

Selective Enrichment Coupled with Proteomics to Identify S-Acylated Plasma Membrane Proteins in *Arabidopsis*

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Protein S-acylation, predominately in the form of palmitoylation, is a reversible lipid post-translational modification on cysteines that plays important roles in protein localization, trafficking, activity, and complex assembly. The functions and regulatory mechanisms of S-acylation have been extensively studied in mammals owing to remarkable development of high-resolution proteomics and the discovery of the S-acylation-related enzymes. However, the advancement of S-acylation studies in plants lags behind that in mammals, mainly due to the lack of knowledge about proteins responsible for this process, such as protein acyltransferases and their substrates. In this article, a set of systematic protocols to study global S-acylation in *Arabidopsis* seedlings is described. The procedures are presented in detail, including preparation of *Arabidopsis* seedlings, enrichment of plasma membrane (PM) proteins, ensuing enrichment of S-acylated proteins/peptides based on the acyl-biotin exchange method, and large-scale identification of S-acylated proteins/peptides via mass spectrometry. This approach enables researchers to study S-acylation of PM proteins in plants in a systematic and straightforward way. © 2020 Wiley Periodicals LLC.

Basic Protocol 1: Preparation of *Arabidopsis* seedling materials

Basic Protocol 2: Isolation and enrichment of plasma membrane proteins

Support Protocol 1: Determination of protein concentration using BCA assay

Basic Protocol 3: Enrichment of S-acylated proteins by acyl-biotin exchange method

Support Protocol 2: Protein precipitation by methanol/chloroform method

Basic Protocol 4: Trypsin digestion and proteomic analysis

Alternate Protocol: Pre-resin digestion and peptide-level enrichment

Keywords: acyl-biotin exchange (ABE) • palmitoylation • plasma membrane proteins • proteomics • S-acylation

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INTRODUCTION

Protein S-acylation, whereby a 16-carbon (palmitate) or 18-carbon (stearic acid) fatty-acid chain is covalently linked to a cysteine residue through a thioester bond, is a reversible post-translational modification, which makes it unique among lipid modifications (Turnbull & Hemsley, 2017). The S-acylation state of proteins can dynamically regulate protein-membrane affinity and thus impact a variety of biological events in eukaryotes, e.g., cellular signaling, metabolism, and pathogenesis (Li & Qi, 2017). Although progress has been made toward understanding many aspects of S-acylation in yeast and mammals, little is known regarding the role or extent of S-acylation in plants. Identification and characterization of functional S-acylated proteins will enhance our understanding of the biological roles of S-acylation in plants.

In the last two decades, S-acylproteomic approaches have greatly accelerated the characterization of S-acylated proteins and enabled researchers to study the dynamics of protein S-acylation at a global level. An acyl-biotin exchange (ABE) method (Drisdell & Green, 2004) coupled with mass spectrometry (MS) has been widely used to achieve proteomic profiling of S-acylated proteins and to validate individual proteins' S-acylation (Collins, Woodley, & Choudhary, 2017b; Hemsley, Weimar, Lilley, Dupree, & Grierson, 2013; Yang, Di Vizio, Kirchner, Steen, & Freeman, 2010). The current set of protocols reflects our efforts to optimize the ABE method for plants and to achieve better enrichment in a facile manner. For example, although S-acylation of a large number of plasma membrane (PM) proteins (e.g., receptor-like kinases, or RLKs) may be of biological interest, they are difficult to detect due to their relatively low expression levels. Considering this, we adopted the Brij-58 treatment method (Collins, Leslie, Peck, & Heese, 2017) to enrich the PM proteins prior to ABE and proteomics. Through this approach, our protocols successfully identify a number of PM proteins with potential S-acylation sites.

The approach described here includes four parts, as shown in Figure 1: (i) methods for preparation of *Arabidopsis* seedling materials in Murashige and Skoog agar medium (MS agar medium; Basic Protocol 1); (ii) isolation of total proteins from plant tissues and enrichment of PM proteins based on a simplified detergent-dependent method (Basic Protocol 2) and measurement of protein concentration using BCA assay (Support Protocol 1); (iii) optimized enrichment of S-acylated proteins based on the ABE method (Basic Protocol 3) and protein precipitation using methanol/chloroform (Support Protocol 2); and (iv) S-Trap trypsin digestion and MS-based identification of S-acylated proteins (Basic Protocol 4) and pre-resin digestion and peptide-level enrichment for the identification of specific sites of S-acylation (Alternate Protocol).

BASIC PROTOCOL 1

PREPARATION OF ARABIDOPSIS SEEDLING MATERIALS

To obtain sufficient seedling materials for PM protein enrichment (Basic Protocol 2) and subsequent S-acylation enrichment (Basic Protocol 3), a practical method of germinating and growing *Arabidopsis* on MS agar medium in a plant growth chamber is described. The protocol includes surface sterilization of seeds with bleach and a method of placing single seeds onto MS agar medium rapidly and evenly. This protocol makes it possible to maintain consistency between genotypes and between different sets of experiments and also simplifies the biological treatment procedure if required.

Materials

Arabidopsis Col-0 seeds

Bleach solution: 20% (v/v) commercial bleach and 0.1% (v/v) Triton X-100

Sterile water

$\frac{1}{2}$ MS agar medium plates (see recipe)

Liquid nitrogen

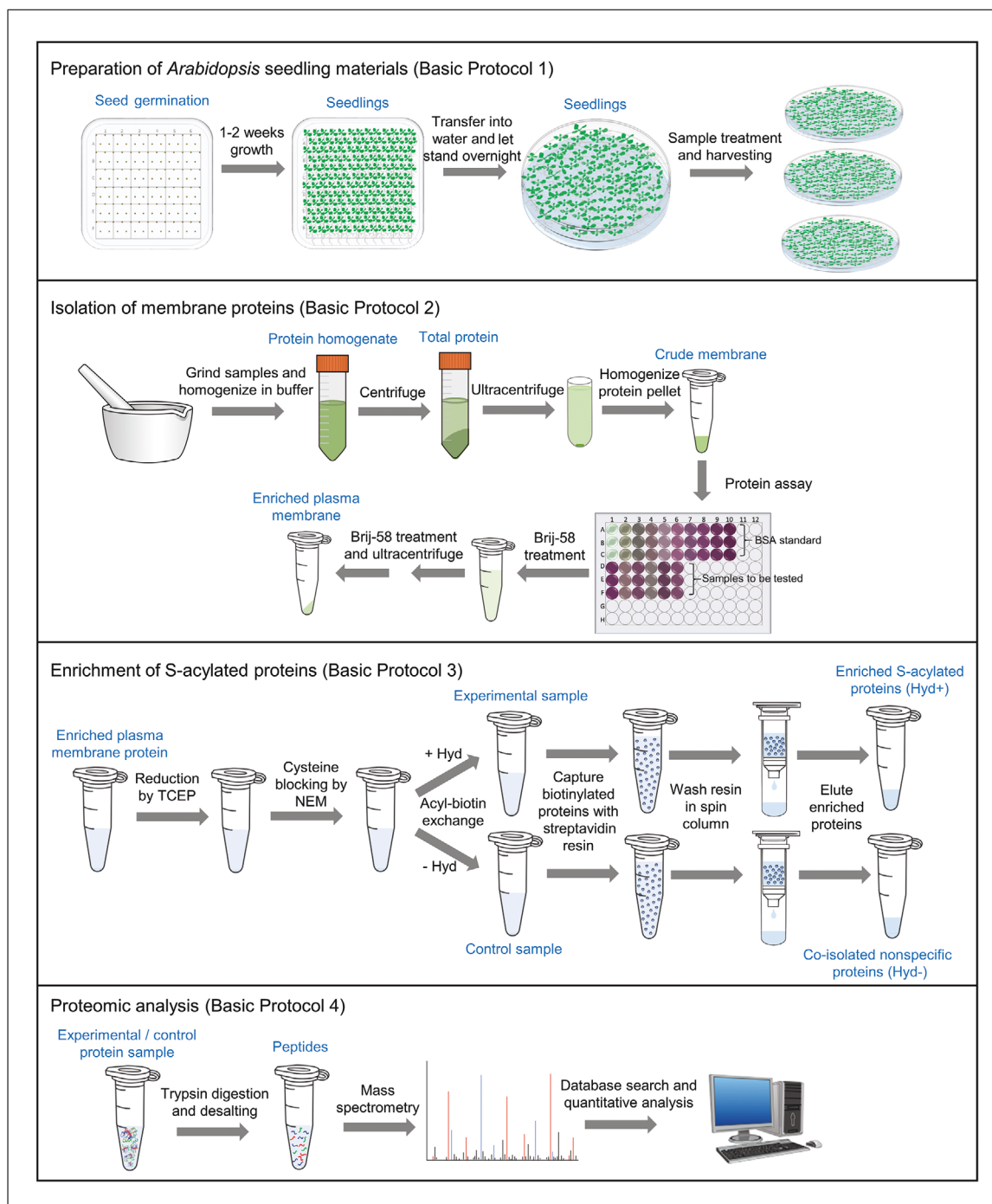


Figure 1 Workflow for purification and identification of S-acylated proteins in *Arabidopsis* seedlings. *Arabidopsis* seeds are germinated and grown on $\frac{1}{2}$ MS agar medium plates in a growth chamber and harvested after treatment with the required conditions (Basic Protocol 1). Membrane proteins are isolated and enriched using a simplified method involving Brij-58 treatment and ultracentrifugation (Basic Protocol 2). Proteins with S-acylation are reduced by TCEP, alkylated by NEM and DTDP, and subsequently enriched by acyl-biotin exchange (ABE) and streptavidin resin purification (Basic Protocol 3). Enriched proteins are trypsin-digested and identified by mass spectrometry (Basic Protocol 4).

