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Arabidopsis leaf protoplasting V.1

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Protocol status: Working
We have used this protocol to successfully measure gene expression in protoplasts under stress conditions.

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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.

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).

Materials

MATERIALS

- D-Mannitol **Merck MilliporeSigma (Sigma-Aldrich)**
- Vacuum system
- KCl
- CaCl2
- MgCl2
- NaCl **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**53014
- BSA **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**A7906
- b-mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**M3148-25ML
- MES, sodium salt **Bio Basic Inc.** **Catalog #**MB0611.SIZE.250g
- Syringe Filtration - Aqueous Solutions, Sterile, Individual Pack, 0.45UM, 13mm, 10/Pk **Bio Basic Inc.** **Catalog #**SFA1345S.SIZE.1
- Macerozyme R-10 **Gold Biotechnology** **Catalog #**M8002
- Razor blades **Fisher Scientific** **Catalog #**12-640
- Centrifuge
- Compound Microscope
- Cellulase R10
- BRAND® counting chamber BLAUBRAND® Neubauer improved New without clips, double ruled **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**BR717805
- Round bottom glass centrifuge tubes
- Miracloth **Merck Millipore (EMD Millipore)** **Catalog #**475855

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 $MgCl_2$.

Keep plants in dark overnight (the day before)

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)

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

Enzyme digestion of leaf material

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).

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

5 Vacuum infiltrate 3 × 10 min.

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).

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

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

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

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

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.

11 Use haemocytometer to calculate the concentration of protoplasts.

12 Re-suspend in **W5 solution** to a concentration of 2×10^5/mL.

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 2×10^5/mL in **MMG solution** kept at room temperature (will need to do another count using haemocytometer before resuspension).