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Title: Efficient Regeneration-Based *Agrobacterium*-Mediated Transformation of an Asexual Amphibious Brassicaceae Species, *Rorippa aquatica*

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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? **Yes**

If **Yes**, how far apart are the locations? Roughly 30 feet (two separate labs on the same floor, relatively close to one another)

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No.**

Current Protocol Length

Number of Steps: 17

Number of Shots: 41

Introduction

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

INTRODUCTION:

~~What research gap are you addressing with your protocol?~~

- 1.1. **Page Gonzalez:** We address the lack of a simple, scalable *Agrobacterium* transformation pipeline for asexual plants, enabling molecular analyses of gene functions.

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

~~What are the most recent developments in your field of research?~~

- 1.2. **Page Gonzalez:** Recently, whole-genome and physiological/gene-expression studies introduced *Rorippa aquatica* as a model for heterophyly. It is useful in studying the molecular basis of land–water adaptation.

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

CONCLUSION:

~~What advantage does your protocol offer compared to other techniques?~~

- 1.3. **Page Gonzalez:** Our protocol bypasses sterile tissue culture steps and provides a transformation-regeneration pipeline for asexual, vegetatively propagated plants lacking seeds or flowers.

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

~~What significant findings have you established in your field?~~

- 1.4. **Page Gonzalez:** Our studies on ethylene pathway activation in *Rorippa aquatica* under submergence provide key insights into terrestrial plant submergence responses.

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

~~What new scientific questions have your results paved the way for?~~

- 1.5. **Page Gonzalez:** Our method opens the door to investigating how light- and hormone pathways enable this amphibious plant to metamorphose under water.
- 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.

Protocol

2. Transformation of *Rorippa aquatica* Leaf Cuttings

Demonstrator: Page Gonzalez

- 2.1. To begin, select 2-month-old stock plants that exhibit healthy leaves with entire margins [1].
 - 2.1.1. WIDE: Talent examining plants placed on a bench.
- 2.2. Prepare a plant propagation tray measuring 20 by 30 centimeters with 2 layers of autoclaved paper towels [1]. Fold the paper towels neatly [2] and saturate them with sterile water until a thin layer of water remains on the surface [3].
 - 2.2.1. Talent placing autoclaved paper towels into the propagation tray.
 - 2.2.2. Talent folding the paper towels inside the tray.
 - 2.2.3. Talent pouring sterile water to saturate the paper towels, showing a thin water layer on top.
- 2.3. Ensure that the laboratory bench is equipped with 100 to 500 milliliters of double-distilled water [1], sterile containers suitable for the anticipated volume of resuspended inoculum [2], a micropipette, and appropriate pipette tips [3].
 - 2.3.1. Talent placing a container of double distilled water on the bench.
 - 2.3.2. Talent arranging sterile containers on the bench.
 - 2.3.3. Talent placing a micropipette and tips on the bench.
- 2.4. Now, quickly excise 10 leaves at the petiole base from the selected stock plants using scissors [1] and place them into a Petri dish containing sterile water [2].
 - 2.4.1. Talent excising leaves at the petiole base from stock plants.
 - 2.4.2. Talent placing leaf explants into a Petri dish filled with sterile water.
- 2.5. After 2.5 hours, pipette 50 microliters of surfactant per liter of resuspension solution to achieve a 0.005 percent surfactant concentration [1]. Gently swirl the flask to mix the solution [2] and ensure a prepared propagation tray is accessible for explant placement after inoculation [3].

- 2.5.1. Talent pipetting surfactant into the resuspension solution.
- 2.5.2. Talent gently swirling the flask to mix the solution.
- 2.5.3. Talent positioning the prepared propagation tray nearby.

- 2.6. Now, place a leaf with the abaxial side facing upward [1]. Using a scalpel, cut perpendicularly along the leaf's midrib into pieces measuring 1 centimeter in length [2] and place them in a 50-milliliter tube suitable for use in a rotating tube mixer to serve as inoculation explants [3].
 - 2.6.1. Talent positioning a leaf abaxial side up on a cutting surface.
 - 2.6.2. Talent cutting the leaf perpendicularly along the midrib into uniform sections with a scalpel.
 - 2.6.3. Talent placing freshly cut leaf pieces into a 50 milliliter tube.

