Quantitative Analysis of Plasma Membrane Proteome Using Two-Dimensional Difference Gel Electrophoresis

Wenqiang Tang

Abstract

The plasma membrane (PM) controls cell’s exchange of both material and information with the outside environment, and PM-associated proteins play key roles in cellular regulation. Numerous cell surface receptors allow cells to perceive and respond to various signals from neighbor cells, pathogens, or the environment; large numbers of transporter and channel proteins control material uptake or release. Quantitative proteomic analysis of PM-associated proteins can identify key proteins involved in signal transduction and cellular regulation. Here, we describe a protocol for quantitative proteomic analysis of PM proteins using two-dimensional difference gel electrophoresis. The protocol has been successfully employed to identify new components of the brassinosteroid signaling pathway, and should also be applicable to the studies of other plant signal transduction pathways and regulatory mechanisms.

Key words: Signal transduction, Proteomics, Plasma membrane, 2-D DIGE, Protein prefractionation, Brassinosteroids

1. Introduction

Signal transduction is important for plants because plants are sessile and rely on signal transduction pathways to regulate gene expression and to adapt to the changing environment. However, our knowledge of plant signal transduction pathways is still very limited. Although genetic studies in model systems such as Arabidopsis have identified key components of many signaling pathways, there are often gaps in these genetically defined pathways largely because many genes’ functions cannot be identified by traditional genetic studies due to genetic redundancy or lethality. On the other hands, recent progresses in proteomic technologies have provided important tools for identifying new signal transduction proteins (1).
Compared with the traditional genetic studies which rely on phenotypes caused by altering gene activity, proteomic studies identify protein functions based on the changes of protein’s abundance or modification associated with cellular or physiological perturbation or protein’s interaction with other known proteins. Proteomics not only identifies proteins’ function, but also reveals their mechanisms of function and regulation, such as posttranslational modifications and protein-protein interaction (2, 3).

While proteomics is a powerful approach for biological research, it has its own challenges and limitations. A major challenge is the high complexity of the proteomes. The most recent release of Arabidopsis genome annotation indicates that the Arabidopsis genome contains 27,416 protein-encoding genes. Each gene can encode different proteins due to alternative splicing and each protein can be regulated by a wide range of posttranslational modifications, such as phosphorylation, methylation, ubiquitination, and acetylation. Therefore, the complexity of cellular protein species is actually much higher. Moreover, the abundance of the proteins in a cell may differ by up to ten orders of magnitude, and proteins involved in signal transduction or regulation normally exist in low abundance and thus are difficult to detect. Due to high sample complexity and limited analytical power, current proteomic technologies cannot analyze all the proteins of a eukaryotic sample, unlike genomic analysis of the whole genome by microarray or high-throughput sequencing. Therefore, reducing the complexity of protein profiles by sample prefractionation strategies, such as subcellular fractionation and affinity purification, is considered critical for studying low-abundance signal transduction proteins by proteomic approaches (4, 5).

Two types of proteomic approaches have been widely used. Two-dimensional gel electrophoresis (2-DE) separates proteins based on charge by isoelectric focusing (IEF) electrophoresis in first dimension and based on size by SDS-PAGE in second dimension (6). Traditional 2-DE separates one sample in each gel and relative quantification is achieved by comparison between gels, which is not accurate because of gel-to-gel variations. A significant improvement was introduced about a decade ago by a new method called 2-D difference gel electrophoresis (2-D DIGE) (7, 8). 2-D DIGE uses different fluorescence dyes to covalently label proteins before proteins are mixed together and then separated in the same 2-DE gel. Images of different samples acquired from the same gel have the same spot patterns and can be directly compared to quantify abundance ratios. 2-D DIGE allows direct comparison of up to three samples within the same gel. By using a pooled reference sample in all gels, cross-gel comparison and multiplexing can be performed when large numbers of samples need to be compared (8).
The proteins of spots of interest can be identified by in-gel digestion followed by mass spectrometry analysis.

Another proteomic approach is gel-free and based on direct mass spectrometry analysis of digested peptides. This approach has become more and more popular due to improvement of MS technology, such as powerful tandem mass spectrometry (MS/MS). Typically, liquid chromatography of tryptic peptides is performed before MS analysis (LC–MS/MS), and isotope or isobaric labeling improves quantitative comparison between samples (3).

