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Dear editor,

We thank all reviewers for their thoughtful comments and suggestions for improving the manuscript.

We have carefully edited the manuscript according to the reviewer's comments and sent it out for Language editing by a nature speaker. We revised the *Keywords*, *Short Abstract*, *Abstract* sections as required. We have defined all the abbreviations and rephased all the sentences in the imperative terms in the manuscript. We replaced all the potential commercial terms with generic terms through this manuscript accordingly.

We have edited the *Protocol* section according to the editor's suggestion and provides more details in the step mentioned in the comments. We have revised the *Results* section to demonstrate the advantages of this workflow in plant phosphoproteomics. We have re-generate Figure 1, and now it is differing from our previous paper. We added the Disclosure section in the manuscript and updated the materials table accordingly.

We are grateful to the reviewers for raising excellent points that have enabled us to further strengthen our manuscript. We hope you share our interest and enthusiasm in this work and look forward to hearing from you.

Thanks and best regards,

Pengcheng Wang

TITLE:

Phosphoproteomic Strategy for Profiling Osmotic Stress Signaling in *Arabidopsis*

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KEYWORDS:

mass spectrometry, phosphoproteomics, protein phosphorylation, stage tips approach, Tandem Mass Tag labeling, *Arabidopsis*, osmotic stress

SUMMARY:

Presented here is a phosphoproteomic approach, namely stop and go extraction tip based phosphoproteomic, which provides high-throughput and deep coverage of *Arabidopsis* phosphoproteome. This approach delineates the overview of osmotic stress signaling in *Arabidopsis.*

ABSTRACT:

Protein phosphorylation is crucial for the regulation of enzyme activity and gene expression under osmotic condition. Mass spectrometry (MS)-based phosphoproteomics has transformed the way of studying plant signal transduction. However, requirement of lots of starting materials and prolonged MS measurement time to achieve the depth of coverage has been the limiting factor for the high throughput study of global phosphoproteomic changes in plants. To improve the sensitivity and throughput of plant phosphoproteomics, we have developed a stop and go extraction (stage) tip based phosphoproteomics approach coupled with Tandem Mass Tag (TMT) labeling for the rapid and comprehensive analysis of plant phosphorylation perturbation in response to osmotic stress. Leveraging the simplicity and high throughput of stage tip technique, the whole procedure takes approximately one hour using two tips to finish phosphopeptide enrichment, fractionation, and sample cleaning steps, suggesting an easy-to-use and high efficiency of the approach. This approach not only provides an in-depth plant

phosphoproteomics analysis (> 11,000 phosphopeptide identification) but also demonstrates the superior separation efficiency (< 5% overlap) between adjacent fractions. Further, multiplexing has been achieved using TMT labeling to quantify the phosphoproteomic changes of wild-type and *snrk2* decuple mutant plants. This approach has successfully been used to reveal the phosphorylation events of Raf-like kinases in response to osmotic stress, which sheds light on the understanding of early osmotic signaling in land plants.

INTRODUCTION:

High salinity, low temperature, and drought cause osmotic stresses, which is a major environmental factor that affects plant productivity[1,2]. Protein phosphorylation is one of the most significant post-translational modifications mediating signal perception and transduction in plant response to osmotic stress[3-5]. SNF1-related protein kinase 2s (SnRK2s) are involved in the osmotic stress signaling[6]. Nine of ten members of the SnRK2 family show significant activation in response to osmotic stress[7,8]. The *snrk2.1/2/3/4/5/6/7/8/9/10* decuple (*snrk2-dec*) mutant having mutations in all ten SnRK2 displayed hypersensitivity to osmotic stress. In *snrk2-dec* mutant, the osmotic stress-induced accumulation of inositol 1,4,5-trisphosphate (IP_3), abscisic acid (ABA) biosynthesis, and gene expressions are strongly reduced, highlighting the vital role of SnRK2s in osmotic stress responses[6]. However, it is still unclear how SnRK2s kinases regulate these biological processes. Profiling the phosphoproteomic changes in response to osmotic stress is an efficient way to bridge this gap and to delineate the osmotic stress-triggered defense mechanisms in plants.

Mass spectrometry (MS) is a powerful technique for mapping plant phosphoproteome[9]. Characterization of plant phosphoproteomics, however, remain a challenge due to the dynamic range of plant proteome and the complexity of plant lysate[4]. To overcome these challenges, we developed a universal plant phosphoproteomic workflow, which eliminates unwanted interferences such as from photosynthetic pigments and secondary metabolites, and enabling the deep coverage of plant phosphoproteome[10]. Several phosphopeptide enrichment methods such as immobilized metal ion affinity chromatography (IMAC) and metal oxide chromatography (MOC) have been developed for enriching phosphopeptides prior to MS analysis[11-16]. Acidic non-phosphopeptides co-purifying with phosphopeptides are the major interferences for phosphopeptide detection. Previously, we standardized the pH value and organic acid concentration of IMAC loading buffer to eliminate the binding of non-phosphopeptides, to obtain more than 90% enrichment specificity bypassing the pre-fractionation step[11].

Sample loss in the multi-step process of phosphopeptide enrichment and fractionation hampers the sensitivity of phosphopeptide identification and the depth of phosphoproteomic coverage. Stop-and-go-extraction tips (stage tips) are pipette tips that contain small disks to cap the end of the tip, which can be incorporated with chromatography for peptide fractionation and cleaning[17]. Sample loss during the stage tip procedure can be minimized by avoiding sample transfer between the tubes. We have successfully implemented stage tip in Ga^{3+}-IMAC and Fe^{3+}-IMAC to separate low abundant multiple phosphorylated peptides from singly phosphorylated peptides, which improved the depth of human phosphoproteome[15]. In addition, the use of high pH reversed-phase (Hp-RP) stage tip has demonstrated the wider coverage of human membrane

proteome compared to that of strong cation exchange (SCX) and strong anion exchange (SAX) chromatography[18]. Therefore, integrating IMAC and Hp-RP stage tip techniques can increase plant phosphoproteome coverage with simplicity, high specificity, and high throughput. We have demonstrated that this strategy identified more than 20,000 phosphorylation sites from *Arabidopsis* seedlings, representing an enhanced depth of plant phosphoproteome[19].

Here, we report a stage tip-based phosphoproteomic protocol for phosphoproteomic profiling in *Arabidopsis.* This workflow was applied to study the phosphoproteomic perturbation of wild-type and *snrk2-dec* mutant seedlings in response to osmotic stress. The phosphoproteomic analysis revealed the phosphorylation sites implicated in kinase activation and early osmotic stress signaling. Comparative analysis of wild-type and *snrk2-dec* mutant phosphoproteome data leaded the discovery of a Raf-like kinase (RAF)-SnRK2 kinase cascade which plays a key role in osmore stress signaling in high plants.

PROTOCOL:

1. Sample preparation

1.1. Harvest the control and stress treated seedlings (1 g) in an aluminum foil and flash freeze the samples in liquid nitrogen.

