectiles. Tungsten particles (0.7–1.6 µm diameter on average) were used as microprojectiles to bind the actual DNA molecules on their surface. The vacuum chamber included a stopping plate and shelves for the target tissues. Plasmid DNA binding on the surface of tungsten particles was carried out with the $Ca(NO_3)_2$ precipitation of DNA.

Callus proliferation and selection: Shot parts of calli – not larger than 5mm in diameter -were removed from the original callus bombarded with the two plasmids' DNA, three days after gene shooting. Shot parts of calli were cultured on a selective medium containing 50mg/l kanamycin for callus proliferation for one month. Selective test was made by transferring the healthy green and white callus on three selective media containing 50mg/l kanamycin, 0.8M/l mannitol and (50mg/l kanamycin + 0.8M/l mannitol) for three months, subcultured every 3–4 weeks.

Transformation tests

Selection test: For transformation test, the bombarded callus cells were first placed on selective medium containing 50mg/l kanamycin. Resistant calli were transferred to three selective media containing 50mg/l kanamycin, 0.8M/l mannitol and (50mg/l kanamycin+0.8M/l mannitol) for callus selection. To screen for transformed cells, calli were cultured on selective medium containing 60, 70 and 150mg/l kanamycin, 0.8M/l mannitol and (70mg/l kanamycin + 0.8M/l mannitol) for three months, and later on selective medium containing (150mg/l kanamycin+0.8M/l mannitol). The selective test was repeated four or more times using (150mg/l kanamycin+0.8M/l mannitol) for 4 months. It was necessary to keep the callus in direct contact with the selective medium to develop transformed callus. Some parts from the control callus were cultured in the same bottle.

DNA isolation and PCR test: Genomic DNA from transformed and non-transformed control calli was extracted according to the QIAGEN Dneasy Plant System Mini Kit protocol (*Qiagen, GmbH* 2000). Klenow DNA polymerase reactions, and the double stranded sequencing of connecting

fragments of plasmid constructions were executed by the standard protocol (*Sambrook* et al. 1989), with minor changes based on the recommendations of the enzyme producing companies.

Statistical analysis: The difference among different genotypes and concentrations of kanamycin and mannitol were calculated using analysis of variance and the levels of significance were assessed by Duncan's test using *SPSS program* (1999).

Results and discussion

Callus induction and maintenance: In the case of Fönix and Maxidor bean cultivars, calli were induced from roots, leaf with petiole, leaf discs, hypocotyl segments and shoot apex explants cultured on B5 medium supplemented with 1mg/l kinetin and 2mg/l 2,4-D within one week. Calli were friable, pieces of the most friable calli were transferred to B5 and MS media in order to establish a callus culture. Further growth produced callus proliferation in large quantities within three weeks, so that several subcultures could be made from one initial culture. Much more bean calli were obtained by using roots, leaf with petiole or hypocotyl segments explant culture than using shoot apex or leaf discs culture in the two cultivars. Vertical explant culture in the case of hypocotyl segments or shoot apex gave more calluses than the horizontal culture.

B5 medium containing 1mg/l KIN and 2mg/l 2,4-D produced good and much callus from hypocotyl segment explant. Callus should be subcultured every 3–4 weeks. The protocol was repeated several times with similar results. Most of the media supplemented by (KIN+2,4-D) or (BA+NAA) stimulated rapid proliferation of callus. Hypocotyl segment explant gave the best results, and they were superior. This will help in introducing foreign DNA into cells. Callus formation was observed within one week in 80% of the cultured immature cotyledons of bean on Schenk & Hildebrandt medium (*Dixon* 1985) supplemented with 0.4mg/l 2,4-D, 1mg/l BAP (*Giovinazzo* et al. 1993). Similar callus induction systems were reported in common and tepary beans by *Mohamed* et al. (1993). Our observations are in agreement with previous studies. Callus was induced from bean hypocotyl and epicotyl (*Velich* et al. 1994). B5 medium completed with 1mg/l KIN and 2mg/l 2,4-D proved to be optimal in callus induction from hypocotyl and epicotyl in *P. vulgaris* L (*Gémesné Juhász* et al. 1995).

The results indicated that by using hypocotyl explant and vertical culture on B5 medium containing 1mg/l KIN and 2mg/l 2,4-D, we obtained good and much bean calli. On the other hand, when we have used B5 medium supplemented with the same plant growth regulators and MS medium supplemented with the others, we found that the B5 medium supplemented with 1mg/l KIN and 2mg/l 2,4-D induced much more callus than half strength MS medium supplemented with 0.5 or 0.75mg/l BA and 0.1mg/l NAA. In general, we can assume that cytokinin (KIN or BA) and auxin (2,4-D) in a ratio of 1:2 or 1:1 are useful for rapid callus induction in common bean.

