We share this protocol freely with laboratories conducting basic research. A simple registration form is required to help us keep a record of the users of this protocol. For commercial purposes, please contact Corporate Sponsored Research & Licensing Office at MGH (617-428-0200)

For citing this protocol:
Sheen, J. 2002, A transient expression assay using *Arabidopsis* mesophyll protoplasts.
http://genetics.mgh.harvard.edu/sheenweb/

The protocol has been streamlined and can be applied to different types of plant materials. The growth condition of the plants seems most critical for experimental reproducibility. Each lab may need to work out the best plant growth conditions. The quality of DNA and PEG is critical. The protocol is simple and the approach is powerful. However, it only awards success to the scientists who are willing to take time to master the system with patience and faith. Potential wounding and stress problems could be minimized during the experimental process to avoid high background. The protoplasts generated using this protocol have been used to study hormone, sugar, stress and defense responses using reporter genes that show similar responses in intact plants. RT-PCR is routinely used to confirm that the endogenous gene response is similar to that of the corresponding reporter gene. Good luck with your experiments!

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Updated on 9/26/97, 2/28/98, 9/11/00, 12/16/00, 4/6/01, 9/1/01, 9/29/01, 10/3/01, 4/21/02, 5/27/02, 10/10/03, 12/23/03, 3/22/04

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A Transient Expression Assay Using *Arabidopsis* Mesophyll Protoplasts

A. Protoplast Isolation

Plant Materials:
BE plants grown on the B5 medium
Greenhouse-grown BE, Col, Ler and C24 plants are fine

Use well expanded leaves from 3-4 weeks old plants (the second and/or third/fourth pair, 1-2 cm) before flowering. Shorter photoperiod (12-13 h light or less, 50-150 μE) is recommended for Col and Ler that flower earlier under long-day condition.

Protoplast Isolation Procedure:
Cut 0.5-1 mm leaf strips with fresh razor blades without wounding. This is perhaps the most tedious part for most people. However, I consider it easier and more efficient than peeling the lower epidermis of the leaves one by one. It takes some practice to do a good job in cutting leaves. A very good preparation yields around 10^7 protoplasts/g fresh weight (about 100 to 150 leaves digested in 40-60 ml of enzyme solution). For a practice or for most small scale experiments, 10-20 leaves digested in 5-10 ml cellulase/macerozyme solution will give 0.5 -1 x 10^6 protoplasts that are enough for more than 50-100 samples (1-2 x 10^4 protoplasts per sample). Note that it is not necessary to use 10^6 protoplasts per sample for gene expression analysis as commonly recommended in other protoplast protocols. The experiments can be easily scaled up or down as long as the recommended DNA/protoplast ratio is followed (see below).

Use a flask (125 ml flask for 10 ml enzyme solution) with a side arm for leaf digestion and apply vacuum infiltration for 5-30 min or just put leaf strips in a Petri dish with enzyme solution and put it into to a vacuum desiccator. Continue the digestion for about 3 h without shaking in the dark (digestion time is depending on the experimental goals and desirable responses). This step needs to be tested empirically for your own assay. The usual prolonged incubation of leaves for 16-18 h in the dark for protoplast isolation is stressful and might eliminate physiological responses of leaf cells. However, the stress might be potentially important for the dedifferentiation and regeneration processes. The enzyme solution should turn green after a gentle swirling motion, which indicates the release of round protoplasts (check under microscope, the size of Arabidopsis mesophyll protoplasts is around 30 to 50 μm). We do not intend to release protoplasts 100%. Be gentle with protoplasts but you can handle them with regular pipets and pipet tips.