2-ml Eppendorf microcentrifuge tubes
 3D rotator (Fisher Scientific, cat. no. 260100F)
 Mini centrifuge (BioExpress)
 Laminar flow hood (Labconco)
 Sterile 1-ml pipet tips
 Micropore tape (3M, cat. no. 1530-0)

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Plant growth chamber (22°C, 16-hr light/8-hr dark)
Forceps
Deep petri dishes (25 × 100 mm; Fisher, cat. no. 08-757-10E)

Additional reagents and equipment for biological treatment (e.g., biotic or abiotic stress, nutrition, light)

Seed sterilization

1. Put required volume of *Arabidopsis* Col-0 seeds in a 2-ml Eppendorf microcentrifuge tube.

To sufficiently sterilize the seeds (step 2), put a ≤ 300 - μ l volume of seeds in one tube. The basal volume of seeds will expand two-fold during the sterilization procedures. Divide the seeds into multiple tubes for sterilization if a large number of seeds is needed.

2. Sterilize seeds with 1 ml bleach solution for 15 min with gentle shaking on a 3D rotator at room temperature.
3. After a short low-speed spin in a mini centrifuge, in a laminar flow hood, remove bleach solution with a pipet and discard.

Execute steps 3 to 5 in a laminar flow hood to maintain aseptic conditions.

4. Wash seeds thoroughly with 1 ml sterile water as in step 3.
5. Repeat step 4 five more times. Add 1 ml sterile water to seeds after the final wash.
6. Store surface-sterilized seeds at 4°C for 3 days for vernalization to synchronize germination.

Seed germination and seedling growth

7. Plate seeds on $\frac{1}{2}$ MS agar medium plates, spaced evenly, with sterile 1-ml pipet tips.

To place individual seeds on a plate, draw 150 μ l seed suspension into a 1-ml pipet tip. This works best if the density of seeds in the seed suspension is adjusted with water to about 300 to 400 seeds per 150 μ l. Hold up the plate and gently touch the end of the pipet to the plate, and the seeds should flow out uniformly via surface tension. Put white printer paper under the plate as a background to help distinguish seeds.

A density of 13 × 13 seeds per square plate (as shown in Fig. 1) is preferred in this experiment. Using this density, the seedlings can grow healthily, and moreover, you can harvest plenty of seedling materials to accomplish the protein enrichment procedures (Basic Protocols 2 and 3).

8. Dry plates in the hood with the lids slightly off for 10 min. Then, seal plates with micropore tape to prevent desiccation.
9. Place plates vertically in the plant growth chamber. Grow seedlings for 1 to 2 weeks at 22°C with a 16-hr light/8-hr dark cycle.

We can obtain ~2.5 g of seedlings per square plate if seeds are placed with a density of 13 × 13 per square plate and grown for 2 weeks under normal conditions.

10. Using forceps, gently transfer seedlings to deep petri dishes containing 40 ml sterile water. Place transferred seedlings in the growth chamber overnight.

This step is necessary if a large number of seedlings is needed for protein extraction. The step also reduces the wound response of seedlings, homogenizes the samples, and benefits the following biological treatments (step 11).

11. Conduct biological treatment (e.g., biotic or abiotic stress, nutrition, light) as needed.
12. Take seedlings out of the treatment solution, remove excess water with a paper towel, and immediately freeze samples in liquid nitrogen. Store at –80°C until further use.

We need ~10 g of seedlings per sample for this experiment. Growth to 10 g of seedlings requires four square plates if seedlings are planted for 2 weeks at the suggested density.

**BASIC
PROTOCOL 2**

ISOLATION AND ENRICHMENT OF PLASMA MEMBRANE PROTEINS

This protocol is used only if there is a specific interest in analyzing S-acylation of PM proteins. Enrichment of PM is essential in order to identify more S-acylated PM proteins, which often are present in whole-cell extracts at low levels. A number of PM enrichment methods are available, but most are time consuming and result in low recovery. As with many techniques, there is a trade-off of increased enrichment versus overall yield. Here, we employ a simplified enrichment procedure using the detergent Brij-58 and ultracentrifugation (Collins, Leslie, et al., 2017). The method is straightforward, easy to follow, and reproducible. Our testing of various methods indicates that although this method may not provide the greatest enrichment of PM proteins, it is sufficient for subsequent S-acylation analysis while maintaining good yield.

Materials

Liquid nitrogen
Frozen *Arabidopsis* seedling samples (see Basic Protocol 1)
Brij-58 (Sigma, cat. no. P5884)

50-ml conical tubes (Thermo Fisher, cat. no. 339650), room temperature or 4°C
Mortar and pestle
Sorvall LYNX 6000 Superspeed Centrifuge with F14-14 × 50cy fixed-angle rotor, 4°C
Miracloth (Millipore, cat. no. 475855-1R)
30-ml thick-walled polycarbonate ultracentrifuge tubes (Seton, PN-2005-30 ml)
Sorvall Discovery 100SE Ultracentrifuge with Surespin 630 rotor and 36-ml swinging buckets, 4°C
Paint brush with ~1-cm-long synthetic brush
1.5- and 2-ml Eppendorf microcentrifuge tubes
Platform shaker (New Brunswick™ Innova® 2100)
1.5-ml Eppendorf safe-lock tubes (Fisher, cat. no. 05-402-11)
Sorvall Discovery M150 SE Micro-Ultracentrifuge with S45-A fixed-angle rotor

Additional reagents and equipment for preparing homogenization buffer (H buffer; see recipe) and determining protein concentration (see Support Protocol 1)

Isolation of crude membrane

1. On the day of protein isolation, add proteinase inhibitors to pre-prepared H buffer without inhibitors (see recipe).

Do not add DTT as a reducing reagent because DTT can break the thioether bond of the S-acylation. TCEP, a potent reducing agent that does not cleave thioester bonds, will be used to break disulfide bonds in the protocol for enrichment of S-acylated proteins (see Basic Protocol 3).

2. Dispense 20 ml H buffer into 50-ml conical tubes and keep on ice.

Calculate the volume of H buffer as 2 ml H buffer per 1 g tissue.

3. Take seedlings out of the –80°C freezer and keep them in liquid nitrogen.

Ensure that you can protect your hands completely from the liquid nitrogen while handling the materials within. Using a pair of forceps or wearing a waterproof, insulating glove can protect your skin.

4. Chill a mortar and pestle with liquid nitrogen.

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5. Place a frozen *Arabidopsis* seedling sample (~10 g) into chilled mortar. Grind seedlings with the pestle in liquid nitrogen for > 10 min, until the seedlings become a fine powder.

Keep the samples frozen by slowly adding liquid nitrogen during this procedure. To be consistent, use similar grinding force and time for different samples.

6. Scoop finely ground frozen seedling powder into a 50-ml conical tube containing H buffer on ice (see step 2).

From this point on, all steps must take place on ice or at 4°C.

7. Let seedling powder thaw in the H buffer on ice. Mix homogenate well by gently inverting the tubes every 10 min during the thawing process.

Avoid vortexing because it can break the membranes.

It takes ~1 hr to fully thaw and homogenize the cell lysates.

8. Centrifuge total homogenate for 10 min at $10,000 \times g$, 4°C, in a Sorvall LYNX 6000 Superspeed Centrifuge with an F14-14 $\times$ 50cy fixed-angle rotor.

9. Filter supernatant through one layer of Miracloth into a new, pre-chilled 50-ml conical tube. Squeeze Miracloth layer to collect all the cleared homogenate.

10. Centrifuge filtered homogenate for 20 min at $10,000 \times g$, 4°C.

The supernatant of the homogenate is considered to be total proteins (T). Save ~100 μ l T fraction in a 1.5-ml microcentrifuge tube for further immunoblot analysis. Keep it on ice or store at -20°C.

11. Transfer rest of the total homogenate to a 30-ml thick-walled polycarbonate ultracentrifuge tube. Balance tubes before loading into the 36-ml swinging buckets of the Surespin 630 rotor.

In order to run an ultracentrifuge smoothly and safely at its operating speed, always follow the manufacturer's instruction manual, use compatible ultracentrifuge tubes, and load the proper sample volume. All opposing loads must balance within a certain weight range, as specified by the centrifuge manual.

12. Centrifuge samples for 4 hr at $121,000 \times g$, 4°C, in a Sorvall Discovery 100SE Ultracentrifuge with the Surespin 630 rotor and 36-ml swinging buckets.

Speed (rpm) should be calculated from RCF ($\times g$). Online tools can help with the calculation (e.g., <https://www.sciencegateway.org/tools/rotor.htm>). For the Surespin 630 rotor, a speed of $121,000 \times g$ equates to 260,000 rpm.

Caution should be used when operating any high-speed centrifuge, regardless of brand. Training should be given to users before operating any ultracentrifuge.

13. After ultracentrifugation, gently transfer tubes onto ice.

14. Carefully remove all supernatant without disturbing pellet.

Most of the cytosolic and solubilized proteins are present in the supernatant fraction (S_{121}), and crude membrane proteins (CM) are enriched in the pellet. Save ~100 μ l S_{121} fraction in a 1.5-ml microcentrifuge tube for further immunoblot analysis. Keep it on ice or store at -20°C.

