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Plant Transformation via Protoplast Electroporation

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1. Introduction

In electroporation, cells are permeabilized by the application of very short, high-voltage electric pulses. Molecules ranging in size from small organic metabolites and reporter dyes to large macromolecules—including antibodies and plasmids—can be introduced into cells by electroporation. Electroporation is effective on virtually any type of cell, and is now the method of choice for the genetic transformation of bacteria and certain animal cell lines. The primary application of electroporation to plants has been for DNA uptake for studies of transient gene expression and for stable transformation. However, electroporation has also been used to introduce RNAs *(1,2)*, antibodies *(3)*, and small molecules *(4)* into plant cells and isolated organelles *(5)*.

Because the thick plant cell wall restricts macromolecule movement, most work on plant cell electroporation utilizes protoplasts. This has limited the use of electroporation for stable transformation to species whose protoplasts are regenerable. As protoplast regeneration systems are improved, reports of new species of plants transformed by electroporation continue to appear (for example, **ref.** *6*). One advantage of electroporation over particle bombardment for stable transformation is that electroporation results predominantly in single-copy plasmid insertions (Bates, unpublished observations), whereas particle bombardment tends to introduce large plasmid concatemers. However, the main use of protoplast electroporation is in transient expression assays for studies of transcriptional regulation (for example, *7,8*). These studies do not require protoplast regeneration. A growing number of recent reports indicate that electroporation can be used to introduce DNA into walled plant cells and plant tissues *(9,10)*. Tissue electroporation does not work in all cases, and the parameters for successful plant tissue electroporation are not yet clear.

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However, the success of tissue electroporation in crops, such as maize and soybean, reopens the use of electroporation for stable transformation in major crops.

This chapter provides a protocol for protoplast electroporation, a protocol for the selection of stable, kanamycin-resistant transformants, and notes on how to optimize these protocols for both stable transformation and transient expression. These protocols have been used for many years in the author's laboratory for the transformation of tobacco protoplasts, but they can be readily modified for use with protoplasts of other species and for the uptake of molecules other than DNA.

2. Materials

2.1. Protoplast Electroporation

1 Instrumentation· Electroporation equipment is available from a variety of commercial manufacturers and can also be homemade Lists of commercial manufacturers and instrument specifications, as well as a general discussion of homemade equipment can be found in Chassy et al. *(11)*.

   Two types of DC electrical pulses, square-wave pulses and capacitive discharges, may be used for electroporation. However, because the equipment is less expensive, most laboratories use capacitive-discharge electroporation systems The equipment presently used in the author's laboratory is the Cell-Porator® Electroporation System I manufactured by Gibco BRL Life Technologies Inc. (Gaithersburg, MD). This capacitive-discharge instrument allows the pulse voltage to be adjusted from 0 to 400 V, pulse length can be varied by selecting one of eight different-sized capacitors (from 10–1980 µF) (*see* **Note 1**) The Gibco BRL Cell-Porator utilizes presterilized, disposable electroporation chambers to hold the cells during electroporation For work with plant protoplasts, electroporation chambers should be selected that have a 0.4-cm electrode gap Chambers with 0 1-cm electrode gaps can also be purchased, but are designed for electroporation of bacteria

2. Protoplasts: Protoplasts isolated by standard procedures are suitable for electroporation. Protoplasts from a wide range of species, organs, and cell cultures have been successfully electroporated However, it is important that the protoplasts be of high quality. Even in high-quality protoplast preparations, electroporation kills a substantial fraction of the protoplasts Preparations of marginal or low-quality protoplasts are likely to be completely killed by the electric shocks

3 DNA· The plasmid DNA used in electroporation does not have to be highly purified, but should be free of RNA and proteins (such as RNase and restriction enzymes) that might affect the viability of the protoplasts The author's laboratory routinely uses plasmids isolated by alkaline lysis *(12)*. RNA is removed from the plasmid preparations by RNaseA digestion followed by phenol extraction and ethanol precipitation (*see* **Note 2**)

   Before electroporation, the DNA must be sterilized. DNA that has been ethanol-precipitated and redissolved in autoclaved water or TE is probably suffi-

ciently sterile. However, to be certain the DNA is sterile it can be passed through a 0.2-μm pore, low-binding, cellulose acetate syringe filter (e.g., Nalgene #190-2520). It is convenient to filter-sterilize the DNA after diluting it into electroporation medium at the start of an experiment

For transient expression studies, linear and supercoiled plasmids are equally effective *(13)*. However, for stable transformation, linear DNA is 3- to 10-fold more efficient than supercoiled *(14)*.

