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[Abstract] We established and elaborated on a method to enrich the membrane proteome of mature pollen from economically relevant crop using the example of *Solanum lycopersicum* (tomato). To isolate the pollen protein fraction enriched in membrane proteins, a high salt concentration (750 mM of sodium chloride) was used. The membrane protein-enriched fraction was then subjected to shotgun proteomics for identification of proteins, followed by *in silico* analysis to annotate and classify the detected proteins.
Keywords: Membrane proteome, Pollen, Tomato, Proteomics

[Background] As proper distribution of proteins and solutes between different cellular compartments or the insertion of newly-synthesized proteins into membranes is largely dependent on membrane proteins, the membrane proteome is central for maintenance of cellular and organellar homeostasis (Paul *et al.*, 2013; 2014 and 2016a). Considering the importance of membrane proteins in general, these are also essential for pollen function and development (Paul *et al.*, 2016b). Many global pollen proteomic studies have been performed in the past (Chaturvedi *et al.*, 2013 and 2016); however, information about the intracellular distribution of proteins and the composition of the membrane proteome in pollen is rarely discussed (Pertl *et al.*, 2009). One reason might be the low abundance and solubility of membrane proteins. Here we describe a protocol to isolate and analyze a protein fraction enriched in membrane proteins from mature pollen, which was established for tomato (Figure 1).
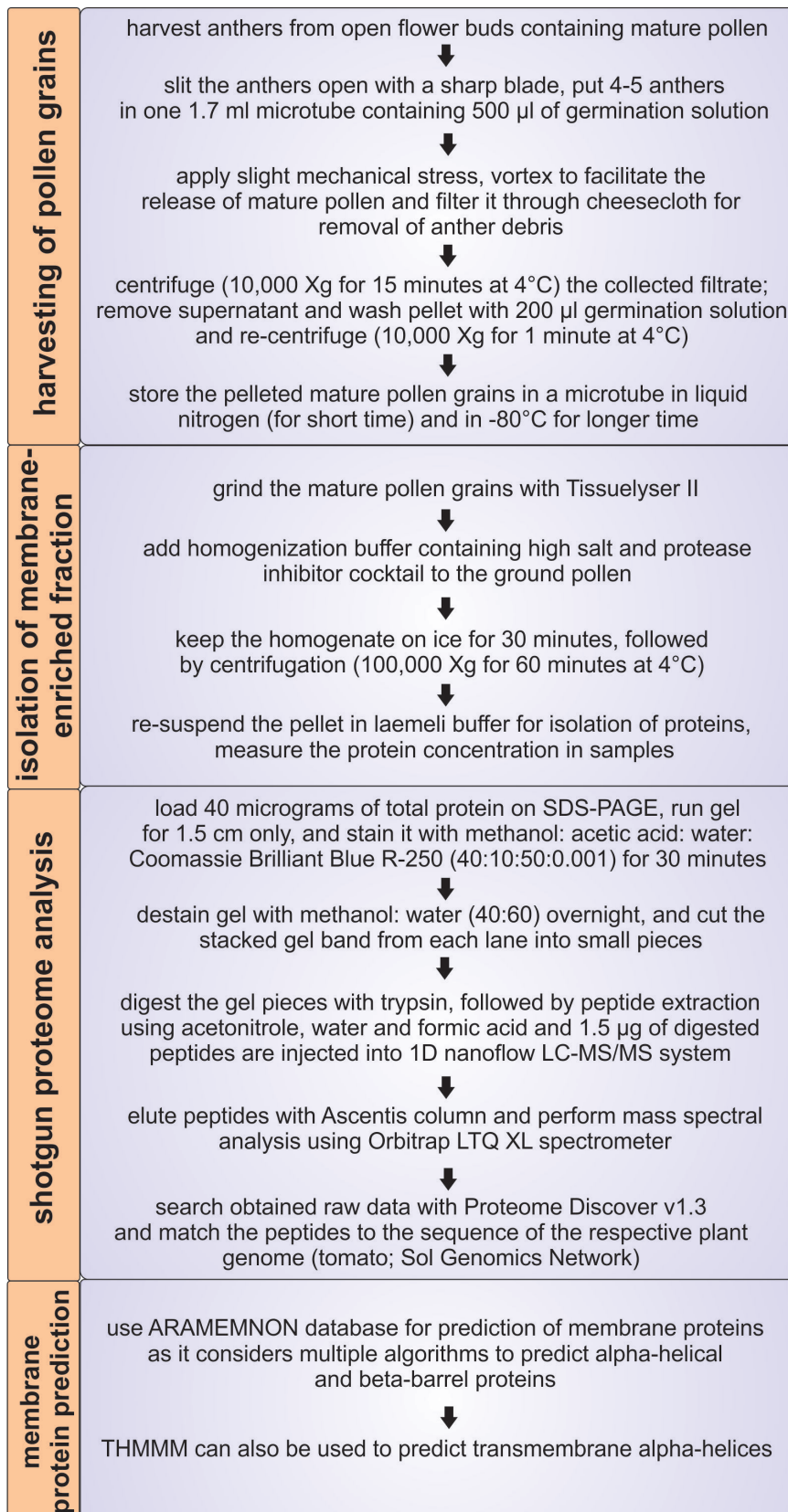
harvesting of pollen grains

