

PEG-Mediated Protoplast Transformation with Naked DNA

Jaideep Mathur and Csaba Koncz

1. Introduction

Direct introduction of DNA into plant protoplasts facilitates a rapid analysis of transient gene expression, as well as the generation of stably transformed transgenic plants. Transient gene expression assays performed after DNA transformation permit a comparative analysis of *cis*-acting regulatory sequences and their function in transcriptional control of plant genes by signaling pathways mediating cellular responses to different environmental and hormonal stimuli (1). There are a number of methods for introducing DNA into plant protoplasts, but the most commonly used technique is the polyethylene glycol (PEG)-mediated DNA uptake. The PEG-mediated transformation is simple and efficient, allowing a simultaneous processing of many samples, and yields a transformed cell population with high survival and division rates (2). The method utilizes inexpensive supplies and equipments, and helps to overcome a hurdle of host range limitations of *Agrobacterium*-mediated transformation. The PEG-mediated DNA transfer can be readily adapted to a wide range of plant species and tissue sources.

In *Arabidopsis thaliana*, several methods of direct gene transfer to leaf mesophyll (3–5) and root-derived protoplasts (6) have been reported. They are all derived from a PEG-mediated direct gene transfer technique established originally for tobacco protoplasts by Negrutiu et al. (7). This chapter describes a method for PEG-mediated transformation of protoplasts derived from leaves, roots, and cell suspensions of *A. thaliana*. Leaf mesophyll protoplasts are able to regenerate after embedding into alginate, but their yield is relatively low. In comparison, cell suspensions provide an unlimited source of rapidly dividing protoplasts that can be obtained within 2–3 h and show a transient expression

of introduced genes within 24 h. Root-derived protoplasts also feature a high division and regeneration capability. A low autofluorescence of cell suspension and root-derived protoplasts is of particular importance, when light emitting enzymes, such as the green fluorescence protein (GFP) or luciferases, are being used as reporter proteins in nondestructive in vivo gene expression assays (8–9). In leaf protoplasts, the red fluorescence of chloroplasts is a deterrent for effective monitoring of the *GFP* reporter gene activity. Nonetheless, the different protoplast systems provide a choice of material according to the specific questions addressed in the experiments.

2. Materials

2.1. Materials and Equipment

Items listed in Chapter 6 are required along with the following:

1. Fluorimeter.
2. Fluorescence microscope with FITC filters.
3. Diapositive films (Kodak Ektachrome 320T; Kodak Panther 1600, Braunschweig, Germany).

2.2. Media and Solutions

The pH is adjusted to 5.8 with 1M KOH or 1N HCl. The protoplast medium (PM), and the enzyme solution are filter sterilized. Growth regulator stocks (1 mg/mL) are filter sterilized and added separately to sterilized media. Solutions 14, 16, 17, and 18 should be filter sterilized and stored at -20°C .

1. Basal medium (BM): MS medium (5) (pH 5.8) containing B5 vitamins (6), with or without gelling agents (0.8% agar or 0.2% gelrite), and 3% sucrose, if not stated otherwise.
2. 0.5 BM Medium: consisting of half concentration of MS macroelements (5), B5 vitamins, and 3% sucrose with and without gelling agents (0.8% agar or 0.2% gelrite, pH 5.8).
3. MSAR I medium (7): BM medium containing 2.0 mg/L indole-3-acetic acid (IAA), 0.5 mg/L 2,4-dichloro-phenoxyacetic acid (2,4-D), 0.5 mg/L 6-(γ,γ -dimethylallylamino)-purine riboside (IPAR) (pH 5.8).
4. MSAR II medium (7): BM medium containing 2.0 mg/L IPAR, 0.05 mg/L α -naphthaleneacetic acid (NAA) with 0.2% gelrite (pH 5.8).
5. MSAR III medium (7): BM medium containing 1.0 mg/L (IAA), 0.2 mg/L indole-3-butyric acid (IBA), 0.2 mg/L 6-furfurylaminopurine (kinetin), 0.2% gelrite (pH 5.8).
6. Protoplast medium (PM): 0.5X BM medium with 0.45M sucrose or 0.45M mannitol (pH 5.8).
7. Enzyme solution: 1.0% Cellulase (Onozuka R-10; Serva), 0.25% Macerozyme (R10; Serva) dissolved in PM medium.