NOTE: Higher protein concentration is usually observed from two-week-old seedlings than that from mature plants. One gram of seedlings generates approximately 10 mg of protein lysate, which is enough for the MS analysis. All centrifugation steps take place at 16,000 *x g* in step 1.

1.2. Grind frozen seedlings into a fine powder using a mortar and pestle filled with liquid nitrogen.

1.3. Add 1 mL of lysis buffer ((6 M guanidine-HCl in 100 mM Tris-HCl, pH 8.5) with 10 mM Tris (2-carboxyethyl)phosphine hydrochloride (TCEP), 40 mM 2-chloroacetamide (CAA), protease inhibitor and phosphatase inhibitor cocktails) to the mortar and mix with the aid of pestle.

1.4. Transfer the plant lysate to a 1.5 mL tube and heat at 95 °C for 5 min.

1.5. Place the tube on ice for 10 min. Sonicate the tube on ice for 10 s, pause for 10 s, and repeat thrice.

1.6. Centrifuge the tube for 20 min and aliquot 150 µL of plant lysate into a 1.5 mL tube.

1.7. Add 600 µL of 100% methanol into the tube. Vortex and spin down the tube.

1.8. Add 150 µL of 100% chloroform into the tube. Vortex and spin down the tube.

1.9. Add 450 µL of ddH_2O into the tube. Vortex and centrifuge the tube for 3 min.

1.10. Discard the upper aqueous layer. Add 600 µL of 100% methanol into the tube. Centrifuge the tube for 3 min and then discard the solution.

1.11. Wash protein pellets with 600 µL of 100% methanol. Discard the solution and air dry the protein pellet.

1.12. Resuspend protein pellets into 600 µL of digestion buffer (12 mM sodium deoxycholate (SDC)/12 mM sodium lauroyl sarcosinate (SLS) in 100 mM Tris-HCl, pH 8.5). Sonicate the tube until the suspension is homogenized.

1.13. Measure the protein concentration using a BCA kit and adjust the concentration to 4 µg/µL with the digestion buffer. Transfer 100 µL of the lysate (400 µg proteins) into a new tube.

1.14. Add 292 µL of 50 mM triethylammonium bicarbonate (TEAB) and 8 µL of Lys-C (2.5 Unit/mL) to the tube. Incubate the tube at 37 °C for 3 h.

1.15. Add 100 µL of 50 mM TEAB with 8 µg trypsin to the tube. Incubate the tube at 37 °C for 12 h.

1.16. Add 25 µL of 10% trifluoroacetic acid (TFA) to the tube. Vortex and centrifuge the tube for 20 min at 4 °C.

1.17. Transfer the supernatant into a conditioned desalting column for desalting. Dry the eluate using a vacuum centrifuge concentrator.

NOTE: Activate the resin with 1 mL of methanol followed by 1 mL of 0.1% TFA in 80% acetonitrile (ACN). Wash out the organic solvent with at least 3 mL of 0.1% TFA in 5% ACN. Load acidified peptide samples prepared in step 1.16 into the column and then wash the column with at least 3 mL of 0.1% TFA in 5% ACN. More washes may be needed for samples with high salt concentrations. Elute the phosphopeptides with 1 mL of 0.1% TFA in 80% ACN.

2. Tandem Mass Tag (TMT) labeling

2.1. Resuspend the dried peptides into 100 µL of 200 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 8.5.

2.2. Dissolve the TMT reagent of each channel (0.8 mg) in 40 µL of anhydrous ACN. Vortex the TMT reagent tube for 5 min and spin it down.

2.3. Transfer 40 µL of TMT to the sample tube and incubate for 1 h at room temperature.

NOTE: In this experiment, the channel 126 to 128 were labeled with three biological replicates of plants without mannitol treatment, and the channel 129 to 131 were labeled with three biological replicates of plants with mannitol treatment.

2.4. Add 8 µL of 5% hydroxylamine and incubate for 15 min.

2.5. Mix 6 samples in a 5 mL tube and add 14 µL of 10% TFA.

2.6. Add 3,876 µL of 0.1% TFA to the tube. Vortex and spin down.

2.7. Transfer the solution to a conditioned desalting column for desalting. Dry the eluate using an evaporator.

3. Preparation of IMAC stage tip

3.1. Use a 16 G blunt-ended needle to penetrate a polypropylene frit disk.

NOTE: Hold the cutter perpendicular to the surface of the disk and roll the cutter a couple of times to make sure that the disk is completely excised. Place the disk in a Petri dish for storage. Place the needle into a 200 µL pipette tip and push the frit into the tip using a plunger.

3.2. Press the frit gently into the tip using a plunger to cap the end of tip.

3.2.1. Place the frit disk in tips with the same pressure, which provides better reproducibility. The reproducibility of stage tips production is important for constant back pressure, which affects the timing of the centrifuge steps and reproducibility between technical replicates.

3.2.2. If the stage tips show high back pressure during conditioning step, discard the tips to avoid potential tip clogging when while loading samples. It could be that the disk might have been pressed too hard into position.

3.3. Invert Ni-NTA spin column and place on a 1.5 mL tube.

3.4. Use a plunger to press the frit of Ni-NTA beads gently and push the beads into the tube.

NOTE: Ensure to push the frit gently to avoid the potential bead loss or adsorption on the spin column.

4. Preparation of Hp-RP stage tip

4.1. Prepare Hp-RP stage tip as described for IMAC stage tip using a C8 disk.

NOTE: Do not apply large force to the disk because it may result in a densely packed frit and increase the back pressure of the stage tip. All centrifugation steps take place at 1,000 *x g* for 5 min in step 4.

4.2. Suspend 1 mg of C18 beads in 100 µL 100% methanol and pass the beads solution through the tip.

4.3. Add 20 µL of buffer 8 (80% ACN/20% 200 mM NH_4HCO_2, pH 10.0) to the stage tip and pass buffer 8 through the tip by centrifugation.

4.4. Add 20 µL of Buffer A (200 mM NH_4HCO_2) to the stage tip and pass buffer A through the tip by centrifugation.

NOTE: Balance the centrifuge during use and ensure that the Hp-RP stage tip is not completely dried.

5. Preparation of spin adaptor

5.1. Use sharp end tweezers to puncture a hole at the center of the lid of a 1.5 mL tube.

5.2. Insert the IMAC stage tip or Hp-RP stage tip into the hole of the spin adaptor.

NOTE: Ensure that the stage tip is not close to the bottom of spin adaptor.

6. Phosphopeptide enrichment using IMAC stage tip

6.1. Suspend the 10 mg Ni-NTA beads with 400 µL of loading buffer (6% acetic acid (AA), pH 3.0) and load all of the beads solution into per tip. Pass the solution through the tip by centrifugation.

NOTE: All centrifugation steps take place at 200 *x g* for 3 min in step 6 besides step 6.8.

6.2. Load 100 µL of 50 mM ethylenediaminetetraacetic acid (EDTA) to strip the nickel ions from IMAC stage tip and pass the solution through the tip by centrifugation.

