A Transient Expression Assay Using Maize Mesophyll Protoplasts

First draft on 1/8/2000 and updated on 11/19/2012

Reference

Sheen, J. 1991. Molecular mechanisms underlying the differential expression of maize pyruvate, orthophosphate dikinase genes. Plant Cell. 3: 225-245. PMCID: PMC159995.

Soak seeds in water for overnight. Grow plants under standard greenhouse conditions or in the dark (preferred condition for protoplast manipulation, but some corn strains do not grow well in the dark) until the second leaf is about 10-15 cm above the 1st leaf. Use half vermiculite and half peat moss and germinate seeds under light for 3 days (1-2 cm shoots visible) before moving to the dark chamber. It takes about 10 to 11 days at 25 ^{o}C. Only give water but no nutrients (lots of goodies in the seed) because excess salts can make the manipulation of mesophyll protoplasts more difficult. It is not necessary to grow plants under sterile conditions and do surface sterilization. Amp (50-100 ug/ml) can be added to the culture medium to prevent bacterial growth. When growing "green" plants under light for protoplast isolation, low light (30-75 μmole/m^{2}sec) and short photoperiod (<12 hr) is generally recommended. Protoplasts isolated from plants grown under excess light are very fragile and break easily after electroporation. Etiolated and greening protoplasts are the best for most experiments. We have used commercial corn seeds from Illinois Foundation seeds, Inc. (phone 217-485-6260; 217-485-6420) for the past 20 years. The best seeds I can recommend for control experiments are FR992 x FR697 and FR27 x FRM017. The corn seeds for the original hybrid line FR9 x FR37 are no longer available. The dry seeds can be stored at 4^{o}C for up to 10-15 years without losing the germination vigor.

Cut the middle part (8-10 cm) of the second leaves. Pile 20 to 40 leaves (press gently) and cut 0.5 mm strips without bruising (wounding. No juice or "blood") the leaves. This is perhaps the most difficult part for most people. I usually prepare several fresh razor blades (rinsed with 95% ethanol and wipe them dry) and change razor blades when a dull edge can be sensed. A good preparation yields around 5 x10^6 protoplasts/g fresh weight which are enough for 50 electroporation samples (10^5 each).

Use a flask (250 ml flask for 10-20 ml enzyme solution) with a side arm for leaf digestion and apply vacuum for 30 min for infiltration. Continue the digestion for another 120 min with gentle shaking (40 rpm on a platform shaker). 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. The enzyme solution should now turn green for green and greening leaves but remain yellow for etiolated leaves grown in the dark which indicates the release of round protoplasts (check under microscope, the size of maize mesophyll protoplasts is around 25 to 35 μ m). Release the protoplasts thoroughly by shaking at 80 rpm for 5 min. No intention is made to release protoplasts 100%.

Filter the enzyme solution containing protoplasts with a 35 μm nylon mesh. Spin at 150xg to pellet the protoplasts in a round-bottomed tube for 2 min (speed 3-4 with an IEC clinical centrifuge). Higher speed may be used if the protoplast recovery is poor. The protoplast pellet should be resuspended easily by gentle shaking within seconds. Wash protoplasts once in cold electroporation solution and resuspend in the same solution at 1-2 x 10^6/ml. Keep the protoplasts on ice. (However, keep all steps at room

temperature if cold treatment will abolish the desired physiological responses; e.g. phytochrome response). Although the protoplasts can be kept on ice for at least 24 h, freshly prepared protoplasts should be used for the study of gene regulation, signal transduction, and protein trafficking, processing and localization. These protoplasts are also a good source for the isolation of intact nuclei and chloroplasts.

Electroporation

Use 1-2 x 10^5 protoplasts per electroporation in 300 µl with 25 to 50 µg of plasmid DNA of 5 kb in size (10 µg/kb). The efficiency may decrease when the plasmid size is larger than 10 kb. The use of carrier DNA is unnecessary. The optimal electroporation condition is 5 msec, 400 V and 200 µF, and one pulse. Two to three pulses can be used if an extremely high transfection efficiency (50-80%) is desired. Promega X-cell 450 (no longer available), Hoefer Progenetor II, Biorad Gene Pulser with a capacitance extender (not the best one) and even a home-made machine can provide the condition. Keep electroporated samples on ice for 10 min before protoplast resuspension and transferring to a 6-well tissue culture dish (Falcon 3046) at 0.5-2 x 10^5/ml. The dish can be coated with 5% calf serum for 10 sec before use to prevent the sticking of the protoplasts to the plastic surface (BSA is not good for this purpose!). The protoplast incubation time is 3-6 h for RNA analysis and labeling, and 6-24 h for enzyme activity analysis and protein labeling. About 10^3-10^4 protoplasts are enough for enzyme assay but 0.5 x 10^6 protoplasts are required for RNA analysis. Please try out smaller experiments to become familiar with the procedure and be observant and flexible.

