Chapter 2

Quantitative Measurement of Phosphopeptides and Proteins via Stable Isotope Labeling in *Arabidopsis* and Functional Phosphoproteomic Strategies

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Abstract

Protein phosphorylation is one type of posttranslational modification, which regulates a large number of cellular processes in plant cells. As an emerging powerful biotechnology that integrates all aspects of advantages from mass spectrometry, bioinformatics, and genomics, phosphoproteomics offers us an unprecedented high-throughput methodology with high sensitivity and dashing speed in identifying a large complement of phosphoproteins from plant cells within a relatively short period of time. Needless to say, phosphoproteomics has become an integral portion of life sciences, which penetrates various research disciplines of biology, agriculture, and forestry and irreversibly changes the way by which plant scientists study biological problems.

Because phosphorylation/dephosphorylation of protein is dynamic in cells and the amount of phosphoproteins is low, the preservation of a phosphor group onto phosphosite throughout protein purification as well as enrichment of these phosphoproteins during purification has become a serious technical issue. To overcome difficulties commonly associated with phosphoprotein isolation, phosphopeptides' enrichment, and mass spectrometry analysis, we have developed a urea-based phosphoprotein purification protocol for plants, which instantly denatures plant proteins once the total cell content comes into contact with the UEB solution. To measure the alteration of phosphorylation on a phosphosite using mass spectrometer, an in vivo ^{15}N metabolic labeling method (SILIA, i.e., stable isotope labeling in *Arabidopsis*) has been developed and applied for *Arabidopsis* differential phosphoproteomics. Thus far, hundreds of signaling-specific phosphoproteins have been identified using both *label-free* and ^{15}N-labeled differential phosphoproteomic approach. The phosphoproteomics has allowed us to identify a number of signaling components mediating plant cell signaling in *Arabidopsis*. It is envisaged that a huge number of phosphosites will continue to be uncovered from phosphoproteomics in the near future, which will become instrumental for the development of plant phosphor-relay networks and molecular systems biology.

Key words: Plant, Functional phosphoproteomics, Mass spectrometry, Stable isotope labeling in *Arabidopsis*, In vivo stable isotope ^{15}N labeling, Quantitative proteomics, Site-directed mutagenesis

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1. Introduction

Reversible protein phosphorylation plays a central role in cell signaling, regulation of gene expression, controlling of the growth and development of an organism, and its adaptation to environmental changes (1). Plants also make use of phosphor-relay mechanism for ethylene signaling (2). Phosphoproteomics (3) has been developed for profiling global protein phosphorylation at a given developmental stage or in response to a specific external cue. Identification of protein phosphorylaiton has always been technically difficult in the past due to the relatively low abundance as well as the labile nature of the phosphorylation site. With the emerging powerful phosphoproteomic technology, i.e., the immobilized metal-ion affinity chromatography (IMAC)-based phosphopeptides' enrichment coupled with liquid chromatography mass spectrometric sequencing (LC–MS/MS) of phosphorylated peptides, we are now able to profile phosphoproteins at large scale and determine the phosphorylation sites associated with a developmental cue or an environmental inducer (3–8). Ever since Nühse et al. (4) have identified more than 300 phosphorylation sites from *Arabidopsis* membrane proteins using the phosphoproteomic approach, nearly 30,430 phosphopeptides have been characterized thus far from the model plant *Arabidopsis* according to PhosphAT3.0 (http://phosphat.mpimp-golm.mpg.de/statistics.html).

With the advent of breakthroughs in quantitative differential proteomics, i.e., the *label-free* approach (9–11) and the isotope-assisted approach (12–18), the mass spectrometry-based quantitation of phosphorylation level of a large number of phosphosites has become the focus of quantitative phosphoproteomics. The advantages of the in vitro isotope-labeling methods, such as isotope-coded affinity tags (ICATs) (12), isotope tagging for relative and absolute protein quantitation (iTRAQ) (17), and ^{18}O-enriched water (13, 19), are well recognized because they are quite versatile and readily used to incorporate peptides isolated from virtually any proteins despite their known shortcomings, such as their susceptibility to sample manipulation error (20–23).

To further advance the study on quantitative proteomics and phosphoproteomics and to efficiently measure the phosphorylation levels of a large number of phosphosites using mass spectrometer, an in vivo stable isotope-labeling method has been introduced into this field. The differential peptide abundance can be measured through in vivo metabolic ^{15}N labeling, in which a heavy isotopic tag, either ^{15}N or ^{13}C, in the form of salt, amino acid, or sugar, is mixed into the food or medium for an organism. When this organism such as *Arabidopsis* grows on the labeled supporting media, it presumably assimilates the heavy stable isotopes into the entire protein complement. Because the in vivo metabolic labeling has little measureable

