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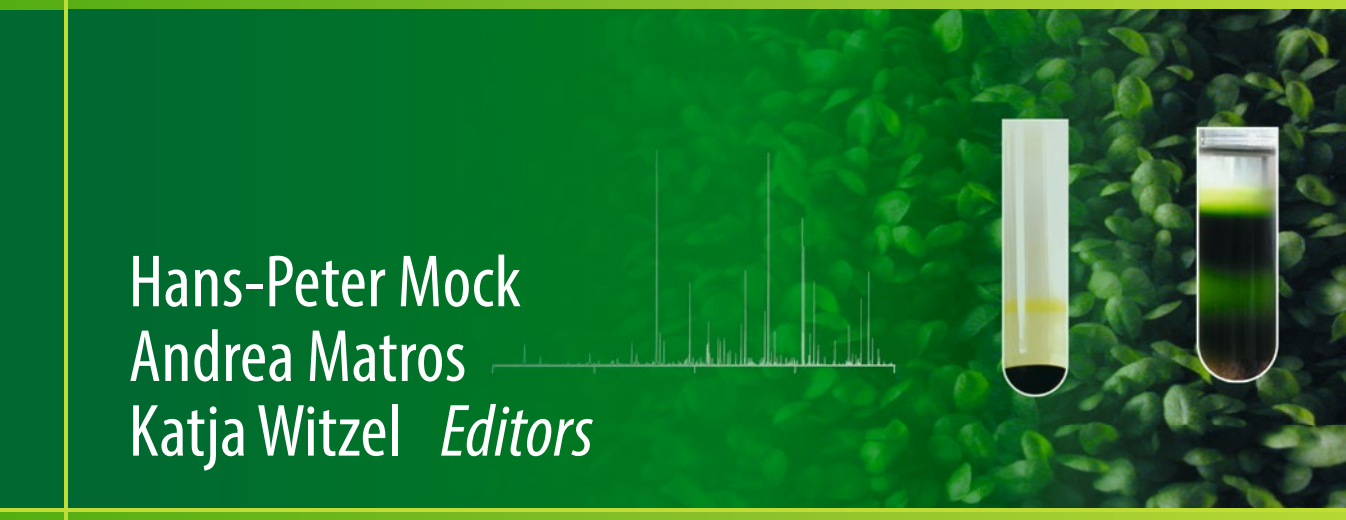

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Plant Membrane Proteomics

Methods and Protocols

Humana Press

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Plant Membrane Proteomics

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Edited by

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Preface

Proteomics has found its way into many plant science laboratories and is complementing other large-scale "omics" approaches. Separation of complex protein samples has been achieved using 2-D gel electrophoresis long before suitable mass spectrometrical techniques were available for routine analysis. Only with the progress of nucleotide sequencing techniques the necessary databases were established to allow proper identification of relevant protein candidates. The advancement of liquid chromatography (LC)-based peptide separation and of mass spectrometry (MS) enabled a shift from 2-D gel-based separation toward LC-MS as the central technology for the analysis and quantitative comparison of protein samples. Reliable separation of complex peptide mixtures resulting from the digest of proteins also helped to overcome one of the major problems of 2-D gels, the missing suitability for separation of membrane proteins. Another major advantage of LC-MS-based proteome analysis lies in the much lower sample amounts needed in comparison with the amounts needed for a large 2-D gel. As an example, the proteome analysis of plasma membranes strongly benefits from these technical developments. Despite the still ongoing rapid further development of mass spectrometry, separation capacity is still not sufficient to cope with the complexities of whole tissue proteomes of plants, e.g., generated by the extraction of leaves and roots. Hence the analysis of subcellular fractions is one strategy to study plant cellular networks on the level of proteins, for which a number of examples are provided in this volume. The availability of many resources, such as mutant lines as well as the first available genome, has made Arabidopsis the prime model in plant research. However, adaptation of protocols for subcellular fractionation has often been challenging. With the rapid progress in nucleotide sequencing now genomic information is available for many plant species, allowing spreading the application of proteomic techniques into a larger palette of crops or biochemical model organisms. We intended to respect this advancement by including protocols from a range of species. Mass spectrometrical analysis of samples and subsequent data evaluation are important aspects also covered by select contributions in this volume. However, we also would like to refer the reader to other recent volumes in this series related to the topic. Proteomic techniques are also covered in volumes 1295 (Proteomic Profiling; Ed. A. Posch), 1394 (Proteomics in Systems Biology; Ed. J. Reinders), and 1410 (Quantitative Proteomics by Mass Spectrometry; Ed. S. Sechi). Protocols related to plant endosomes are presented in volume 1209 (Ed. M.S. Otegui) and protocols for the isolation of organelles and subcellular fractions are provided in volume 1511 (Eds. N.L. Taylor & A.H. Millar).

We hope that this volume will contribute to promote the use of plant membrane proteomics in the future.

Gatersleben, Germany — *Hans-Peter Mock*
Gatersleben, Germany — *Andrea Matros*
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Chapter 1

Free Flow Zonal Electrophoresis for Fractionation of Plant Membrane Compartments Prior to Proteomic Analysis

Bronwyn J. Barkla

Abstract

Free flow zonal electrophoresis (FFZE) is a versatile, reproducible, and potentially high-throughput technique for the separation of plant organelles and membranes by differences in membrane surface charge. It offers considerable benefits over traditional fractionation techniques, such as density gradient centrifugation and two-phase partitioning, as it is relatively fast, sample recovery is high, and the method provides unparalleled sample purity. It has been used to successfully purify chloroplasts and mitochondria from plants but also, to obtain highly pure fractions of plasma membrane, tonoplast, ER, Golgi, and thylakoid membranes. Application of the technique can significantly improve protein coverage in large-scale proteomics studies by decreasing sample complexity. Here, we describe the method for the fractionation of plant cellular membranes from leaves by FFZE.

Key words Membranes, Fractionation, Organelles, Plants, Sample complexity, Tonoplast, Plasma membrane, Surface charge

1 Introduction

We all can acknowledge that looking for a needle in a haystack is a near impossible task. This can also be true in proteomics, where low abundant proteins in a complex sample are analogous to needles, especially when there is no prior knowledge available for these proteins. The large dynamic range of proteins in samples can make accurately and sensitively quantifying all the proteins a challenge, yet it is a fundamental requirement for proteomics to deliver biologically relevant information. One strategy to help overcome this is to utilize the biochemical properties of proteins/peptides to separate complex samples into multiple, simpler fractions [1].

Free flow zonal electrophoresis (FFZE) is a versatile technique that allows for the separation of cells, organelles, cellular membranes, and proteins [2]. Because the technology uses no organic solvents, high salts, or supporting media (such as gels or paper), and only minimal, non-denaturing, buffer components, it does not

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compromise the sample; maintaining sample integrity, including enzyme activity and protein stability [3]. This also facilities high sample recovery with estimates on the order of 95% [4].

During FFZE, the sample is injected continuously into a dedicated sample port (Fig. 1), allowing it to enter a thin chamber (<0.5 mm) between two sealed 50 cm long vertical plates lined with polymethyl methacrylate. Prior to sample application the chamber is filled with separation media which enters the chamber via seven inlet ports (nine inlet ports on the newest model) and is maintained under laminar flow (Fig. 1). An electric field is generated by the application of high voltage perpendicular to the flow of separation media and sample causing the sample components to deflect within the chamber according to their charge (Fig. 1). Once the sample has traveled through the chamber it is collected into

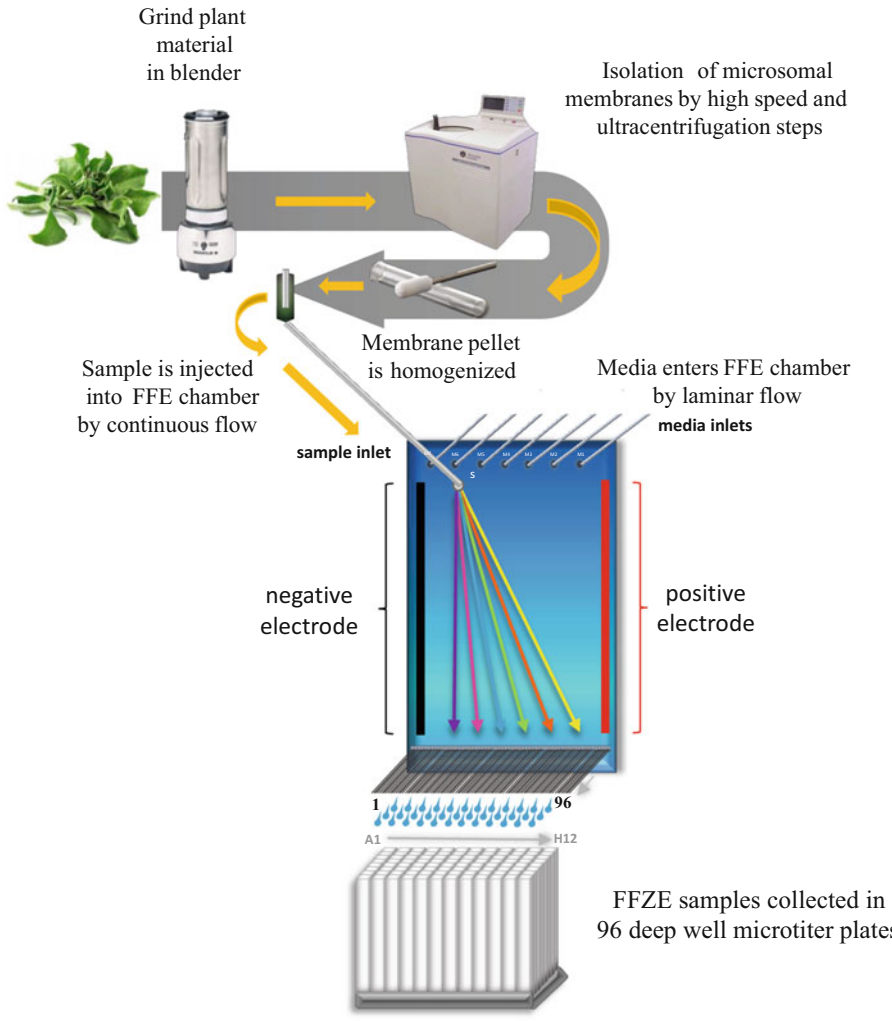

Fig. 1 Membrane preparation and Free flow zonal electrophoresis schematic

96 distinct fractions based on the sample position in the chamber following separation (Fig. 1). The ability to continuously inject sample and collect separated fractions allows for preparative applications [5].

The technique of FFZE was introduced in the late 1950s primarily to separate biopolymers [6], but with the commercialization of the Free Flow Electrophoresis (FFE) system has since been applied to broad applications in biology, medicine, and chemistry. In plants, FFZE was first used to separate chloroplasts [7, 8], and has since been applied to successfully separate a wide range of plant cellular membranes and organelles as detailed in Table 1. Here, we describe the method for fractionation of plant membranes by Free Flow Zonal electrophoresis which we have applied to several different plant species with successful outcomes [18, 19].

2 Materials

Analytical grade reagents should be used for all buffers and solutions. All solutions should be prepared using freshly obtained (not stored) ultrapure water (prepared by purifying deionized water to attain 18 MΩ cm at 25 °C—referred to here as MilliQ water). Solutions for Free Flow Zonal Electrophoresis should be filtered through biologically inert nitrocellulose filters with a pore size of 0.22 μm prior to use. Solutions for the preparation of microsomal membranes should be stored at 4 °C prior to use. Solutions for FFZE should be stored at room temperature.

2.1 Buffers for Isolating Microsomal Membranes

1. Homogenization Buffer: 400 mM mannitol, 10% (w/v) glycerol, 5% (w/v) polyvinylpyrrolidone (PVP)-10, 0.5% (w/v) bovine serum albumin (BSA), 1 mM phenylmethyl sulfonyl fluoride (PMSF), 30 mM Tris, 2 mM dithiothreitol (DTT), 5 mM ethylene glycol tetraacetic acid (EGTA), 5 mM MgSO4, 0.5 mM butylated hydroxytoluene, 0.25 mM dibucaine, 1 mM benzamidine, and 26 mM K^+-metabisulfite, adjust to pH 8.0 with H_2SO_4 (*see* **Note 1**).
2. Suspension Buffer: 400 mM mannitol, 10% (w/v) glycerol, 6 mM Tris/MES pH 8.0 and 2 mM DTT.

2.2 Solutions for Free Flow Zonal Electrophoresis

1. Separation medium: Media inlets 2–6 (or 2–9 in new model), and counterflow inlets 1–3; 10 mM triethanolamine (TEA), 10 mM acetic acid, 2 mM KCl, and 250 mM sucrose.
2. Stabilization medium: Media inlets 1 and 7; 40 mM TEA, 40 mM acetic acid, 8 mM KCl, and 180 mM sucrose.
3. Cathodic and anodic circuit electrolyte solutions; 100 mM TEA, 100 mM acetic acid, and 20 mM KCl adjusted to

Table 1
Reports in the literature of Free Flow Zonal Electrophoresis of plant organelles and membranes

Fraction purified	Species	Reference
Chloroplast	*Spinacia oleracea*	[8]
Thylakoid membrane	*Arabidopsis thaliana*	[9]
Plasma membrane	*Glycine max*	[10]
Plasma membrane	*Arabidopsis thaliana*	[11]
Plasma membrane	*Glycine max*	[12]
Plasma membrane	*Nicotiana tabacum*	[13]
Plasma membrane	*Nicotiana tabacum*	[14]
Plasma membrane	*Catharanthus roseus*	[15]
Plasma membrane	*Lepidium sativum*	[16]
Plasma membrane	*Cucurbita pepo*	[16]
Tonoplast	*Cucurbita pepo*	[16]
Tonoplast	*Lepidium sativum*	[16]
Tonoplast	*Catharanthus roseus*	[15]
Tonoplast	*Nicotiana tabacum*	[13]
Tonoplast	*Nicotiana tabacum*	[14]
Tonoplast	*Arabidopsis thaliana*	[11]
Tonoplast	*Glycine max*	[17]
Tonoplast	*Glycine max*	[12]
Tonoplast	*Mesembryanthemum crystallinum*	[18]
Tonoplast	*Thellungiella halophilia*	[18]
Tonoplast	*Arabidopsis thaliana*	[18]
Tonoplast	*Ananas comosus*	[18]
Tonoplast	*Mesembryanthemum crystallinum*	[19]
Mitochondria	*Arabidopsis thaliana*	[20]
Mitochondria	*Oryza sativa*	[21]
Peroxisome	*Arabidopsis thaliana*	[22]
Golgi	*Arabidopsis thaliana*	[23]
Golgi	*Arabidopsis thaliana*	[24]
Endoplasmic reticulum	*Arabidopsis thaliana*	[24]

pH 7.4 with NaOH, with 0.4% formaldehyde added to the anodic solution to prevent loss of chloride by anodic oxidation.

4. Laminar flow stripe testing solution: Dilute 0.5 ml of 2-(4-Sulfophenylazo)1,8-dihydroxy-3,6-naphthalenedisulfonic acid trisodium salt (SPADNS) in 50 ml of Milli-Q water.
5. Electrode membrane storage solution: 50% (v/v) glycerol and 50% (v/v) isopropanol.

3 Methods

3.1 Isolation of Microsomal Membranes

1. Leaves or roots of plants of interest are harvested and sliced into small pieces and mid veins are removed.
2. Plant material (30 g fresh weight) is immersed in 300 ml of ice-cold homogenization medium and all subsequent operations are carried out at 4 °C.
3. Tissue is homogenized in a commercial blender with five pulses of 20 s each with 20 s rest periods in between each pulse (*see* **Note 2**).
4. The homogenate is filtered through four layers of cheesecloth, aliquoted into centrifuge tubes, and centrifuged at 10,000 × *g* (20 min at 4 °C) using a JA20 rotor (Beckman) in a high-speed J2-HS centrifuge (Beckman).
5. Pellets are discarded and the supernatants centrifuged at 80,000 × *g* (50 min at 4 °C) using a fixed angle 45Ti rotor (Beckman) in an L8-M ultracentrifuge (Beckman).
6. The supernatant is aspirated, and the microsomal pellets are resuspended in a minimal volume of suspension medium (*see* **Note 3**) and homogenized 5× in a 15 ml Potter-Elvehjem glass tissue homogenizer with a teflon pestle (*see* **Note 4**).
7. One milliliter of membrane homogenate is then aliquoted into 2 ml conical tubes and the samples are immediately frozen in liquid N_2 for storage at −80 °C prior to FFZE.

3.2 Measurement of Protein in Membranes

1. Protein content of microsomes is measured by a modification of the Bradford dye-binding method [25] in which membrane protein (2–5 μl) is partially solubilized with 30 μl 0.5% (v/v) Triton X-100 for 5 min before the addition of 800 μl of Milli-Q H_20 and 200 μl of the dye reagent concentrate (Bio-Rad, México).
2. Bovine serum albumin (Fraction V; BSA) is employed as a protein standard for generation of a standard curve.
3. Adjust the protein concentration of the sample to 3 mg/ml (maximum 5 mg/ml) (*see* **Note 5**).

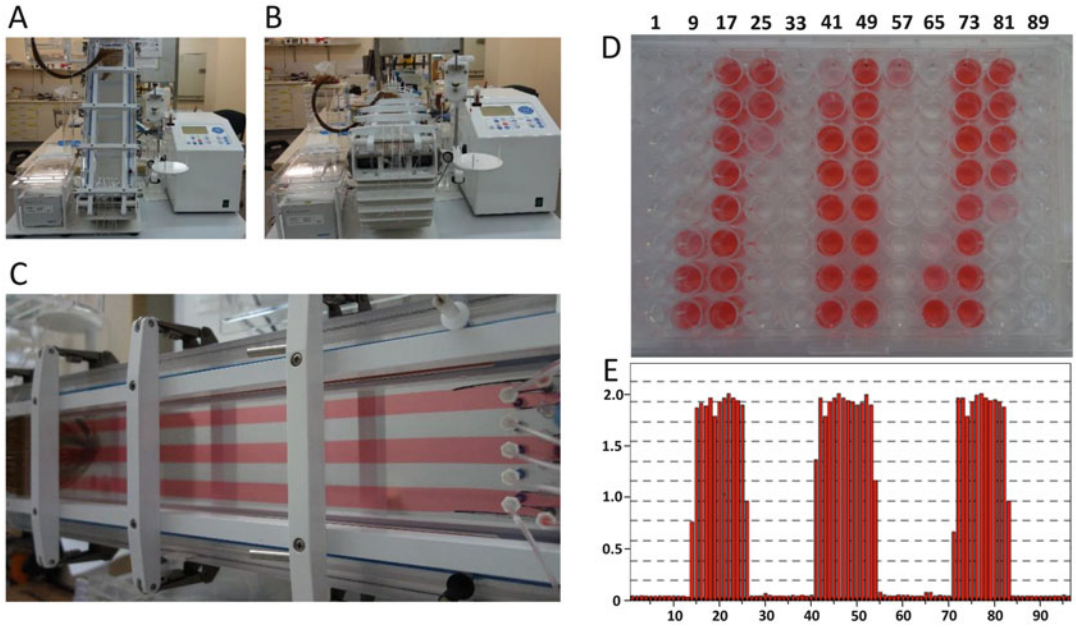

Fig. 2 Free flow electrophoresis equipment and quality control test. The free flow electrophoresis apparatus is shown in the vertical position (**a**) and the horizontal position (**b**). To test for laminar flow of the separation media in the chamber prior to running sample, 0.5% SPADNS solution in Milli-Q water is added to media inlet tubes 2, 4, and 6, (2, 3, 5, 7 and 8 for new 9 sample inlet model), while other inlet tubes remain in Milli-Q water. The dye is allowed to pass through the chamber and should form three straight lines within the chamber (**c**). Fractions are collected in a microtiter plate once the red dye is seen to emerge from the fraction collecting tubing (**d**). The absorbance of the collected fractions can then be read in a microplate reader at a wavelength of 517 nm (**e**)

3.3 Preparation of the Free Flow Electrophoresis Unit

1. Turn on the main power to the FFE unit and turn on the circulating water bath which has been filled with antifreeze at the appropriate dilution (*see* **Note 6**), and set the temperature to 5 °C.
2. Place the separation chamber in the upright position (*see* Fig. 2a), and open the chamber. Clean the inside and outside plates with 1× Milli-Q water, 1× isopropanol, and 1× Milli-Q water (*see* **Note 7**).
3. Lay the anode and cathode membranes, smooth side down, onto the appropriate electrode chamber seal, and then place the pre-wetted (with Milli-Q water for 20 min) 0.8 mm filter paper strips over the membranes, ensuring that they are properly aligned and do not extend over the gaskets (*see* **Note 8**).
4. Place the rigid 0.5 mm plastic spacer, which determines the chamber separation space thickness, onto the chamber lower plate and ensure that it does not overlap with either the internal electrode gaskets or external chamber gaskets (*see* **Note 9**).

5. For FFZE separation mode ensure that the sample injection port tubing extends into the chamber by 1 cm and is pointed in the direction of the media flow.
6. Close the chamber and latch the clamps starting from the middle and working out by turning the clamps two turns. Ensure that the clamps are tightened evenly and tighten the pairs of clamps simultaneously (*see* **Note 10**).
7. Place the seven media inlet tubes (9 in newer model), along with the three counterflow tubes into a reservoir filled with Milli-Q water. Close the media pump cartridges for all media inlet tubes making sure they are all clamped to the same position to ensure consistent pressure on the tubing, but leave the counterflow tubing cartridges open. Place the chamber/counterflow waste tubing into the fraction collection housing so that liquid flow will drain and be collected into the waste reservoir container. Turn on the media pump at 250 ml/h and fill the separation chamber until the liquid level reaches the halfway point. Reverse the media pump flow and empty the chamber until the liquid level is just below the entry point. This step allows for all air bubbles to be flushed from the media tubing and chamber. Then switch the media pump to the forward position and make sure, as the water enters the chamber, it is bubble free.
8. Turn on the sample pump and put it in reverse mode to flush the tubing and ensure there is no blockage. Fill the chamber and eliminate all air bubbles from the chamber and counterflow manifold by reversing water flow if necessary.
9. Close the pump cartridges on the three counterflow tubes and flush the air out of the counterflow tubing. Once both the chamber and counterflow manifold are free of air bubbles (*see* **Note 11**) stop the media pump and close the three levers on the counterflow manifold (down position). Restart the media pump and put on maximum to flush air out of the collection tubes and check that all the 96 fraction collection tubes are dripping (*see* **Note 12**).
10. Place the separation chamber in the horizontal position (*see* Fig. 2b) and perform a Stripe Test on the equipment to ensure laminar flow (*see* Fig. 2c–e). Turn off media pump and sample pump, and place media tubing 2, 4, and 6 (use tubing 2, 3, 5, 7, and 8 for new 9 sample inlet model) into a bottle containing 100 ml 0.5% SPADNs solution, all other media tubing remains in Milli-Q water. Reverse the media pump for 1 s and then fast flow the sample pump in the forward direction until red dye is observed entering the chamber. Set the pump for a flow rate of 250 ml/h. Once the dye has traveled through the chamber and red dye is seen to be emerging from fraction collection tubing

(approximately 10 min), place a microtiter plate into the fraction collector housing, and collect the fractions until the wells are approximately 2/3 full (*see* **Note 13** and Fig. 2d). Inspect the microtiter plate to ensure that the dye deposit has been uniform. Each red dye stripe should fill 15 ± 2 wells with 13 ± 2 wells filled with water in between. Additionally, the microtiter plate can be scanned in a spectrophotometer at 517 nm and data graphed. Results should appear as shown in Fig. 2e.

3.4 Sample Loading and Running

1. Turn off the media pump and sample pumps. Place the Free Flow Electrophoresis solutions, made in Subheading 2.2 above, onto the media housing tray. Place media inlet tubes 1 and 7 into the stabilization media. Place media inlet tubes 2–6, and counter flow tubes 1–3, into the separation medium. Quick reverse the media pump for at least 1 s to expel any possible air bubbles that may have been trapped when tubing was moved between solutions and then run the media pump in the forward direction at a flow rate of 250 ml/h for at least 15 min to expel all the water and fill the chamber and fraction collection tubing with separation medium. Once again check that all fraction tubes are dripping (*see* **Note 12**).
2. Position the bottles containing the Anodic and Cathodic solutions into the electrode housing and place the electrode tubing into the appropriate solution (*see* **Note 14**). Close the electrode housing cover and turn on the electrode pump to circulate the electrode solutions.
3. While the chamber is filling with the separation medium (*see* **step 1** above), prepare the sample by diluting 1:1 with separation medium. Centrifuge the sample at 10,000 × *g* for 10 min at 4 °C to remove all precipitates and transfer the supernatant to a clean 2 ml tube. Place the sample on ice and place the sample tubing into the sample so that the tubing rests just above the bottom of the tube. Reverse the sample pump for 1 s to eliminate air at the entrance.
4. Secure the high voltage cover in place over the chamber. Set the desired running parameters. For routine FFZE we use 750 V (118 mA) and a media flow rate of 250 ml/h. Turn on the high voltage and allow the voltage to stabilize for 15 min (*see* **Note 15**).
5. Set the sample pump to run at 1200 µl/h and inject the sample using the anodic sample inlet (*see* **Note 16**).
6. Once the sample has traveled through the chamber and fraction tubing (approximately 10 min) place the required microtiter plate in the fraction collection housing (*see* **Note 17**) and collect the sample as needed. Multiple deep well plates can be collected to increase the yield of fractions if necessary (*see* **Note 18**)

and will depend on the amount of sample. When changing plates make sure to tap the collection tube housing plate before removing the microtiter plate from the housing and quickly position the next plate before drops begin to fall.

