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Title: Wide-Field, Real-Time Imaging of Local and Systemic Wound Signals in Arabidopsis

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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? **Yes**

   If **Yes**, can you record movies/images using your own microscope camera? **Yes**

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

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 11
Number of Shots: 17

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Masatsugu Toyota:** This protocol allows plant-wide, real-time imaging of the activity of the plant systemic signaling system through monitoring the dynamics of Ca^{2+} and apoplastic glutamate in response to wounding.

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

1.2. **Masatsugu Toyota:** This plant-wide real-time imaging method provides a robust tool to understand the dynamics of rapid and long-distance signals in plants, combining high spatiotemporal resolution and ease of use.

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

OPTIONAL:

1.3. **Masatsugu Toyota:** This protocol offers the potential to provide new insights into the spatial and temporal characteristics of systemic Ca^{2+} signaling in both biotic and abiotic stress responses in other plant species.

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

Introduction of Demonstrator on Camera

1.4. **Masatsugu Toyota:** Demonstrating the procedure will be Takuya Uemura, a postdoctoral fellow from my laboratory.

1.4.1. INTERVIEW: Author saying the above.

1.4.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Protocol

2. Microscope setting and conducting real-time imaging

2.1. Begin by turning on the motorized fluorescence stereomicroscope equipped with a 1x objective lens and a sCMOS *(pronounce 'S-C-moss')* camera **[1]**.

2.1.1. WIDE: Establishing shot of talent turning on the microscope.

2.2. Configure the device settings to irradiate with excitation light centered on 470 nanometers, selected using a filter that transmits light between 450 and 490 nanometers. Acquire emission light using a filter that transmits between 510 and 560 nanometers **[1]**.

2.2.1. Talent configuring the irradiation and excitation.

2.3. Remove the lid from the dish containing the plant and place it under the objective lens **[1]**. Check the fluorescence signal from the plant, then wait for approximately 30 minutes in the dark until the plants are adapted to the new environmental conditions **[2]**.

2.3.1. Talent placing the dish on the stage.

2.3.2. Talent checking the fluorescence signal.

2.4. Adjust the focus and magnification to see the whole plant in the field of view **[1]**, then set the acquisition parameters to detect the fluorescence signals using the microscope's imaging software. Set the recording time to 11 minutes **[2-TXT]**.

2.4.1. Talent adjusting focus.

2.4.2. SCREEN: 62114_screenshot_1. 0:00 – 0:40. **TEXT: See text manuscript for suggested imaging settings**

2.5. Image for 5 minutes prior to starting the experiment to acclimate the plant to the blue light irradiation from the microscope, then start recording. To determine the average baseline fluorescence, record at least 10 frames before wounding or glutamate application **[1]**.

2.5.1. SCREEN: 62114_screenshot_1. 0:41 – 1:41. *Video Editor: Speed up after 0:50.*

2.6. For real-time imaging of wound-induced cytosolic calcium ion and apoplastic glutamate concentration changes, cut the petiole or the middle region of leaf L1 with scissors **[1]**. *Videographer: This step is important!*

2.6.1. Talent cutting the petiole of the leaf.

2.7. For real-time imaging of glutamate-triggered cytosolic calcium changes, cut approximately 1-millimeter from the tip of leaf L1 across the main vein with scissors

[1]. After at least 20 minutes, apply 10 microliters of 100 millimolar glutamate to the leaf's cut surface **[2]**. *Videographer: This step is difficult and important!*

2.7.1. Talent cutting across the main vein of the leaf.

2.7.2. Talent applying glutamate to the cut.

3. Data analysis

3.1. For fluorescence intensity analysis over time, define a region of interest at the place where fluorescence intensity is to be analyzed. Define 2 ROIs for the velocity calculation of the calcium wave **[1]**.

3.1.1. SCREEN: 62114_screenshot_2. 0: 00 – 0:05.

3.2. In the imaging software, click on **Time Measurement**, **Define**, and **Circle**. Measure the distance between the 2 regions by clicking on **Annotations and Measurement**, **Length**, and **Simple Line [1]**.

3.2.1. SCREEN: 62114_screenshot_2. 0: 05 – 0:40. *Video Editor: Speed up after 0:25 – 0:35.*

3.3. Measure the raw fluorescence values in each ROI over time by clicking on **Measure**, then export the raw data to spreadsheet software to convert the fluorescence signal into numbers at each time point **[1]**.

