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Title: A Stepwise Guide to the Isolation and Analysis of Leaf Surface and Apoplastic RNA Using Arabidopsis Rosettes

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage?

   No

3. **Filming location:** Will the filming need to take place in multiple locations?

   No (or Maybe)

   The locations may be one floor up or down depending on whether we want to show where we grow the plants

Current Protocol Length
Number of Steps: 25
Number of Shots: 47

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Roger Innes:** We are trying to understand how plants regulate their microbiomes and whether RNAs secreted by plants regulate gene expression in both prokaryotic and eukaryotic microbes that colonize plants.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera *Suggested B roll: Figure 2*

What technologies are currently used to advance research in your field?

1.2. **Lucía Borniego:** We use a combination of high-resolution gel electrophoresis and Illumina-based RNA-seq analyses to assess the RNAs present on leaf surfaces and how they change in response to biotic stress.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera *Suggested B roll: 4.3.1*

What significant findings have you established in your field?

1.3. **Meenu Singh**: We have shown that tRNA and tRNA-derived fragments, or tDRs, are especially abundant in extracellular RNA, likely due to their resistance to degradation. Also, we believe that tDRs likely regulate gene expression in bacteria.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera

What research gap are you addressing with your protocol?

1.4. **Roger Innes:** While recent studies suggest that extracellular RNAs are taken up by fungal and bacterial pathogens, the underlying mechanisms of RNA uptake and functional consequences of this uptake remain largely unexplored.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera

What research questions will your laboratory focus on in the future?

1.5. **Megha Hastantram Sampangi-Ramaiah:** We wish to identify the cellular sources of leaf surface RNA and the biological roles this leaf surface RNA might be playing in the context of plant-microbe interactions.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

~~How do you think publishing with JoVE will enhance the visibility and impact of your research?~~

~~1.6. Meenu Singla-Rastogi, Postdoctoral Research Associate: (authors will present their testimonial statements live)~~

~~1.6.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera~~ NOTE: Not filmed

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

1.7. Meenu Singla-Rastogi, Postdoctoral Research Associate: (authors will present their testimonial statements live)

1.7.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera

Protocol

2. Collection of Leaf Surface RNA

Demonstrator: Meenu Singla-Rastogi

2.1. To begin, take 6- to 7-week-old Arabidopsis plants for each replicate **[1]**. Add Silwet-L77 *(Silwet-L-Seventy-Seven)* to the sterile VIB *(V-I-B)* before starting the isolation of leaf surface wash **[2-TXT]**. Then, using a measuring cylinder, transfer 100 milliliters of the prepared VIB solution into a 500-milliliter spray bottle **[3]**.

2.1.1. WIDE: Talent selecting healthy Arabidopsis plants that are 6 to 7 weeks old.

2.1.2. Talent adding 1 microliter of Silwet-L77 to 100 milliliters of sterile VIB using a micropipette. **TXT: VIB: Vesicle Isolation Buffer; Final concentration: 0.001%**

2.1.3. Talent pouring the VIB solution into a 500-milliliter spray bottle using a measuring cylinder.

2.2. Using fine scissors, carefully detach the Arabidopsis rosettes from their roots **[1]**. After that, use a soft brush to remove any excess soil from the petioles **[2]**.

2.2.1. Talent using fine scissors to cut the rosettes away from the roots. NOTE: 2.2.1, 2.2.2 & 2.3.1 were filmed together

2.2.2. Talent using a soft brush to gently remove soil from the petiole area.

2.3. Place the detached rosettes on a flat tray with the abaxial surface facing upwards **[1]**. Then, spray the abaxial surface using five pumps of the spray bottle filled with VIB and wetting agent **[2]**. Carefully flip the rosettes over and spray the adaxial surface **[3]**.

2.3.1. Talent positioning rosettes on a flat tray with the undersides facing up.

2.3.2. Talent spraying the abaxial surface of the rosettes with five firm pumps. NOTE: 2.3.2 and 2.3.3 were filmed together

2.3.3. Talent flipping the rosettes and spraying the adaxial surface five times.

2.4. Prepare modified syringes with holes at the bottom by wrapping them with parafilm around the neck to suspend the tip above the bottom of the bottle **[1].** To recover the leaf surface wash, gently insert the sprayed rosettes into the needleless 60-milliliter modified syringes, placed inside 250-milliliter centrifuge bottles **[2],** and spray two firm pumps of buffer into the syringe **[3]**. NOTE: The VO is edited for the additional shot

Added shot: Talent sealing the neck of the modified syringe with parafilm to suspend it

in a centrifuge bottle.

