Production of transgenic kidney bean shoots by electroporation of intact cells

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Abstract

We obtained transformed bean shoots by electroporation of intact bean cells with the plasmid pDPG165 containing bar gene conferring herbicide resistance to plants. Transformed shoots were selected from electroporated callus on herbicide containing media. Data of molecular analysis (PCR and Southern blotting) confirmed the insertion of bar gene in the genome of herbicide resistant shoots. Detailed procedures for obtaining regenerative bean callus, optimization of electroporation of intact cells and transgenic shoots are given.

Additional key words: herbicide resistance, PCR, *Phaseolus vulgaris*, regeneration, Southern blotting, transformation.

Introduction

The use of Ti plasmid of *Agrobacterium tumefaciens* as an effective vector to introduce foreign genes into cells of dicotyledonous plants has encouraged the wide application of this technique for legume crops (Hall and DeRose 1988, Kung 1993, Yang 1993). Several successful transformation and regeneration systems have been reported in legumes, *e.g.* bean (Badr *et al.* 1998, Franklin *et al.* 1993), alfalfa (Thomas *et al.* 1992), lentil (Warkentin and McHughen 1992), and cicer (Fontana *et al.* 1993). However, several factors including bacterial strain, origin of explant, plant genotype and cultivar as well as pH of the medium are known to influence the efficiency of *Agrobacterium*-mediated transformation (Badr *et al.* 1998, Holford *et al.* 1992, Lulsdorf *et al.* 1991, Pickardt *et al.* 1991).

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Abbreviation: MS - Murashige and Skoog; NAA - α-naphthalene acetic acid; BAP - benzyl aminopurine; 2,4-D - 2,4 dichlorophenoxy acetic acid.
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Electroporation, *i.e.* electric field mediated membrane permeabilization (Van Wert and Sundars 1992), is known as an appropriate method that can be used for altering genetic make up of genotypes recalcitrant for *Agrobacterium*-mediated transformation. The main advantage of electroporation is the elimination of the typical host specificity problems for *Agrobacterium*. On the other hand, the regeneration of transformed protoplasts to fertile plants remains a complicated procedure in many cases. Recently, some progress has been made when intact plant cells instead of isolated protoplasts were transformed successfully by electroporation, as in the case of sugar cane (Arencibia *et al.* 1992, 1995), maize (D'Halluim *et al.* 1992), rice (Xu and Li 1994), and kidney bean (Dillen *et al.* 1995). Up to now there has been published only a limited number of reports and the potential of this techniques has to be investigated in more detail using various plant species and experimental conditions.

For successful transformation two prerequisites should be optimized, namely the genotype independent gene delivery system and the availability of plant regeneration. So herein, we describe a protocol for the transformation of an Egyptian kidney bean variety through electroporation of intact callus cells and subsequent regeneration of transgenic kidney bean shoots from electroporated callus.

Materials and methods

Production of regenerative kidney bean callus: Seeds of kidney bean (*Phaseolus vulgaris* L. cv. Giza 3) were surface sterilized and germinated onto MS medium containing 1 mg dm^{-3} BAP. Decapitated shoot tip explants were cultured onto MS medium supplemented with 2 mg dm^{-3} 2,4-D and 0.5 mg dm^{-3} BAP, for callus proliferation. The proliferated callus (1-month-old) was used for cell electroporation. Electroporated callus was transferred to regeneration medium which contained MS basal salts, 2 mg dm^{-3} BAP and 0.5 mg dm^{-3} NAA. These conditions were optimized after a series of preliminary experiments.

Plasmid: The *E. coli* strain harboring the plasmid pDPG165 which carries the bar gene, conferring herbicide resistance to plants, was kindly provided by Dr. A. Arenciba, Center for Genetic Engineering and Biotechnology, Plant and Fertilizer Division, Havana, Cuba. Plasmid DNA was purified following to the protocol of *Qiagen* for plasmid maxi preparation and its final concentration was estimated by gel electrophoresis.