Effectiveness of kanamycin on the growth of control callus: Prior to transformation, an effective concentration of antibiotic kanamycin for the selection of transformants was determined by culturing callus on B5 medium, containing various concentrations of kanamycin. The kanamycin sensitivity of callus growth was studied in the two cultivars of Fönix and Maxidor to investigate the suitability of kanamycin-resistance as a selectable marker for callus transformation. Phenotypically, we observed that 50mg/l kanamycin caused almost total inhibition of callus growth. This concentration was sufficient to kill the control callus. After repeating this experiment 3 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 from non-transgenic callus. We suggest that this is the minimum concentration that should be necessary to select transformed callus. So we recommend the use higher concentration to differentiate the transgenic callus with *npt* II gene from non-transgenic *(Figure 1)*.

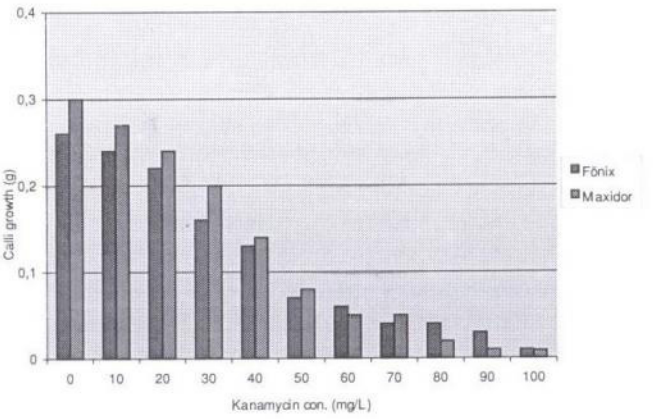

Figure 1 Effect of different concentrations of kanamycin on the growth of Fönix and Maxidor calli cultures growth on B5 medium

Effectiveness of mannitol on the growth of control callus: The mannitol sensitivity of callus growth was studied in the two cultivars of Fönix and Maxidor to investigate the suitability of mannitol-resistance for callus transformation. Phenotypically, we observed that 0.8M/l mannitol was sufficient to kill the control callus in the two cultivars. After repeating this experiment 3 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 from non-transgenic callus. So we recommend using this concentration to differentiate the transgenic from non-transgenic control callus *(Figure 2)*.

Transformation: The gene shooting system is well established in our laboratory. For genetic transformation we

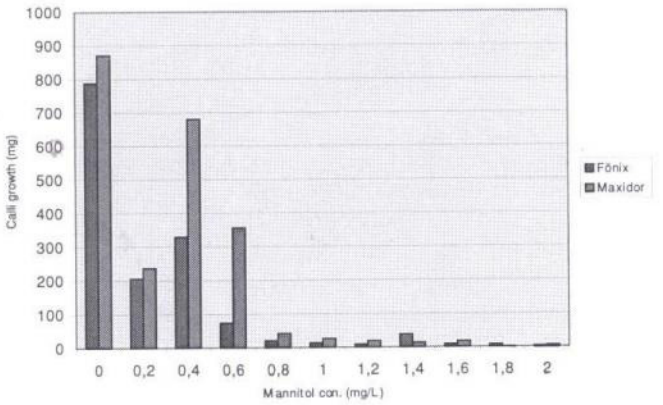

Figure 2 Effect of different concentrations of mannitol on the growth of Fönix and Maxidor callus cultures on a B5 medium

used the standard physical factors as it were described by *Jenes* et al. (1996 and 1997) and used by *Eissa* et al., (2001). More than 200 small callus parts were obtained after shooting and were transferred to selective media. This gives us the chance of using it to introduce useful genes into bean. We started to improve bean genotypes for drought tolerance using a mannitol-dehydrogenase gene (*mtl*) isolated from *E. coli*. When this gene is active in transgenic bean, the mannitol content of the cytoplasm is higher than in normal cells. These tissues can better tolerate dehydration stress.