Although there has been a trend in the field to shift from 2-DE to gel-free MS approaches, each method has its own strength and limitations. 2-DE is believed to have limitations for certain types of proteins, such as hydrophobic proteins and proteins of extreme molecular weight or charge. In particular, 2-DE is widely considered ineffective for separating transmembrane proteins; however, proteins containing single transmembrane domain or peripheral membrane-associated protein can be readily resolved in 2-DE. Gel-free methods in principle can detect any proteins/peptides. However, in reality, peptides representing only a few thousands of proteins can be detected in typical LC–MS/MS analysis. Major advantages of 2-D DIGE include reliable quantitative analysis of not only protein abundance but also posttranslational modifications/processings that cause spot shifts in the gel. For example, phosphorylation shifts a protein spot to the acidic side in the IEF dimension and protein cleavage or modification by ubiquitination would shift protein spot along the SDS-PAGE dimension (9). In LC–MS/MS, a change of posttranslational modification can only be analyzed when the peptide containing the modification is detected, and usually only a few peptides are detected in LC–MS/MS for each low-abundance protein. Therefore, 2-D DIGE has some unique advantages in studying signal transduction.

Following the protocol described here, we have analyzed the early brassinosteroid (BR)-regulated plasma membrane (PM) proteins using 2-D DIGE, and we were able to identify two major components of the BR signal transduction pathway: the BAK1 receptor kinase previously identified by molecular genetics and the BR-signaling kinases (BSKs) as new components that transduce signal downstream of the BR receptor kinase BRI1. We also identified a number of other novel BR-regulated PM-associated proteins (5, 10). Our data demonstrated that 2-D DIGE coupled with prefractionation is a very powerful approach in studying signal transduction pathway. Although the protocol is described for comparison between BR-treated and untreated samples, it can be used for comparison between any biological samples.
2. Materials

2.1. Growth of Arabidopsis Seedlings and Cell Lysis

1. Growth media: 1/2 Murashige and Skoog basal salt mixture and 1.5% sucrose; adjust pH to 5.7 with 1 M KOH.

2. 2 mM brassinolide (BL) in 85% ethanol; aliquote and store at −20°C.

3. 1,000× Protease inhibitor cocktail: 1 mM E-64 (Sigma), 1 mM bestatin (Sigma), 1 mM pepstatin (Sigma), and 2 mM leupeptin (Sigma) in dimethyl sulfoxide (DMSO); aliquote and store at −80°C.

4. 1,000× Phenylmethanesulfonyl fluoride (PMSF, Sigma): Dissolve 1 M PMSF in isopropanol. Store in aliquots at −20°C.

5. 100 mM cantharidin (Sigma) in DMSO; aliquote and store at −20°C.

6. 1 M dithiothreitol (DTT, Sigma) in double-distilled water (ddH₂O); aliquot and store at −20°C.

7. 200 mM sodium orthovanadate in ddH₂O (see Note 1).

8. Tissue grinding buffer (buffer H): 25 mM HEPES, pH 7.5 with NaOH, 0.33 M sucrose, 10% glycerol, 0.6% polyvinylpyrrolidone, 5 mM ascorbic acid, 5 mM Na-EDTA, 50 mM NaF, 2 mM imidazole, and 1 mM sodium molybdate. Store aliquots at −20°C and thaw on ice before use (see Note 2). Right before grinding, add protease inhibitor cocktail, PMSF, DTT, cantharidin, and activated sodium orthovanadate stock solution to make the final grinding buffer containing 1 μM PMSF, 5 mM DTT, 0.1 mM cantharidin, and 1 mM sodium orthovanadate.

2.2. Plasma Membrane Isolation

1. Dextran stock solution (20%, w/w) is prepared by adding 780 g of ddH₂O to 220 g of Dextran T-500 (average molecular weight 500,000, Fisher scientific) on a balance. The mixture can be incubated at 50°C water bath to facilitate dissolving. Aliquot and store at −20°C (see Note 3).

2. PEG stock solution (40%, w/w) is prepared by adding 300 g of ddH₂O to 200 g of polyethylene glycol (PEG)-3350 (average molecular weight 3,350, Sigma). Store in aliquots at −20°C.

3. Potassium phosphate buffer (0.2 M, pH 7.8): Mix 0.2 M K₂HPO₄ with 0.2 M KH₂PO₄ by constant stirring until the pH of the solution reaches 7.8.

4. 1 M potassium chloride in ddH₂O.

5. Na-EDTA 50 mM in ddH₂O, pH 7.8 with NaOH.

6. 2 M Sucrose in ddH₂O.