4. Electroporation medium: Hepes-buffered saline (HBS): 150 m*M* KCl, 4 m*M* $CaCl_2$, 10 m*M* HEPES (pH 7.2), and enough mannitol to balance osmotically the protoplasts For tobacco mesophyll protoplasts, 0 21 *M* mannitol is used. The HBS can be prepared in advance, sterilized by autoclaving, and stored at 4°C.

2.2. Selection of Stable Transformants

1. Media for protoplast culture and selection of stable transformants: Tobacco mesophyll protoplasts may be cultured in K_3G medium (K_3 salts, vitamins, and hormones *[15]* containing 0.4 *M* glucose as the carbon source and osmotic stabilizer). K_3G may be sterilized by autoclaving, and stored at 4°C
2. Callus medium (CM)· Murashige and Skoog salts and vitamins *(16)* plus 100 mg/L inositol, 3% sucrose, 1 mg/L benzyladenine, and 1 mg/L α-naphthaleneacetic acid For selection of kanamycin-resistant stable transformants, CM is amended by addition of various amounts of mannitol, Sea plaque agarose (FMC Corp , Rockland, ME), and kanamycin as described below in the **Subheading 3.** CM and CM amended with mannitol and Sea plaque agarose can be prepared in advance, sterilized by autoclaving, and stored at 4°C Kanamycin should not be autoclaved. A 1000X stock of kanamycin can be prepared by dissolving 100 mg/mL kanamycin sulfate in water; the stock should be filter-sterilized and stored at –20°C.

3. Methods

The following procedures must be carried out under sterile conditions, preferably in a laminar flow hood. All solutions should be at room temperature (*see* **Note 3**).

3.1. Protoplast Electroporation

1. Freshly isolated protoplasts should be used for electroporation: After washing the protoplasts free of the enzymes used for cell-wall digestion, pellet the protoplasts and resuspend them in 10 mL HBS + mannitol (*see* **Note 4**).
2. Determine the number of protoplasts/mL using a hemacytometer.
3. Place aliquots of 1×10^6 protoplasts into 15-mL conical centrifuge tubes, and pellet the protoplasts by centrifugation (50*g* for 5 min).
4. Discard the supernatant. Using a disposable plastic transfer pipet, gently resuspend each protoplast sample in 0.5 mL HBS + mannitol + DNA. The plasmid DNA concentration in the electroporation medium should be 10–100 μg/mL (*see* **Note 5**).

5 Use a disposable plastic transfer pipet to transfer the protoplast samples to electroporation chambers Let stand for 5 min.
6 Resuspend the protoplasts by gentle agitation of the electroporation chamber, and then immediately apply a single electric pulse (325 μF, 300 V; *see* **Note 6**) If you are using the Gibco BRL Cell-Porator, make sure the instrument is on the low Ω setting.
7. Wait 2–3 min, and then resuspend the protoplasts by agitation of the electroporation chamber. Transfer the protoplasts to a conical centrifuge containing 5 mL of K_3G Rinse the electroporation chamber with 0. 5 mL HBS + mannitol, and combine the rinse with the protoplast sample (*see* **Note 7**)
8 When all the protoplast samples have been electroporated, pellet the protoplasts, and resuspend each sample in 5 mL of culture medium.
9 Transfer the protoplast samples to a 60 × 15 mm Petri dish, and culture the protoplasts at 27°C.
10. Protoplasts may be sampled after 24 h for transient gene expression or maintained in culture for later selection of stable transformants

3.2. Selection of Stable Transformants

The following procedure for selection of stable transformants is based on the agarose-bead culture technique of Shillito et al. *(17)*. In this procedure, the protoplast culture is diluted and solidified by addition of low-melting-point agarose. When properly diluted, the embedded protoplasts are well separated from each other and grow into individual calli. Selection for transformants is carried out after embedding the cells. This procedure permits calculation of plating efficiency and transformation efficiency, and allows isolation of individual transformed clones. The method outlined here describes selection of kanamycin-resistant clones, but it can easily be adapted for use with other selectable markers or reporter genes. During selection, the culture is progressively diluted and the osmotic strength of the medium is reduced. The sequence of medium changes described here has been optimized for work with protoplasts of *Nicotiana tabacum,* but can be modified for work with other species.