Preparation of callus tissue for electroporation: Friable callus obtained as described previously was subjected to the following steps: *1)* 150 mg of fresh callus tissue was suspended in electroporation buffer EPB (5 mM $CaCl_2$, 10 mM HEPES, 10 % glucose, pH 7.2) according to Dekeyser *et al.* (1990), which was replaced every hour. *2)* After 4 h of preplasmolysis, the cell aggregates were collected by centrifugation and resuspended in 0.6 cm^3 of EPB containing 0.2 M spermidine, then mixed with the desired amount of plasmid (*i.e.* 20, 40, 80, 160 and 320 μg) and the samples were kept 3 h on ice in the dark. *3)* 0.055 cm^3 of 3 M NaCl (final concentration 156 mM) were added to each sample prior to the electric discharge as described by Arencibia *et al.* (1995). *4)* Samples (approximately 0.8 cm^3 were transferred into sterile 0.4 cm^3 electrode gap cuvettes (*Bio-Rad*). *5)* The electroporation was carried out by discharging one pulse with desired field strength (200, 250, 300, 350, 400 V cm^{-1}) and capacitance (300, 500, 700, 900 μF). *6)* After electroporation, samples were transferred immediately to ice and incubated for 10 min, then transferred to the selective and regeneration medium, containing 4 mg dm^{-3} *Basta* (*Sigma*, USA), 2 mg dm^{-3} BAP and 0.5 mg dm^{-3} NAA. *7)* The efficiency of electroporation was evaluated based on the number of callus colonies that survived and proliferated on selective medium per electroporated sample.

DNA isolation and PCR analysis: DNA isolation was performed using the CTAB method of Doyle and Doyle (1990). One gram fresh sample was ground to powder in liquid nitrogen with a prechilled pestle and mortar, suspended in 5 cm^3 preheated CTAB buffer, and incubated at 65 °C for 1 h with occasional shaking. The suspension was then mixed with 1/3 volume of chloroform, mixed gently, centrifuged and the upper phase was transferred to a new sterilized tube. Extraction was repeated with an equal volume of chloroform. The aqueous layer was transferred to a new tube, 2/3 volume of isopropanol was added and nucleic acids were either spooled using a Pasteur pipette or sedimentated by centrifugation. The pellet was washed carefully twice with 70 % ethanol, dried at room temperature and resuspended in 0.5 cm^3 TE buffer. DNA was purified by incubation of the resuspended sample at 37 °C for 30 min with 20 μg cm^{-3} RNase (*Boehringer* Mannheim, Germany). DNA concentration was determined by electrophoresis of 0.005 cm^3 of sample along with serial dilutions of Lambda DNA in 0.8 % agarose (Castiglione *et al.* 1993).

PCR amplification of target bar gene, using the complementary primer 5′AGAACGACGCCCGGCCGACA 3′ and reverse primer 5′CCGTATAGGCTCGCGGAGC 3′, was performed in a 0.02 cm^3 reaction mixture as follows: 0.002 cm^3 10 × amplification buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.002 cm^3 (200 μM) dNTP mix (each of dATP, dCTP, dGTP and dTTP), 0.001 cm^3 of each primer, 0.002 cm^3 (40 ng) DNA sample, 0.0001 cm^3 (0.5 unit) Taq polymerase (*Promega*) and 0.0119 cm^3 sterilized distilled water. The reaction mixture was assembled on ice, overlaid with a drop of mineral oil and amplification conducted for 35 cycles using *MJ Research, Inc.* (USA) thermal controller preheated to 92 °C as follows: denaturation at 92 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 2.5 min. Amplification products were electrophoresed in 2 % agarose (1 % Nusieve GTG, 1 % Seakam LE, *FMC Bioproducts*) in TAE buffer for 3 h at 3 V cm^{-1} and visualized under UV-light after staining in 0.2 μg dm^{-3} ethidium bromide.

Southern blot analysis: Genomic DNA isolated from transformed and non transformed kidney bean shoots was digested with both Hind III and EcoR I, then electrophoresed overnight in 0.8 % agarose gel at 2 V cm^{-1}. The entire blotting procedure was performed as recommended by *Amersham* (Germany). The radioactive probe was prepared by the random primed labelling method according to Feinberg and Vogelstein (1984) using α-^{32}P dATP. The hybridization probe was the 1.9 kb Hind III/EcoR I fragment from the plasmid pDPG 165 containing the entire bar gene.

Results and discussion

Electroporation conditions and recovery of transgenic shoots: With a capacitance of 300 μF increasing field strength from 200 to 400 V cm^{-1} resulted in enhancement of electroporation efficiency with a maximum (3.66) at 400 V cm^{-1}. The same behavior was also recorded for capacitance, *i.e.* raising the capacitance from 300 to 900 μF is associated with increment in electroporation efficiency. Thus the highest electroporation efficiency of kidney bean cells can be achieved at 400 V cm^{-1} and 900 μF (Table 1).

Table 1. Effect of capacitance [μF] and field strength [V cm^{-1}] on the efficiency of electroporation of kidney bean callus cells. Efficiency of electroporation (transformation) is evaluated based on the number of callus colonies survived and proliferated onto the selective medium per electroporated sample. Three calli samples were electroporated with each combination of capacitance and field strength. Values represent the mean of three experiments ± S.E.