Selection: The toxic levels of kanamycin and mannitol to non-transformed callus tissue had been determined in preliminary experiments *(Eissa* et al., 2001). The bombarded cells were incubated for 3 days without selection to allow expression of the resistance genes. After selection on kanamycin containing media (50, 60, 70, 100 and 150mg/l), several kanamycin-resistant calli have been obtained, they survived and grew. Control, non-transformed calli died. After selection on 0.8M/l mannitol-containing media, drought resistant calli had been obtained. After selection on (150mg/l kanamycin+0.8M/l mannitol)-containing media kanamycin and drought resistant calli had been obtained, while control and non-transformed calli were not able to grow in the presence of (150mg/l kanamycin+0.8M/l mannitol). In a previous attempt for bean callus transformation *(Eissa* et al., 2001) no mannitol tolerant callus was obtained. Now, the growth of putative transformed calli was rapid, doubled in size during two weeks and appeared normal (white and green friable) up to 150mg/l kanamycin+0.8M/l mannitol. Since the transformed calli showed a fresh weight increased on medium containing 150mg/l kanamycin+0.8M/l mannitol, we concluded to high level of kanamycin and mannitol-resistance in these calli. Resistance of the selected calli was verified by their ability to grow on selective-media containing 150mg/l kanamycin+0.8M/l mannitol repeatedly.

These results showed that kanamycin is an effective selection marker for *P. vulgaris*. Stable kanamycin and mannitol-resistant-transgenic lines were obtained after introduction (co-transformation) of chimeric genes encoding the enzymes neomycin phosphotransferase and mannitol-1-phosphate dehydrogenase. The presence of 50mg/l kanamycin inhibits callus induction and/or growth and causes a rapid browning and degeneration of the bean tissues (*Mariotti* et al. 1989). *Christou* et al. (1988) used 50 and 100mg/l kanamycin for selection kanamycin-resistant colonies of soybean and cultures were amplified on 100mg/l. Resistant bean calli could be distinguished 3–4 weeks after selection started by adding kanamycin to the medium at a concentration of 75mg/l (*Eapen* et al. 1987). The transformed callus colonies were further cultured in the presence of 100mg/l kanamycin. After direct gene transfer (*Köhler* et al. 1987) detected kanamycin-resistant calli of moth bean on a medium supplemented with 75mg/l kanamycin.

Similar results were reported by *Genga* et al. (1990) after selection on 100mg/l kanamycin-containing medium several kanamycin-resistant-bean calli had been obtained. To verify callus transformation, kanamycin-resistant calli were maintained by successive regular subcultures on the kanamycin-containing medium. *McClean* et al. (1991) obtained callus capable of growing in the presence of up to 500µg/ml kanamycin from the bean tissues whereas control callus ceased growing at 200µg/ml kanamycin. Callus production of transformed dry bean explants continued on the MS media and control explant did not produce callus at 20mg/l kanamycin. The kanamycin concentration was increased to 50mg/l over a 2 month period (*McClean* et al. 1988). Kanamycin at a concentration of 50mg/l was chosen by (*Franklin* et al. 1993) for selecting transformed callus from hypocotyl explants of *P. vulgaris*. The transformed callus proliferated rapidly on a kanamycin-selection medium. After co-cultivation bud explants of *P. acutifolius*, (*Dillen* et al. 1997) put the explants on a medium containing 300mg/l of kanamycin or 20mg/l of geneticin as a selective agent, that antibiotic concentration completely inhibited callus formation on non-transformed leaf discs.

PCR: The presence of the mannitol dehydrogenase gene (*mtl*) in the transformed cells was detected by PCR method using specific primers for the gene. Among 12 lines of kanamycin resistant calli, five lines contained the *mtl* based on the PCR test (*Figure3*).

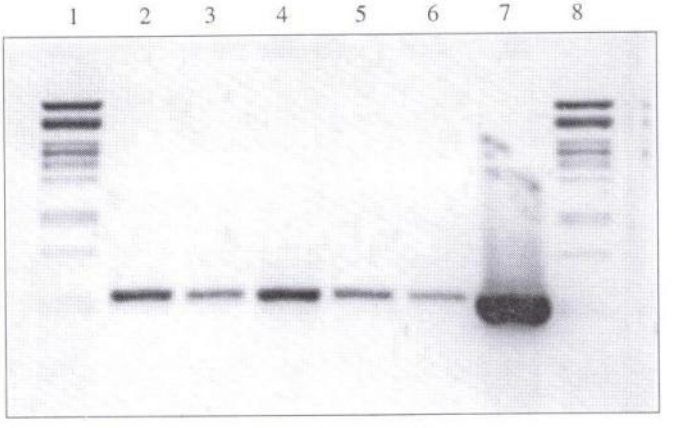

Figure 3 PCR for detection of mt1 gene
Notes: **1, 8** lanes = ladder; **2, 3, 4, 5, 6,** streaks = genomic DNA purified from the transgenic calli; **7** lane = pFF19-mtl plasmid

According to our experiments, it is possible to introduce osmosis-protective genes into bean (*P. vulgaris* L.) by a special Genebooster™ equipment-mediated DNA transfer.

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