The experiment can be paused here. Freeze the membrane pellet at -20°C overnight.

15. Resuspend CM pellet in 50 μ l cold H buffer using a paint brush with a ~1-cm-long synthetic brush. To thoroughly resuspend the CM proteins, slowly and gently swirl paint brush in the CM in circles, avoiding making bubbles. During this resuspension

step, slowly add more H buffer in 200- μ l increments, up to an approximate total volume of 800 μ l. Rinse paint brush with the last addition.

The pellet appears to be thick and sticky because of the large amount of membrane lipids. It is necessary to add the H buffer in increments because it will be difficult to completely resuspend the pellet if too much buffer is added at once: small pellet pieces will appear and will be difficult to disperse.

16. Transfer resuspended CM to a 1.5-ml Eppendorf microcentrifuge tube. Keep on ice.
17. Determine protein concentration for the CM fraction using a BCA protein assay kit (see Support Protocol 1).

After completely resuspending the CM proteins, save ~ 50 μ l in a 1.5-ml microcentrifuge for further immunoblot analysis. Keep it on ice or store at -20°C .

Enrichment of plasma membrane proteins

18. On the same day as PM enrichment, add proteinase inhibitors to 30 ml pre-prepared H buffer without inhibitors (see recipe). Prepare 10 ml of 1% Brij-58 buffer by dissolving 100 mg Brij-58 in 10 ml H buffer (10 mg/ml) with vigorous shaking on a platform shaker at room temperature. Prepare 10 ml of 0.2% Brij-58 buffer by diluting 2 ml of 1% Brij-58 buffer with H buffer. Chill all buffers on ice.
19. Pipet 8 mg CM proteins into a 1.5-ml Eppendorf safe-lock tube. Add calculated volumes of cold H buffer and 1% Brij-58 buffer to achieve a final Brij-58 volume of 0.15 μ l of 0.2% Brij-58 buffer per microgram of CM protein and a total volume of 1.2 ml. Invert tube several times and incubate on ice for 30 min.

For example, if the protein concentration of the CM fraction is 15 $\mu\text{g}/\mu\text{l}$, $\text{Vol}_{\text{CM}} = 533$ μl ; $\text{Vol}_{1\% \text{ Brij-58}} = 240$ μl ; and $\text{Vol}_{\text{H buffer}} = 1200$ $\mu\text{l} - 533$ $\mu\text{l} - 240$ $\mu\text{l} = 427$ μl .

Add the reagents in the following order: CM fraction, H buffer, 1% Brij-58 buffer.

20. Balance samples with cold H buffer during the incubation.
21. Centrifuge samples for 30 min at $100,000 \times g$, 4°C (45,000 rpm in a Sorvall Discovery M150 SE Micro-Ultracentrifuge with a S45-A fixed-angle rotor).
22. Discard supernatant. For the second Brij-58 incubation, resuspend protein pellet in 40 μ l cold 0.2% Brij-58 buffer with the paint brush. During the resuspension step, add more cold 0.2% Brij-58 buffer in 200- μ l increments to a total volume of 1.2 ml. Rinse paint brush with the last addition. Incubate on ice for 30 min.
23. Balance samples with cold 0.2% Brij-58 buffer during the incubation.
24. Centrifuge samples for 30 min at $100,000 \times g$, 4°C .
25. Use same method as in step 22 to resuspend the enriched membrane pellet (ePM) in cold H buffer (no Brij-58) to a final volume of 400 μ l.

If ePM proteins will be directly used for global proteomic analysis, two wash steps with H buffer should be conducted (as in steps 22 to 24), and 30-min incubation on ice is not needed.

26. Determine protein concentration for the final ePM proteins using a BCA protein assay kit (see Support Protocol 1).
27. Pipet 2 mg ePM proteins into a new 2-ml Eppendorf microcentrifuge tube for S-acylation enrichment (see Basic Protocol 3), keeping volumes consistent across samples by adjusting with H buffer.

The rest of the ePM proteins can be stored at -20°C for further SDS-PAGE or immunoblot analysis.

28. Store ePM proteins at -80°C until further use.

DETERMINATION OF PROTEIN CONCENTRATION USING BCA ASSAY

Quantitation of proteins is a common protocol. We specifically describe the BCA protein assay here because it is compatible with detergent [e.g., sodium dodecyl sulfate (SDS)].

Materials

BCA protein assay kit (Pierce, cat. no. 23225), including bovine serum albumin (BSA) and reagents A and B

Distilled, deionized H₂O (ddH₂O)

Protein samples (see Basic Protocol 2)

Vortex mixer (Vortex-Genie 2, Fisher Scientific, cat. no. 12-812; optional)

96-well clear microplate (Fisher, cat. no. 07-200-95)

Plate shaker

37°C incubator

Microplate reader (Synergy HT, BioTek)

Microsoft Excel

1. Prepare 200 µl of 1 µg/µl bovine serum albumin (BSA) from the BCA protein assay kit in ddH₂O as a working standard.
2. Determine total volume of BCA working reagent required. Prepare BCA working reagent by mixing reagents A and B at a ratio of A/B of 50:1. Mix well by inverting or vortexing.

Volume of BCA working reagent required = (# standards + # unknown samples) × (# replicates) × 200 µl.

3. For BSA standards, pipet 0 to 10 µl of 1 µg/µl BSA into wells of a 96-well clear microplate in duplicate or triplicate and add ddH₂O to bring volume to 10 µl.
4. For samples to be tested, pipet 2 µl protein sample and 8 µl ddH₂O into wells in duplicate or triplicate.

To make sure the amount of tested protein is in the range of the BSA standards, dilute samples 5 or 10 times before loading if the predicted concentration of the proteins is >5 µg/µl.

5. Add 200 µl BCA working reagent (see step 2) to each well and mix plate thoroughly on a plate shaker for 30 s.
6. Cover plate and incubate at 37°C for 30 min.
7. Cool plate to room temperature. Measure absorbance at 562 nm using a microplate reader.
8. Prepare a standard curve based on absorbance values of the BSA standards in Microsoft Excel. Use standard curve to determine the protein concentration of each sample.

ENRICHMENT OF S-ACYLATED PROTEINS BY ACYL-BIOTIN EXCHANGE METHOD

The protocol described here employs a low-background ABE (LB-ABE) method that can be applied to any type of tissue and biological treatment and that results in fewer background contaminants (Zhou et al., 2019). The protocol involves reduction of disulfide bonds by TCEP, free-thiol blocking by NEM and DTDP, ABE with HPDP-biotin, and capture of biotinylated proteins by streptavidin resin. It allows studies of the global S-acylproteome by proteomics and validation of the S-acylation state of a specific protein by western blot.

NOTE: Conduct the experiment at room temperature unless otherwise specified.

Materials

2-mg ePM protein samples (see Basic Protocol 2)
2% (w/v) SDS buffer (2SB buffer; see recipe)
0.5 M Tris (2-carboxyethyl) phosphine hydrochloride (TCEP; Sigma-Aldrich, cat. no. C4706)
50 mM Tris-HCl (Fisher, cat. no. BP152-10), pH 7.4
3.33 M *N*-ethylmaleimide (NEM; Sigma-Aldrich, cat. no. 04259; in ethanol)
50 mM 2,2'-dithiodipyridine (DTDP; Sigma-Aldrich, cat. no. D5767; in dimethylformamide, or DMF)
2 M hydroxylamine (Sigma-Aldrich, cat. no. 159417; make fresh), pH 7.4, 4°C
1 mM HPDP-biotin (EZ-Link™ HPDP-Biotin, Thermo Fisher, cat. no. 21341)
High-capacity streptavidin agarose resin (Pierce, cat. no. 20357)
Equilibration buffer (E buffer; see recipe)
Dilution buffer (see recipe)
50 mM TCEP (Sigma-Aldrich, cat. no. C4706) in E buffer (see recipe)

Sterile 1-ml pipet tips
ThermoMixer R (Eppendorf, cat. no. 5355)
Vortex mixer (Vortex-Genie 2, Fisher Scientific, cat. no. 12-812)
3D rotator (Fisher Scientific, cat. no. 260100F)
1.5- and 2-ml microcentrifuge tubes
Snap-cap spin columns with collection tubes (Pierce, cat. no. 69725)
Microcentrifuge (accuSpin Micro 17, Fisher Scientific)

Additional reagents and equipment for the methanol/chloroform precipitation method (see Support Protocol 2)

Protein reduction and free-thiol blocking

1. Take out 2-mg ePM protein samples (400 µl in volume) from the –80°C freezer and thaw proteins on ice.
2. Precipitate proteins using the methanol/chloroform precipitation method (see Support Protocol 2).

This step will remove DNAs, RNAs, lipids, and small-molecule metabolites.

The volume of starting protein for the precipitation procedure should be ≤200 µl to fit into 2-ml microcentrifuge tubes. Divide each 400-µl ePM protein sample into two 2-ml microcentrifuge tubes, 200 µl each, for precipitation. Combine the two aliquots during the process of precipitation (see Support Protocol 2).

3. To re-solubilize the protein pellets, agitate proteins in 50 µl 2SB buffer with a sterile 1-ml pipet tip. After breaking up the big pellet pieces, add another 150 µl 2SB buffer. Incubate tube in a ThermoMixer R at 37°C with shaking at 1200 rpm until no obvious protein clumps are visible. Add 160 µl 2SB buffer up to a 360-µl volume and mix well by inverting tube.

