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This work is dedicated to the memory of Professor Jacek Augustyniak
Communication

Electroporated intact BY-2 tobacco culture cells as a model of transient expression study★✪

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Transfer of foreign genes into plant cells can be accomplished by several methods: agrobacterium-mediated, microinjection, biolistic particle bombardment and electroporation. The last one is frequently used for transfection of plant protoplasts for transient gene expression. Electroporation is a simple procedure and allows transfecting a large number of cells at one time. Square wave-modulated porators are the most efficient for introducing expression cassettes into plant protoplasts. Based on a protocol developed by Wu & Feng (*Plant Cell Reports*, 1999, 18, 381–386), we optimized conditions for transfection of intact *Nicotiana tabacum* BY-2 cells using square wave-modulated electroporator. To simplify screening for transfected gene expression we used constructs with a GFP marker gene.

Electroporation of cells in the presence of DNA has been widely used in recent years in molecular biology for studying transient gene expression. It consists in subjecting cells to an electric field, which forms pores in the lipid bilayer of the cell membrane, allowing DNA molecules to enter into the cytoplasm [1]. Pore formation is reversible and cell survival is maintained, thus such a method of introducing foreign DNA into cells is fast, simple, efficient, non-toxic and applicable to a great variety of cells. However, in spite of all its advantages electroporation has not been applied equally successfully in experiments with plant

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Abbreviation: GFP, green florescent protein.

cells (except those with protoplasts) because of the cell wall. There are some earlier reports indicating that the cell wall does not prevent DNA molecules from being internalized [1–3]. In 1999 Wu and Feng [4] described an effective method of electroporation applicable to the intact plant cells. In this method plant cells are subjected to plasmolysis prior to electroporation. The modifications of these procedure are presented in this paper.

MATERIALS AND METHODS

Plant material. Culture cells of *Nicotiana tabacum* bright yellow (BY-2) were maintained in BY medium (E. Kraszewska, personal communication, [5]) composed of: MS [6] (ICN, Murashige & Skoog, Plant Salt Mixture), 8 g/l (0.23 M) sucrose (USB), 1 mg/l thiamine (Sigma), 100 mg/l *myo*-inositol (Sigma), 255 mg/l KH_2PO_4 (POCh, Poland) and 0.2 mg/l 2,4-dichlorophenoxyacetic acid (Sigma) and cultivated in a 7-day cycle.

Construction of plasmid. The plasmid for transient expression assay was obtained as follows. Plasmid pBIN 35S-mgfp5-ER carrying the green fluorescent protein (GFP) expression cassette [7] was used as a template for PCR reaction with CaMV 10 (GAC TGC AGT TTC AGA AAG AAT GCT AAC CC) and NOS 02 (GAG AGC TCG ATC TAG TAA CAT AGA TGA CAC C) as primers. The GFP expression cassette (CaMV 35S-mgfp5-ER-NOSter) obtained in PCR was cloned directly into pGEM T-easy (Promega). The desired plasmid was linearized before transfection experiments using *Sal*I enzyme (Boehringer) and analysed by electrophoresis. In addition, the GFP expression cassette was cut out by *Bam*HI and *Sac*I enzymes (Boehringer).

Transfection of intact* N. tabacum *BY-2 cells. Electroporation of BY-2 cells was performed according to the method described earlier by Wu & Feng [4] with several changes. On the 5-th day of cell growth 20 ml of culture suspension was removed from a flask and centrifuged at 100 ***g*** for 10 min. Then the cells were washed twice in the culture medium and centrifuged again. The final volume of the cell pellet was about 600 μl. The plasmolysis was done by adding to the cell pellet 600 μl of EP solution (5 mM $CaCl_2$, 10 mM NaCl, 8.7% glycerol, 0.4 M sucrose, 10 mM Pipes buffer, pH 6.8) in the presence of 30 μg linearized plasmid DNA. After 15–20 min of plasmolysis, suspension of the cells was transferred to four standard BTX 4 mm cuvettes (300 μl to each). The cuvettes were placed under vacuum at 670 mbar for 1 min, incubated at room temperature for 10 min and then placed on ice for 5 min. In the next step the electroporation was carried out using BTX Electro Square Porator T 820. The samples were pulsed once at 2 kV/cm, the pulse lasting 80 μsec. After the electroporation the cuvettes were kept on ice for 10 min and incubated at room temperature for another 10 min. Then the cells were deplasmolysed by adding BY medium without sucrose. This was done in three steps with 5-min intervals that made possible to decrease sucrose concentration from 0.4 M to 0.05 M. The deplasmolysed cells were transferred into BY medium and incubated for several days.

