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TRANSFORMATION OF PETUNIA PROTOPLASTS BY ELECTROPORATION

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ABSTRACT

Transformation efficiency of electroporated protoplasts of petunia was determined by assaying β-glucuronidase (GUS) activity. The uptake of foreign DNA by protoplasts of petunia was shown to be dependent on the age of the cell culture from which the protoplasts were isolated and the concentration of DNA used. The maximum GUS activity was discernible when high voltage electric pulse (1000 v/cm) was applied to the solution containing protoplasts and DNA. Transformant calli were selected using kanamycin.

Key words : Petunia, protoplast, electroporation

Genes may be transferred into plant cells by a number of approaches (Davey *et al.*, 1980). Two different approaches have been followed to transfer genes into plant protoplasts, viz., electroporation and polyethylene glycol (PEG) which induce DNA uptake (Larkin *et al.*, 1990). A wide range of plants and crop species have been transformed by employing the protoplasts as the recipient system (Finch, 1994). Electroporation of protoplasts to transfer foreign DNA is hampered by the low frequency of transformation (Hain *et al.*, 1985). There has been limited work on determining the factors affecting the efficiency of transformation by electroporation. This communication describes some of the factors that determined the efficiency of transformation of petunia protoplasts by electroporation.

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MATERIALS AND METHODS

The experiment was conducted during 1993-94 in the Department of Plant Science, Cook College, The State University of New Jensey, USA.

Cotyledons from *in vitro* grown seedlings of *Petunia hybrida* var. Ultra Blue were cultured in MS medium (Murashige and Skoog, 1962) containing 2, 4-D (2.00 mg/1) and 2% sucrose. The cultures were incubated in the dark at 25°C for four weeks. Callus formed from the cotyledons was excised and placed in MS liquid medium containing 2, 4-D (1.00 mg/1) and incubated at 22°C under continuous light (125 Em^{-2} s^{-1}) with continuous agitation (120 rpm).

Suspension cultures were subcultured every week according to Otsuki (1990).

Protoplasts were prepared from suspension cultures of different ages by washing with electroporation solution and treating with enzymes as described by Otsuki (1990). The protoplasts was filtered through 30m nylon mesh and centrifuged at 200 x g for 3 min. The supernatant was discarded and pellet washed thrice with electroporation solution and used for electroporation.

The protoplasts were suspended in phosphate - buffered saline (PBS; 1 x PBS containing 8 g of NaCl, 0.2 g of KCl, 0.2 of KH_2PO_4 and 1.15 g Na_2 HPO_4 per litre) containing 0.2 M mannitol at a density of 2 x 10^{-6} ml^{-1}. One ml of the protoplast suspension was chilled briefly on ice and heat shocked at 45°C for 5 min prior to the addition of plasmid DNA. Electroporation was carried out as described by Potter *et al.* (1984). Protoplasts were held at 0°C for 20 min after electroporation. The protoplasts were then diluted with MS medium containing 3.5% sucrose, 0.2 M mannitol and 1.5 mg/l 2, 4-D and incubated at 26°C. Protoplast viability was determined by Evans blue exclusion (Kanai and Edward, 1973).

The plasmid pBI 121 containing β-glucuronidase gene (GUS) driven by CaMV 35 S promoter was used in the present study.

The GUS activity was measured by *in situ* staining (Jefferson *et al.*, 1987) of electroporated and non-electroporated cells.

Total DNA was isolated from eight independent kanamycin - resistant calli according to established procedure (Sambrook *et al.*, 1989). DNA was digested with Hind III, fractionated on 0.7% agarose gel and transferred to Genescreen plus membrane (Dupont). The probe (GUS insert) was radio - labelled with ^{32}p using the random primer method and used for hybridization.

RESULTS AND DISCUSSION

The protoplasts isolated from 1, 2, 4 and 6 weeks old suspension cultures were electroporated to determine the optimum stage for transformation. The expression of GUS was highest in protoplasts isolated from 4 weeks old suspension cultures (Fig. 1). The expression of GUS was almost negligible in protoplasts from 6 weeks old cultures.

The effect of DNA concentration on the expression of GUS activity was examined by using 10 to 100 g/ml of pBI 121 during electroporation. Maximum GUS activity was observed when DNA concentration was 50 g/ml (Fig. 2). The levels of GUS expression were examined at various times of incubation after electroporation. The GUS expression was detected as early as 13 h and as late as 90 h after electroporation, with the maximum level discernible between 24 and 48 h (data not shown).

