Video Article

Isolation of Protoplasts from Tissues of 14-day-old Seedlings of *Arabidopsis thaliana*

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Abstract

Protoplasts are plant cells that have had their cell walls enzymatically removed. Isolation of protoplasts from different plant tissues was first reported more than 40 years ago [1] and has since been adapted to study a variety of cellular processes, such as subcellular localization of proteins, isolation of intact organelles and targeted gene-inactivation by double stranded RNA interference (RNAi) [2-5]. Most of the protoplast isolation protocols use leaf tissues of mature Arabidopsis (e.g. 35-day-old plants) [2-4]. We modified existing protocols by employing 14-day-old Arabidopsis seedlings. In this procedure, one gram of 14-day-old seedlings yielded 5 10^6-10^7 protoplasts that remain intact at least 96 hours. The yield of protoplasts from seedlings is comparable with preparations from leaves of mature Arabidopsis, but instead of 35-36 days, isolation of protoplasts is completed in 15 days. This allows decreasing the time and growth chamber space that are required for isolating protoplasts when mature plants are used, and expedites the downstream studies that require intact protoplasts.

Protocol

Part 1. Preparation of solid medium for growing plants.

We typically culture plants on concentrated Murasige and Skoog (MS) medium supplemented with 1% sucrose and 0.7% agar. It contains all necessary ingredients to keep plants healthy.

For preparing 1L of MS, media pH 5.7

1. Add 2.15 g of MS powder into 800 ml of water in 1.5 -2.0L autoclavable bottle.
2. Place a stirring bar into the bottle and dissolve MS powder by stirring medium on a stirring plate.
3. While stirring, add, drop by drop, 1N KOH to adjust pH of MS medium to 5.7.
4. Add 10 g of sucrose, keep stirring.
5. Add 7 g of agar *.
6. Adjust the final volume of the prepared medium to 1000 ml and sterilize by autoclaving.*
7. Place autoclaved medium on a stirring plate and cool it down while stirring, so that agar will not precipitate at the bottom of the bottle. Cool down medium to ~ 60 °C.*
8. Pour 100 mm Petri plates. Keep plates at 4 °C.

*Tip 1: Do not attempt to dissolve agar. It will solubilize during autoclaving.
*Tip 2: Keep the stirring bar in a bottle during autoclaving, you will need it later.
*Tip 3: If you can keep you hands on the autoclaved bottle for 10 s, it means that the medium has cooled down sufficiently and you can start pouring plates.

Part 2. Plant material and growth conditions.

1. Sterilize seeds of *Arabidopsis thaliana*
    1. Place 100 mg of seeds into eppendorf microcentrifuge tubes and add 1 ml of 70% ethanol. Mix well and incubate for 2 min. Spin seeds down, aspirate the supernatant.
    2. Add 1 ml of 1.8 % bleach solution* containing 0.1% Tween-20. Mix for 10 min. Spin down seeds and aspirate bleach solution.
    3. This step has to be done in a laminar flow hood. Add 1 ml of sterile water to seeds, mix well. Spin down seeds, aspirate water. Repeat this step 5 times.
2. Spread seeds onto the prepared in step 1 plates (100 seeds/plate).
3. Keep seeded plates for 24-48 h at 4 °C in the dark for stratification.
4. Grow plants for 14 days in the growth chamber with 8 h light/16 h dark photoperiod (at a photosynthetic photon flux density of 250 µmol $m^{-2}s^{-1}$) at 23 /19 °C light/dark temperature regime and 75% relative humidity.

*Tip 1: Bleach solution is made-up by dilution a household Clorox, containing 6% sodium hypochlorite and adding Tween-20 to a final concentration of 0.1%.

Part 3. Isolation of protoplasts from *Arabidopsis* seedlings.

1. Slice 2 g of 14-day-old seedlings with a fresh razor blade in 15 ml of filter-sterilized TVL Solution. We chop plant material in sterile disposable Petri dishes.

2. Transfer chopped tissues into 200 ml beaker, add 20 ml of filter-sterilized Enzyme Solution, swirl the beaker to let tissues mix with Enzyme Solution, cover with parafilm and aluminum foil.
3. Shake plant tissues at 35 rpm in the dark at room temperature for 16-18 h.
4. Collect the released protoplasts into 50 ml Falcon tube by sieving through 8 layers of the cheese cloth, pre-wet in W5 Solution.
5. Sieve protoplasts from the cheese-cloth one more time by washing the cloth with 15 ml of W5 Solution.
6. Carefully overlay protoplasts with 10 ml of W5 Solution, do not disturb the sugar gradient; centrifuge for 7 min at 100 g.
7. Collect 10 ml of protoplasts at the interface of Enzyme Solution and W5 Solution (Fig. 1B) and transfer to a new 50 ml Falcon tube.
8. Add 15 ml W5 Solution, centrifuge for 5 min at 60 g. Remove the supernatant.
9. Wash protoplasts free from Enzyme Solution by resuspending protoplasts in 15ml of W5 Solution, centrifuge for 5min at 60 g
10. Remove the supernatant, resuspend pelleted protoplasts in 1-3ml W5 Solution.
11. Evaluate protoplast yield by cell counting with a hemocytometer.