3.5 Shutdown and Cleaning of the Free Flow Electrophoresis Unit

1. Turn off the high voltage, electrode pump, media pump, and sample pump. Move the chamber from the horizontal position to the vertical position. Immerse all tubing; media tubing 1–7 (1–9 in newer model) and counterflow tubing 1–3 in a beaker of Milli-Q water. Reverse the pump for 1 s and then set the media pump flowrate at 300 ml/h to actively rinse the chamber and tubing. Turn on the sample pump in the negative flow mode. Flush the chamber for 30 min with water.
2. Empty the electrode circuits of electrode buffer by removing tubing from electrode solutions, placing them in an empty container and turn the electrode pump on for 1 min. Place the electrode tubing into a beaker of water and turn on the electrode pump allowing the circuit to rinse for 10 min. Following rinsing stop the pump and place tubing once again into an empty beaker and run pump until the electrode chambers and tubing is free of water.
3. Lower the media pump flow rate to 50 ml/h, turn off the sample pump, and place the collection tube housing over the container of Milli-Q water containing the media tubes such that the water flushed through the chamber will be recycled back into the beaker. The equipment can be left in this mode overnight and will be ready for use again in the morning.
4. If the equipment is not going to be reused within 3 days the chamber should be opened. Open all media pump cartridges for media tubing 1–7 (1–9 in newer model) and counterflow tubing 1–3. Open the separation chamber clamps starting from both ends and working to the middle clamps, loosening the clamps by two turns then relatching them. Open the chamber and carefully remove the spacer, filter paper strips and membranes. Place the membranes into the glycerol/isopropanol solution for storage and allow the spacer and filter paper strips to dry on a flat surface. Clean the chamber as described in Subheading 3.3, **step 2** above and turn off the circulating water bath cooler. Leave the chamber open until completely dry and then close the chamber and clamp the middle set of clamps loosely to secure chamber.

4 Notes

1. The homogenization solution can be made the day before. Add the PVP-10 after the pH of the solution has been adjusted and it has been brought up to volume to avoid coating of the pH

electrode with PVP. Add the PMSF, DTT, benzamidine and K^+ metabisulfite immediately before use.

2. It is recommended to place the blender vessel in the fridge for 10–15 min following homogenization to allow for all bubbles to settle prior to filtering the homogenate otherwise sample can be lost.
3. Resuspend the six microsomal membrane pellets in a total of 2 ml of suspension buffer by sequentially resuspending each pellet using the same 1 ml of suspension media and then using an additional 1 ml of suspension buffer to clean out the remaining membrane material in each of the tubes. This gives a highly concentrated extract for FFZE as it needs to be diluted into the FFE separation buffer.
4. During the homogenization it is important not to make bubbles so do not draw the pestle out of the membrane mixture.
5. It is critical to maintain the concentration in this range as if the sample is too concentrated it will precipitate within the Free Flow Electrophoresis Chamber. This will alter the deflection of the sample in the chamber.
6. The circulating water bath is filled with antifreeze liquid at the manufacturer's recommended dilution.
7. Use a lint free cloth like Kimberley Clark Kimwipes.
8. The anode and cathode membranes are stored in a solution of 50% glycerol/ 50% isopropanol. Before placing the membranes onto the electrode seal use your thumb and index finger to slide off excess storage solution. The electrode gasket edges should be slightly visible outside the membrane at the top and bottom and along the sides.
9. Leave sufficient space between the internal electrode gasket and the spacer for media flow. Check that the positioning of the spacer does not block any of the sample inlets at the bottom of the chamber or the counterflow inlets at the top of the chamber.
10. Check that the cooler has reached the correct operating temperature before closing the chamber. Make sure there is relatively equal pressure on all clamps; otherwise, this will distort the flow of media and sample within the chamber.
11. Check for air bubbles at the top of the electrode gaskets as well as at the three counterflow chamber inlets at the top of the chamber as these are the most troublesome areas. If bubbles remain at the counterflow inlets it is possible to release the outlet tubing from the chamber adapter quickly which will draw out the bubble. It is important to make sure that the tubing is reattached tightly to the adapter.

12. If a fraction collection tube is not dripping, as a first measure, fast forward the media pump and if this does not solve the problem use a vacuum pump with tubing attached to suck out any air bubbles that may be trapping the liquid flow in the tube. Leave the media pump running for 15 min to ensure the system is working optimally.
13. The red dye should enter the chamber and travel in a straight line toward the collection tubing (*see* Fig. 2). Air bubbles in the chamber and uneven clamp tension can cause the red dye lines to distort. If this occurs the chamber needs to be emptied, opened and clamps readjusted or air bubbles removed by refilling.
14. The anodic solution goes on the left side and the cathodic solution on the right side of the electrode housing.
15. Do not turn the high voltage on if the media pump is off or if the chamber temperature is above 10 °C. This could result in serious damage to the equipment through overheating as the high voltage generates significant amounts of heat.
16. The choice of sample inlet port depends upon the separation need and can be changed.
17. Either 250 µl standard microplates, 250 µl UV microplates, or 2–4 ml deep well plates can be used depending on output volume required. To monitor separation during a run we routinely collect a UV compatible microtiter plate and measure the absorbance at 280 nm to determine the protein profile.
18. When changing plates make sure to tap the collection tube housing plate before removing the microtiter plate from the housing and quickly position the next plate before drops begin to fall or re-tap the housing prior to sliding in the new plate.

References

1. Gundry RL, White MY, Murray CI, Kane LA, Fu Q, Stanley BA, Van Eyk JE (2009) Preparation of proteins and peptides for mass spectrometry analysis in a bottom up proteomics workflow. Curr Protoc Mol Biol 88:1–23
2. Islinger M, Eckerskorn C, Völkl A (2010) Free-flow electrophoresis in the proteomic era: a technique in flux. Electrophoresis 31:1754–1763
3. Roman MC, Brown PR (1994) Free flow electrophoresis. Anal Chem 66:86A–93A
4. Kuchumov A, Weber G, Eckerskorn C (2005) Reducing protein sample complexity with free flow electrophoresis (FFE). In: Smejkal GB, Lazarev A (eds) Separation methods in proteomics. CRC Press, FL, pp 187–204
5. Wagner H (1989) Free-flow electrophoresis. Nature 341:669–670
6. Hannig K (1958) Preparative, continuous diffraction electrophoresis. Clin Chim Acta 3:10–16
7. Klofat W, Hannig K (1967) Electrophoretic isolation of chloroplasts. Hoppe Seylers Z Physiol Chem 348:739–741
8. Dubacq J-P, Kader J-C (1978) Free flow electrophoresis of chloroplasts. Plant Physiol 61:465–468
9. Eichacker LA, Weber G, Sukop-Koppel U, Wildgruber R (2015) Free flow electrophoresis for separation of native membrane protein complexes. Methods Mol Biol 1295: 415–425

10. Canut H, Brightman A, Boudet AM, Morré DJ (1988) Plasma membrane vesicles of opposite sidedness from soybean hypocotyls by preparative free-flow electrophoresis. Plant Physiol 86:631–637
11. Bardy N, Carrasco A, Galaud JP, Pont-Lezica R, Canut H (1998) Free-flow electrophoresis for fractionation of *Arabidopsis thaliana* membranes. Electrophoresis 19:1145–1153
12. Sandelius AS, Penel C, Auderset G, Brightman A, Millard M, Morré DJ (1986) Isolation of highly purified fractions of plasma membrane and tonoplast from the same homogenate of soybean hypocotyls by free-flow electrophoresis. Plant Physiol 81:177–185
13. Maurel C, Tacnet F, Güclü J, Guern J, Ripoche P (1997) Purified vesicles of tobacco cell vacuolar and plasma membranes exhibit dramatically different water permeability and water channel activity. Proc Natl Acad Sci U S A 94:7103–7108
14. Gerbeau P, Güçlü J, Ripoche P, Maurel C (2002) Aquaporin Nt-TIPa can account for the high permeability of tobacco cell vacuolar membrane to small neutral solutes. Plant J 18:577–587
15. Canut H, Baudracco S, Cabane M, Boudet AM, Marigo G (1991) Preparation of sealed tonoplast and plasma-membrane vesicles from *Catharanthus roseus* (L.) G. Don. cells by free-flow electrophoresis. Planta 184:448–456
16. Scherer GF, Vom Dorp B, Schöllman C, Volkmann D (1992) Proton-transport activity, sidedness, and morphometry of tonoplast and plasma-membrane vesicles purified by free-flow electrophoresis from roots of *Lepidium sativum* L. and hypocotyls of *Cucurbita pepo* L. Planta 186:483–494
17. Canut H, Brightman A, Boudet AM, Morré DJ (1990) Tonoplast vesicles of opposite sidedness from soybean hypocotyls by preparative free-flow electrophoresis. Plant Physiol 94:1149–1156
18. Barkla BJ, Vera-Estrella R, Pantoja O (2007) Enhanced separation of membranes during free flow zonal electrophoresis in plants. Anal Chem 79:5181–5187
19. Barkla BJ, Vera-Estrella R, Hernandez-Coronádo M, Pantoja O (2009) Quantitative proteomics of the tonoplast reveals a role for glycolytic enzymes in salt tolerance. Plant Cell 41:4044–4058
20. Eubel H, Lee CP, Kuo J, Meyer EH, Taylor NL, Millar AH (2007) Free-flow electrophoresis for purification of plant mitochondria by surface charge. Plant J 52:583–594
21. Huang S, Taylor NL, Narsai R, Eubel H, Whelan J, Millar AH (2009) Experimental analysis of the rice mitochondrial proteome, its biogenesis, and heterogeneity. Plant Physiol 149:719–734
22. Eubel H, Meyer EH, Taylor NL, Bussell JD, O'Toole N, Heazlewood JL, Castleden I, Small ID, Smith SM, Millar AH (2008) Novel proteins, putative membrane transporters, and an integrated metabolic network are revealed by quantitative proteomic analysis of Arabidopsis cell culture peroxisomes. Plant Physiol 148:1809–1829
23. Parsons HT, Christiansen K, Knierim B, Carroll A, Ito J, Batth TS, Smith-Moritz AM, Morrison S, McInerney P, Hadi MZ, Auer M, Mukhopadhyay A, Petzold CJ, Scheller HV, Loqué D, Heazlewood JL (2012) Isolation and proteomic characterization of the Arabidopsis Golgi defines functional and novel components involved in plant cell wall biosynthesis. Plant Physiol 159:12–26
24. Parsons HT, Fernández-Niño SM, Heazlewood JL (2014) Separation of the plant Golgi apparatus and endoplasmic reticulum by free-flow electrophoresis. Methods Mol Biol 1072:527–539
25. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chapter 2

Isolation and Purity Assessment of Membranes from Norway Spruce

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Abstract

Gaining membrane vesicles from different plant species and tissue types is crucial for membrane studies. Membrane vesicles can be used for further purification of individual membrane types, and, for example, in studies of membrane enzyme activities, transport assays, and in proteomic analysis. Membrane isolation from some species, such as conifers, has proved to be more difficult than that of angiosperm species. In this paper, we describe steps for isolating cellular membranes from developing xylem, phloem, and lignin-forming tissue-cultured cells of Norway spruce, followed by partial enrichment of plasma membranes by aqueous polymer two-phase partitioning and purity analyses. The methods used are partially similar to the ones used for mono- and dicotyledonous plants, but some steps require discreet optimization, probably due to a high content of phenolic compounds present in the tissues and cultured cells of Norway spruce.

Key words Aqueous polymer two-phase partitioning, Developing xylem, Lignin-forming tissue culture, Membrane vesicles, Norway spruce, Phenol-binding agent, Phloem, Plasma membrane, Purity assessment

Abbreviations

BSA	Bovine serum albumin
DTT	Dithiothreitol
PEG	Polyethylene glycol
PVP	Polyvinylpyrrolidone
PVPP	Insoluble polyvinylpolypyrrolidone
SB	Suspension and storage buffer
UDP-Glc	UDP-glucose

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1 Introduction

Cellular organization and function depend on compartmentalization of biochemical reactions by membranes. Each membrane has a characteristic composition of proteins and lipids that alter in dependence on plant development and external factors. The outer cellular membrane (plasma membrane) is involved in ion transport, signal transduction, cell wall synthesis, and other functions. Gaining membrane vesicles from different plant species and tissue types is crucial for membrane studies. Crude membrane vesicles can be used, for example, for further purification of individual membrane types [1], in studies of membrane enzyme activities and transport assays [2, 3] and in proteomic analysis [4]. At present, the most commonly used method for plasma membrane isolation from plant materials is partitioning in an aqueous dextran/polyethylene glycol (PEG) two-phase system [5, 6]. By this method, membrane vesicles are separated according to their surface charge rather than according to their size and density. Right-side-out (apoplastic side out) plasma membranes preferentially migrate into the PEG-rich upper phase, whereas inside-out plasma membrane vesicles and intracellular membranes partition at the interface and into the dextran-rich lower phase. The method is relatively rapid and, in case of angiosperm species, gives plasma membrane vesicles of high purity (~90–95%; [5, 6]). Membrane isolation from conifers, however, is more demanding than from angiosperms [7], and extra modifications and further optimization are required to gain any membranes for further studies [7, 8]. The plasma membranes obtained by aqueous polymer two-phase partitioning from developing xylem and lignin-forming tissue-cultured cells [9] of Norway spruce (*Picea abies* Karst.) were partially enriched, since vacuolar membranes co-migrated with plasma membranes into the upper phase, whereas other intracellular membranes were preferentially distributed into the lower phase [8].

The optimum polymer concentration of the two-phase systems differs depending on plant species and the tissues used [4, 5]. The reason for this is related to the hydrophobic/hydrophilic properties of the membrane surfaces. The concentration of dextran and PEG needs to be optimized for every material.

In this paper, we present protocol modifications that are essential to obtain cellular membranes from Norway spruce tissues and cells rich in phenolic compounds. The type of a phenol-binding agent in the homogenization buffer has a clear effect on the yield of cellular membranes from lignin-forming tissues [8]. Without addition of soluble polyvinylpyrrolidone (PVP) K-30 we were unable to get any cellular membranes from tissue-cultured spruce cells and phloem tissues, whereas insoluble polyvinylpolypyrrolidone (PVPP, Polyclar AT) was the preferred phenol-binding agent with

developing xylem. We also present methods to enrich plasma membranes using aqueous polymer two-phase partitioning. The distribution of the obtained membranes is determined with specific antibodies against marker proteins of several membrane types in western blots. Also enzyme activity assays for marker enzymes of each membrane type are described, as well as chlorophyll *a* measurement to detect thylakoid membranes. The methods used are partially similar to those for mono- and dicotyledonous plants, but some steps require optimization, probably due to the high content of phenolic compounds present in developing xylem, phloem, and lignin-forming tissue-cultured cells of Norway spruce.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Store the reagents at +4 °C (unless indicated otherwise).

2.1 Cell Extraction

1. 50 mM EDTA, pH 7.5. Adjust pH of EDTA solution to 7.5 with NaOH.
2. Homogenization buffer: 50 mM MOPS-KOH, pH 7.5 (*see* **Note 1**), 5 mM EDTA, 500 mM sucrose and freshly added 5 mM dithiothreitol (DTT), 5 mM ascorbic acid, 4 mM cysteine and protease inhibitors.
3. Phenol-binding agent: 1.5% (w/v) polyvinylpyrrolidone (PVP) K-30 or 1.5% (w/v) insoluble polyvinylpolypyrrolidone (PVPP). Make a pretest to assay which one is better for your material (*see* Subheading 3.2.1). Dissolve PVP K-30 in the homogenization buffer (*see* **Note 2**). If you use tissue powder ground in liquid nitrogen, add PVPP directly with the sample to the extraction mixture.
4. Basic mill with the ability to be cooled with liquid nitrogen.
5. Nylon cloth for filtering.
6. Centrifuge (+4 °C).

2.2 Aqueous Polymer Two-Phase Partitioning Components

1. 20% (w/w) Dextran T500. Since dextran is very hygroscopic, dissolve all powder when opening a new lot. Aliquot and store at −20 °C (*see* **Note 3**).
2. 40% (w/w) polyethylene glycol (PEG) 3350. Aliquot and store at −20 °C (*see* **Note 3**).
3. 0.2 M K-phosphate buffer, pH 7.8. Prepare 0.2 M *ortho*-phosphoric acid, adjust pH with KOH to 7.8. Alternatively, prepare 0.2 M K_2HPO_4 and 0.2 M KH_2PO_4 solutions, and adjust pH of the K_2HPO_4 solution to 7.8 with the KH_2PO_4 solution. Store at room temperature.

4. 0.2 M KCl.
5. K-phosphate-sucrose buffer: 5 mM K-phosphate buffer, pH 7.8, supplemented with 330 mM sucrose and 3 mM KCl. Dilute from 0.2 M buffer, and add sucrose and KCl. Check pH, fill to the final volume, and store at −20 °C in 50 ml tubes.
6. Suspension and storage buffer (SB): 10 mM MOPS-KOH, pH 7.5, supplemented with 330 mM sucrose.
7. 500 mM DTT, freshly made in 50 mM EDTA.
8. Centrifuge with a swinging bucket rotor (+4 °C).
9. Ultracentrifuge (+4 °C).
10. Glass homogenizer.

2.3 Membrane Washing

1. Washing buffer: 10 mM MOPS-KOH buffer, pH 7.5, supplemented with 150 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA and 0.01% (w/v) Triton X-100 (*see* **Note 4**). Aliquot in 50 ml portions and store at −20 °C.

2.4 Protein Quantitation

1. 10 mM MOPS-KOH, pH 7.5, supplemented with 330 mM sucrose and 0.01% (w/v) Triton X-100 (*see* **Note 4**).
2. Make a stock solution (1 mg/ml) of bovine serum albumin (BSA). Dilute BSA standard solutions (2, 5, 10, 15, 20, 25 μg/ml) in SB (use serial dilutions). Store in aliquots at −20 °C.
3. Bradford protein assay [10].
4. Multichannel pipette.
5. Microtiter plate reader.

2.5 SDS-PAGE Gel Electrophoresis

1. 10% (w/v) SDS stock solution.
2. Membrane protein solubilization and loading buffer (4×): 100 mM Tris–HCl, pH 6.8, supplemented with 20% (v/v) glycerol, 0.008% (w/v) bromophenol blue, and 100 mM DTT (freshly added). While dissolving membrane proteins, add SDS to a final concentration of 4% (*see* Subheading 3.6.1).
3. Ready-made SDS-PAGE gels.
4. Running buffer (10×): 250 mM Tris base, 1.9 M glycine, 1% (w/v) SDS. For run, dilute to 1× with water.
5. SDS-PAGE apparatus.

2.6 Immunoblotting

1. Transfer buffer (Towbin): 25 mM Tris base, 200 mM glycine, 20% (v/v) methanol (*see* **Note 5**).
2. tTBS buffer: 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% (w/v) Tween 20. Dilute from the 10× TBS stock solution, and add Tween 20. Prepare 1 L per gel.
3. Nitrocellulose membrane.

4. Blocking buffer: 5% (w/v) nonfat milk powder in tTBS. Prepare 50 ml per gel and mix in a magnetic stirrer for 30 min. Prepare fresh every time.
5. Antibody buffer: 2.5% (w/v) nonfat milk powder in tTBS. Prepare fresh every time.
6. Primary antibodies (rabbit polyclonal serum; Agrisera, Vännäs, Sweden) against (1) H^+-ATPase (AS07 260), a marker for plasma membrane; (2) cytochrome *c* oxidase subunit II (COX II; AS04 053A) for inner mitochondrial membrane, (3) vacuolar H^+-pyrophosphatase (V-PPase, AS12 1849) for tonoplast, and (4) binding immunoglobulin protein (BiP; AS09 481) for endoplasmic reticulum (ER) (*see* **Note 6**).
7. Secondary antibody goat anti-rabbit IgG (H&L) horseradish peroxidase conjugate.
8. ECL Western Blotting Detection reagent.
9. Hyperfilm ECL.
10. Immunoblotting apparatus.
11. Film developer, or solutions and tools to develop films manually.

2.7 Chlorophyll a Measurement

1. 100% acetone.
2. 80% (v/v) acetone supplemented with 0.01% (w/v) $MgCO_3$. Magnesium protects the central atom of chlorophyll.
3. Glass cuvettes (semimicro; *see* **Note 7**).
4. Spectrophotometer.

2.8 Enzyme Assays

1. 50 mM MOPS-KOH, pH 7.2, supplemented with 330 mM sucrose. Aliquot and store at −20 °C.

2.8.1 Glucan Synthase II (a Plasma Membrane Marker)

2. Buffer mixture: Add 5 mM spermine, 50 mM cellobiose and 0.5 mM $CaCl_2$ to 50 mM MOPS-KOH, pH 7.2, supplemented with 330 mM sucrose. Aliquot (e.g., 1 ml aliquots), and store at −20 °C. Thaw the volume you need for the assay, and add DTT (5 mM) just before use.
3. 0.1% (w/v) Digitonin in 50 mM MOPS-KOH, pH 7.2, supplemented with 330 mM sucrose. This is a 10× stock solution, final concentration of digitonin will be 0.01% (w/v).
4. 10 mM UDP-glucose (UDP-Glc) in 50 mM MOPS-KOH, pH 7.2, supplemented with 330 mM sucrose. This is a 5× stock solution, final concentration will be 2 mM.
5. [^{14}C]UDP-Glc. Use 0.5 kBq/reaction.
6. Formic acid (98%).
7. 0.5 M ammonium acetate, pH 3.6. Adjust pH of 0.5 M acetic acid solution to 3.6 with ammonia (in the fume hood). Just

before use, mix ammonium acetate with ethanol in a mixing ratio 7:3 (v/v).

8. Cellulose filter discs, Whatman 3MM, 25 mm diameter.
9. Glass fiber discs, 25 mm diameter.
10. Scintillation liquid.
11. Scintillation vials (20 ml).
12. Water bath at +25 °C.
13. Scintillation counter.

2.8.2 Latent Uridine Diphosphatase (UDPase, a Golgi Marker)

1. 34.6 mM MES-KOH, pH 6.5, supplemented with 3.46 mM $MgSO_4$ and 381 mM sucrose.
2. 34.6 mM MES-KOH, pH 6.5, supplemented with 3.46 mM $MgSO_4$, 381 mM sucrose and 0.035% (w/v) Triton X-100 (*see* **Note 4**).
3. 90 mM Uridine 5′-diphosphate (UDP, disodium salt) in water. Prepare fresh each time.
4. 10% (w/v) SDS (phosphate free).
5. Ames reagent [11]:
   (a) Ammonium molybdate in 1 N H_2SO_4. Add 28.6 ml concentrated H_2SO_4 and 4.2 g ammonium molybdate × 4 H_2O to make 1 L (*see* **Note 8**). This solution is stable at room temperature.
   (b) 10% (w/v) ascorbic acid. Prepare ascorbic acid solution fresh every time, keep on ice.
   (c) Combine ascorbic acid and ammonium molybdate at a mixing ratio of 1:6 (v/v) just before the assay. Keep the Ames reagent on ice.
6. To make a standard curve with a known amount of inorganic phosphate, prepare 5 mM stock solution of KH_2PO_4 in SB. Prepare the following dilutions: 0, 50, 100, 250, 500, 1000 nmol KH_2PO_4/ml. Aliquot and store at −20 °C for further use.

2.8.3 Cytochrome c Oxidase (a Marker for Mitochondrial Inner Membrane)

1. 50 mM MOPS-KOH buffer, pH 7.4, supplemented with 0.025% (w/v) Triton X-100 (*see* **Note 4**).
2. Cytochrome *c*.
3. 400 mM ascorbic acid in water (prepare fresh each time).
4. Gel filtration column (Sephadex G-25).
5. 50 mM MOPS-KOH buffer, pH 7.4.

2.8.4 Antimycin Resistant NADH: Cytochrome **c** *Reductase (a Marker for ER)*

1. 54.3 mM MOPS-KOH, pH 7.5, supplemented with 360 mM sucrose and 0.016% (w/v) Triton X-100 (*see* **Note 4**).
2. 5 mM oxidized cytochrome *c* solution in water. Make larger volume and store in aliquots at −20 °C.
3. 100 mM Na-azide in water (*see* **Note 9**).
4. Antimycin A from *Streptomyces* sp. (*see* **Note 9**). Prepare 10 mM antimycin A stock solution in absolute ethanol. Store at −20 °C in a firmly closed vial. For measurements, dilute with ethanol to 1 mM.
5. 30 mM β-NADH in water. Make fresh each time, keep on ice.

2.8.5 Cl^--Stimulated, NO_3^--Sensitive Mg^{2+}-ATPase: a Marker for Tonoplast

1. 37.5 mM Tris–HCl buffer, pH 8.0, supplemented with 413 mM sucrose. Use Tris-base to make 37.5 mM solution, adjust pH with HCl to 8.0. Add sucrose and fill to the final volume. Store in aliquots at −20 °C.
2. 1.5 M KCl.
3. 1.5 M KNO_3.
4. 90 mM Adenosine 5′-triphosphate (ATP, magnesium salt) in water. Adjust pH to 8 with KOH.
5. Ames reagent [11]:
   (a) Ammonium molybdate in 1 N H_2SO_4. Add 28.6 ml concentrated H_2SO_4 and 4.2 g ammonium molybdate × 4 H_2O to make 1 L (*see* **Note 8**). This solution is stable at room temperature.
   (b) 10% (w/v) ascorbic acid. Prepare ascorbic acid solution fresh every time, keep on ice.
   (c) Combine ascorbic acid and ammonium molybdate at a mixing ratio of 1:6 (v/v) just before the assay. Keep the Ames reagent on ice.
6. To make a standard curve with a known amount of inorganic phosphate, prepare 5 mM stock solution of KH_2PO_4 in SB. Prepare the following dilutions: 0, 50, 100, 250, 500, 1000 nmol KH_2PO_4/ml. Aliquot and store at −20 °C for further use.

3 Methods

Plant material preparation, cell homogenization, and membrane preparation have been described for developing xylem, phloem and lignin-forming, tissue-cultured cells of Norway spruce [8, 9], but the methods can also be applied to other plant tissues containing a high content of phenolic compounds. Slight modifications may be needed for membrane preparation for each species and

tissues. Perform all the procedures for the preparation of membranes on ice or at +4 °C unless mentioned otherwise. Before the work, cool down solutions, beakers, tubes, and centrifuge rotors. Especially, while working with aqueous polymer two-phase partitioning, avoid changes of temperature during the work, since separation of membranes to upper and lower phases is temperature-dependent [12].

3.1 Plant Material

3.1.1 Developing Xylem

1. Harvest a Norway spruce tree with active secondary growth with the owner's permission.
2. Peel the bark with phloem off from the trunk (Fig. 1a). This is easy as cells separate along the cambial layer during secondary growth.
3. Collect developing xylem using a sharp knife or a razor blade (Fig. 1b).
4. Freeze the scrapings immediately in liquid nitrogen, and wrap in aluminum foil on dry ice. Transfer to the laboratory on dry ice, and store at −80 °C.
5. Precool the mill with liquid nitrogen. Grind the material three times for 20 s. Mix the material with a cold spoon, and keep cooling the mill and the xylem material with liquid nitrogen in between. Weigh the ground powder in pre-weighed 50 ml plastic tubes. Store at −80 °C before use (*see* **Note 10**).

