Submission ID #: 61489
Scriptwriter Name: Bridget Colvin
Project Page Link: https://www.jove.com/account/file-uploader?src=18754193

Title: Phosphoproteomic Strategy for Profiling Osmotic Stress Signaling in *Arabidopsis*

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Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **N**

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **55**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Chuan-Chih Hsu**: This strategy is a powerful tool for comprehensively studying the global changes of plant phosphoproteomes in response to biotic or abiotic stresses **[1]**.

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

REQUIRED:

1.2. **Chuan-Chih Hsu**: This technique allows in-depth phosphoproteomics analysis in an easy-to-use and high throughput manner **[1]**.

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

Protocol

2. Sample Preparation

2.1. To prepare the samples, freeze 1 gram each of control and stress-treated seedling samples wrapped in aluminum foil in liquid nitrogen **[1]**.

2.1.1. WIDE: Talent dipping foil in LN2

2.2. Use a mortar and pestle to grind the frozen treated seedling sample into a fine powder in a mortar filled with liquid nitrogen **[1 TXT]**.

2.2.1. Seedling being ground **TEXT: Repeat control sample preparation in same manner**

2.3. Use the pestle to mix 1 milliliter of lysis buffer with the sample **[1 TXT]** and transfer the resulting plant lysate to a 1.5-milliliter tube **[2]**.

2.3.1. Sample being mixed, with buffer container visible in frame **TEXT: See text for all buffer and solution preparation details**
2.3.2. Talent adding lysate to tube

2.4. Heat the sample at 95 degrees Celsius for 5 minutes **[1]** followed by a 10-minute incubation on ice **[2]** before sonicating the sample on ice three times for 10 seconds per sonication **[3 TXT]**.

2.4.1. Talent placing tube at 95 °C
2.4.2. Talent placing tube on ice
2.4.3. Tube being sonicated on ice **TEXT: Rest 10 s between sonications**

2.5. After the last sonication, collect the sample by centrifugation **[1 TXT]** and aliquot 150 microliters of lysate into a new 1.5-milliliter tube **[2]**.

2.5.1. Talent placing tube(s) into centrifuge **TEXT: 10 min, 16,000 x g, RT**
2.5.2. Talent adding lysate to tube

2.6. Add 600 microliters of 100% methanol to the tube **[1]** and vortex the sample before a second centrifugation **[2]**.

2.6.1. Talent adding methanol to tube, with methanol container visible in frame
2.6.2. Talent vortexing tube

2.7. After discarding the supernatant, add 150 microliters of 100% chloroform to the tube for vortexing and centrifugation **[1]**.

2.7.1. Talent adding chloroform to tube, with chloroform container visible in frame

2.8. Add 450 microliters of double distilled water to the pellet **[1]** and vortex before centrifuging the sample for 3 minutes **[2]**.

2.8.1. Talent adding water to tube
2.8.2. Talent vortexing tube

2.9. Discard the upper aqueous layer **[1]** and add 600 microliters of 100% methanol to the tube **[2]**.

2.9.1. Upper aqueous layer being removed
2.9.2. Talent adding methanol to tube, with methanol container visible in frame

2.10. After another centrifugation, wash the pellet with 600 microliters of 100% methanol **[1]** and allow the plant protein pellet to air dry for 10-15 minutes **[2]**.

2.10.1. Talent adding methanol to tube, with methanol container visible in frame
2.10.2. Talent placing tube to air dry

2.11. Resuspend the pellet in 600 microliters of digestion buffer **[1]** and sonicate the tube until the suspension is homogenized **[2]**.

2.11.1. Talent adding digestion buffer to tube, with digestion buffer container visible in frame *Videographer: Important step*
2.11.2. Tube being sonicated *Videographer: Important step*

2.12. Use a BCA (B-C-A) kit according to the manufacturer's instructions to measure the protein concentration **[1-TXT]** and adjust the concentration to 4 micrograms of protein/microliter of digestion buffer **[2]**.

2.12.1. Talent opening kit, with sample tube visible in frame **TEXT: BCA: bicinchoninic acid**
2.12.2. Talent adding buffer to tube, with buffer container visible in frame

2.13. Transfer 100 microliters of the lysate to a new tube **[1]** and add 292 microliters of 50-millimolar TEAB (T-E-A-B) and 8 microliters of Lys-C (lyse-C) to the tube for a 3-hour incubation at 37 degrees Celsius **[2-TXT]**.