1. Choose a plasmid containing a functional neomycin phosphotransferase II gene such as pMON200 *(18)* or pBI121 (Clonetech, Palo Alto, CA). Linearize the plasmid by digestion with a suitable restriction enzyme. It is convenient to cut enough of the plasmid for several experiments. Then phenol-extract and ethanol-precipitate the DNA, and resuspend it in water at a concentration of 1 μg/μL.
2 Electroporate the protoplasts in the presence of 10–100 μg/mL of linearized DNA, and culture them in 60 x 15 mm Petri plates in 5 mL of K_3G (*see* **Note 8**)
3. After 1 wk of culture, the protoplasts will have grown into small-cell clusters (*see* **Note 9**) At this point, the protoplast-derived colonies are immobilized by a 1:1 dilution of the culture with medium containing agarose Prepare in advance CM medium + 0.23 *M* mannitol + 2 4% Sea plaque agarose, and sterilize it by

autoclaving. Just before the transfer, melt this medium, and let it cool to just above its gelling temperature. While the agarose is cooling, scrape any adhering protoplast colonies off of the Petri plate with a micropipet tip Transfer half of the culture (2.5 mL) to a new 60 × 15 mm Petri plate Now add 2 5 mL of the agarose-containing medium to both Petri plates of protoplasts Mix thoroughly by swirling the plates. Place the Petri plates in the refrigerator for 15 min to solidify the agarose. Culture the protoplasts at 27°C

4. At the end of the second week of culture, divide the solidified culture into wedges using a spatula, transfer the wedges to 100 x 15 mm Petri plates, and add 5 mL of liquid CM + 0.13 *M* mannitol supplemented with 100 µg/mL kanamycin (*see* **Note 10**). Culture at 27°C.
5. At the end of the third week, add 5 mL CM supplemented with 100 µg/mL kanamycin to each Petri plate.
6. Thereafter, at weekly intervals, remove 5 mL of liquid from each Petri plate, and replace it with 5 mL of fresh CM supplemented with 100 µg/mL kanamycin Transformed colonies should be visible after 4–5 wk of culture.
7. When the transformed colonies are 2–3 mm in diameter (4–6 wk of culture), they can be picked out of the agarose using a spatula, and cultured on CM + 0.8% agar + 100 µg/mL kanamycin. After another 2 wk of growth, they should be large enough to be transferred to regeneration medium.

4. Notes

1. Pulse voltage, or more precisely electric field strength, and pulse length are two critical parameters in electroporation, and must be optimized for each species and cell type Field strength depends on the voltage applied to the electroporation chamber and the distance between the electrodes in the chamber. The appropriate units for field strength are V/cm. Application of a 100 V pulse to a chamber with a 0.4-cm electrode gap results in a field strength of 250 V/cm.

   Pulse length is determined by the size of the capacitor and the resistance of the electroporation medium. Discharging a capacitor produces an exponentially decaying pulse. The length of such pulses is best described by their RC time constant, which is the time required for the pulse voltage to drop to 37% of its initial value
2. Both RNA and proteins present in a crude plasmid preparation can be introduced into the protoplasts by electroporation along with the DNA and may affect the experimental outcome. For example, in some early experiments in the author's laboratory, protoplasts were electroporated in the presence of a plasmid that had been treated with RNase, but not phenol-extracted. No transient gene expression was observed until the RNase was removed from the plasmid preparations
3. Some electroporation protocols call for chilling the cells in an ice bath during and immediately after electroporation. The pores that form in the plasma membrane owing to electric shocks have been shown to stay open longer if the cells are maintained at a temperature below the membrane's phase transition temperature. Chilling the cells would be expected to improve the efficiency of transformation

by electroporation, because it would allow more time for DNA uptake However, several studies of transient and stable gene expression have shown that chilling does not improve electroporation efficiency It turns out that the uptake of DNA during electroporation is electrophoretic (and not diffusive) and occurs during the electric pulse itself Thus, chilling the cells is unnecessary if transformation is the goal of electroporation Chilling may be useful, however, when electroporation is used to induce the uptake of molecules other than nucleic acids.