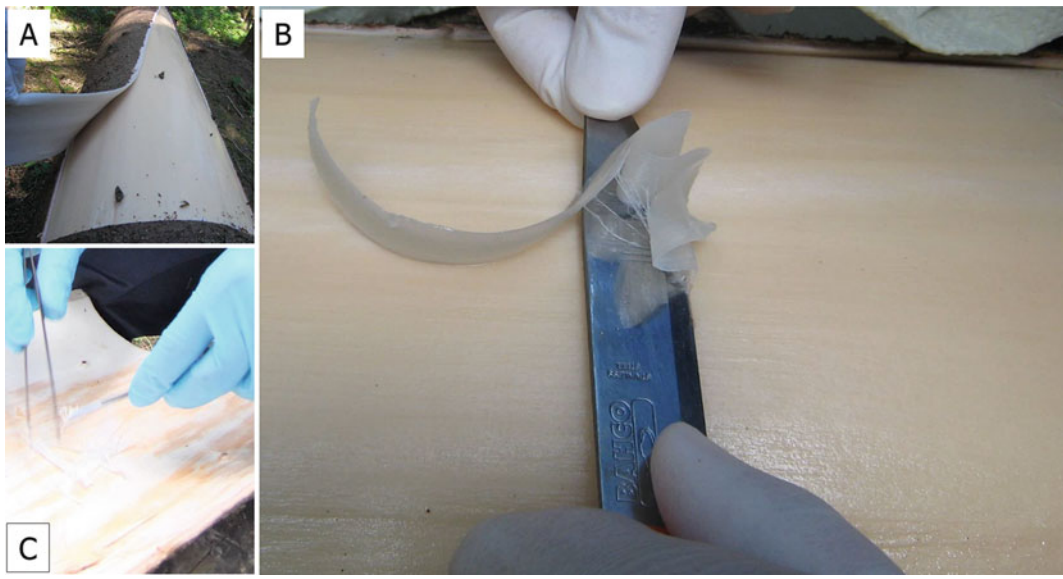

Fig. 1 (**a**) Peeling off bark from a Norway spruce trunk at the time of active secondary growth in late June. (**b**) Collecting developing xylem from the top of mature secondary xylem. (**c**) Collecting phloem from the inner bark

3.1.2 Phloem

Phloem and bark stay together when the bark is peeled off from the trunk at the time of active secondary growth (*see* Subheading 3.1.1).

1. Scrape thin layer of phloem from the inner surface of the bark using sharp surgeon's knife (Fig. 1c).
2. Freeze, store and grind similarly to developing xylem material (*see* **Note 10**).

3.1.3 Lignin-Forming Cell Culture

An extracellular lignin-forming cell culture of Norway spruce [9] was used as a source for cellular membranes.

1. Collect tissue-cultured cells from the liquid culture with active lignin formation. Wash the cells briefly in a Büchner funnel with cold water, wrap in pre-weighed pieces of aluminum foil. Weigh, freeze in liquid nitrogen, and store at −80 °C.
2. Grind the cells in a big mortar with pestle. Precool them with liquid nitrogen, and add liquid nitrogen several times during grinding. Cells (20–30 g) require grinding for 30 min to make a homogenous powder.
3. Store the powder in 50 ml plastic tubes at −80 °C (*see* **Note 10**).

3.2 Membrane Preparation

3.2.1 Testing the Suitability of the Phenol-Binding Agent

1. To assay the preference of the phenol-binding agent, prepare cellular membranes (microsomal vesicles) (1) without any phenol-binding agent; (2) with 1.5% (w/v) soluble PVP K-30; and (3) with 1.5% (w/v) insoluble PVPP in the homogenization buffer.
2. Use 15 g of the ground tissue/cell powder. Proceed as described in Subheading 3.2.2, and evaluate the membrane yield after pelleting the microsomal vesicles in ultracentrifugation. In spruce, the preferential phenol-binding agent was easily determined by judging from the size of the membrane pellet after ultracentrifugation.

3.2.2 Preparation of Microsomal Vesicles

To get reasonable amounts of microsomal membranes for further work, it is recommended to start with ca. 130–160 g of xylem and phloem powder, and 200 g of powdered cultured cells.

1. Prepare homogenization buffer ready by adding the freshly added ingredients (*see* **Notes 11** and **12**). If you start from the amounts of material described above, 500 ml buffer is required.
2. Mix the plant powder gradually with the homogenization buffer in a big beaker, since the plant powder should thaw in the buffer. In case of xylem that prefers insoluble PVPP as the phenol-binding agent, add the plant powder and the buffer gradually into a beaker containing PVPP. Mix with a glass rod until the plant powder is properly thawed (takes ca. 20 min).

Then transfer the beaker on ice, do not let the mixture warm up. Save a small volume of the homogenization buffer for balancing the tubes.

3. Filter the homogenate through a nylon cloth into a big beaker on ice. Use clean nitrile gloves, press and wring the package with hands until the plant residue is as dry as possible.
4. Transfer the filtrate into precooled centrifuge tubes, equilibrate, and centrifuge for 10 min at 10,000 × *g* at +4 °C. Cell walls and intact cell organelles are pelleted, and membranes remain in the supernatant.
5. Transfer the supernatant into new tubes by pipetting, and pellet the membranes in an ultracentrifuge for 45 min at 100,000 × *g* at +4 °C (*see* **Note 13**).
6. In case of phloem and cultured cells, conduct an extra washing step for microsomal membranes, because soluble PVP used in the homogenization buffer interferes with the following aqueous polymer two-phase partitioning [8]. Suspend the pellets into K-phosphate-sucrose buffer. Use a glass homogenizer to resuspend the pellets into the buffer so that PVP K-30 that remained in the pellet dilutes out (*see* **Note 14**). Transfer the sample into ultracentrifuge tubes. Fill the tubes with the buffer and mix so that the washing procedure is as efficient as possible. Repeat centrifugation (100,000 × *g*, 45 min, +4 °C). The pellet contains the cellular membranes and is called "microsomal fraction" or "microsomal vesicles."
7. If you continue to enrich plasma membranes, suspend the pellet into K-phosphate-sucrose buffer using a glass homogenizer. Estimate the volume of the buffer needed, keeping in mind that the pellet will increase the final volume substantially (*see* **Note 15**). If you need microsomal vesicles in your experiments, suspend the pellet into SB (or the buffer of your own choice).

3.3 Enrichment of Spruce Plasma Membranes with Aqueous Polymer Two-Phase Partitioning

For each material, you need to optimize the polymer and KCl concentration. This is done with a small-scale phase partitioning system by combining a 3.0 g phase mixture and 1.0 g of the sample in a 15 ml tube to make a 4 g phase system (Table 1).

3.3.1 Combining Two-Phase Systems

1. Thaw dextran and PEG solutions well before preparing the phases (overnight at +4 °C). Mix all stock solutions (especially dextran and PEG) well (e.g., in a rotating shaker for at least 0.5 h) before pipetting.
2. Weigh the components by using a balance. Use wide-tip Pasteur pipettes for dextran and PEG as they are very viscous. Start from sucrose, and then add stock solutions starting from the densest one: (1) dextran, (2) PEG, (3) K-phosphate buffer,

Table 1
Optimization of the concentration of polymers in a 4-g phase system: combining components of the phase system

Components	6.0/6.0%	6.1/6.1%	6.2/6.2%	6.3/6.3%	6.4%/6.4%
20% Dextran T500	1.20 g	1.22 g	1.24 g	1.26 g	1.28 g
40% PEG 3350	0.60 g	0.61 g	0.62 g	0.63 g	0.64 g
Sucrose (powder)	0.339 g	0.339 g	0.339 g	0.339 g	0.339 g
0.2 M K-phosphate, pH 7.8[a]	75 µl	75 µl	75 µl	75 µl	75 µl
0.2 M KCl (3 mM final)[a]	45 µl	45 µl	45 µl	45 µl	45 µl
0.5 M DTT in 50 mM EDTA[b]	8 µl	8 µl	8 µl	8 µl	8 µl
Water to sum the weight to	3.00 g	3.00 g	3.00 g	3.00 g	3.00 g
Sample	1.0 g	1.0 g	1.0 g	1.0 g	1.0 g
Final weight of the phase system	4.0 g	4.0 g	4.0 g	4.0 g	4.0 g

[a]Note that the sample contains K-phosphate and KCl making the final concentration 5 mM and 3 mM, respectively
[b]Use of DTT and EDTA depends on the further use of your sample. If you study redox enzymes, you should omit these

(4) KCl, and (5) water (*see* **Note 16**). Do not tare the balance while weighing.

3. Mix properly, and make sure that sucrose is well dissolved. Remember to make extra tubes for the preparation of fresh lower phases (*see* Subheading 3.3.2).
4. Let the phase mixtures cool to +4 °C in a fridge and make the last mixing (*see* **Note 17**). Store at +4 °C overnight.
5. Continue as described below for the large-scale purification, except that start with 5 g of material for each phase concentration, and use 1.0 g sample and a 3.0 g phase mixture (Table 1).
6. Assay the markers for each membrane type (*see* Subheading 3.6), and select the concentration of the polymers for large-scale purification. You may also need to optimize the KCl concentration for optimal plasma membrane enrichment.
7. For large-scale purification, combine the phase mixtures in 50 ml plastic tubes (Table 2). After adding all components except the sample, add water to sum up the weight exactly to 27.00 g (*see* **Note 16**). Mix properly until sucrose is well dissolved.
8. Let it cool to +4 °C in the fridge and make the last mixing (*see* **Note 17**). Store at +4 °C overnight.

*3.3.2 Aqueous Polymer Two-Phase Partitioning (See **Note 18**)*

Use freshly prepared microsomal vesicles for aqueous polymer two-phase partitioning. Work on ice all the time.

Table 2
Preparation of the 36 g aqueous polymer two-phase partitioning system

Components	6.15/6.15%	6.3/6.3%
20% Dextran T500	11.07 g	11.34 g
40% PEG 3350	5.535 g	5.670 g
Sucrose (powder)	3.05 g	3.05 g
0.2 M K-phosphate, pH 7.8[a]	0.675 g	0.675 g
0.2 M KCl (3 mM final)[a]	405 µl	405 µl
0.5 M DTT in 50 mM EDTA[b]	72 µl	72 µl
Water to sum the weight to	27.00 g	27.00 g
Sample	9.0 g	9.0 g
Final weight of the phase system	36.0 g	36.0 g

The optimum concentration of dextran, PEG, and KCl needs to be determined for each material. For Norway spruce, the optimized concentration of dextran/PEG was determined to be 6.15% (w/w) and 3 mM KCl for the tissue-cultured cells, and 6.3% (w/w) and 3 mM KCl for xylem and phloem. Other incredients (final concentrations): 330 mM sucrose, 5 mM K-phosphate, 1 mM DTT and 0.1 mM EDTA

[a]Note that the sample contains K-phosphate and KCl making the final concentration 5 and 3 mM, respectively

[b]Use of DTT and EDTA depends on the further use of your sample. If you study redox enzymes, you should omit these

1. Pipette 9.0 g of microsomal fraction on the top of a 27 g phase mixture (now called tube A) resulting in a 36 g phase system (*see* **Note 19**). Remember to keep a small volume of the microsomal fraction for purity assessment.
2. Prepare fresh lower phases for further purification by pipetting 9.0 g of K-phosphate-sucrose buffer on the top of two other 27 g phase mixtures (tubes B and C; *see* **Note 20**).
3. Close the lids, and mix the phases properly by inverting the tubes 30 times.
4. Centrifuge in a swinging bucket rotor for 10 min (1000 × *g*, +4 °C, with no brake; *see* **Note 21**).
5. Dextran and PEG phases are now well separated (Fig. 2a), make a note of their volumes.
6. Remove as much as possible the upper phases from tubes B and C without disturbing the lower phase; these are the fresh lower phases that are used in the following plasma membrane enrichment steps.
7. From the tube A, pipette as much upper phase as you can without disturbing the intermediate phase (~90% of upper phase) on the top of the fresh lower phase in tube B. Close and invert the tube 30 times, and centrifuge as described above (Fig. 2b).
8. Repeat the pipetting, inverting, and centrifugation using the lower phase of tube C (Fig. 2c; *see* **Note 20**).

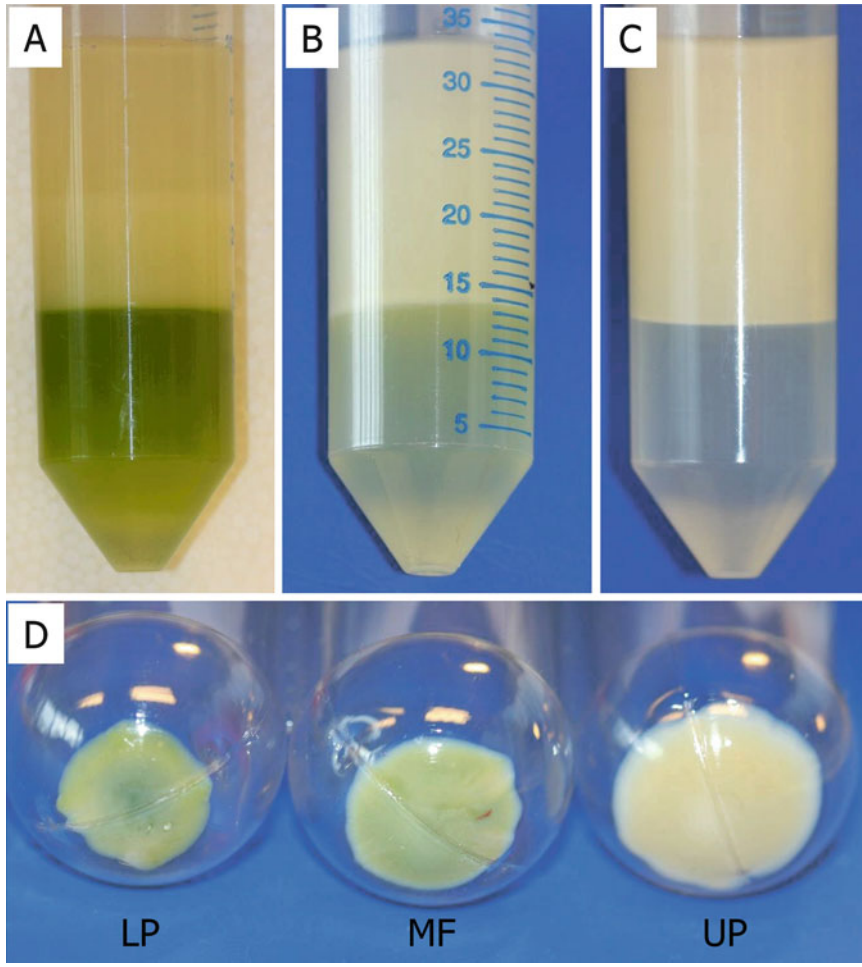

Fig. 2 Aqueous polymer two-phase partitioning of cellular membranes of tissue-cultured Norway spruce cells as an example. The migration of thylakoid membranes into the lower phase is easy to follow as this cell culture is green with well-developed chloroplasts. (**a**) Microsomal fraction was mixed with the phase system: the first phases after the separation of the PEG (*upper phase*) and dextran phases (*lower phase*). (**b**) For further purification, the first upper phase was pipetted on the top of the fresh lower phase. The second phases after phase separation: some green color is still visible in the lower phase. (**c**) The third phases in the consecutive purification of the second upper phase with a fresh lower phase. (**d**) Membranes pelleted by ultracentrifugation. LP: A subsample of lower and intermediate phases from the first phase separation containing the intracellular membranes; MF: A subsample of microsomal fraction containing all cellular membranes; UP: The upper phase sample after three phase separations. Thylakoid membranes have diminished drastically, visible as absence of the green color in the upper phase membranes

9. Transfer the last upper phase to an ultracentrifuge tube without disturbing the intermediate phase. Fill with SB and mix properly, e.g., with a glass rod or by inverting. Dilution of the upper phase that contains PEG should be at least twofold.
10. Mark down the combined volume of the lower and the intermediate phase (if any) in tube A.

11. Take a sample for purity assessment: mix the lower and the interphase well, and pipette, e.g., 1 ml of the mixture into an ultracentrifuge tube. Add SB and pipette up and down several times to collect all sample from the pipette tip. Mix well. Dilution of the lower phase containing dextran should be at least tenfold.
12. Also, take a certain volume of the sample from the original microsomal fraction, and pipette this to an ultracentrifuge tube. Fill with SB and mix properly.
13. Pellet the membranes at 100,000 × *g* for 45 min at +4 °C (Fig. 2d).
14. Add a small volume of SB on the top of the pellet (*see* **Note 22**). Suspend the pellet into the solution with a glass rod, transfer into a 1.5 ml tube by pipetting with a cut tip (wider tip hole). You can rinse the centrifuge tube with a small volume of the buffer, and combine it to the sample. Record the exact volume of the sample at this point. Use a small, plastic pestle to suspend the membranes properly.
15. Aliquot the membranes into 1.5 ml tubes (e.g., 100 µl/tube), and add DTT to 1 mM final concentration from 500 mM stock solution. Do not add DTT to aliquots that you plan to use in redox reactions (e.g., cytochrome *c* oxidase, antimycin-resistant cytochrome *c* reductase).
16. Freeze the aliquots in liquid nitrogen and store at −80 °C.

3.4 Washing Membranes

Washing of cellular membranes is needed to remove loosely attached membrane proteins and cytoplasmic proteins that were trapped inside the vesicles during membrane isolation ([4, 13]; *see* **Note 23**). You need to wash the membranes also for marker enzyme analyses.

1. Thaw the membrane samples in a warm water bath (ca. +45 °C) with slow motion. Transfer on ice immediately when the sample is thoroughly melted.
2. Resuspend the membranes into cold washing buffer (10 mM MOPS-KOH buffer, pH 7.5, supplemented with 150 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA and 0.01% Triton X-100). Use ca. 12 ml of the buffer for maximum of 5 mg membrane protein.
3. Incubate the mixture on ice for 30 min and mix regularly by inverting the tube.
4. Pellet the membranes by centrifugation (100,000 × *g*, 45 min, +4 °C) and suspend the pellet in SB using a glass rod and a small, plastic pestle. Note down the exact volume of the sample after resuspending the pellet.
5. Use immediately for the assays.

3.5 Protein Quantitation

1. Dilute the membrane samples with 10 mM MOPS-KOH, pH 7.4, supplemented with 330 mM sucrose and 0.01% (w/v) Triton X-100 (*see* **Note 24**).
2. Pipette 160 µl of samples, BSA standard solutions or buffer-only blanks into microtiter plate wells.
3. Add 40 µl of Bradford dye concentrate by using a multichannel pipette.
4. Incubate for 10 min at room temperature, and measure absorbance at 595 nm.
5. Subtract blank (buffer-only) values from the sample and BSA standard values. Compare the sample absorbance values with those of the BSA standard curve, and calculate the protein concentration (*see* **Note 25**).

3.6 Purity Assessment

3.6.1 Western Blotting

Washed membranes are used in western blotting to test the distribution and relative abundance of membranes in different phases of the aqueous polymer two-phase partitioning system. First, determine the optimum amount of protein to be added onto the gel by using pretests (*see* Table 3 for spruce protein amounts). Equal amounts of protein (based on protein quantification) are used for different membrane fractions to create a valid comparison (*see* **Notes 26** and **27**).

1. Solubilize membrane proteins by adding membrane protein solubilization and loading buffer with freshly added DTT onto the pellet (1/4 of the final volume of the reaction). Add SDS dropwise from the 10% stock solution to 4% final concentration (other final concentrations: 25 mM Tris–HCl, pH 6.8, 5% glycerol, 0.002% bromophenol blue and 25 mM DTT). Incubate for 10 min at +70 °C.
2. Pellet the membranes by centrifugation (17,000 × *g*, 40 min, +15 °C; *see* **Notes 28** and **29**).
3. Separate equal amounts of solubilized proteins from the microsomal fraction, upper and lower phases in a SDS-PAGE gel electrophoresis. Optimized amounts of protein for each spruce material are shown in Table 3.
4. Transfer the proteins to nitrocellulose membranes with a transfer system according to the manufacturer's instructions.
5. Incubate the nitrocellulose membranes in boxes (*see* **Note 30**) containing tTBS for 5 min at room temperature.
6. Block the membranes for 1 h at room temperature with the blocking buffer (25 ml/membrane) with agitation.
7. Wash the membranes with tTBS (100 ml, 10 min).
8. Incubate the membranes for 1 h at room temperature with the primary antibody diluted in the antibody buffer (10 ml/

Table 3
Optimized amounts of spruce proteins to be used in Western blotting, and optimized dilutions of the primary antibodies for various membrane types (*see* Note 6)

	Antibody against H^+-ATPase		Antibody against COX II		Antibody against V-PPase		Antibody against BiP	
Spruce material	μg protein	Antibody dilution	μg protein	Antibody dilution	μg protein	Antibody dilution	μg protein	Antibody dilution
Developing xylem	5	1:2500	5	1:3000	0.2–0.4	1:5000	10	1:300,000
Phloem	10	1:2500	10	1:3000	1	1:5000	10	1:300,000
Cultured cells	10	1:2500	10	1:3000	1	1:5000	10	1:300,000

H^+-ATPase, a marker for plasma membranes; cytochrome *c* oxidase subunit II (COX II), a marker for inner mitochondrial membranes; vacuolar H^+-pyrophosphatase (V-PPase), a marker for tonoplast; binding immunoglobulin protein BiP, a marker for ER membranes

membrane; *see* **Note 31**). Optimized dilutions for each primary antibody for spruce proteins are shown in Table 3 (*see* **Note 6**).

9. Wash the nitrocellulose membranes three times for 10 min with tTBS (á 100 ml).
10. Incubate the membranes with the secondary antibody (dilution 1:10,000 in the antibody buffer, 10 ml/membrane; *see* **Note 31**) for 1 h at room temperature with agitation.
11. Wash the membranes four times for 10 min with 100 ml tTBS.
12. Detect the signals by Western Blotting Detection reagent according to the manufacturer's instructions by variating the exposure times (1 s—several minutes depending on the signal).
13. Develop, and photograph or scan the films, and evaluate the band intensities from the images (16 bit TIFF) with ImageJ. Alternatively, measure the band intensities by taking an image with ChemiDoc Touch Imaging System, or similar equipment.

3.6.2 Chlorophyll *a* Measurement

To determine the amount of thylakoid membranes, a spectrophotometric assay for chlorophyll *a* is used [14].

1. Zero the spectophotometer with 100% acetone (*see* **Note 7**).
2. Dilute the membrane sample (in SB) with 80% (v/v) acetone supplemented with $MgCO_3$. Test the volume of the sample needed (e.g., 30 µl; final volume needed for semimicro cuvettes is 900 µl).
3. Measure absorbance at 663 and 645 nm.
4. Calculate the amount of chlorophyll *a* by using the following equation:

$$\text{mg chl } a/\text{g fresh weight} = (12.7^{*}\text{A663} - 2.69^{*}\text{A645})^{*} (V/(1000^{*}\ W))^{*}\text{dilution factor in the cuvette}^{*}\text{dilution factors during sample preparation}$$

where:

V = *sample volume (ml)*.
W = *cell fresh weight (g)*.

In calculations, take into account all dilutions that you made during membrane isolation.

3.6.3 Glucan Synthase II Activity Assay

Glucan synthase II (callose synthase) is used as a marker for plasma membranes [15, 16]. The enzyme catalyzes formation of β-1,3-glucan from UDP-glucose. The assay, however, is not specific for plasma membrane, as also intracellular enzymes may contribute to

the measured activity [17]. For each reaction, use 15 μl of membrane sample containing 1–13 μg protein (*see* **Note 32**).

1. Make a [^{14}C]UDP-Glc mixture. Calculate the volume of substrate needed (10 μl/reaction) and make one extra. Add [^{14}C]UDP-Glc to a mixture containing 10 mM UDP-Glc in 50 mM MOPS-KOH, pH 7.2, 330 mM sucrose so that one reaction contains 0.5 kBq. Non-labeled UDP-Glc (at 10 mM) is included in the mixture to make sure that the substrate amount does not limit the reaction.
2. Prepare four different reactions in 1.5 ml tubes (final volume 50 μl) with three replicates. Add all the components except [^{14}C]UDP-Glc mixture, as that will start the reaction. Keep the tubes still on ice at this point.
   (a) 20 μl Buffer mixture, 5 μl digitonin, 15 μl sample, 10 μl [^{14}C]UDP-Glc mixture.
   (b) 20 μl Buffer mixture, 5 μl MOPS-KOH + 330 mM sucrose, 15 μl sample, 10 μl [^{14}C]UDP-Glc mixture.
   (c) 20 μl Buffer mixture, 5 μl digitonin, 15 μl MOPS-KOH + 330 mM sucrose, 10 μl [^{14}C]UDP-Glc mixture.
   (d) 20 μl Buffer mixture, 5 μl digitonin, 15 μl sample, 5 μl MOPS-KOH + 330 mM sucrose.
3. Start the reaction with the addition of [^{14}C]UDP-Glc mixture, incubate for 30 min at +25 °C water bath (exact time).
4. Stop the reaction by adding 5.5 μl 98% formic acid (to 10% final concentration), mix properly, and spin down.
5. Pipette 53 μl of the reaction mixtures (a)–(c) onto cellulose filter discs on aluminum foil.
6. Let the filter papers dry properly.
7. Transfer the filters into a 1 L flask, and wash on a platform shaker two times 45 min in a solution containing ethanol–ammonium acetate, pH 3.6 (60 disks/500 ml). The sugar polymers remain attached to the filter paper due to hydrogen bonding to cellulose, whereas unused UDP-Glc is washed out.
8. Transfer the filter papers onto the foil on a tray. Detach the filter papers from each other, otherwise they will stick together, and let them dry at room temperature or in an oven (at +45 °C).
9. Incubate the reaction vials (d) at +25 °C similarly to reactions (a)–(c).
10. After stopping the reaction, add 5 μl [^{14}C]UDP-Glc mixture to the reaction vials (d). Mix.
11. Pipette 53 μl of the reaction mixtures (d) on top of glass fiber discs pre-wetted with 150 μl water, and let them dry. Reactions

(d) show the total radioactivity used in each reaction (remember to multiply the values by two as half of the volume of [^{14}C] UDP-Glc mixture is used in these samples). Use glass fiber filters for these samples, since in cellulose filters UDP-Glc would absorb in between the cellulose fibers leading to quenching of the radioactivity. There are no sugar polymers in samples (d), so do not wash the glass fiber discs.

