Co-transformation of bean callus using high-velocity microprojectiles-mediated DNA transfer

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Eissa Ahmed E., Bisztray Gy. and Velich I.

Szent István University, Faculty of Horticultural Science, Department of Genetics and Horticultural Plant Breeding, H-1118 Budapest, Ménesi út 44. Hungary

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Summary: We have found that 50 mg/l kanamycin and 0.8 Mol/l mannitol concentration was sufficient to kill the control callus of bean (*Phaseolus vulgaris* L.) and differentiate transgenic from the non-transgenic cells. The GeneBooster particle delivery system was used for the bombardment of bean callus. The kanamycin resistance gene was used as a selectable marker. The test was made by transferring the healthy white callus, subcultured for three months on selective and non-selective medium. After selection on kanamycin containing media, several kanamycin resistant calli had been obtained, survived and grew. After selection on mannitol containing media no drought resistant calli had been obtained. Resistance of the selected calli were verified by their ability to grow repeatedly on selective medium containing 150 mg/l kanamycin. Selective pressure was maintained over a period of 8 months.

Introduction

Today great effort is being directed towards the development of drought-tolerant crop genotypes through the use of plant breeding strategies (*Velich* et al. 1994), involving the integression of genetic material from saline-tolerant wild species into cultivated plants. With recent developments in biotechnology, the possibility is also given to obtaining drought-tolerant crop genotypes by the use of somatic cell selection or protoplast fusion or by gene transformation using the recombinant DNA method. Optimal yield, even under extreme environmental conditions is highly desirable in bean production and an objective of breeding. They tested mannitol treatment of *in vitro* bean callus to study of drought stress reaction complex. They omitted the mannitol treatment of callus from the comparison as it is not suitable to the rapid determination of a high number of lots. *Gémesné Juhász* et al. (1995) studied the effect of non-ionic osmotic stress on bean callus cultures. They maintained callus cultures of 26 varieties of bean (*Phaseolus vulgaris* L.) on media containing various concentration of mannitol. The induced non-ionic osmotic stress inhibited growth, the relative growth rate is lower and dry matter content is higher in stress sensitive calli than in less sensitive one. We wanted to see the effect of an *E. coli* mannit dehydrogenase gene *(mtl)* on the sugar composition and drought or saline tolerance of bean callus cells.

The most promising procedure is gene shooting which is still under significant development (*Jenes* et al. 1997). The process involves the high velocity acceleration of microprojectiles carrying foreign DNA, penetration of the cell wall and membrane by microprojectiles and delivery of the DNA into plant cells (*Klein* et al. 1987). Microprojectiles have been used to carry RNA or DNA into plant tissue and these molecules were subsequently expressed genetically. Theoretically any type of cell or tissue can be used as a target for gene transfer. *Mariotti* et al. (1989) obtained transformed cells of *Phaseolus vulgaris* L. and *P. coccineus* L. with kanamycin resistance. Cells were able to form growing callus when cultured on medium containing 50 mg/l kanamycin.

Material and methods

Plant material: Seeds of *Phaseolus vulgaris* cv. *Fönix* and *Maxidor* -two commercialized bean varieties -were used as source of plant material. Materials were obtained from the breeder (*Prof. I. Velich*).

Preparation of the explants and callus induction: Bean *in vitro* cultures and callus induction were obtained by the methods of *Eissa Ahmed* et al. (2000). After explant preparation cultured on B5 medium according to *Gamborg* et al. (1968). The medium contains 1 mg/l kinetin and 2 mg/l

2,4-D, callus was produced within one week and subcultured every 3-4 weeks.

Kanamycin sensitivity test of bean callus: Calli were treated by 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 and 100mg/l kanamycin. To define which concentration was sufficient to kill the control callus and differentiate transgenic from the nontransgenic cells.

Mannitol sensitivity test of bean callus: In another experiment calli were treated by 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2 Mol/l mannitol. To define which concentration was sufficient to kill the control callus and differentiate transgenic from the nontransgenic cells.

Plasmid: We used plasmid pFF19K carries the kanamycin resistance gene as selectable marker and plasmid pFF19-mtl carries the mannit dehydrogenase gene (*mtl*) (from G. Dallmann, ABC Gödöllő, Hungary).

Gene shooting: We used the standard physical factors as it was described by *Jenes* et al. (1997). The appropriate pressure of N_2 gas for the bombardment of the tissue, the shooting distance and the size of the Tungsten particles used as microprojectiles. The microprojectiles were accelerated using high-pressure Nitrogen gas. 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.

Culture media, callus proliferation and selection: After 3 days from gene shooting, shot calli were removed from the original bombarded with 2 plasmid. Cultured on a selective medium containing 50 mg/l kanamycin for one month. Selective test was made by transferring the healthy white callus on three selective medium containing 50mg/l kanamycin, 0.8 Mol/l mannitol and 50 mg/l kanamycin+0.8 Mol/l mannitol for three months. Calli were subcultured every 3–4 weeks.

Transformation tests: The bombarded callus cells were first placed on selective medium containing 50 mg/l kanamycin. The resistant calli were transferred to selective medium containing 50 mg/l kanamycin or 0.8 Mol/l mannitol and or 50 mg/l kanamycin+0.8 Mol/l mannitol. To screen for transformants cells, calli were cultured on selective medium containing 60, 70 and 150 mg/l kanamycin for three months. The selective test was repeated three or more times using 150 mg/l kanamycin for four months. We cultured parts from control callus in the same bottle or tube as a control.