7. Microsome resuspension solution (buffer R): Prepare 4× stock containing 20 mM potassium phosphate (pH 7.8), 1 M W. Tang
sucrose, 32 mM KCl, and 0.4 mM EDTA using stock solution listed on items 3–6. For resuspension of microsome, add protease inhibitor cocktail and 1 mM DTT to 1× buffer R right before use. KCl concentration in buffer R should match the final KCl concentration chosen for the two-phase mixture. In this case, the author used a two-phase mixture with 6.0% polymers and 8 mM KCl.

8. T3 upper-phase dilution solution (buffer D): 5 mM potassium phosphate (pH 7.8) and 0.1 mM EDTA. Add protease cocktail and 1 mM DTT right before use.

9. Two-phase mixture preparation: One day before PM purification, according to Table 1, on a balance, mix 12 g of 20% (w/w) Dextran T-500, 40% (w/w) PEG 3350, 4× buffer R, and ddH2O in a 25-ml glass centrifuge tube for a 16 g of two-phase mixture. Three 12 g of two-phase mixtures should be prepared for each sample and labeled with T1, T2, and T3, since two wash steps are to be used for PM purification. Leave T1 for future microsomal resuspension solution, and weight 4 g of 1× buffer R to each T2 and T3 tube to make a full strength 16 g of two-phase mixture. Vortex and spin T1, T2, and T3 at 1,000 × g for 5 min in a swinging rotor to facilitate phase settling (see Note 4). Store T1, T2, and T3 at 4°C until used for PM preparation.

### Table 1
Two-phase system with 6.0% Dextran T-500, PEG 3350, and 8 mM KCl

<table>
<thead>
<tr>
<th>Phase mixture</th>
<th>4 g</th>
<th>16 g</th>
<th>20 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% (w/w) Dextran T-500 (g)</td>
<td>1.2</td>
<td>4.8</td>
<td>6</td>
</tr>
<tr>
<td>40% (w/w) PEG 3350 (g)</td>
<td>0.6</td>
<td>2.4</td>
<td>3</td>
</tr>
<tr>
<td>4× Buffer R (g)</td>
<td>0.75</td>
<td>3</td>
<td>3.75</td>
</tr>
<tr>
<td>ddH2O to the weight of (g)</td>
<td>3</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>Microsome in buffer R (or buffer R alone) to the weight of (g)</td>
<td>4</td>
<td>16</td>
<td>20</td>
</tr>
</tbody>
</table>

*aTwo-phase systems with various Dextran concentrations can be prepared by varying the amount of 20% Dextran, 40% PEG 3350 and ddH2O

2.3. Sample Labeling and Two-Dimensional Gel Electrophoresis

Ideally, two-dimensional electrophoresis should be performed using the 2-D DIGE system of GE Healthcare. 2-DE equipment of other companies, such as Bio-Rad, can also run 2-D DIGE with some modifications: the glass plates for SDS-PAGE should be low-fluorescence glass and one of the two glass plates for each
gel must be treated with Bind Silane so that the gel will attach to one plate and will not change shape during staining and spot picking. Precast SDS-PAGE gels are commercially available, but we routinely cast our own gels to reduce cost. We recommend using the 24-cm IPG strips and 26 x 20-cm SDS-PAGE gels because large gels produce higher resolution. In general, the number of protein spots that can be detected is correlated to the size of the gels. A fluorescence scanner, such as the Typhoon Trio (GE Healthcare) or similar equipment, is required for acquiring the CyDye images. Image analysis should be performed using DeCyder software (GE Healthcare). Other 2-DE software can be used, but software that performs spot detection on separate images from the same gel tends to introduce errors in quantitation. Spot picking should be performed using the Ettan Spot picker of GE Healthcare, which is designed to pick spots based on fluorescence images with two reference spots. Manual spot picking is possible by post-staining the gel with Coomassie Blue or silver after acquiring the fluorescence images.

1. 2-DE buffer: 7 M urea, 2 M thiourea, and 4% CHAPS in ddH$_2$O. Filter through a 0.22-µm filter and store at −20°C as 1 ml of aliquots to avoid repeated freeze and thaw.

2. CyDye working solution: 0.1 mM CyDye DIGE Fluor minimal dyes (GE Healthcare) in high quality anhydrous dimethylformamide (DMF, Sigma) (see Note 5).

3. 10 mM Lysine in ddH$_2$O. Aliquot and store at −20°C.