6.3. Load 100 µL of loading buffer to the stage tip and pass the solution through the tip by centrifugation.

6.4. Load 100 µL of 50 mM $FeCl_3$ in 6% AA to the stage tip and pass the solution through the tip by centrifugation. Fe^{3+} ions will chelate to IMAC stage tip.

6.5. Load 100 µL of loading buffer to condition the stage tip and pass the solution through the tip by centrifugation.

6.6. Load 100 µL of loading buffer with sample peptides prepared in step 2.7 to the stage tip and pass the solution through the tip by centrifugation.

NOTE: Take a new tube as a spin adaptor to collect the flow-through of sample and store the flow-through in the freezer for future analysis.

6.7. Load 100 µL of washing buffer (4.5% AA and 25% ACN) to the stage tip and pass the solution through the tip by centrifugation.

6.8. Perform the second wash with loading buffer. Load 100 µL of loading buffer to the stage tip and pass the solution through the tip using 1000 × g centrifuge for 3 min.

NOTE: Ensure the washing buffer is replaced by loading buffer in the stage tip to eliminate the loss of phosphopeptides during elution step. Equilibrate the IMAC stage tip prior to phosphopeptide elution to ensure the concentration of ACN is below 5%.

6.9. Trim the IMAC stage tip at the front with a scissors and place the trimmed IMAC stage tip inside the Hp-RP tip. Ensure that the two layers of stage tips do not touch the lid of the centrifuge.

7. Phosphopeptide fractionation using Hp-RP C18 stage tip

7.1. Add 100 µL of elution buffer (200 mM ammonium phosphate ($NH_4H_2PO_4$)) to the trimmed IMAC stage tip and pass the solution through the two layers of stage tips by centrifugation.

NOTE: If the solution does not pass through the stage tip, increase the speed of spin down. All centrifugation steps take place at 1,000 *x g* for 5 min in step 7. All the Hp-RP buffers are listed in **Table 1**.

7.2. Discard the IMAC stage tip using a tweezers and add 20 µL of Buffer A to the Hp-RP stage tip and pass the buffer through the tip by centrifugation.

NOTE: Ensure all the elution buffer pass through the IMAC stage tip before discarding it.

7.3. Add 20 µL of Buffer 1 to elute fraction 1 and collect the eluate in a new tube by centrifugation. Repeat the process with Buffers 2, 3, 4, 5, 6, 7, and 8 to collect each fraction in a new tube. Dry the final eluate of each fraction using a vacuum concentrator.

NOTE: Prepare fresh fractionation buffers. Take 8 new tubes as the spin adaptor of each fraction. Collect the eluate of each fraction in a different spin adaptor. Phosphopeptides are not stable under basic conditions, so dry the eluates by a concentrator or acidified by 10% TFA immediately.

8. LC-MS/MS analysis and data analysis

8.1. Add 5 µL of 0.1% formic acid (FA) and analyze the sample by a mass spectrometer.

8.2. Run a 90 min gradient with 6-30% buffer B (80% ACN and 0.1% FA) for each fraction.

8.3. Fragment the top 10 labeled peptides using high collision energy dissociation (HCD). Complete a database search to identify phosphorylation sites.

8.4. Load the raw files into the MaxQuant software, name the experiments, and set fractions and PTM.

NOTE: The tutorial of MaxQuant software can be found at https://www.maxquant.org/.

8.5. Select reporter ion MS2 in the type of LC-MS/MS run and 6plex TMT as isobaric labels. Enable filter by PIF function and set the min PIF as 0.75.

8.6. Select Acetyl (Protein N-term), Oxidation (M), and Phospho (STY) to the panel of variable modifications. Select Carbamidomethyl (C) as fixed modifications.

8.7. Set digestion mode as specific, select Trypsin/P as digestion enzyme, and two missed cleavage.

8.8. Set PSM false discovery rate (FDR) and protein FDR as 0.01. Set the minimum score for modified peptides as 40.

8.9. Add *Arabidopsis thaliana* database to the panel of fasta files and run database searching.

8.10. Load the txt file of Phospho (STY) sites to the Perseus software as the search is done.

NOTE: The detailed tutorials of Perseus software can be found at https://www.maxquant.org/perseus/.

8.11. Filter out the reverse phosphorylation sites. Filter out the unlocalized phosphorylation sites using localization probability 75% as the cut-off.

8.12. Use the intensities of phosphorylation sites for statistical analysis.

REPRESENTATIVE RESULTS:

To demonstrate the performance of this workflow, we exploited IMAC stage tip coupled with Hp-RP stage tip fractionation to measure the phosphoproteomic changes in wild-type and *snrk2-dec* mutant seedlings with or without mannitol treatment for 30 minutes. Each sample was performed in biological triplicates, and the experimental workflow is represented in **Figure 1**. The digested peptides (400 µg) of each sample were labeled with one TMT-6plex channel, pooled and desalted. The phosphopeptides were further enriched using an IMAC stage tip, and the purified phosphopeptides were subsequently fractionated into eight fractions by a Hp-RP stage tip. Each fraction was analyzed by a 90 min LC gradient analysis. The raw files were searched using a search engine against *Arabidopsis thaliana* database.

A total of 11,077 unique phosphopeptides were identified corresponding to 3,630 phosphoproteins with 6,852 localized phosphorylation sites (Class I, localization probability > 0.75), indicating the wide coverage of *Arabidopsis* phosphoproteome. A total of 8,107 and 7,248

phosphopeptides were identified from wild-type and *snrk2-dec* mutant sample, respectively. This illustrates the efficiency of the workflow in providing in-depth coverage for delineating the global view of signal transduction in *Arabidopsis*. We compared the number of identified phosphopeptides across 8 fractions. A few phosphopeptides were identified in the first two fractions, the fraction 1 and 2. However, majority of phosphopeptides were evenly distributed in the rest 6 fractions (**Figure 2A**), suggesting this approach provides the capability to separate complex phosphopeptides from the plant phosphoproteome. To further demonstrate the separation efficiency of this workflow, we evaluated the overlap of phosphopeptides between two adjacent fractions (eq. F1-to-F2). Less than 5% phosphopeptides overlap in the adjacent fractions, indicating robust fractionation efficiency of the Hp-RP stage tip (**Figure 2B**).

Using the data obtained by the workflow, we compared the phosphoproteomic profiles of wild-type and *snrk2-dec* mutant upon mannitol treatment. A total of 433 and 380 phosphorylation sites were increased after mannitol treatment in wild-type and *snrk2-dec* mutant sample, respectively (**Figure 3**). Among that, 312 phosphosites showed induction (FDR < 0.01) in wild-type, but not in *snrk2-dec* mutant plants. The Gene Ontology (GO) analysis revealed that the function of phosphorylation and activation of protein kinase, regulation of phosphate metabolic process, signal transduction, are significantly enriched in the SnRK2-dependent phosphoproteins. Interestingly, GO term related to root development was also enriched in SnRK2-dependent group, consistent with the phenotype of root growth retardation under osmotic stress[6]. We also identified 116 phosphosites up-regulated by mannitol treatment in both wild-type and *snrk2-dec* mutant. These phosphoproteins were independent of SnRK2, or candidates that mediate osmotic stress-triggered signaling prior to SnRK2s activation. We observed that several B4 subgroup RAFs such as RAF18 (AT1G16270), RAF24 (AT2G35050), and RAF42 (AT3G46920), were significantly up-regulated by osmotic stress. Further study revealed that RAF kinases are quickly activated by osmotic stress and required for phosphorylation and activation of SnRK2s[20].