To avoid clogging the pipet tip and to accelerate breaking up the pellet pieces, cut 2 to 3 mm off the end of the pipet tip.

To re-solubilize the protein well using the ThermoMixer, it may take 20 min or longer. The time needed for protein re-solubilization will be reduced in later steps because the protein amount will be largely decreased due to loss in the precipitation step.

4. Add 40 µl of 0.5 M TCEP to a final concentration of 50 mM. Vortex and then incubate at room temperature for 60 min with rotation on a 3D rotator.

5. Remove excess TCEP by methanol/chloroform precipitation (see Support Protocol 2).

6. Re-solubilize protein pellets in 2SB buffer as described in step 3, with a total volume of 2SB buffer of 200 μ l.

The final volume of 2SB buffer can be scaled up, but the volume will determine the number of aliquots for further protein precipitation. More 2SB buffer may need more aliquots for protein precipitation, which leads to more operations and an enhanced possibility of losing protein.

7. Add 194 μ l of 50 mM Tris-HCl, pH 7.4 [97% (v/v) final], and 6 μ l of 3.33 M NEM [3% (v/v) final]. Vortex and then incubate in a ThermoMixer R in the dark at 55°C with shaking at 300 rpm for 60 min.

Protect NEM from light.

8. Remove excess NEM by methanol/chloroform precipitation (see Support Protocol 2).

9. Re-solubilize protein pellets in 2SB buffer as described in step 3, with a total volume of 2SB buffer of 100 μ l.

10. Add 100 μ l of 50 mM DTDP. Vortex and then incubate at room temperature for 60 min with rotation on a 3D rotator.

11. Remove excess NEM and DTDP by three sequential methanol/chloroform precipitations (see Support Protocol 2).

Complete removal of the free-cysteine-blocking reagents (NEM and DTDP) is critical for enriching S-acylated thiols. You may be tempted to reduce the number of precipitations in order to reduce protein loss; however, experience indicates that sequential precipitations are needed as described.

The experiment can be paused here. Store the protein pellet at -80°C overnight.

S-acylation-site labeling by acyl-biotin exchange

12. Re-solubilize protein pellets in 2SB buffer as described in step 3, with a total volume of 2SB buffer of 400 μ l.

13. Equally divide protein solution into two 2-ml microcentrifuge tubes (200 μ l each). Add the following reagents to the tubes, followed by incubation at room temperature for 60 min with rotation on a 3D rotator:

- a. Experimental sample (Hyd+): 100 μ l of 2 M hydroxylamine, pH 7.4, and 100 μ l of 1 mM HPDP-biotin.
- b. Control sample (Hyd-): 100 μ l of 50 mM Tris-HCl, pH 7.4, and 100 μ l of 1 mM HPDP-biotin.

The control samples (Hyd-), absent of hydroxylamine are processed in parallel with the experimental sample (Hyd+) for all the following procedures. To effectively distinguish S-acyl-proteins from nonspecific contaminant background, the Hyd- sample is necessary.

14. During the incubation in step 13, pre-equilibrate high-capacity streptavidin agarose resin:

- a. Equilibrate required volume of resin to room temperature.

Each protein sample (including Hyd+ and Hyd-) needs ≥ 100 μ l resin in storage solution (50 μ l settled resin).

- b. Pipet resin into snap-cap spin columns. Place columns into the collection tubes supplied with the columns.

To sufficiently equilibrate the resin, transfer $\leq 400\ \mu\text{l}$ resin into one spin column.

- c. Centrifuge 1 min at $400 \times g$ and discard storage solution from the collection tubes.
- d. Add $600\ \mu\text{l}$ E buffer on top of resin bed. Press hard to close snap cap. Holding tightly to keep the column and collection tube leak-proof, mix well by inverting 10 times.

The bottom cap is not needed when washing the resin with E buffer if the column and collection tube can be tightly connected. If the connection is not fluid-tight, buffer will leak into the collection tube from the bottom of the column by gravity. In this case, perform gentle pipetting instead of inverting to mix the solution well.

- e. Centrifuge 1 min at $400 \times g$ and discard E buffer from the collection tubes.
 - f. Repeat steps 14d and 14e two more times.
 - g. Add $100\ \mu\text{l}$ E buffer per $100\ \mu\text{l}$ bed resin to column. Transfer resuspended resin to a new 2-ml microcentrifuge tube by pipetting from the top of the column.
 - h. Repeat step 14g to collect remaining resin. Combine all resin.
 - i. Equally divide resin into new 2-ml microcentrifuge tubes according to the required number of samples.
15. Remove excess biotin-HPDP by two sequential methanol/chloroform precipitations (see Support Protocol 2).

Purification of biotinylated proteins with streptavidin resin

16. Re-solubilize protein pellets in $50\ \mu\text{l}$ 2SB buffer as described in step 3, without adding more 2SB buffer.
17. Add $950\ \mu\text{l}$ dilution buffer to decrease concentration of SDS to 0.1%. Vortex and then centrifuge 5 min at $16,000 \times g$.

Save $50\ \mu\text{l}$ supernatant in a new 1.5-ml microcentrifuge tube as the input of the enrichment for further immunoblot analysis. Store at -20°C .

18. Transfer supernatant to a 2-ml microcentrifuge tube containing pre-equilibrated high-capacity streptavidin agarose resin (see step 14) and incubate at room temperature for 60 min with rotation on a 3D rotator.

The protein amount cannot exceed the resin's binding capacity.

19. Transfer solution containing streptavidin resin to a new snap-cap spin column that has been placed in a collection tube. Label column. Centrifuge 1 min at $400 \times g$ and discard flow-through.

If the solution volume is such that the entire sample cannot be added at once, centrifuge the column to allow some of the solution to pass through; then, add more protein solution.

20. Add $600\ \mu\text{l}$ E buffer. Press hard to close snap cap. Holding tightly to keep the column and collection tube leak-tight, wash resin thoroughly by inverting 10 times and then centrifuge 1 min at $400 \times g$. Discard E buffer from the collection tube.
21. Repeat step 20 five more times.
22. Place column in a new 1.5-ml microcentrifuge tube (used as the collection tube) and centrifuge 2 min at $400 \times g$ to remove excess E buffer. Discard microcentrifuge tube.
23. Place bottom cap onto the column (use the white tip supplied with the column) and place column into a new 1.5-ml microcentrifuge tube. Label tube.

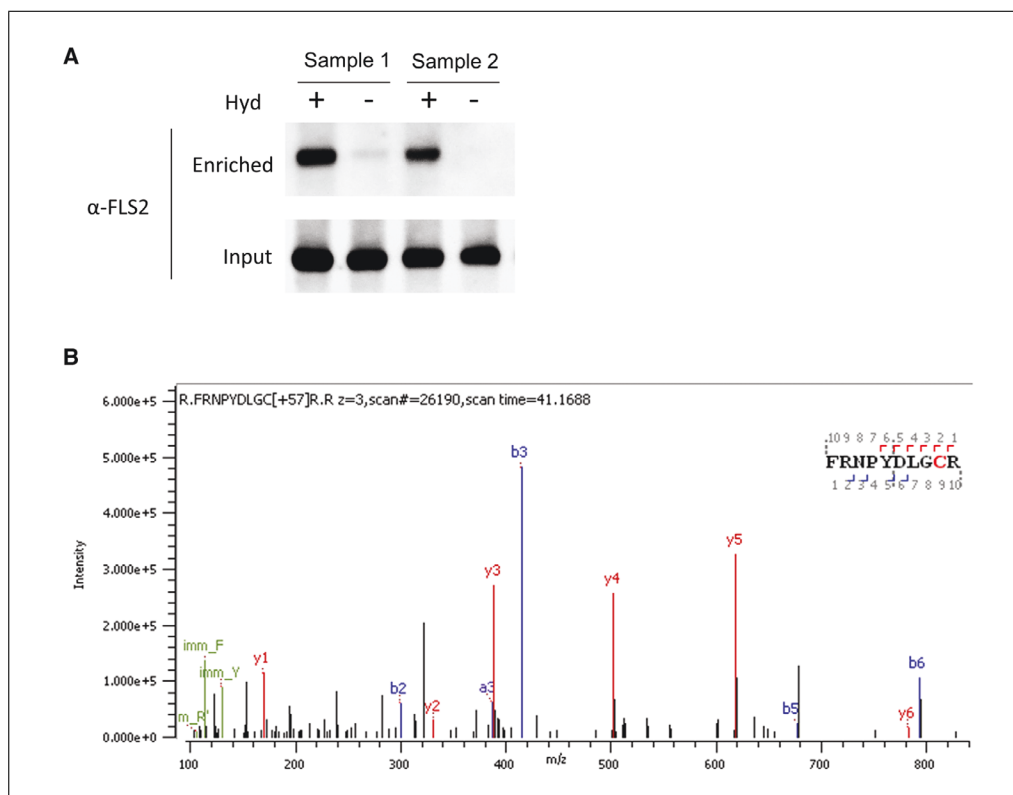


Figure 2 Enrichment and identification of S-acylated proteins using mass spectrometry. **(A)** Successful enrichment of the known S-acylated protein AtFLS2 (AT5G46330) in Hyd+ samples shown by western blotting. Hyd+, hydroxylamine present; Hyd-, hydroxylamine absent. **(B)** Representative MS/MS spectrum of the S-acylated cysteine peptide #FRNPYDLGCR# from AtTIP1 (AT5G20350, a known protein acyltransferase) with IAM labeling on cysteine.

