High-velocity microprojectile mediated DNA delivery into *Phaseolus vulgaris* callus cells

INTERNATIONAL JOURNAL OF HORTICULTURAL SCIENCE

AGROINFORM
Publishing House, Hungary

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

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

Key words: *Phaseolus vulgaris*, bean, callus, transformation, hygromycin, gene-gun

Summary: We report the method for the establishment of rapidly growing callus cultures of Phaseolus vulgaris and the conditions required for efficient transformation using high velocity microprojectiles and high level of transient gene expression. Using hypocotyl explant and vertical culture on B5 medium with 1mg/l kinetin and 2 mg/l 2,4-D, we can recommend to get a rapidly growing callus from bean which is a good starting material to introduce foreign DNA into bean cells. The GeneBooster particle delivery system was used for the bombardment of bean callus and the Hgm resistance gene (Hgmr) was used as a selectable marker gene. 25mg/l hygromycin (Hgm) concentration was sufficient to kill the control callus. We used the standard physical factors, the appropriate pressure of N2 gas for the bombardment of the callus tissue, the shooting distance and the size of tungsten particles used as microprojectiles. Selective and nonselective tests were made by transferring the healthy green and white calluses, subcultured for 4 months on selective and nonselective medium. Several Hgm resistant calli had been obtained. Selective pressure was maintained over a period of 10 months.

Introduction

Difficulties in obtaining plants from somatic cells or protoplasts in *Phaseolus* species have till now hampered the production of transgenic plants by the application of the most common methods available for the introduction of foreign DNA into cells *(Genga* et al., 1990). Gene transfer technology in fact, requires reproducible protocols for in vitro culture and efficient plant regeneration from protoplasts, cells or organs. In case of bean, no stable transformation and regeneration method is routinely available *(Giovinazzo* et al., 1993). The most promising procedure is "gene shooting" which is still under significant development *(Jenes* et al., 1997).

The process of gene delivery by the bombardment of DNA into intact plant cells has been described in detail *(Sanford,* 1990) and several types of shooting devices have been reported. 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. This approach therefore can be used to study the transient or stable expression of foreign genes in an intact tissue. Theoretically any type of cell or tissue can be used as a target for gene transfer. Some target tissues such as embryogenic suspension culture *(Vasil* et al., 1992; and *Jenes* et al., 1996), and meristematic tissue *(McCabe* et al., 1988) have proven to be transformable and are able to grow into transgenic plants.

In case of legumes *(McCabe* et al., 1988) used particle acceleration by electric discharge to introduce DNA-coated gold particles into meristems of immature soybean seeds. *Allavena & Bernacchia* (1991) attempted genetic transformation of bean by high velocity microprojectiles. They used cotyledons with the adaxial face exposed to the flight path of the microprojectiles and embryo axis with primary leaves were also exposed to bombardment. They found that 60% of the apical meristems showed at least one GUS expressing unit after three shots on the same plant material. Brasileiro et al. (1996) made susceptibility of common and tepary beans to *Agrobacterium* spp. strains using microprojectile bombardment. Apical meristems of *Phaseolus vulgaris* cv. Jalo were bombarded with Tungsten microprojectile then inoculated with an *A. tumefaciens* wild type strain (Ach5). One month later, the explants showed 50 to 70% a tumor formation. Similarly, when bombarded meristems were inoculated with an *A. tumefaciens* disarmed strain, 44% of them showed substantial sectors of GUS activity, suggesting the expression of introduced gene. *Mariotti* et al. (1989) obtained transformed cells of

Phaseolus vulgaris L. and *P. coccineus* L. with kanamycin (Km) resistance. Cells were able to form growing callus when cultured on medium containing 50mg/l Km.

In this paper we report the establishment of a rapidly growing callus culture of *Phaseolus vulgaris* cv. Fönix and Maxidor whereas the conditions required for the successful transformation of callus using GeneBooster for delivering gene into callus cells as well as conditions for high level of transient gene expression.