Analysis of transient gene expression. For monitoring the transient expression of GFP the cells were observed for 3 days after electroporation under an Olympus fluorescence microscope using U-MNB filters (BP 470-490, BA 515, DM 500). Viability of the cells was measured using standard trypan blue test [8] and efficiency of transfection was estimated under the fluorescence microscope.

RESULTS AND DISCUSSION

The method of the plant cell transfection used in our work consists of four stages: (1) partial plasmolysis of the cells to create "temporary protoplasts", (2) incubation under vacuum, (3) electroporation, (4) deplasmolysis of the cells. We found optimal conditions of Wu

& Feng [4] method and adapted them to our needs. The optimization of the method concerned all these stages. We analysed and tried to change certain parameters so as to achieve a high level of gene expression and high cells' survival (Table 1). A number of controls were performed in each experiment. The tobacco cells were observed under a microscope at every step of transfection (Fig. 1). The observation enabled us to evaluate the amount of the living cells and their condition. Linearized plasmid pGEM carrying the CaMV35S-mgfp5-NOSter cassette or only the expression cassette were introduced into the cells. In both cases green fluorescence appeared after electroporation and the efficiency of transfection was the same (Fig. 2). After transfection many cells were permanently plasmolysed as seen under a microscope. Therefore various concentrations of sucrose in the plasmolytic solution and the incubation time of tobacco cells in this solution were tested (Table 1). Decreasing the sucrose concentration from 0.4 M to 0.2 M resulted in clumping of the cells. A similar phenomenon occurred when strength of the vacuum was reduced. We focused our attention on the plasmolysis process and the incubation under vacuum because of very low vitality of the cells after these two stages. Next, various electrical field conditions were tested and various length and strength of electric pulses were examined. At last the deplasmolysis process and volume of the medium in which the cells were incubated after transfection were improved. The established optimal electroporation parameters are presented in Materials and Methods. Electroporated tobacco BY-2 cells are shown in Fig. 2. The efficiency of the method was very high – up to 50%. The viability of the cells just after electroporation was about 70% but decreasing during the following days.

Plasmid DNA was introduced into the plant cells by a modified protocol of Wu & Feng [4] who reported that effective gene transfer into intact plant cells can be achieved by plasmolysing them in the presence of plasmid DNA prior to electroporation. We analysed the conditions under which delivering of plasmid DNA into intact *N. tabacum* BY-2 cells

Table 1. Analysis of parameters influencing the electroporation process

Stages of electroporation	Performed changes and their results										
Plasmolysis	Ttime of incubating tobacco cells										
	20 min						5 min				
	Concentration of sucrose in the plasmolysis solution (EP)										
	0.4 M			0.2 M			0.4 M			0.2 M	
	+ + +			+ +			+			0	
Vacuum	Strength (mb) (1)					Time (2)				Skipping of the vacuum stage	
	500	600	650	700	800	1 min		2 min		– [b]	
	–	+	+ + +	+ + +	+	+ + + [a]		–			
Electroporation	Voltage (V) (3)			Length of pulses (μs) (4)					Number of pulses (5)		
	280 V	600–700	800–900	10	40	50	80	90	1	2	
	–	+ +	+ + +	–	+	+ +	+ + +	+	+ + +	+ +	

–, experiment not successful; +, low and ++, better efficiency of electroporation, respectively; +++, optimal electroporation parameters; 0, not analysed; [a], the time measured since the vacuum pump was turned on; [b], skipping of the vacuum stage resulted in clumping of the cells; (1), constant parameters: 0.4 M sucrose and time of incubation t = 20 min; (2), constant parameters: as above and strength of vacuum 670 mb; (3), constant parameters: as above and time of vacuum t = 1 min; (4), constant parameters: as above and voltage 800–900 V; (5), constant parameters: as above and length of pulses t = 80 μs.

