High-efficiency transformation of *Agrobacterium tumefaciens* with plasmid DNA by electroporation

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SUMMARY

We describe a very simple and highly efficient procedure for direct transformation of *Agrobacterium tumefaciens* with plasmid DNA.

The protocol is based on high-voltage electropermeabilization of whole cells, and reproducible yields of 10^7 to 5 x 10^8 transformants per μg of plasmid DNA are obtained. Basically, electroporation is performed in 15% polyethylene glycol using an electrical field strength of 12.5 kV/cm. The utility of this transformation method is demonstrated by the direct establishment in *Agrobacterium tumefaciens* of a representative genomic library from the plant *Arabidopsis thaliana*. Our results offer interesting prospects for the transfer of gene banks and for gene complementation in plants without the use of *Escherichia coli* as an intermediate host.

RESUMEN

Describimos un método simple y altamente eficiente para transformar directamente *Agrobacterium tumefaciens* con ADN plasmídico. El protocolo se basa en la electropermeabilización de la pared celular bajo la acción de un campo eléctrico de alto voltaje y se obtienen resultados reproducibles de 10^7 a 5 x 10^8 transformantes por microgramo de ADN plasmídico. Usualmente, la electroporación se realiza en una solución de polietilén-glicol al 15% y una magnitud del campo eléctrico de 12.5 kV/cm.

La utilidad de este método de transformación es demostrada mediante el establecimiento de una genoteca de *Arabidopsis thaliana* directamente en *Agrobacterium tumefaciens*. Nuestros resultados ofrecen interesantes perspectivas para la transferencia de bancos de genes y la complementación génica en plantas prescindiendo del uso de *Escherichia coli* como hospedero intermediario.

INTRODUCTION

In recent years, high-voltage mediated electropermeabilization or electroporation has proven a very useful tool for introducing DNA into various types of cells. Particularly with bacteria, many previously inaccessible species can be transformed by the electroporation technique, albeit with widely different efficiencies (Bone *et al.*, 1989; Chassy *et al.*, 1988; Desomer *et al.*, 1990; Luchansky *et al.*, 1988; Trevors *et al.*, 1990; Wirth *et al.*, 1989). One particularly interesting species is *Agrobacterium tumefaciens*, the causative agent of crown gall, which has become an established tool in the genetic engineering of plants (for reviews, see Gheysen *et al.*, 1989). Using a freeze-thaw method, Holsters *et al.* (1978) have obtained transformation frequencies of 10^3 transformed cells/μg DNA. Later modifications of this procedure did not increase markedly this rather low efficiency (Nishigushi *et al.*, 1987; Höfgen and Willmitzer, 1988).

Hence, most currently used procedures for establishing gene libraries in *Agrobacterium tumefaciens* involve *Escherichia coli* as an intermediate cloning host, and subsequent interspecies transfer by conjugation (Simoens *et al.*, 1986; Olszewksi *et al.*, 1988). It would be advantageous to directly construct large gene libraries (e.g. from plants) in *A. tumefaciens*. This in turn requires a high transformation efficiency. Recent studies demonstrate that electroporation can be used for *A. tumefaciens* transformation with high efficiency (Mattanovich *et al.*, 1989; Mersereau *et al.*, 1990; Shen Wen-ju and Forde, 1989).

Here we report the successful application of high-voltage electroporation for direct plasmid DNA transformation of *A. tumefaciens*. Efficiencies of 5 x 10^8 transformed cells/μg DNA were routinely obtained. We studied the effect of physiological conditions of the cell cultures and electroporation parameters on transformation efficiency. We describe the construction of a representative *Arabidopsis thaliana* gene library in *A. tumefaciens* and a simple protocol of colony hybridization to screen the transformed population.

As far as we know, these results promote *Agrobacterium tumefaciens* to being the second most efficient bacterial cloning system available to date.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains *E. coli* MC1061 (Casabadan and Cohen, 1980) and *A. tumefaciens* C58ClRif^r, containing plasmid pGV2260 lacking the total T-region (Van Larebeke *et al.*, 1974; Deblaere *et al.*, 1985) were used in this work.

Plasmids

The binary cosmid vector pDE1004, used in this work, was constructed as follows. First, the 1.78-kb BglII fragment of pHC79 (Hohn and Collins, 1981),

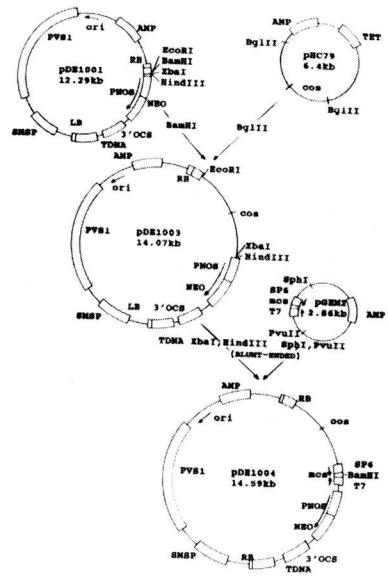

FIG. 1. Construction of pDE1004 vector. This vector was used to establish a representative genomic library of *A. tumefaciens*. It contains both origin of replication for *E. coli* Col E1 (ori), and for *A. tumefaciens* (PVS1), the genes conferring resistance to ampicillin (AMP) and streptomycin-spectinomycin (SMSP) for selection of transformed cells in *E. coli* and *A. tumefaciens* respectively and also the neomycin phospho-transferase gene (NEO) for selection in plants. See the text for further explanations.