4. SDS equilibration buffer: 6 M urea (BioUltra, for molecular biology, >99.5%, Sigma), 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS, >99%, Sigma), and 50 mM Tris–HCl, pH 8.8. Aliquot and store at −20°C. Add 0.5% (w/v) DTT or 4.5% (w/v) iodoacetamide (GE Healthcare) right before use.

5. Bind-Silane ($\gamma$-methacryloxypropyltrimethoxysilane) working solution: Dissolve 20 µl of Bind-Silane solution (GE Healthcare) in 80% (v/v) ethanol and 0.02% (v/v) glacial acetic acid. Prepare right before use.

6. Low-fluorescence glass plates for SDS-PAGE.

7. 1.5 M Tris–HCl, pH 8.8.

8. 10% (w/v) SDS in ddH$_2$O.

9. Acrylamide stock solution (30.8% w/v): Dissolve 300 g of high-quality acrylamide (for molecular biology, >99.5%, Sigma) and 8 g of N,N'-methylenebisacrylamide (Sigma) in 800 ml ddH$_2$O. Add ddH$_2$O to the volume of 1,000 ml and filter the solution through a 0.2-µm membrane to remove any insoluble substance. Store at 4°C and away from light.
10. 10% (w/v) Ammonium persulfate in ddH$_2$O. Aliquot and store at $-20^\circ$C.

11. Water-saturated isobutanol: Mix 800 ml of isobutanol with 200 ml of water in a glass bottle, shake violently, and sit still to allow liquid separation. Use the top layer. Store at room temperature.

12. SDS Running buffer (10×): 250 mM Tris, 1,920 mM glycine, and 1% (w/v) SDS. Store at room temperature.

13. Agarose overlay solution: 0.5% (w/v) low melting agarose (Promega) in SDS running buffer; store at room temperature. Microwave to melt all the agarose, and allow the solution to cool slightly before use.

14. Front dye solution: 1% (w/v) bromophenol blue (Sigma) in SDS running buffer.

2.4. Deep Purple Staining

1. Fixation solution: 15% (v/v) ethanol; 1% (w/v) citric acid (pH ~2.3) in water.

2. Borate buffer: 100 mM sodium borate in water, pH to 10.5 with NaOH.

3. Staining solution: Right before use, add 1 ml Deep Purple (GE Healthcare) to 500 ml of borate buffer.

4. Washing solution: 15% (v/v) ethanol in water.

3. Methods

3.1. Liquid Culture of Arabidopsis Seedlings

1. Surface sterilize Arabidopsis seeds in 40 ml of bleach containing 5.25% sodium hypochlorite for 10 min (see Note 6).

2. Extensively wash the seeds at least five times each with >40 ml sterilized water. Add seeds to flask containing growth medium at a ratio of 50 mg of seeds/250 ml of medium. The growth medium should not exceed 1/4 volume of the flask to ensure enough aeration for the seedlings.

3. Keep imbibed seeds at 4°C for 2 days to synchronize germination, and then move the culture to light chamber to grow under continuous light for 7 days on a shaker at a shaking speed of 90 rpm.

4. Mix all the culture in a large container and split culture mix into two 250-ml beakers for hormone and mock treatment. To minimize sample variation, evenly split seedlings together with old culture medium to avoid sudden osmotic or nutrient shock.
5. For treatment with BL (the most active form of BR), add BL stock solution to 100 nM to one of the beakers to start the treatment, and add the same volume of 85% ethanol to the mock-treated sample. Shake gently for 2 h.

6. Harvest tissue by filtering through a nylon mesh. Use different nylon mesh for BL-treated and mock-treated tissue, or harvest the mock sample first, to avoid cross-BL contamination of the mock sample.

3.2. Purify Plasma Membrane from Liquid-Grown Arabidopsis Seedlings

1. To reduce variations, process BL-treated sample and mock-treated sample in parallel for PM isolation. Reverse the handling order of sample and control in repeat experiments.

2. Homogenize 30–50 g of material (mock-treated sample first) with PowerGen 700D grinder in buffer H (for 1 g tissue, 1–2 ml of grinding buffer should be used) at 10,000 rpm for 5 min. Starting from here, all steps should be performed at 4°C (see Note 7).

3. Filter homogenate through two layers of miracloth, and spin at 10,000 × g for 10 min to remove insoluble debris and organelles. Keep the supernatant.