detrimental effect on the growth and development of an organism, this in vivo labeling approach can be especially useful for experiments involving smaller model plants, such as *Arabidopsis*. The key advantage of this in vivo labeling approach is that the mixing of a pair of in vivo metabolically labeled plant protein samples at the earliest step of manipulation possible eliminates the deviation of a peptide ion measurement resulting from multiple steps of sample manipulation throughout an extensive peptide preparation process. Stable isotope labeling with amino acids in cell culture (SILAC) (14) is an in vivo metabolic labeling technique used frequently, in which isotope-coded amino acids (such as Lys or Arg) labeled with ^{15}N or ^{13}C are incorporated into proteins of an organism (24–26). Alternatively, ^{15}N- or ^{13}C-labeled salts or sugars are being incorporated into the organism studied (27). This type of stable isotope metabolic-labeling approach has been applied successfully onto numerous model organisms for quantitative proteomic studies (9, 18, 28–34). Moreover, ^{15}N metabolic labeling has been applied for top-down proteomics and serves as a standard to evaluate other quantitative proteomics techniques, such as DIGE (35) and spectral counting (36). In some cases, both ^{13}C and ^{15}N labeling are combined together to measure protein abundance of three different biological samples (37).

Thus, the advancement in quantitative proteomics in general and phosphoproteomics in specific prompts us to establish a practical protocol for the study of differential phosphoproteomics. Our protocol integrates the recent advancement in proteomics and depicts the processes of both *label-free* (11) and stable isotope labeling in *Arabidopsis* (SILIA) labeling methods. Both are specially designed for *Arabidopsis* plant growing on the solid agar medium because this solid medium is a common growth condition widely used by many laboratories around the world to carry out *Arabidopsis* mutant screens and physiological studies. This protocol should be a useful application of functional phosphoproteomics in plant cell signaling and pioneer an alternative workflow, in addition to genetic screening of *Arabidopsis* mutants,for identification of signaling components mediating the intricate phosphor-relay network during plant cell signaling: i.e., *MS/MS- and bioinformatics-based phosphosite identification → validation by in vitro kinase assay → in planta validation of the functional role of putative phosphorylation site by site-directed mutagenesis.*

2. Materials

2.1. Plant Growth and Harvest

1. MS medium for *label-free* plant growth (11): Murashige and Skoog (MS) basal salt mixture (Sigma-Aldrich, St. Louis, MO) 4.33 g/L, sucrose 10 g/L, 1 mg/L thiamine HCL, 0.1 mg/L

pyridoxine, 0.1 mg/L nicotinic acid, 100 mg/L myo-inositol, and 0.8% bacteriological agar. Adjust pH to 5.7 by KOH.

2. Plant growth medium for in vivo SILIA (38): 9 mM KNO_3 or $K^{15}NO_3$, 0.4 mM $Ca_5OH(PO_4)_3$, 2 mM $MgSO_4$, 1.3 mM H_3PO_4, 50 μM Fe-EDTA, 70 μM H_3BO_3, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM Na_2MoO_4, 10 μM NaCl, 0.01 μM $CoCl_2$, 10 g/L sucrose, 1 mg/L thiamine HCL, 0.1 mg/L pyridoxine, 0.1 mg/L nicotinic acid, 100 mg/L myo-inositol, and 0.8% bacteriological agar. Adjust pH to 5.7 by KOH.

This medium formula was modified from MS medium and specially designed for *Arabidopsis* growing on solid medium in plate or jar. The medium should be made in two separate sets and labeled clearly as a light nitrogen medium (^{14}N, from normal KNO_3) or a heavy nitrogen medium (^{15}N, from $K^{15}NO_3$).

2.2. Protein Sample Preparations

1. Urea extraction buffer (UEB) is designed for the initial dissolving of plant cell lysate (38), which is 150 mM Tris–HCl, pH 7.6, 8 M urea, 0.5% SDS, 1.2% TritonX-100, 20 mM EDTA, 20 mM EGTA, 50 mM NaF, 2 mM $NaVO_3$, and 1% Glycerol 2-phosphate disodium salt hydrate, stored at 4°C. Add the following compounds immediately to make a final concentration of 1 mM PMSF, 5 mM DTT, 0.5% *phosphatase inhibitors cocktail 2* (and *phosphatase inhibitors cocktail 1*, which can be purchased only in some regions and countries), 1× complete EDTA-free protease inhibitors cocktail, 5 mM ascorbic acid, and 2% PVPP. The final UEB solution looks brownish and can be stored at −80°C for experimental use for at least 3 months.
2. Resuspension buffer (RSB): 50 mM Tris–HCl, pH 7.6, 8 M urea, 10 mM DTT, 1% SDS, and 10 mM EDTA. Stored at 4°C.
3. Precipitation solution: Acetone:methanol (12:1), precooled at −20°C.
4. Rinse solution: Acetone:methanol:H_2O (12:1:1.4), precooled at −20°C.

2.3. SDS-PAGE and In-Gel Digestion

1. Coomassie staining buffer: 0.2% Brilliant Blue G250 in 20% methanol, 0.5% acetic acid.
2. Destain buffer: 20% methanol, 0.5% acetic acid.
3. Wash buffer: 50% acetonitrile (ACN)/25 mM NH_4HCO_3.
4. DTT solution: 10 mM DTT in Milli-Q water.
5. IAA solution: 55 mM iodoacetamide (IAA) in 25 mM NH_4HCO_3. Use freshly made solution every time and keep solution in dark.