Material and methods

Plant material

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

Preparation of the explants and callus induction

Dry bean seeds were sterilised and germinated by the methods of *(Eissa Ahmed* et al. 1999). After 7–10 days, two types of explants were prepared: shoot apex and hypocotyl. Explants were cultured on B5 medium *(Gamborg* et al., 1968) vertically or horizontally in 55x90mm glass bottles. On the medium containing 1mg/l kinetin 2mg/l dichlorophenoxyacetic acid (2,4-D), 20g/l sucrose, 8g/l agar-agar, the pH was adjusted to 5.7 before autoclaving. Callus was produced within one week. After 3-4 weeks, callus was transferred to fresh medium and subcultured every 3-4 weeks. Sometimes the explants were cultured on medium: half MS major salts+ minor elements (Murashige and Skoog, 1962) supplemented with B5 vitamins + 0.5 or 0.75mg/l benzyladenine (BA) and 0.1mg/l naphthaleneacetic acid (NAA).

Hygromycin B (Hgm) sensitivity test of bean callus

Calli were treated by 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35 and 40mg/l Hgm on the same media using 8 replicates per each treatment, and the calli were weighted after 57 days. Statistical analysis was made to define which dose was effective to kill the control callus.

Plasmid

We used plasmid pFF19H. This plasmid carries the Hgm resistance gene *(Timmermans* et al., 1990).

Gene shooting

We used the standard physical factors as it was described by *Jenes* et al., (1996) for the bombardment. The macroprojectiles (plastic macrocarriers) were acceleratedy using high-pressure Nitrogen gas. Tungsten particles (0.7–1.6M diameter on average) were used as microprojectiles (microcarriers) 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 callus were removed from the original callus and cultured directly on a selective medium containing 20mg/l Hgm B. Healthy green and white calluses were subcultured every 3–4 weeks for 4 months. To screen for transformed cells, calli were cultured on selective medium containing 25mg/l Hgm B for 1 month. Then calli were transferred to non-selective medium for 1 month and finally to selective media again containing 25mg/l Hgm B. The selective non-selective test was repeated two or more times using 30mg/l Hgm B. During all these periods, calli were subcultured every 3–4 weeks. We cultured parts from control callus in the same bottles or tubes as a control.

Results and discussion

Callus induction

In Fönix and Maxidor cultivars, calli were induced from hypocotyl and shoot apex explants on B5 medium supplemented with 1mg/l kinetin and 2mg/l 2,4-D within one week. Results showed that more bean calli were obtained by using hypocotyl explant culture than by shoot apex culture. Vertical explant culture of hypocotyl or shoot apex gave more callus than horizontal culture. The B5 medium supplemented with 1mg/l kinetin and 2mg/l 2,4-D induced much more callus than half MS medium supplemented with 0.5 or 0.75mg/l BA and 0.1mg NAA. Callus should be subcultured every 3–4 weeks. This will help in introducing foreign DNA into cells. These results are comparable to the other ones found in the literature. *Giovinazzo* et al. (1993) induced calli from immature cotyledons sliced in three pieces, placed on SH medium supplemented with 2mg/l pCH, 0.4mg/l 2,4-D, 1mg/l 6BAP and 0.6% agar. They found that callus formation occurred within 1 week approximately on 80% of the cultured cotyledons. Callus was subcultured every 20 days. *Mohamed* et al. (1993) prepared cotyledonary leaf explants from 2 weeks old seedlings. The explants were cultured on medium containing 4% sucrose and $0.2g/l^{-1}$ casein hydrolysate and supplemented with combinations of 2,4-D (4, 8 or 16M) and kinetin (0, 1, 2 or 4M) for callus induction. In general we can assume that auxin (2,4D) and cytokinin (kinetin or BA) in a ratio of 2:1 or 1:1 are useful for rapid callus induction of common bean.