2.13.1. Talent adding lysate to tube
2.13.2. Talent adding TEAB and Lys-C to tube, with TEAB and Lys-C containers visible in frame **TEXT: TEAB: triethylammonium bicarbonate**

2.14. At the end of the incubation, add 100 microliters of 50-millimolar TEAB and 8 micrograms of trypsin to the tube for a 12-hour incubation at 37 degrees Celsius **[1]**.

2.14.1. Talent adding TEAB and trypsin to tube, with TEAB and trypsin containers visible in frame

2.15. Then add 25 microliters of 10% TFA (T-F-A) to the tube **[1 TXT]** and vortex the tube before centrifuging **[2]**.

2.15.1. Talent adding TFA to tube, with TFA container visible in frame **TEXT: TFA: trifluoroacetic acid**
2.15.2. Talent vortexing tube **TEXT: 20 min, 16,000 x g, 4 °C**

3. Tandem Mass Tag (TMT) Labeling

3.1. For tandem mass tag labeling, resuspend the dried peptides from six samples grown under the same treatment condition in 100 microliters of 200-millimolar HEPES **[1 TXT]** and pool the samples in a 5-milliliter tube **[2]**.

3.1.1. WIDE: Talent adding HEPES to tube **TEXT: HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid**
3.1.2. Talent adding sample(s) to tube

3.2. Add 14 microliters of 10% TFA to the tube **[1]** and transfer the solution to a conditioned desalting column **[2]**.

3.2.1. Talent adding TFA to tube, with TFA container visible in frame
3.2.2. Talent adding solution to column

3.3. Then dry the eluate using an evaporator **[1]**.

3.3.1. Eluate being placed into evaporator/being dried

4. Immobilized Metal Ion Affinity Chromatography (IMAC) and High pH Reversed-Phase (Hp-RP) Stage Tip Preparation

4.1. For IMAC (eye-mack) stage tip preparation, use a 16-gauge, blunt-ended needle to penetrate a polypropylene frit disk **[1]** and use a plunger to gently press the frit into the tip **[2]**.

4.1.1. WIDE: Talent penetrating disk *Videographer: Important step*
4.1.2. Frit being pressed into tip *Videographer: Important step* **TEXT: Discard tips that show high back pressure during conditioning**

4.2. Then place an inverted nickel-NTA (N-T-A) spin column onto a 1.5-milliliter tube **[1-TXT]** and use the plunger to press the frit of nickel-NTA beads gently into the tube **[2]**.

4.2.1. Inverted column being placed onto tube **TEXT: NTA: nitriloacetic acid**
4.2.2. Frit being pressed into tube **TEXT: Prepare Hp-RP tips in same manner but with C18 disk**

5. IMAC Stage Tip Phosphopeptide Enrichment

5.1. For phosphopeptide enrichment using an IMAC stage tip, add 400 microliters of loading buffer to the nickel-NTA beads **[1]** and load the entire volume of bead solution into one IMAC stage tip **[2]**.

5.1.1. WIDE: Talent adding loading buffer to beads, with loading buffer container visible in frame
5.1.2. Talent loading volume onto tip

5.2. Pass the solution through the tip by centrifugation **[1-TXT]** and load 100 microliters of 50-millimolar EDTA to strip the nickel ions from the tip by centrifugation **[2]**.

5.2.1. Talent placing tip into centrifuge **TEXT: 3 min, 200 x g, RT**
5.2.2. Talent adding EDTA to tip, with EDTA container visible in frame

5.3. Treat the tip with 100 microliters of loading buffer by centrifugation **[1]**.

5.3.1. Talent adding buffer onto tip, with buffer container visible in frame

5.4. Next, treat the tip with 100 microliters of 50-millimolar ferric chloride in 6% acetic acid by centrifugation **[1-TXT]**.

5.4.1. Talent placing tip into centrifuge **TEXT: Fe^{3+} ions chelate to IMAC stage tip**

5.5. Condition the stage with 100 microliters of loading buffer by centrifugation **[1]** followed by centrifugation with 100 microliters of loading buffer supplemented with the sample peptides **[2]**.

5.5.1. Talent adding loading buffer to tip, with loading buffer container visible in frame

5.5.2. Talent adding loading buffer to tip, with sample peptide container visible in frame

5.6. After centrifugation, rinse the tip two times with 100 microliters washing buffer per wash **[1]** followed by one rinse with 100 microliters of loading buffer **[2-TXT]**.

5.6.1. Talent adding washing buffer to tip, with washing buffer container visible in frame
5.6.2. Talent placing tip into centrifuge **TEXT: 3 min, 1000 x g, RT**

5.7. At the end of the centrifugation, use scissors to trim the front of the IMAC stage tip **[1]** and place the trimmed tip inside the high pH reversed-phase tip **[2]**.