harvest anthers from open flower buds containing mature pollen

⬇

slit the anthers open with a sharp blade, put 4-5 anthers
in one 1.7 ml microtube containing 500 µl of germination solution

⬇

apply slight mechanical stress, vortex to facilitate the
release of mature pollen and filter it through cheesecloth for
removal of anther debris

⬇

centrifuge (10,000 Xg for 15 minutes at 4°C) the collected filtrate;
remove supernatant and wash pellet with 200 µl germination solution
and re-centrifuge (10,000 Xg for 1 minute at 4°C)

⬇

store the pelleted mature pollen grains in a microtube in liquid
nitrogen (for short time) and in -80°C for longer time

isolation of membrane-enriched fraction

grind the mature pollen grains with Tissuelyser II

⬇

add homogenization buffer containing high salt and protease
inhibitor cocktail to the ground pollen

⬇

keep the homogenate on ice for 30 minutes, followed
by centrifugation (100,000 Xg for 60 minutes at 4°C)

⬇

re-suspend the pellet in laemeli buffer for isolation of proteins,
measure the protein concentration in samples

shotgun proteome analysis

load 40 micrograms of total protein on SDS-PAGE, run gel
for 1.5 cm only, and stain it with methanol: acetic acid: water:
Coomassie Brilliant Blue R-250 (40:10:50:0.001) for 30 minutes

⬇

destain gel with methanol: water (40:60) overnight, and cut the
stacked gel band from each lane into small pieces

⬇

digest the gel pieces with trypsin, followed by peptide extraction
using acetonitrole, water and formic acid and 1.5 µg of digested
peptides are injected into 1D nanoflow LC-MS/MS system

⬇

elute peptides with Ascentis column and perform mass spectral
analysis using Orbitrap LTQ XL spectrometer

⬇

search obtained raw data with Proteome Discover v1.3
and match the peptides to the sequence of the respective plant
genome (tomato; Sol Genomics Network)

membrane protein prediction

use ARAMEMNON database for prediction of membrane proteins
as it considers multiple algorithms to predict alpha-helical
and beta-barrel proteins

⬇

THMMM can also be used to predict transmembrane alpha-helices

Figure 1. Overview of the protocol for isolation of the membrane proteome of mature tomato pollen