24. To elute the enriched biotinylated proteins, add 100 μ l of 50 mM TCEP in E buffer to each column, incubate in a ThermoMixer R at 37°C with rotation at 800 rpm for 20 min, and then centrifuge for 2 min at 400 $\times$ g. Keep flow-through.

25. Repeat step 24. Combine flow-through.

Save 20 μ l S-acylation enriched proteins in a 1.5-ml microcentrifuge tube and store in -20°C for further immunoblot analysis. The success of the enrichment experiment can be validated by western blotting against a known acylated protein marker (e.g., AtFLS2). In a successful experiment, when loading the same amount of eluted proteins, the signal of the marker protein should be much stronger in the Hyd+ sample than the Hyd- sample (as shown in Figure 2A).

26. Precipitate proteins using the methanol/chloroform method (see Support Protocol 2).

The experiment can be paused here. Store the protein pellet at -80°C .

SUPPORT PROTOCOL 2

PROTEIN PRECIPITATION BY METHANOL/CHLOROFORM METHOD

Some reagents may impair the downstream procedures (Basic Protocol 4 and Alternate Protocol). Removing interfering contaminants is critical to these steps. We choose to use the methanol/chloroform precipitation method because it does not require sample cooling and is simple to do, inexpensive, and time saving. Protein precipitation with chloroform and methanol results in dry protein material with high recovery, free of salt and detergent (Fic, Kedracka-Krok, Jankowska, Pirog, & Dziedzicka-Wasylewska, 2010).

NOTE: All steps are conducted at room temperature in a fume hood (Labconco).

Materials

Protein samples (see Basic Protocol 3)
Methanol (Fisher, cat. no. A412-500)
Chloroform (Fisher, cat. no. C606SK-4)
ddH₂O

Vortex mixer (Vortex-Genie 2, Fisher Scientific, cat. no. 12-812)
Microcentrifuge (accuSpin Micro 17, Fisher Scientific)
SpeedVac concentrator (Thermo Scientific, cat. no. SC250EXP)

1. To protein sample of 200- μ l starting volume, add 800 μ l methanol and mix well by vortexing for 20 s.

The method can be scaled up or down. To simplify our operations, we perform the precipitation within 2-ml microcentrifuge tubes. For a sample of ≥ 400 μ l total volume, divide the proteins into two or more microcentrifuge tubes and precipitate separately. After step 5, combine the floating-protein aliquots by pouring all protein solutions into one tube and continue with step 6.

2. Add 200 μ l chloroform and mix well by vortexing for 20 s.
3. Add 600 μ l ddH₂O and mix well by vortexing for 30 s.

The sample should look cloudy.

4. Centrifuge 2 min at 14,000 $\times$ g.
5. Pipet off upper layer, leaving a small volume of upper phase behind.

Proteins are suspended between the two liquid phases (top phase, methanol/ddH₂O; bottom phase, chloroform).

6. Add 1 ml methanol and mix well by inverting.
7. Centrifuge 5 min at 16,000 $\times$ g.
8. Carefully pipet off supernatant as much as possible without disturbing the pellet.
9. Dry proteins using a SpeedVac concentrator.

Avoid drying too long, as this makes the pellet harder to re-solubilize.

TRYPSIN DIGESTION AND PROTEOMIC ANALYSIS

A variety of proteomic approaches and equipment can be used to analyze the control and S-acylated proteins, as long as they are compatible with the samples. By way of example, we present the specific methods that we have used. In this protocol, protein trypsin digestion is performed via the suspension trap (S-Trap) method. S-Trap digestion tolerates a high concentration of SDS, provides efficient digestion, and results in peptide samples ready for MS. Subsequently, the digested peptides are separated and identified using a Waters nanoACQUITY liquid chromatography (LC) instrument coupled with a Thermo Orbitrap Fusion Lumos high-resolution mass spectrometer.

NOTE: Conduct the experiment at room temperature unless otherwise specified.

Materials

Protein pellets (see Basic Protocol 3)
SDS lysis buffer: 5% (w/v) SDS (from 20% stock; Invitrogen, cat. no. AM9820)
and 50 mM Tris, pH 7.55 (from 1 M Tris, pH 8.0; Invitrogen, cat. no. AM9855G)

BASIC PROTOCOL 4

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500 mM iodoacetamide (IAM; No-Weigh Format; Pierce, cat. no. A39271)
 12% (v/v) phosphoric acid (from 25% stock; RICCA Chemical Company, cat. no. 5851-16)
 S-Trap binding buffer: 90% (v/v) methanol (Optima LC/MS Grade; Fisher Chemical, cat. no. A456-212) and 100 mM Tris, pH 7.5 (from 1 M Tris, pH 8.0; Invitrogen, cat. no. AM9855G)
 Digestion buffer: 50 mM Tris, pH 8.0 (from 1 M Tris, pH 8.0; Invitrogen, cat. no. AM9855G), with or without sequencing-grade modified trypsin (Promega, cat. no. V5111; see step 11 for concentration)
 0.2% (v/v) formic acid (FA; Sigma, cat. no. F0607) in water (LC/MS grade; Fisher Chemical, cat. no. W6-4)
 0.2% (v/v) FA (Sigma, cat. no. F0607) in 50% (v/v) acetonitrile (ACN; Fisher Chemical, cat. no. A955-4) in water (LC/MS grade; Fisher Chemical, cat. no. W6-4)
 0.1% (v/v) FA (Sigma, cat. no. F0607) in 3% (v/v) ACN (Fisher Chemical, cat. no. A955-4) in water (LC/MS grade; Fisher Chemical, cat. no. W6-4)
 Mobile phase A (MPA): 0.1% (v/v) FA (Sigma, cat. no. F0607) in water (LC/MS grade; Fisher Chemical, cat. no. W6-4)
 Mobile phase B (MPB): 0.1% (v/v) FA (Sigma, cat. no. F0607) in ACN (Fisher Chemical, cat. no. A955-4)

 Microcentrifuge (Centrifuge 5415D, Eppendorf)
 1.5-ml low-retention microcentrifuge tubes (Fisherbrand, Fisher Scientific, cat. no. 02-681-331)
 ThermoMixer C (Eppendorf)
 Vortex mixer (Vortex-Genie 2, Fisher Scientific, cat. no. 12-812)
 S-TrapTM micro spin columns (PROTIFI, cat. no. C02-micro-10)
 SpeedVac concentrator (Thermo Scientific, cat. no. SC250EXP)
 LC system (Waters nanoACQUITY or equivalent, including dual pump for online desalting)
 Online solid-phase extraction (SPE) trap column (Phenomenex Jupiter C18 3 μ m particle, column ID 150 μ m, OD 360 μ m, length $\sim$ 5 cm)
 Reversed-phase LC (RPLC) column (packed in-house; Phenomenex C18 3 μ m particle, pore size 300 Å, column ID 75 μ m, OD 360 μ m, length $\sim$ 60 cm)
 MS system (Thermo Orbitrap Fusion Lumos or other high-resolution mass spectrometer compatible with proteomics workflows)
 Proteomics software (e.g., MS-GF+, MaxQuant, Proteome Discoverer, Byonic, PEAKS Studio)

Alkylation and trypsin digestion

1. Resuspend protein pellet ($\sim$ 20 μ g protein) in 50 μ l SDS lysis buffer.
The initial volume of SDS lysis buffer can be larger if needed; adjust the volume of S-Trap binding buffer (see step 5) accordingly.
2. Centrifuge 8 min at $13,000 \times g$. Transfer supernatant to a new 1.5-ml low-retention microcentrifuge tube.
3. Alkylate unlabeled cysteines by adding 1 μ l of 500 mM IAM to a final concentration of 10 mM. Incubate in a ThermoMixer C in the dark at 25°C with shaking at 300 rpm for 45 min.
4. Add 5 μ l of 12% phosphoric acid per 50 μ l SDS lysis buffer for a final concentration of 1.2% phosphoric acid. Mix well by vortexing and then centrifuge solution via a short spin.

Make sure the SDS-solubilized lysate is acidified to exactly 1.2% (v/v) phosphoric acid final. The pH should be about 2 to 3. The sample volume is low, so checking pH may not be

practical. If possible, take an aliquot for pH measurement to confirm. The highly acidified SDS-solubilized lysate is necessary for the formation of colloidal protein particulate and further protein capture in the protein-trapping matrix.

5. Add 385 μl S-Trap binding buffer per 55 μl acidified SDS lysis buffer (total volume of 440 μl). Mix well by vortexing.

For starting volumes of SDS lysis buffer >50 μl , maintain a 1:7 v/v ratio of SDS lysis buffer to S-Trap binding buffer.

6. Place S-TrapTM micro spin column in a 1.5-ml low-retention microcentrifuge tube and add acidified SDS lysate/S-Trap binding buffer mixture to the spin column.

No column pre-equilibration is necessary. Do not exceed volume of the spin column when loading solution. If the initial SDS lysate volume is higher, load the column multiple times until the full volume has been bound. To load 440 μl total solution, load 220 μl , centrifuge through, and then repeat with the next 220 μl .

7. Centrifuge spin column for ≥ 30 s at $4000 \times g$, until all SDS lysate/S-Trap binding buffer has passed through the column.

Proteins will be bound and trapped within the protein-trapping matrix of the spin column.