2.4.1. Talent placing two sprayed rosettes inside each modified 60 milliliter syringe positioned in a centrifuge bottle.

2.4.2. Talent spraying two firm pumps of buffer into the syringe with plants. NOTE: This shot is modified

2.5. Then, centrifuge the loaded bottles at 100 *g* for 10 minutes at 4 degrees Celsius **[1]**.

2.5.1. Talent placing the centrifuge bottles with syringes into the rotor and starting the centrifuge.

2.6. Now, filter the recovered leaf surface wash through a 0.22-micrometer syringe filter **[1]** and collect the filtrate in a 50-milliliter centrifuge tube kept on ice **[2]**.

2.6.1. Talent attaching a 0.22 micrometer syringe filter to a syringe. NOTE: 2.6.1 & 2.6.2 were filmed together

2.6.2. Talent collecting the filtrate into a 50 milliliter centrifuge tube placed in an ice box.

2.7. For the leaf surface swab method, spray the detached rosettes with the VIB and wetting agent solution in the same manner as described in the previous step **[1]**.

2.7.1. Talent spraying detached rosettes with VIB and wetting agent on both sides using the same procedure. NOTE: 2.7.1, 2.8.1 & 2.8.2 were filmed together

2.8. To recover the leaf surface swab, use sterile cotton-tipped sticks to swab both the adaxial and abaxial surfaces of the rosettes **[1]**. Then, press the soaked tips against the inner wall of a 15-milliliter centrifuge tube to extract the liquid **[2]**.

2.8.1. Talent swabbing the leaf surfaces thoroughly using sterile cotton-tipped sticks.

2.8.2. Talent pressing the wet cotton tips against the inside of a 15 milliliter centrifuge tube.

2.9. Filter the extracted leaf surface swab sample through a 0.22-micrometer syringe filter into a 2-milliliter tube placed on ice **[1]**.

2.9.1. Talent filtering the liquid using a 0.22 micrometer syringe filter into a chilled 2 milliliter centrifuge tube.

3. Collection of Apoplastic Wash Fluid (AWF)

3.1. Place the same set of plants previously used for leaf surface wash or swab isolation into a clean beaker **[1]** and gently rinse them twice with distilled water **[2]**.

3.1.1. Talent placing Arabidopsis plants into a beaker. NOTE: 3.1.1 & 3.1.2 were filmed together. The next clip is the second shot of this step, at the sink

3.1.2. Talent pouring distilled water over the plants and decanting the water to perform two rinses.

3.2. To vacuum infiltrate the rosettes with VIB, carefully place the rinsed rosettes into a French press coffee maker containing 300 to 500 milliliters of VIB **[1]**. Secure the lid and gently lower the plunger until the rosettes are fully submerged **[2]**. Insert the French press into a vacuum chamber and apply a vacuum for 20 seconds using a vacuum pump until the plants are well infiltrated **[3]**.

3.2.1. Talent loading the French press with rosettes and 300 to 500 milliliters of VIB. NOTE: 3.2.1 & 3.2.2 were filmed together

3.2.2. Talent placing the lid on the press and lowering the plunger slowly to submerge the rosettes.

3.2.3. Talent placing the French press inside a vacuum chamber and operating the vacuum pump.

3.3. After releasing the vacuum, remove the French press from the chamber **[1],** and take off the lid **[2]**. Pour the VIB into a 500-milliliter plastic beaker **[3]** and remove the rosettes. Then, gently shake the rosettes and brush the leaves across a paper towel to remove excess buffer **[4]**.

3.3.1. Talent lifting the French press out of the chamber,

3.3.2. Talent removing the lid of the French press. NOTE: 3.3.2 & 3.3.3 were filmed together

3.3.3. Talent pouring the liquid into a separate beaker~~. and removing the rosettes.~~ NOTE: This part is filmed in the next shot

3.3.4. Talent removing the rossettes, and shaking and brushing them across paper towels to remove excess liquid. NOTE: removing the rosettes from 3.3.3 were filmed in this step

3.4. Now, using blotting paper, gently blot the leaf surfaces to remove any remaining buffer **[1]**.

3.4.1. Talent dabbing the rosettes gently with blotting paper to remove residual buffer.

3.5. To collect the apoplastic wash fluid, insert the VIB-infiltrated rosettes into 60-milliliter

needleless syringes placed inside 250-milliliter centrifuge bottles as done previously **[1]**. Centrifuge the bottles at 600 *g* for 30 minutes at 4 degrees Celsius with slow acceleration **[2]**.