containing the cos site, was inserted into the BamHI site of pDE1001(*). The resulting plasmid, pDE1003, was linearized with HindIII and XbaI and the protruding ends filled in by Klenow polymerase activity. This DNA was ligated with the 520-bp SphI-PvuII, blunt-ended fragment of pGem2 (Riboprobe Gemini System, Promega Biotec), resulting in the final construction pDE1004 (see figure 1).

Media and antibiotics

A. tumefaciens was grown at 28°C in YEB medium. The *E. coli* was grown at 37°C in LB medium. When necessary, YEB and LB media were solidified with 1.5% Difco agar and supplemented with antibiotics such as ampicillin, rifampicin, spectinomycin, or streptomycin at a concentration of 100 μg/ml.

Electroporation equipment

In this work we used the Gene-Pulse™ apparatus (Bio-Rad, Richmond CA, USA) equipped with a pulse controller and cuvettes of 0.2 cm electrode distance.

DNA preparations

For large and small scale plasmid DNA preparation the procedure of Birnboim and Doly (1979) was used. Total *A. thaliana* DNA was obtained according to Lemmers *et al.* (1980). All DNA manipulations were carried out using standard procedures (Maniatis *et al.*, 1982).

Construction of a genomic library of *A. thaliana*

Binary vector pDE1004 was digested with BamHI and dephosphorilated with calf intestinal alkaline phosphatase.

Total *A. thaliana* DNA was partially digested with Sau3A. An enriched fraction of high molecular weight fragments between 20 and 25 kb, obtained from a sucrose gradient, was ligated in five molar excess to the vector at a final concentration of 50 μg/ml. After 12 hours incubation at 16°C, the ligation mixture was dialyzed using a Millipore membrane (o 0.025 μm) and electroporated into *A. tumefaciens*.

Colony hybridization procedure for *A. tumefaciens*

Colonies from transformed *A. tumefaciens* were grown onto nylon filters (Hybond N, Amersham) placed on the surface of YEB plates. After an overnight incubation at 28°C, the filter colonies were placed side up on a pad of absorbent filter paper soaked in a solution of pronase P (0.5 mg/ml) and sarkosyl(2% w/v) and incubated 1 hour at 37°C. Later, the filters were treated with denaturing and neutralizing solutions and washed in 2 x SSC. Thereafter, the filters were dried at room temperature. Hybridizations were carried out using standard procedures (Maniatis *et al.*, 1982).

RESULTS AND DISCUSSION

Establishment of a highly efficient transformation procedure for *A. tumefaciens*

The recent success in transformation of recalcitrant bacteria by electroporation (Chassy *et al.*, 1988; Langer, 1988; Luchansky *et al.*, 1988; Desomer *et al.*,1990) stimulated the development of a highly efficient transformation procedure for *A. tumefaciens*. For reasons described later, the transformation procedure was optimized with a newly constructed binary vector pDE1004. This vector contains, besides the T-DNA borders and a selectable NPTII gene, the highly stable pVS1 origin of replication, the lambda cos site and flanking the cloning sites, the promoters SP6 and T7.

The lambda cos site was included in order to have an alternative way to establish the *A. thaliana* genomic library in case of negative results in the direct transformation of *A. tumefaciens* by electroporation. The SP6 and T7 will be very useful in further studies of gene expression and phenotypic complementation in plants by

* J. Denecker, unpublished results.

transformation of mutants with complete gene libraries.

All electroporation experiments were performed with a constant capacitance of 25 μF, known to give the best results with a large number of different bacteria (Luchansky *et al.*, 1988).

In a first series of experiments, we evaluated the influence of the conductivity of the electroporation medium on the transformation efficiency. A 24-hr preculture of *A. tumefaciens* in YEB medium was diluted into 250 ml YEB medium and shaked at 200 rpm at 28°C. After incubating 6 hrs until $O.D._{600} = 0.3$, cells were collected by a 6 000-rpm centrifugation at 4°C (Sorvall GS3 rotor). Cells were washed with 250 ml and then with 1/10 volume of cold sterile water and harvested in each case by 6 000-rpm centrifugation at 4°C (Sorvall SS34 rotor). Finally, the cells were resuspended in 500 μl of different electroporation media.