FIGURE LEGENDS:

Figure 1: Workflow of stage tip-based phosphoproteomic method. Protein was extracted and digested from wild-type and *snrk2-dec* mutant seedlings treated with or without mannitol. The digested peptides of each replicate were labeled with a unique TMT6-plex channel. Phosphopeptides were pooled and then enriched using an IMAC stage tip. The purified phosphopeptides were separated by a Hp-RP stage tip. Each fraction was analyzed by mass spectrometer.

Figure 2: Phosphoproteomic profiling of wild-type and *snrk2* decuple mutant seedlings by IMAC stage tip and Hp-RP fractionation. (**A**) The number of identified phosphopeptides per fraction. (**B**) The separation efficiency of the stage tip-based Hp-RP chromatography. The overlap between the adjacent fractions is represented by the percentage of the same peptide identified in the adjacent fractions.

Figure 3: Quantification of the phosphoproteomic changes in response to osmotic stress. Volcano plots show the log2 fold change of phosphorylation sites in (**A**) wild-type and (**B**) *snrk2-dec* mutant seedlings in response to mannitol treatment. The black circle represents the kinase

phosphorylation sites induced by mannitol. The red circle indicates the phosphorylation sites of RAFs up-regulated in response to mannitol.

Table 1: Buffers for Hp-RP stage tip fractionation.

DISCUSSION:

The dynamic range and complexity of plant proteome and phosphoproteome are still a limiting factor to depth of phosphoproteomics analyses. Despite the capability of single run LC-MS/MS analysis to identify 10,000 phosphorylation sites[21,22], the coverage of the whole plant phosphoproteome is still limited. Therefore, a phosphoproteomic workflow that provides high sensitivity and superior separation efficiency is required in profiling the global view of plant signaling networks in response to environmental stress. Commercial high-pressure liquid chromatography (HPLC) column-based chromatography is a common method for reducing the peptide complexity prior to MS analysis[23,24]. However, tedious sample collection steps and large elution volume are the major challenges of HPLC-based methods in terms of sensitivity and throughput. stage tip is an alternative approach to execute peptide pre-fractionation or enrichment for high-sensitivity and high-throughput works. This new phosphoproteomic workflow exploiting isobaric labeling coupled with phosphopeptide enrichment and fractionation provides better coverage and depth of plant phosphoproteomics over other phosphoproteomic workflows[25,26].

The pH value of samples is the crucial factor that determines the enrichment specificity of phosphopeptides. The pH value may be affected by the previous step of sample preparation, so it is essential to check the pH value before sample loading. If the pH is shifted, adding acetic acid or sodium hydroxide to samples to adjust the pH to 3.0. We execute this protocol for simultaneous elution and loading of phosphopeptides onto C18 stage tip. Therefore, it is important to equilibrate the IMAC stage tip prior to phosphopeptide elution to ensure the concentration of ACN is below 5%.

The number of fractions is dependent on the complexity and sample sizes. Typically, 5 to 8 fractions are enough to provide broad coverage of phosphopeptide identification in plants. The concentration of ACN in fractionation buffers can be adjusted according to the hydrophobicity of samples. Most of the phosphopeptides are eluted from C18 beads using the range of 5% to 25% ACN concentration. Phosphopeptides typically are more hydrophilic compared to non-phosphorylated peptides due to the additional negative charges of the phosphate group. However, tagging TMT reagent on N-terminus of peptide leads to an increase of hydrophobicity of labeled peptides[27], that may explain why fewer phosphopeptides were identified in the first two fractions in our case (**Figure 2A**). Thus, a test using a TMT-zero reagent and an aliquot of samples can be used to evaluate the number of fractions and the ACN concentration in each fractionation buffers. The optimized buffers may result in better separation efficiency of TMT-6plex labeled phosphopeptides.

The limitations of stage tip-based fractionation are the number of fractions and the capacity of tips. It is difficult to expand the number of fractions in stage tip separation because discontinuous

gradient buffers are used for stage tip fractionation. On the other hand, a continuous gradient can be applied to HPLC columns to achieve a high fraction number. The capacity of tips is another factor that limits the application of stage tips. For large-scale phosphoproteomics analysis (> 10 mg proteins) it is better to use HPLC-based enrichment with longer column length packed with more C18 beads and fractionation, which is beyond the analytical scale of stage tips. Taken together, stage tip-based phosphopeptide enrichment and fractionation is a useful method for small to medium scale of phosphoproteomics analyses. This approach can be integrated with isobaric labeling to achieve multiplexed quantification. The results also demonstrated that it could be used for in-depth plant phosphoproteomics profiling and quantification. This workflow is applicable to other species and different tissue types for the delineation of specialized signaling networks.

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DISCLOSURES

The authors declare no conflict of interest.

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21 Humphrey, S. J., Azimifar, S. B., Mann, M. High-throughput phosphoproteomics reveals in vivo insulin signaling dynamics. *Nature Biotechnology.* **33** (9), 990-995 (2015).
22 Bekker-Jensen, D. B. et al. An optimized shotgun strategy for the rapid generation of comprehensive human proteomes. *Cell Systems.* **4** (6), 587-599 e584 (2017).
23 Possemato, A. P. et al. Multiplexed phosphoproteomic profiling using titanium dioxide and immunoaffinity enrichments reveals complementary phosphorylation events. *Journal Proteome Research.* **16** (4), 1506-1514 (2017).
24 Hogrebe, A. et al. Benchmarking common quantification strategies for large-scale phosphoproteomics. *Nature Communications.* **9** (1), 1045 (2018).
25 Wong, M. M. et al. Phosphoproteomics of Arabidopsis Highly ABA-Induced1 identifies AT-Hook-Like10 phosphorylation required for stress growth regulation. *Proceedings of the National Academy of Sciences of the United States of America.* **116** (6), 2354-2363 (2019).
26 Yang, F., Melo-Braga, M. N., Larsen, M. R., Jorgensen, H. J., Palmisano, G. Battle through signaling between wheat and the fungal pathogen *Septoria tritici* revealed by proteomics and phosphoproteomics. *Molecular & Cell Proteomics.* **12** (9), 2497-2508 (2013).

27 Tsai, C. F. et al. Tandem Mass Tag labeling facilitates reversed-phase liquid chromatography-mass spectrometry analysis of hydrophilic phosphopeptides. *Analytical Chemistry.* **91** (18), 11606-11613 (2019).