Effectiveness of Hgm B on the growth of control callus

Our initial experiments focused on the demonstration of the concentration of Hgm B added to the callus culture that was effective to kill the callus. We observed that 25mg/l Hgm B was sufficient to kill the control callus of Fönix and Maxidor *(Fig. 1)*. So we recommend using this

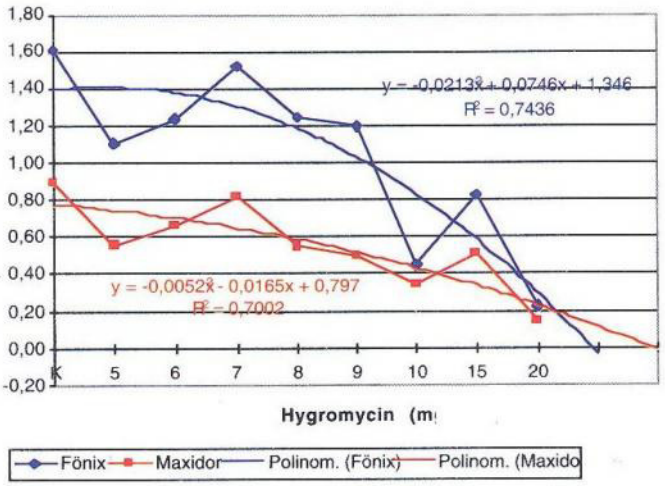

Figure 1 Effect of varying concentrations of hygromycin 0, 5, 6, 7, 8, 9, 10, 15 and 20 mg/l media on the growth of Fönix and Maxidor callus cultures grown into B5 medium.

concentration to differentiate the transgenic callus from the non-transgenic control callus.

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 and membranes, thus enter intact plant cells without killing them. We introduced the plasmid carrying Hgm resistance gene into the target tissues. After selection on Hgm-containing media, several (Hgm^r) callus had been obtained, found to survive and grow. Control, non-transformed calli were not able to grow in the presence of Hgm *(Fig. 2)*.

Mariotti *et al.* (1989) found that root tips or segments from bean plants can be induced to form an actively growing callus on media supplemented with 2mg/l 2,4-D and 0.1mg/l kinetin. The presence of 50mg/l kanamycin inhibits callus induction and/or growth, and causes a rapid browning and degeneration of the tissues. Similar results were obtained by *(Jenes* et al., 1997) on rice. They used rice suspension cultures derived cell clusters bombarded with plasmid DNA. Transformed and non-transformed cells could be selected at a relatively low concentration of antibiotic, on a medium containing 50mg/l Hgm B. We used 25mg/l hygromicin and that concentration was sufficient to kill non-transformed cells while transformed cells were able to grow even at 30mg/l hygromycin concentration *(Fig. 3)*. The presence of the resistance gene in the cells was detected by PCR method using specific primers for the gene. Transformed calli were maintained on selective medium for 10 months.

The methods described here are useful for the establishment of rapidly growing callus cultures of bean and to transform bean cells for transient or stable gene expression by gene shooting.

Figure 2 Selection of Maxidor callus for resistance to 25mg/l hygromycin after approximately 5 weeks on selective medium (in the center) yield callus resistant to the antibiotic, whereas control callus do not (around).

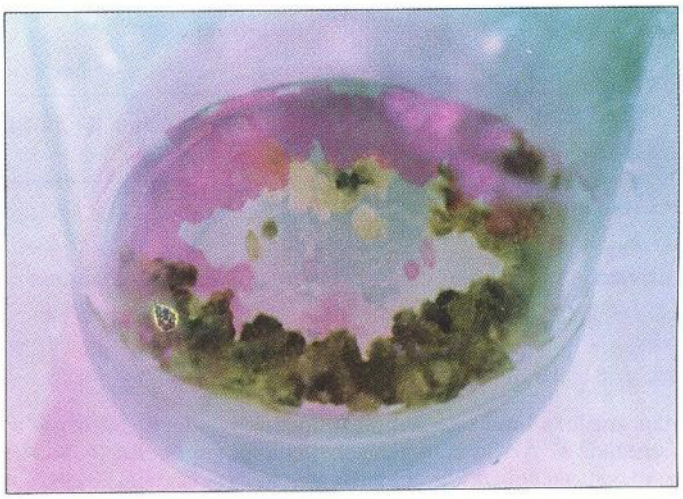

Figure 3 Maxidor callus, non-transformed callus were not able to grow in the presence of 30mg/l hygromycin (around center) while transgenic callus was resistante it could grow (at the center).