6. Trypsin solution: 30 ng/μl TPCK-treated trypsin in 25 mM NH_4HCO_3.
7. Extraction buffer: 1% formic acid in 50% ACN.

2.4. Ion Exchange Chromatography and IMAC/TiO_2 Enrichment

1. Ion exchange chromatography (SCX) buffer set: (A) 5 mM KH_2PO_4, pH 2.65, 30% ACN (*v*/*v*), (B) 5 mM KH_2PO_4, 350 mM KCl, pH 2.65, and 30% ACN (*v*/*v*).
2. Ion exchange gradient: 0–1 min, 0% Buffer B; 1–12 min, 15% Buffer B; 12–18 min, 35% Buffer B; 18–22 min, 100% Buffer B; 22–26 min, 100% Buffer B; 26–27 min, 0% Buffer B; and 27–40 min, 0% Buffer B. Flow rate: 1 ml/min.
3. C18 reverse phase (RP) column (Oasis HLB, waters) for peptide desalt.
4. NTA-agarose beads (Sigma).
5. $FeCl_3$ solution: 0.1 M $FeCl_3$ in Milli-Q water, freshly made before use.
6. IMAC loading buffer: 6% acetic acid/30% ACN. pH must be less than 3.0.
7. IMAC elution buffer: 200 mM ammonium phosphate, pH 4.5.
8. TiO_2 equilibration/washing buffer: 1 M glycolic acid, 5% TFA, 80% ACN.
9. TiO_2 elution buffer: 1% ammonium hydroxide.
10. TiO_2 beads (GL science Inc, Tokyo, Japan).

2.5. Ziptip and LC–MS/MS Data Acquisition/Analysis

1. Ziptip equilibrium/wash solution: 0.1% formic acid.
2. Ziptip elution solution: 1% formic acid in 50% methanol.
3. Buffer A for LC/MS RP column: 0.1% formic acid in water.
4. Buffer B for LC/MS RP column: 0.1% formic acid in ACN.

2.6. Buffer for In Vitro Kinase Assay

1. 20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM $Na_4P_2O_7$, 1 mM glycerophosphate, and 1 mM NaF; store in 4°C. Add 1 mM Na_2MoO_4, 1 mM Na_3VO_4, 1× complete EDTA-free protease inhibitors cocktail, and 1 mM PMSF freshly before use.
2. Activation mix: 10 ml 50% glycerol, 0.5 ml 50 mM ATP, 0.6 ml 1 M $MgCl_2$, and 0.15 ml 10 mg/ml BSA.
3. Trypsin digestion buffer: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1 mM $CaCl_2$, and 10 mM $MgCl_2$.
4. Synthetic peptides with the target sequences were synthesized by peptide synthesis companies on the market.

3. Methods

3.1. Plant Growth and Harvest

1. The *Arabidopsis* seeds are surface sterilized before imbibed at 4°C for 3 days in dark. Glass jars with 9 cm in diameter and 15 cm in height are autoclaved. Plant nutrient agar medium (40 ml) are poured into each jar and cooled to dry overnight in hood. For *label-free* method, only MS medium is used and not necessary to label the jar. For metabolic labeling with ^{15}N, jars were labeled to distinguish the ^{15}N medium from the ^{14}N medium.
2. Seeds are then suspended in 0.1% (*w*/*v*) agar and sown in rows on plant nutrient agar medium within the jar. Plant about 25 seeds in each jar. The distance between each seed is about 0.8 cm (see Note 1).
3. Jars with planted seeds are transferred to plant growth chambers (16-h light/8-h dark cycle, with constant temperature of 20°C). After 3 weeks, the seedlings are placed in airflow chamber for 5 h to eliminate endogenous ethylene (see Note 2).
4. Adjust the gas flow rate to fill one cultivation jar within 4 s. For *label-free* experiment, divide the jars into two fractions, treated and untreated, respectively, for 15 min or any other time frames. For SILIA experiment, seedlings grown on ^{14}N are treated with air and ^{15}N-labeled seedlings are treated with a hormone or an external inducer for a period of time. To avoid variance induced by different isotopic incorporation, two sets of reciprocal labeling are required.
5. Harvest the seedlings with liquid nitrogen and preserve the tissue in −80°C freezer.

3.2. Protein Sample Preparation

1. Seedlings (10 g) are ground into powders with liquid nitrogen in a precooled mortar. To effectively denature plant proteins for the purpose of freezing phosphor group onto phosphosite and to prevent in vitro nonspecific enzyme catalysis by kinase and phosphatase during protein preparation, a phosphoprotein extraction buffer UEB is employed during plant protein isolation. The tissue powder is then mixed with 50 ml UEB buffer and ground for 2 min. The cell lysate is transferred to centrifuge tubes and centrifuged at 10°C for 2 h (RCF = 110,000 × *g*, rpm = 39,359 for TST 60.4 Sorvall rotor). The high-speed centrifugation is designed to remove cell debris, DNA, cell wall, lipid, and RNA. The presence of these macromolecules interferes protein resuspension and separation on SDS-PAGE.
2. The supernatant is mixed with 3× volumes of precipitation solution and kept at −20°C for 2 h to precipitate proteins.

