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Transcriptome and morphological analyses in *Selaginella apoda*
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Introduction:
This guide details the germination of *S. apoda* spores, the isolation and storage of plant tissue, and the optimized procedure of transcriptome library preparation from tissue samples. In addition, I present here our protocol for tissue sample preparation for scanning electron microscopy (SEM).

I. Spore germination:

Materials:

Harvested *S. apoda* strobili
Wax paper
Tap water
50 mL beaker
C-fern media petri dishes (4)

1) Place the strobili in a container lined with wax paper. Cover the container such that contaminants may not enter but air can still circulate. Allow material to dry fully, 2 to 4 weeks.
2) After removing the dried vegetative material, carefully pour off the larger megaspores into a suitable container. The smaller microspores will adhere to the wax and will be visible as a fine, reddish dust. Tap to dislodge above a separate container.
3) Recombine micro- and megaspores in an approximately 1:10 mass ratio, respectively, and submerge in tap water in the beaker. Allow to rest for 15 minutes.
4) Shake beaker to disperse the spores in solution then pour approximately equal volumes of water across each of the sterile petri dishes.
5) Seal with surgical tape to maintain humidity within the dish, then place in indirect sunlight. Allow 2+ weeks for germination and 2+ months for the development of adult plants.

II. Tissue harvesting and storage

Materials:

S. apoda adult specimens
Liquid nitrogen
Sterile 1.5 mL epitubes

Equipment:

Dissecting scissors
Plastic test tube rack

1) Submerge the plastic rack fully in liquid nitrogen in a Styrofoam container or ice bucket of suitable size. Place as many clean, labeled 1.5 mL tubes in the rack as individual tissues being collected.
2) Allow liquid to evaporate fully before repeating Step 1 twice for a total of three applications of liquid nitrogen, making sure to bathe fully the inside of the tubes. For the rest of the procedure, do not allow the remaining liquid nitrogen to fully evaporate; replenish as necessary so as to maintain temperature.
3) Collect tissue of interest, using separate scissors for each type. Carefully place in the appropriate tube, adding small volumes of liquid nitrogen as necessary to flush the tissue down the side of the tube.
4) Following collection, store at -80°C.

III. Transcriptome library preparation

Nb: the procedure below has been optimized for tissue samples containing secondary metabolites. Our RNA extraction protocol is lightly modified from the one developed by Cone *et al.* (1986) for tissues with high start content.

i. RNA extraction

Materials:

Plant tissue (<0.1 g)
Liquid nitrogen
Lysis buffer
Chloroform:phenol:isoamyl alcohol extraction solution
Precipitation solution

Equipment:

Spatula (autoclaved)
Mortar and pestle (autoclaved)
Centrifuge

1) Fill the mortar with liquid nitrogen, submerging the pestle in the volume. Allow to fully evaporate, then fill twice more.
2) After cooling spatula, scrape plant tissue into the cooled mortar and cover with a small volume of liquid nitrogen. Grind into a fine powder, adding more liquid nitrogen as necessary to maintain the temperature and wash tissue down the sides of the mortar.
3) Add 200 μL lysis buffer to the mortar, grinding the now-frozen solution into a fine slurry. Using the cooled spatula, scrape into an appropriately-sized tube.
4) Add equal volume chloroform:phenol:isoamyl alcohol (10:4:1) and invert several times to mix. Let sit at room temperature for 5 minutes.
5) Centrifuge at 10,000 rpm for 5 minutes, then carefully remove aqueous phase to a new, clean tube.
6) Repeat steps 4 and 5 for a total of two extractions.
7) Precipitate RNA pellet with 1 mL precipitation solution, allowing tube to sit at room temperature for 5 minutes before centrifugation at 14,000 rpm for 10 minutes. Carefully remove solution without disturbing RNA pellet.
8) Add equal volume 4M $LiCl_2$ solution (1:1 DEPC H_2O) and allow to sit overnight at 4ºC.
9) Recover the RNA by centrifugation at 10,000 rpm for 10 minutes, carefully removing the $LiCl_2$ solution.
10) Repeat step 7 for a total of two precipitations.
11) Wash pellet with 70% EtOH solution then centrifuge at 14,000 rpm for 10 minutes. Carefully remove supernatant and allow pellet to air-dry for 5-10 minutes before resuspending in DEPC-treated water.

ii. mRNA purification

poly-A(+), rRNA(-) mRNA was purified from pooled total RNA samples of minimum 5 μg using 1.8X Dynabeads (Invitrogen) with a slightly modified protocol: all volumes of kit reagents were halved and only one round of purification was performed to minimize loss of RNA.

iii. cDNA library preparation

cDNA libraries were prepared using the ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre). We found that more than the recommended 15 rounds of PCR amplification were necessary to guarantee a sufficient final yield. Additionally, we found that samples of the highest quality were produced by cDNA purification first with the MinElute PCR Purification Kit (Qiagen) and finally with the Agencourt AMPure XP system (Beckman Coulter).

IV. SEM sample preparation

Materials:

Fresh plant tissue
FAA solution
Ethanol

Equipment:

Vacuum infiltrator
Critical point dryer (Denton DCP-1, Denton Vacuum)
Sputter-coater (Hummer 6.2 Sputter System, Anatech)

1) Isolate plant material and immediately store in fresh FAA solution at room temperature.
2) Conduct two rounds of vacuum filtration to 67 kPa for 5 minutes each to perfuse tissue.
3) Move samples through a graded ethanol series at room temperature as follows: 50% EtOH rinse; 70%, 85%, 95%, 100%, 100%, 100% EtOH for 1 hour each. Store in 100% EtOH at 4ºC overnight.
4) Dry samples in a critical point dryer according to the manufacturer's directions.
5) After mounting samples, sputter-coat at 15 mA for 3 minutes or according to the manufacturer's directions.

Solutions

Formalin-acetic acid-alcohol (FAA)
Makes 100 mL. Store at room temperature.

Ethanol (100%)	50 mL
Glacial acetic acid	5 mL
Formaldehyde (40%)	10 mL
dH_2O	to 100 mL

Lysis buffer
Store at room temperature.

0.1 M NaCl
50 mM Tris-HCl (pH 7.4)
50 mM EDTA
2% $NaDodSO_4$
200 µg/mL proteinase K

Precipitation solution
Makes 25 mL. Store at 4ºC.

Ethanol (100%)	24 mL
4M $LiCl_2$	1 mL

References

Cone, K. C., F. A. Burr, and B. Burr. 1986. Molecular analysis of the maize anthocyanin regulatory locus C1. Proceedings of the National Academy of Sciences **83**:9631-9635.