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Isolation and Efficient Maize Protoplast Transformation

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[Abstract] Establishing plant gene function and the study of gene expression regulation often require that genes (or gene variants) are introduced into plants and their activity somehow assayed. Some plants (*e.g.*, *Arabidopsis*) are easily transformed, while for others (*e.g.*, maize) making stable transgenic plants remains challenging, is expensive and time-consuming. An alternative solution to generating transgenic plants is to assay gene function transiently. In some plants, it is possible to do this by Agro-infiltration. We have adapted a maize protoplast isolation protocol that permits high-efficiency transformation by electroporation, irrespective of the genetic background. Transformed protoplasts have been used to assay gene function using metabolic profiling, to explore protein-DNA interactions using chromatin immunoprecipitation (ChIP), and to assay the activation of reporter constructs by transcription factors. Here, we describe the protocol for efficient maize protoplast isolation and transformation by electroporation.

Keywords: Maize, Protoplast, Electroporation

[Background] Maize (*Zea mays)* is one of the most cultivated crops in the world, with a productivity that continues to increase thanks to genetic advances and to improved agronomic practices (Haarhoff and Swanepoel, 2018). In addition to the grain, the stover is used for the production of biofuels and electricity (Trivedi *et al.*, 2015). Maize is also an important model plant for fundamental research (Cone *et al.*, 2002; Nannas and Dawe, 2015). While a large number of maize genes have been cloned and characterized, functional information on many genes is still lacking (Jin *et al.*, 2017; Cao *et al.*, 2018). One of the strategies most commonly used to determine gene function is the characterization of the phenotypes of loss-of-function mutants. Unless multiple alleles are available, the usual practice is to complement the mutants with the wild-type gene to confirm the nature of the mutation. When multiple candidate genes must be validated, in a plant like maize, this can become prohibitively expensive and very time-consuming. In some instances, for example, to identify the gene responsible for a missing step in a biosynthetic pathway, transformation of protoplasts derived from the mutant with the candidate genes, combined if necessary with precursor feeding, can narrow down, or even identify, the responsible gene (Casas *et al.*, 2016). We have also successfully used maize protoplast transformation to confirm transcription factor target genes (Yang *et al.*, 2017), and to establish the organization of transcriptional complexes (Hernandez *et al.*, 2007). We describe here a fast and simple protocol to generate and

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transform protoplasts from maize seedlings, irrespective of the genotype. This protocol is largely based on Sheen (1991 and 2001) and Jang and Sheen (1994).

Materials and Reagents

1. Pipette tips (Fisher brand, catalog number: 02707414)
2. Aluminum foil (Reynolds Kitchens, REYNOLDS WRAP, catalog number: 353224)
3. Filter paper (Fisher Scientific, catalog number: 34155)
4. Parafilm (Fisher Scientific, catalog number: 13-374-12)
5. 35 µm Mesh (Carolina biological supplies, catalog number: 652222R)
6. Single Edge Blade 009 (Accutec blades, catalog number: 94-0491-0000)
7. Glass cutting board
8. Electroporation Cuvettes (BIO-RAD, catalog number: 1652088)
9. 50 ml Falcon tubes (Thermo Fisher, catalog number: 14-432-22)
10. 1.7 ml Microcentrifuge tubes (Fisher Scientific, catalog number: 21-402-905)
11. 25 seeds of maize
12. Cellulase “ONOZUKA” RS (Yakult Pharmaceutical Industry Co., Ltd)
13. Macerozyme R-10 (Yakult Pharmaceutical Industry Co., Ltd)
14. D-Mannitol (Fisher Scientific, catalog number: M120-3)
15. Potassium Chloride (KCl) (Sigma-Aldrich, catalog number: P3911)
16. MES free acid monohydrate (GoldBIO, catalog number: M-090-500)
17. Calcium chloride ($CaCl_2$) (Sigma-Aldrich, catalog number: C2661).
18. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A3059)
19. Maxiprep Kit (Invitrogen, catalog number: K210017)
20. Protoplasts buffer (see Recipes)
21. Enzyme suspension (see Recipes)

Equipment

1. Flask with side arm
2. Pipettes
3. Hemocytometer (Hausser & Son LEVY ULTRA PLANE Improved NEUBAUER MINT)
4. Centrifuge (Thermo Scientific™ Sorvall™ Legend™ XTR)
5. Electroporator (BTX electro-square-Porator ECM 830)
6. Fluorescence microscope (Leica DM IL LED Inverted Phase Contrast Microscope with the camera Leica DFC 3000 G)
7. Optical microscope (Reichert Scientific Jung microscope series 150)
8. Refrigerated incubator (Fisher Scientific, catalog number: 3720A)
9. Benchtop Orbital Shakers (Fisher Scientific, catalog number: SHKE3000)