8. 0.45M Mannitol and 0.45M sucrose solutions (pH 5.8).
9. Sodium alginate solution: 1% (w/v) solution in BM medium containing 0.45M sucrose.
10. Calcium agar plates: 20 mM calcium chloride, 0.45M sucrose and 1% agar.
11. 0.5M MaMg solution: 0.5M mannitol, 15 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2% MES (morpholinoethane sulfonic acid, pH 5.8).
12. PEG solution (PEG 1450): 40 g PEG in 100 mL MaMg solution.
13. GUS extraction buffer: 0.1M potassium phosphate (pH 7.8), 2 mM Na_2EDTA , 2 mM dithiothreitol, 5% glycerol.
14. GUS substrate: 1 mM crystalline 4-methylumbelliferyl- β -D-glucuronide (Sigma M 9130, St. Louis, MO) in GUS extraction buffer.
15. GUS stop buffer: 0.2M Na_2CO_3 (store at 4°C).
16. MU standard: 1 mM 4-methylumbelliferone in GUS stop buffer. Prepare dilutions for calibration in stop buffer.
17. X-Gluc solution for tissue staining: for preparing 100 mL solution, dissolve 100 mg X-Gluc in approx 50 μL N,N-dimethylformamide and add 98 mL (0.1M) potassium phosphate buffer (pH 7.0), 1 mL of potassium ferricyanide (5 mM), 1 mL of potassium ferrocyanide (5 mM), and 0.1 mL Triton X-100.
18. X-gluc solution for staining of protoplasts: For 100 mL solution add to 100 mg X-gluc dissolved in 50 μL of N,N-dimethylformamide 100 mL of CaCl_2 (125 mM) solution containing 0.45M mannitol.
19. Hygromycin B solution: 1 g dissolved in 20 mL PBS may be obtained from Boehringer Mannheim (Mannheim, Germany, cat. no. 843555). Dilute to 15 mg/mL concentration in sterile water. Store at -20°C .

3. Methods

3.1. Isolation of Protoplasts

Methods for the isolation of protoplasts from leaf mesophyll tissue, root and cell suspension cultures are described in Chapter 6.

3.2. PEG-Mediated Transformation of Protoplasts

1. After washing with 0.45M mannitol (see Chapter 6), resuspend the protoplast pellet in 1 mL of MaMg solution.
2. Count the number of protoplasts and adjust the protoplast density to approx 1×10^6 cells/mL (see Note 1).
3. Place the protoplast suspension on ice for 35 min (see Note 2).
4. Centrifuge the protoplasts at 60g for 5 min.
5. Resuspend the protoplast pellet in 0.3 mL of MaMg solution and carefully transfer them as a single droplet in the middle of a 9-cm, glass Petri dish (see Notes 3 and 4).
6. Add slowly 25–35 μg of plasmid DNA dissolved in water into the drop of protoplast suspension. Shake the Petri dish very gently to mix the DNA well with the protoplast suspension (see Notes 5 and 6).
7. After 5 min add 0.3 mL of PEG solution at the circumference of the drop of protoplast suspension.

8. Tilt the Petri dish gently to allow mixing of the PEG solution with the protoplasts. (Alternatively, carefully mix the drops of PEG solution surrounding the drop of protoplast suspension by a sterile micropipet tip.)
9. After 10 min add from the sides of the protoplast droplet 1 mL of 0.45M mannitol solution.
10. Add at 2 min intervals 2 mL of 0.45M mannitol solution with gentle shaking until about 12 mL are present in the Petri dish (see **Note 7**).
11. Collect the protoplasts in a 12 mL centrifuge tube and centrifuge them at 60g for 5 min.
12. Assay for transient gene expression, using reporter (GUS or GFP) gene constructs, after 24–48 h (see **Subheading 3.3.**) or start embedding of the protoplasts into alginate, in order to select for stably transformed cells and process them further to plant regeneration (see **Subheading 3.4.** and **3.5.**).

3.3. Transient Gene Expression Assays with GUS and GFP Reporters

3.3.1. Application of the GUS Reporter Gene

Qualitative and quantitative assays of GUS reporter enzyme activity are carried out as described by Jefferson (13).

1. Transient expression of the GUS reporter gene may be assayed 24–48 h after the PEG-mediated DNA uptake. For this, collect the protoplasts by centrifugation at 60g for 5 min.
2. Resuspend the protoplast pellet in approx 1 mL of X-gluc solution for protoplasts and incubate them at room temperature for 6–12 h.
3. Take a drop of protoplast suspension (e.g., 10 μ L) and count the number of cells using a hemocytometer and an inverted microscope. Transformed protoplasts appear blue (see **Notes 8** and **9**, **Fig. 1A**).
4. The ratio between the total number of cells plated and the number of GUS expressing cells indicates the relative transformation efficiency expressed in percentage.