3. Centrifuge in Beckman JA10.5 rotor with 15,000 × g at 10°C for 20 min to pellet the protein.
4. Pour off supernatant, and rinse the pellet with 10 ml cold rinse solution to remove residue pigment and precipitated urea.
5. Pellet is dried in open air until there is no significant liquid droplet (about 10 min).
6. Protein pellet is then dissolved with 10 ml of RSB (see Note 3). Precipitate the protein again with 3× volumes of RSB at −20°C for 2 h.
7. Centrifuge with Beckman JA25.5 rotor (11,000 rpm at 10°C for 20 min) to pellet protein.
8. Pour off supernatant, and rinse the pellet with 10 ml cold rinse solution to remove residue pigment and precipitated urea. The pellet is dried in air. Resuspend the pellet in RSB with a final volume of 6 ml.
9. Freeze and keep the protein sample in −80°C. The protein concentration is determined by protein DC assay (BioRad).

3.3. SDS-PAGE and In-Gel Digestion

For *label-free* experiment, skip the sample mixing step (step 1 in this section) and start from step 2 to run the protein sample directly on SDS-PAGE gel.

1. Mix both ^{14}N- and ^{15}N-labeld protein samples extracted from ^{14}N-labeled tissue (untreated) and that from ^{15}N-labeled tissue (treated) at 1:1–1.5 ratio (depending on the actual ^{15}N labeling efficiency).
2. Load 40 mg of proteins onto four preparative SDS-PAGE (180 × 190 × 1–1.5 mm, 10%) gels evenly. For *label-free* experiment, both treated and untreated samples need to be loaded onto two separate sets of gels. Electrophoresis is stopped when the bromophenol blue dye migrates approximately 10 cm into the resolving gel.
3. Each gel is lightly stained with Coomassie blue (immersed in staining buffer for 10 min with gentle shake), destained, and fixed with destain buffer for 30 min (see Note 4).
4. Each gel is evenly cut into 5–50 strips. Identical strips from different gels are combined and further diced into 1-mm^3 cubes (see Note 5). The cubes from the same strip are collected into a 50-ml falcon tube.
5. Gel cubes are washed with 8 ml wash buffer for 15 min with shake. The wash buffer is then pipetted out and the wash step is repeated for two times.
6. Dehydrate the gel with 5 ml 100% ACN, pour off the solution, and dry the gel completely by flushing with compressed air.
7. Gel cubes are immersed in 5 ml of 10 mM DTT at 56°C for 1 h for reduction.

8. Remove DTT solution. Gel cubes are alkylated with 5 ml IAA solution at room temperature for 1 h. This step should be conducted in dark.
9. Wash the gel with 8 ml of wash buffer for additional two times. Dehydrate and dry the gel as in step 6.
10. The gel cubes is rehydrated in 2.5 ml trypsin solution on ice for 30 min (see Note 6) and then digested overnight at 37°C.
11. Sonicate the falcon for about 15 min, and then collect the supernatant. To further extract the digested peptides, 2 ml extraction buffer is added into the falcon. Falcon tube is sonicated for 15 min again and supernatant is collected. Repeat the extraction step for additional three times.
12. Pour off the supernatant of the same stripes together. The peptide solution is then flushed by compressed air until all ACN is removed from the solution.
13. The peptide solution is frozen by liquid nitrogen and concentrated by lyophilizer. The peptide powder could be stored at −80°C.

3.4. Ion Exchange Chromatography and IMAC/TiO_2 Enrichment

1. Peptide powder is reconstituted with 1.04 ml of 5 mM KH_2PO_4 (pH 2.65) and centrifuged at 4°C for 5 min with a maximum speed of a benchtop centrifuge to remove the insoluble.
2. The mixture of 20 μl is reserved for quality control and peptide concentration measurement.
3. Not more than 1.6 mg of peptide is loaded onto the ion exchange chromatography column to avoid overloading. Run the SCX in the program described in Subheading 2.4. Twelve fractions were collected from 2 to 26 min (2 min per fraction).
4. Fraction no. 1–3 and 10–12 are combined due to their low abundance of peptide. All the fractions are evaporated to remove the existence of ACN, then frozen by liquid nitrogen, and lyophilized to half of the original volume.
5. Activate the C18 RP column (Oasis) with 1 ml ACN.
6. The column is then equilibrated with 1 ml 0.1% formic acid.
7. Load the peptide samples from step 4 onto C18 column with syringe and reload for at least three times for maximum binding.
8. Wash the column with 1 ml 0.1% formic acid.
9. The peptide bound to the column is eluted with 1 ml 80% ACN/0.1% FA. Use spin vacuum to concentrate the eluted sample.
10. Pipette 200 μl NTA agarose beads into a 1.5-ml Eppendorf tube, and centrifuge at 5,000 rpm in a benchtop centrifuge for 30 s to remove storage buffer.