10. Microcentrifuge (Thermo Scientific, model: Sorvall™ Legend™ Micro 17, catalog number: 75002403)
11. Water bath (VWR, model: 1230)

Procedure

A. Etiolated plant growth
   1. Soak maize seeds in water O/N at room temperature.
   2. Transfer the seeds to wet filter papers in a covered beaker for 2 days at room temperature.
   3. Move the germinated seeds to soil and grow in an incubator in the dark for 10-12 days at 26 °C, 60% relative humidity.

   Note: Etiolated maize leaves are used because green leaves are more brittle (Sheen, 2001), and chloroplasts interfere with the detection of the GFP signal.

B. Protoplasts buffer preparation (Total volume: 50 ml)

   In a Falcon tube, mix: 48.75 ml of 0.6 M D-Mannitol, 0.25 ml of 2 M KCl,1 ml of 0.5 M MES (pH 5.7).

   Note: Keep the buffer on ice.

C. Protoplast Isolation
   1. Enzyme suspension: Weight 0.3 g of cellulase and 0.07 g of macerozyme in a 50 ml Falcon tube. Add 10 ml of protoplasts buffer and disperse along the wall of the tube using a spatula or glass rod.

      Note: Do not vortex. Avoid at all times the foam production, it can affect the activity of the enzyme.
   2. Incubate the enzyme suspension in a water bath for 5 min at 55 °C, and then cool on ice for 5 min.
   3. Add 50 µl 1 M $CaCl_2$ and 100 µl 10% BSA to the enzyme suspension, and mix gently. Keep in the dark (by wrapping the tube in aluminum foil) on ice.
   4. Transfer the enzyme suspension to a flask with a vacuum arm, and keep in the dark (by wrapping the flask with aluminum foil).
   5. Cut 12 leaves from the 10-12 day-old maize etiolated seedlings. Select the second in each seedling when they are 10-15 cm higher than the first leaf (Figure 1A). Stack the leaves on top of each other and discard of 1/3 from each end, keeping only the middle part (6-8 cm).
   6. Using a new (never used) razor blade cut the leaves in very fine strips on the cutting glass board (Figure 1B) (Usually three new blades are used). Make sure you do not squeeze/damage the leaves while cutting. As you cut them, add the cut leaves to the flask that contain the enzyme suspension.

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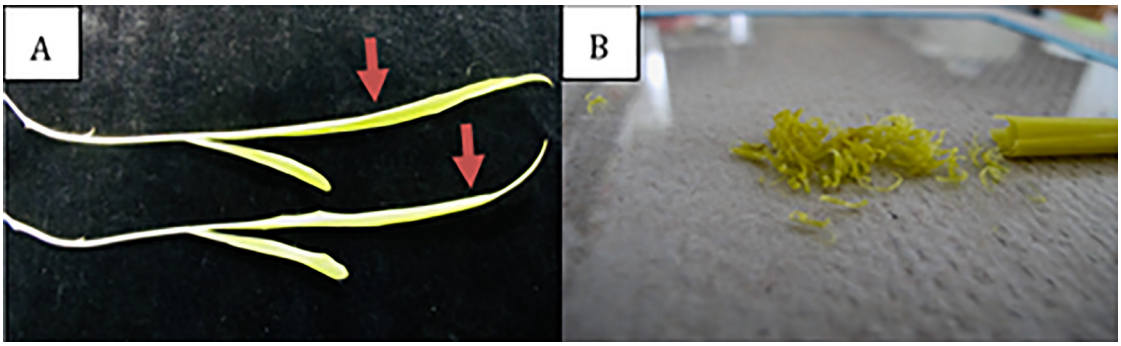

Figure 1. Etiolated maize leaves. A. Second leaves from etiolated 10-day-old maize plants. B. Cut strips on the glass board.