4. Microsomal membranes are pelleted by centrifugation at 60,000 × g for 30 min (higher speed or longer spin time makes pellet hard to resuspend).

5. Resuspend pellet in 4–5 ml of buffer R by gently pipetting up and down 150 times. Keep the pipette tip below surface to avoid generating bubbles (see Note 8).

6. On a balance, add 4 g of resuspended microsomal solution (about 20–30 mg microsomal protein) to a 12 g of Dextron T-500/PEG 3350 two-phase mixture (T1) which is prepared the day before PM purification (see Subheading 2). Invert the centrifuge tube 30 times (see Note 9).

7. Spin T1 and 1× buffer R-added T2 at 1,000 × g, 4°C, for 5 min to separate the two phases.

8. Remove 8 ml of upper phase from T2 without disturbing the interface to a clean tube for a later balancing purpose. Transfer the same amount (8 ml) of upper phase from T1 to the low phase of T2. Invert the centrifuge tube 30 times to mix solutions.

9. Spin T2 and 1× buffer R-added T3 at 1,000 × g, 4°C, for 5 min.

10. Remove 8 ml of upper phase from T3 to a clean tube without disturbing the interface. Transfer the same amount (8 ml) of upper phase from T2 to T3, and invert 30 times.

11. Spin T3 at 1,000 × g, 4°C, for 5 min. Transfer 8 ml T3 upper phase from each BR-treated or untreated sample to two to
three clean ultracentrifuge tubes, and dilute with 5–10 volumes of buffer D. Invert ten times to mix.

12. Spin at 100,000 \( \times g \) for 1 h to pellet the PM.

13. Resuspend the PM in 100 \( \mu l \) of buffer D by vortexing (or slowly pipetting up and down without generating bubbles). Alternatively, the PM pellet can be resuspended/solubilized in another buffer suitable for the next analysis step.

### 3.3. Two-Dimensional Difference Gel Electrophoresis

**3.3.1. Label Proteins with CyDye**

1. Mix the purified PM in buffer D with five volumes of cold 100% methanol at \(-20^\circ C\) overnight to precipitate the protein.

2. Centrifuge at 20,000 \( \times g \) for 15 min to pellet the protein, and discard supernatant.

3. Add 1 \( ml \) of \(-20^\circ C\) methanol to the pellet, and vortex for 1 min to remove residual salt in the pellet.

4. Centrifuge at 20,000 \( \times g \) for 15 min to pellet the protein, and discard supernatant carefully; the pellet is very loose now.

5. Quick spin and remove residual methanol with a sharp pipette, and immediately add 40 \( \mu l \) 2-DE buffer to the pellet. The pellet should dissolve instantly into the buffer. It is important not to overdry the protein pellet; otherwise, the pellet becomes very difficult to dissolve by the 2-DE buffer in the next step. If the pellet does not dissolve, sonicate at the lowest power for 5–10 s. During sonication, the temperature of 2-DE buffer should be controlled under 37\(^\circ C\). Elevated temperature above 37\(^\circ C\) can hydrolyze urea to isocyanate, which modifies proteins by carbamylation.

6. Determine the protein concentration by Bio-Rad protein assay reagent (Bio-Rad) using BSA dissolved in 2-DE buffer as standard.

7. Adjust samples to equal protein concentrations of 3–5 \( \mu g/\mu l \) with 2-DE buffer.

8. Aliquot 5–10 \( \mu l \) of 2-DE buffer-dissolved PM protein solution, and mix with 0.2 \( \mu l \) of Tris–HCl buffer (1.5 M, pH 8.8) to adjust the pH of the protein solution to around 8.8. Add 50–100 pmol of CyDye (0.5–1 \( \mu l \) of 0.1 mM CyDye working solution) to the protein solution, and incubate the mixture on ice for at least 2 h in the dark for protein labeling (see Note 10).

9. Terminate the labeling reaction by adding 1 \( \mu l \) of 10 mM lysine and incubating on ice for 10 min.

**3.3.2. Isoelectric Focusing**

1. Combine Cy3- and Cy5-labeled pair of control and treated samples in a 1.5-ml Eppendorf tube.

2. Add 9 \( \mu l \) of 1 M DTT and 4.5 \( \mu l \) of IPG buffer, pH 4–7. Add 100 \( \mu g \) of unlabelled protein each from mock- and BL-treated
sample to make the total protein amount in the mixture around 250–300 μg. Use 2-DE buffer to adjust the final volume of the protein mixture to 450 μl for IEF separation using the 24-cm Immobiline DryStrips (GE Healthcare), pH 4–7 (see Notes 11 and 12).