- 2.7. Pour the *Agrobacterium* inoculum into the tube at a volume sufficient to fully saturate all explant material [1]. Place the tube onto a rotary mixer [2] and agitate at room temperature for 10 minutes to perform inoculation [3].
 - 2.7.1. Talent pouring inoculum into the tube containing explants.
 - 2.7.2. Talent placing the tube onto the rotary mixer.
 - 2.7.3. Talent starting the rotary mixer set to room temperature.

- 2.8. Then, using forceps, remove all inoculated leaf explants from the solution [1] and directly place them in the prepared propagation tray [2].
 - 2.8.1. Talent removing inoculated leaves from the solution with forceps.
 - 2.8.2. Talent placing inoculated explants into the propagation tray.

- 2.9. Separate and spread the soaked explants evenly [1] so that they are partially immersed but not fully submerged in the thin layer of water atop the saturated paper towels [2].
 - 2.9.1. Talent separating and spreading explants across the tray.
 - 2.9.2. Close-up showing explants partially immersed in water on the paper towels.

- 2.10. Next, seal the propagation tray with plastic wrap [1]. Place the trays in a dark environment and incubate at an ambient temperature of 22 degrees Celsius for 3 days [2].

2.10.1. Talent sealing the tray with plastic wrap.

2.10.2. Talent placing the sealed tray into a dark incubator.

2.11. After the completion of the dark incubation period, confirm that the trays remain sealed with transparent plastic wrap [1]. Then, add double-distilled water to the trays as needed to maintain moisture levels [2].

2.11.1. Talent inspecting sealed trays.

2.11.2. Talent adding double distilled water to maintain moisture.

2.12. Transfer the inoculation trays to a growth chamber and maintain them under vegetative propagation conditions [1].

2.12.1. Talent moving trays into a growth chamber.

3. Regeneration of Rosette Plantlets and Establishment of the R1-T1 Generation

3.1. Visually inspect regenerated rosette plantlets for transplant suitability [1]. Identify ideal regenerants by confirming the presence of an intact shoot with multiple leaves [2] and roots measuring at least 2 to 3 millimeters in length [3].

3.1.1. Talent examining multiple regenerated rosette plantlets on a bench.

3.1.2. Close-up of pointing to a plantlet showing an intact shoot with multiple leaves.

3.1.3. Close-up of pointing roots measuring at least 2 to 3 millimeters in length.

3.2. Then, excise intact transgenic rosette plantlets using forceps [1]. Transplant the excised plantlets under the same conditions used for wild-type clonal regenerants [2].

3.2.1. Talent excising an intact transgenic rosette plantlet.

3.2.2. Talent transplanting the plantlet into the growth medium.

3.3. Excise a rosette plantlet while carefully avoiding contact with any transgenic leaves [1]. Trim excess foliage immediately surrounding the transgenic leaf [2] and transplant a single plantlet per pot [3].

3.3.1. Talent excising a rosette plantlet while avoiding the transgenic leaves.

3.3.2. Talent trimming excess surrounding foliage.

- 3.3.3. Talent transplanting one plantlet into an individual pot. *Videographer: Please obtain multiple reusable shots for this step. It will be used again*

- 3.4. After fertilizing each transplanted plant, water them thoroughly [1], mist the foliage [2], and place the plants under the appropriate conditions [3].
 - 3.4.1. Talent watering the plant.
 - 3.4.2. Talent misting the plant leaves.
 - 3.4.3. Talent placing the plant into the designated holding area.

- 3.5. Finally, return explants containing transformants that lack shoots and roots, or appear too small for transplantation to the propagation tray [1]. Continue screening these explants [2] and transplant them once they have grown sufficiently [3].
 - 3.5.1. Talent placing undersized or undeveloped explants back into the propagation tray.
 - 3.5.2. Shot of explants maintained in the tray in the designated chamber.
 - 3.5.3. *Reuse 3.3.3.*

Results

4. Results

- 4.1. The majority of one-month-old R1-T1 seedlings exhibited chimeric RUBY (*ruby*) expression patterns, while a small number showed entirely RUBY-expressing leaves [1].
 - 4.1.1. LAB MEDIA: Figure 3A. Video editor: Show the seedling with leaves that are partly red and partly green.
- 4.2. Maintained mature, six-month-old R1-T1 seedlings expressed chimeric RUBY expression [1].
 - 4.2.1. *Added shot: Two mature R1-T1 seedlings.*
- 4.3. Chimeric RUBY-pigmented leaf sectors excised from R1-T1 seedlings were capable of asexual propagation [1].
 - 4.3.1. LAB MEDIA: Figure 3B.
- 4.4. Excised chimeric leaf pieces regenerated into either fully RUBY-expressing or chimeric R1-T1 rosette plantlets within 2 to 4 weeks after excision [1], with visible root formation during regeneration [2].
 - 4.4.1. LAB MEDIA: Figure 3C.
 - 4.4.2. LAB MEDIA: Figure 3C. Video editor: Zoom in on the thin pink root indicated by the arrow.
- 4.5. After one month, most R2-T1 seedlings displayed predominantly RUBY leaves [1].
 - 4.5.1. LAB MEDIA: Figure 3D,E