Materials and Reagents

1. 1.7 ml microtubes (Corning, Axygen®, catalog number: MCT-175-C)
2. Pipette tips
3. Cheesecloth Miniwipes (Fisher Scientific, catalog number: 06-665-28)
4. Sharp blade (Red Devil, catalog number: 3272)
5. Bond-Elute C-18 SPEC plate (Agilent Technologies, Santa Clara, CA, USA)
6. Seeds of the cultivar of choice. For the method development–seeds of *Solanum lycopersicum* cv. Moneymaker (Accesion: LA2706) and cv. Red setter were used
7. Liquid nitrogen
8. Sodium chloride (NaCl) (Fisher Scientific, catalog number: S271-500)
9. Protease inhibitor cocktail (Sigma-Aldrich, catalog number: P9599)
10. 6x Laemmli buffer (Alfa Aesar, catalog number: J61337)
11. Urea (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 29700)
12. Acrylamide/bis-acrylamide (Fisher Scientific, catalog number: BP1408-1)
13. TEMED (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 17919)
14. Ammonium persulfate (Acros Organics, catalog number: 327081000)
15. Acetonitrile (Fisher Scientific, catalog number: A9561)
16. Formic acid (Fisher Scientific, catalog number: A117-50)
17. Boric acid (Fisher Scientific, catalog number: A73-500)
18. Calcium nitrate tetrahydrate (Fisher Scientific, catalog number: C109-500)
19. Magnesium sulphate anhydrous (Fisher Scientific, catalog number: M65-500)
20. Potassium nitrate (Fisher Scientific, catalog number: P263-500)
21. Potassium chloride (Fisher Scientific, catalog number: P217-500)
22. Dithiothreitol (DTT) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: R0861)
23. Ethylenediaminetetraacetic acid, disodium salt dihydrate (EDTA) (Fisher Scientific, catalog number: S311-100)
24. Tris base (Fisher Scientific, catalog number: BP152-500)
25. Methanol (Fisher Scientific, catalog number: A456-1)
26. Acetic acid (Fisher Scientific, catalog number: A507-P500)
27. Double distilled water
28. Coomassie Brilliant Blue R-250 (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 20278)
29. Trypsin solution sequencing grade (Roche Diagnostics, catalog number: 11418475001)
30. Ammonium bicarbonate (Fisher Scientific, catalog number: A643-500)
31. Calcium chloride (Fisher Scientific, catalog number: C70-500)
32. Trifluoroacetic acid (TFA) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 28904)
33. Germination solution (see Recipes)

34. Homogenization buffer (see Recipes)

35. Staining solution (see Recipes)

36. Destaining solution (see Recipes)

37. Trypsin buffer (see Recipes)

Equipment

1. Vortex (*e.g.*, Fisher Scientific, model: Fisher Scientific™ Analog Vortex Mix, catalog number: 02-215-365)

2. Centrifuge (*e.g.*, Eppendorf, model: 5424 R)

3. Ultracentrifuge (*e.g.*, Thermo Fisher Scientific, model: Sorvall® Discovery™ 90 SE)

4. Daisy BBs (Daisy, model: 24, catalog number: 980024-001)

5. Tissuelyser II (QIAGEN, model: Tissuelyser II, catalog number: 85300)

6. Speed-Vac Labogene ApS (LabGene™, model: ScanVac CoolSafe 110-4, catalog number: H01130032)

7. Pipette

8. Sonicator (BANDELIN electronic, model: Sonorex RK 100, catalog number: 301.00044253.024)

9. Precolumn (Eksigent, Redwood City, CA, USA)

10. Ascentis column (Sigma-Aldrich, Supelco, model: Ascentis® Express Peptide ES-C18 HPLC Column, catalog number: 53552-U), dimension 15 x 100 µm, pore size 2.7 µm

11. Orbitrap LTQ XL mass spectrometer (Thermo Fisher Scientific, model: LTQ XL™)

Software

1. Proteome Discoverer 1.3 (Thermo, Germany) or other software like MaxQuant (http://www.maxquant.org/), Protmax (http://www.univie.ac.at/mosys/software.html) and MASCOT (http://www.matrixscience.com/)

2. PROMEX (http://promex.pph.univie.ac.at/promex/) or other software like ProteomeXchange (http://www.proteomexchange.org/)

Procedure

A. Harvesting of mature pollen grains

1. Grow plants (*e.g.*, tomato cv. Moneymaker–MM hereafter and cv. Red setter–RS hereafter) under controlled greenhouse conditions (for tomato: 24 °C/20 °C, day/night; 60% humidity (Fragkostefanakis *et al.*, 2015).
   Note: Growth conditions need to be adapted for the plant species or cultivar in use.