3. Perform IEF following standard rehydration loading protocol. The typical running conditions are as follows: rehydration for 2 h, active rehydration at 50 V for 10 h, step and hold at 500 V, 1,000 V for 1 h each, and then hold at 8,000 V until reaching a total of 80,000 V-h.

3.3.3. SDS-PAGE

Skip steps 1 and 2 if using precast gels.

1. Treat one of the two glass plates for a gel cassette with Bind Silane. Soak the small low-fluorescence glass plate (the plate without the spacer, GE Healthcare) overnight in laundry bleach, which contains 5.25% sodium hypochlorite, to help scrape off any residual bound gel or CyDye from previous experiments. Wash the plate with Decon solution (GE Healthcare), rinse thoroughly with ddH₂O₂, and air dry in a dust-free environment. In the chemical hood, lay the dried plate flat on a layer of clean KimWipes. Add 2–3 ml of freshly made Bind-Silane working solution on the plate, and wipe over all areas of the plate with KimWipes or other lint-free cleaning tissue until the solution is completely dry. Cover the plate with KimWipes and leave in the hood for 2 h for excess Bind Silane to evaporate. Wipe clean the Bind Silane-treated surface with 95% ethanol using KimWipes, attach two reference markers on Bind Silane-treated surface, and leave the plate in a dust-free environment for another hour. The reference marker stickers should be placed half way along the edge and 1 cm away from the spacer (see instructions of GE Healthcare for more details).

2. Casting SDS-polyacrylamide gel: Assemble the gel caster according to Ettan DALT electrophoresis unit user’s manual (GE Healthcare) using one Bind Silane-treated low-fluorescence glass plate with reference markers and one untreated plate for each gel (if both plates are treated with Bind Silane, you will not be able to open the gel cassette after gel polymerizes). Following standard protocol, prepare 10% SDS-polyacrylamide gel solution using the stock solution listed in sect. 2.3, steps 7–9. Degas the solution with a vacuum pump for 5 min. Add appropriate volume of ammonium persulfate, mix thoroughly by stirring, and pour the acrylamide gel solution into the gel caster until the gel surface is 2 cm below the upper edge of the small glass plate. Add water-saturated isobutanol to cover the acrylamide surface and let the solution polymerize at room temperature for at least 2 h. Best
polymerization can be achieved by letting the gel polymerize overnight at room temperature. Gels can be stored at 4°C for up to 2 weeks; however, best results are achieved using freshly prepared SDS-polyacrylamide gels.

3. At the end of the IEF run, carefully remove the IPG strip from the strip holder and place it in individual equilibration tubes (GE Healthcare) with gel side away from the tube wall (this can be stored in ~80°C freezer if the SDS-PAGE gels are not ready). Add 10 ml of SDS equilibration buffer containing 0.5% (w/v) DTT to each tube.

4. Incubate the strips for 15 min with gentle agitation in the dark.

5. Pour off the equilibration buffer in the tube and replace with 10 ml of SDS equilibration buffer containing 4.5% (w/v) iodoacetamide.

6. Incubate the strips for another 15 min with gentle agitation in dark.

7. Briefly rinse the strips by submerging in an equilibration tube containing SDS running buffer.

8. Get the SDS-PAGE gel ready by removing all liquid on top of the gel. Holding one end of the strip with forceps, carefully place the strip in between the two glass plates on top of the SDS-polyacrylamide gel.

9. Add warm agarose overlay solution to cover the strip. Add from one side to avoid trapping of air bubbles. Use a thin flat spatula or spacer to push the strip gently until it reaches the surface of the polyacrylamide gel. Try not to leave any space between the strip and the surface of the polyacrylamide gel, as the space will increase horizontal streaking of gel spots (see Note 13).

10. For each polyacrylamide gel, cut two 5 × 5-mm filter papers. On one filter paper, add 2 μl of PageRuler plus prestained protein ladder (Fermentas). On the other filter paper, add 2 μl of front dye solution. Place the filter papers on top of the SDS-PAGE gel next to each side of the IPG strip (protein ladder on acidic side and the front dye on basic side). Using a spatula or spacer, push the filter papers gently down through the agarose until they touch the surface of the gel.

11. Wait until the agarose solidifies completely, and then start the second dimension SDS gel electrophoresis at 50 V for 2 h, and then at 110 V until the bromphenol blue front reaches the end of the gel (this usually takes 8–12 h).