Figure 1

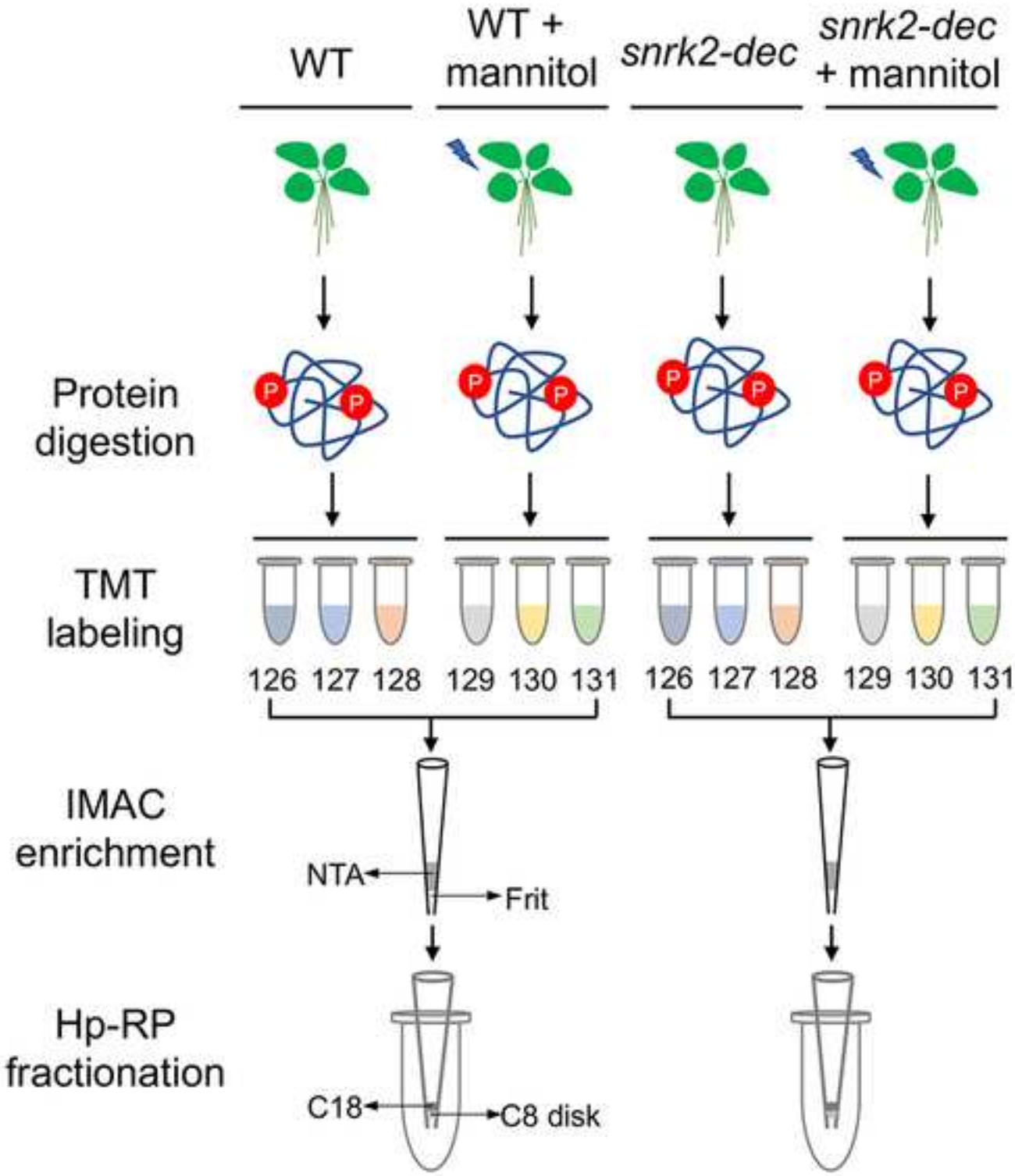

Figure 2

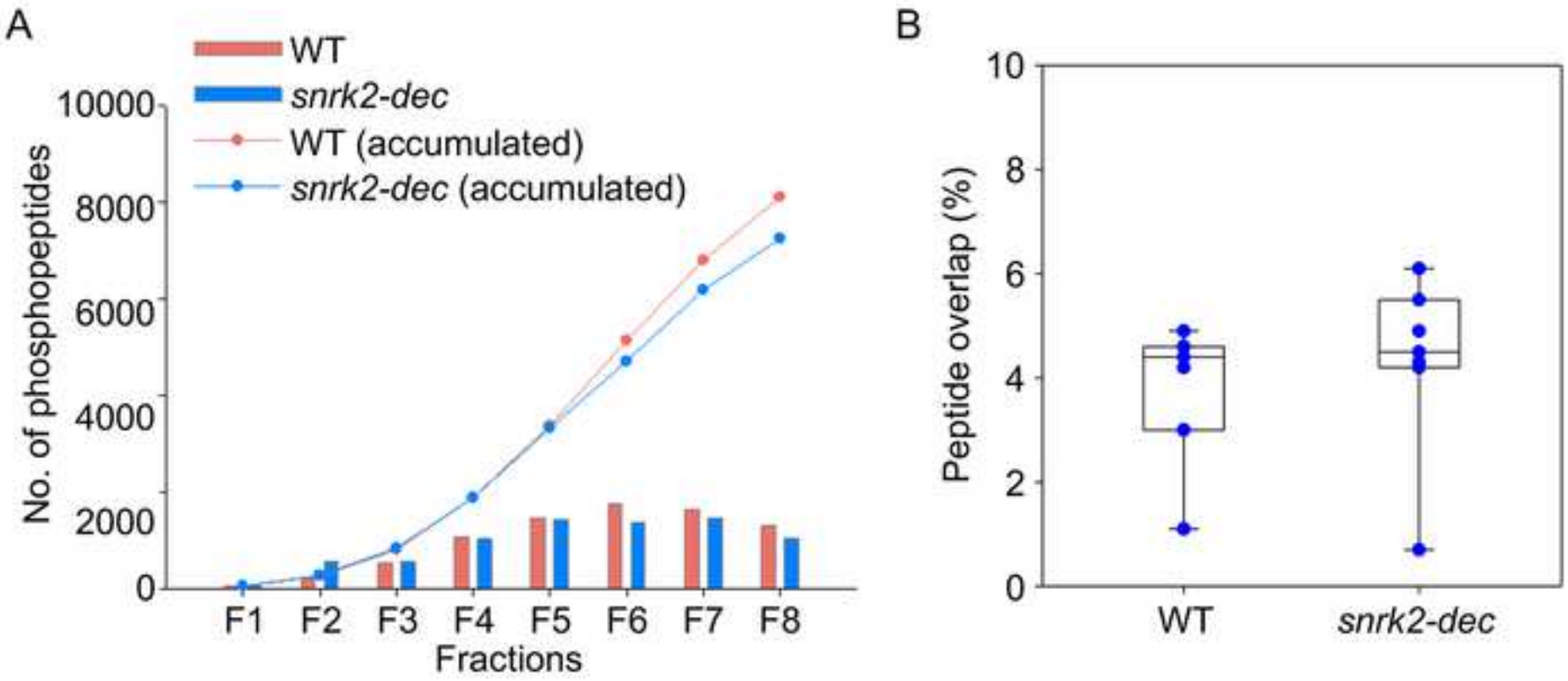

Figure 3

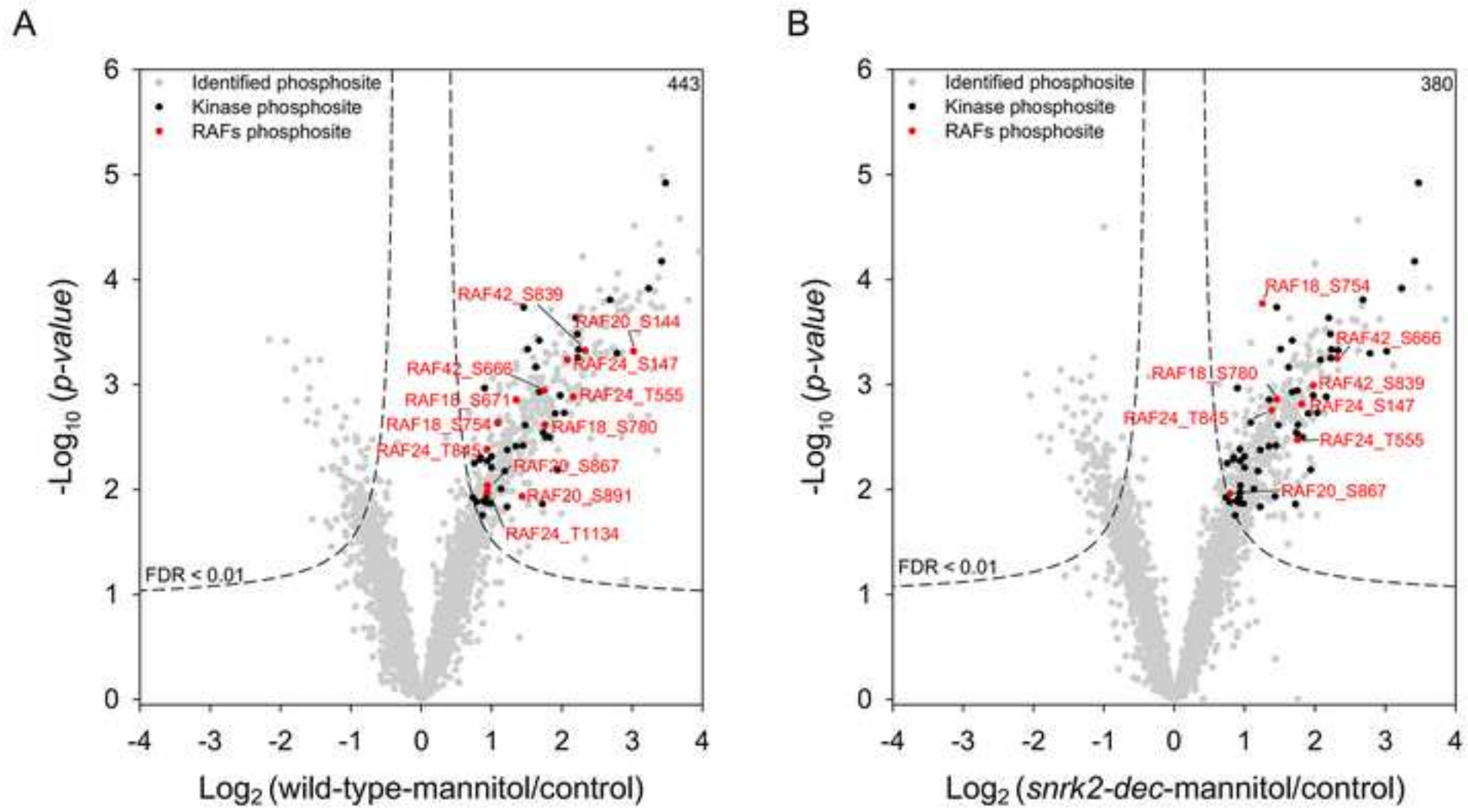

Table 1	**Buffers for Hp-RP stage tip fractionation**
Buffer A	200 mM ammonium formate (NH4HCO2), pH 10.0.
Buffer B	100% ACN.
Buffer 1	5% Buffer B, 95% Buffer A.
Buffer 2	8% Buffer B, 92% Buffer A.
Buffer 3	11% Buffer B, 89% Buffer A.
Buffer 4	14% Buffer B, 86% Buffer A.
Buffer 5	17% Buffer B, 83% Buffer A.
Buffer 6	20% Buffer B, 80% Buffer A.
Buffer 7	23% Buffer B, 77% Buffer A.
Buffer 8	80% Buffer B, 20% Buffer A.

Table of Materials

Click here to access/download;Table of Materials;Materials 042220.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 mL tube	eppendorf	22431081	Protein LoBind, 1.5 mL, PCR clean, colorless, 100 tubes
200 µL pipet tip	Gilson	F1739311	
2-chloroacetamide	Sigma-Aldrich	C0267	
acetic acid	Sigma-Aldrich	5438080100	
acetonitrile	Sigma-Aldrich	271004	
ammonium hydroxide	Sigma-Aldrich	338818	
ammonium phosphate monbasic	Sigma-Aldrich	216003	
BCA Protein Assay Kit	Thermo Fisher Scientific	23227	
blunt-ended needle	Hamilton	90516	Kel-F hub (KF), point style 3, gauge 16
C18-AQ beads	Dr. Maisch	ReproSil-Pur-C18-AQ 5 µm	
C8 Empore disk	3 M	2214	47 mm
Centrifuge	eppendorf	22620444	
chloroform	Sigma-Aldrich	CX1058	
data analysis software		Perseus 1.6.2.1	https://maxquant.net/perseus/
ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich		
formic acid	Sigma-Aldrich	5330020050	
Frits	Agilent	12131024	Frits for SPE Cartridges
Guanidine hydrochloride	Sigma-Aldrich	50933	
H2O	Sigma-Aldrich	1153334000	
HEPES	Sigma-Aldrich	H3375	
Iron (III) chloride	Sigma-Aldrich	157740	
LTQ-orbitrap	Thermo Fisher Scientific	Velos Pro	
mass spectrometer	Thermo Fisher Scientific	LTQ-Orbitrap Velos Pro	
methanol	Sigma-Aldrich	34860	
nano LC	Thermo Fisher Scientific	Easy-nLC 1000	
Ni-NTA spin column	Qiagen	31014	
N-Lauroylsarcosine sodium salt	Sigma-Aldrich	L9150	
plunger	Hamilton	1122-01	Plunger assembly N, RN, LT, LTN for model 1702 (25 µl)

search engine software		MaxQuant 1.5.4.1	https://www.maxquant.org
SEP-PAK Cartridge 50 mg	Waters	WAT054960	
sodium deoxycholate	Sigma-Aldrich	D6750	
SpeedVac	Thermo Fisher Scientific	SPD121P	
TMT 6-plex	Thermo Fisher Scientific	90061	
Triethylammonium bicarbonate buffer	Sigma-Aldrich	T7408	
Trifluoroacetic acid	Sigma-Aldrich	91707	
Tris(2-carboxyethyl)phosphine hydrochloride	Sigma-Aldrich	C4706	
Trizma hydrochloride	Sigma-Aldrich	T3253	

Point to point responses to editor and reviewers' comments

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Response: The revised manuscript was edited by a native speaker to check the spelling and grammar issues.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points
Response: The revised manuscript was reformed as required.