Results and discussion

Callus induction: The results showed that bean callus were induced from roots, hypocotyl and shoot apex explants on B5 medium supplemented with 1 mg/l kinetin and 2 mg/l 2,4-D within one week. Callus should be subcultured every 3–4 weeks. This will help in introducing foreign DNA into cells. *Velich* et al. (1994) induced callus from bean hypocotyls and epicotyls. Callus cultures were prepared from 26 bean cultivars and candidates representing bean types in Hungary (*Gémesné Juhász* et al. 1995). They cut first-leaf, epicotyl and hypocotyl of the 7-day-old seedlings into parts 5 to 8 mm-long sections. They placed all explants on initiation medium: the B5 medium (*Gamborg* et al. 1968) supplemented with 2% (w/v) sucrose, 0.7% agar, 2,4-dichlorophenoxy acetic acid (2,4-D) and kinetin in different concentrations, 2 mg/l 2,4-D only or 2 mg/l 2,4-D+0.5 mg/lkinetin or 2 mg/l 2,4-D+1 mg/l kinetin. Callus initiation B5 medium containing 2mg/l 2,4-D and 1mg/l kinetin proved the best. Though 2,4-D alone could induce callus but in the absence of kinetin calli got brown, stopped developing and died in 2–3 weeks. In the efficiency of callus development no differences were found in respect to different inocula. In the callus maintenance media the decrease of kinetin content (B5, 2 mg/l 2,4-D and 0.6 mg/l kinetin) favoured the prolongation of cultures in contrast to the induction media.

The effect of kanamycin on the growth of control callus: Our initial experiments focused on the demonstration of the concentration of kanamycin added to the callus culture that was effective to kill the callus. We observed that 50 mg/l kanamycin was sufficient to kill the control callus of *Fönix* and *Maxidor*, so we recommend using this concentration to differentiate the transgenic from the non-transgenic control callus.

The effect of mannitol on the growth of control callus: Also our initial experiments focused on the demonstration of the effect of mannitol concentration (added to the callus culture) that was effective to kill the callus. We observed that 0.8 Mol/l mannitol was sufficient to kill the control callus of *Fönix* and *Maxidor*, so we recommend to use this concentration to differentiate the transgenic from the non-transgenic control callus. *Velich* et al. (1994) tested the mannitol treatment of *in vitro* bean callus to study of drought stress reaction complex. They transferred the induced callus to a medium containing 9% mannitol for 16 days and determined the relative growth rate and soluble solids. They omitted the mannitol treatment of callus from the comparison, as it is not suitable to the rapid determination of a high number of samples. *Gémesné Juhász* et al. (1995) found that the growth of callus cultures decreases considerably in the treatment with 9% mannitol. The relative growth rate decreased in treatments of 3%, 5%, 7%, 9% and 11% mannitol in every genotype. Mannitol concentration surpassing 9% resulted in a negative growth rate value in every genotype. It means at the same time the upper limit of the treatment concentration. On media containing 9% mannitol differences between genotypes can be seen clearly. In the treatment of 9% mannitol concentration the negative growth rate indicates the high water loss of cells resulting in senescence and death of cells. Due to the high percentage of cell death, differentiation between genotypes cannot be

made. Calli varieties of negative rate can be taken for sensitive to osmotic stress. Genotypes with positive growth rate at 9% mannitol concentration seem to be less sensitive to osmotic stress.

Transformation of callus: *Klein* et al. (1987) reported that nucleic acids can be delivered into plant cells using high-velocity microprojectiles. After being accelerated, small Tungsten particles pierce cell walls, membranes and enter plant cells without killing them. We used the standard physical factors as it was described by *Jenes* et al. (1997). Shot calli were removed from the original bombarded plate after three days and cultured on a selective medium containing 50 mg/l kanamycin. After selection on kanamycin containing media, several kanamycin resistant calli have been obtained, found survived and grew. Control, non-transformed calli were not able to grow in the presence of 50 mg/l kanamycin *Figure 1*.

Similar results were obtained by *Mariotti* et al. (1989). They found that root tips or segments from bean plants can be induced to form an actively growing callus on UM solidified medium supplemented with 2 mg/l 2,4-D and 0.1 mg/l kinetin. The presence of 50 mg/l kanamycin inhibits callus induction and/or growth and causes a rapid browning and degeneration of the tissues.

After selection on mannitol containing media no drought resistant calli had been obtained, found brown and died. After selection on kanamycin and mannitol containing media no kanamycin and drought resistant calli had been obtained, found brown and died. The presence of 0.8 Mol/l mannitol inhibits callus growth and causes a rapid browning and degeneration of the cells. Controlled and non-transformed calli died. Resistance of the selected calli was verified by their ability to grow repeatedly on selective medium containing 150 mg/l kanamycin *(Figure 2)*.

The presence of the kanamycin resistance gene and the *mtl* gene in the cells is detected by PCR method using specific primers for the genes and the sugar content of the cells are monitored by OPLC analysis are in progress.

According to our experiment it is possible to introduce genes into *Phaseolus vulgaris* bean by high-velocity microprojectiles-mediated DNA transfer. The method can be used in the future for transformation of bean.

Figure 1 Shot *Fönix* callus growing into selective medium containing 50 mg/l kanamycin after one month

Figure 2 Transient expression from the pFF19K gene in *Fönix* callus after 7 months subcultured and gene fusion was tested by selection on kanamycin stable transgenic lines cultured on media containing 150 mg/l kanamycin.

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