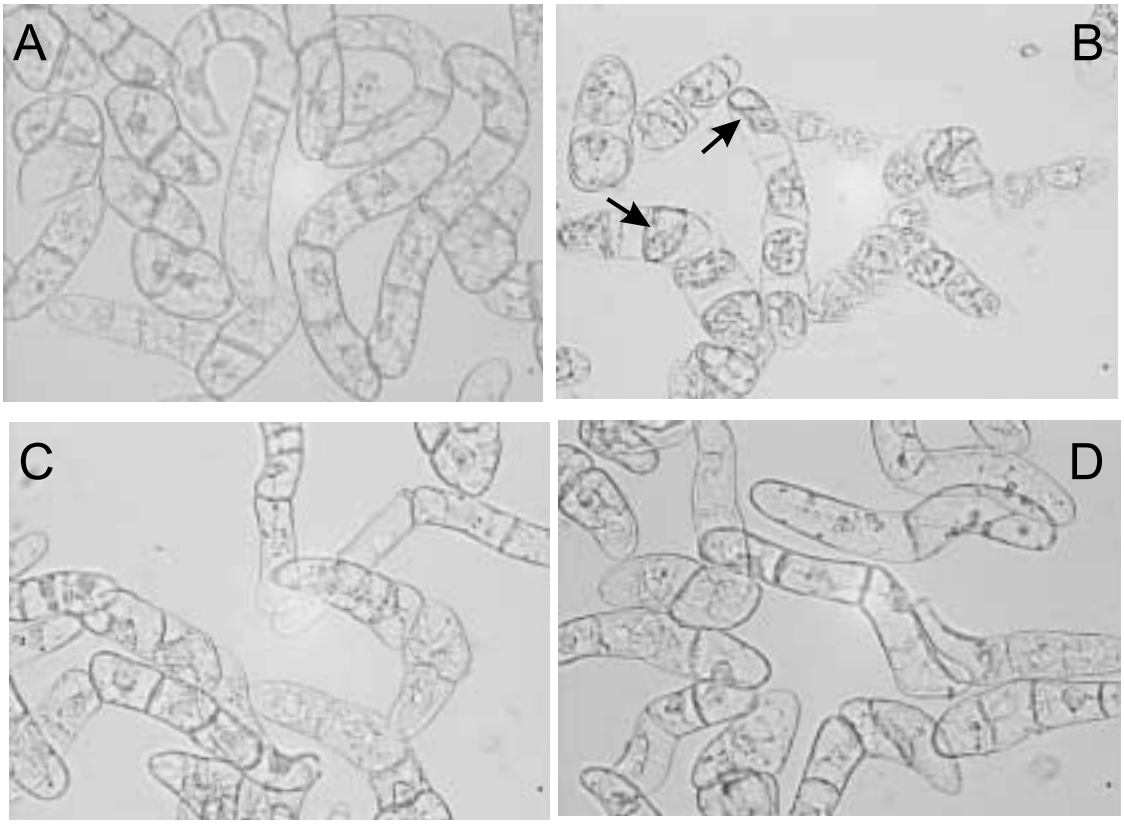

Figure 1. Photography of *N. tabacum* BY-2 cells (of suspension culture) under light microscopy.

(A) After 5 days cultivation; (B) after plasmolysis; (C) after incubation in vacuum; (D) after electroporation and deplasmolysis. Arrows indicate cell membrane surrounding "temporary protoplasts" formed during plasmolysis.

would be successful. The efficiency of transfection, cells' survival and vitality of protoplasts it saves time and money. Intact cells were cultured without special treatment.

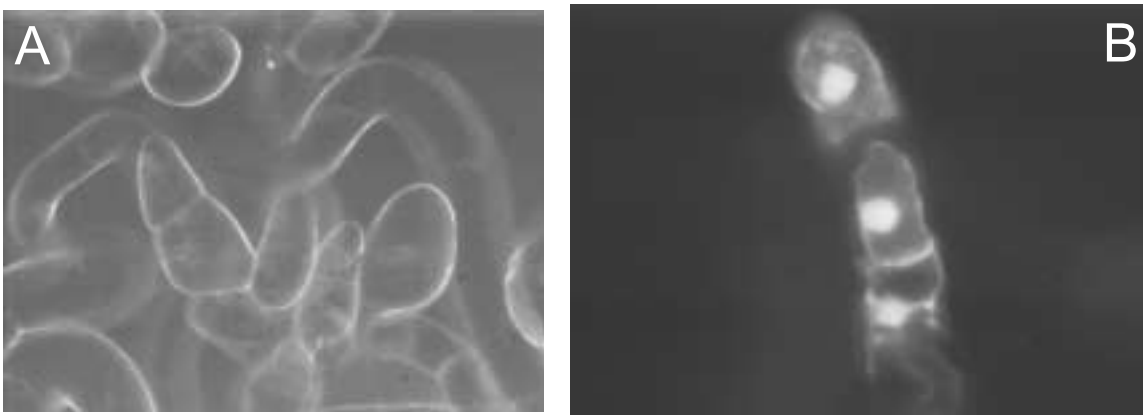

Figure 2. Photography of transfected *N. tabacum* BY-2 cells under fluorescence microscopy.

(A) "No DNA" control; (B) cells transfected with GFP cassette.

transfected tobacco cells were very high. Working with the intact plant cells instead of protoplasts is very convenient. Contrary to standard method based on preparation of After electroporation they quickly returned to their natural walled state and could be used to further analysis. The use of intact plant cells and a square porator allow to perform the ex-

periments rapidly and with a high electroporation efficiency. Advantages of the system are simplicity of the procedure and reproducibility of the results. The system can be useful for transient gene expression studies. The experiments using GUS reporter gene are under way.

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