Electroporation of Agrobacterium tumefaciens

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CHAPTER 4

Electroporation of *Agrobacterium tumefaciens*

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1. Introduction

Agrobacterium tumefaciens is a soil bacterium that causes tumors on dicotyledonous plants. Virulent strains harbor a large plasmid, the Ti (tumor-inducing) plasmid, which is involved in tumorigenesis. A small segment of this plasmid, the T-DNA, is transferred to the plant cell and becomes integrated into one of the chromosomes in the nucleus. The T-DNA contains genes for the production of phytohormones viz an auxin and a cytokinin. Therefore, expression of the T-DNA in the plant cell leads to tumor formation (for review, *see* ref. *1*). Deletion of the *onc* genes within the T region of the Ti plasmid results in nononcogenic strains. However, if the 24-bp border repeat, which surrounds the T region in the Ti plasmid, is kept intact, the mutated T-DNA is still delivered to plant cells by *Agrobacterium.* This natural plant vector system is used for the genetic engineering of plants (for review, *see* ref. *2*). If genes are added to the T region of the Ti plasmid, these are cotransferred to the plant cell. An important finding was that separating the T region from the remaining part of the Ti plasmid did not prevent transfer of the T-DNA to the plant cell *(3).* On the basis of this principle, the binary vector system was developed. Binary vectors are wide host-range plasmids that are maintained by both *E. coli* and *A. tumefaciens* and contain an artificial T region into which genes of interest can be cloned. Traditionally,

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cloning with binary vectors is done in *E. coli.* The resulting vector is then introduced into an *A tumefaciens* helper strain for delivery of the T-DNA to plant cells.

The efficient introduction of plasmids into *A. tumefaciens* is thus of great practical importance for plant molecular biology. There are several methods for introducing plasmid DNA into *A. tumefaciens* The classical method is conjugative transfer by triparental mating *(4).* Although good results are obtained with this method, the procedure is very time-consuming. Alternatively, plasmids may be introduced into *A. tumefaciens* via transformation. However, calcium chloride treatment, generally used for transformation of *E. coli,* is not effective for *A. tumefaciens.* A freeze–thaw treatment *(5)* is effective, but the transformation efficiency is low (maximally 10^3 transformants/µg DNA).

Electroporation can be used to introduce plasmids efficiently into *A. tumefaciens.* Transformation frequencies of up to 10^7 transformants/µg DNA have been reported *(6–10).* After electroporation, transformants are selected by plating the pulsed cell–DNA mixture on selective plates. Owing to the possible growth of spontaneous antibiotic resistant mutants, it is necessary to check for the presence of the electroporated plasmid in the *A. tumefaciens* cell by performing a small-scale plasmid isolation *(6,11).* In this chapter, detailed protocols are described for electroporation as well as plasmid isolation. In Section 4., various applications of electroporation for gene transfer to *A. tumefaciens* are described (*see* Notes 5 and 6).

2. Materials

2.1. Bacterial Strains and Plasmids

A commonly used bacterial strain (LBA288) is a rifampicin resistant derivative of *A. tumefaciens* strain C58 that is cured of its Ti plasmid *(12).* Other derivatives of LBA288 include strain LBA1100, containing the spectinomycin resistant (Sp^r) Ti helper plasmid pAL1100 *(13)* and strain LBA1143, containing the carbenicillin resistant (Sp^rCb^r) Ti helper pAL1143 *(13).* Strain LBA4404, containing the streptomycin resistant (Sm^r) Ti helper plasmid pAL4404 *(3),* is a derivative of wild-type Ach5 (*see* Note 4). Kanamycin resistance (Km^r) can be conferred by DNA of the 7-kbp binary vector pSDM14 *(14)* and the 11-kbp vector pBin19 *(15).*