11. Equilibrate the beads with 1 ml 6% acetic acid. Wash the beads for 30 s and centrifuge at 5,000 rpm for 30 s. Remove the supernatant.
12. Add 1 ml 0.1 M $FeCl_3$ to the Eppendorf. Incubate the beads at 4°C with end-over-end rotation for 2 h.
13. Spin down the beads, and remove the charge buffer. Wash the beads with 1 ml 6% acetic acid for three times to remove free iron ions.
14. Equilibrate the beads with 1 ml IMAC loading buffer for three times.
15. The concentrated peptide sample from step 9 is reconstituted with Fe-IMAC loading buffer. For each milligram of peptide sample, 200 µl Fe-IMAC loading buffer is used to dissolve the sample in a new Eppendorf. The peptide is then incubated with 30 µl Fe^{3+}-NTA beads (from step 14) via a 45-min end-over-end incubation.
16. Spin down the beads. Remove and save the flow-through fraction. Fe-IMAC loading of 350 µl buffer is added to the Eppendorf to wash away the nonspecific binding peptides. Wash twice with Fe-IMAC loading buffer and once with water.
17. The phosphopeptides enriched by Fe-NTA beads are eluted with 50 µl elution buffer. Save the eluted sample in −20°C.
18. Add 800 µl of TiO_2 equilibration/washing buffer to flow-through fraction from step 16, and then incubate the solution with 5 mg of TiO_2 beads for 45 min (see Note 7).
19. The TiO_2 beads are washed twice with TiO_2 equilibration/washing buffer and once with water (200 µl for each step).
20. The enriched phosphopeptides are eluted with 50 µl elution buffer and combined with the samples obtained from step 17 and saved at −20°C before being passed through Ziptip enrichment.

3.5. Ziptip and LC–MS/MS Data Acquisition/Analysis

1. Acidify the peptide sample with 20 µl formic acid (see Note 8).
2. Aspirate 12 µl ACN into Ziptip, dispense to waste, and repeat three times.
3. Aspirate 12 µl Ziptip equilibrium/wash solution, dispense to waste, and repeat five times.
4. Aspirate the acidified sample and dispense 20 rounds for efficient binding.
5. Aspirate 12 µl Ziptip equilibrium/wash solution, dispense it to the waste, and repeat five times.
6. Elute the sample with 40 µl elution solution. Freeze and dry the sample in a spin vacuum. The sample is then reconstituted in 10 µl 0.1% formic acid and ready for LC/MS analysis.

7. LC–MS/MS is performed with a nanoflow LC (nano AcquityTM, Waters) coupled to an ESI-hybrid quadrupole time-of-flight (Q-TOF) Premier tandem mass spectrometer (Waters). The program MassLynx (version 4.1, Waters) is used for data acquisition and instrument control. A 180 μm × 20 mm Symmetry C18 trap column and 75 μm × 250 mm BEH130 C18 analytical column are used. The mobile phases are 0.1% HCOOH/H2O (A) and 0.1% HCOOH/CH3CN (B). LC gradient elution condition is initially 1–5% B (5 min), 40% B (90 min), 99% B (94–109 min), and then initial concentration 1% B (110–120 min), with a flow rate of 200 nl/min.
8. The mass spectrometer (Waters Q-TOF Premier) is operated in a positive ion mode with following basic parameters: source temperature is 80°C, capillary voltage is 2.4 kV, sample cone voltage is 35 V, and collision cell gas flow rate is 0.50 ml/min. The collision energy is variable during MS/MS scan according to *z* and *m/z* and the exact values are from factory instructions. Data-dependent analysis is set as below: 1-s MS *m/z* 250–2,000 and max 3-s MS/MS *m/z* 50–2,000 (continuum mode), 30-s dynamic exclusion. Three most abundant, +2, +3, or +4 charged ions, whose intensity rising above 40 counts/s, are selected in each MS/MS scan.
9. Raw data are processed using ProteinLynx 2.2.5 (smooth 3/2 Savitzky Golay and center 4 channels/80% centroid), and the resulting MS/MS dataset is searched against TAIR database (download from www.arabidopsis.org and specific for *Arabidopsis*) using MASCOT search engine. The settings in the workflow template are as follows: trypsin digestion with up to two missed cleavage sites are allowed; 100 ppm mass tolerance for MS precursor ions and 0.1 Da mass tolerance for MS/MS fragment ions; carbamidomethylation (C) is specified as a fixed modification and phosphorylation (S, T, and Y), deamidation (N, Q), and oxidation (M) are allowed as variable modifications. Figure 1 is an example to show the phosphopeptides discovered in a phosphoproteomics analysis (11). Figure 2 shows an example of light and heavy isoforms of a single phosphopeptide.

