Arabidopsis leaf protoplasting

Diep Ganguly,Peter Crisp

Abstract

Method for isolating mesophyll protoplasts from Arabidopsis as a system for monitoring RNA stability using transcriptional inhibitors (e.g. cordycepin) under stress treatments (e.g. high-light or hydrogen peroxide treatment).

Adapted from Yoo S.-D., Cho Y.-H. & Sheen J. (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nature Protocols 2, 1565–1572.

Citation: Diep Ganguly,Peter Crisp Arabidopsis leaf protoplasting. **protocols.io** dx.doi.org/10.17504/protocols.io.p9adr2e
Published: 13 Jun 2018

Guidelines

Adapted from Yoo S.-D., Cho Y.-H. & Sheen J. (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nature Protocols 2, 1565–1572.

Ideally, preps will be done on 3-4 week old *Arabidopsis* plants (before flowering). Choose well-expanded leaves (usually #5 & #7).

Protoplasting 80 leaves (using ~ 65 - 75 mL enzyme solution) from 3 week old plants yielded approx. 1.5 million protoplasts (diluted to 20,000/mL) from which 1.4 - 2.8 ug total RNA (of high quality) could be extracted using TRIzol.

Higher RNA yields were obtained using fewer leaves and more enzyme solution (40-50 leaves of 3 week old plants gave ~4 ug total RNA).

Before start

Before starting experiment, it is considered ideal to keep intended *Arabidopsis* plants for protoplast extraction in the dark overnight. This is so that all the starch is used up, making the isolation procedure easier (less junk in extract).

Prepare the following solutions:
Enzyme Solution: Make solution of 20mM MES (pH 5.7), 0.4M Mannitol and 20mM KCl. Heat solution to 70°C for 3-5min. To this add cellulose R10 (1.5%) and macerozyme R10 (0.4%). Warm solution to 55°C for 10 min (inactivates DNAse and proteases and aids solubilising enzyme). Cool to room temperature and add 10mM Ca Cl_2, 1-5mM ß-mercaptoethanol (optional) and 0.1% BSA. Filter the final enzyme solution through a 0.45um syringe filter into a petri dish (enzyme solution should be made fresh, however, can be stored overnight at 4°C).

W5 Solution: 2mM MES (pH 5.7), 154mM NaCl, 125mM $CaCl_2$ and 5mM KCl.
MMG Solution: 4mM MES (pH5.7), 0.4M Mannitol and 15mM $MgCl_2$.

Materials

D-Mannitol View by Sigma Aldrich

✓ Vacuum system by Contributed by users

✓ KCl by Contributed by users

✓ CaCl2 by Contributed by users

✓ MgCl2 by Contributed by users

NaCl 53014 by Sigma Aldrich

BSA A7906 by Sigma Aldrich

b-mercaptoethanol M3148-25ML by Sigma-aldrich

MES, sodium salt MB0611.SIZE.250g by Bio Basic Inc.

Syringe Filtration - Aqueous Solutions, Sterile, Individual Pack, 0.45UM, 13mm, 10/Pk SFA1345S.SIZE.1 by Bio Basic Inc.

Macerozyme R-10 M8002 by Gold Biotechnology

Razor blades 12-640 by Fisher Scientific

✓ Centrifuge by Contributed by users

✓ Compound Microscope by Contributed by users

✓ Cellulase R10 by Contributed by users

BRAND® counting chamber BLAUBRAND® Neubauer improved New without clips, double ruled BR717805 by Sigma

✓ Round bottom glass centrifuge tubes by Contributed by users

Miracloth 475855 by Merck Millipore

Protocol

Keep plants in dark overnight (the day before)

Step 1.

Before starting experiment, it is considered ideal to keep intended *Arabidopsis* plants for protoplast extraction in the dark overnight. This is so that all the starch is used up, making the isolation procedure easier (less junk in extract).

Use 3-4 week old *Arabidopsis* plants (before flowering). Choose well-expanded leave (usually #5 & #7). Approx. 40 leaves (in 50ml enzyme solution) should give a yield of roughly 1, 500, 000 protoplasts (can extract almost 3ug RNA from this using TRIzol method). This is suitable for routine experiments. For larger scale: Sheen et al. suggests 100 – 150 leaves in 40 – 60ml enzyme solution yielding 10^7.

Prepare buffers (the day before)

Step 2.

Enzyme Solution: Make solution of 20mM MES (pH 5.7), 0.4M Mannitol and 20mM KCl. Heat solution to 70°C for 3-5min. To this add cellulose R10 (1.5%) and macerozyme R10 (0.4%). Warm solution to 55°C for 10 min (inactivates DNAse and proteases and aids solubilising enzyme). Cool to room temperature and add 10mM Ca Cl_2, 1-5mM ß-mercaptoethanol (optional) and 0.1% BSA. Filter the final enzyme solution through a 0.45um syringe filter into a petri dish (enzyme solution should be made fresh, however, can be stored overnight at 4°C).

W5 Solution: 2mM MES (pH 5.7), 154mM NaCl, 125mM $CaCl_2$ and 5mM KCl.

MMG Solution: 4mM MES (pH5.7), 0.4M Mannitol and 15mM $MgCl_2$.

Enzyme digestion of leaf material

Step 3.

Cut 0.5-1mm leaf strips of the leaf using a fresh sharp razor blade (on a nice cutting surface) without tissue crushing at the cutting site (i.e. try not to push down on the tissue with the blade, rather try to “glide” the blade to make the cut).

Enzyme digestion of leaf material

Step 4.

Transfer leaf cuts quickly and gently into enzyme solution and make sure leaf strips are submerged nicely.

Enzyme digestion of leaf material

Step 5.

Vacuum infiltrate 3 x 10 min.

Enzyme digestion of leaf material

Step 6.

Continue digestion, **without shaking,** in the dark for at least 3 hours @ room temperature (enzyme solution should turn from a light brown colour to green after gentle swirling which indicates protoplast release).

Enzyme digestion of leaf material

Step 7.

Check for release of protoplasts in solution under the microscope (after gentle swirling). *Arabidopsis* mesophyll protoplasts are approximately 30 - 50 µm in diameter.

Resuspend protoplasts

Step 8.

Dilute the enzyme/protoplast solution with an equal volume of **W5 solution**.

Resuspend protoplasts

Step 9.

Rinse 2 clean layers of miracloth in **W5.**

Filter the enzyme solution containing the protoplasts with these layers of miracloth.

Resuspend protoplasts

Step 10.

Centrifuge the flow-through at 150 g/rcf for 2 minutes (brake ON/hard stop) in a 30ml-round-bottom glass centrifuge tube.

Remove as much supernatant as possible and re-suspend protoplasts by gentle swirling.

Resuspend protoplasts

Step 11.

Use haemocytometer to calculate the concentration of protoplasts.

Resuspend protoplasts

Step 12.

Re-suspend in **W5 solution** to a concentration of $2x10^5$/mL.

Resuspend protoplasts

Step 13.

Rest protoplasts on ice for at least 30 minutes. Protoplasts should begin to settle to the bottom of the tube by gravity. Remove as much W5 solution as possible without touching the pellet. Re-suspend at $2x10^5$/mL in **MMG solution** kept at room temperature (will need to do another count using haemocytometer before resuspension).