3.3.2. The Use of Green Fluorescent Protein as Reporter

The mGFP4 gene construct described by Haseloff and Amos (14), as well as its mutated derivative (Reichel et al., 17) work effectively in all *Arabidopsis* protoplast preparations (see **Note 10**).

Fig. 1. (opposite page) GUS and GFP reporter gene expression assays with *Arabidopsis* protoplasts. (A) GUS staining of PEG-transformed protoplasts derived from roots of *Arabidopsis* ecotype Columbia after incubation with X-gluc for 6 h at room temperature. (B) Leaf mesophyll protoplasts from *Arabidopsis* ecotype Columbia transformed with pCK-GFPs65c exhibit green fluorescence when illuminated with blue light. The chloroplasts emit red fluorescence, whereas the yellow fluorescence results from overlapping red and green areas. (See color insert following p. 208.)

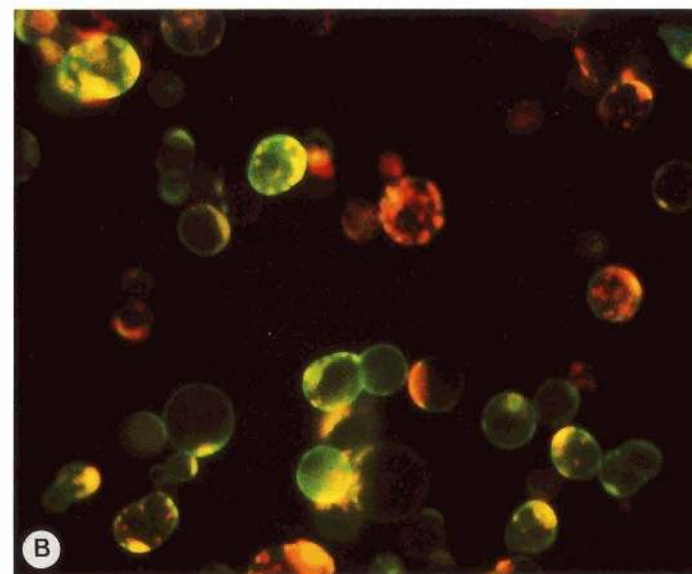
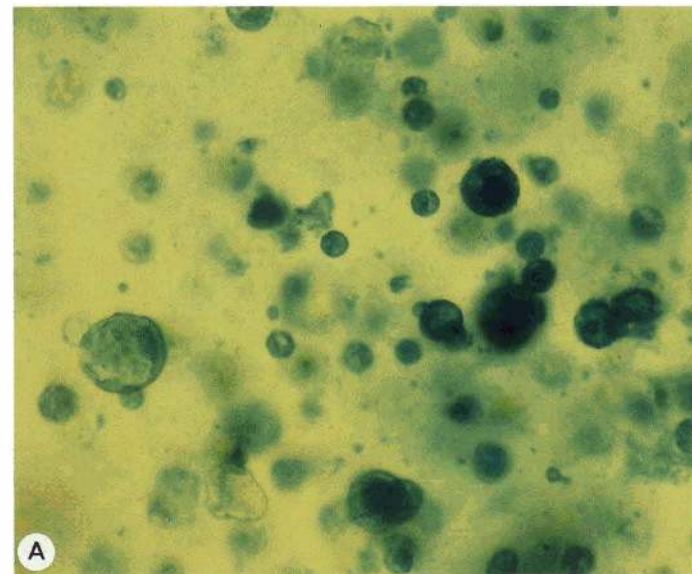


Plate 2 (Fig. 1; see full caption on p. 270 and discussion in Chapter 29). GUS and GFP reporter gene expression assays with *Arabidopsis* protoplasts. (A) GUS staining of PEG-transformed protoplasts derived from roots of *Arabidopsis* ecotype Columbia after incubation with X-gluc for 6 h at room temperature. (B) Leaf mesophyll protoplasts from *Arabidopsis* ecotype Columbia transformed with pCK-GFPs65c exhibit green fluorescence when illuminated with blue light. The chloroplasts emit red fluorescence, whereas the yellow fluorescence results from overlapping red and green areas.

1. For assaying the transient expression of GFP reporter gene, take an aliquot of 200 μL from the transformed protoplasts and transfer into a hemocytometer (*see Note 11*).
2. Observe and count the number of green fluorescent cells using a fluorescence microscope equipped with fluorescein isothiocyanate (FITC) filters (*see Fig. 1B* and *Notes 12* and *13*).
3. Take pictures in bright field and UV/blue light of the same field for records, using Kodak 320T and P-1600 slide films, respectively (*see Note 14*).