2. Harvest anthers (> 10 mm length for MM and RS) from flower buds after anthesis as they contain mature pollen grains (preferable time is 0-72 h post anthesis).

www.bio-protocol.org/e2315

3. Make a horizontal cut on the collected anther and put four anthers in one 1.7 ml microtube containing 500 µl of germination solution.

4. Squeeze anther tissue with slight mechanical stress using pipette tips and vortex for 15-20 sec to release the mature pollen from anther locules.

5. Further, pass germination solution containing isolated mature pollen grains through cheesecloth to remove anther debris.

6. Centrifuge the collected pollen grains at 10,000 *x g* for 15 min at 4 °C.

7. Remove the supernatant, wash the pelleted pollen grains with 200 µl of germination solution and centrifuge at 10,000 *x g* for 1 min at 4 °C.

8. Remove the supernatant and repeat the washing step A7.

9. Remove the supernatant, immerse the collected pollen in liquid nitrogen and store at -80 °C till further usage.

B. Isolation of membrane-enriched fraction

1. Add metal beads (Daisy BB) that help to homogenize the tissue to microtubes and grind the collected mature pollen grains with TissueLyser II (at 25/sec for 1 min).

2. Add 10-20 ml homogenisation buffer containing sodium chloride (NaCl, 750 mM) and protease inhibitor cocktail to the ground mature pollen, re-suspend and leave it on ice for 30 min.

3. Centrifuge the homogenised mixture at 100,000 *x g* for 60 min at 4 °C.

4. Re-suspend the pellet in Laemmli buffer and quantify the protein concentration.
   Note: The supernatant is enriched in soluble proteins and can also be subjected to shotgun proteomics protocol, if needed.

C. Shotgun proteomics

1. Load 40 µg of total proteins onto an SDS-PAGE (12.5% with maximum thickness 1 mm) and run the gel at 80 V constant for 1.5 cm in resolving gel (Valledor and Weckwerth, 2014). Laemmli buffer is used as loading buffer.

2. Stain the gel with methanol:acetic acid:water:Coomassie Brilliant Blue R-250 (40:10:50:0.001) for 30 min and de-stain it overnight in methanol:water (40:60). Afterwards, slice the thick stacked protein band from the remaining gel and chop it into smaller pieces with a sharp blade. Immerse the chopped gel pieces into double distilled water until all samples are processed.

3. Afterwards, remove water and add 1 ml of sodium bicarbonate (25 mM) and incubate at 37 °C for 15 min. Discard supernatant and add 1 ml of 25 mM sodium bicarbonate and 50% acetonitrile. Incubate the samples at 37 °C for 15 min. Repeat this step.

4. Remove the supernatant and dehydrate gel pieces in 300 µl 100% acetonitrile for 5 min at room temperature. Discard supernatant and dry out in a Speed-Vac for 5 min.
   Note: Gel pieces should have a 'transparent-plastic' look. If the gel-pieces are still translucent, repeat the step.

5. Digest the chopped gel pieces with 50 µl trypsin solution (10 ng/µl) and incubate for 15 min at 37 °C. If required, add more trypsin solution until the gel pieces are completely rehydrated. Incubate for 14-16 h at 37 °C and avoid shaking during incubation. Afterwards, stop the reaction by adding 5 µl of 1.0% trifluoroacetic acid (pH should be < 4 after addition of TFA).
    Note: Add 1 ml of trypsin buffer to dilute trypsin to 10 ng/µl. Incubate on ice for 10 min and then pipette up and down to completely re-suspend the pellet. After dilution, trypsin can be stored at -20 °C for one month. It is highly recommended to store it in aliquots (Valledor and Weckwerth, 2014).