Note on electroporators:

Promega stopped selling the X-cell 450 machine for a while. Some scientists have been able to produce homemade ones for $300 with fixed "optimal" capacitance (200-400 µF), pulse time (5-10 msec) and flexible voltage (up to 500 V). Protoplasts are large cells (25-60 µm) and cannot tolerate high voltage. Monocot protoplasts cannot tolerate NaCl salt but tobacco and Arabidopsis protoplasts do well with 100-200 NaCl or KCl salt, which reduces protoplast burst after electroporation. You may try 1, 2 or up to three pulses to gain the best efficiency with minimal cell burst (need to try it yourself).

BTX T820
ELETRO SQARE PORATOR
This machine is available now on the market but more expensive. You may get it cheaper from eBay!

The operation condition is very different from the other electroporation machines described above.

Please consult the manufacture for the best condition. It uses much faster and stronger pulses and generates square-wave pulse with more precisions. You will need to try up to 20-25 continued pulses (10-50 micro-sec pulses but not mini-sec), which can be delivered automatically by the machine and at higher voltage. Please follow the manufacture instruction and try it out yourself. It's important to try out experiments to see what works for your protoplasts.

BEX has many types of electroporators

http://www.bexnet.co.jp/english/product/device/index.html?_kk=electroporation%20transfection&_kt=5aa6ee4c-72ec-4337-a668-ee14bb49a3e8&gclid=CLGU_rniwrICFcJo4Aodn3YAKQ

CAT assay

Harvest protoplasts by centrifugation at 500 x g for 2 min. Remove the supernatant. Add 100 μl of hypotonic buffer (10 mM Tris, pH 8 and 2 mM $MgCl_2$) and vortex vigorously for 5 sec to lyse the protoplasts. Freeze and store samples at -70^{o}C until ready for analysis. 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 is unnecessary.

GUS assay

The GUS assay has been described by Jefferson (Add the cell extract into 100 μl of 1 mM MUG, incubate for 60-90 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).

LUC assay

We use a Promega kit for LUC assay with either a luminometer or a scintillation counter.

In this system, photosynthetic gene promoters show tissue-specific and developmental (greening and normal green leaf development) regulation. In addition, regulated gene expression in response to metabolites, light, heat shock, phytohormone, redox, photooxidation, elicitor has been observed. Reagents as agonists/antagonists of cellular functions and processes can be introduced into protoplasts by incubation or electroporation. Molecular genetic manipulation of cloned genes and the introduction of a dominant mutant activity can be performed. However, antisense approach is unlikely to work in transient assays if the targeted protein is very stable in the absence of mRNAs. Transfection efficiency is about 30-50% with two electroporation pulses. Co-transfection efficiency is very high (almost 100%). We have co-transfected up to four different plasmid DNA together. Transient protoplast expression assay using mutant maize leaves can also be carried out.

Enzyme solution:

1.5% cellulase R10 for green leaves and RS for etiolated or greening leaves, 0.3% macerozyme R10 (Yakult Honsha), 0.6 M mannitol, 10 mM MES, pH 5.7. Heat the enzyme solution at 50-55^{o}C for 10 min (to inactivate proteases and accelerate enzyme solubilization) and cool it to room temperature before adding 1 mM $CaCl_2$, 5 mM β -mercaptoethanol, and 0.1% BSA. The enzyme solution is light brown but clear. Pass the enzyme solution through a 0.45 μm filter.

Electroporation, washing, and incubation solutions:

0.6 M mannitol. 4 mM MES, pH5.7, and 20 mM KCl for washing and electroporation, and 4 mM KCl for incubation. Protoplasts can be incubated under light (15-50 $\mu E/m^2s$) or in the dark depending on the purpose of the experiments. About 50 (green) to 99% (etiolated and greening) of protoplasts will survive electroporation.