3.6. In Vitro Kinase Assay

The in vitro kinase assay is used as a useful tool to validate the phosphorylation sites identified by phosphoproteomics and bioinformatics-predicted sites (14). Once the phosphopeptides are identified, a short of stretch (21–30 amino acids long) of polypeptide containing the newly identified phosphosite (S, T, or Y), the highly conserved amino acid sequence motif surrounding the phosphosite, is fused to a HisTag (6× histidine) at C-terminus. This hybrid peptide is synthesized chemically and used as a substrate for in vitro plant kinase assay.

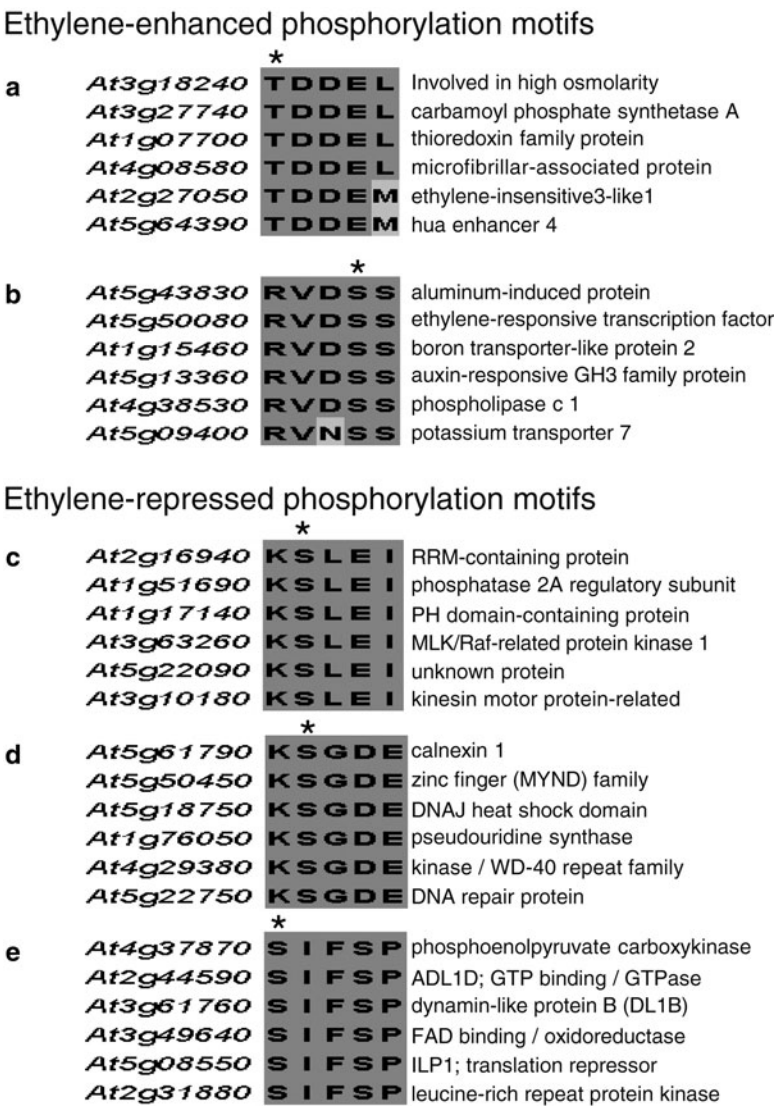

Fig. 1. Bioinformatics analysis of phosphopeptides and construction of phosphorylation motifs using the protein sequence database. (**a**) *T*DDEL, (**b**) RVD*S*S, (**c**) K*S*LEI, (**d**) K*S*GDE, and (**e**) *S*IFSP are five conserved phosphorylation motifs built from both authentic phosphopeptides and protein sequences deposited in the database. The phosphopeptide sequences determined by phosphoproteomic analysis are placed on the top of each group, whereas the rest are the proteins with annotations found in databases. Proteins with unknown functions were omitted (11).

1. Fresh tissue powder of 100 mg is mixed with 300 μl extraction buffer. The mixture is vortexed and incubated on the ice for about 10 min. The cell lysate is then centrifuged at 4°C for 10 min at 14,000 × *g*.