The optimal effect varying the field strength during electroporation is presented in Fig. 3. The maximum activity of GUS was obtained with 1000 v/cm electric field, 0.5 ms time constant (resistance 668 @, capacitance 3mF), with 50 ug of DNA in electroporation solution (Fig. 3). It is possible that such a combination of electrical parameters induces more pores, larger pores or long - lived pores in the plasma membrane, allowing more DNA to be taken

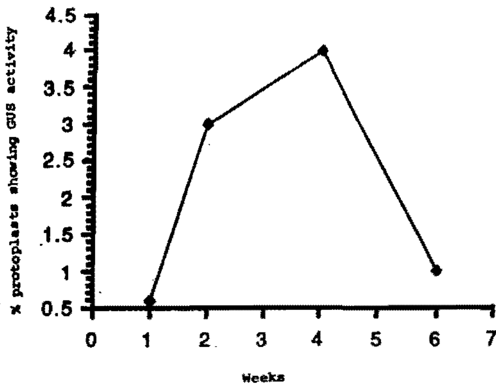

Fig. 1. Effect of the age of suspension culture of GUS activity

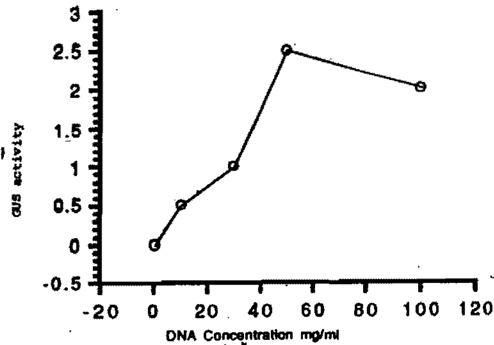

Fig. 2. Effect of DNA concentration on GUS activity in protoplasts

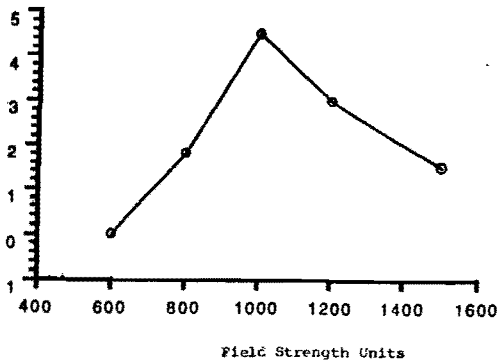

Fig. 3. Effect of pulse field strength on GUS activity in suspension culture of protoplasts

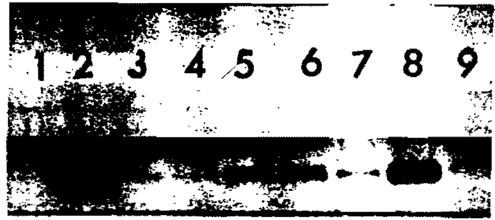

Fig. 4. Southern blot analysis of GUS positive calli Transformed calli were tested for integration of the GUS gene. All the eight independent kanamycin resistant calli tested, contained insertions of the GUS gene into genomoic DNA. Lane 2 to 8 represent the kanamycin resistant calli with plasmid containing GUS gene. Lane 1 is non-transformed control.

up, or perhaps a longer pulse duration facilitates the electrophoretic movement of DNA molecules into the nucleus. Survival percentage of protoplasts after electroporation under optimal conditions was 60.6% as judged by staining with fluorescein diacetate. This is an agreement with the report that most efficient uptake of DNA by protoplasts often occurs at the voltage which kills 30 - 50% of the protoplasts (Hoffmann, 1987). Similar general observations have also been made by Hauptmann *et al.* (1987). Subsequent to electroporation, the protoplasts were allowed to form calli on selection medium contaiing kanamycin (100 mg/l). The kanamycin resistant colonies continued to grow and reached a size of 100-200 µm or more after four weeks in culture. During selection, visible colonies developed in 0.7% agarose beads as well as in the liquid medium. Transformed colonies were also observed when kanamycin was present only in the liquid medium indicating that both selection methods can yield large number of resistant colonies. However, the advantage of

embedding the protoplasts in agarose beads over the liquid selection method is that the culture medium can be replaced without disturbing the developing colonies. The Southern analysis of the transformed calli showed positive signals corresponding to hybridization with GUS gene (Fig. 4). This observation confirms that the gene was integrated into the genome of petunia.

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