3.5.1. Talent loading rosettes into modified syringes placed inside centrifuge bottles.

3.5.2. Talent placing the bottles into a centrifuge and running at 600 g for 30 minutes at 4 degrees Celsius.

3.6. Then, filter the pooled apoplastic wash fluid through a 0.22-micrometer syringe filter in a 2-milliliter tube placed on ice **[1-TXT]**.

3.6.1. Talent attaching a 0.22 micrometer syringe filter and filtering the AWF into a new tube. **TXT: Record plant fresh weight post-isolation to normalize RNA/sample**

4. RNA Gel Electrophoresis for Quantification and Comparison

Demonstrator: Lucía Borniego

4.1. Prepare or purchase a mini gel containing either 10 or 15 percent polyacrylamide and 7 molar urea in Tris-Boric Acid EDTA buffer using an Acrylamide and Bis solution **[1-TXT]**.

4.1.1. Shot of the talent with the prepared gel. **TXT: 40% Acrylamide/Bis Solution (37.5:1)**

4.2. Then, mix the RNA samples with 2 times denaturing loading buffer **[1]**. Heat the mixture at 65 degrees Celsius for 5 minutes **[2]** and immediately place it on ice **[3]**.

4.2.1. Talent adding 2x loading buffer to RNA samples and pipetting to mix.

4.2.2. Talent incubating the RNA mixture in a heat block at 65 degrees Celsius.

4.2.3. Talent transferring tubes to ice.

4.3. Now, resolve the RNA samples in 0.5 times TBE running buffer at room temperature by applying 220 volts for approximately 1 hour and 15 minutes **[1-TXT]**.

4.3.1. Talent loading RNA samples into the gel wells and running the gel at 220 volts. **TXT: Use 1:1 mix of low-range and 14–30 nt ssRNA ladders as size standards**

4.4. Then, stain the gel with SYBR *(Syber)* Gold nucleic acid gel stain in 0.5 times TBE for 10 minutes **[1]**, rinse the gel twice with distilled water **[2]**, and image it using an imaging system **[2]**.

4.4.1. Talent immersing the gel in SYBR Gold stain solution.

4.4.2. Talent washing the stained gel twice with distilled water.

4.4.3. Talent placing the gel on the imaging system platform and capturing the fluorescence image.

4.5. To quantify the resolved RNA bands, use the gel analysis method from the ImageJ documentation under the Analyze and Gel Analysis menu **[1-TXT]**.

4.5.1. Open ImageJ software and show navigation to Analyze > Gel Analysis. **TXT: Compare band patterns to 100 ng/µL cytoplasmic RNA control**

Videographer: Please film the computer screen for this shot

5. RNA Quantification Using a Microtiter Plate-Based Method

Demonstrator: Lucía Borniego

5.1. Prepare RNA standards for two technical replicates by diluting cytoplasmic lysis RNA in RNase- and DNase-free water **[1-TXT]**.

5.1.1. Talent pipetting cytoplasmic RNA into RNase/DNase-free water to create standard dilutions. **TXT: Final RNA concentration: 0, 6.75, 12.5, 25, 50, 100, 200 ng/µL**

5.2. To prepare 1.5 times SYBR *(Syber)* Gold nucleic acid gel stain, dissolve it in 500 millimolar Tris-HCl buffer at pH 8 **[1]**.

5.2.1. Talent preparing 1.5x SYBR Gold stain solution in a beaker using 500 millimolar Tris-HCl buffer.

5.3. For the microplate setup, add 99 microliters of distilled water to each well **[1]**. Next, add 1 microliter of RNA to each well, using the same volume for both RNA samples and standards **[2]**. Finally, add 50 microliters of SYBR Gold solution to each well **[3]**.

5.3.1. Talent pipetting 99 microliters of distilled water into all wells of a black-bottom microplate.

5.3.2. Talent adding 1 microliter of RNA sample or standard to the respective wells.

5.3.3. Talent adding 50 microliters of 1.5x SYBR Gold stain to each well.

5.4. To set the microplate reader parameters, choose either Linear or Orbital shaking for 3 seconds **[1]**. Set the microplate reader to Fluorescence Endpoint mode with an

excitation wavelength of 496 nanometers and an emission wavelength of 540 nanometers **[2].**

5.4.1. Microplate reader interface showing the setting of shaking mode to Linear or Orbital for 0:03 (MM:SS).

5.4.2. Microplate reader display showing selection of Fluorescence Endpoint mode and input of excitation at 496 nanometers and emission at 540 nanometers.