8. Wash captured protein by adding 150 μl S-Trap binding buffer and centrifuging the spin column for 30 s at $4000 \times g$.

9. Repeat step 8 three more times.

10. Transfer spin column to a new 1.5-ml low-retention microcentrifuge tube.

Use of a new tube prevents contamination of the resulting digestion.

11. Add 20 μl digestion buffer containing trypsin at 1:10 (w/w) trypsin/protein to top of the spin column.

The amount of trypsin should be ≥ 0.75 μg . Ensure that there is no air bubble between the protease-containing digesting solution and the protein trap.

12. Close cap of the spin column loosely to limit evaporative loss.

13. Incubate in a ThermoMixer C at 47°C for 1 hr for trypsin digestion. Do not shake.

14. Add 40 μl digestion buffer without trypsin to spin column.

15. Centrifuge 60 s at $4000 \times g$ to elute peptides.

This aqueous elution contains most peptides.

16. Add 40 μl of 0.2% FA in water to spin column. Centrifuge 60 s at $4000 \times g$.

17. Add 35 μl of 0.2% FA in 50% ACN in water. Centrifuge 60 s at $4000 \times g$.

This elution assists in recovery of hydrophobic peptides.

18. Pool all eluted peptide solutions and dry peptides in a SpeedVac concentrator.

19. Resuspend peptides in 0.1% FA in 3% ACN in water at 0.1 $\mu\text{g}/\mu\text{l}$ for MS analysis.

Proteomic analysis

20. Configure LC system for 5-min online desalting in the online SPE trap column with MPA at 5 $\mu\text{l}/\text{min}$.

The online desalting is achieved with a dual-pump LC configuration. After sample is injected onto the SPE trap column, the “wash” pump flows at 5 $\mu\text{l}/\text{min}$ with MPA to desalt the sample. After desalting, the “wash” pump is diverted, and the “analytical” pump flows into the SPE. The gradient starts (see step 21), and peptides are gradually eluted

Table 1 Representative Gradient of Mobile Phase B (MPB) for Peptide Separation

Time (min)	% MPB
0	1
2	8
20	12
75	30
97	45
100	95
110	95
115	1
150	1

from the SPE into the analytical column. Please consult the liquid chromatograph's manufacturer for details on the setup.

21. Configure LC method for 150-min separation with MPA and MPB at 0.3 µl/min using the RPLC column.

A representative gradient table is shown in Table 1. % MPA = 100% – % MPB.

22. Configure data-dependent acquisition for MS system.

Representative settings for the Orbitrap instrument are listed below:

Ion source settings: electrospray voltage 2.2 kV, s-lens RF 30%, capillary temperature 250°C. No source gas.

MS1 settings: 60k resolution, AGC target 4E5, maximum injection time 50 ms, scan range 400-2000 m/z, profile spectra.

MS2 settings: 15k resolution, AGC target 1E5, maximum injection time 22 ms, isolation window 1 m/z, normalized collision energy 30 ± 5, centroid spectra.

Data-dependent acquisition: cycle time 3 s, intensity threshold 5E4, include charge state 2-6, dynamic exclusion of 45 s.

23. Inject 5 µl peptide solution (0.1 µg/µl; see step 19) for each sample and collect MS data.
24. Process data with proteomics software. Download protein FASTA from UniProt for all *Arabidopsis* protein sequences. Append common contaminant protein sequences, such as sequences for trypsin and human keratin.

Some proteomics software has a built-in function to add contaminant proteins. If not, manually add the protein sequences from the FASTA.

25. Include the following dynamic modifications in the peptide search: carbamidomethyl (+57.0215) on Cys, N-ethylmaleimide (+125.0477) on Cys, dithiodipyridine (+108.9986) on Cys, methionine oxidation, protein N-terminal acetylation, deamidation (Asn, Gln).

Peptides with carbamidomethyl (labeled with IAM) are most likely S-acylated in vivo. Example data are shown in Figure 2B using Byonic software for peptide identification. Mass error tolerance is 10 ppm.

Non-S-acylated cysteines, which are not effectively blocked by NEM and DTDP (see steps 7 to 11 in Basic Protocol 3), will be labeled by IAM. In addition, proteins with an S-acylation site may also generate peptides without cysteine residues (and thus without

carbamidomethyl modification). Therefore, the results need to be cautiously interpreted. Comparing the proteomics data between control and enriched samples quantitatively can reveal potential S-acylated proteins. Alternatively, enrichment at the peptide level (see Alternate Protocol) can be performed.

PRE-RESIN DIGESTION AND PEPTIDE-LEVEL ENRICHMENT

ALTERNATE PROTOCOL

After performing the protein reduction, the free-cysteine blocking, and the ABE procedures as described in Basic Protocol 3, steps 1 to 15, the biotinylated proteins are directly digested into peptides in this protocol using an in-solution digestion method. Biotinylated peptides are then purified using streptavidin resin. Compared to the enrichment at the protein level in Basic Protocol 3, although this protocol identifies less total potential S-acylated protein, it allows the identification of much more specific S-acylation sites. You should choose one protocol (Basic Protocol 3 or Alternate Protocol) depending on your requirements. This protocol is used specifically when identification of the specific acylated site is desired.

NOTE: Conduct the experiment at room temperature unless otherwise specified.

Additional Materials (also see Basic Protocol 3)

Ammonium bicarbonate (NH_4HCO_3 ; Sigma-Aldrich, cat. no. A6141)
Urea (Fisher, cat. no. U15-500)
Protein pellets (see Basic Protocol 3)
1 M calcium chloride (CaCl_2 ; Fisher, cat. no. C79-3)
Trypsin (Promega, cat. no. V5111)
2× E buffer (see recipe)
Washing buffer: 2 M urea (Fisher, cat. no. U15-500) and 100 mM Tris (Fisher, cat. no. BP152-10), pH 7.4
Elution buffer: 50 mM TCEP (Sigma-Aldrich, cat. no. C4706) and 100 mM Tris (Fisher, cat. no. BP152-10), pH 7.4
500 mM IAM (Sigma-Aldrich, cat. no. I1149)

37°C incubator
C18 clean-up tips (Pierce, cat. no. 87784)

Pre-resin trypsin digestion

1. Freshly prepare 100 mM NH_4HCO_3 and 8 M urea/100 mM NH_4HCO_3 , pH 8, and keep at room temperature.
2. Re-solubilize protein pellets in 50 μl of 8 M urea/100 mM NH_4HCO_3 , pH 8, in a ThermoMixer R at 60°C with rotation at 1000 rpm for 30 min.
3. Dilute sample with 450 μl of 100 mM NH_4HCO_3 to reduce the urea concentration to a final concentration of <1 M. Vortex and then centrifuge 5 min at $16,000 \times g$ to remove un-solubilized protein aggregates.
4. Transfer supernatant to a new 1.5-ml microcentrifuge tube. Add 0.5 μl of 1 M CaCl_2 to a concentration of 1 mM CaCl_2 .
5. Digest sample for 3 hr or overnight with trypsin at 37°C at a concentration of 1 μg trypsin per 50 μg protein.

Purification of biotinylated peptides

6. Add 500 μl of 2× E buffer to achieve final concentrations of 50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 0.2% Triton X-100, 0.4 M urea, and 50 mM NH_4HCO_3 .

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7. For each sample, equilibrate 100 μ l streptavidin agarose resin in storage solution (50- μ l bed volume) with E buffer as in step 14 in Basic Protocol 3.
8. Transfer resin to the samples and incubate at room temperature for 60 min with rotation on a 3D rotator.
9. Transfer above solution containing streptavidin resin to a new spin column placed in a new collection tube supplied with the spin column. Label column. Centrifuge 1 min at $400 \times g$ and discard flow-through.

If the solution volume is such that the entire sample cannot be added at once, centrifuge the column to allow some of the solution to pass through, discard the flow-through, and then proceed with the rest of the solution.

10. Wash resin with E buffer three times. To wash the resin thoroughly, add 600 μ l E buffer on top of resin bed, press hard to close snap cap, hold tightly to keep column and collection tube connected to prevent leakage, and mix well by inverting 10 times. Centrifuge 1 min at $400 \times g$ and discard E buffer from the collection tubes.
11. Wash resin with 600 μ l washing buffer three times as in step 10.
12. Place column in a new 1.5-ml microcentrifuge tube and centrifuge 2 min at $400 \times g$. Discard microcentrifuge tube.
13. Place bottom cap onto the column (use the white tip supplied with the column) and place column into a new 1.5-ml microcentrifuge tube. Label tube.
14. To elute the enriched biotinylated peptides, add 100 μ l elution buffer to each column and incubate in a ThermoMixer R at 37°C with rotation at 800 rpm for 20 min and then centrifuge tubes for 2 min at $400 \times g$. Keep flow-through.
15. Repeat step 14. Combine flow-through.

The flow-through contains the enriched peptides with potential S-acylation sites. After thiol-acyl cleavage by hydroxylamine, biotin labeling by HPDP-biotin, thiol-biotin capture by the streptavidin resin, and thiol-biotin cleavage by TCEP, the S-acylation sites are present as free thiols on cysteines at this time point. The sites will be further alkylated by IAM and detected by MS.