Capacitance [μF] Field strength [V cm^{-1}]	300	500	700	900
200	0.0	1.0 ± 0.8	4.3 ± 0.8	5.3 ± 1.6
250	0.3 ± 0.5	3.0 ± 0.9	4.6 ± 0.5	6.6 ± 0.4
300	2.0 ± 0.8	4.3 ± 0.5	6.0 ± 0.8	8.3 ± 1.6
350	2.3 ± 1.0	4.3 ± 0.9	6.3 ± 0.9	10.3 ± 0.4
400	3.6 ± 1.2	4.6 ± 1.2	7.0 ± 0.8	11.6 ± 1.2

A proportional relationship between the efficiency of electroporation and the concentration of DNA was recorded. The highest efficiency of electroporation was obtained with 160 μg DNA. Higher concentration of DNA (320 μg) had no significant enhancement effect (Table 2).

Table 2. Effect of plasmid DNA concentration [μg] on the efficiency of electroporation of kidney bean callus cells. The electroporation conditions were 900 μF and 400 V cm^{-1}.

DNA [μg]	20	40	80	160	320
Efficiency of electroporation	4.0 ± 0.8	8.6 ± 1.2	14.6 ± 1.2	17.6 ± 2.0	17.0 ± 1.6

The proliferation of kidney bean calli from electroporated cells and the subsequent recovery of transgenic shoots is the most interesting outcome of these experiments. Fig. 1*A* shows callus colonies with shoot primordia onto a selective medium containing 4 mg dm^{-3} of the herbicide *Basta*, 2 mg dm^{-3} BAP and 0.5 mg dm^{-3} NAA.

In our previous work, *Basta* at 4 mg dm^{-3} was found to be a lethal for Egyptian kidney bean callus *in vitro* (Badr *et al.* 1998). Formation of kidney bean shoots from callus proliferated from electroporated cells was obtained after two cycles of subculturing onto the same fresh selective medium (Fig. 1*B*). It should be mentioned that callus proliferated from non electroporated cells died after one week of cultivation on *Basta* containing medium (data not shown). Work is going on to multiply the selected transgenic bean shoots and to induce their root formation.

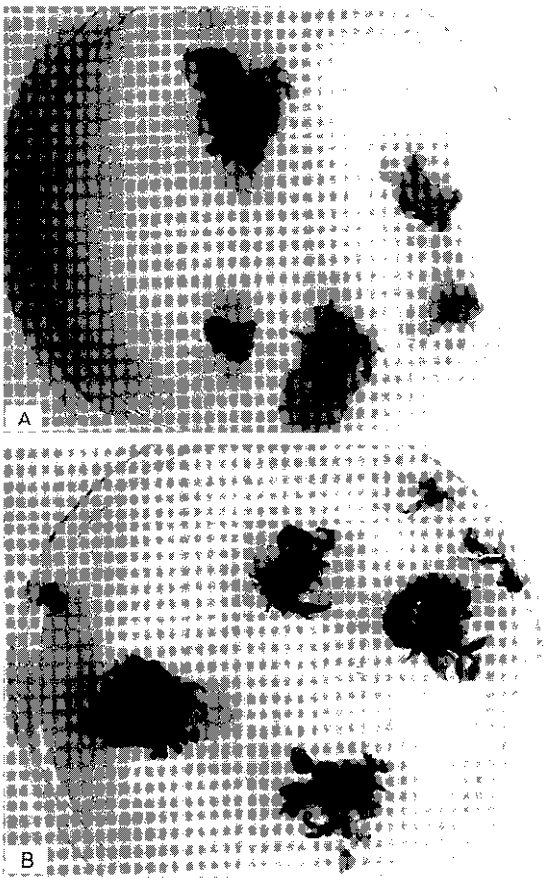

Fig. 1*A*. Calli proliferated from electroporated cells on selective medium (MS, 2 mg dm^{-3} BAP, 0.5 mg dm^{-3} NAA, 4 mg dm^{-3} *Basta*), age of the culture is two months. *B*. Transgenic bean shoots on the same medium, age of the culture is three months.

Molecular analysis of transgenic shoots: Genomic DNA isolated either from transgenic shoots (based on the selection) or from control shoots was analyzed by PCR amplification for the presence of the bar gene. Examined shoots, recovered from electroporated cells on selective medium, gave the expected amplification product as compared with the corresponding product of the plasmid pDPG 165, while in control shoots, no amplicons were detected (Fig. 2). PCR positive shoots were further analyzed by Southern hybridization. The hybridization signals obtained with DNA from transgenic shoots show the presence of a 1.9 kb band, as it is expected from the map for Hind III/EcoR I restricted pDPG 165 (Fig. 3). This indicates the integration of bar gene in the genome of kidney bean shoots. Moreover, it should be mentioned that the results of hybridization experiments confirmed the PCR analysis.

