

An Efficient Procedure for Protoplast Isolation from Mesophyll Cells and Nuclear Fractionation in Rice

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[Abstract] Plant protoplasts, a proven physiological and versatile cell system, are widely used in high-throughput analysis and functional characterization of genes. Green protoplasts have been successfully used in investigations of plant signal transduction pathways related to hormones, metabolites and environmental challenges. This protocol, adapted from Zhang *et al.* (2011), describes a procedure for the isolation of rice protoplasts from green tissue and shows an efficient and rapid method for isolation of nuclei from these protoplasts which are commonly used in a variety of experimental procedures including the isolation of high-molecular-weight DNA (Watson and Thompson, 1986), *in vitro* DNA synthesis (Roman, 1980), isolation of labeled transcripts for differential screening of cDNA libraries (Somssich *et al.*, 1989), preparation of nuclear extracts for *in vitro* transcription systems (Roberts and Okita, 1991), isolation of nuclear proteins (Harrison *et al.*, 1992) and studies of protein targeting to the nucleus (Hicks and Raikhel, 1993).

Materials and Reagents

1. 12-day-old plants with 1-2 cm leaves before flowering
2. Cellulase RS (Yakult Pharmaceutical)
3. Macerozyme R-10 (Yakult Pharmaceutical)
4. D-Mannitol (Sigma-aldrich, catalog number: M4125)
5. KCl (Sigma-aldrich, catalog number: P9333)
6. MES hydrate (Sigma-aldrich, catalog number: M8250)
7. CaCl₂ (Sigma-aldrich, catalog number: C1016)
8. BSA Bovine serum albumin (Sigma-aldrich, catalog number: A2153)
9. Spermidine (Sigma-aldrich, catalog number: S2626)
10. EDTA (Sigma-aldrich, catalog number: EDS)

11. Sucrose (Sigma-aldrich, catalog number: 84097)
12. Triton X-100 (Sigma-aldrich, catalog number: X100)
13. DTT (Sigma-aldrich, catalog number: 43815)
14. Enzyme solution (ES) (see Recipes)
15. Washing and incubation solution (WS1) (see Recipes)
16. Washing solution (WS2) (see Recipes)
17. MMG solution (see Recipes)
18. Nuclei isolation solution (NIB) (see Recipes)

Equipment

1. Razor blades (No18 sterile stainless steel scalpel blade) (Swann Morton, catalog number: 0323)
2. Micro-dissecting forceps (Sigma-aldrich, catalog number: F3767)
3. Nunc petri dishes (diam 90 mm × H 15 mm, surface area size 58 cm², vented) (Sigma-aldrich, catalog number: Z717223)
4. 50 ml conical tubes (Bioland scientific, catalog number: A02-04)
5. Orbitron Rotator I (115 V) (Boekel Scientific)
6. Nylon mesh CX-60 (120 μm) and CX-400 (38 μm) (Carolina Biological Supplies, catalog number: 65-2222N)
7. Glass funnel
8. Hemocytometer [Mfr Part Number: 3100; Cell depth: 0.100mm +/- 2% (1/10mm); Volume: 0.1 Microliter] (PGC Scientific, catalog number: 1907353).
9. Stereo Microscope (Olympus, catalog number: SZ51)
10. 25G5/8 gauge needle (Kendall, catalog number: KND8881511235)

Procedure

A. Prepare protoplast

1. Weigh 1 g cut leaves, removing the petiole (see Figure 1A).
2. On the flat glass, chop leaves into 0.5-1 mm strips with fresh razor blades or with flamed scissors without shredding.
Note: Strips don't need to be very small to avoid the accumulation of excessive cell debris on the filter. Leaves can be easily cut using a plate surface like a petri dish.
3. Using a forceps, transfer immediately the plant material to a deep petri dish filled with washing solution WS1 (enough to cover the strips) and after gentle shaking (1-3 min by hand) remove all liquid by pipetting.

4. Add freshly prepared enzyme solution (ES). 5 ml of ES is generally used per 1 g of cut leaf material. Incubate 4-5 h in the dark, at room temperature, with gentle shaking (60-80 rpm) in the rotator.