3.3.1. SCREEN: 62114_screenshot_2. 0: 41 – 1:07.

3.4. Determine the baseline fluorescence value, which is defined as F_0, by calculating the average of F over the first 10 frames in the recorded data **[1]**. Then, normalize the F data as described in the text manuscript **[2-TXT]**.

3.4.1. SCREEN: 62114_screenshot_3. 0:00 – 0:20.

3.4.2. SCREEN: 62114_screenshot_3. 0:21 – 1:01. **TEXT: $\Delta F / F = (F - F_0) / F_0$; ΔF = time-dependent change in fluorescence** *Video Editor: Zoom in on formula being entered and speed up until 0:50.*

3.5. For calcium velocity wave analysis, define a significant signal rise point above the pre-stimulated values as representing detection of a calcium increase in each ROI. Calculate the time difference of the calcium increase between the 2 ROIs and the distance between them to determine the velocities of any calcium wave **[1]**.

3.5.1. SCREEN: 62114_screenshot_3. 1:02 – 2:30. *Video Editor: Speed up as much as possible, but make sure the actions are still discernable.*

Results

4. Results: Ca^{2+} transmission and elevation of apoplastic glutamate in response to mechanical wounding

4.1. Propagation of a wound triggered change in the concentrations of the cytosolic calcium ion and apoplastic glutamate is shown here **[1]**. Cutting the petiole of a leaf in plants expressing GCaMP3 *(pronounce 'G-camp-3')* led to a significant increase in calcium that was induced locally and then spread through the vasculature **[2]**.

4.1.1. LAB MEDIA: Figure 3.

4.1.2. LAB MEDIA: Movie S1.mp4.

4.2. The signal was rapidly propagated to neighboring leaves within a few minutes **[1]**. Upon cutting a leaf in plants expressing basic-chitinase-eye-glue-sniffer, a rapid apoplastic glutamate increase was observed around the cut region **[2]**. Within a few minutes, the signal also propagated through the vasculature **[3]**.

4.2.1. LAB MEDIA: Movie S2.mp4.

4.2.2. LAB MEDIA: Figure 4. *Video Editor: Emphasize the 2 s image.*

4.2.3. LAB MEDIA: Figure 4. *Video Editor: Emphasize the 160 s image.*

4.3. For real-time imaging of calcium signal propagation triggered by the application of glutamate, the edge of a leaf in plants expressing GCaMP3 was cut **[1]**. This caused a local cytosolic calcium ion concentration increase **[2]** but the signal disappeared within a few minutes **[3]**.

4.3.1. LAB MEDIA: Movie S3.mp4.

4.3.2. LAB MEDIA: Figure 5. *Video Editor: Emphasize the 40 s image.*

4.3.3. LAB MEDIA: Figure 5. *Video Editor: Emphasize the 124 s image.*

4.4. After approximately 10 minutes, glutamate was applied to the cut surface, causing a rapid, local increase in the concentration of cytosolic calcium and then propagation of this signal to distal leaves **[1]**.

4.4.1. LAB MEDIA: Movie S4.mp4.

4.5. To measure the changes in cytosolic calcium concentration induced by wounding in the systemic leaf, the time course change of GCaMP3 signal intensity was measured **[1]** in 2 regions of interest **[2]**.

4.5.1. LAB MEDIA: Figure 6 A and B.

4.5.2. LAB MEDIA: Figure 6 A and B. *Video Editor: Emphasize the 1 and 2 in A.*

4.6. Apoplastic glutamate concentration changes in response to mechanical damage were measured as well **[1]**. The glutamate signature exhibited a single peak at approximately 100 seconds after wounding **[2]**.

4.6.1. LAB MEDIA: Figure 7 A.

4.6.2. LAB MEDIA: Figure 7 B.

Conclusion

5. Conclusion Interview Statements

5.1. **Takuya Uemura:** This experiment should be conducted under temperature- and humidity-controlled conditions because Ca^{2+} signals are elicited by changes in these environmental conditions.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.2.*

5.2. **Takuya Uemura:** This protocol offers the potential to provide insights into the molecular mechanisms underlying long-distance wound signaling through using mutants that are defective in putative elements of the rapid signaling system.

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