3.4. Selection of Stable Transformants

1. For embedding of the cells into alginate after PEG-mediated DNA uptake, adjust the density of protoplasts to $3\text{--}5 \times 10^5$ cells/mL and create alginate gel-drops of 250–500 μL on calcium agar plates (*see Notes 15* and *16*).
2. After 45 min remove the alginate drops containing the embedded protoplasts and place them in 55-mm Petri dishes containing 5 mL of PM medium.
3. Culture the protoplasts in low light (500–700 lx) conditions at 25°C.
4. After 7 and 14 d remove 2.5 mL of the PM medium and add 2.5 mL of fresh PM medium containing appropriate antibiotic(s) for selection (*see Notes 17* and *18*).
5. The frequency of stable transformation is determined at different time points, following the application of relevant antibiotic selection (*see Note 18*). When using GFP, the transformation efficiency can also be determined without applying an antibiotic selection (*see Note 19*). Take out small aliquots or sections of growing cells or tissues respectively in a Petri dish. Count the total number of cells in a field using white light and the number of cells showing GFP fluorescence under UV/blue light after transformation with the GFP reporter gene. This ratio expressed on a percentage basis indicates the relative transformation frequency (*see Notes 19* and *20*). Similarly, when using GUS reporter constructs, small aliquots from dividing cell cultures or microcalli are incubated for 6–12 h in X-gluc solution supplemented with appropriate osmoticum, and the ratio of GUS-stained cells vs the total cell number is determined.
6. Proceed for regeneration of stable transformants after applying a proper antibiotic selection (**Subheading 3.5**). Remove 1 mL of PM medium and add 1 mL of MSAR I medium containing antibiotic(s) on d 21, 28, and 35 (*see Note 21*).

3.5. Plant Regeneration from Protoplast-Derived Transformed Calli

1. Remove the liquid medium from the Petri dishes using a pipet and transfer the alginate beads carrying microcalli, after dividing them into four to five pieces, into MSAR II medium containing the selective antibiotics in Petri dishes (*see Notes 21* and *22*).
2. Place the Petri dishes in an illuminated culture chamber (3000 lx) set for 16 h of light and 8 h of dark at 25°C.
3. Transfer green calli and regenerating shoots to MSAR II medium containing the selective antibiotics and grow the shoots in glass jars until they attain a size of about 2 cm (*see Note 23*).

4. Transfer the shoots into MSAR III medium to induce root formation for 3–6 d, then place the plantlets in culture tubes containing 0.5 BM agar medium with only 0.5% sucrose for flowering and seed setting (*see Note 24*).

4. Notes

1. If the number of protoplasts exceeds 100 cells/square in the hemocytometer, dilute the protoplast suspension.
2. Do not leave the protoplasts, especially those obtained from cell suspensions, too long (> 60 min) on ice.
3. A glass test tube can also be used for PEG-mediated DNA uptake. However, we found the use of Petri dishes better because the addition of PEG solution, the consequent clumping, and the subsequent restoration of protoplasts can easily be monitored under an inverted microscope.
4. The use of glass Petri dishes is recommended for this step, because the PEG-treated protoplasts tend to adhere to the surface of plastic Petri dish and therefore often get damaged during the process of reconstitution following the dilution of PEG.
5. The use of carrier DNA has been recommended in a number of protocols and suggested to increase the efficiency of transformation. However, we found no significant increase in the transformation rates by applying carrier DNA. On the other hand, unnecessary clumping of protoplasts occurs in the presence of excessive amounts of carrier DNA. The washing step that removes the enzymes seems to have a greater bearing on the transformation efficiency, because protoplast samples that have not been washed very well always yield lower transformation, division, and survival rates.
6. The DNA should be sterile (ethanol-precipitated and dissolved in sterile water). Do not incubate DNA for too long with the protoplasts because it may result in lower transformation efficiency (**16**) owing to nuclease digestion.
7. It is worthwhile to include DNA untreated controls that are processed through the same PEG treatment and washes as the DNA-treated protoplasts. The PEG-treated protoplasts will clump up and look shrunken. However, they should not burst. If they appear to be damaged, the PEG concentration of 20% is probably too high and should be reduced. Following dilution of the PEG, the protoplasts should declump and regain their former shape.
8. Use the microscope with a fully open diaphragm and at low magnifications, since at either higher magnifications or low light, an illusion of a blue tinge in cell suspension and root derived protoplasts may lead to erroneous counts.
9. The indigo-colored reaction product of the β -glucuronidase enzyme and the substrate X-gluc (5-bromo-4-chloro-3-indolyl glucuronide) is cytotoxic and may sometimes lead to a collapse of transformed protoplasts. This may cause errors in counting of the transformed cells because the dye diffuses into the vicinity of the collapsed protoplast.
10. In the modified GFP, available from J. Haseloff, the cryptic splice sites were removed. This GFP is detectable in both UV (excitation filter 340–380 nm, reflection short pass 400 nm, long pass 430 nm) and blue light (450–490 nm,

short pass 510 nm, long pass 520 nm), whereas the GFP protein translated from the modified pCK-GFPs65c gene construct (Reichel et al., 17) works optimally in blue light with no visible green fluorescence in UV light.