Acknowledgments

The authors wish to thank Pedryc Andrzej, for his help with the statistical analysis of data and his assistance with the manuscript.

References

Allavena, A. & Bernacchia, G. (1991): Attempting genetic transformation of bean by high velocity microprojectiles. Annual Report of the Bean Improvement Cooperative. BIC. 34: 137–138.

Brasileiro, A. C. M., Aragáo, F. J. L., Rossi, S., Dusi, D. M. A., Barros, L. M. G. & Rech, E. L. (1996): Susceptibility of common and tepary beans to *Agrobacterium* spp. strains and improvement of *Agrobacterium*-mediated transformation using microprojectile bombardment. J. Amer. Soc. Hort. Sci. 121(5): 810–815.

Eissa Ahmed E., Bisztray Gy. & Velich I. (1999): Attempting regeneration from cultured cotyledons and plant regeneration from

cotyledonary nodes in common bean (*Phaseolus vulgaris* L.). International J. of Hort. Sci., 5(1-2): 57–60.

Gamborg, O. L., Miller, R. A. & Ojima, K. (1968): Nutrient requirements of suspension cultures of soybean root cells. J. Exp. Res. 50: 151–158.

Genga, A., Ceriotti, A., Bollini, R. & Allavena, A. (1990): Preliminary approaches for genetic transformation of bean. Annual Report of the Bean Improvement Cooperative. BIC. 33: 75.

Giovinazzo, G., Greco, V. & Bollini, R. (1993): Optimization of cell suspension culture, protoplasts isolation, and transient transformation of *Phaseolus vulgaris*, L. Annual Report of the Bean Improvement Cooperative. BIC. 36: 14.

Jenes B., Bittencourt, P. A. L., Csányi Á., Pauk J, Nagy I., Toldi O. & Balázs E. (1996): The GENEBOOSTER – a new microparticle bombardment device – for genetic transformation of plants. Plant Tissue culture and Biotechnology. 2: 42–51.

Jenes B., Toldi O., Bittencourt P., Nagy I., Csányi Á. & Balázs E. (1997): The GENEBOOSTER -designed and developed by the Agricultural Biotechnology Center, Gödöllõ. Hungarian Agricultural Research. 3: 14–17.

Klein, T. M., Wolf, E.D., Wu, R. & Sanford, J. C. (1987): High-velocity microprojectiles for delivering nucleic acids into living cells. Nature. 327(7):70–73.

McCabe, D. E., Swain, W. F., Martinell, B. J. & Christou, P. (1988): Stable transformation of soybean (*Glycine max*) by particle acceleration. Bio/Technology. 6: 923–926.

Mariotti, D., Fontana, G. S. & Santini, L. (1989): Genetic Transformation of grain legumes: *Phaseolus vulgaris* L. and *P. coccineus* L. J. Genet. and Breed. 43: 77–82

Mohamed, M. F., Coyne, D. P. & Read, P. E. (1993): Enhanced differentiation of somatic embryoids in callus cultures of common bean. Annual Report of the Bean Improvement Cooperative. BIC. 36: 16–17.

Murashige, T. & Skoog, F. (1962): A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15: 473–497.

Sanford, J. C. (1990): Biolistic plant transformation. Physiol. Plant. 79: 206–209.

Timmermans, M. C. P., Maliga P. Vieira, J. & Messing, J. (1990): The pFF plasmids: casettes utilising CaMV sequences for expression of foreign genes in plants. J. of Biotechnology, 14: 333–344.

Vasil, V., Castillo, A. M., Fromm, M. E. & Vasil, I. K. (1992): Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Bio/Technology. 10: 667–674.