12. Insert the cellulose and glass fiber discs into scintillation vials (*see* **Note 33**). Add scintillation liquid (6 ml): the discs need to be fully soaked.
13. Scintillation count two times for 5 min to detect the radioactivity in the samples.
14. Subtract the background radioactivity (sample c) from sample values before calculating the results. Take into account all dilutions that you made during membrane isolation.

Digitonin indicates whether the membrane vesicles are right-side out or inside-out, i.e., enzyme latency [17]. In aqueous polymer two-phase partitioning, the right-side out plasma membranes preferentially partition into the upper PEG-phase. As the active site of the glucan synthase II enzyme is considered to be on the cytoplasmic side [17], a detergent, digitonin, induces increase in the enzyme activity as the substrate has an easier access to the active site. If the plasma membrane vesicles were inside-out, they preferentially partition into the lower dextran phase. The active site is already available for the substrates, and hence, digitonin does not induce activation in the enzymic activity (non-latent activity; [17]).

3.6.4 Latent Uridine Diphosphatase (UDPase)

Latent uridine diphosphatase (UDPase) is used as a marker for Golgi membranes [18]. Remember to prepare a blank reaction that contains SB instead of sample/standard to zero the spectrophotometer. Prepare also a boiled control sample (10 min boiling) for each material.

1. Let the buffer warm up to the reaction temperature (+25 °C), keep UDP and the samples on ice (*see* **Note 32**).
2. Pipette into 1.5 ml tubes:

   200 µl buffer (34.6 mM MES-KOH, pH 6.5, 3.46 mM $MgSO_4$, 381 mM sucrose, with or without 0.035% Triton X-100; final concentrations in the reaction mixture 30 mM, 3 mM, 330 mM and 0.03% or 0%, respectively). You need to prepare two lots for the assay: one with and another without Triton X-100. Make three replicates for each sample/standard/blank reaction.
3. Add 7.5 µl 90 mM UDP (3 mM final concentration), mix.
4. Add 22 µl sample (4–9 µg protein)/KH_2PO_4 standard dilutions in SB/SB buffer only.

5. Mix properly and incubate for 30 min at +25 °C (e.g., in a water bath; exact time).
6. Stop the reaction by adding 40 µl 10% SDS (1.5% final concentration).
7. Detect the liberated phosphate by adding immediately 690 µl Ames reagent [11]. Mix and incubate for 60 min at room temperature (+20 °C).
8. Measure absorbance at 820 nm.
9. Use the standard curve to calculate the amount of liberated phosphate in your samples (*see* **Note 25**).
10. Subtract the boiled sample value from the sample values as that shows the non-enzymatic release of inorganic phosphate due to the plant material. Take into account all dilutions that you made during membrane isolation.

Latent UDPase activity represents the difference in activity with or without Triton X-100.

3.6.5 Cytochrome c Oxidase Assay

Cytochrome *c* oxidase assay is used as a marker for mitochondrial inner membrane [19]. Oxidation of reduced cytochrome *c* is followed at 550 nm by using a kinetics program in the spectrophotometer. Do not add DTT to samples to be analyzed for cytochrome *c* oxidase assay.

For the assay, reduce cytochrome *c* with ascorbic acid [20].

1. Prepare 250 µl 10 mM cytochrome *c* solution in water.
2. Prepare 250 µl 400 mM ascorbic acid solution in water. Prepare fresh each time.
3. Add cytochrome *c* and ascorbic acid solutions to 2 ml water so that the final volume of cytochrome-ascorbic acid mixture is 2.5 ml (final concentrations 1 mM and 40 mM, respectively).
4. Incubate for 10 min at room temperature to reduce all cytochrome *c*.
5. Remove ascorbic acid in a gel filtration column (PD-10, Sephadex G-25, GE Healthcare). For this, equilibrate the column at +4 °C with 25 ml 50 mM MOPS-KOH-buffer, pH 7.4, according to the manufacturer's instructions. Pipette cytochrome-ascorbic acid mixture (2.5 ml) on the top of the column, and let it go into the column. Then elute the high-molecular-weight cytochrome *c* with 3.5 ml buffer. Cytochrome *c* is diluted 1.4-fold (3.5 ml/2.5 ml), i.e., the final concentration is 714 µM. Wash the column with 25 ml of buffer. Store the column at +4 °C for the next use as it can be used for five to six times.
6. Store the reduced cytochrome *c* in aliquots at −20 °C.

7. For the enzyme activity determination, thaw the reduced cytochrome *c*.
8. Make sure that cytochrome *c* is totally reduced by adding a few crystals of Na-dithionite ($Na_2S_2O_4$) into the tube with a spatula. Follow whether any color change occurs. The oxidized cytochrome *c* is orange, and the reduced one is pink.
9. Remove the excess dithionite by aeration (blow some air through the solution with a Pasteur pipette for a couple of minutes). This will lead to oxidation of the remaining dithionite.
10. To assay the enzyme activity (*see* **Notes 32** and **34**), pipette directly into the semimicro cuvette:

    890 µl 50 mM MOPS-KOH buffer, pH 7.4, with 0.025% (w/v) Triton X-100. Place the cuvette into the cuvette chamber, and zero the spectrophotometer by pressing auto zero.
11. Add 70 µl reduced cytochrome *c* (final concentration 50 µM in the final volume of 1 ml). Close the cuvette with a piece of parafilm, mix by inverting.
12. Measure the change in A550 nm for 1 min (background oxidation).
13. Add 40 µl of the membrane sample containing ca. 10–50 µg protein (dilute with SB if needed; *see* **Note 35**). Mix by inverting the cuvette, and immediately follow the change in A550 nm for at least 1 min.
14. Subtract the background values from the sample values before calculating the final results. Calculate the activity as katals (mol/sec). Use equation

$$\varepsilon = A/(c^* l)$$

    where:

    ε550 (reduced cytochrome *c*) = 18.5 mM^{-1} cm^{-1} [19], A = change in A550 nm/min, c = concentration (mol/l), l = length of the light path (1 cm).

In calculations, take into account all dilutions that you made during membrane isolation.

3.6.6 Antimycin Resistant NADH: Cytochrome c Reductase (A Marker for ER)

Antimycin resistant NADH:cytochrome *c* reductase is used to assay the presence of ER membranes (modified from [21]). With Norway spruce samples, the concentration of antimycin A commonly used in this assay (1 µM; [21]) did not fully inhibit the antimycin sensitive part of the activity, hence, a concentration showing maximum inhibition (30 µM) was chosen [8]. Ethanol concentration in the reaction (3%, v/v, final) did not affect the enzyme activity.

Make replicates for each sample. Do not have DTT in the samples for this assay (*see* **Notes 32** and **34**).

1. Pipette into the semimicro cuvette:

   920 µl 54.3 mM MOPS-KOH, pH 7.5, supplemented with 360 mM sucrose and 0.016% (w/v) Triton X-100 (final concentrations: 50 mM, 330 mM and 0.015%, respectively). Place the cuvette into the cuvette chamber, and zero the spectrophotometer by pressing auto zero.
2. Add

   10 µl 5 mM oxidized cytochrome *c* (50 µM final).
   10 µl 100 mM Na-azide (1 mM final).
   30 µl 1 mM antimycin A (30 µM final).
   Close the cuvette with a piece of parafilm, and mix by inverting.
3. Add 20 µl sample (ca. 5–15 µg protein). Mix and follow cytochrome *c* reduction at 550 nm for 2 min. This shows the non-enzymatic reaction, and the inhibitors have time to bind to corresponding sites.
4. Start the enzymatic reaction by adding 10 µl 30 mM NADH (0.3 mM final). Mix and follow reduction of cytochrome *c* at 550 nm.
5. Use the first minute linear increase for calculating the results (*see* **Note 35**). Subtract the non-enzymatic background before calculating the final results. Calculate the activity as katals (mol/s). Use equation

$$\varepsilon = A/(c^{*}l)$$

   where:

   ε550 (cytochrome *c*) = 18.5 mM^{-1} cm^{-1} [19], A = change in A550 nm/min, c = concentration (mol/l), l = length of the light path (1 cm).

In calculations, take into account all dilutions that you made during membrane isolation.

3.6.7 Cl^{-}-Stimulated, NO_3^{-}-Sensitive Mg^{2+}-ATPase: A Marker Enzyme for Tonoplast

Cl^{-}-stimulated, NO_3^{-} sensitive Mg^{2+}-ATPase activity is used as a marker for vacuolar membranes [18]. The activity is not inhibited by *ortho*-vanadate [22]. Release of inorganic phosphate is determined by the method of Ames [11].

1. Pipette into a 1.5 ml tube (final volume 224.5 µl; make three replicates for each reaction (*see* **Notes 32** and **34**):

   180 µl buffer (37.5 mM Tris–HCl, pH 8.0, supplemented with 413 mM sucrose).
   7.5 µl 1.5 M KCl *or* 1.5 M KNO_3 (50 mM final).
   7.5 µl 90 mM ATP (for samples) *or* 7.5 µl H_2O (for standard reactions).
   7.5 µl H_2O.
   Mix.
2. Start the reaction with 22 µl sample (containing 3–9 µg protein) or KH_2PO_4 standard solution (*see* **Note 36**). Mix the

components, and incubate for 30 min at +25 °C in a water bath (exact time). Remember to make a blank (SB only instead of a sample) to zero the spectrophotometer.

3. Stop the reaction by adding 40 μl 10% SDS followed immediately by 690 μl Ames reagent. Mix, incubate for 60 min at room temperature.
4. Measure absorbance at 820 nm.
5. In calculations, take into account all dilutions that you made during membrane isolation. Difference in activity observed between KCl and KNO_3 is the nitrate-sensitive ATPase activity.

4 Notes

1. You can make a stock solution (5×) of MOPS (250 mM, pH 7.5). Before cell extraction, dilute to onefold and add the required components.
2. It takes ca. 30 min to dissolve PVP K-30 in a magnetic stirrer.
3. Freeze and store the dextran and PEG stock solutions (e.g., in 50 ml aliquots) at −20 °C. Dextran is very hygroscopic (i.e., it absorbs water easily), hence, the exact concentration can be estimated by polarimeter only [5]. Store the powder in a dry place, and dissolve the whole lot at the same time. Aliquot and store in the freezer. This way you guarantee the reproducibility of the aqueous polymer two-phase partitioning in the following purification rounds, since small differences in the stock solutions used to prepare the two-phase systems affect the phase separation. Ideally, the dextran and PEG concentration for the aqueous polymer two-phase partitioning need to be optimized again every time new stock solutions are made. Optimization can be done in a small scale starting with 5 g ground material for each phase concentration. In aqueous two-phase partitioning, use 3.0 g phase system and 1.0 g of sample (Table 1). Optimization is time-consuming, hence, preparation of large volumes of the stock solutions is justified.
4. Weigh Triton X-100 on a balance as it is sticky liquid, and rinse carefully into the final solution.
5. Collect Towbin buffer after use, as you can use this buffer for several (5–6) times.
6. If you are using a species with no experience of the antibody in question, the suitability (specificity) and dilution of the antibody need to be tested according to the manufacturer's instructions.
7. Plastic cuvettes are not suitable with acetone.

8. Addition of H_2SO_4 to water is an exothermic reaction, i.e., it generates heat. Add H_2SO_4 to water in portions, let it cool to room temperature before adjusting the final volume. Use safety glasses when preparing the solution.
9. Na-azide and antimycin A are very toxic. Take care when using these compounds.
10. After grinding the plant material in liquid nitrogen, put the ground powder in 50 ml plastic tubes and store at −80 °C. Do not, however, close the lids very tightly, as the tubes might explode in the freezer or when you remove them from the freezer.
11. To make the day of membrane preparation as easy and fluent as possible, make all possible preparations in the previous day. For example, weigh the ingredients of the homogenization buffer, put the centrifuge rotors to a cold room, and mark the tubes.
12. Use a fume hood as DTT is smelly.
13. Equilibrate centrifuge tubes carefully on a balance, especially for the ultracentrifuge. Check that the ultracentrifuge tubes and the rotor can be used at the *g* forces that you intend to use.
14. Be gentle when re-suspending the membrane vesicles into the buffer. Only use just enough force and time to resuspend, do not grind the membranes at this point.
15. If you started with 130–160 g xylem or phloem powder, or with 200 g of cultured cells, and you continue with aqueous polymer two-phase partitioning, 19–20 ml is a good final volume to aim into when suspending the pellet.
16. By having the pipetting order from the densest solution to the least dense, you can correct small mistakes since the addition of a new component goes on the top of the previous solution.
17. Combining of the phases can be done in temperature other than that where the real separation of membranes is conducted. But since the temperature affects phase separation, the final mixing and aqueous polymer two-phase partitioning need to be done at constant temperature (+4 °C).
18. If you prepare membranes in a large scale (e.g., start from 130–200 g of plant material) you need two persons working, as it is good to be as fast as possible (especially if you later intend to use the vesicles to study enzyme activities). Still, the procedure takes a long working day. Do all possible preparations beforehand (e.g., prepare homogenization buffer and two-phase mixtures, mark tubes, and plan the volumes of membrane aliquots you will store).
19. If you start with 160 g xylem powder, you will have ~19–20 ml of microsomal fraction for aqueous polymer two-phase

partitioning. This means that you can conduct the whole two-phase protocol in two sets of tubes A, B, and C.

20. In pretests, you need to determine the optimum number of phases to be used for each material.
21. Use of a brake leads to a formation of a big intermediate phase.
22. The volume of the buffer to be added has to be estimated by the size of the pellets. In general, 0.5 ml is a good volume to start with.
23. Enzyme activities are retained better if washing of the membranes is conducted just prior to assaying the activities.
24. Membrane preparations done according to our procedure should be diluted 50–1000 times for the protein assay. Use serial dilutions.
25. Use only sample values that are in the linear region of the standard curve.
26. For solubilization of membrane proteins, calculate the volumes for pipetting ready, and treat all samples (microsomal fraction, upper phase, lower phase) as similarly as possible since you compare the band sizes between these samples. When planning of the pipetting volumes, take into account the maximum volume that can be loaded onto the gel.
27. Protein amount to be used in western blot needs to be optimized for each plant tissue and for each antibody.
28. Pellet the membranes by centrifugation at +15 °C to avoid precipitation of SDS.
29. In solubilization with SDS, different plant membranes behave differently. For example, xylem proteins are easily solubilized, whereas phloem and cell culture membranes form a big pellet in centrifugation after incubation at +70 °C.
30. Empty pipette tip boxes are useful in western blotting as membrane washing containers.
31. It is advisable to roll the nitrocellulose membrane in a loose roll and insert it into the 50 ml tube. By this way, 10 ml of antibody solution is enough for the whole membrane, when the tube is incubated on its side on a rotating shaker.
32. Thaw the samples just immediately before the washing procedure and the assay. It is recommended to thaw enzyme samples quickly in a warm water bath (ca. +45 °C). Keep the tubes in motion so that the ice block keeps the enzyme sample cool while melting. Transfer the tubes on ice immediately after all ice has disappeared.
33. Be careful that the sample side is always in the same way, e.g., upward.

34. Let the buffer warm to the measuring temperature before the start of the assay. Use a temperature-controlled cuvette chamber in the spectrophotometer, or keep the buffer in a water bath to keep the temperature constant during the enzyme assay.
35. If change in absorbance per minute is too high (over 100 mA units), dilute the sample with SB and measure again.
36. Reaction supplemented with KCl is enough for the KH_2PO_4 standard measurement, as no enzyme activity is followed with the standard reactions.

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References

1. Findlay JBC, Evans WH (1987) Biological membranes: a practical approach. IRL Press, Oxford
2. Johansson F, Olbe M, Sommarin M, Larsson C (1995) Brij 58, a polyoxylethylene acyl ether, creates membrane vesicles of uniform sideness. A new tool to obtain inside-out (cytoplasmic side-out) plasma membrane vesicles. Plant J 7:165–173
3. Menckhoff M, Lüthje S (2004) Transmembrane electron transport in sealed and NAD(P)H-loaded right-side-out plasma membrane vesicles isolated from maize (*Zea mays* L.) roots. J Exp Bot 55:1343–1349
4. Lüthje S, Meisrimler CN, Hopff D, Schütze T, Köppe J, Heino K (2014) Class III peroxidases. In: Jorrin-Novo JV et al (eds) Plant proteomics: methods and protocols, methods in molecular biology, vol 1072. Springer Science & Business Media, LLC, New York, pp 687–706
5. Larsson C, Widell S, Kjellbom P (1987) Preparation of high-purity plasma membranes. Methods Enzymol 148:558–568
6. Larsson C, Sommarin M, Widell S (1994) Isolation of highly purified plant plasma membranes and separation of inside-out and right-side-out vesicles. Methods Enzymol 228:451–469
7. Toll E, Castillo FJ, Crespi P, Crèvecoeur M, Greppin H (1995) Purification of plasma membranes from leaves of conifer and deciduous tree species by phase partitioning and free-flow electrophoresis. Physiol Plant 95:399–408
8. Kärkönen A, Meisrimler CN, Takahashi J, Väisänen E, Laitinen T, Jiménez Barboza LA, Holmström S, Salonvaara S, Wienkoop S, Fagerstedt KF, Lüthje S (2014) Isolation of cellular membranes from lignin-producing tissues of Norway spruce and analysis of redox enzymes. Physiol Plant 152:599–616
9. Kärkönen A, Koutaniemi S, Mustonen M, Syrjänen K, Brunow G, Kilpeläinen I, Teeri TH, Simola LK (2002) Lignification related enzymes in *Picea abies* suspension cultures. Physiol Plant 114:343–353
10. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
11. Ames BN (1966) Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol 8:115–118
12. Sandelius AS, Morré DJ (1990) Plasma membrane isolation. In: Larsson C, Møller IM (eds)

The plant plasma membrane. Springer-Verlag, Berlin, Heidelberg, pp 44–75

13. Bérzci A, Asard H (2003) Soluble proteins, an often overlooked contaminant in plasma membrane preparations. Trends Plant Sci 8:250–251
14. Bruinsma J (1963) The quantitative analysis of chlorophylls *a* and *b* in plant extracts. Photochem Photobiol 2:242–249
15. Kauss H, Jeblick W (1986) Synergistic activation of 1,3-β-glucan synthase by Ca^{2+} and polyamines. Plant Sci 43:103–107
16. Palmgren MG, Askerlund P, Fredrikson K, Widell S, Sommarin M, Larsson C (1990) Sealed inside-out and right-side-out plasma membrane vesicles. Plant Physiol 92:871–880
17. Larsson C (1985) Plasma membranes. In: Linskens HF, Jackson JF (eds) Modern methods of plant analysis, new series, vol 1. Springer-Verlag, Berlin, pp 85–104
18. Briskin DP, Leonard RT, Hodges TK (1987) Isolation of the plasma membrane: membrane markers and general principles. Methods Enzymol 148:542–558
19. Hodges TK, Leonard RT (1974) Purification of a plasma-membrane bound adenosine triphosphatase from plant roots. Methods Enzymol 32:392–406
20. Møller IM, Palmer JM (1982) Direct evidence for the presence of a rotenone-resistant NADH dehydrogenase on the inner surface of the inner membrane of plant mitochondria. Physiol Plant 54:267–274
21. Sterling JD, Quigley HF, Orellana A, Mohnen D (2001) The catalytic site of the pectin biosynthetic enzyme α-1,4-galacturonosyltransferase is located in the lumen of Golgi. Plant Physiol 127:360–371
22. Churchill KA, Sze H (1984) Anion-sensitive, H^+-pumping ATPase of oat roots; direct effects of Cl^-, NO_3^-, and a disulfonic stilbene. Plant Physiol 76:490–497

Chapter 3

Nuclear Proteome: Isolation of Intact Nuclei, Extraction of Nuclear Proteins, and 2-DE Analysis

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Abstract

Proteome profiling aims to unravel the mystery of biological complexity encoded by the genome. The successful proteome profiling largely depends upon analytical approaches because single-step proteome characterization of eukaryotic cells is difficult due to the large number of proteins expressed and their complex physiochemical properties. Organellar proteomics helps in identifying a refined set of proteins by pinpointing certain activities to specific organelles, thereby increasing our knowledge of cellular processes. The reliability of a plant organelle proteome is intimately dependent on the purity of the organelle preparation. Methodological improvements in sample handling, organelle fractionation, and protein extraction are therefore crucial to plant subcellular proteomics. The nuclear proteins are organized into complex regulatory networks and perform varied cellular functions. Therefore, characterization of the nuclear proteome is an important step toward accumulating knowledge about regulation of gene expression and function. In this chapter, we present methods for the isolation of nuclei, purification of nuclear proteins, and proteome profiling that have been adapted for proteomic characterization of economically important crop species, such as chickpea.

Key words Cellular contaminants, Nuclear proteins, DAPI staining, Marker enzymes, Organelle proteome

1 Introduction

The protein repertoire of any crop is not just dependent on the species per se, but is also organ as well as organelle specific. Proteomic characterization of individual plant subcellular components reduces protein complexity, thereby increasing technical resolution and provides focused information on protein expression and possible function in relation to cellular location under specific developmental and/or environmental conditions. The nucleus is the largest and also one of the most easily identifiable membrane-bound organelle in eukaryotic cells, wherein it serves as the principal information and administrative center. The composition of the nucleus gives it a markedly greater refractive index than the contiguous cytoplasm, making the monitoring of the isolation procedure

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easier through visualization by phase contrast optical microscopy. The nucleus not only stores the cell's hereditary material, or DNA, but it controls the activities of the cell by regulating gene expression, thereby influencing protein synthesis, intermediary metabolism, growth, as well as cell division. Nuclear proteins have been shown to be implicated in different cellular functions including structural integrity, gene regulation, translation, signaling, defense, and a variety of RNA-associated functions [1–6]. Increasing evidence suggests the presence of nearly 25% of total cellular proteins in the nucleus, entailing a variety of roles to this organelle [3, 7]. Isolation of intact nuclei is essential, both for functional studies and for biochemical analysis of the integral macromolecules. The separation of nuclei from plant tissues is difficult essentially due to the tight association of the nuclear membrane with the endoplasmic reticulum in addition to similar densities of nuclei and chloroplasts [2, 3, 8]. During the past decades, a number of plant organelle proteomes, including nucleus, have been characterized [9–14], which highlights the need for modification, optimization, and refinement of the various isolation protocols to suit isolation of different organelles from a variety of tissues/species. In general, the protocol for the isolation of nuclei should not only be as simple as possible and easy to repeat, but should also yield intact and pure nuclei so that maximal and reproducible protein characterization is achieved. Here, we present a protocol for isolation and proteomic characterization of plant nucleus, which has been successfully replicated for both legume and cereal crops. Through optimization of this protocol, enriched nuclei fractions were successfully isolated from the aerial parts of fully hydrated as well as dehydrated seedlings [3, 4, 6, 15–17] (Subheadings 3.1 and 3.2). Application of percoll/sucrose gradient for the purification of nuclei resulted in a lower yield of non-aggregated intact nuclei, which was insufficient for further proteomic analysis and hence, this approach was not pursued further. The isolated nuclear fraction was checked for contamination at two levels: the organellar and the protein level. At the organellar level, the nuclear pellet was subjected to microscopic analysis (Subheading 3.3) as well as checked for chlorophyll contamination (Subheading 3.4). At the protein level, enrichment was checked by immunoblotting for nuclear resident proteins (Subheading 3.5) and by enzyme assays (Subheading 3.6). The nuclear protein so obtained was further used for proteomic characterization (Subheading 3.7).

2 Materials

2.1 Plant Growth, Maintenance, and Tissue Harvesting

Chickpea (*Cicer aritienum* L.) seedlings are grown in pots containing a mixture of soil and soil rite (2:1, w/w) in an environmentally controlled growth room and maintained at 25 ± 2 °C, 50 ± 5%

relative humidity under 16 h photoperiod (340 μmol/m^2/s light intensity). Routinely, we harvest aerial parts of 3-weeks-old seedlings as experimental material and store it at −80 °C after quick-freezing in liquid nitrogen until further use.

2.2 Nuclei Isolation

All the solutions need to be freshly prepared, either directly or as stock solutions, without protease inhibitors and polyamines (which should be added fresh before use, after the dilution of the original buffer).

1. 10× homogenization buffer (HB): 0.1 M Tris base, 0.8 M KCl, 0.1 M EDTA, 10 mM spermidine, 10 mM spermine, pH 9.5 adjusted with NaOH. The stock can be prepared the previous day and stored at 4 °C (*see* **Note 1**).
2. HB (1×): 10× HB diluted ten times plus 0.5 M sucrose and 0.15% β-mercaptoethanol.
3. HB (1×) plus detergent: Triton X-100 was mixed with 1× HB up to the concentration of 0.50% (*see* **Note 2**).
4. Wash Buffer: 1× HB without the addition of Triton X-100. Add protease inhibitor cocktail (Sigma; P9599) according to the manufacturer's instructions.
5. Other requirements: Polyvinyl polypyrrolidone (PVPP), Miracloth (Calbiochem).

2.3 Nuclear Protein Extraction and Quantification

1. Guanidine hydrochloride solution: 0.3 M guanidine hydrochloride in 95% ethanol.
2. 2-DE sample buffer: 8 M urea, 2 M thiourea, and 2% (w/v) CHAPS.
3. TriPure Reagent (Roche).
4. Chloroform 100% stock.
5. Absolute ethanol.
6. Acetone 100% stock.
7. 2-D Quant Kit (GE Healthcare).

2.4 DAPI Staining

1. Prepare stock solution of 4,6-diamidino-2-phenylindole (DAPI) at a concentration of 1 mg/mL in ddH_2O. Aliquot the solution and store in the dark at −20 °C.
2. 1× phosphate-buffered saline (PBS): 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl.
3. Prepare 1 μg/mL working DAPI solution by diluting 1 μL of the DAPI stock solution in 999 μL of PBS. The working DAPI solution can be stored at 4 °C in dark and used within a week.