11. Optimal fluorescence is observable only after 48 h, although some transformed protoplasts start exhibiting GFP fluorescence 24 h after transformation.
12. A filter set specifically designed for the observation of GFP (GFP-41014; HQ GFP-LP 41015) is available from the Chroma Technology (Houston, Texas). However, the commonly available FITC filter set is also adequate for most purposes.
13. When viewed on a Leitz Aristoplan fluorescence microscope (Leitz, Bensheim, Germany), using the filter sets described in **Note 10**, the *Arabidopsis* cell suspension and root-derived protoplasts exhibit a faint blue autofluorescence, whereas dead protoplasts display an intense yellow-orange fluorescence. Under blue light, the chloroplasts emit a bright red fluorescence. In certain cases, an overlapping of the green and red fluorescence results in yellow fluorescence in the pictures taken (see **Fig. 1B**).
14. Some photobleaching occurs in the UV light and therefore, when using the original GFP construct from J. Haseloff, it is advisable to take pictures first in normal light, then under blue light, and finally in UV light. The use of a fast film is recommended for the same reason. The problem of photobleaching is not encountered with the improved pCK-GFPs65c GFP construct.
15. A certain proportion of protoplasts will invariably break during and following the PEG treatment. The debris of dead cells is detrimental for a continued liquid culture of surviving protoplasts. Therefore, embedding of the PEG-treated protoplasts is necessary for obtaining stable transformants.
16. Larger drops may be prepared and later cut into smaller pieces. However, we find it easier to use small drops which avoid the problem of spreading.
17. Depending on the quality of protoplast preparations, up to 75% of the protoplasts survive and 20–40% of cells will undergo divisions during the first 5–7 d of culture.
18. After 14 d of culture, the dividing cells should form colonies of 4–16 cells. In the case of leaf mesophyll protoplasts, this time may be longer by another 7 d. Selection pressure should be applied at this stage to inhibit the growth and development of untransformed calli. We use 15 µg/mL hygromycin (Boehringer Mannheim) when selecting for constructs carrying the *hpt* (hygromycin phosphotransferase) selectable marker gene under the Cauliflower Mosaic Virus (CaMV) 35S promoter.
19. Although epifluorescence illumination adapted for use with an inverted microscope would be ideal for viewing of transformed cell clumps and regenerating shoots, in the absence of such a lighting system, the following method may be used: Remove a single alginate drop carrying protoplasts from the culture plate and place in a sterile Petri dish. After sealing the Petri dish with parafilm, invert the plate, and observe the cells in the alginate gel directly under the fluorescence microscope. The same procedure can be adopted for microcalli and regenerating structures.
20. In certain leaf pieces, the green fluorescence is entirely masked by the chlorophyll pigment. In case of doubts about the transgenic nature of regenerated plant, a

small portion of leaves may be used to make protoplasts in 1 mL of enzyme solution and the protoplasts may directly be observed as they are released. Alternatively, the sample tissue may be fixed in formalin:acetic acid:70% ethanol (FAA 5:5:90) for an hour and then observed after washing out the fixative with few rinses of distilled water.

21. By d 35, microcalli are visible and in some, the differentiation of roots and somatic embryo-like structures may already be observed.
22. Alternative approaches involve either depolymerisation of the alginate gel in 20 mM sodium citrate solution containing an appropriate osmoticum (4), or transferring the microcalli from the alginate gel into regeneration medium (9). Since we use a lower concentration of alginate than earlier reported (3–4), the regeneration of calli consisting of more than 64 cells is not hindered by the alginate embedding.
23. Care must be taken to remove the dead cells from the regenerating cultures.
24. The culture tubes are capped with loose cotton to facilitate proper aeration necessary for seed setting. Take care not to place the tubes close to the light source because this will cause moisture condensation inside the tubes and result in a low pollination rate. Rooted plants can be transferred to soil and, after proper hardening, will flower and set seed.

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