6. For peptide extraction, add 150 µl of 50% acetonitrile (v/v) and 1% formic acid (v/v) into the microtube containing the gel pieces and incubate for 5 min at room temperature followed by sonication for 3 min (low intensity ultrasound bath).

7. Spin microtubes (1,000 *x g* for 1 min) and collect the supernatant in a new microtube 'A'. Repeat step C6 and transfer the supernatant to microtube 'A'.

8. Add 100 µl of 90% acetonitrile (v/v) and 1% formic acid (v/v) to the microtube containing the gel pieces and incubate for 5 min at room temperature. Transfer the supernatant to microtube 'A'.

9. Dry the samples in Speed-Vac (microtube A). For long-term storage of extracted peptides -80 °C is preferred.

10. Afterwards, proceed with peptide desalting using spec 96 well C18, Agilent (Valledor and Weckwerth, 2014).

11. Dissolve peptides in 4% (v/v) acetonitrile, 0.1% (v/v) formic acid.

12. Inject digested peptides into a one-dimensional nanoflow LC-MS/MS system that has a precolumn.

13. Elute the peptides with an Ascentis column during 80 min and a gradient ranging from 5% to 50% (v/v) acetonitrile, 0.1% (v/v) formic acid and perform mass spectral analysis on an Orbitrap LTQ XL mass spectrometer with a controlled flow rate of 500 nl per minute and specific tune settings for MS as follows: spray voltage set to 1.8 kV; temperature of the heated transfer capillary set to 180 °C.

14. After each full MS scan, perform ten MS/MS scans, and dynamically select ten most abundant peptide molecular ions, with a dynamic exclusion window set to 90 sec.

15. Omit ions with a +1 or unidentified charge state in the full MS from MS/MS analysis.

Data analysis

A. Shotgun proteome analysis
    1. Search the obtained raw data (in form of mass spectra) with SEQUEST algorithm present in Proteome Discoverer version 1.3. For this, set identification confidence to 5% FDR (false discovery rate) and the variable modifications to acetylation of N-terminus and oxidation of methionine, with a mass tolerance of 10 ppm for the parent ion and 0.8 Da for the fragment ion.

2. Match peptides to the genome sequence of the respective plant species, tomato in this case (Sol Genomics Network), and consider a significant hit when the peptide confidence is at medium or high and an Xcorr threshold is established at 1 per charge (2 for +2 ions, 3 for +3 ions, *etc.*).

Note: Choose high thresholds to minimize false detection of proteins.

B. Prediction of membrane proteins

First, use a database like ARAMEMNON for prediction of membrane proteins (Schwacke *et al.*, 2003). ARAMEMNON considers 20 and 11 different databases (or algorithms) to predict alpha-helical and beta-barrel proteins, respectively.

1. If the plant species of your interest is not listed in ARAMEMNON, you can predict *Arabidopsis* co-orthologs of the detected protein by well-established approaches (Simm *et al.*, 2015).

2. The detected proteins should be verified by a second program like THMMM (http://www.cbs.dtu.dk/services/TMHMM/), which predicts transmembrane alpha helices based on hidden Markov models (Krogh *et al.*, 2001).

Recipes

1. Germination solution
   2 mM boric acid
   2 mM calcium nitrate tetrahydrate
   2 mM magnesium sulphate anhydrous
   1 mM potassium nitrate
   For preparing 100 ml mix:
   0.012 g of boric acid
   0.047 g of calcium nitrate tetrahydrate
   0.024 g of magnesium sulphate anhydrous
   0.010 g of potassium nitrate

2. Homogenization buffer
   100 mM potassium chloride
   5 mM DTT
   1 mM EDTA
   50 mM Tris pH 7.2
   750 mM sodium chloride
   For preparing 100 ml mix:
   0.745 g of potassium chloride
   0.077 g of DTT
   0.029 g of EDTA
   0.605 g of Tris pH 7.2

Note: Always add sodium chloride in the last place as per the homogenization buffer; for 20 ml homogenization buffer, add 0.876 g of sodium chloride.