2.5 Chlorophyll Assay

1. Acetone 100%.

2.6 Immunoblot Analysis

1. Western blot transfer buffer: 0.025 M Tris, 0.192 M glycine, and 20% methanol.
2. Tris-buffered saline (TBS; 10×): 0.1 M Tris–HCl (pH 7.4), with 1.5 M NaCl.
3. TBS containing Tween-20 (TBST): 1× TBS with 0.05% Tween-20.
4. Ponceau S: 0.1% (w/v) Ponceau S stain dissolved in 5% acetic acid.
5. Blocking solution: 5% nonfat milk in 1× TBS (*see* **Note 3**). Blocking solution can be stored at 4 °C for a week.
6. Mouse anti-fibrillarin (Abcam Limited) and sheep anti-histone core (Abcam Limited) antibodies.
7. Secondary antibodies (anti-mouse and anti-sheep) conjugated to alkaline phosphatase.
8. Alkaline phosphatase buffer: 100 mM diethanolamine (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2$.
9. Nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP): Dissolve 1 g NBT in 20 mL of 70% dimethylformamide (DMF). Dissolve 1 g BCIP in 20 mL of 100% DMF. Add 66 μL of NBT and 33 μL of BCIP to 10 mL of alkaline phosphatase buffer just before adding to membrane.

2.7 Enzyme Assays

Stock solutions for the buffers can be made a day before and stored at 4 °C. On the day of the experiment, the buffers can be diluted as required and used.

1. Potassium phosphate buffer (70 mM): Make 70 mM solutions of both monobasic (KH_2PO_4) and dibasic (K_2HPO_4) potassium phosphate. For pH 7.5, take dibasic solution and add monobasic solution until the desired pH is achieved.
2. 10 mM Tris–HCl buffer: Make 10 mM Tris solution and maintain pH 9.0 with HCl.
3. 100 mM HEPES-KOH buffer: Prepare 100 mM HEPES solution and maintain pH 7.5 with KOH.
4. 100 mM TES-NaOH buffer: Make 100 mM TES (N-[Tris (hydroxymethyl)methyl]-2-aminoethanesulfonic acid) solution and maintain pH 9.0 with HCl.
5. 5 mM NAD: Immediately before use, prepare 5 mM solution in 3 mL ddH_2O using ß-nicotinamide adenine dinucleotide hydrate (NAD).
6. 50 mM NADP: Prepare 1 mL solution with NAD phosphate hydrate immediately before use.
7. 100 mM fumarate: Prepare 1 mL solution using fumaric acid.

8. Potassium phosphate (KH_2PO_4): Prepare 100 mM solution and use in desired dilution.
9. 0.1 M glucose-6-P (G6P): Prepare 0.1 M G6P solution and use as required.
10. $MgCl_2$ solution (1 M).
11. Triton X-100 stock.
12. NADP-malic enzyme: Immediately before use, prepare a solution containing 1 U/mL of malic enzyme in cold ddH_2O.

2.8 1-DE and 2-DE SDS Polyacrylamide Gel Components

Prepare all the solutions with ultrapure water and analytical grade reagents. Store all reagents at room temperature (unless indicated otherwise).

1. Resolving gel buffer: 1.5 M Tris–HCl, pH 8.8.
2. Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8.
3. Acrylamide solution: Dissolve 30% acrylamide +0.8% methylene bis-acrylamide in 60 mL ddH_2O. Add a spatula of AG 501-X8 (D) mixed-resin beads (Bio-Rad) and mix for about 30 min. Make up to 100 mL with ddH_2O. Store at 4 °C, in a bottle wrapped with aluminum foil (*see* **Note 4**).
4. 20% SDS (sodium dodecyl sulfate).
5. 10% APS (ammonium persulfate). Keep at 4 °C and make fresh every week.
6. 10× SDS running buffer (1 L): 30 g Tris, 144 g glycine, and 10 g SDS dissolved in ddH_2O and made up to 1 L (*see* **Note 5**).
7. Coomassie Blue stain: 10% (v/v) acetic acid, 0.006% (w/v) Coomassie Blue dye, and 90% ddH_2O.
8. Isopropanol fixing solution: 10% (v/v) acetic acid, 25% (v/v) isopropanol, and 65% ddH_2O.
9. SDS sample loading buffer (10 mL): 1.25 mL Tris–HCl (0.5 M, pH 6.8), 2 mL glycerol (50%), 1 mL SDS (20%), 0.5 mL β-mercaptoethanol (add immediately before use), 5 mL ddH_2O, and a pinch of bromophenol blue.
10. Destaining solution: 10% (v/v) acetic acid.
11. IEF rehydration buffer: 8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 20 mM DTT, 0.5% (v/v) pharmalyte (pH either 4–7 or 6–11; GE Healthcare) and 0.05% (w/v) bromophenol blue.
12. Equilibration buffer: 6 M urea, 50 mM Tris–HCl (pH 8.8), 30% (v/v) glycerol and 2% (w/v) SDS. Aliquot and store at −20 °C until use.
13. Agarose sealing solution: 0.5% agarose in 1× SDS running buffer with a pinch of bromophenol added as tracking dye. Heat in a microwave oven so that agarose dissolves completely. Prepare just before use.

14. Other requirements: IPG strips (pH 4–7 and 6–11; GE Healthcare), IPG buffer (pH 4–7 and 6–11; GE Healthcare), dithiothreitol (DTT), iodoacetamide (IAA), and Silver Stain Plus kit (Bio-Rad).

3 Methods

3.1 Isolation of Intact Nuclei

All solutions, glassware, tubes, and equipment should be precooled to 4 °C and kept on ice all the time. Centrifuge rotors should be cooled down to the same temperature.

1. Ground 20 g tissue into powder in a prechilled mortar and pestle using liquid nitrogen (*see* **Note 6**). Add 0.3% (w/w) PVPP to the ground powder and mix thoroughly (*see* **Note 7**).
2. Transfer the ground powder (*see* **Note 8**) immediately into a 1 L ice-cold conical flask containing 400 mL chilled 1× HB plus detergent buffer (*see* **Note 9**).
3. Gently stir the contents for 30 min at 4 °C for the complete lysing of the organellar membranes.
4. Filter this suspension through four layers of cheesecloth and two layers of Miracloth into an ice-cold 500-mL centrifuge bottle (*see* **Note 10**). Pellet the homogenate by centrifugation with a fixed-angle rotor (1800 × *g*) at 4 °C for 20 min.
5. Separate the supernatant, and gently suspend the pellet with the assistance of a small paintbrush soaked in ice-cold wash buffer. Finally, add another 30 mL of the ice-cold wash buffer.
6. To remove the particulate matter remaining in the suspension, filter the resuspended nuclei into a 50 mL centrifuge tube through two layers of Miracloth by gravity. Centrifuge (57 × *g*) the contents at 4 °C for 2 min to remove intact cells and tissue residues.
7. Transfer the supernatant into a fresh centrifuge tube, and pellet the nuclei by centrifugation (1,800 × *g*) at 4 °C for 15 min in a swinging bucket centrifuge. Wash the pellet two additional times by gentle resuspension in wash buffer followed by centrifugation (1,800 × *g*) at 4 °C for 15 min.
8. Suspend the resulting nuclei pellet either in 1 mL of 1× HB without β-mercaptoethanol and store on ice for further purity experiments or directly use for protein extraction.

3.2 Nuclear Protein Extraction and Quantification

Nuclear proteins can be prepared from the nuclei-enriched pellet using TriPure Reagent (Roche) according to the manufacturer's instructions with few modifications.

1. Resuspend the nuclei pellet completely in minimum amount of TriPure reagent. Dissolve the pellet by repeated pipetting of

the suspension. Dispense 1 mL of the suspension into 1.5 mL reaction tubes and incubate for 5 min at room temperature for complete dissociation of nucleoprotein complexes.

The amount of TriPure used depends on the quantity of the isolated nuclei obtained through the previous step, which again varies according to the quality of the starting sample used.

2. Add 0.2 mL of chloroform to each tube and vortex the tubes for 15 s so as to form a homogenous mixture. Allow the sample to stand for 10 min at room temperature before centrifugation at 12,000 × *g* for 15 min at 4 °C.
3. Following centrifugation, the mixture gets separated into a lower red (phenol-chloroform) phase, an interphase, and a colorless upper aqueous phase. Remove all of the aqueous (upper) phase completely using a pipette. Use the remaining interphase (white) and the lower phase (red) for protein isolation.
4. Add 0.3 mL of 100% ethanol to the tube so as to precipitate the DNA. Mix the sample thoroughly by vigorous tapping of the tubes. Incubate for 3 min at room temperature and centrifuge at 2,000 × g for 5 min at 4 °C.
5. Transfer the phenol-ethanol supernatant to a fresh 2.0 mL reaction tube. Add 1.5 mL of acetone to each tube and mix by inversion. Complete precipitation of proteins can be achieved by incubation for 10 min at room temperature. Recover the protein pellet by centrifugation at 12,000 × *g* for 10 min at 4 °C.
6. Remove the supernatant and add 2 mL of guanidine hydrochloride reagent to each tube so as to cover the pellet (*see* **Note 11**). After 20 min incubation, collect the protein pellet by centrifugation at 7,500 × *g* for 5 min at 4 °C.
7. Wash the pellet two to three times with guanidine hydrochloride before a final 20 min wash in 2 mL of 100% ethanol. Centrifuge at 7,500 × *g* for 5 min at 4 °C. Discard the supernatant and air-dry the pellet. Resuspend the protein pellet in minimum amount of 2-DE sample buffer (*see* **Note 12**). The protein concentration can be determined using the 2-D Quant kit (GE Healthcare) according to the manufacturer's instructions.
8. Prepare a standard curve using 2 mg/mL BSA standard solution provided with kit. Prepare the tubes with 10–50 μL of the sample to be assayed. Add 500 μL of precipitant to each tube including standard curve tubes and vortex for proper mixing. Add 500 μL co-precipitant to each tube, and mix thoroughly. Centrifuge the content at 10,000 × *g* for 5 min and decant the supernatant completely. Add 100 μL copper solution and

400 μL ddH_2O to each tube followed by vortexing. Next add 1 mL color reagent (100 parts color reagent A: 1 part color reagent B) to each tube and incubate for 20 min at room temperature. Read the absorbance of standard and sample at 480 nm using ddH_2O as the reference. Generate a standard curve by plotting the absorbance of the standards against the quantity of protein and determine protein concentration of the samples.

3.3 DAPI Staining of Isolated Nuclei

1. Mix 6 μL of nuclei suspension (Subheading 3.1, protocol **step 8**) with 6 μL of working DAPI solution.
2. Allow the suspension to stain for 15 min before washing it twice with PBS.
3. For microscopic analysis, place a small volume of the suspension on a microscopic slide, cover with a coverslip, and record the images with and without a UV filter (*see* **Note 13**; Fig. 1a).

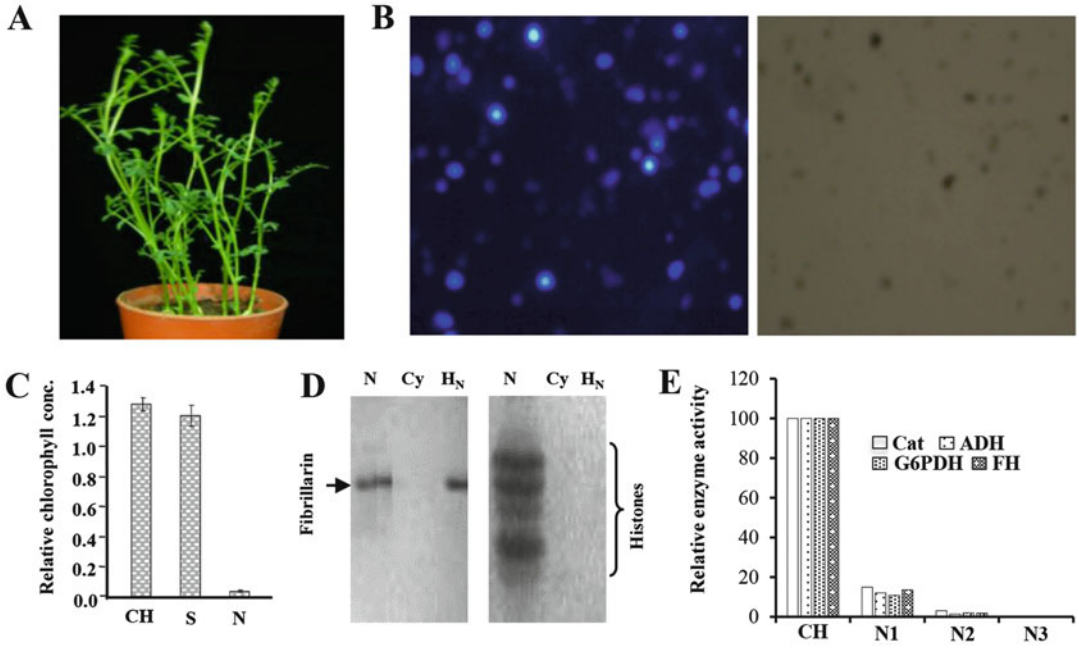

Fig. 1 Purity assessment of the isolated nuclear fraction. (**a**) A representative photograph of 3-week-old chickpea seedlings grown in pot. (**b**) The DAPI-stained nuclei as visualized by epifluorescence (*left panel*) and the corresponding bright field image (*right panel*). (**c**) Total chlorophyll content (mg/g fresh weight) in crude homogenate (CH), supernatant (S), and nuclear fraction (N). (**d**) Immunoblot analysis with nuclear-resident proteins, fibrillarin and histone core. Cytosolic fraction served as negative control, while Hela nuclear extract served as positive control for fibrillarin. *N* nuclear enriched fraction, *Cy* cytosolic fraction and *HN* Hela nuclear extract. (**e**) Determination of contaminating non-nuclear proteins by monitoring the activity of catalase (Cat), alcohol dehydrogenase (ADH), glucose-6-phosphate dehydrogenase (G6PDH), and fumarate hydratase (FH) at different stages of purification. *CH* represents crude homogenate, *N1* represents the fraction after first centrifugation, while *N2* and *N3* represent the subsequent fractions during purification

3.4 Chlorophyll Assay

The chlorophyll content in the crude homogenate (Subheading 3.1, protocol **step 4**), the supernatant (Subheading 3.1, protocol **step 5**), and the nuclei suspension (Subheading 3.1, protocol **step 8**) can be determined by the colorimetric method.

1. Prepare the samples by pipetting 1 mL of each fraction into a 15 mL centrifuge tube and adding 8 mL of acetone and 1 mL of ddH_2O to it and centrifuge at 1000 × *g* for 5 min. Gently collect the supernatant in a fresh tube.
2. Record the absorbance of these samples at 652 nm. Perform the assay in triplicates and calculate the amount of chlorophyll in 1 mL of the suspension as mg chlorophyll/mL = Absorbance/34.5.
3. Finally, determine the amount of chlorophyll as mg per g fresh tissue weight. The purity of the nuclear fraction can be evaluated on the basis of the difference in chlorophyll content in supernatant and the nuclear suspension (*see* **Note 14;** Fig. 1b).

3.5 Immunoblot Analysis

1. Resolve proteins on 12.5% SDS-PAGE (as detailed in Subheading 3.7.1) and electrotransfer onto nitrocellulose membranes using standard procedures [18].
2. Upon completion of the transfer, remove the membrane from the blotting apparatus and subject to Ponceau S stain to verify transfer efficiency and equal loading of proteins.
3. Scan the stained protein bands and immediately wash two to three times for 10 min each with 1× TBS with shaking at room temperature to remove the stain.
4. Incubate the membrane with blocking solution for 1 h at room temperature or overnight at 4 °C with shaking.
5. Wash in TBST, three times each for 10 min.
6. Incubate with primary antibodies specific for nucleus (anti-fibrillarin (1:3000 dilution) and anti-histone core (1:2000 dilution)) in TBS for 1 h at room temperature with shaking.
7. Wash in TBST, three times each for 10 min.
8. Incubate with secondary antibodies (1:10,000 dilution) conjugated to alkaline phosphatase for 1 h at room temperature.
9. Wash in TBST, three times each for 10 min.
10. Develop the blot in the dark with NBT/BCIP added to alkaline phosphatase buffer (Fig. 1c).
11. Stop the reaction by decanting the buffer and adding ddH_2O.

3.6 Enzyme Assays

The purity of the nuclear fraction can be also determined on the basis of marker enzyme activities specific for different organelles, viz., alcohol dehydrogenase (ADH) for cytosol, catalase for microbodies, fumarase or fumarate hydratase for mitochondria, and

glucose-6-phosphate dehydrogenase (G6PDH) for plastids (Fig. 1d). Perform all the assays in triplicates.

1. The catalase enzyme assay can be performed using 10 μg of organellar protein for each reaction. Prepare the reaction mixture by adding 50 μL of protein extract to 940 μL of 70 mM potassium phosphate buffer. Initiate the reaction by adding 10 μL of H_2O_2 (3% v/v) and monitor the decrease in absorbance at 240 nm for 5 min. Baseline correction can be done by subtracting the absorbance taken without the addition of H_2O_2.
2. Determine the activity of ADH with ethanol as substrate by measuring NADH production from NAD by increase in absorbance at 340 nm at 25 °C. A typical reaction mixture contains 750 μmol Tris–HCl (pH 9.0), 3 μmol NAD, and 1% (v/v) ethanol and 10 μg of organellar protein in a final volume of 5.0 mL. Initiate the reaction by adding ethanol and note the absorbance changes.
3. The assay for G6PDH should contain 10 mM $MgCl_2$, 0.1% Triton X-100, 0.17 mM NADP, 0.33 mM G6P, and 20 mM TES-NaOH (pH 7.5) and 10 μg of organellar protein in a final volume of 3 mL. Measure the reduction of $NADP^+$ by monitoring the absorbance at 340 nm.
4. Measure the activity of fumarate hydratase as increase in absorbance at 340 nm due to NADPH formation. The reaction mixture consists of 10 mM fumarate, 25 mM Hepes-KOH buffer (pH 7.5), 0.2 U malic enzyme/mL, 0.27 mM NADP, 4 mM $MgCl_2$, and 5 mM potassium phosphate and 10 μg of organellar protein in a final volume of 5 mL. Initiate the reaction by adding fumarate and incubate at 37 °C. Monitor the absorbance changes over time.

3.7 Gel Based Separation and Staining of Nuclear Proteome

3.7.1 1-DE

1. Clean the glass plates with detergent and water followed by ethanol. Assemble the gel cassette (10 × 12 cm) and attach this assembly in the gel electrophoresis apparatus.
2. Prepare polyacrylamide gel according to standard protocol (for 12.5% gel, resolving gel solution (15 mL): 6.255 mL acrylamide/bis-acrylamide solution, 3.75 mL resolving gel buffer, 150 μL 20% SDS, 75 μL 10% APS, 6 μL TEMED, and 4.764 mL ddH_2O). Add TEMED and APS at the end. Pipette the solution leaving enough space for stacking gel. Add 0.3 mL of n-butanol. A very sharp liquid interface will be visible within 10–20 min. Allow the gel to polymerize for another half an hour at least. Rinse the surface of the gel with ddH_2O before pouring the stacking gel (4% stacking gel (5 mL): 0.67 mL acrylamide/bis-acrylamide solution, 1.26 mL stacking gel buffer, 50 μL 20% SDS, 25 μL 10% APS, 4 μL TEMED, and

2.991 mL ddH_2O). Pour the stacking gel solution and insert the 10-well comb taking care not to trap any bubbles below the teeth.

3. Prior to adding the sample buffer, keep samples at 4 °C. Add the SDS sample loading buffer to the sample (50 μg) and boil at 100 °C for 3–5 min. Once heated, sample could sit at room temperature for a short time until loading or at −20 °C for a week or more if required.
4. Load samples and run gel at 25 mA in 1× SDS running buffer.
5. At this point, the gel can either be transferred to a membrane (for immunoblotting *see* Subheading 3.5) or stained with Coomassie blue (*see* below).
6. Place gel in a container. Cover with isopropanol fixing solution and shake at room temperature for 30–60 min.
7. Pour off fixing solution. Cover with Coomassie blue staining solution and shake at room temperature for 2 h.
8. Decant the staining solution. Wash gel with 10% acetic acid to destain, shaking at room temperature. Digitize the gel image (Fig. 2a).

3.7.2 2-DE

The authors' laboratory performs 2-DE according to standard procedure with few modifications. The entire protocol is described in brief.

1. Perform isoelectric focusing (IEF) on 13 cm IPG strips with 150 μg of protein for pH range 4–7 and 100 μg of protein for pH 6–11 strips.
2. For pH gradient strip 4–7, dilute aliquots of proteins with IEF rehydration buffer and load 250 μL solution by the in-gel rehydration method (*see* **Note 15**). However, for 6–11 pH strips, rehydration can be performed with only rehydration buffer without the addition of protein sample. Load the protein sample (100 μg protein in 100 μL rehydration buffer) using the anodic cup-loading method.
3. Perform IEF at 20 °C up to 30,000 and 70,000 Vh for 4–7 and 6–11 pH IPG strips, respectively (*see* **Notes 16** and **17**). Avoid both underfocussing and overfocussing.
4. For reduction, treat the focused strips with 1% (w/v) DTT in 10 mL of equilibration buffer for 15 min.
5. Treat the strips with 2.5% (w/v) iodoacetamide in equilibration buffer for another 15 min.
6. Dip the strips in 1× running buffer and then load on the top of 12.5% polyacrylamide gels for SDS-PAGE. Position the IPG strips between the plates on the surface of the second-dimension gel with the plastic backing against one of the glass

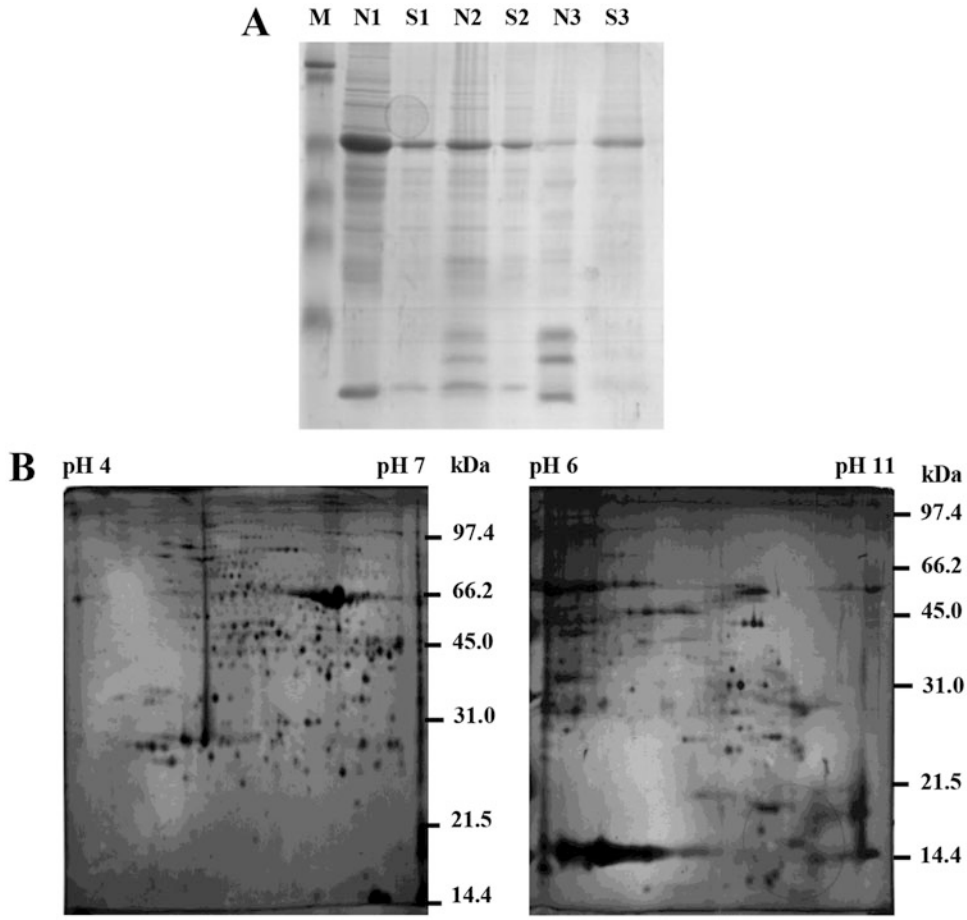

Fig. 2 Nuclear proteome profile of chickpea. (**a**) Nuclear proteins resolved onto 12.5% 1-DE gel. *M* molecular weight marker, *N1* initial nuclear pellet, *S1* initial supernatant obtained after centrifugation. *N2* and *N3* represent subsequent nuclear pellets, while *S2* and *S3* represent subsequent supernatant obtained after each washing step. (**b**) 2-DE profile of nuclear enriched fraction

plates (*see* **Note 18**). Add the molecular weight markers to Whatman paper in a volume of 15–20 μL and apply to the top of the gel at one end of the IPG strip. Seal the strips in place using the agarose sealing gel.

7. Perform electrophoresis at constant current in two steps, initially fixed at 15 mA per gel (during transfer of protein from IPG strip to second dimension gel) and then 30 mA per gel till the dye front reaches approximately 1 mm from the bottom of the gel.
8. Disassemble the gel stacks. Electrophoresed proteins can be stained with Silver Stain Plus kit (Bio-Rad) as per the manufacturer's instructions. Gel images can be digitized with a Bio-Rad FluorS equipped with a 12-bit camera.

4 Notes

1. Chromatin contains Mg^{2+} bound to the negatively charged phosphates in the DNA. However, this can cause problems in nuclear isolation. Polyamines, spermine, and spermidine in the presence of metal chelators or divalent cations help to stabilize the chromatin structure, thereby preventing aggregation of nuclei bodies [19].
2. The addition of nonionic detergent such as Triton X-100 facilitates the release of nuclei from cells and helps to dissolve chloroplast and mitochondrial membranes, among others. The nuclear membrane is relatively stable owing to its double unit structure; however, the concentration of Triton X-100 to be used is critical.
3. Mix milk solution well and filter. Failure to filter can lead to spotting where tiny dark grains will contaminate the blot during color development.
4. Mixed resin AG 501-X8 (D) (anion and cation exchange resin) removes charged ions (e.g., free radicals) and allows longer storage of acrylamide solution. Upon exhaustion of the exchange capacity, the color of resin changes from blue-green to gold. The used mixed resin can be filtered out before storage.
5. Initially add only Tris and glycine to 800 mL of water. Let it dissolve and then add SDS and make up the volume.
6. According to the literature, freezing of plant tissues may lead to disintegration of nuclei and thus fresh leaf material should be preferred. However, our protocol was equally effective with the use of frozen plant materials.