Alkylation and desalting

16. Add 4 μ l of 500 mM IAM solution to a final concentration of 10 mM (1:50 dilution) and mix by mild vortexing. Incubate in a ThermoMixer in the dark at 25°C with rotation at 300 rpm for 45 min.
17. Use C18 clean-up tips to desalt sample prior to MS by following the product manual.
18. As step 19 in Basic Protocol 4, resuspend peptides in 0.1% FA in 3% ACN in water at 0.1 μ g/ μ l for MS analysis (see Basic Protocol 4, steps 20 to 25).

REAGENTS AND SOLUTIONS

$\frac{1}{2}$ MS agar medium plates

- 2.22 g/L Murashige and Skoog Basal Salt Mixture (with vitamins; Caisson, cat. no. MSP09)
- 20 g/L sucrose
- Adjust pH to 5.7 with 1 N KOH
- Aliquot liquid medium into small conical flasks and add 0.5% (w/v) Phytigel (2.5 g/500 ml; Sigma, cat. no. P8169)
- Autoclave for 20 min at 15 psi, 121°C
- Cool down medium until not too hot to handle
- In laminar flow hood, add appropriate antibiotic to medium and shake medium well

In laminar flow hood, dispense medium into 100 × 100-mm square petri dishes (Fisher, cat. no. 08-757-11A) and let agar medium dry
Store plates ≤6 months at 4°C without antibiotic and ≤1 month at 4°C with antibiotic

Dilution buffer

50 mM Tris·HCl (Fisher, cat. no. BP152-10), pH 7.4
150 mM NaCl (Fisher, cat. no. S271-3)
5 mM EDTA (Sigma-Aldrich, cat. no. ED2SS)
0.2% (v/v) Triton X-100 (Fisher, cat. no. BP151-500)
Store ≤1 week at room temperature

Equilibration buffer (E buffer)

50 mM Tris·HCl pH 7.4 (Fisher, cat. no. BP152-10)
150 mM NaCl (Fisher, cat. no. S271-3)
5 mM EDTA (Sigma-Aldrich, cat. no. ED2SS)
0.1% (w/v) SDS (Fisher, cat. no. BP166-500)
0.2% (v/v) Triton X-100 (Fisher, cat. no. BP151-500)
Store ≤1 week at room temperature

Equilibration buffer (E buffer), 2 ×

100 mM Tris·HCl (Fisher, cat. no. BP152-10), pH 7.4
300 mM NaCl (Fisher, cat. no. S271-3)
10 mM EDTA (Sigma-Aldrich, cat. no. ED2SS)
0.2% (w/v) SDS (Fisher, cat. no. BP166-500)
0.4% (v/v) Triton X-100 (Fisher, cat. no. BP151-500)
Store ≤1 week at room temperature

Homogenization buffer (H buffer)

50 mM HEPES-KOH (Fisher, cat. no. BP310-100), pH 7.5
250 mM sucrose (Fisher, cat. no. BP220-212)
5% (v/v) glycerol (Fisher, cat. no. BP229-1)
10 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, cat. no. ED2SS), pH 8.0
0.5% (w/v) soluble polyvinylpyrrolidone (PVP-10; Sigma-Aldrich, cat. no. PVP-10)
50 mM sodium pyrophosphate (NaPP; Sigma-Aldrich, cat. no. P8010)
1 mM sodium molybdate dihydrate (NaMo; Sigma-Aldrich, cat. no. 331058)
25 mM sodium fluoride (NaF; Sigma-Aldrich, cat. no. S7920)
Filter-sterilize solution
Store ≤6 months at 4°C
Just prior to use, add the following proteinase inhibitors per 10 ml H buffer:
100 µl 100 mM phenylmethylsulfonyl fluoride (PMSF; Sigma, cat. no. 93842; 1 mM final in ethanol)
10 µl 10 mM leupeptin (Sigma, cat. no. L2884; 10 µM final)
10 µl 10 mM calyculin (Sigma, cat. no. C5552; 10 nM final in dimethyl sulfoxide, or DMSO)

Proteinase inhibitor cocktail for plant tissues (e.g., Sigma, cat. no. P9599) can be used to replace the proteinase inhibitors listed in the recipe.

SDS buffer, 2% (2SB buffer)

50 mM Tris·HCl (Fisher, cat. no. BP152-10), pH 7.4
2% (w/v) SDS (Fisher, cat. no. BP166-500)

5 mM EDTA (Sigma-Aldrich, cat. no. ED2SS)

Store ≤ 1 week at room temperature

COMMENTARY

Background Information

Protein S-acylation is catalyzed by protein acyltransferases (PATs), and de-S-acylation relies on acyl-protein thioesterases (APTs) (Zheng, Liu, Liu, Wang, & Dong, 2019). Due to its reversible characteristic, dynamic S-acylation can impact proteins' function by altering their membrane affinity in a flexible and smart manner. Twenty-four PAT genes have been identified in *Arabidopsis* (Zheng et al., 2019). However, only *PAT4*, *PAT5*, *PAT9*, and *PAT10* have been characterized and their substrates studied (Wan et al., 2017; Zhou et al., 2013; Chen, Ahsan, Thelen, & Stacey, 2019). MtAPT1 is the only reported protein thioesterase that has been characterized in plants (Duan et al., 2017). Identification and functional characterization of the S-acylation enzymes and their substrates will elevate our understanding of S-acylation in plants.

Approaches for large-scale identification of S-acylated proteins mainly depend on proteomic methods in combination with different S-acylation-site labeling and capture methods. Three major methods have been employed to achieve proteomic profiling of S-acylated proteins: metabolic labeling followed by click chemistry (MLCC) (Kostiuk et al., 2008), ABE (Drisdell & Green, 2004), and resin-assisted capture (acyl-RAC) (Forrester et al., 2011). In MLCC, a palmitate analog is added to cell culture medium and is metabolically incorporated into native S-acylation sites during culturing. After protein isolation, palmitate analog-labeled proteins are conjugated to a biotin analog by click chemistry. The biotinylated proteins are then enriched by streptavidin affinity purification (Zhou, An, Freeman, & Yang, 2014). In ABE, free thiols are blocked by an alkylating reagent (e.g., NEM), and S-acylated cysteines are then hydrolyzed into free cysteines by neutral hydroxylamine. The newly exposed cysteines are conjugated to a biotin analog (e.g., HPDP-biotin), so the formerly S-acylated proteins can be enriched by streptavidin affinity purification and identified through MS or verified by western blotting. Basic Protocol 3 uses the LB-ABE method (Zhou et al., 2019), which employs additional blockage of free cysteine with DTDP. In the acyl-RAC strategy, a thiol-reactive resin, which can capture free thiols, replaces the biotin exchange and avidin purification steps.

Using this strategy, S-acylated peptides can be more rapidly purified.

Compared to MLCC, ABE does not require metabolic labeling, so it can be used to analyze S-acylated proteins in any plant tissues or cell types. In addition, ABE has the potential to capture the full S-acylproteome. A combination of PM enrichment by Brij-58 treatment (Basic Protocol 2) and S-acylation enrichment by the LB-ABE method (Basic Protocol 3) enables us to identify more PM proteins with S-acylation.

Critical Parameters and Troubleshooting

Protein amount

Too little final protein and tryptic peptide will lead to unfavorable MS results (Basic Protocol 4 and Alternate Protocol). Almost all steps will impact the final protein levels.

- Compared to in a general global proteomic analysis, much more starting plant material (Basic Protocol 1) is needed due to the two stages of protein enrichment (PM enrichment, in Basic Protocol 2, and S-acylation enrichment, in Basic Protocol 3).

- Excessive Brij-58 in PM enrichment (Basic Protocol 2) will result in a big loss of proteins. Using the appropriate volume of Brij-58 is crucial.

- A small amount of protein will be lost at each ultracentrifugation and precipitation step (Basic Protocol 2). Thus, avoid conducting unnecessary ultracentrifugation and protein precipitation/re-solubilization procedures.

- Sufficient ABE exchange by HPDP-biotin and purification of S-acylated proteins (Basic Protocol 3) are essential to retain the S-acylated proteins. To achieve this, adequate volumes of HPDP-biotin and streptavidin resin are needed.

Chemical contaminants

Carryover of some reagents may impair downstream procedures. Removing interfering contaminants is critical for these steps.

- The thiol-blocking reagents NEM and DTDP (Basic Protocol 3) will further alkylate newly exposed thiol by hydroxylamine cleavage and will prohibit biotin labeling. We recommend using sequential methanol/chloroform precipitation to completely remove the alkylating reagents.

Table 2 Time Considerations for Each Protocol

Protocol	Procedure	Time
Basic Protocol 1: Preparation of <i>Arabidopsis</i> seedling materials	Seed sterilization and cold treatment	3 days
	Seed germination and seedling growth	7-14 days
Basic Protocol 2: Isolation and enrichment of plasma membrane proteins	Isolation of crude membrane	8 hr
	Enrichment of plasma membrane protein	8 hr
Support Protocol 1: Determination of protein concentration using BCA assay	BCA protein assay	1 hr
Basic Protocol 3: Enrichment of S-acylated proteins by acyl-biotin exchange method	Protein reduction and free-thiol blocking	10 hr
	Acyl-biotin exchange	3 hr
	Purification of biotinylated proteins	5 hr
Support Protocol 2: Protein precipitation by methanol/chloroform method	Protein precipitation	30 min
Basic Protocol 4: Trypsin digestion and proteomic analysis	Alkylation and trypsin digestion	4 hr
	Mass spectral data acquisition	3 hr
	Database searching	1 hr
Alternate Protocol: Pre-resin digestion and peptide-level enrichment	Pre-resin trypsin digestion	4-12 hr
	Purification of biotinylated peptides	5 hr
	Alkylation and desalting	2 hr

• The biotinylating reagent HPDP-biotin (Basic Protocol 3) can bind and occupy the binding sites of streptavidin agarose resin. As a result, the streptavidin resin's capacity to capture S-acylated proteins is decreased. Sequential methanol/chloroform precipitation can help remove the excess HPDP-biotin.