3. Staining solution

   methanol:acetic acid:water:Coomassie Brilliant Blue R-250 (40:10:50:0.001)

4. Destaining solution

   Methanol and water (40:60)

5. Trypsin buffer

   25 mM sodium bicarbonate

   10 % (v/v) acetonitrile

   5 mM calcium chloride

   For preparing 100 ml mix:

   0.21 g of sodium bicarbonate

   100 µl of pure acetonitrile

   645 µl of water

   Note: Always add calcium in the last place as per the trypsin buffer; for 10 ml of trypsin buffer, add 0.005 g of calcium chloride.

Acknowledgments

References

1. Chaturvedi, P., Ghatak, A., and Weckwerth, W. (2016). Pollen proteomics: from stress physiology to developmental priming. *Plant Reprod* 29(1-2): 119-32.

2. Chaturvedi, P., Ischebeck, T., Egelhofer, V., Lichtscheidl, I. and Weckwerth, W. (2013). Cell-specific analysis of the tomato pollen proteome from pollen mother cell to mature pollen provides evidence for developmental priming. *J Proteome Res* 12(11): 4892-4903.

3. Fragkostefanakis, S., Simm, S., Paul, P., Bublak, D., Scharf, K. D. and Schleiff, E. (2015). Chaperone network composition in *Solanum lycopersicum* explored by transcriptome profiling and microarray meta-analysis. *Plant Cell Environ* 38(4): 693-709.

4. Krogh, A., Larsson, B., von Heijne, G. and Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden markov model: application to complete genomes. *J Mol Biol* 305(3): 567-580.

5. Paul, P., Chaturvedi, P., Selymesi, M., Ghatak, A., Mesihovic, A., Scharf, K. D., Weckwerth, W., Simm, S. and Schleiff, E. (2016a). The membrane proteome of male gametophyte in *Solanum lycopersicum.* *J Proteomics* 131: 48-60.

6. Paul, P., Roth, S. and Schleiff, E. (2016b). Importance of organellar proteins, protein translocation and vesicle transport routes for pollen development and function. *Plant Reprod* 29(1-2): 53-65.

7. Paul, P., Simm, S., Blaumeiser, A., Scharf, K. D., Fragkostefanakis, S., Mirus, O. and Schleiff, E. (2013). The protein translocation systems in plants - composition and variability on the example of *Solanum lycopersicum*. *BMC Genomics* 14: 189.

8. Paul, P., Simm, S., Mirus, O., Scharf, K. D., Fragkostefanakis, S. and Schleiff, E. (2014). The complexity of vesicle transport factors in plants examined by orthology search. *PLoS One* 9(5): e97745.

9. Pertl, H., Schulze, W. X. and Obermeyer, G. (2009). The pollen organelle membrane proteome reveals highly spatial-temporal dynamics during germination and tube growth of lily pollen. *J Proteome Res* 8(11): 5142-5152.

10. Schwacke, R., Schneider, A., van der Graaff, E., Fischer, K., Catoni, E., Desimone, M., Frommer, W. B., Flugge, U. I. and Kunze, R. (2003). ARAMEMNON, a novel database for *Arabidopsis* integral membrane proteins. *Plant Physiol* 131(1): 16-26.

11. Simm, S., Fragkostefanakis, S., Paul, P., Keller, M., Einloft, J., Scharf, K. D. and Schleiff, E. (2015). Identification and expression analysis of ribosome biogenesis factor co-orthologs in *Solanum lycopersicum*. *Bioinform Biol Insights* 9: 1-17.

12. Valledor, L. and Weckwerth, W. (2014). An improved detergent-compatible gel-fractionation LC-LTQ-Orbitrap-MS workflow for plant and microbial proteomics. *Methods Mol Biol* 1072: 347-358.