   We have used up to a month old batch of frozen tissue for our studies. However, this period is easily extendable if the storage conditions are fairly stable.
7. The buffer for isolation of nuclei is supplemented with PVPP for absorbing phenolic compounds specifically in the case of chickpea, which is rich in phenolics.
8. Some available protocols use further homogenization techniques. However, from our own experience, it is not advisable to further disrupt the tissue as it may lead to breakage of the nuclear membrane and spillage of chromatin.
9. The ratio of homogenization buffer to tissue weight is critical for the subsequent fractionation of nuclei and needs to be optimal, as insufficient buffer amount may result in impure nuclear preparations. In fact, the volume of flask used is also critical as large volume translates into greater surface area so as to achieve optimum shaking for lysis of contaminating organellar membranes.

10. It is important to use as small a pad of cheesecloth and Miracloth as possible to reduce the loss of sample.
11. At this step, the centrifuge tube could be stored overnight at 4 °C, and the rest of the steps could be undertaken the following day.
12. This protocol yields fairly good amounts of protein but did not achieve a complete dissolution of the precipitate at final step in rehydration buffer. The trick to achieve maximum dissolution is to not over-dry the pellet.
13. Optimally, the nuclei observed under epifluorescence should resemble round spheres with a diameter of about 10–15 μm, with few or no broken or torn structures found nearby. The authors' laboratory could achieve purified nuclei as intact uniform spheres approx. 10 μm in size with little or no impurity.
14. A chlorophyll assay should be carried out in triplicate to evaluate possible contamination of the nuclear fractions. The crude homogenate and the supernatant, which contains cytoplasmic and chloroplast proteins, should retain maximum chlorophyll content, while the chlorophyll content in the semi-pure nuclei preparations should be comparatively negligible.
15. Always use the recommended amount of rehydration buffer for the IPG strips. Also ensure that there are no bubbles between the rehydration solution and the gel surface for effective rehydration.
16. As isoelectric focusing proceeds, the bromophenol blue tracking dye migrates toward the anode. If the dye does not migrate, no current is flowing. If this occurs, check the contact between the external face of the strip holder electrodes and the electrode areas on the IEF system, and between the rehydrated gel and the internal face of the electrode.
17. Rehydrated IPG strips should at all times be under IPG cover fluid so as to prevent dehydration of the strips which would lead to improper focusing.
18. The entire lower edge of the IPG strip should be in contact with the top surface of the second dimension gel for effective protein transfer. No air bubbles should be trapped between the IPG strip and the gel surface or between the gel backing and the glass plate.

Acknowledgments

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References

1. Bae MS, Cho EJ, Choi EY, Park OK (2003) Analysis of the *Arabidopsis* nuclear proteome and its response to cold stress. Plant J 36:652–663
2. Khan MK, Komatsu S (2004) Rice proteomics: recent developments and analysis of nuclear proteins. Phytochemistry 65:1671–1681
3. Pandey A, Choudhary MK, Bhushan D, Chatttopadhyaya A, Chakraborty S, Datta A, Chakraborty N (2006) The nuclear proteome of chickpea reveals predicted and unexpected proteins. J Proteome Res 5:3301–3311
4. Pandey A, Chakraborty S, Datta A, Chakraborty N (2008) Proteomics approach to identify dehydration-responsive nuclear proteins from chickpea (*Cicer arietinum* L.) Mol Cell Proteomics 7:88–107
5. Repetto O, Rogniaux H, Firnhaber C, Zuber H, Kuster H, Larre C, Thompson R, Gallardo K (2008) Exploring the nuclear proteome of *Medicago truncatula* at the switch towards seed filling. Plant J 56:398–410
6. Choudhary MK, Basu D, Datta A, Chakraborty N, Chakraborty S (2009) Dehydration-responsive nuclear proteome of rice (*Oryza sativa* L.) illustrates protein network, novel regulators of cellular adaptation and evolutionary perspect. Mol Cell Proteomics 8:1579–1598
7. Moriguchi K, Suzuki T, Ito Y, Yamazaki Y, Niwa Y, Kurata N (2005) Functional isolation of novel nuclear proteins showing a variety of subnuclear localizations. Plant Cell 17:389–403
8. Rossignol M, Peltier J, Mock H, Matros A, Maldonado AM, Jorrin JV (2006) Plant proteome analysis: a 2004–2006 update. Proteomics 6:5529–5548
9. Millar AH, Taylor NL (2014) Subcellular proteomics-where cell biology meets protein chemistry. Front Plant Sci 5:55
10. Agrawal GK, Bourguignon J, Rolland N, Ephritikhine G, Ferro M, Jaquinod M, Alexiou KG, Chardot T, Chakraborty N, Jolivet P, Doonan JH, Rakwal R (2011) Plant organelle proteomics: collaborating for optimal cell function. Mass Spectrom Rev 30:772–853
11. Baginsky S, Gruissem W (2004) Chloroplast proteomics: potentials and challenges. J Exp Bot 55:1213–1220
12. Erhardt M, Adamska I, Franco OL (2010) Plant nuclear proteomics – inside the cell maestro. FEBS J 277:3295–3307
13. Albenne C, Canut H, Jamet E (2013) Plant cell wall proteomics: the leadership of Arabidopsis thaliana. Front Plant Sci 4:111
14. Huang S, Jacoby RP, Millar AH, Taylor NL (2014) Plant mitochondrial proteomics in plant proteomics: methods and protocols, 2nd edn. Springer, New York, pp 499–526
15. Subba P, Kumar R, Gayali S, Shekhar S, Parveen S, Pandey A, Datta A, Chakraborty S, Chakraborty N (2013) Characterisation of the nuclear proteome of a dehydration-sensitive cultivar of chickpea and comparative proteomic analysis with a tolerant cultivar. Proteomics 13:1973–1992
16. Jaiswal DK, Ray D, Choudhary MK, Subba P, Kumar A, Verma J, Kumar R, Datta A, Chakraborty S, Chakraborty N (2013) Comparative proteomics of dehydration response in the rice nucleus: new insights into the molecular basis of genotype-specific adaptation. Proteomics 13:3478–3497
17. Kumar R, Kumar A, Subba P, Gayali S, Barua P, Chakraborty S, Chakraborty N (2014) Nuclear phosphoproteome of developing chickpea seedlings (*Cicer arietinum* L.) and protein-kinase interaction network. J Proteomics S105:58–73
18. Shaw CE, Zheng J (1998) Western immunoblot analysis. Methods Mol Biol 105:295–306
19. Willmitzer L, Wagner KG (1981) The isolation of nuclei from tissue-cultured plant cells. Exp Cell Res 135:69–77

Chapter 4

Identification of Plant Nuclear Proteins Based on a Combination of Flow Sorting, SDS-PAGE, and LC-MS/MS Analysis

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Abstract

In the plant nucleus, the majority of cellular DNA content is stored and maintained. This makes this highly specialized organelle the major coordinator of almost all essential processes in plant cells such as transcription, DNA replication, and repair. None of these biological pathways can be fully understood without a comprehensive characterization of nuclear proteins. Nevertheless, the interest of the proteomic community in the plant nuclear proteome has been very limited so far. This is probably due to the high integrity of plant cell, presence of many interfering metabolites, and considerable endogenous proteolytic activity which make the sample preparation problematic. Hereby, we describe a novel protocol for the high-throughput plant nuclear protein identification that combines a flow cytometric sorting of formaldehyde-fixed nuclei with protein and peptide separation and their subsequent LC-MS/MS analysis.

Key words Cell cycle, Flow cytometry, Gel electrophoresis, In-gel digestion, Mass spectrometry, Nuclear proteome, Plant nucleus, Protein analysis

Abbreviations

ACN	Acetonitrile
AmBic	Ammonium bicarbonate
APS	Ammonium persulfate
CBB	Coomassie Brilliant Blue
CHCA	α-Cyano-4-hydroxycinnamic acid
DAPI	4′,6-Diamidino-2-phenylindole
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
FA	Formic acid
FSC	Forward-scattered light
GO	Gene ontology

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HPLC	High-performance liquid chromatography
LC-ESI-MS	Liquid chromatography coupled to electrospray ionization mass spectrometry
LC-MALDI-MS	Liquid chromatography coupled to matrix-assisted laser desorption/ionization mass spectrometry
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LSB	Loading sample buffer
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
PMSF	Phenylmethanesulfonylfluoride
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
StageTip	Stop-and-go Tip
TFA	Trifluoroacetic acid
UHR-Q-TOF	Ultra-high-resolution quadrupole time-of-flight mass spectrometer

1 Introduction

The nucleus is a dynamic, complex, and highly organized structure in eukaryotic cells, which harbors the majority of the genetic information. Therefore, nearly all DNA-related processes including the corresponding regulatory pathways take place in this important and unique organelle.

The structure and composition of the cell nucleus is intimately linked with its biological functions. There are two main structural parts: the nucleoplasm and nuclear envelope. The nuclear envelope encloses the nucleus and is composed mainly of the inner and outer nuclear membranes, nuclear pore complexes, and nuclear lamina. Its major function is to facilitate the trafficking of macromolecules between the nucleoplasm and cytosol, but it also plays an important role in chromatin organization and function. The dominant component of nucleoplasm is chromatin, which is arranged into chromosomes and comprises both the condensed heterochromatin and more loosened interchromatin and euchromatin. Other constituents of the nucleoplasm are membrane-less sub-nuclear organelles such as the nucleolus, Cajal bodies, and nuclear speckles [1].

The basis of these structures is constituted by proteins that also represent highly efficient effectors of all biological phenomena occurring in the nucleus. Hence, for the complete understanding of nuclear organization and nuclear-related functions, it is necessary to fully characterize the nuclear proteome. Indeed, many proteomic publications from the last decade have been focused on

animal nuclei. On the other hand, only sparse proteomic reports have dealt with nuclear proteins from plants so far, probably due to troublesome sample preparation. The most commonly used protocol for the isolation of cell nuclei involved the following steps: mechanical homogenization of samples, filtration of the homogenate in order to remove large cell debris, pelleting, suspension in nonionic detergent, and a final separation of the nuclei by density gradient centrifugation (or differential centrifugation). This strategy is time-consuming, risky regarding possible contamination by other cellular organelles or their components and technically demanding. As a result, only modest numbers of identified plant nuclear proteins have been reported in previously published proteomic studies [2, 3].

To overcome the aforementioned obstacles and explore the unique characteristics of plant nucleus, we have developed a novel approach that combines initial nuclei isolation by flow sorting with DNase-assisted protein extraction, proteolytic digestion, and protein identification by LC-MS/MS of peptides. This method allowed unambiguous identification of 3818 peptides matching to 803 proteins extracted from G1 cell cycle phase nuclei in a single proteomic experiment [4]. Furthermore, a gene ontology (GO) enrichment analysis confirmed that the vast majority of the proteins were nucleus-related. Altogether, this data clearly demonstrated that the coupling of flow cytometry with proteomic methods provides an elegant and powerful means of mapping the plant nuclear proteome. In the following sections, the procedure is described in detail. Specifically, a protocol for proteomic characterization of nuclei from barley (*Hordeum vulgare* L., cv. Morex), which is an economically important cereal characterized by a large repeat-rich genome of 5100 Mbp/1C [5], is presented.

In the first steps, seeds are germinated in the dark and the emerging roots are cut out and fixed in a formaldehyde solution. Formaldehyde introduces both intra- and inter-molecular cross-links between proteins and proteins and nucleic acids. Thus, it preserves these molecules in their mutual spatial relationships [6, 7]. The fixation also helps to avoid unwanted contamination and protects nuclear proteins from artificial changes. Then, a crude preparation of nuclei is obtained by mechanical homogenization and the purification is accomplished by a flow cytometric sorting employing staining by 4′,6-diamidino-2-phenylindole (DAPI). DAPI was chosen because it provides a high-resolution quantification of DNA and hence it allows for an efficient and sensitive separation between G1, S and G2 cell cycle phase nuclei. Moreover, DAPI does not stain dsRNA.

Protein extraction was designed to: (a) remove the major contaminant represented by DNA, (b) release cross-links formed during fixation, and (c) efficiently solubilize most of the nuclear protein mass. To this end, a procedure comprising DNase digestion

in a lysis buffer [8] was adapted. DNase I is an endonuclease that cleaves DNA at phosphodiester linkages yielding mono- and oligonucleotides with a phosphate group at the position 5′. This enzyme acts on single-stranded DNA, double-stranded DNA, and chromatin and as such, it appeared as a tool of choice. Moreover, when a mild heat (37 °C) is applied for DNase treatment, the formaldehyde cross-links are reversed [9, 10]. Nuclear protein solubilization is further enhanced by a modified radioimmunoprecipitation assay buffer [11], which disrupts the nuclear envelope.

In the final part of the protocol, a classical gel-based proteomic approach is employed for the identification of extracted proteins [12]. Namely, the nuclear proteins are separated according to their molecular mass using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), stained with Coomassie Brilliant Blue (CBB) dye, excised and digested directly in the gel pieces. The obtained peptides are desalted, analyzed by LC-MS/MS and the acquired data are searched against a barley protein database with the Mascot search engine [13]. In this setup, a standard SDS-PAGE is utilized because it enables good protein fractionation, while it still retains sample processing time on a reasonable level. Anyway, if desired, the extracted proteins can be easily either separated by 2-D electrophoresis or dissolved and digested in-solution (unpublished data).

2 Materials

2.1 Plant Material

1. Dried mature seeds of barley (*Hordeum vulgare* L.).

2.2 Reagents and Solutions (See Note 1)

2.2.1 Preparation of Intact Nuclei Suspension and Its Flow Sorting

1. Tris buffer: 10 mM Tris/HCl, pH 7.5, 10 mM Na_2EDTA, 100 mM NaCl.
2. Formaldehyde fixative: 2% (v/v) formaldehyde in the Tris buffer. Prepare just before use!
3. 10× LB01-P stock solution: 15 mM Tris/HCl, pH 9.0, 2 mM Na_2EDTA, 0.5 mM EGTA, 80 mM KCl, 20 mM NaCl, 0.2 mM spermine·4HCl, 0.5 mM spermidine·3HCl, 0.1% (v/v) Triton X-100.
4. LB01-P buffer: Mix 1 mL of the 10× LB01-P stock solution with 9 mL deionized water. Add 10 μL of 2-mercaptoethanol and mix well. Prepare just before use!
5. DAPI stock solution: 0.1 mg/mL DAPI.
6. Phenylmethanesulfonylfluoride (PMSF) stock solution: 100 mM PMSF dissolved in isopropanol.
7. Sheath fluid: 10 mM NaCl.
8. P5 buffer: 10 mM Tris/HCl, pH 8.0, 50 mM KCl, 2 mM $MgCl_2.6H_2O$, 5% (w/v) sucrose.

2.2.2 Protein Extraction, Separation, and Detection

1. Nuclear lysis buffer: 25 mM Hepes/NaOH, pH 8.0, 150 mM NaCl, 1% (v/v) IGEPAL CA-630, 0.1% (w/v) sodium deoxycholate, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 1 mM dithiothreitol (DTT), 1 mM PMSF, 1 mM Na_3VO_4, 10 mM NaF, Roche cOmplete EDTA free inhibitor cocktail (Hoffmann-La Roche, Basel, Switzerland).
2. DNase I solution (1 unit/μL; one unit completely digests 1 μg of plasmid DNA to oligonucleotides at 37 °C in 10 min).
3. Ice-cold acetone.
4. Loading sample buffer (LSB): 62.5 mM Tris/HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) 2-mercaptoethanol, 0.05% (w/v) Bromophenol Blue.
5. Molecular weight marker for SDS-PAGE.
6. Acrylamide/*N*,*N*′-methylenebisacrylamide solution: 30% T, 2.67% C.
7. 4× Resolving Buffer: 1.5 M Tris/HCl, pH 8.8.
8. 4× Stacking Buffer: 0.5 M Tris/HCl, pH 6.8.
9. SDS solution: 10% (w/v) SDS.
10. Ammonium persulfate (APS) solution: 10% (w/v) APS. Use only freshly prepared!
11. *N*,*N*,*N*′,*N*′-tetramethylethylenediamine.
12. 10× Electrode Running Buffer: 0.25 M Tris/HCl, pH 8.3, 1.92 M glycine, 1% (w/v) SDS.
13. CBB stock solution: 12.5% (v/v) H_3PO_4, 12.5% (w/v) $(NH_4)_2SO_4$, 0.15% (w/v) CBB G-250.
14. Methanol.
15. CBB working solution: Mix 40 mL of the CBB stock solution with 10 mL of pure methanol.
16. De-staining solution: 5% (v/v) acetic acid.

2.2.3 Protein Digestion and Peptide Desalting

1. Ammonium bicarbonate buffer: 100 mM ammonium bicarbonate (AmBic).
2. De-staining solution: 50% (v/v) acetonitrile (ACN) in 100 mM AmBic.
3. ACN.
4. DTT solution: 10 mM DTT in 100 mM AmBic. Use only freshly prepared!
5. Iodoacetamide solution: 55 mM iodoacetamide in 100 mM AmBic. Use only freshly prepared!
6. 25 mM AmBic.
7. Trypsin solution: 1 μM thermostable trypsin modified by raffinose (its application is described in ref. [4]; or another

proteomics grade trypsin) in 25 mM AmBic. Use only freshly prepared (from a 200 μM stock solution in 0.1% (v/v) formic acid (FA) stored in aliquots at −80 °C)!

8. Extraction buffer: 30% (v/v) ACN containing 5% (v/v) FA.
9. Isopropanol.
10. Equilibration solution: 5% (v/v) FA.
11. Elution solution: 50% (v/v) methanol containing 2.5% (v/v) FA.

2.2.4 NanoLC-ESI-MS and MS/MS Analysis of Peptides

1. 5% FA (v/v) containing 5% (v/v) methanol.
2. Loading solvent: 2% (v/v) FA.
3. Mobile phase A: 2% (v/v) ACN containing 0.4% (v/v) FA.
4. Mobile phase B: 90% (v/v) ACN containing 0.4% (v/v) FA.

2.2.5 NanoLC-MALDI MS and MS/MS Analysis of Peptides

1. Loading solvent: 2% (v/v) ACN containing 0.05% (v/v) trifluoroacetic acid (TFA).
2. Mobile phase A: 0.05% (v/v) TFA.
3. Mobile phase B: 80% (v/v) ACN containing 0.05% (v/v) TFA.
4. Matrix stock solution: saturated α-cyano-4-hydroxycinnamic acid (CHCA) in 90% (v/v) ACN containing 0.1% (v/v) TFA.
5. Matrix working solution: 748 μL of 95% (v/v) ACN containing 0.1% (v/v) TFA is mixed with 36 μL of the saturated CHCA solution, 8 μL of 10% (v/v) TFA and 8 μL of 100 mM $(NH_4)H_2PO_4$.
6. Peptide standard stock solution: the protocol has been optimized with the Peptide Calibration Standard II (Bruker Daltonik, Bremen, Germany); the whole content of the tube is dissolved in 125 μL of 0.1% (v/v) TFA according to the manufacturer's instructions; 10 μL aliquots are stored frozen at −80 °C. Any other peptide calibration standard applicable for MS of peptides is applicable.
7. Solvent for peptide standard: 748 μL of 85% (v/v) ACN containing 0.1% (v/v) TFA is mixed with 36 μL of the saturated CHCA solution, 8 μL of 10% (v/v) TFA, and 8 μL of 100 mM $(NH_4)H_2PO_4$.
8. Peptide standard working solution: 2 μL of the Peptide Calibration Standard II aliquot are added to 300 μL of the above solvent.
9. Solvent for dissolving peptides from digests (LC-MALDI): 0.1% (v/v) TFA.
10. Washing solvent for external pump at the spotter: isopropanol.
11. Washing solvents for MALDI target: (a) isopropanol and (b) 30% (v/v) ACN containing 0.1% (v/v) TFA.

2.3 Consumables, Tools and Instruments

1. Non-powdered protective gloves.
2. Pipettes and appropriate tips.
3. Plastic tubes (0.5, 1.5, and 2 mL) with appropriate racks.
4. Biological incubator with a temperature control.
5. Glass Petri dishes (18 cm diameter) with filter paper cut to fit the diameter for seed germination.
6. Mechanical tissue homogenizer.
7. Nylon mesh filters (50 and 20 μm pore size), cut to 4 × 4 cm squares.
8. Sample tubes for flow cytometer.
9. Sample tube holder.
10. Ice container.
11. pH meter.
12. Flow cytometer and sorter equipped with blue (488 nm, 100 mW) and UV (355 nm, 100 mW) lasers.
13. Microscopic slides with coverslips.
14. Fluorescence microscope with optical filter sets for DAPI fluorescence (for checking of nuclei integrity after flow sorting).
15. Bio-safety hood.
16. Analytical scales.
17. Pipette tips for SDS-PAGE sample loading.
18. Syringe with a stainless steel needle with a blunt end (21 G).
19. Temperature-controlled shaker.
20. Centrifuge for sample tubes.
21. Thermoblock.
22. Vortex.
23. Electrophoretic cell with accessories.
24. Power supply.
25. Orbital shaker.
26. Disposable surgical lancet.
27. Laminar flow box.
28. Vacuum centrifuge.
29. Ultrasonic bath.
30. Stainless steel syringe needle (21 G) with a plunger.
31. Stop-and-go Tip (StageTip): to prepare a StageTip, place an octadecyl (C_{18})-bonded silica Empore extraction disk (cat. no. 2215, 3M, St. Paul, MN, USA) into a clean Petri dish in a dust-free environment. Gently press a 21 G needle into the disk and doing so, cut a small piece of the disk. Repeat once more (two

pieces of the disk should remain in the needle). Place the needle inside a 200 μL pipette tip and release the disks using a suitable plunger. Always press the disk gently! If you apply a big pressure, the disks will be packed densely and the back pressure of the StageTip will be too high. Also, if the disks are positioned sideways, discard the StageTip.

32. 1 mL glass vials for HPLC.
33. Pre-column for nanoLC-ESI-MS/MS: the protocol has been optimized using a reversed-phase pre-column (75 μm × 20 mm) ended with a frit (IntegraFrit™, cat. no: IF360-75-50-N-5; New Objective Inc., Woburn, MA, USA) and packed with 5 μm C18 sorbent (ReproSil-Pur 200, C18-AQ; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) using a pressure vessel; any other nano column of the same or similar parameters is applicable.
34. Analytical column for nanoLC-ESI-MS/MS: The protocol has been optimized using a reversed-phase analytical column (75 μm × 200 mm) ended with a pointed emitter (PicoTip™, cat. no: FS360-50-8-N-5-C25; New Objective Inc., Woburn, MA, USA) and packed with 5 μm C18 sorbent (ReproSil-Pur 200, C18-AQ; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) using a pressure vessel; any other nano column of the same or similar parameters is applicable. Both nano columns for nanoLC-ESI-MS/MS (pre-column and analytical column) are routinely prepared in our laboratory using a pressure vessel "Sample and Column Loader" (Proxeon Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) pressurized with helium.
35. Dionex UltiMate3000 RSLCnano liquid chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) or another analogous chromatograph.
36. Ultra-high-resolution quadrupole time-of-flight (UHR-Q-TOF) mass spectrometer maXis equipped with a nano electrospray ion source (Bruker Daltonik, Bremen, Germany) coupled on-line to the Dionex chromatograph or any other instrument with similar parameters.
37. Millicup™ HV disposable vacuum bottle top filtration funnels (300 mL) for HPLC with 47 mm PVDF membrane filters (0.45 μm pore size) by Millipore (Bedford, MA, USA).
38. Dionex UltiMate3000 RSLCnano liquid chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) connected to a Proteineer fc II fraction collector (Bruker Daltonik, Bremen, Germany) or any other system with the same or similar parameters.
39. Syringe: Precision glass syringe for solvents, capacity of 1 mL.

40. Pre-column for nanoLC-MALDI-MS/MS: the protocol has been optimized using a reversed-phase Nano Trap column (100 μm × 20 mm; nanoViper inlet/outlet) packed with Acclaim PepMap® C18 silica particles (5 μm particle size, 100 Å pore size) by Thermo Fisher Scientific (Waltham, MA, USA); any other nano column of the same or similar parameters is applicable.
41. Analytical column for nanoLC-MALDI-MS/MS: The protocol has been optimized using a reversed-phase Acclaim PepMap® RSLC column (75 μm × 150 mm; nanoViper inlet/outlet) with C18 silica particles (2 μm particle size, 100 Å pore size) by Thermo Fisher Scientific (Waltham, MA, USA); any other nano column of the same or similar parameters is applicable.
42. MALDI target: The protocol has been optimized with an MTP AnchorChip™ 800-384 target (Bruker Daltonik, Bremen, Germany) but any other comparable target can be used depending on the available mass spectrometer.
43. ultrafleXtreme MALDI-TOF/TOF mass spectrometer equipped with a smartbeam-II laser (Bruker Daltonik, Bremen, Germany) or any other instrument with similar parameters.
44. Computers for database searches: first with the database search engine Mascot Server (Matrix Science, London, UK) and in-house versions of protein databases such as Swiss-Prot (Swiss Institute of Bioinformatics, Geneva, Switzerland) or NCBInr (National Center for Biotechnology Information, Bethesda, MD, USA); second with a proteomic software platform such as ProteinScape 3.1 (Bruker Daltonik, Bremen, Germany).

3 Methods

3.1 Seed Germination (See Note 2)

1. Leave seeds in a beaker filled with deionized H_2O and let them to soak at laboratory temperature for 15 min. Keep the seeds aerated in the dark at 25 °C (in a biological incubator) overnight.
2. Germinate the seeds in a glass Petri dish on a layer of wet paper towels sandwiched by two layers of filter paper in the dark at 25 °C (in a biological incubator), until the root length reaches 2–3 cm (Fig. 1a).

3.2 Preparation of Suspension of Intact Nuclei (See Note 3)

1. Cut out the roots and transfer them into a beaker with deionized H_2O (Fig. 1b). The number of barley roots needed for the preparation of a 1 mL nuclei sample (*see* further) is estimated to a value of 60.