2.2. Medium and Antibiotics Used for Selection of Transformed Cells

1. LC medium: 10.0 g tryptone (Difco, Detroit, MI), 5.0 g yeast extract, 8.0 g NaCl, distilled water to 1 L. Sterilize by autoclaving for 20 min at 120°C. When indicated, supplement medium with 0.1% glucose. For solid medium in plates, add 1.8% agar before sterilization. Agar medium can be stored in liquid form for up to 1 wk in a 55°C incubator. When stored for a longer period of time, the medium must be kept in a solid form. It can then be liquified with the aid of a microwave oven before use.
2. Rifampicin: 10 mg/mL dissolved in methanol. This solution can be stored at 4°C for at least 6 mo.
3. Spectinomycin: 50 mg/mL in sterile H_2O.
4. Carbenicillin: 50 mg/mL in sterile H_2O.
5. Kanamycin: 50 mg/mL in sterile H_2O—for use in bacterial selection plates: No further sterilization of the drugs is needed. Solutions remain stable for at least 6 mo at –20°C.
6. Selection plates. Liquid LC agar medium, supplemented with the required antibiotics to final concentrations of 20 μg/mL rifampicin, 100 μg/mL kanamycin, 100 μg/mL spectinomycin, or 75 μg/mL carbenicillin. For LBA4404, however, carbenicillin is used at 10 μg/mL.

2.3. Solutions for Electroporation

1. Washing solution: 1 m*M* HEPES, adjust to pH 7.0 with NaOH. Autoclave 20 min at 120°C.
2. Electroporation solution: 10% glycerol in distilled H_2O. Filter-sterilize through a 0.22-μm membrane.
3. SOC medium: 2% bacto-tryptone, 0.5% yeast extract, 10 m*M* NaCl, 2.5 m*M* KCl, 10 m*M* $MgSO_4$, 10 m*M* $MgCl_2$, 20 m*M* glucose.

2.4. Solutions for Small-Scale Plasmid Isolations

1. Solution 1: 50 m*M* glucose, 10 m*M* EDTA, 25 m*M* Tris-HCl, pH 8.0, 4 mg/mL lysozyme. Add lysozyme just before use.
2. Solution 2: 1% SDS, 0.2*N* NaOH. This solution must be freshly prepared from stock solutions of 20% SDS and 4*N* NaOH.
3. Solution 3: 1 mL H_2O-saturated phenol plus 15 μL 4*N* NaOH.
4. Solution 4: 3*M* sodium acetate, adjust to pH 4.8 with acetic acid.
5. Phenol-chloroform: 1 vol of 100 m*M* Tris-HCl, pH 8.0, saturated phenol plus 1 vol of chloroform:isoamylalcohol (24:1).
6. TE buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.

3. Methods

3.1. Preparation of *A. tumefaciens* Cells for Electroporation

1. Inoculate an LC plate with bacteria, and incubate for 3 d at 29°C.
2. Use a loop to transfer bacteria into 2 mL of LC medium, and incubate at 29°C for 6 h with agitation.
3. Inoculate 100 mL of LC medium, supplemented with 0.1% glucose, with 100 µL of the preculture. Grow the cells overnight at 29°C with vigorous shaking to an OD_{660} of 1.0–1.5 (*see* Note 1).
4. Chill the culture on ice for 15 min, and harvest the cells by centrifugation in a cold rotor at 4000*g* for 20 min.
5. Resuspend the pellet in 10 mL of 1 m*M* HEPES, pH 7, and centrifuge as above. Repeat this washing step three times.
6. Wash the pellet in 10 mL of 10% glycerol.
7. Resuspend the pellet in a final volume of 500–750 µL of 10% glycerol The cell concentration should be $1–5 \times 10^{11}$ cells/mL.
8. Distribute the bacterial suspension in 40-µL aliquots, freeze in liquid nitrogen, and store at –70°C. The cells are usable for electroporation for at least a year under these conditions without significant loss of transformation efficiency.