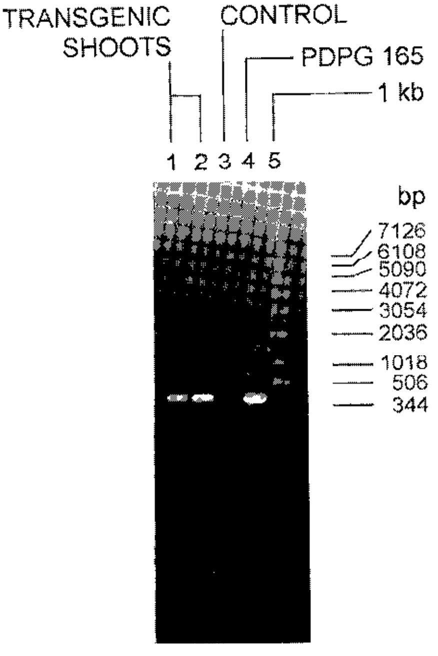

Fig. 2. PCR amplification products of genomic DNA isolated from transgenic (lanes 1, 2) and non transformed bean shoots (control, lane 3), PCR product of pDPG 165 plasmid, used in electroporation (lane 4), 1 kb DNA ladder as size marker (lane 5).

Based on the results of the present study and other work dealing with electroporation of intact cells (Arencibia *et al.* 1995, D'Halluim *et al.* 1992, Xu and Li 1994, Dillen *et al.* 1995) it may be concluded that for obtaining maximum electroporation efficiency in kidney bean, field strength, capacitance, and concentration of plasmid DNA should be taken into consideration. Electroporation conditions reported here for the Egyptian cultivar are different from those optimized by Arencibia *et al.* (1995) for sugar cane, D'Halluim *et al.* (1992) for maize, Xu and Li (1994) for rice and resemble those reported by Dillen *et al.* (1995) for seedling tissue of kidney bean. Accordingly, we suggest that optimal electroporation conditions must be determined experimentally for each species and we attribute these variations to size and concentration of plasmid DNA to transfer, the physical character of recipient plant cells and experimental conditions. Concentration of plasmid DNA seems to be a critical factor. Herein we noticed that 160 μg of DNA is necessary to achieve highest efficiency. However, in most previous publications low amounts (20 - 30 μg) of DNA were used, with exception of Arencibia *et al.* (1995) who applied 150 μg for electroporation.

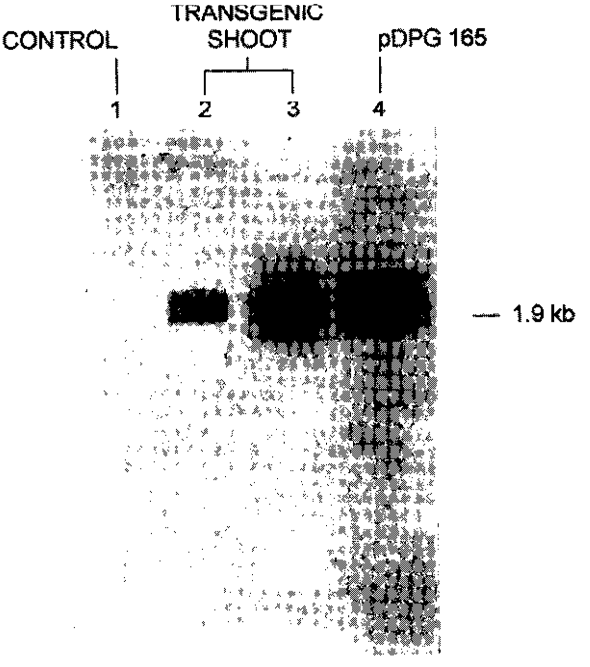

Fig. 3. Results of Southern blot hybridization analysis performed on Hind III/EcoR I digested genomic DNA isolated from non transformed bean shoots (1, control), transgenic shoots (2, 3) and Hind III/EcoR I digested pDPG 165 plasmid (4).

From the foregoing results, it can be also concluded that a suitable regeneration system represents the basis for successful recovery of transgenic kidney bean shoots. That can be achieved by optimizing of a simple protocol. Electroporation of organized, regenerable callus tissue and a short callus phase allowed us to recover shoots from electroporated tissue. Some factors optimized here may be a guide for other plant species.

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