B. Harvesting protoplasts

1. After the enzymatic digestion, transfer the digestion mixture to a 50 ml Falcon tube, add an equal volume of WS2 solution and shake vigorously by hand for 10 sec.
2. Release the protoplasts from undigested leaf tissue by filtering the mixture through 120 μm nylon mesh sieves on glass funnel into a round bottom tubes. Two sequential filtering steps, first using CX-60 (120 μm) and then CX-400 (38 μm), are sufficient for protoplasts being isolated from leaf tissue.

Note: We usually go through a 3 step filtration procedure when working with suspension culture material since the sieves clog quickly. The sieves are used in the order CX-60, CX-150 (104 μm) and finally CX-400.

3. Wash the protoplasts 3-5 times using 2 ml WS2 solution.
4. Spin, after each washing, the tube for 10 min at 50 x g. Discard supernatant and wash protoplasts again with 2 ml WS2 solution.
5. After washing, collect the pellets by centrifugation at 50 x g for 3 min with a swinging bucket rotor and re-suspend in MMG solution for counting or in NIB solution for the Isolation of nuclei.

C. Evaluating the quality (viability) and counting the protoplasts

Evaluating the quality

The protoplast isolation procedure often damages or kills a percentage of cells. To determine the viability of the cells, we used fluorescein diacetate (FDA) staining to estimate the percentage of viable protoplast (green protoplast) in a preparation.

1. Mix 0.25 ml of 0.05% fluoriscin diacetate (dissolved in acetone) with 20 ml of the appropriate isolation medium to make the "stain" solution.
2. Pipete 0.25 ml of the protoplast suspension into 1.0 ml of the stain solution.
3. Swirl gently to mix and mount some of the protoplasts on a glass slide. First, look at the protoplasts using a 10 or 20 x objectives lens. Now view the protoplasts using the UV source (420- to 490 nm excitation). In the rice green tissue protoplasts, the chloroplasts could be easily identified by their typical chlorophyll autofluorescence under a confocal microscope, while they could not be clearly observed in etiolated protoplasts.
4. Observe at least 50 protoplasts and calculate the percentage of living cells. Multiply the estimate of number of protoplasts by this percentage to obtain an estimate of the number of living protoplasts.

Example: 5×10^5 protoplasts per milliliter multiplied by 0.95 viable protoplasts, equals 4.75×10^5 living protoplasts per milliliter. A good isolation will yield between 90 and 95 % viable protoplasts.

Counting protoplasts

1. Place a cover glass over the hemocytometer counting chambers.
2. Gently mix the protoplast sample thoroughly to insure a uniform distribution of the cells and withdraw a sample with a Pasteur pipet.

Note: Dilute the sample if the cell count is greater than 10^7 cells/ml.

3. Examine at the appropriate magnification (100 x should work for the protoplasts) and count the number of protoplasts in four, appropriately-sized grids in each chamber.

Note: Include all cells touching the middle line along the left or top margin of a square, but not the right or bottom. At 100 x, a 1 mm^2 square will fill the field.

4. Count a total of 16 grids from 4 chambers
5. Record the data in table 1 & 2 and calculate the number of cells per mL using the following equation (to be significant at the 5% level of confidence, the 8 counts cannot deviate more than 7% from the mean [Klein and Klein (1970)]): $\text{Cells/ml} = \text{Cells/square} \times 1 \text{ square/volume (mm}^3) \times 1000 \text{ mm}^3/\text{ml} \times \text{dilution factor}$

Table 1: Hemocytometer data

Grid size counted	0.1 mm x _____ mm x _____ mm
volume of grid counted (mm^3)	
Dilution (if any)	

Table 2: Protoplast density in a partially purified suspension of Red cabbage protoplasts measured with a hemocytometer

Grid	protoplasts grid^{-1}	protoplasts ml^{-1}
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		

11		
12		
13		
14		
15		
16		
mean +/- std		

D. Isolation of nuclei

1. Re-suspend pellet at 10^6 /ml of cold NIB in 2 ml eppendorf tube or bigger tubes.
2. Put on ice for 7 min.
3. Fill a large syringes with protoplasts and pass it 4 times through a 25G5/8 gauge needle.
4. Filter the lysate through 20 μ m filter into a new tube. Centrifuge at 400 x g for 10 min.
5. Discard supernatant
6. Re-suspend in NIB + glycerol or SDS solution for SDS gel electrophoresis.