3.2. Electroporation of *A. tumefaciens*

1. Gently thaw the cells on ice. This takes 10–15 min.
2. Chill the electroporation cuvets with 0.2-cm electrode gap on ice (*see* Note 7).
3. Add 1–5 µL of plasmid DNA (10 ng) to 40 µL of cell suspension, and mix well or use the cells for direct transfer (*see* Note 5).
4. Transfer the mixture to a prechilled electroporation cuvet. Take care that the suspension is in contact with both electrodes of the cuvet.
5. Apply an electric pulse at 2.5 kV, 25 µF, and 200 Ω (*see* Note 2). This should result in a pulse of 12.5 kV/cm with a time constant of approx 4.7 ms.
6. Immediately add 1 mL of SOC medium, and gently but quickly resuspend the cells with a sterile Pasteur pipet.
7. Transfer the cell suspension to a 1.5-mL tube, and incubate at 29°C for 1–1.5 h.
8. Plate 100-µL aliquots of appropriate dilutions on selective medium. Incubate for 3 d at 29°C.

3.3. Small-Scale Plasmid Isolations

1. Inoculate fresh bacteria in 2 mL of LC medium, and incubate overnight at 29°C with agitation.

2. Centrifuge 0.5 mL of the bacterial culture for 5 min at 12,000*g*, and discard the supernatant.
3. Suspend the pellet by vortexing in 100 µL of solution 1. Incubate 10 min at room temperature
4. Add 200 µL of solution 2, mix by inverting the tube four times, and incubate for 10 min at room temperature.
5. Add 30 µL of solution 3, and mix by brief and gentle vortexing.
6. Immediately add 150 µL of solution 4, mix by inversion, and incubate at –20°C for 15 min.
7. Centrifuge for 5 min at 12,000*g*, and transfer the supernatant to a fresh 1.5-mL tube.
8. Add 400 µL of phenol-chloroform, and mix by brief vortexing.
9. Centrifuge for 3 min at 12,000*g*, and transfer the aqueous (top) layer to a 1.5-mL tube.
10. Add 800 µL of ice-cold 96% ethanol, and mix by inversion.
11. Precipitate the DNA by incubation at –70°C for 15 min.
12. Centrifuge for 10 min at 12,000*g*, and discard the supernatant.
13. Wash the pellet with 250 µL of 70% ethanol, and dry briefly in a vacuum centrifuge.
14. Dissolve the pellet in 25 µL of dH_2O or TE buffer (*see* Note 3).
15. Use 5 µL of the DNA solution to check plasmid presence in a 0.6% agarose gel, 10 µL for restriction analysis, and 1–10 µL for transformation of *E. coli* or *Agrobacterium*, e.g., by electroporation (*see* Note 4).

4. Notes

1. Some workers prepare electrocompetent cells from overnight cultures with an OD_{660} of 1–1.5 *(4)*, whereas others use early log-phase cultures with an OD_{660} of 0.5 *(7)*. It is possible that cellular competence for transformation is influenced by the growth phase of the bacterial culture. Therefore, we compared electroporation of two different cell suspensions of LBA288. One suspension was derived from cells that had been cultured overnight as described in Section 3. To obtain a suspension of log-phase cells, an overnight culture was diluted 1:100 in LC medium supplemented with 0.1% glucose and incubated further for about 6 h until the cells had reached an OD_{660} of 0.3. Electrocompetent cell suspensions of 1.1×10^{11} and 2.5×10^{10} cells/mL were thus obtained. Forty-microliter aliquots were used for electroporation with 100 ng pSDM14 DNA. Results are shown in Table 1. The total number of transformants was equal for both cell suspensions. However, the number of transformants per surviving cells was almost 10-fold higher for the early log-phase cells. A higher initial cell density of

Table 1
Effect of Growth Phase on Transformation Frequency[a]

Growth phase, OD_{660}	Cell density, cells/mL	Total number of transformants	Number of transformants per surviving cell
1.1	1.1×10^{11}	2.1×10^{4}	7.2×10^{-6}
0.3	2.5×10^{10}	2.2×10^{4}	8.1×10^{-5}

[a]LBA288 cells were grown to an OD_{660} of 1.1 (overnight culture) or to an OD_{660} of 0.3 (early log phase). Transformation was carried out by using 100 ng of pSDM14 DNA, a field strength of 12.5 kV/cm, a capacitance of 25 µF, and a resistance of 200 Ω.

early log-phase cells will probably lead to an increase in the total number of transformants that can be obtained. However, it is more convenient to use overnight cultures. For most purposes, cells prepared from overnight cultures are sufficiently competent.