7. Vacuum the enzyme suspension containing the cut leaves at 20 mm Hg for 40 min to allow sufficient infiltration. Keep in the dark as much as possible.
   Note: A central vacuum line is used.
8. Cover the holes in the flask with Parafilm and shake at 60 rpm for 3 h at 26 °C to digest the cell walls. Keep in the dark.
9. Shake at 90 rpm for 5 min the enzyme suspension to release the protoplasts.
10. Filter the enzyme suspension containing the protoplasts into a 50 ml Falcon tube using a 35 µm nylon mesh.
11. Collect the protoplasts by centrifugation at 150 *x g* for 1.5 min at room temperature, and then remove the supernatant using a vacuum system.
12. Wash protoplasts twice with 10 ml protoplasts buffer. Spin at 150 *x g* for 1.5 min after each wash, and discard the supernatant.
    Note: Be very careful when resuspending the protoplasts, do not vortex, mix by inverting the tube gently.
13. Finally, resuspend the protoplast in 2-3 ml protoplasts buffer. Keep on ice.

D. Counting protoplast

Load 15 µl of the protoplast suspension into a hemocytometer and count cells under a light microscope using the 20x objective (Figure 2). The expectation is to obtain ~10^5 protoplasts/ml (8 protoplasts per square).

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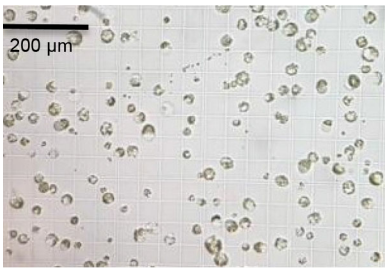

Figure 2. Protoplasts isolated from etiolated maize leaves. Image was taken at 20x magnification using a Reichert Scientific Jung microscope, and the bar (top left corner) corresponds to 200 µm.

E. Protoplast transformation

1. Plasmid DNA preparation from *E. coli* cells harboring the relevant plasmids: Plasmid DNA is isolated using the Invitrogen Kit (following the protocol provided by Invitrogen), which yields good-quality DNA with values A_{260}/A_{280} = 1.7-2.0. Dilute 15-40 µg DNA in protoplasts buffer and take to a total volume of 150 µl.
2. Electroporation: Transfer the 150 µl of DNA suspension to the cuvette, and add 150 µl protoplast suspension (1-2 x 10^5/ml). Mix very gently. The optimal electroporation conditions are 5 ms, 200 µF (0.5-1 kV/cm) and one pulse. For instance, 100 V would be used to achieve 0.75 kV/cm if a 2 mm cuvette is used.
3. After electroporation, place the cuvette back on ice, add 700 µl of protoplasts buffer, mix very gently and transfer to a fresh 1.7 ml Eppendorf tube.
4. Store tubes for 18-22 h at room temperature in the dark, laying on the side.

F. Transformation efficiency

Transformation efficiency is estimated as a percentage of transformed cells following GFP expression (Figure 3), for example when driven from a constitutive promoter, such as the CaMV35 promoter (p35S::GFP). Using the equation:

$$\frac{\#\ protoplast\ expressing\ GFP}{total\ \#\ protoplast} * 100 = \%\ trasnformation\ efficiency$$

The transformation efficiency can reach 30-70% irrespective of the genotype.

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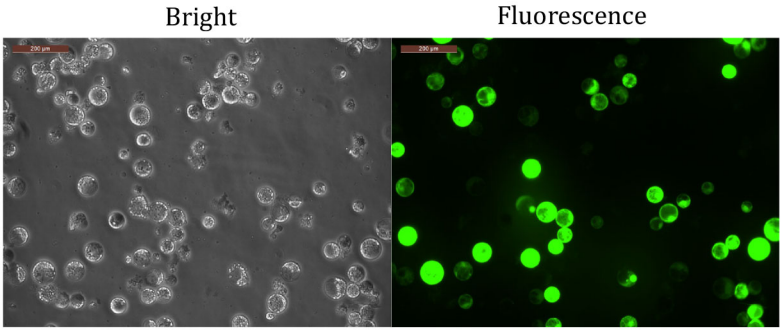

Figure 3. Protoplasts were prepared from etiolated maize leaves and transformed as described in "Procedure" with a plasmid expressing GFP. Images were taken at 20x magnification, and the bar (top left corner) corresponds to 200 µm.

Recipes

1. Protoplasts buffer
   0.6 M D-Mannitol
   2 M KCl
   0.5 M MES (pH 5.7)
2. Enzyme suspension
   Protoplasts buffer
   1 M $CaCl_2$
   10% BSA
   3% Cellulase "ONOZUKA" RS
   0.7% Macerozyme R-10

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We declare no conflicts of interest or competing interests.

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