Fig. 1 Preparation of intact barley nuclei suspension. (**a**) Seed germination in a Petri dish. (**b**) Cutting of roots and their transfer into a beaker with deionized water. (**c**) Fixation of roots in formaldehyde solution. (**d**) Cutting of meristem root tips prior to their mechanical homogenization. (**e**) Release of nuclei into LB01-P lysis buffer by mechanical homogenization. (**f**) Filtration of the suspension of nuclei. (**g**) Nuclei sorting and collection into tubes (**h**) or onto microscopic slides for integrity verification (**i**)

2. Place the roots into a beaker with the formaldehyde fixative solution and keep them immersed at 5 °C for 10 min (Fig. 1c).
3. Rinse the roots three times in the Tris buffer with EDTA at 5 °C for 5 min. After the last rinse, keep the roots in the Tris buffer on ice.

4. Cut root tips (in a length of 1–2 mm) and transfer them into a 5 mL polystyrene tube containing 1 mL of LB01-P buffer (Fig. 1d).
5. Grind the root tips using a mechanical homogenizer at 15,000 rpm for 13 s (Fig. 1e).
6. Filter the crude suspension of nuclei through a 50 μm nylon mesh and collect the filtrate into another 5 mL polystyrene tube (Fig. 1f).
7. Keep the suspension on ice until the flow sorting.

3.3 Nuclei Sorting Using Flow Cytometry

1. Set up the flow sorter according to the manufacturer's instructions.
2. Filter the suspension of nuclei through a 20 μm nylon mesh.
3. Add DAPI to the filtered suspension of nuclei to achieve a final concentration of 2 μg/mL (e.g., 20 μL of the DAPI stock solution is used for 1 mL nuclei sample).
4. Launch the control software of the flow sorter to create all appropriate histograms and dot plots. First, use a dot plot of forward-scattered light (FSC) vs. DAPI to visualize populations representing nuclei (Fig. 2a). Mark a region surrounding the population of nuclei (R) and use this gating on the remaining dot plots and histograms. For sorting, build dot plots DAPI-W vs. DAPI-A (Fig. 2b). Make a histogram for DAPI staining showing the distribution of relative DNA content among nuclei (Fig. 2c).
5. Run the sample. Analyze at least 20,000 nuclei and save the data.
6. Create sorting regions (G1, S, and G2, according to the respective cell cycle phase) surrounding the population of nuclei of interest (Fig. 2b).
7. Sort the nuclei in a required number (Figs. 1g and 2c) and collect them in appropriate collection tubes containing LB01-P supplemented with 10 μM PMSF (Fig. 1h; *see* **Note 4**), and onto microscopic slides into P5 buffer for a verification of nuclei integrity (Fig. 1i).
8. Analyze the prepared slide using a fluorescence microscope.

3.4 Nuclear Protein Extraction (See Note 5)

1. Remove the LB01 buffer from the sorted and pelleted nuclei using a pipette with a tip for SDS-PAGE sample loading.
2. Add 50 μL of the nuclear lysis buffer to the nuclei and vortex the sample briefly.
3. Sonicate the sample in an ultrasonic bath for 15 min and disperse the nuclei thoroughly by squeezing ten times against the tube wall with a syringe needle.

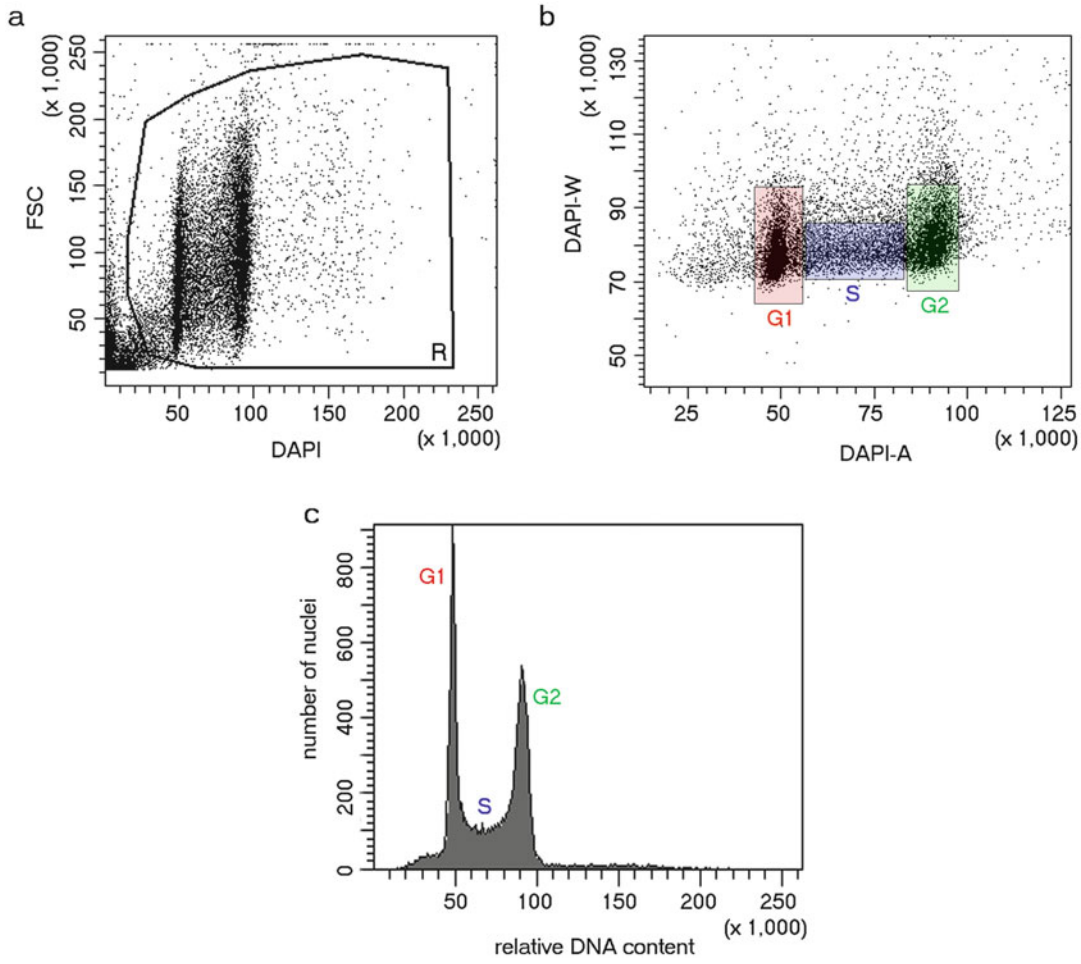

Fig. 2 Flow cytometric analysis of barley nuclei. (**a**) A dot plot FSC vs. DAPI, which is used for separating populations of nuclei (region R). (**b**) A dot plot DAPI-W vs. DAPI-A created for analysis and sorting nuclei stained with DAPI. DAPI-A and DAPI-W represent the total fluorescence and size of the particle, respectively. There are three regions G1, S, and G2 chosen for sorting nuclei of interest. (**c**) A histogram of relative fluorescence intensity of DAPI-stained nuclei. It consists of the three groups of nuclei (according to the G1, S, and G2 phase of the cell cycle)

4. Add 10 μL of 1 U/μL DNase I stock solution, mix the sample by pipetting (*see* **Note 6**), and incubate it at 37 °C for 2 h.
5. Add another 10 μL aliquot of DNase I and continue with the incubation overnight.
6. Centrifuge the sample at 25,000 × *g* for 15 min and move the supernatant to a new tube.
7. Precipitate proteins in the supernatant by adding 4 volumes of ice-cold acetone and perform incubation at −20 °C for 24 h.
8. Collect the precipitate by a centrifugation at 25,000 × *g* for 15 min and discard the supernatant.

9. Add 50 μL of LSB to the protein precipitate and the original nuclear pellet (*see* **step 6**).
10. Re-suspend the samples by a careful pipetting, sonicate in the ultrasonic bath for 15 min, and incubate with heating at 100 °C for 15 min.
11. Spin down the liquid condensed at the tube lid and walls using the centrifuge.

3.5 SDS-PAGE [14] and Protein Detection (See Note 7)

1. Prepare a discontinuous vertical polyacrylamide gel slab composed of a 10% resolving gel and 4% stacking gel. Alternatively, you may use a commercial precast gel of the same or similar parameters.
2. Assemble the electrophoretic cell according to the manufacturer's instructions and place the gel slab inside.
3. Load molecular weight marker (according to the manufacturer's instructions) and the prepared samples into selected wells and run the separation at 80 V for 30 min and then at 120 V until tracking dye reaches the bottom end of the gel.
4. Disassemble the electrophoretic cell, pull out the gel slab, remove one of the covering glass plates, and cut off the stacking gel.
5. Place the resolving gel into a glass Petri dish (or a disposable plastic Petri dish) filled with 50 mL of the CBB working solution. Stain the gel overnight under continuous shaking on an orbital shaker.
6. Transfer the gel into a Petri dish with deionized water and rinse it for 5 min under continuous shaking on the orbital shaker.
7. Carefully remove water by a decantation and add enough de-staining solution to keep the gel immersed.
8. De-stain the gel using continuous shaking on the orbital shaker for 1 h.
9. Check the gel. If the background is still bluish, replace the de-staining solution. Perform this replacement repeatedly until the gel background is almost clear.

3.6 In-gel Protein Digestion [15]

1. Excise protein bands or larger slices from the stained gel using a clean disposable surgical lancet (Fig. 3; *see* **Notes 8** and **9**).
2. Cut the excised bands or slices into cubic pieces (*see* **Note 10**) and transfer them into clean tubes.
3. Spin the gel pieces down using a centrifuge.
4. To de-stain proteins, add 100 μL of the de-staining solution (*see* **Note 11**) and incubate the suspension under a gentle shaking (about 500 rpm) for 30 min.

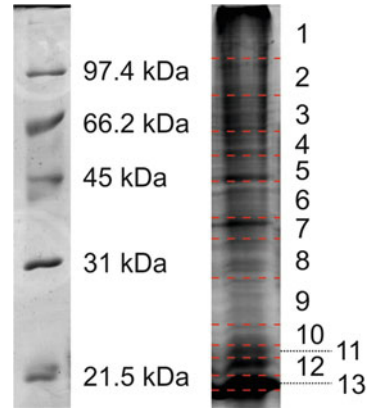

Fig. 3 SDS-PAGE separation of barley nuclear proteins isolated from G2 nuclei. The *left lane* shows a protein marker (with the indicated molecular mass values), the *right lane* belongs to the proteins from nuclear pellet fraction boiled in LSB. The numbers at the right lane label gel slices (i.e., fractions) selected for the subsequent procedure of in-gel digestion of proteins

5. Spin the gel pieces down in the centrifuge and remove all liquid.
6. Add 100 μL of pure ACN and incubate the suspension with an occasional vortexing until the gel pieces turn white and shrink.
7. Spin the gel pieces down in the centrifuge and remove all liquid.
8. Add 100 μL of the DTT solution and incubate the suspension at 56 °C for 30 min.
9. Repeat **steps 5**–7.
10. Cover the gel pieces with 100 μL of the iodoacetamide solution and incubate at laboratory temperature in the dark for 20 min.
11. Repeat **steps 5**–7.
12. Add 100 μL of 25 mM AmBic and incubate the suspension at laboratory temperature under continuous shaking (1000 rpm) for 10 min.
13. Repeat **steps 5**–7.
14. Add as much trypsin solution as needed to cover completely the shrunk gel pieces (usually 30–40 μL or more; this is based on the size of the excised gel band or slice) and incubate the tubes on ice or in a fridge.
15. After 30 min, check the gel pieces. If all trypsin solution was soaked up, add some more.
16. Keep the samples on ice or in the fridge for another 90 min to enable trypsin to diffuse into the gel pieces.

17. Spin the gel particles down in the centrifuge and aspirate the liquid. Add 25 mM AmBic without trypsin (usually 30–40 μL) to prevent from an excessive presence of trypsin autolytic peptides in the final digest. This step is optional and its inclusion in the protocol is based on researcher's experience with autolysis of a particular trypsin preparation (*see* **Note 12**).
18. Incubate the samples at 37 °C overnight.
19. To extract peptide digestion products, add 100 μL of the extraction buffer and incubate the suspension at 37 °C with continuous shaking (1,400 rpm; *see* **Note 13**) for 30 min.
20. Transfer the supernatant into a new tube.
21. To complete the extraction of peptides, add 100 μL of the extraction buffer to the gel pieces and incubate the suspension at 37 °C with continuous shaking (1,400 rpm) for another 30 min (*see* **Note 14**).
22. Combine the supernatant with that one from the **step 20**.
23. Dry down the pooled supernatants in a vacuum centrifuge. Store the tubes with peptides at −20 °C until the next step of peptide desalting is done (if it is not done immediately).

3.7 Peptide Desalting [16]

1. Dissolve the peptide samples in 50 μL of the equilibration solution and sonicate the tubes in an ultrasonic bath for 5 min.
2. Shake the samples at a maximum speed for 5 min and spin them down in a centrifuge.
3. Insert the necessary number of StageTips into clean 2 mL tubes by fixing them in punched tube lids and place the resulting cartridge in the centrifuge (*see* **Note 15**).
4. Add 50 μL of pure isopropanol into the StageTips and activate the reverse phase by centrifugation at 4,000 × *g* for 2.5 min (*see* **Note 16**). Repeat this step once more.
5. To equilibrate the reverse phase, add 50 μL of the equilibration solution into the tips and centrifuge the cartridges at 2,000 × *g* for 2.5 min. Repeat this step once more.
6. Place the equilibrated StageTips into new, empty 2 mL tubes.
7. Load the samples into the tips and centrifuge the cartridges at 1,500 × *g* for 10 min.
8. Add 50 μL of the equilibration solution into the loaded StageTips and centrifuge at 2,000 × *g* for 2.5 min. Repeat once more.
9. Place the loaded and washed StageTips into new, empty 1.5 mL tubes (*see* **Note 17**).
10. To elute the retained peptides, add 50 μL of the elution solution into the tips and centrifuge the cartridges at 1,500 × *g* for 10 min. Repeat this step once more.

11. Transfer the eluted samples into 1 mL glass vials for HPLC and dry down the content in a vacuum centrifuge. Close the vials by screw neck caps with PTFE/silicone septa and store at −20 °C until the MS analysis (*see* **Note 18**).

3.8 NanoLC-ESI MS and MS/MS Analysis of Peptides (See Note 19)

1. Add 15 μL of 5% (v/v) FA acid containing 5% (v/v) methanol to each sample in a glass vial and mix the content thoroughly by a repeated pipetting and aspirating to dissolve the desalted peptides. Close the vials by screw neck caps with PTFE/silicone septa.
2. Transfer the vials into an ultrasonic bath and sonicate the content for 5 min. Spin down the liquid in a centrifuge and place the vials into the cooled autosampler of the nano-flow liquid chromatograph (thermostated at 8 °C).
3. Prepare the nano-flow liquid chromatograph according to the manufacturer's instruction.
4. Inject 5 μL of the peptide solution into the nano-flow liquid chromatograph. Peptides are first loaded on the pre-column thermostated at 45 °C using the loading buffer at a flow rate of 3 μL/min in a time period of 10 min. They are retained whereas unbound impurities are washed out.
5. Elute the retained peptides from the pre-column to the analytical column using a multistep gradient. The gradient is prepared by a programmed mixing of the mobile phases A and B (Table 1) at a flow rate of 200 nL/min. The total run time is 75 min.
6. The eluent from the analytical column is sprayed directly using nanoESI on-line source into UHR-Q-TOF maXis mass spectrometer. The source conditions were optimized as follows: End Plate Offset: 0 V; electrospray capillary voltage: 2150 V; desolvation gas: 6 L/min; desolvation temperature: 130 °C. The other settings were optimized as recommended by the manufacturer for a typical proteomic workflow.
7. The mass spectrometer UHR-Q-TOF maXis is controlled by Compass for QTOF series v. 1.9 (Bruker Daltonik) and operated in a data-dependent mode A "dynamic method" is applied with a fixed cycle time of 2 s. The mass range for both the MS and MS/MS scans is set at m/z 100–1600. The dynamic exclusion duration is set at 0.3 min after two spectra are acquired. Isolation of precursor ions is performed using a variable isolation window of 3–10 Da depending on the value of precursor mass. The collision energy is adjusted between 17 and 46 eV as a function of the m/z value and charge state of each precursor ion.
8. Analyze and process raw data using an appropriate software, such as DataAnalysis v. 4.3(×64), and save the extracted MS

Table 1
A multistep gradient for reversed-phase nanoLC separation of peptides prior to coupled ESI-MS and MS/MS on UHR-Q-TOF maXis mass spectrometer

Time (min)	Mobile phase A	Mobile phase B	Gradient curve
Initial condition	92	8	Linear
0 Start of sample loading onto the pre-column	92	8	Linear
9	92	8	Linear
10 End of sample loading and start of gradient elution	92	8	Linear
13	90	10	Linear
36	83	17	Linear
50	74	26	Linear
58	68	32	Linear
61	35	65	Linear
62	15	85	Linear
65	15	85	Linear
68 Re-equilibration of the analytical column	92	8	Linear
85 End of gradient	92	8	

and MS/MS spectra into MGF (Mascot Generic Format) and XML formatted files.

9. Upload the MGF and XML files, each containing a list of precursor ions and the corresponding fragmentation data, to ProteinScape v3.1. Using Mascot v2.4, perform searches against a custom database of 105,041 barley (*Hordeum vulgare*) protein sequences downloaded from the Uniprot depository (http://www.uniprot.org) and supplemented with sequences of common contaminants such as keratins, proteolytic enzymes, standard proteins (bovine serum albumin) and reversed sequences of all barley proteins for the determination of false discovery rate. The database search is usually performed in two steps. The first round is done with wide mass tolerances for both precursors and the corresponding fragment ions (±50 ppm and ±0.1 Da, respectively). The resulting list of peptides identified with a high confidence is used for the subsequent re-calibration of precursor and fragment masses in the corresponding MGF and XML files. Searching is then repeated with narrower mass tolerances (±4 ppm and ±0.01 Da for precursors and fragments, respectively). The search parameters include trypsin as a protease (up to two

missed cleavage sites are allowed), carbamidomethylation of cysteine as a fixed modification and N-terminal protein acetylation and oxidation of methionine as variable modifications. A minimum ion peptide score of 15 with a minimum peptide length of seven amino acids at a significance threshold of $p < 0.05$ and a false discovery rate of <1% is commonly used to validate the MS and MS/MS data.

3.9 NanoLC-MALDI MS and MS/MS Analysis of Peptides

1. Add 15 μL of 0.1% (v/v) TFA to each sample in a glass vial and mix the content thoroughly by a repeated pipetting and aspirating to dissolve the desalted peptides. Close the vials by screw neck caps with PTFE/silicone septa.
2. Transfer the vials into an ultrasonic bath and sonicate the content for 10 min. Then spin down the liquid on a centrifuge.
3. Place the vials with samples to be analyzed into the cooled autosampler of the nano-flow liquid chromatograph (5 °C).
4. Filter the loading solvent and mobile phases using Millicup filters and perform a degassing step by placing the bottles into the ultrasonic bath for 15 min (*see* **Note 20**).
5. Place the loading pump tubing into the loading solvent and the tubing leading to both nano pumps into the respective mobile phases. Purge the pumps (via the control software or manually, if applicable) and adjust the loading solvent and mobile phase A flow rates at 10 μL/min and 300 nL/min, respectively. Keep the pumps running for at least 1 h in order to equilibrate the trap column as well as the analytical column. When new columns are attached, it is recommended to wash them consecutively by both mobile phases with a final equilibration in the mobile phase A.
6. Prepare the MALDI target according to the manufacturer's instructions. The washing steps include wiping off impurities with a tissue paper wetted by isopropanol, sonication of the target submerged in isopropanol for 15 min repeated by a sonication in 30% (v/v) ACN containing 0.1% (v/v) TFA. Finally, the target is dried on air or using a flow of nitrogen gas.
7. Transfer the working solution of peptide calibration standard by pipette to the respective calibration spots (aliquots of 0.5 μL) on the target and wait for drying out the stuck drops: matrix-peptide cocrystals are formed.
8. Place the target into the spotting device. Launch the control software (e.g., Hystar by Bruker Daltonik). Using the software, adjust a correct horizontal as well as vertical positioning of the spotting needle at sample spots. Mount the syringe filled with the working matrix solution to the internal pump and attach it to the tubing (*see* **Note 21**). Perform priming of the internal

pump system. Check the level of the washing solvent at the external pump.

9. Open the sample table as a new window in the software and input information about samples in the autosampler. Select a method to be used, which contains important parameters for separation runs such as the injection mode, flow rate values, gradient programming, and eluate collection time window (including how many fractions are collected in total and what is the time interval for each spotting). The procedure has been optimized for injecting 5 μL sample aliquots, the flow rates are as above and the gradient has the following composition: 0 min, 4% mobile phase B; 7 min, 4% B; 45 min, 60% B; 48 min, 90% B; 57 min, 90% B; 59 min, 4% B; 70 min, 4% B. The eluate is usually collected in 17-s fractions (120 fractions in total) starting from 20 min and spotted after mixing with the working matrix solution. The total drop volume is 420 nL. The total run time is 70 min.
10. Run automatic separations and collect eluate fractions. This results in obtaining matrix-peptide cocrystals for each fraction at the surface of the target (sample spots).
11. Launch the acquisition software for MALDI mass spectrometry (MALDI MS), e.g., flexControl by Bruker Daltonik. Place MALDI target into the instrument and wait for re-establishing vacuum in the ion source. Adjust target geometry by a manual or automatic position teaching, select a method for acquisition peptide mass spectra, and check the quality of selected calibration spots by switching on the laser and acquiring a few hundred calibration spectra. Optionally you may do this briefly for selected sample spots.
12. Set up automatic spectra acquisition runs. This is done by opening the corresponding LC-MALDI control software (such as the WarpLC by Bruker Daltonik) and customizing parameters. Select sample spots to be analyzed, indicate the positions of the calibration spots. Select a folder into which data will be stored on the computer for each sample (referring to a single digest) and a folder in the proteomic platform (e.g., ProteinScape by Bruker Daltonik), where database search results will appear. Select proper acquisition methods for calibration, MS and MS/MS measurements, spectra processing, and database searches.
13. Switch on automatic spectra acquisition runs (*see* **Note 22**). During the procedure, mass spectra are acquired and calibration repeatedly checked. MS peaklists are built, which is followed by selecting precursors for fragmentation. Then tandem mass spectra are acquired and MS/MS peaklists are built.

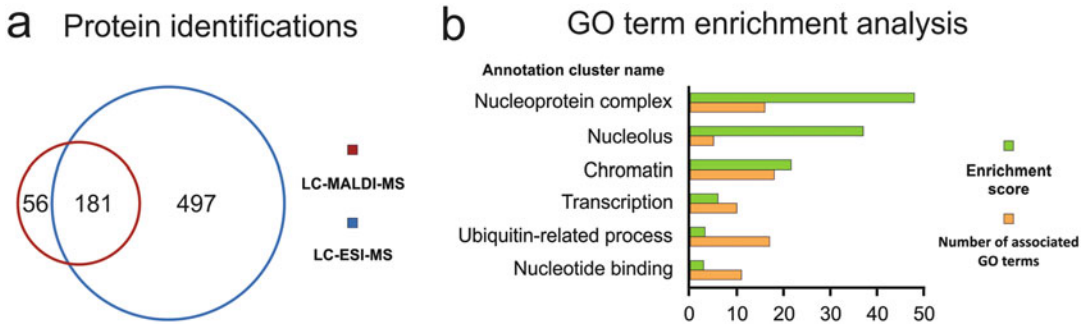

Fig. 4 A brief look inside the G2 barley nuclear proteome. (**a**) This panel shows numbers of proteins identified with ESI- and MALDI-based mass spectrometric approaches. The numbers originate from a recent analysis performed in our laboratory. The depicted Venn diagram shows both overlapping and instrument-unique identifications. (**b**) Most significantly enriched GO annotation clusters (enrichment score > 3) resulting from bioinformatic processing of the respective dataset

14. Database searches utilize the MS/MS peaklists and result in lists of proteins assigned for the collected eluate of each sample (spread, e.g., over 120 spots) in the database.

3.10 Example of Results

Using the described protocol, nuclear proteins were extracted from five million barley nuclei sorted in the G2 phase, separated by SDS-PAGE (Fig. 3), digested with trypsin and analyzed on two different mass spectrometers. In this simple experiment, 734 proteins were identified (Fig. 4a) covering a wide range of protein abundances. GO term enrichment analysis of the identified proteins using the gene functional classification tool DAVID [17, 18] revealed more than 30 enriched annotation clusters that were almost exclusively connected with nucleus and its functions (Fig. 4b). These results are in a good agreement with our previous results obtained from barley G1nuclei [4] and demonstrate that the combination of flow sorting with proteomic techniques is a promising and powerful tool for detailed exploration of plant nuclear proteomes.

4 Notes

1. The highest available purity of all chemicals is a prerequisite for achieving best results.
2. The use of viable and healthy seeds is necessary. The number of seeds, which are needed for the preparation of a 1 mL sample, depends on the number of roots per seedling and size of root tips.
3. During homogenization of root tips and the subsequent flow sorting, nuclei are exposed to shearing forces. The preceding

mild fixation of roots with formaldehyde thus makes the nuclei more resilient. Formaldehyde-fixed roots should be stored on ice and processed within a few hours. Always wear non-powdered protective gloves and work in a bio-safety hood when using formaldehyde.

4. Sorting of approx. five million nuclei is usually performed into 1 mL of LB01-P with 100 μM PMSF in 15 mL collection tubes.
5. The rough estimation of protein content in five million nuclei is 150 μg. This is based on the approximate estimation of the total weight of five million barley nuclei, which is 250 μg, and on knowledge that DNA and protein content of a plant nucleus is around 20% and 60% of the nucleus dry weight, respectively [19].
6. DNase I is extremely sensitive to a physical denaturation. Always mix any DNase solution only by a gentle pipetting or repeated turning the tube upside down and back. Do not vortex!
7. Always wear non-powdered protective gloves and work in a bio-safety hood while working with electrophoresis chemicals. Do not touch the equipment and polyacrylamide gels by bare hands in order to prevent a keratin contamination of the samples.
8. As the majority of the contamination by human keratins emerges during the procedure of protein digestion, always wear non-powdered protective gloves and work in a laminar flow box with. Pay a special attention to this step. Use only chemicals of the highest purity and visually check all the laboratory glassware for impurities.
9. Each sample lane from the polyacrylamide gel should be processed for excisions of gel bands or slices according to the staining intensity pattern. To avoid a future suppression of proteins with lower abundance by abundant ones during nanoLC-MS/MS of peptides from digests, it is advantageous to excise the most intense protein bands separately and not within larger slices. *See* Fig. 3 as an example.
10. A typical size of the gel pieces should be about 1 × 1 × 1 mm. Smaller pieces can easily clog pipette tips.
11. From this point on, if any liquid is added, the gel pieces have to be completely submerged. The volumes provided in text fit well to protein bands with a width up to 5 mm. For processing larger gel slices, these volumes have to be increased accordingly. There is an optimal ratio of 1:1.5 between the volumes of the gel matrix and liquid phase.