Filter the enzyme solution containing protoplasts with a 35-75 μm nylon mesh. Spin at 100 x g to pellet the protoplasts in a round-bottomed tube for 1-2 min (speed 3 with an IEC clinical centrifuge). Higher speed or the addition of $CaCl_2$ (50 mM) may be used if the protoplast recovery is poor. The pelleted protoplasts should be resuspended easily by gentle shaking. Wash protoplasts once in cold washing/incubation (WI) solution for electroporation or W5 solution for PEG transfection, and resuspend protoplasts in the same solution at 1-2 x 10^5/ml. Keep the protoplasts on ice (30 min) in WI or W5 solution. For some experiments, protoplasts could be kept at room temperature before use (Please test). Although the protoplasts can be kept on ice for at least 24 h, freshly prepared protoplasts should be used for the study of regulated gene expression, signal transduction, and protein trafficking, processing and localization. We use these protoplasts to study leaf cell responses to sugars, auxin, ABA, cytokinin, heat, EtOH, H_2O_2, heat, and elicitors. The responses in protoplasts are usually similar to those observed in intact plant leaves. These protoplasts are also a good source for the isolation of intact nuclei and chloroplasts. If W5 solution is used, spin down protoplasts (speed 3 for 1 min) and resuspend in MMg solution (1-2 x 10^5/ml) before PEG transfection.

B. PEG Transfection

All steps are carried out at 23^{o}C
Add 10 µl DNA (10-20 µg of plasmid DNA of 5 kb in size)
Add 100 µl protopolasts to a microfuge tube (2 x 10^4 protoplasts), mix well
Add 110 µl of PEG/Ca solution, mix well (handle 6-10 samples each time)
Incubate at 23^{o}C for 5-30 min (Please test)
Dilute with 0.44 ml W5 solution and mix well gently
Spin at speed 3 in a Clinical centrifuge for 1 min, remove PEG
Resuspend protoplasts gently, dilute in 100 µl, add to 1 ml WI or W5 (6-well plates)
This procedure can be scaled up or down depending on the experimental needs

Comments:
Due to the "transient" nature of the experiments, it is not necessary to perform experiments under sterile conditions. The addition of Amp (50 µg/ml) can prevent bacterial growth if it is necessary.
The system is most suitable for the study of early events in diverse signal transduction pathways, gene regulation, and cell death (Asai et al., Plant Cell 12:1823-1835, 2000).
The use of carrier DNA is unnecessary.
Use 6-well tissue culture dishes (Falcon 3046) for protoplast incubation. We now also use 12-well or 24-well plates for large experiments (with 50-200 samples) with reduced volume.
The dish can be coated with 5% calf serum for 1 sec before use to prevent the sticking of the protoplasts to the plastic.
The protoplast incubation time is 2-6 h for RNA analysis and 2-16 h for enzyme activity analysis and protein labeling. About 100-10^3 protoplasts are sufficient for reporter enzyme assays, 10^4-10^5 protoplasts are required for protein labeling & immunoprecipitation or western blot analysis, and 10^6 protoplasts for RNA analysis.
These protoplasts can be cultured for cell wall regeneration and cell cycle initiation with proper medium and plant hormones (Damm et al., MGG 217: 6-12. 1989; Masson & Paszkowski, 1992, Plant J. 2: 829-833).
PEG transformation efficiency is 50-90% based on GFP expression. If your protoplasts are healthy (from healthyleaf materials. Please test), most protoplasts remain intact.
Electroporation efficiency is 10-30% (depending on plant conditions). More than 50% protopolasts can be killed by electroporation. However conditions could be adjusted to reduce killing (Please test). Protoplasts produced from different plant species and tissues or growth conditions may react differently to electroporation. For instance, etiolated or greening maize mesophyll protoplasts tolerate electroporation extremely well.

Harvest protoplasts by centrifugation at 100 x g for 2 min. Remove the supernatant. Freeze and store samples at -80^{o}C until ready for analysis.
Add 100 µl of hypotonic buffer (10 mM Tris, pH 8 and 2 mM MgCl2) or LUC lysis buffer (Promega), vortex vigorously for 2 sec to lyse the protoplasts. (The LUC lysis buffer contains 1% Triton X-100. Thus, gentle vortex is sufficient.)