Representative data

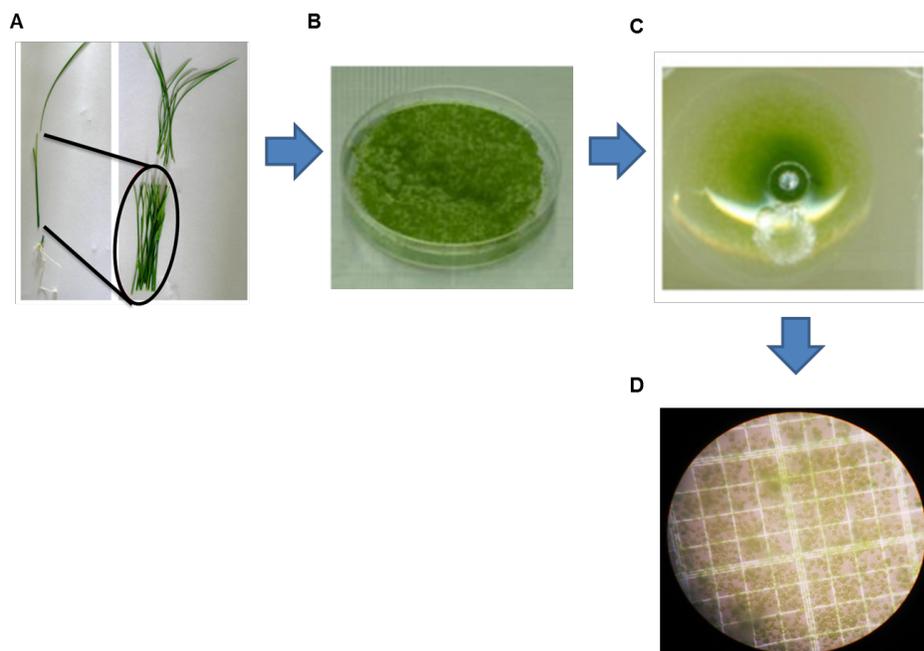


Figure 1. Isolation of protoplasts from rice green tissue. A. A representative healthy 12-day-old rice seedling used for protoplast isolation. Black markers indicate the optimal sections of seedlings (stem and sheath) yielding protoplasts. B. Cut strips were digested by enzyme solution (ES). C.

Protoplasts collected after the filtration step. D. Image of protoplasts obtained using a Leica digital camera under an Olympus microscope with a 40x objective.

Recipes

1. Enzyme solution (ES)

1.5% (w/v) cellulase R10

0.4% (w/v) macerozyme R10

0.4 M mannitol

20 mM KCl

20 mM MES (pH 5.5)

Heat to 55 °C for 10 min, then cool to room temperature before adding 10 mM CaCl₂

0.1% BSA

Note: For longer-term storage, the enzyme powder should be aliquoted and stored at -20 °C in tightly capped plastic tubes. For regular use, a frozen 5 g tube is "thawed" and stored at 4 °C.

2. Washing and incubation solution (WS1)

0.5 M mannitol

4 mM MES-KOH (pH 5.5)

20 mM KCl

3. Washing solution (WS2)

154 mM NaCl

125 mM CaCl₂

5 mM KCl

2 mM MES at pH 5.7

4. MMG solution

0.4 M mannitol

15 mM MgCl₂

4 mM MES at pH 5.7

Note: Solutions WS1, WS2 and MMG can be prepared before the experiment, autoclaved and stored at 4 °C. Just check the pH before using them.

5. Nuclei isolation solution (NIB)

0.1 mM spermidine

10 mM MES-KOH (pH 5.5)

2.5 mM EDTA

10 mM NaCl

10 mM KCl

0.2 M sucrose

0.15% Triton X-100
 2.5 mM DTT (add fresh just before using)

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