3.3.4. Gel Scanning, Image Analysis, and Spot Picking

1. At the end of electrophoresis, take out the gel cassettes. Rinse the surface of the glass plates, and scan the gel for Cy3- and Cy5-labeled images using a Typhoon 8600 scanner or a newer model if Cy2 is used (GE Healthcare) with PMT power set at
600. Rescan with different PMT power to obtain images with strong signal without spot signal saturation.

2. Visually examine the image for reddish or greenish spots. Analyze the DIGE image with DeCyder 6.5 software (GE Healthcare) to find the spots that are statistically different between samples and control. Generate a list of spots of interest for gel picking.

3. Open the gel cassette. The SDS-polyacrylamide gel will stick to the Bind Silane-treated glass plate.

4. Fix 2-D DIGE gels in 1,000 ml fixation solution (gel side up) for a minimum of 1.5 h with gentle rocking to avoid scratching the surface of the glass plates. Usually, we do fixation overnight to reduce the background.

5. Move the gels from fixation solution into 500 ml of Deep Purple (GE Healthcare) staining solution (gel side up); try to minimize carryover of the fixation solution. Stain the gels for 1 h in dark with gentle rocking.

6. Wash the gels with 1,000 ml of washing solution for 30 min by gentle rocking.

7. Place the gels in 1,000 ml of fixation solution and rock gently for 30 min. For long-term storage (up to 12 months) of the gels, replace the fixation solution with 1% citric acid containing 1:500 diluted Deep Purple and store the gels at 4°C in the dark.

8. Scan the gel on Typhoon scanner (gel side up) to acquire the Deep purple staining images and Cy5 image. Use Cy5 image as reference gel to mark the spots of interest on Deep Purple image. Use the Decyder software to generate spot picking list.

9. Excise the spots of interest from the gel using an Ettan Spot Picker (GE Healthcare), and process the gel plugs for in-gel digestion and protein identification by mass spectrometry analysis, which can be done by a mass spectrometry facility or service provider.

4. Notes

1. Phosphatase inhibitors (such as cantharidin and sodium orthovanadate) are essential for preventing protein dephosphorylation during sample preparation. Sodium orthovanadate should be activated for maximal inhibition of protein phosphatases. To activate sodium orthovanadate, prepare 200 mM of sodium orthovanadate in ddH₂O and adjust the pH to 10.0 using either 1 N NaOH or 1 N HCl. At pH 10.0, the sodium orthovanadate solution is yellow. Boil the solution until it
turns colorless. Cool to room temperature, and adjust the pH to 10.0 again. Repeat the procedure until the solution remains colorless and the pH stabilizes at pH 10.0. Store the activated sodium orthovanadate as aliquots at −20°C. Add sodium orthovanadate to buffer right before use.

2. In some PM isolation protocols, 0.5% (w/v) Casein and/or 0.5% (w/v) bovine serum albumin (BSA) are included to protect the proteins from degradation after lysis. Since casein and BSA are copurified with the PM vesicles, their relative high abundance creates serious contamination problem later on. In our experience, the casein and/or BSA spots in 2-D DIGE can be used as reference for reproducibility between the samples, but can contaminate other spots in mass spectrometry analysis.

3. Dextran T-500 usually contains 5–10% water, so the concentration of the solution is not 22% but somewhere between 20 and 21%. Exact concentration should be determined with a polarimeter with specific rotation of Dextran at 199° ml/g/dm. If a polarimeter is not available, assuming there is 5% of water in Dextran T-500, large stock solutions (2–3 L) should be prepared so that the same dextran stock is used throughout the whole experiment to ensure reproducible results.

4. For details about the PM purification procedures, readers should read refs. 11, 12. It is advisable to begin with a two-phase mixture with 6.5% polymers and 4–5 mM KCl. Such a two-phase mixture usually yields high-quality PM preparation. Once you are familiar with the methods, you can test different concentrations of polymers and KCl to achieve a good balance between the purity and the yield of the purified PM.