• Salts (e.g., NaCl) and detergents (e.g., SDS) can disrupt MS analysis (Basic Protocol 4 and Alternate Protocol). Salts can usually be effectively removed by offline and online SPE prior to MS. Strong ionic detergent (e.g., SDS) contamination following protein solubilization is a frequent cause of failed MS analysis. SDS can be removed at the protein level using S-Trap or size-based filters. However, SDS is extremely difficult to remove from digested peptides. Avoid using SDS in the final buffer before desalting.

False positives and background control

False positives can be recognized as putative sites for S-acylation during the downstream analysis (Basic Protocol 4 and Alternate Protocol). Most of the false positives come from insufficient blocking of free cysteine residues. Complete blocking of original and TCEP-induced free cysteines is crucial. NEM is used to primarily block the majority of the free cysteines. In addition to this, DTDP is employed in Basic Protocol 3 for further blocking. Some non-S-acylated proteins can be captured due to hydroxylamine-independent nonspe-

cific binding with biotin or resin during purification with streptavidin resin; these co-enriched proteins will be identified as false positives.

A sample without hydroxylamine treatment (Hyd-) should be processed in parallel with the experimental sample (Hyd+) as a background control (Basic Protocol 3). The proteins exclusively identified from the Hyd+ samples are considered to be potential S-acylated proteins. Distinguishing S-acylated proteins from substantial contaminant background is a big challenge. A Hyd+/Hyd-ratio of spectral counting (SC) or label-free quantitation can be referred to in order to determine the candidates.

Understanding Results

Following the protocol for preparation of *Arabidopsis* seedlings (Basic Protocol 1), we can harvest 2.5 g of seedlings per agar plate if grown in a growth chamber for 2 weeks. Four plates are combined as one sample for protein extraction. Following the protocol for enrichment of PM proteins (Basic Protocol 2), the final yield is approximately 10 to 15 mg CM protein after the first ultracentrifugation step. The final enriched PM proteins (ePM) are approximately 2 to 3 mg per 8 mg starting CM. A total of 2 mg ePM is used for enrichment of S-acylated proteins/peptides in Basic Protocol 3. The rest of the ePM can be used for global proteomic or phospho-proteomic analysis (Basic Protocol 4).

Following the protocols for protein-level enrichment (Basic Protocol 3) and proteomic analysis (Basic Protocol 4), we identified about 30,000 peptides in a single LC-MS/MS run, with only 1% of peptides containing cysteines with IAM alkylation. Hyd+ samples identified more peptides than Hyd- samples. Using the protocol for peptide-level enrichment and proteomic analysis (Alternate Protocol), thousands of peptides were identified, with over 80% of the peptides containing cysteines with IAM alkylation. Hyd+ samples identified more peptides than Hyd- samples.

Please note that not all identified proteins in Hyd+ samples are putative S-acylated proteins; on the contrary, a large portion of proteins are nonspecific background proteins. Likewise, not all IAM-alkylated cysteines are S-acylation sites. These background proteins/peptides are false positives. Comparison of spectral counting between Hyd+ and Hyd- samples enables us to remove many of the false positives. We use the following criteria for considering potential S-acylated proteins:

1. The Hyd+/Hyd- ratio is >2 ;
2. At least two tryptic peptides are detected; and
3. The above two criteria are fulfilled in at least two replicates.

Confidence levels can be classified based on the Hyd+/Hyd- ratio. For example, potential S-acylated proteins can be categorized into high confidence ($\text{Hyd+}/\text{Hyd-} \geq 10$), medium confidence ($5 \leq \text{Hyd+}/\text{Hyd-} < 10$), and low confidence ($2 < \text{Hyd+}/\text{Hyd-} < 5$). Protein S-acylation can be validated by western blot.

Time Considerations

See Table 2 for time considerations for the protocols.

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Author Contributions

Lijuan Zhou: Data curation; formal analysis; investigation; methodology; writing-original draft. **Mowei Zhou:** Data curation; formal analysis; investigation; software; writing-review & editing. **Marina A. Gritsenko:** Investigation; writing-review & editing. **Gary Stacey:** Conceptualization; funding acquisition; project administration; supervision; writing-review & editing.

Literature Cited

- Chen, D., Ahsan, N., Thelen, J. J., & Stacey, G. (2019). S-acylation of plant immune receptors mediates immune signaling in plasma membrane nanodomains. *bioRxiv*, 720482. doi: 10.1101/720482.
- Collins, C. A., Leslie, M. E., Peck, S. C., & Heese, A. (2017). Simplified enrichment of plasma membrane proteins from *Arabidopsis thaliana* seedlings using differential centrifugation and Brij-58 treatment. *Methods in Molecular Biology*, 1564, 155–168. doi: 10.1007/978-1-4939-6813-8_13.
- Collins, M. O., Woodley, K. T., & Choudhary, J. S. (2017). Global, site-specific analysis of neuronal protein S-acylation. *Scientific Reports*, 7, 4683. doi: 10.1038/s41598-017-04580-1.
- Drisdel, R. C., & Green, W. N. (2004). Labeling and quantifying sites of protein palmitoylation. *Biotechniques*, 36, 276–285. doi: 10.2144/04362RR02.
- Duan, M., Zhang, R., Zhu, F., Zhang, Z., Gou, L., Wen, J., ... Wang, T. (2017). A lipid-anchored NAC transcription factor is translocated into the nucleus and activates glyoxalase I expression during drought stress. *Plant Cell*, 29, 1748–1772. doi: 10.1105/tpc.17.00044.
- Fic, E., Kedracka-Krok, S., Jankowska, U., Pirog, A., & Dziedzicka-Wasylewska, M. (2010). Comparison of protein precipitation methods for various rat brain structures prior to proteomic analysis. *Electrophoresis*, 31, 3573–3579. doi: 10.1002/elps.201000197.
- Forrester, M. T., Hess, D. T., Thompson, J. W., Hultman, R., Moseley, M. A., Stamler, J. S., & Casey, P. J. (2011). Site-specific analysis of protein S-acylation by resin-assisted capture. *Journal of Lipid Research*, 52, 393–398. doi: 10.1194/jlr.D011106.
- Hemsley, P. A., Weimar, T., Lilley, K. S., Dupree, P., & Grierson, C. S. (2013). A proteomic approach identifies many novel palmitoylated proteins in *Arabidopsis*. *New Phytologist*, 197, 805–814. doi: 10.1111/nph.12077.
- Kostiuk, M. A., Corvi, M. M., Keller, B. O., Plummer, G., Prescher, J. A., Hangauer, M., ... Berthiaume, L. G. (2008). Identification of palmitoylated mitochondrial proteins using a bio-orthogonal azido-palmitate analogue. *The*

- FASEB Journal, 22, 721–732. doi: 10.1096/fj.07-9199com.
- Li, Y., & Qi, B. (2017). Progress toward understanding protein S-acylation: Prospective in plants. *Frontiers in Plant Science*, 8, 346. doi: 10.3389/fpls.2017.00346.
- Turnbull, D., & Hemsley, P. A. (2017). Fats and function: Protein lipid modifications in plant cell signalling. *Current Opinion in Plant Biology*, 40, 63–70. doi: 10.1016/j.pbi.2017.07.007.
- Wan, Z. Y., Chai, S., Ge, F. R., Feng, Q. N., Zhang, Y., & Li, S. (2017). Arabidopsis protein S-acyl transferase 4 mediates root hair growth. *Plant Signaling and Behavior*, 90, 249–260. doi: 10.1111/tj.13484.
- Yang, W., Di Vizio, D., Kirchner, M., Steen, H., & Freeman, M. R. (2010). Proteome scale characterization of human S-acylated proteins in lipid raft-enriched and non-raft membranes. *Molecular & Cellular Proteomics*, 9, 54–70. doi: 10.1074/mcp.M800448-MCP200.
- Zheng, L., Liu, P., Liu, Q., Wang, T., & Dong, J. (2019). Dynamic protein S-acylation in plants. *International Journal of Molecular Sciences*, 20, 560. doi: 10.3390/ijms20030560.
- Zhou, B., An, M., Freeman, M. R., & Yang, W. (2014). Technologies and challenges in proteomic analysis of protein S-acylation. *Journal of Proteomics and Bioinformatics*, 7, 256–263. doi: 10.4172/jpb.1000327.
- Zhou, B., Wang, Y., Yan, Y., Mariscal, J., Di Vizio, D., Freeman, M., & Yang, W. (2019). Low-background acyl-biotinyl exchange largely eliminates the co-isolation of non-S-acylated proteins and enables deep S-acylproteomic analysis. *Analytical Chemistry*, 91, 9858–9866. doi: 10.1021/acs.analchem.9b01520.
- Zhou, L. Z., Li, S., Feng, Q. N., Zhang, Y. L., Zhao, X., Zeng, Y. L., ... Zhang, Y. (2013). Protein S-acyltransferase10 is critical for development and salt tolerance in *Arabidopsis*. *Plant Cell*, 25, 1093–1107. doi: 10.1105/tpc.112.108829.