3. Please provide at least 6 keywords or phrases.
Response: Now, it has seven keywords.

4. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Presented here is a protocol …"
Response: We rephrase the Short Abstract to 'Presented here is a phosphoproteomic approach namely StageTip-based phosphoproteomic, which provides high-throughput and deep coverage of Arabidopsis phosphoproteome. This approach delineates the overview of osmotic stress signaling in Arabidopsis.'

5. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.
Response: The revised Abstract is 210 words.

6. Please define all abbreviations during the first-time use.
Response: We re-checked the manuscript and defined all abbreviations during the first-time used.

7. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: LTQ-Orbitrap Velos Pro mass spectrometer, MaxQuant software, TAIR10 database, Perseus software, IMAC StageTip, SEP-PAK, Empore extraction disk, etc.
Response: The revised manuscript was reformed as required. MaxQuant and Perseus are free downloaded software for academic usage but not commercial software.

8. Is the term StageTip commercial, if yes please remove and use generic terms.
Response: The term StageTip is not commercial.

9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."
Response: The manuscript was revised as required.

10. The Protocol should contain only action items that direct the reader to do something including all specific details associated with the step in complete sentences.

Response: The manuscript was revised as required.

11. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.
Response: The manuscript was revised as required.

12. Some of the subheadings can be combined. E.g., step 1-3 can be combined under protein preparation.
Response: The manuscript was revised as required.

13. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? For this please include all the button clicks in the software, knob turns, mechanical actions, etc.
Response: We added some details to the revised protocol.

14. 1: which plants, how old? Any specifications? For grinding you use the complete plant including roots, stem, leaves, flowers, etc?
Response: We use two-week-old seedlings, now this information was added in the revision.

15. 3: Volume of the digestion buffer used?
Response: We added the volume of digestion buffer (600 µL) in the revision.

16. 3.7 How is this done?
Response: We added more details in the note of step (1.17) to present the workflow of peptide desalting in the revision.

17. 4.1: Where did you obtain the dried peptides from? Step 3 ends with total protein.
Response: After Lys-C and Trypsin digestion (1.14 to 1.17 in the revision), the total protein was digested to peptides.

18. 9.4- 9.10: How do you perform the elution?
Response: We elute the phosphopepetides from C18 StageTip by adding 20 µL of the elution Buffer and pass through the solution by centrifugation the StageTip at 1,000 × g for 5 min. We detailed it in step 7.3 in the revision.

19. 10: how do you perform the analysis?
Response: We added more details to the revised protocol (step 8 in the revision).

20. It is unclear where samples are being processed. Please bring out link from one step to the next.
Response: We added more details to the revised protocol (step 8 in the revision).

21. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Response: We suggest recording step 6.1 to 9.4, which are the more essential steps we optimized.

22. Please ensure that the results are described with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title.
Response: We revised the result part, as suggested.

23. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that

allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
Response: We re-draw Figure 1 and now it is different from our previous publication and no copyright permission is needed.

24. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.
Response: We added a Disclosures section in the revision.

25. Please sort the materials table in alphabetical order and remove all trademark (™) and registered (®) symbols.
Response: The materials table is sorted by in alphabetical order and all trademark and registered symbols are removed.

26. Please upload each Figure individually to your Editorial Manager account. Please combine all panels of one figure into a single image file.
Response: The figures were uploaded as required.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript entitled "A phosphoproteomics strategy for profiling osmotic stress signaling in Arabidopsis" by Wang et al describes the methodology to carry out mass spectrometry based phosphoproteomics analysis in the model plant Arabidopsis thaliana. The authors developed a simplified StageTip protocol for phosphopeptide enrichment and fractionation and coupled it with TMT labeling for peptide quantification. Further, as a proof of concept the authors carried out a comparative phosphoproteomics study of wild-type Arabidopsis and snrk2 decuple mutant under osmotic stress. The authors delineated the early signaling pathways activated under mannitol stress. The study is useful for researchers who are relatively new to the field of mass spectrometry based phosphoproteome analysis. The manuscript is technically sound. However, the manuscript needs to be checked thoroughly for grammatical errors prior to acceptance for publication. My comments about the manuscript are as follows:

Major Concerns:
1. Order of authors mentioned in page 1 and page 2 are different.
Response: The order of the authors was corrected.

2. Reference 7 is missing in the introduction section
Response: The reference 7 is added in the revision.

3. Search parameters for analysis using MaxQuant should be discussed in greater detail
Response: The search parameters are detailed in the revision (step 8.4 to 8.10).

Minor Concerns:
1. Page 1 and Page 2: Key words section: 'Protein' should be replaced with 'protein'.
2. Page 1: Abstract section: 'systemwide' should be replaced with 'system-wide'
3. Page 1 and Page 2: Abstract section: 'one hour using one StageTips' should be replaced with 'one hour using one StageTip'
4. Page 1 and Page 2: Abstract section: Suggest replacing 'suggesting the easy-to-use and efficiency of