5.5. Construct a calibration line using the fluorescence intensity values from the RNA standards **[1-TXT]**.

5.5.1. Graphing software or spreadsheet showing fluorescence values plotted against RNA standard concentrations. **TXT: Use the calibration equation to calculate the RNA concentrations of the samples**

Videographer: Please film the computer screen for this shot

Results

6. Results

6.1. Denaturing RNA gel analysis revealed that both leaf surface wash and apoplastic wash fluid samples contain a broad range of RNA sizes, including both long and small RNA species **[1].** The total RNA amounts isolated from the apoplastic wash fluid and leaf surface wash were similar when normalized to plant fresh weight **[2].**

6.1.1. LAB MEDIA: Figure 3. *Video editor: Highlight the AWF and LSW lanes*

6.1.2. LAB MEDIA: Figure 3. Video editor: Highlight the numerical values below the AWF and LSW lanes, showing similar values (0.89 and 0.79)

6.2. The RNA profiles of leaf surface wash, apoplastic wash, and cell lysate differ markedly, confirming minimal intracellular RNA contamination **[1]** and showing that apoplastic RNA is highly processed, with diffuse low-molecular-weight bands **[2].**

6.2.1. LAB MEDIA: Figure 3. *Video editor: Highlight the distinct band patterns in AWF and LSW compared to CL*

6.2.2. LAB MEDIA: Figure 3. *Video editor: Zoom in on the lower region of the AWF lane, where diffuse smearing is prominent below 80 nucleotides*

6.3. Addition of 0.001% Silwet L-77 to the vesicle isolation buffer nearly doubled the RNA yield from leaf surfaces compared to the buffer without the wetting agent **[1],** but increasing this concentration to 0.01% did not further enhance RNA recovery **[2].**

6.3.1. LAB MEDIA: Figure 4. *Video editor: Highlight the band between the VIB and "VIB + 0.001% Silwet L-77" lanes*

6.3.2. LAB MEDIA: Figure 4. *Video editor: Highlight the similar band intensity between "VIB + 0.001% Silwet L-77" and "VIB + 0.01% Silwet L-77" lanes*

6.4. A second wash of the same leaves recovered approximately 63% of the RNA obtained in the first wash**,** indicating ongoing RNA secretion onto the leaf surface **[1].**

6.4.1. LAB MEDIA: Figure 4. *Video editor: Highlight L1 and L2 lanes, showing substantial RNA present in the second wash*

6.5. RNA quantification using fluorescence-based microtiter plate assays demonstrated high linearity with RNA concentration for both SYBR Gold and Ribo488 detection methods

[1].

6.5.1. LAB MEDIA: Figure 5. *Video editor: Highlight the linear trend on the left and right plot*

Words & Pronunciations

1. **Arabidopsis**
   Pronunciation link: https://www.howtopronounce.com/arabidopsis (How To Pronounce)
   IPA: /ˌær.ə.bɪˈdɒp.sɪs/
   Phonetic Spelling: AIR-uh-bi-DOP-sis
2. **Silwet-L77**
   (No standard dictionary entry for "Silwet-L77"; chemical/trade name)
   IPA: /sɪlˈwɛt ɛl ˈsɛv.ən sɛv.ən/
   Phonetic Spelling: sil-WET el SEV-un SEV-un
3. **Vesicle** (as in *Vesicle Isolation Buffer*)
   Pronunciation link: https://www.merriam-webster.com/dictionary/vesicle (How To Pronounce)
   IPA: /ˈvɛs.ɪ.kəl/
   Phonetic Spelling: VES-i-kul
4. **Petiole**
   Pronunciation link: https://www.merriam-webster.com/dictionary/petiole (Merriam-Webster)
   IPA: /ˈpiː.ti.oʊl/
   Phonetic Spelling: PEE-tee-ohl
5. **Apoplast**
   Pronunciation link: https://www.howtopronounce.com/apoplast (How To Pronounce)
   IPA: /ˈæ.pə.plæst/
   Phonetic Spelling: A-puh-plast
6. **Centrifuge**
   Pronunciation link: https://www.merriam-webster.com/dictionary/centrifuge (Wikipedia)
   IPA: /ˈsɛn.trə.fjuːʒ/
   Phonetic Spelling: SEN-tru-fyo͞ozh
7. **Blotting** (as in *blotting paper*)
   (Common word; you may find it in any dictionary)

IPA: /ˈblɒt.ɪŋ/
Phonetic Spelling: BLOT-ing

8. **Electrophoresis** (if applicable in your context, e.g. RNA gel electrophoresis)
Pronunciation link: https://www.merriam-webster.com/dictionary/electrophoresis (Wikipedia)
IPA: /ɪˌlɛk.troʊ.fəˈriː.sɪs/
Phonetic Spelling: i-LEK-troh-fuh-REE-sis