Part 4. Reagents:

TVL: 0.3 M sorbitol; 50 mM $CaCL_2$ Filter-sterilize and store at -20 °C.

Enzyme Solution: 0.5 M sucrose, 10 mM MES-KOH [pH 5.7], 20 mM $CaCl_2$, 40 mM KCl, 1% Cellulase (Onozuka R-10), 1% Macerozyme (R10). Filter-sterilize and freshly use.

W5 Solution: 0.1% (w/v) glucose, 0.08% (w/v) KCl, 0.9% (w/v) NaCl, 1.84% (w/v) $CaCl_2$, 2 mM MES-KOH pH 5.7. Filter-sterilize and store at room temperature.

Part 5: Representative Results:

Using Arabidopsis culture conditions described in Parts 1 and 2 renders healthy seedlings, suitable for isolating protoplasts (Fig. 1A). Protoplast are purified by sucrose density gradient centrifugation and collected at the interface of W5 and Enzyme solutions (Fig. 1B). Using protoplast isolation procedure described in Part 3 we typically harvest 5-10 10^6 of intact protoplasts (Fig. 1C) from one gram of fresh seedlings.

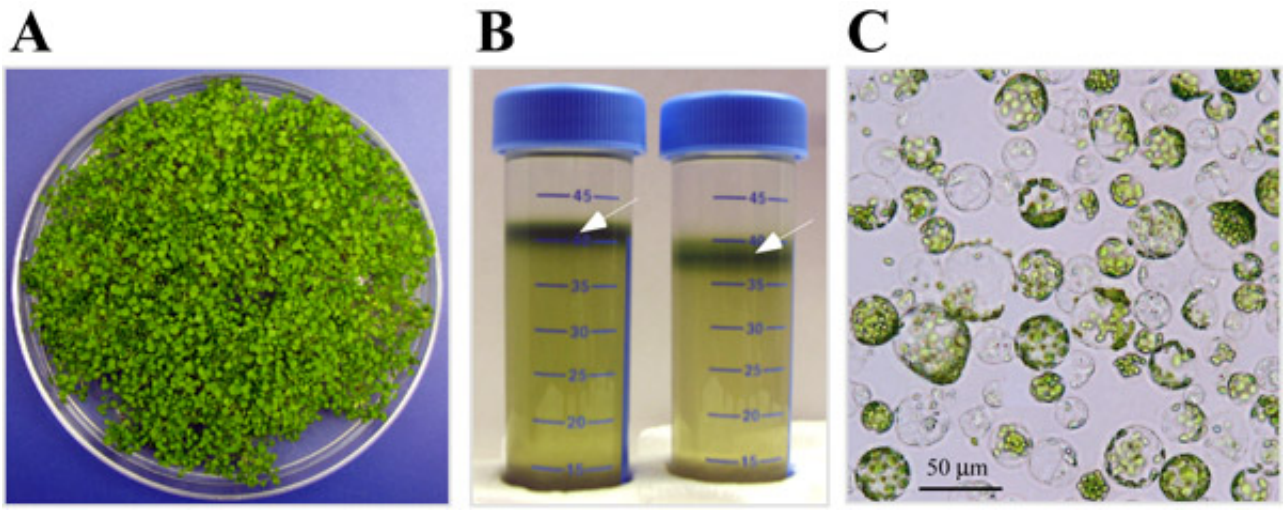

Figure 1. Isolation of protoplasts from Arabidopsis seedlings. A, Tissues from 14-day-old seedlings of Arabidopsis were collected and converted to protoplasts by a modified procedure of (Chen and Halkier, 2000). **B**, Protoplasts were purified by sucrose density gradient centrifugation and collected at the interface of enzyme solution and W5 uffer (*White arrows*). **C**, Bright-field microscopy of protoplasts. Microphotographs were collected using a cooled CCD camera interfaced with the Zeiss Axioscope 2 plus microscope.

Discussion

To ensure the high yield of intact protoplast it is very important to start-up with healthy plants. Use filter-sterilize solutions for isolating protoplasts. Remember that protoplasts are fragile. Therefore, when you are handling protoplasts, do not mix, pipet or vortex them vigorously since it will brake them. Instead, mix protoplasts by slowly rotating or taping the centrifuge tube. The described procedure yield intact protoplasts that are viable for at least 96 h. Therefore, protoplasts can be used to study a variety of cellular processes, such as subcellular localization of proteins, isolation and analyses of intact organelles and targeted gene-inactivation by double-stranded RNA interference (RNAi), etc [3-5].

Disclosures

The provisional patent describing this procedure for its use in functional analyses of plant genes have been filed with Cornell Center For Technology Enterprise and Commercialization (CCTEC), June 2008. Docket No 50341/015001: Vatamaniuk, O.K., and Zhai, Z. Method for rapid functional analyses of plant genes.

Acknowledgements

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