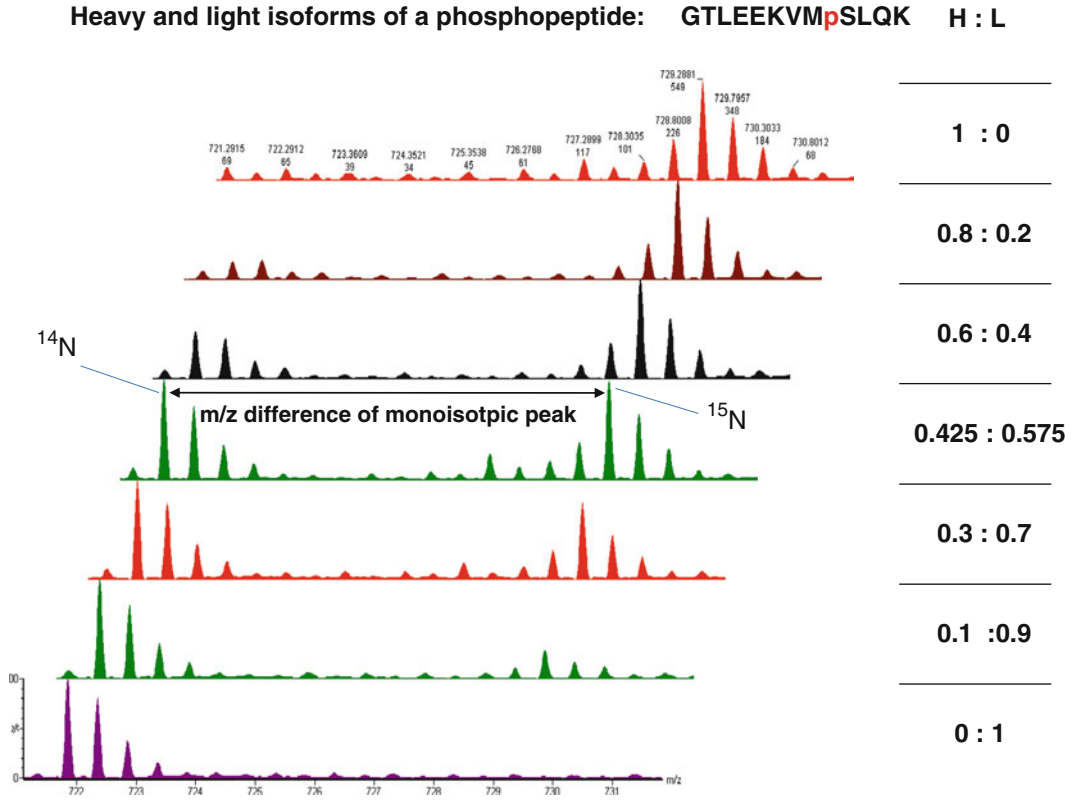

Fig. 2. The mass shift of $^{14}N/^{15}N$-labeled $[M+2H]^{2+}$ precursor ion is 6.9852 *m/z*, corresponding to 13.9704 Da, indicating that the peptide has 14 nitrogen atoms. $^{14}N/^{15}N$-labeled total protein mixtures were extracted from *Arabidopsis thaliana* tissue separately, mixed with different ratios (16:1, 8:1, 2:1, 1:1.3, 1:2, 1:8, and 1:16), and in-solution digested. The resulting peptide was desalted. Fe^{3+}-NTA beads were used to purify phosphopeptides in each sample. The eluting peptides are then subjected to reverse-phase LC–MS/MS analysis. The MS spectrums were acquired by ultra-performance liquid chromatography (nanoAcquity) coupled to a Q-Tof mass spectrometer (micromass, Waters Corporation).

2. Cell lysate of 100 µl is taken out to mix with 25 µl activation mix, 10 µg peptides, and 2.8 µl 50 mM ATP, and incubated at 30°C for 1 h.
3. Ni-NTA beads are firstly equilibrated with extraction buffer. Each sample solution requires 60 µl Ni-NTA slurry. Histidine-tagged substrate peptide is then purified from plant kinase extracts and used for LC–MS/MS analysis. The assayed peptides are purified via an end-over-end rotation for 10 min. Wash with 1 ml trypsin digestion buffer for three times. Add 90 µl trypsin digestion buffer and 1 µg trypsin, and incubate at 37°C for 4 h. Add 10% (*v*/*v*) acetic acid to terminate the reaction and save the eluate solution.
4. Use Ziptip to remove the salts from the digested sample as stated in Subheading 3.5. The sample is then subject to LC/MS MS analysis. Figure 3 is an example of in vitro kinase assay result (14).