2. The electroporation process is influenced by several parameters, such as field strength and pulse length. For *A. tumefaciens,* the highest transformation efficiencies were obtained at a field strength of 12.5 kV/cm, a capacitance of 25 µF, and resistances of 200–400 Ω, giving pulse lengths of 4.5–9 ms *(6–10)*. To test the influence of the pulse length on the transformation efficiency and the survival of LBA288 cells, this strain was electroporated with pSDM14 DNA using resistances of 100, 200, 400, and 600 Ω. As shown in Fig. 1, the optimal pulse length was reached at 200 Ω (time constant = 4.8 ms).
3. DNA solutions used for electroporation must have a low ionic strength. As ionic strength increases, resistance of the suspension decreases. Excess ionic strength will cause arcing in the cuvet. DNA can be prepared either by CsCl density gradient centrifugation or by Qiagen (Diagen GmbH, Düsseldorf, Germany) isolation methods. DNA isolated by minialkaline lysis procedures is also usable, but the efficiency of electroporation is about 2.5-fold lower than with the other preparations *(10)*. Small cloning vectors (10–12 kbp) and large 200–250 kbp Ti plasmids can be reproducibly transformed into *A. tumefaciens* strains *(6)*. Transformation frequency was found to be linearly related to DNA concentration from 0.1 pg up to 1 µg/standard assay *(9)*. However, a reproducible twofold decrease in frequency was found in our experiments when comparing the use of 10 and 100 ng DNA, respectively.
4. Several different *A. tumefaciens* and *A. rhizogenes* strains have been tested in electroporation experiments. Differences in the transformation efficiencies obtained with different stains were observed *(9)*. It is important to take this into account when choosing an *Agrobacterium* strain for an

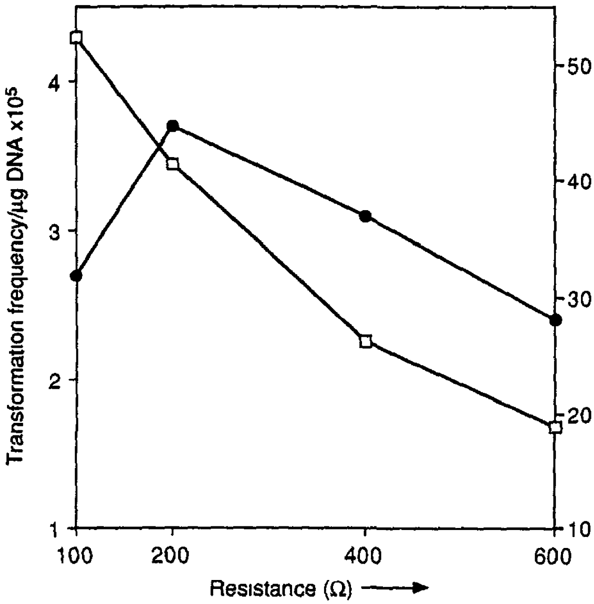

Fig. 1. Influence of pulse time on transformation efficiency and survival. Electroporation was carried out with strain LBA288 using 10 ng of pSDM14 DNA. Pulses were delivered at 12.5 kV/cm, 25 µF, and resistances of 100, 200, 400, and 600 Ω, giving corresponding time constants of 2.4, 4.7, 9.0, and 13.2 ms. Transformation efficiency (●) and survival of cells (□).