5.7.1. Stage tip being trimmed *Videographer: Important step*
5.7.2. IMAC stage Tip being placed into Hp-RP stage tip *Videographer: Important step*

6. Hp-RP C18 Stage Tip Phosphopeptide Fractionation

6.1. For phosphopeptide fractionation using a high pH revese-phase C18 (C-eighteen) stage tip, pass 100 microliters of elution buffer through both layers of stage tips by centrifugation **[1-TXT]**.

6.1.1. WIDE: Talent adding buffer to tip *Videographer: Important step* **TEXT: Ensure tip layers do not touch centrifuge lid**

6.2. At the end of the spin, use tweezers to discard the IMAC stage tip **[1]** and add 20 microliters of Buffer A to the high pH reverse-phase stage tip **[2]**.

6.2.1. Tip being removed with tweezers **TEXT: Confirm complete buffer passage through tip before discarding**
6.2.2. Talent adding buffer A to tip, with buffer A container visible in frame

6.3. After centrifugation, add 20 microliters of Buffer 1 to the collected fraction **[1]** and collect the eluate in a new tube by centrifugation **[2]**.

6.3.1. Talent adding buffer 1 to tube, with buffer container visible in frame
6.3.2. Talent placing tube into centrifuge

6.4. Repeat the process with Buffers 2-8 until all 8 fractions have been collected **[1]**. Then dry the final eluate of each fraction in a vacuum concentrator **[2]**.

6.4.1. Talent adding buffer 2, with buffer container visible in frame
6.4.2. Talent placing fractions into vacuum concentrator

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
2.11., 4.1., 5.7., 6.1.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
4.1. is the most critical step in this protocol. Practicing this step more times to ensure the reproducibility of making stage tips.

Results

7. Results: Representative Osmotic Stress Signaling in *Arabidopsis*

7.1. In this representative analysis, a total of 8107 **[1]** and 7248 phosphopeptides were identified from wild-type and *snrk2* (S-N-R-K-two)-*decuple* mutant samples, respectively **[2-TXT]**, illustrating the efficiency of the workflow in providing in-depth coverage for delineating the global view of signal transduction in *Arabidopsis* **[3]**.

7.1.1. LAB MEDIA: Figure 2A *Video Editor: please emphasize red data line*
7.1.2. LAB MEDIA: Figure 2A *Video Editor: please emphasize blue data line* **TEXT: *snrk2-dec*: Sucrose non-fermenting 1 (SNF1)-related protein kinase 2s**

7.2. Comparison of the number of identified phosphopeptides across 8 fractions **[1]** revealed the presence of few phosphopeptides in the first two fractions **[2]**, but the majority of phosphopeptides were evenly distributed between the last 6 fractions **[3]**, suggesting this approach provides the ability to separate complex phosphopeptides from the plant phosphoproteome **[4]**.

7.2.1. LAB MEDIA: Figure 2A
7.2.2. LAB MEDIA: Figure 2A *Video Editor: please emphasize F1 and F2 data bars*
7.2.3. LAB MEDIA: Figure 2A *Video Editor: please emphasize F3-F8 data bars*
7.2.4. LAB MEDIA: Figure 2A

7.3. Evaluation of the overlap of phosphopeptides between two adjacent fractions indicated that less than 5% of the phosphopeptides overlap occurred in the adjacent fractions **[1]**.

7.3.1. LAB MEDIA: Figure 2B

7.4. After mannitol treatment, 433 phosphorylation sites were increased in the wild-type sample **[1]**, while 380 sites were increased in the *snrk2-decuple* mutant sample **[2]**.

7.4.1. LAB MEDIA: Figure 3 *Video Editor: please emphasize Figure 3A*
7.4.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize Figure 3B*

7.5. Among these, 312 phosphosites showed induction in wild-type, but not in *snrk2-dec* mutant, plants **[1]**.

7.5.1. LAB MEDIA: Figure 3

Conclusion

8. Conclusion Interview Statements

8.1. **Chuan-Chih Hsu**: It is important to use the same amount of pressure to load the frit disk into the tips to ensure a better reproducibility of the analysis **[1]**.

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (Step 4.1)

8.2. **Chuan-Chih Hsu**: Strong cation exchange chromatography can be used as alternative method for fractionating the enriched phosphopeptides to obtain a wider coverage of the plant phosphoproteome **[1]**.

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