4. Because HBS is a nonphysiological, high-salt medium, it is advisable to limit the exposure of the protoplasts to this medium to 30 min. If a large number of samples are going to be electroporated, divide the protoplast preparation into two batches. Leave one batch in the protoplast wash or in culture medium, while the other batch is resuspended in HBS, divided into samples, and electroporated. Then when the first batch of protoplasts has been electroporated and diluted into culture medium (**Subheading 3.1., step 7**), pellet the second batch of protoplasts resuspend them in HBS and process them for electroporation.
5. The efficiency of both transient expression and stable transformation increases linearly with DNA concentration from 10 to 100 µg/mL. Transient expression and stable transformation are also increased by addition of "carrier DNA," such as salmon sperm DNA. For transient gene expression, the author's laboratory uses 10 µg/mL supercoiled plasmid DNA + 50 µg/mL salmon sperm DNA (sheared by sonication). Because the carrier DNA has been found to integrate along with the plasmid, carrier DNA should be avoided for stable transformation When the goal is stable transformation, this laboratory uses 50 µg/mL of linearized plasmid DNA and no carrier
6. The efficiency of electroporation depends on pulse length and voltage A 325-µF capacitive discharge into 0.5 mL of HBS gives a pulse of about 10 ms (RC time constant) For tobacco mesophyll protoplasts, a 10-ms pulse of 300 V (750 V/cm field strength in the electroporation chamber) usually gives optimal results. The optimal setting can vary with species and cell type, and should be determined empirically in preliminary experiments. A quick way to begin to look for effective electroporation parameters is to find pulse settings that result in 50% protoplast death by 24 h after the shocks

   Electroporation efficiency can also vary between batches of protoplasts because of batch-to-batch differences in protoplast viability. Batch-to-batch variability complicates transient expression studies. To handle this variability, replicates and appropriate controls must be included in every experiment. Some transient expression studies also include an internal control. The approach is to add a second plasmid carrying a reporter gene to each sample before electroporation as an internal control. Expression of the second plasmid can be used to normalize both batch-to-batch and sample-to-sample differences in transient gene expression
7. Manufacturers of electroporation equipment intend for the electroporation chambers to be discarded after each use. However, the author finds that the chambers can be reused two or three times without affecting electroporation efficiency or

protoplast viability. Rinsing the chamber after electroporation with 0.5 mL HBS + mannitol not only helps remove all the electroporated protoplasts from the chamber, but also readies the chamber for the next sample.

8. High initial protoplast densities improve survival of the electric shocks. Up to 500 transformed clones can be recovered from a sample of 1 x 10^6 protoplasts This would give an absolute transformation efficiency of 1 transformant for every 2000 electroporated protoplasts. However, the transformation efficiency is actually severalfold higher, because 50% of the protoplasts are killed directly by the electric shock and no more than half of the surviving protoplasts grow into calli

   This laboratory uses 50 µg/mL linear DNA for stable transformation Southern blotting shows that 50–75% of the transformants have a single copy of the plasmid integrated.
9. The timing of the media changes described here should not be adhered too rigidly, but should be modified depending on how fast the protoplasts grow after electroporation. For tobacco protoplasts, the first dilution of the cultures is done when the protoplasts have grown into microcolonies of 5–10 cells, which is sually after 6–7 d of culture This first dilution may have to be delayed if the protoplasts are growing more slowly—as will happen if electroporation kills more than about 75% of the protoplasts Too rapid a dilution of the culture results in death of the protoplast-derived colonies within 24 h of the dilution
10. As described here, the selection pressure starts out at 50 µg/mL of kanamycin and increases to about 100 µg/mL over a period of a few weeks. Selection can be started earlier. For example, it can be conveniently started 1 wk after electroporation by adding kanamycin at the first dilution of the culture This lab obtains the highest number of transformants when addition of kanamycin is delayed until the end of the second week. This may be because plating efficiency depends on cell density, so starting selection early reduces the cell density in the culture, and this in turn, inhibits the growth of some transformed clones Selection for kanamycin resistance is very clean in tobacco. The author never observes untransformed clones growing in the presence of kanamycin.

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