The electroporation was carried out using 1 ng of the previously dialyzed pDE1004 vector. A volume of 100 μl of cell suspension was placed in a sterile Eppendorf tube, mixed with plasmid DNA, incubated for 10 minutes on ice and pulsed at V = 10 kV/cm, R = 200 ohm., and c = 25 μF. After electroporation, the cells were diluted in 1 ml of YEB medium supplemented with 20 mM glucose, 10 mM $MgCl_2$, and 2.5 mM KCl, grown at 28°C and 200 rpm during 2 h, and plated in YEB medium containing rifampicin, streptomycin, and spectinomycin for selection. The plates were incubated for 2 days at 28°C. Results are shown in table 1.

Highest efficiency was observed when H_2O or 15% PEG 6 000 were used as electroporation media. For further experiments only H_2O or 15% PEG 6 000 electroporation media were used and the described cell preparation procedure was performed.

Effect of physiological conditions of cell cultures on transformation efficiency

The effect of cell culture conditions on transformation efficiency was evaluated by harvesting cells at different growth stages.

Table 1
EFFECT OF CONDUCTIVITY OF ELECTROPORATION MEDIUM ON TRANSFORMATION EFFICIENCY

Conductivity of medium (μS)	Viability of cells[a]			Transformation efficiency[a] (transformants per μg DNA)
	(cells/ml) before	(cells/ml) after	(%)	
(μS)	electroporation			
6	3×10^{11}	1.3×10^{11}	43.0	4.8×10^{8}
60	3×10^{11}	1.1×10^{11}	36.6	9.0×10^{7}
300	3×10^{11}	0.5×10^{11}	16.6	2.4×10^{5}
700	3×10^{11}	0.3×10^{11}	10.0	0.5×10^{5}

Electroporation constant parameters were V = 12.5 kV/cm, c = 25 μF, R = 200 Ohms.
[a] The values given are mean of five independent experiments.

The preparation of cells was done as described and in each case the cells were resuspended in the appropriate volume of electroporation medium in order to have the same quantity of cells per ml of cell suspension. The electroporation was done as described and the results are shown in table 2. The best results were obtained when the cells were harvested in an early exponential growth stage ($O.D._{600} = 0.3$). All subsequent experiments were therefore carried out using cells at early exponential growth stage.

cells and the transformation efficiency. In all cases the capacitance was constant ($c = 25\ \mu F$).

We also electroporated the cells with different quantities of DNA, obtaining a non linear relation between the amount of DNA used and the transformation efficiency (data not shown). We observed an efficiency of 5 to 8 x 10^8 transformants per μg of DNA when the procedure was carried out using 1 ng of plasmid pDE1004.

Table 2
EFFECT OF PHYSIOLOGICAL CONDITIONS OF CELL CULTURES ON ELECTROPORATION EFFICIENCY

Step log phase	$O.D._{600}$	Viability of cells[a] (%)	Transformation efficiency[a] (transformants per μg DNA)
Early	0.3	47	4.7×10^8
Middle	0.5	49	2.0×10^7
Late	0.7	55	5.0×10^6
Stationary phase	1.5	60	2.0×10^5

Electroporation constant parameters were V = 12.5 kV/cm, c = 25μF, R = 200 Ohms.
[a] The values given are mean of five independent experiments.

Effect of electroporation parameters on transformation efficiency

In a series of experiments we evaluated both the effect of different values of voltage and the internal resistance on transformation efficiency (table 3). The experimental results show that voltage is directly proportional to transformation efficiency. The increase of internal resistance to values higher than 200 ohm decreases dramatically the viability of

One ng DNA was the standard amount of DNA used as internal control in all subsequent experiments.

On the basis of these results, we established the protocol for high efficiency transformation of *Agrobacterium tumefaciens* by electroporation and a simple procedure of colony hybridization for screening the transformed population. The utility of this transformation was demonstrated by the direct establishment in *A. tumefaciens* of a representative genomic library of the plant *Arabidopsis thaliana*. The construction of this genomic library was made as described (Materials and Methods), we obtained 5 x 10^4 transformants and using different

Table 3
EFFECT OF VOLTAGE AND RESISTANCE ON TRANSFORMATION EFFICIENCY

A

Voltage kV/cm	Capacitance μF	Resistance Ohms	Viability of cell/ml[a] (%)	Transformation efficiency[a] (transformants per μg DNA)
7.5	25	200	95	1.3×10^6
8.75	25	200	91	2.0×10^6
10	25	200	91	6.8×10^6
11.5	25	200	37	33.0×10^6
12.5	25	200	30	64.0×10^6

B

Resistance Ohms	Voltage kV/cm	Capacitance μF	Viability of cell/ml[a] (%)	Transformation efficiency[a] (transformants per μg DNA)
100	12.5	25	80	9.0×10^6
200	12.5	25	31	95.0×10^6
400	12.5	25	10	4.5×10^6
600	12.5	25	3	2.4×10^6
800	12.5	25	1	0.8×10^6

[a] The values given are mean of five independent experiments.

probes we evaluated the representativity of the library (data not shown). Our results offer interesting prospects for the transfer of gene banks and for phenotypic complementation in plants without the use of *E. coli* as an intermediate host. It represents a potential approach for gene complementation in lethal mutant plants where the results of this event can be easily distinguished in the early steps of plant development.

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