Table 2
Comparison of Transformation Frequencies of *A. tumefaciens* Strains LBA288 (C58) and LBA4404 (Ach5)[a]

Strain	Cell density, cells/mL	Total number of transformants	Frequency in transformants/µg DNA
LBA288	2.5×10^{10}	4.0×10^3	4×10^5
LBA4404	1.8×10^{10}	0.9×10^3	9×10^4

[a]Strains were electroporated under the same conditions· Cells of early log-phase growth stage (OD_{660} of 0 3) were used Transformation was carried out by using 10 ng pSDM14 DNA, a field strength of 12.5 kV/cm, a capacitance of 25 µF, and a resistance of 200 Ω (time constant 4.8 ms).

investigation. In Table 2, the transformation frequencies are compared for the strains LBA288 and LBA4404, using the protocol given in Section 3.

5. Direct transfer of bacterial plasmid DNA between two *E. coli* strains by electroporation, so-called electrotransfer, was reported by Summers and

Withers *(16)* Based on these observations, we applied this concept to achieve direct transfer of plasmid DNA between two *A. tumefaciens* strains and from *E coli* to *A. tumefaciens*. We directly transferred the plasmid pBin19 (Km^r) from LBA288 to LBA1143, which contains an Sp^r, Cb^r helper plasmid, as follows: Both strains were prepared for electroporation as described in the protocol. Twenty-microliter aliquots of both strains were mixed and transferred to a prechilled 0.2-cm electroporation cuvet. The mixture was subjected to a pulse of 12.5 kV/cm, 25 µF, and 200 Ω (time constant 4.8 ms). Immediately after the pulse, the cuvet was incubated on ice for 30 s. Then a second pulse (same settings, time constant 4 6 ms) was applied, followed by addition of 1 mL of SOC medium. After a 1-h incubation at 29°C, 100-µL aliquots of the pulsed cell mixture were spread onto LC-agar plates containing kanamycin (100 µg/mL) and spectinomycin (100 µg/mL). A total of 150 colonies were found to be resistant to both antibiotics. As expected, these colonies also turned out to be resistant to carbenicillin, the other marker of the recipient. In a control experiment where no pulses were applied to the mixture, no transfer was observed. Plasmid presence was examined by electrophoresis of minialkaline DNA isolations. On 0.6% agarose gels, two bands were found representing pBin19 and helper plasmid pAL1143, respectively. This shows that electrotransfer between two *A. tumefaciens* strains had indeed occurred. For the electrotransfer of plasmids from *E. coli* to *A. tumefaciens,* cells of the "empty" strain LBA288 were prepared for electroporation. A fresh colony from a plate of the *E. coli* strain CEL247, which contains the Km^r binary vector pBin19, was gently mixed with the cold suspension of thawed *A. tumefaciens* cells. As described above, two electric pulses were applied. Transformed *A. tumefaciens* strains were selected on plates containing rifampicin (20 µg/mL) and kanamycin (100 µg/mL). In the 24 colonies obtained, the presence of plasmid pBin19 was demonstrated by electrophoresis of DNA preparations. Electrotransfer is a very useful application of electroporation because the DNA isolation step can be omitted.

6. Often it is desirable to insert a gene of interest into the Ti plasmid or the *Agrobacterium* chromosome. This can be accomplished by homologous recombination between an introduced plasmid and the *Agrobacterium* genome. The frequency of such transfer is lower than when using a replication-proficient plasmid. Nevertheless, electroporation can be used for this type of transfer. Here we describe the introduction of a mutated *virG* gene into a helper Ti plasmid. The mutated *virG* gene was cloned in the *E. coli* vector pTZ18R (Cb^r), which cannot replicate in *A tumefaciens* DNA (250 ng) of this clone was electroporated into strain LBA1100 *(13),* which contains a helper Ti plasmid with a Sp^r gene. Selection for the Cb

marker after electroporation resulted in growth of several colonies. Southern analysis showed that the clone had integrated into the helper Ti plasmid via homologous recombination by a single crossover event.

7. Although electroporation cuvets are disposable, it is possible to reuse them. The cuvets can be decontaminated by rinsing them twice with 5% dettol, then with distilled H_2O, and finally with 70% ethanol for sterilization. In old cuvets and cuvets that are not cleaned properly, arcing can occur. Replace these cuvets.

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