5. The manufacturer recommends using 400 pmol CyDye DIGE Fluor minimal dye (1 μl of 0.4 mM dye stock) to label 50 μg of protein in a small volume (5–10 μl). We found that satisfying 2-D DIGE images can be obtained with as little as 50–100 pmol/30–50 μg of protein in a small volume (5–10 μl), but very clean low-fluorescence glass plates must be used to improve signal-to-background ratio. This practice greatly reduces the cost of 2D-DIGE experiments. To prepare the CyDye working solution, dissolve each 25 nmol CyDye DIGE Fluor minimal dye in 25 μl of freshly opened high-quality anhydrous DMF. Aliquot CyDye solution to a 1.5-ml centrifuge tube (1 μl each) and speed vacuum for 1 min under dim light or until the solution is completely dried. The aliquoted CyDye can now be stored dry at −80°C for at least 6 months without losing observable labeling activity. With prolonged storage time (more than 1 year), we do see decreased labeling efficiency. In that case, double the CyDye labeling concentration can solve the
problem very well. To prepare the 0.1 mM CyDye working solution, add 10 μl DMF to 1 nmol CyDye aliquot, pipette up and down to mix, and use 0.5–1 μl to label 30–50 μg of protein in small volume (5–10 μl). Store the unused CyDye working solution in −80°C. Since the DMF quality is critical for CyDye labeling efficiency, we also aliquot freshly opened high-quality anhydrous DMF in a 1.5-ml centrifuge tube with crew seal cap (1 ml each) and store in −80°C. The aliquoted DMF should be used only once. Once opened again, the remaining DMF should not be reused for future CyDye dissolving, but still can be used as solvent for other less delicate chemicals.

6. Use a 50-ml falcon tube for seeds’ sterilization. Sterilizing large amount of seeds in a small-volume centrifuge tube (e.g., 1.5-ml Eppendorf tube) will result in contaminated culture. It is also very important to use the seeds harvested from healthy plants. If the seeds were harvested from fungi-contaminated plants, the surface sterilization methods used in this paper are no longer sufficient.

7. The reason we use a grinder to homogenize the tissue at high speed for a relative long time (5 min) is to get similar yield of microsome for two-phase partition when we start with the equal amount of control and treated tissues.

8. Keep the pipette tip in the solution all the times while gently pipetting up and down. This is a tedious work and requires lots of patience. Once compressed together by centrifugation, it is difficult to separate the membrane vesicle by pipetting. We found that resuspension of microsome directly affects the yield of the PM purification.

9. Vortexing two-phase mixture with microsome greatly increases the PM purity, but significantly decreases the yield.

10. It is found that some proteins are differentially labeled by different CyDyes; therefore, running repeat gels that swap the dyes is crucial to avoid any dye-specific effects. For example, to study BR-regulated PM proteomic changes, we run 2-D DIGE gels for at least five biological repeats (one biological repeat means one experiment start from plant culture). Of these five biological repeats, three of the BR-treated samples were labeled with Cy5, and the other two were labeled with Cy3. Technical repeat of 2-D DIGE of the same protein sample is usually unnecessary unless the first run has obvious technical problems.

11. Usually, we first run at least two biological repeat samples in analytical 2-D DIGE using about 50–100 μg of protein to find the spots of interest, and then run a preparative gel using 500–1,000 μg of protein to pick the interesting spots for in-gel digestion and MS analysis. Low amount of loading (50–100 μg of protein in a 24-cm gel) yields highly resolved
gel images, and higher protein loading decreases the image resolution because increased spot size leads to spot overlap and coverage of low-abundance protein spots by high-abundance spots. However, high loading improves protein identification by mass spectrometry, which is critical for identifying low-abundance proteins. To avoid two separate analytical and preparative gels, a compromise is to load 250–300 μg of protein/gel and pool the corresponding protein spots picked from replicate gels for mass spectrometry analysis. One should keep in mind that the spot signal intensity is largely determined by the amount of CyDye used in 2-D DIGE, and by the amount of protein in post-electrophoresis staining.

12. We recommend using 24-cm IPG strips (pH 4–7) for the initial 2D DIGE analysis. However, narrow pH range IPG strips (e.g., pH 3–5.6, 5.3–6.5, 6–11, and 3.5–4.5) give better separation of spots, and thus more protein spots can be detected and less spot overlap and cross-contamination occur when multiple narrow pH range gels are used rather than a single wide-range gel. For basic protein separation, the 18-cm pH 6–11 IPG strips seem to produce better results than the longer 24-cm pH 7–11 or pH 7–10 IPG strips.

13. Tracking the samples and gel orientation are important through the long procedure. Record in notebook the bar code and number on the IPG strip and the sample loaded on the strip.

Acknowledgment

This work was financially supported by National Science Foundation of China (90917008).

References