the approach' with 'suggesting an easy-to-use and efficient approach'
5. Page 1: Abstract section: Suggest replacing 'in depth' with 'in-depth'
6. Page 1: Abstract section: Suggest replacing 'shows the superior separation' with 'demonstrates the superior separation'
7. Replace 'wild type' with 'wild-type' in the entire manuscript
8. Page 2: 'Email addresses of Co-authors' should be replaced with 'Email addresses of co-authors'
9. Page 2: Email of all co-authors should be underlined to maintain uniformity
10. Line 44: Replace 'that' with 'how'
11. Line 44: Replace 'SnRK2s kinase' with 'SnRK2 kinases'
12. Line 45: Replace 'regulates' with 'regulate'
13. Line 49: Suggest replacing 'However, it is still challenging in the analysis of plant phosphoproteomics' with 'Characterization of plant phosphoproteomes however remain a challenge due to….'
14. Line 51: Replace 'the' with 'these'
15. Line 52: Replace 'pigment' with 'pigments'
16. Line 55: Replace 'purifying' with 'enriching'
17. Line 56: Suggest replacing 'Yet acidic non-phosphopeptides co-purified' with 'Acidic non-phosphopeptides co-purifying'
18. Line 57: Replace 'inferences' with 'interferences'
19. Line 58: Add 'Previously' prior to 'We carefully evaluated'
20. Line 61: Replace 'multi-steps' with 'multi-step process' AND delete 'procedure'
21. Line 64: Replace 'chromatographic with 'chromatography'
22. Line 69: Delete 'been'
23. Expand the abbreviation 'Hp-RP' in the first instance
24. Line 71: Replace 'enlarge' with 'increase'
25. Line 73-74: Replace 'the depth coverage' with 'deep'
26. Line 75: Delete 'analyzing'
27. Line 78: Replace 'phosphoproteomic result' with 'phosphoproteomic analysis'
28. Line 84: Suggest replacing 1.1 with 'Harvest the control and stress treated seedlings in an aluminum foil and flash freeze the samples in liquid nitrogen
29. Line 85: Replace 'powders' with 'powder' AND 'mortar' with 'mortar and pestle'
30. Line 86,87: Expand the abbreviations TCEP and CAA
31. Line 87: Suggest adding 'and mix with the aid of pestle'
32. Line 89: Suggest replacing 'tube 10 sec' with 'tube for 10 sec. Repeat thrice'
33. Line 94: Suggest rephrasing to 'Aliquot 150µl plant lysate into a 1.5 mL tube'
34. Line 95,96: Mention the centrifugation speed
35. Line 101-102: Replace with 'air dry the tube' with 'air dry the protein pellet'
36. Line 108: Replace 'by BCA kit' with 'using BCA kit'
37. Replace 'SEP-PAK' with 'Sep-Pak' in the entire text.
38. Line 126: Replace 'Spin' with 'spin'
39. Preparation of Hp-RP StageTip: Lines 148-155 may be deleted and instead written as 'Hp-RP StageTip were prepared as described for IMAC StageTip using C8 Empore extraction disk
40. Line 160-162: What is buffer B, buffer A? Although mentioned at the end of the manuscript under the section 'Materials and Reagents' it would be useful to the readers if the information is added here.
41. Line 161: Replace 'Stagetip' with 'StageTip' AND '1000 g centrifuge' with 'using centrifugation at 1000g'
42. Line 162: Replace 'Stagetip' with 'StageTip'
43. Line 173,174,176,182,184: Replace '200 g centrifuge' with 'using centrifugation at '200g'. Also, please mention the volume of beads to be loaded per tip.
44. Line 250: Replace 'channel' with 'reagents' AND delete 'each'
45. Line 269: Replace 'both' with 'the'

46. Line 289: Replace 'replicates' with 'replicate'
47. Line 301: What is 'Oks'?
48. Line 301: Suggest rephrasing the sentence to 'Despite the ability of one-shot LC-MS/MS to identify more than 10,000 phosphorylation sites, the coverage of the whole plant phosphoproteome is still limited.
49. Line 316: Replace 'workflow' with ''workflows'
50. Line 324: Is it sodium chloride or sodium hydroxide?
51. Line 326: It is unclear what the authors mean by 'washing'?
52. Line 327: Acetonitrile can be abbreviated to ACN in first instance and the abbreviation can be used throughout the remaining text
53. Line 338: Replace 'ACN concentration of' with ''ACN concentration in' AND 'buffers are' with 'buffers can'
54. Line 349: Replace 'can integrate' with 'can be integrated'
55. Table of Materials: To maintain uniformity capitalize the first alphabet of each chemical enlisted
56. Materials and Reagents: Please expand 'AA'
57. Replace 'pipet' with 'pipette' throughout the text
Response to minor concerns: We thank the reviewer for the careful reading and editing of our manuscript. We have carefully edited the manuscript and sent it for language editing. As suggested, we provided more details in the data analysis steps.

Reviewer #2:
i recommend reject...

-Overall, needs significant editing to read better. Various typos and grammatical errors.
Response: We apologize for the typos and grammatical errors. The revised manuscript was carefully edited and sent for language editing.

-Some notes about scaling should be added. Not every prep will yield 4 µg/4µL of protein as noted.
Response: We added the note of step 1.2 to explain that one gram of seedling generates enough starting material for MS analysis.

-Line 166 - Desalting protocol should be detailed.
Response: We added more details of desalting protocol in the note of step 1.17.

Lines 199-128 - Notes about strategy for picking which TMT channels to use for which samples should be detailed.
Response: We added the note of step 2.3 to show the sample we labeled in each TMT channel.

-Overall, the methods need to be detailed for more clearly. The protocol seems to assume a working knowledge of mass spectrometry, phosphopeptide enrichment, and the bioinformatics downstream spectral analysis, none of which are trivial and should be addressed in far more detail if this is meant to be a protocol one could apply one's self.
Response: We thank the reviewer's suggestion and provide more details in the revised protocal.

-I see no supplemental information. Maybe there should be tables of the MS data in full?
Response: Our MS data have been deposited to ProteomeXchange with the dataset identifier PXD014435.

-Is the raw data available somewhere? Should be made publicly available.
Response: Our MS data have been deposited to ProteomeXchange with the dataset identifier PXD014435.

-I don't think this is novel… it's just stage tip based high pH reversed phase. The only thing novel is doing it in a spin tip. But thermos Fisher has high pH spin tips available for purchase. In theory one could just purchase them and take phosphopeptides from a similar large scale phosphoenrichment a separate them on one of those. What advantages does this afford?
Response: We agree with the reviewer's comment that high pH-spin tips are commercially available. However, we found that our workflow within spin tips identified more (at least 20% percent) phosphopeptides than that with commercial high-pH spin tips. As we discussed in the revision, this workflow is applicable to small to medium scale of phosphoproteomics analyses.

Various typos, too many to correct here.
Line 44 - should be SnRK2 kinases, not SnRK2s kinase.
Line 64 - should be chromatographic separation or chromatography, not "chromatographic"
Line 68 - what is Hp? I assume it's high-pH.
Line 88 - degrees C appears to be in a different font.
Line 105 - what is SDC/SLC buffer?
Response: We apologize for the typos and grammatical errors. The revised manuscript was carefully edited and sent for language editing.

Reviewer #3:
Manuscript Summary:
The manuscript describes in detail an optimized protocol to analyze phosphoproteomes of Arabidopsis plants, increasing yield and diversity of the identified proteins.
This protocol will be of interest to the scientific community as it deals with signal transduction, a major point of regulation in response to stress and virtually every key step in plants life. The protocol is clearly described and well written, I do not have any major concerns.

Minor Concerns:
Figure 3B: Please specify in the legend what is "Kinase Phosphosite" and "Ok phosphosite".
Response: We are sorry for the typo. It was corrected in the revision.

In "Table of Materials" Sigma Aldrich is misspelled. Please double check.
Response: We are sorry for the typo. It was corrected in the revision.

In the method description, there are some acronyms that are not specified; e.g. CAA, TCEP, SDC, SLS, TEAB, TMT. Please indicate what these acronyms mean.
Response: We defined all the abbreviations when they are first used.

Please provide details of MaxQuant and Perseus software (internet address)
Response: We added the internet address of the software in the *Table of Materials*.