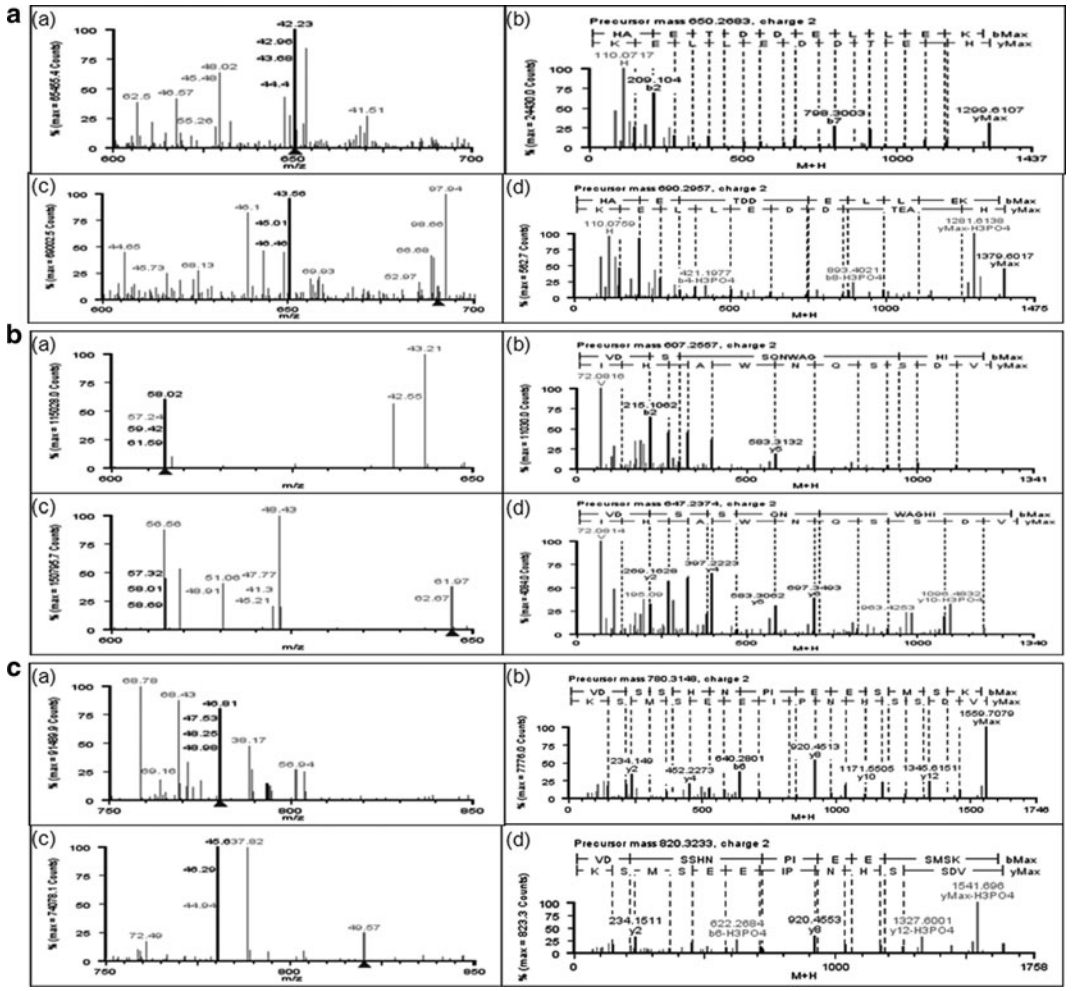

Fig. 3. MS and MS/MS spectra of the phosphorylated peptides produced from the in vitro kinase assays. (**a**) HAETDDELLEK, (**b**) VDSSQNWAGHI, and (**c**) VDSSHNPIEESMSK are three synthetic peptides derived from phosphorylation motifs and used as substrates in the in vitro kinase assays. (**a**, **b**) MS and MS/MS spectra of the substrate peptides, respectively. (**c**, **d**) MS and MS/MS spectra of the *Arabidopsis* kinase-treated synthetic substrate peptides, respectively. Retention time of these phosphopeptides is marked on the top of the mass ion; the precursor ion having the MS/MS spectrum is indicated by an *arrow* underneath, whereas the precursor masses and charges are displayed in the MS/MS spectrum (11).

3.7. Validation of the Functional Role(s) of a Phosphosite in Cell Signaling Using Site-Directed Mutagenesis

Provided that a phosphorylation site has been identified either from MS/MS analysis or bioinformatics-based prediction, the next immediate work is to make point mutations on the phosphosite. Usually, a pair of mutations will be made on the selected phosphosite: S (or T) is mutated to A or I, and Y to F, which is equivalent to a constant dephosphorylation-mimic mutation, and alternatively, S (or T, Y) is mutated to D or E, a constant phosphorylation-mimic mutation. This pair of site-directed mutant genes together with its corresponding wild-type gene are transformed into *Arabidopsis* to examine the in planta function of the phosphorylated protein of

interest. Phenotypes of all three transgenic plants that ectopically express mutant proteins and the control wild-type proteins are analyzed and compared to determine the possible roles of phosphorylation site in planta.

4. Notes

1. Too many seeds per jar will make seedlings too crowded to grow well, while too few seeds per jar will make it hard to collect enough tissue for experiment.
2. Although 150 μM AOA is added to the medium and supposed to remove majority of the endogenous ethylene production, there is still trace amount of ethylene produced. This step intends to remove the endogenous ethylene as much as possible so that the effect of ethylene treatment would be more obvious.
3. Pipette up and down slowly to dissolve. Avoid introducing bubbles into the protein sample to save protein from degradation.
4. Prolonging the time of stain will make the dye hard to be removed and, therefore, interfere the downstream steps.
5. Cube size is crucial for protein digestion efficiency and peptide extraction efficiency. Large pieces of gel make the trypsin hard to be taken into the gel and the digested peptides are hard to be extracted out. However, cube smaller than 1 mm^3 causes trouble when exchanging buffers.
6. This step is crucial for efficient enzyme digestion. Wait until all the gel pieces become rehydrated. Add more trypsin solution if some of the gel pieces still not rehydrate.
7. This is to enrich the leftover phosphor peptides in the flow-through fraction of Fe^{3+}-NTA beads, as TiO_2 beads is less sensitive with salt interference during purification process.
8. Sample's pH needs to be lower than 3 for efficient binding to the Ziptip matrix.

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