Pronunciation Guide:

Agrobacterium

Pronunciation link: <https://www.merriam-webster.com/dictionary/Agrobacterium>

IPA: /,ægroubæk'tɪriəm/

Phonetic Spelling: ag-roh-bak-teer-ee-uhm

Transformation

Pronunciation link: <https://www.merriam-webster.com/dictionary/transformation>

IPA: /,trænsfər'meɪʃən/, /,trænz-/ , /,trɑːns-/

Phonetic Spelling: trans-fer-may-shuhn

Asexual

Pronunciation link: <https://www.merriam-webster.com/dictionary/asexual>

IPA: /eɪ'sekʃuəl/, /,eɪ'sekʃwəl/

Phonetic Spelling: ay-sek-shoo-uhl

Amphibious

Pronunciation link: <https://www.merriam-webster.com/dictionary/amphibious>

IPA: /æm'fɪbiəs/

Phonetic Spelling: am-fib-ee-uhs

Brassicaceae

Pronunciation link: <https://www.howtopronounce.com/brassicaceae>

IPA: /,bræsɪ'keɪsiː.iː/

Phonetic Spelling: bras-ih-kay-see-ee

Rorippa

Pronunciation link: <https://www.howtopronounce.com/rorippa>

IPA: /rɒ:'rɪpə/

Phonetic Spelling: roh-rip-uh

aquatica

Pronunciation link: <https://www.howtopronounce.com/aquatica>

IPA: /ə'kwætɪkə/

Phonetic Spelling: uh-kwat-ih-kuh

Heterophylly

Pronunciation link: <https://www.merriam-webster.com/dictionary/heterophylly>

IPA: /,hetərə'fɪli/

Phonetic Spelling: het-uh-roh-fil-ee

Ethylene

Pronunciation link: <https://www.merriam-webster.com/dictionary/ethylene>

IPA: /'eθə'liːn/

Phonetic Spelling: eth-uh-leen

Submergence

Pronunciation link: <https://www.merriam-webster.com/dictionary/submergence>

IPA: /səb'mɜːdʒəns/

Phonetic Spelling: suhb-mur-juhns

Petiole

Pronunciation link: <https://www.merriam-webster.com/dictionary/petiole>

IPA: /'peti, oʊl/

Phonetic Spelling: pet·ee·ohl

Microliters

Pronunciation link: <https://www.merriam-webster.com/dictionary/microliter>

IPA: /'maɪkrə, li:tər/

Phonetic Spelling: my·kruh·lee·ter

Surfactant

Pronunciation link: <https://www.merriam-webster.com/dictionary/surfactant>

IPA: /sər'fæktənt/

Phonetic Spelling: sur·fak·tuhnt

Abaxial

Pronunciation link: <https://www.merriam-webster.com/dictionary/abaxial>

IPA: /æb'æk.si.əl/

Phonetic Spelling: ab·ak·see·uhl

Inoculum

Pronunciation link: <https://www.merriam-webster.com/dictionary/inoculum>

IPA: /ɪ'nɑ:kjələm/

Phonetic Spelling: ih·nok·yuh·luhm

Rosette

Pronunciation link: <https://www.merriam-webster.com/dictionary/rosette>

IPA: /roʊ'zet/

Phonetic Spelling: roh·zet

Transgenic

Pronunciation link: <https://www.merriam-webster.com/dictionary/transgenic>

IPA: /trænz'dʒenɪk/, /træns-/

Phonetic Spelling: tranz·jen·ik

Chimeric

Pronunciation link: <https://www.merriam-webster.com/dictionary/chimeric>

IPA: /kaɪ'merɪk/, /kɪ-/

Phonetic Spelling: ky·mer·ik

Regenerants

Pronunciation link: <https://www.merriam-webster.com/dictionary/regenerant>

IPA: /rɪ'dʒenərənt/

Phonetic Spelling: ree·jen·uh·ruhnt