A fast and economical xylenes extraction protocol is used for CAT assay (Seed and Sheen, 1988, Gene 67, 271-277; Sheen, in the supplement 9.6.5 of the Current Protocols in Molecular Biology, Ausubel eds). Heating the cell extract at 65^{o}C for 10 min in the presence of 5 mM EDTA might eliminate potential inhibitors for the CAT assay (It seems to be useful for Columbia but not C24). We got good CAT activity without heat treatment. We use a Promega kit for LUC assay with a luminometer.
The GUS assay has been described by Jefferson (Add the cell extract into 100 µl of 1 mM MUG, incubate for 30-90 min at 37^{o}C, add 0.9 ml 0.2 M Na_2CO_3 to stop the reaction, and measure the fluorescence of MU).

C. Solutions

Enzyme solution
1-1.5 % cellulase R10 (RS is too strong)
0.2-0.4% macerozyme R10 (Yakult Honsha, Tokyo, Japan)
0.4 M mannitol
20 mM KCl
20 mM MES, pH 5.7
Heat the enzyme solution at 55^{o}C for 10 min (to inactivate proteases and enhance enzyme solubility) and cool it to room temperature before adding
10 mM CaCl2
5 mM β-mercaptoethanol (optional)
0.1% BSA (Sigma A-6793)
The enzyme solution is light brown but clear (passed through a 0.45 µm filter).

PEG solution (40%, v/v)
4 g PEG4000 (**Fluka, #81240**) ****Very Important!!**
3 ml H_2O
2.5 ml 0.8 M mannitol
1 ml 1M $Ca(NO_3)_2$ or $CaCl_2$

Washing and incubation solution (WI)
0.5 M mannitol,
4 mM MES, pH 5.7
20 mM KCl

W5 solution
154 mM NaCl
125 mM $CaCl_2$
5 mM KCl
2 mM MES (pH 5.7) (no glucose since we use glucose as a signal)

MMg solution
0.4 M mannitol
15 mM $MgCl_2$

4 mM MES (pH 5.7)

D. Electroporation

40 µg plasmid DNA
4-6 x 10^4 protoplasts/300 ul of 0.3 M mannitol/4 mM MES, pH 5.7/150 mM KCl
300-400 V/cm
5 msec, 200 uF, 1-2 pulses

E. Enzymes and nylon filters

The Cellulase and macerozyme are purchased from
Yakult Pharmaceutical IND. CO., LTD.
Shinbashi MCV Building
5-13-5 Shinbashi Minato-Ku
Tokyo, Japan
Tel 03-5470-9811
Fax 03-5470-8921
The purchasing process can take up to a few weeks.

The Nylon filters (35-75 µm) can be purchased from Carolina Biological Supplies

F. Troubleshooting Tips

A list of factors that can be systematically tested if problems occur.
Arabidopsis ecotypes
Arabidopsis growth condition is ecotype-dependent. Since leaves before bolting are used, Col and Ler plants will perform better under shorter photoperiod (10-12 h).
Seed quality (age, growth and storage condition)
Plates (B5 medium)
Soil (water-soaked Metromix 200 or 360, no need for additional nutrient solution). Note that we have had problems with both types of soil occasionally. The quality could vary from batch to batch.
Growth condition: temperature, humidity, light intensity, photoperiod, water, nutrient
Plants, Enzymes & DNA
- Leaf age
- Leaf development & morphology
- Enzyme solution (enzyme quality, heat treatment)
- Digestion time
- Reporter genes
- PEG (quality varies depending on sources and storage)
- Mannitol
- W5

Plasmid DNA quality (We use the more economical CsCl gradient for plasmid DNA purification. Make sure you remove the salt. I do not use the kit due to high price)
Protoplast/DNA ratio (Please test)
Protoplast quality
Resting time
Response time
Incubation time (for gene expression or protein labeling)
Culture plates
Experiment size/duration (Very ambitious experiments tend to fail more often)
Stimuli application (timing, concentration, duration, etc.). We always test them extensively to cover a broad range of signal dosage or concentrations when a new signal is applied for the first time.

Arabidopsis plants are very sensitive to any kind of environmental changes. So, be sensitive to your plants' needs and behavior.