12. When the residual trypsin solution after the soaking up process at 4 °C is not aspirated and the liquid replaced by cold 25 mM AmBic, it is necessary to realize that the original trypsin solution volume should exceed the volume of the gel pieces only slightly! The surface of the liquid phase should appear around 1 mm above the gel pieces.
13. If larger bands are processed, there is an optimal ratio of 1:2 between the volumes of the digest (including the gel pieces) and extraction solution.
14. Never discard the gel pieces after the peptide extraction step. If the digestion fails, you can repeat it with the same samples.
15. Alternatively, the procedure of desalting peptides can be performed using ZipTip C18 pipette tips (Millipore, Bedford, MA, USA) according to the manufacturer's instructions.
16. Any liquid that is applied into the StageTip should never pass completely through. A little residual volume (1–5 μL) above the reverse phase is desirable to prevent from drying out the tip.
17. Do not discard the flow-through, but keep it frozen at −20 °C until the respective sample is analyzed to a satisfaction. If there is any trouble during the peptide desalting process, all peptides are in this fraction and can easily be re-extracted.
18. Alternatively, the vacuum centrifugation step can be done already using tubes with omitting the peptide transfer to HPLC vials. Then the plastic tubes are stored at −20 °C instead.
19. Always use fresh working solutions for all nanoLC-MS/MS analyses!
20. Be sure that the mobile phases and loading solvent for liquid chromatography have been filtered to prevent from a column clogging.
21. NanoLC-MALDI: be sure that the syringe with working matrix solution is properly mounted at the internal pump in the eluate fraction collector (spotter) and the attached tubing is not clogged.
22. Be sure that the ion source of the mass spectrometer used has been cleaned prior to extensive measurements with samples.

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References

1. Meier I (2009) Functional organization of the plant cell nucleus. Springer, Heidelberg
2. Erhardt M, Adamska I, Franco OL (2010) Plant nuclear proteomics – inside cell maestro. FEBS J 277:3295–3306
3. Petrovska B, Sebela M, Dolezel J (2015) Inside a plant nucleus: discovering the proteins. J Exp Bot 66:1627–1640
4. Petrovska B, Jerabkova H, Chamrad I et al (2014) Proteomic analysis of barley cell nuclei purified by flow sorting. Cytogenet Genome Res 143:78–86
5. The International Barley Genome Sequence Consortium (2012) A physical, genetic and functional sequence assembly of the barley genome. Nature 491:711–716
6. Thavarajah R, Mudimbaimannar VK, Elizabeth J et al (2012) Chemical and physical basics of routine formaldehyde fixation. J Oral Maxillofac Pathol 16:400–405
7. Sutherland BW, Toews J, Kast J (2008) Utility of formaldehyde cross-linking and mass spectrometry in the study of protein-protein interactions. J Mass Spectrom 43:699–715
8. Wang F, Zhu J (1990) The effect of DNA intercalators on chromatin of chicken red blood cells – differential extraction on nonhistone proteins. Cell Res 1:105–118
9. Orlando V, Strutt H, Paro R (1997) Analysis of chromatin structure by in vivo formaldehyde cross-linking. Methods 11:205–214
10. Kennedy-Darling J, Smith LM (2014) Measuring the formaldehyde protein-DNA cross-link reversal rate. Anal Chem 86:5678–5681
11. Ngoka LC (2008) Sample prep for proteomics of breast cancer: proteomics and gene ontology reveal dramatic differences in protein solubilization preferences of radioimmunoprecipitation assay and urea lysis buffers. Proteome Sci 6:30
12. Granvogl B, Ploscher M, Eichacker LA (2007) Sample preparation by in-gel digestion for mass spectrometry-based proteomics. Anal Bioanal Chem 389:991–1002
13. Perkins DN, Pappin DJ, Creasy DM et al (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20:3551–3567
14. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
15. Shevchenko A, Tomas H, Havlis J et al (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1:2856–2860
16. Rappsilber J, Mann M, Ishihama Y (2007) Protocol for micro-purification, enrichment, prefractionation and storage of peptides for proteomics using StageTips. Nat Protoc 2:1896–1906
17. Huang DW, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4:44–57
18. Huang DW, Sherman BT, Lempicki RA (2009) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37:1–13
19. Tautvydas KJ (1971) Mass isolation onf pea nuclei. Plant Physiol 47:499–503

Chapter 5

Isolation, Purity Assessment, and Proteomic Analysis of Nuclei

Setsuko Komatsu

Abstract

The integrity of a subcellular proteomics is largely dependent on purity of the isolated compartment away from other contaminants. If high-purity nuclei is isolated, nuclear proteomics is a useful approach for investigating the mechanisms underlying plant physiological function. Although the isolation of high-purity nuclei from tissue or organ in plant is a difficult task, successful purification has been achieved through fractionation processes. For purification, there are five protocols such as (1) differential centrifugation, (2) discontinuous Percoll gradients, (3) continuous sucrose gradients, (4) combined continuous Percoll/sucrose gradients, and (5) continuous Percoll gradients. Furthermore, because purity assessment of purified nuclei is an important step, it is also described in this chapter.

Key words Nuclei isolation, Purity assessment, Nuclear proteins, Proteomics

1 Introduction

The nucleus is the subcellular organelle that functions as the regulatory hub of the cell and is responsible for regulating several critical cellular function, including cell proliferation, gene expression, and cell survival [1, 2]. Nuclear proteomics is a useful approach for investigating the mechanism underlying plant growth and adaptation of environmental stress, including protein-protein interactions and posttranslational modifications [3, 4]. The nuclear proteins of many plant species, such as *Arabidopsis*, rice, maize, barley, wheat, soybean, *Medicago*, chickpea, tomato, potato, apple, *Xerophyta viscosa*, and hot pepper, have been analyzed [1]. To identify the upstream events controlling the regulation of many kinds of proteins in plant, the proteomic analysis of nuclear proteins is a powerful technique.

The separation and enrichment of nuclei from plants is technically challenging, and contamination of the nuclear fraction with proteins from other subcellular organelles impedes the analysis of nuclear proteins [5]. A number of different techniques for the

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isolation of plant nuclei have been developed [1]. Most of the isolation methods consist of similar sequential steps that include disruption, filtration, centrifugation, solubilization, and separation. The final two steps, which involve removal of membranes from contaminating organelles using nonionic detergents and the separation of nuclei by density gradient centrifugation, are critical for obtaining high-purity nuclei. The use of nonionic detergents such as Triton X-100 promotes the release of nuclei from cells and prevents nuclei from forming aggregates [6]. Moreover, detergent application aids in the isolation of nuclei from green leaves, which contain abundant chloroplasts and are sensitive to breakdown and solubilization by detergent [7]. However, the plant nuclear membrane is easily damaged by high concentrations or prolonged exposure to detergent [8]. The density gradient systems used for the isolation of nuclei vary depending on the plant species; however, the density medium of these systems typically consists of sucrose, Percoll, or mixtures of sucrose and Percoll.

After the isolation of nuclei, the purity of the obtained fraction should be assessed. Immuno-blot and enzyme activity analyses of marker nuclear proteins are commonly used to evaluate the purity of isolated nuclei. Histones were used as marker nuclear proteins in immuno-blotting analysis of nuclei isolated from *Arabidopsis* and rice [9, 10]. In chickpea, cytochrome *c* oxidase, histones, and fibrillarin were used as marker proteins to confirm the purity of isolated nuclei [11]. In maize and rice, histone was successfully used as a nuclear marker protein in immuno-blot analysis [12]. Although nuclear enrichment can be evaluated using nuclear marker proteins, contamination in nuclear fractions must be assessed using subcellular marker proteins. For example, the enzyme activities of catalase, alcohol dehydrogenase, fumarate hydratase, and glucose-6-phosphate dehydrogenase in the nuclear fraction obtained from rice were used as markers of contamination with peroxisomes, cytosol, mitochondria, and plastids [13]. The findings from these studies demonstrate that the methods used for nuclei isolation must be modified and optimized according to the type of nuclear materials and intended downstream analyses.

Here, the protocols are described based on the constructed methods for rice [14–16] and soybean [17–21]. Furthermore, as nuclear proteomics, the gel-free/label-free proteomic technique is described because it is useful for the detection of minor proteins.

2 Materials

2.1 Isolation of Nuclei

1. Homogenization buffer: 50 mM HEPES (pH 7.4), 10 mM KCl, 1 mM EDTA, 10 mM ascorbate, 0.1% bovine serum albumin, 20 mM dithiothreitol, 400 mM sucrose, and 1 mM phenylmethylsulfonyl fluoride.

2. 2.0 M sucrose cushion: 37.5 mM Tris-maleate (pH 6.5), 5 mM $MgCl_2$, and 1% dextran T500 (Amersham Biosciences, Piscataway, NJ, USA).

2.2 Purity Assessment of Nuclei

1. Extraction buffer for enzyme assays: 50 mM HEPES-NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 2% polyvinylpyrrolidone, 0.1% Triton X-100, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride.
2. The reaction solution for alcohol dehydrogenase assay: 50 mM MES-NaOH, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM NADH, and 4% acetaldehyde.
3. The reaction buffer for catalase activity: 50 mM potassium phosphate and 15 mM H_2O_2.
4. The reaction buffer for fumarase activity: 70 mM KH_2PO_4-NaOH, 0.05% Triton X-100, and 50 mM malic acid.
5. The reaction buffer for NADH-cytochrome *c* reductase activity: 20 mM potassium phosphate (pH 7.2), 0.2 mM NADH, 0.02 mM cytochrome *c*, and 30 mM NaN_3.
6. SDS sample buffer for immuno-blot: 60 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol.
7. Blocking buffer: 20 mM Tris–HCl (pH 7.5), 500 mM NaCl, and 5% skim milk.

2.3 Proteomic Analysis of Nuclear Proteins

1. Lysis buffer for proteomics: 8 M urea, 2 M thiourea, 5% CHAPS, and 2 mM tributylphosphine.

3 Methods

3.1 Isolation of Nuclei

1. Nuclei are prepared following the sucrose density gradient method. All the steps for the isolation of nuclei are performed on ice or at 4 °C.
2. A portion (5 g) of samples is homogenized in 5 mL of homogenization buffer with a glass mortar and pestle.
3. The homogenate is passed two times through a double-layer Miracloth (Calbiochem, Darmstadt, Germany).
4. The homogenate is transferred to a Falcon tube (15 mL) and centrifuged at 1,000 × *g* for 10 min.
5. The pellet is gently resuspended in the homogenization buffer and layered on the top of a 2.0 M sucrose cushion and centrifuged at 50,000 × *g* for 30 min at 4 °C.
6. The supernatant is carefully removed, and the pellet is gently resuspended again in 5 mL of homogenization buffer.

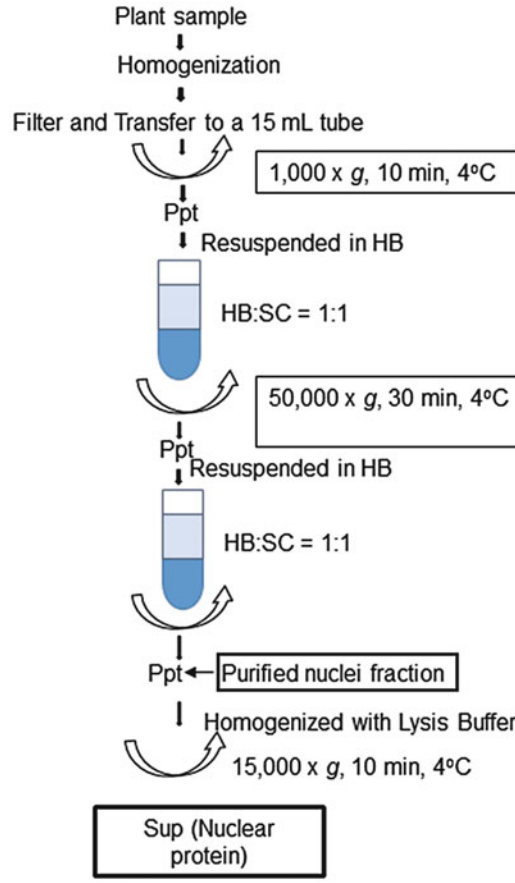

Fig. 1 The procedures of nuclei enrichment. Abbreviations in the figure are as follows: *HB* homogenizing buffer, *SC* sucrose, *Ppt* pellet, *Sup* supernatant

7. The suspension is again layered on the top of a 2.0 M sucrose cushion and centrifuged at 50,000 × *g* for 30 min. The pellet containing the nuclei is resuspended in 100 μL of homogenization buffer.
8. The purified nucleus is vortexed with extraction buffer (for enzyme assay), SDS sample buffer (for immuno-blot), or lysis buffer (for proteomics) and sonicated. After sonication, the homogenate is centrifuged at 12,000 × *g* for 30 min at 4 °C and the supernatant was collected as nuclear proteins (*see* Fig. 1) (*see* **Note 1** with Fig. 2).

3.2 Purity Assessment of Nuclei

1. For enzyme activities, proteins are extracted with extraction buffer; and for immuno-blot, proteins are extracted using SDS sample buffer.
2. Alcohol dehydrogenase activity: Using reaction solution, NADH oxidation is measured at 340 nm at 25 °C for 5 min.

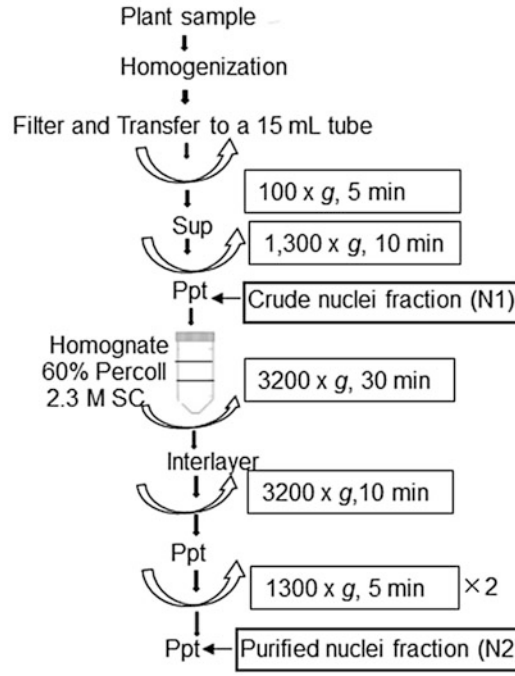

Fig. 2 The procedures of nuclei enrichment. Samples are homogenized with Nuclei Isolation buffer. The homogenates are filtered and centrifuged. The supernatant is collected and centrifuged again. The pellets are collected as crude nuclei fraction (N1). This pellet is resuspended in Nuclei Isolation buffer and layered on the top of a layer which contains 60% Percoll prepared in Nuclei Isolation buffer and 2.3 M sucrose cushion. After centrifugation, the middle layer is collected and washed with Nuclei Isolation buffer. The enriched nuclei is washed two times using Nuclei Isolation buffer. The pellets are collected as final enriched nuclei fraction (N2). Abbreviations in the figure are as follows: *Sup* supernatant, *Ppt* pellet, *SC* sucrose

The enzyme activity is calculated with formula: units/mL = (ΔA340 × total volume × dilution factor)/(6.22 × sample volume) [22].

3. Catalase activity: Using reaction buffer, the subsequent decomposition of H_2O_2 is measured at 240 nm at 25 °C for 5 min. The enzyme activity is calculated with formula: units/mL = (ΔA240 × total volume × dilution factor)/(40 × sample volume) [23].
4. Fumarase activity: Using reaction buffer, the reaction is directly measured at 340 nm. The enzyme activity is calculated with formula: units/mL = (ΔA340 × total volume × dilution factor)/(2.55 × sample volume) [24].
5. NADH-cytochrome *c* reductase activity: Using reaction buffer, the reduction of cytochrome *c* is followed

spectrophotometrically as the absorbance increase at 550 nm. The enzyme activity is calculated with formula: units/mL = (ΔA550 × total volume × dilution factor)/(21.1 × sample volume) [25].

6. Histone enrichment: Proteins are separated on a 12% SDS–polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane using a semidry transfer blotter. The blotted membrane is incubated overnight at 4 °C in blocking buffer. After blocking, the membrane is incubated with a 1:8000 anti-histone H3 antibody (Abcam, Cambridge, UK) for 1 h at room temperature. Anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA, USA) is used as the secondary antibody. After a 1-h incubation with the secondary antibody, signals are detected using an ECL Western blotting detection kit following the manufacturer's protocol, and the signals are visualized using a luminescent image analyzer. Coomassie brilliant blue staining is used as loading control. The relative intensities of bands are calculated using a dedicated software.

3.3 Gel-Free/Label-Free Proteomic Analysis of Nuclear Proteins

1. Enrichment and digestion for nuclear proteins: Proteins (200 μg) are enriched by phase separation in the organic layer. Briefly, 400 μL methanol was added and mixed with each sample before the further addition of 100 μL chloroform and 300 μL water. After mixing, the samples were centrifuged at 20,000 × *g* for 10 min to achieve phase separation. The upper aqueous phase was discarded and 300 μL methanol was slowly added to the lower phase. The samples were further centrifuged at 20,000 × *g* for 10 min, and the obtained pellets were dried.
2. The enriched proteins are reduced, alkylated, and digested [26]. The resulting tryptic peptides are acidified with formic acid (pH < 3), and the mixed solution is centrifuged at 20,000 × *g* for 10 min. The supernatant is collected for liquid chromatography (LC)-mass spectrometry (MS)/MS analysis.
3. Mass spectrometry analysis: Peptides in formic acid are loaded onto nanoLC system (Ultimate 3000; Dionex, Germering, Germany) equip with trap column (C18 PepMap trap column, 300 μm ID × 5 mm; Dionex). The peptides are eluted from the trap column and separate using 0.1% formic acid in acetonitrile at a flow rate of 200 nL/min on nanoLC capillary column (C18 Tip column, 75 μm 1D × 120 mm; Nikkyo Technos, Tokyo, Japan) with a spray voltage of 1.8 kV. The peptide ions in the spray are analyzed on MS (nanospray LTQ XL Orbitrap; Thermo Fisher Scientific, San Jose, CA, USA) operated in data-dependent acquisition mode with the installed Xcalibur software (version 2.0.7, Thermo Fisher Scientific). Full-scan mass spectra are acquired in the MS over 400–1,500 m/z

with a resolution of 30,000. A lock mass function is used to obtain high mass accuracy [27]. As the lock mass, the ions $C_{24}H_{39}O_4^+$ (m/z 391.28429), $C_{14}H_{46}NO_7Si_7^+$ (m/z 536.16536), and $C_{16}H_{52}NO_8Si_8^+$ (m/z 610.18416) are used. The three most intense precursor ions above a threshold of 500 are selected for collision-induced fragmentation in the linear ion trap at a normalized collision energy of 35% after accumulation to a target value of 1,000. Dynamic exclusion is employed within 90 s to prevent the repetitive selection of peptides. Acquired spectra are used for protein identification.

4. Protein identification using acquired mass spectrometry data: Protein identification is performed using the Mascot search engine (version 2.5.1, Matrix Science, London, UK) using suitable database. DTA files are generated from acquired raw data files and convert to Mascot generic files using Proteome Discoverer software (version 1.4.0.288, Thermo Fisher Scientific). The parameters used in the Mascot searches are as follows: carbamidomethylation of cysteine is set as a fixed modification, and oxidation of methionine is set as a variable modification. Trypsin is specified as the proteolytic enzyme, and one missed cleavage is allowed. Peptide mass precursor tolerance is set at 10 ppm, fragment mass tolerance is set at 0.8 Da, and peptide charges are set at +2, +3, and +4. Peptide cut-off score is 10 and for peak filtration the S/N threshold (FT-only) is set at 1.5. An automatic decoy database search is performed as part of the search. Mascot results are filtered with the Mascot percolator to improve the accuracy and sensitivity of peptide identification [28]. False discovery rates (false positive/(false positive + true positive)) for peptide identification of all searches are less than 1.0%. Peptides with a percolator ion score of more than 13 ($p < 0.05$) are used for protein identification. Mascot search results are exported in XML format for comparison analysis.
5. Protein abundance of the identified proteins: Protein abundance is analyzed based on the emPAI values. Briefly, the emPAI value of each identified protein is divided by the sum of the emPAI values for all identified proteins and multiplied by 100. Protein content is estimated by the molar fraction percentage (mol%) [29].
6. Differential analysis of the identified proteins: The acquired Mascot results are exported into SIEVE software (version 2.1.377; Thermo Fisher Scientific) for quantitation analysis between samples. Briefly, the chromatographic peaks detected by MS are aligned and the peptide peaks are detected as a frame on all parent ions scanned by MS/MS using 5 min of frame time width and 10 ppm of frame m/z width. Areas of the chromatographic peak within a frame are compared for each

sample, and the ratios between the samples are determined for each frame. The frames with MS/MS scan are matched to the Mascot results. The peptide ratios between the samples are determined from the variance weighted average of the ratios in the frames, which MS/MS spectrum match to the peptides. The ratios of peptides are further integrated to determine the ratios of the corresponding proteins. Total ion current is used for normalization of differential analysis of protein abundance. The outliers of ratio are deleted in frame table filter based on frame area. The minimum requirement for protein identification is two matched peptides. Significant changes of protein abundance between samples are analyzed ($p < 0.05$).

7. Analyses of protein localization and function: Protein localization is queried using the intracellular targeting prediction programs of YLoc (http://abi.inf.unituebingen.de/Service/YLoc/webloc.cgi) [30], WoLF PSORT (http://wolfpsort.org/) [31], Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi) [32], and NucPred (http://psort.hgc.jp/form2.html) [33].

4 Note

1. Nuclei are also isolated according to the manufacturer's instructions of Plant Nuclei Isolation/Extraction Kit (Sigma, St. Louis, MO, USA) with some modifications. Briefly, a portion (2 g) of samples is ground with buffer. The homogenates are filtered through a double layer of Filter Mesh and centrifuge. The resulting pellet is resuspended in Nuclei Isolation buffer containing protease inhibitor mixture (Roche, Werk Penzberg, Germany), and layered on top of cushions containing 60% Percoll prepared in 1×Nuclei Isolation buffer and 2.3 M sucrose. After centrifugation at 3200 × *g* for 30 min at 4 °C, the middle layer is collected and washed with Nuclei Isolation buffer containing protease inhibitor mixture to remove Percoll and sucrose (Fig. 2).

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References

1. Yin X, Komatsu S (2016) Plant nuclear proteomics for unraveling physiological function. New Biotechnol 33:644–654
2. Wilson KL, Dawson SC (2011) Functional evolution of nuclear structure. J Cell Biol 195:171–181
3. Aebersold R, Mann M (2003) Mass spectrometry based proteomics. Nature 422:198–207
4. Petrovská B, Šebela M, Doležel J (2015) Inside a plant nucleus: discovering the proteins. J Exp Bot 66:1627–1640
5. Pandey A, Choudhary MK, Bhushan D, Chattopadhyay A, Chakraborty S, Datta A et al (2006) The nuclear proteome of chickpea (*Cicer arietinum* L.) reveals predicted and unexpected proteins. J Proteome Res 5:3301–3311
6. Loureiro J, Rodriguez E, Doležel J, Santos C (2007) Two new nuclear isolation buffers for plant DNA flow cytometry: a test with 37 species. Ann Bot 100:875–888
7. Sikorskaite S, Rajamäki ML, Baniulis D, Stanys V, Valkonen JP (2013) Protocol: optimised methodology for isolation of nuclei from leaves of species in the Solanaceae and Rosaceae families. Plant Methods 9:31
8. McKeown P, Pendle AF, Shaw PJ (2008) Preparation of *Arabidopsis* nuclei and nucleoli. In: Hancock R (ed) The nucleus, vol 1. Humana Press, New York, pp 67–75
9. Bae MS, Cho EJ, Choi EY, Park OK (2003) Analysis of the *Arabidopsis* nuclear proteome and its response to cold stress. Plant J 36:652–663
10. Aki T, Yanagisawa S (2009) Application of rice nuclear proteome analysis to the identification of evolutionarily conserved and glucose-responsive nuclear proteins. J Proteome Res 8:3912–3924
11. Kumar R, Kumar A, Subba P, Gayali S, Barua P, Chakraborty S et al (2014) Nuclear phosphoproteome of developing chickpea seedlings (*Cicer arietinum* L.) and protein-kinase interaction network. J Proteome 105:58–73
12. Guo B, Chen Y, Li C, Wang T, Wang R, Wang B et al (2014) Maize (*Zea mays* L.) seedling leaf nuclear proteome and differentially expressed proteins between a hybrid and its parental lines. Proteomics 14:1071–1087
13. Choudhary MK, Basu D, Datta A, Chakraborty N, Chakraborty S (2009) Dehydration-responsive nuclear proteome of rice (*Oryza sativa* L.) illustrates protein network, novel regulators of cellular adaptation, and evolutionary perspective. Mol Cell Proteomics 8:1579–1598
14. Komatsu S (2007) Extraction of nuclear proteins. Methods Mol Biol 355:73–77
15. Khan MM, Komatsu S (2004) Rice proteomics: recent developments and analysis of nuclear proteins. Phytochemistry 65:1671–1681
16. Li M, Yin X, Sakata K, Yang P, Komatsu S (2015) Proteomic analysis of phosphoproteins in the rice nucleus during early stage of seed germination. J Proteome Res 14:2884–2896
17. Komatsu S, Han C, Nanjo Y, Altaf-Un-Nahar M, Wang K, He D, Yang P (2013) Label-free quantitative proteomic analysis of abscisic acid effect in early-stage soybean under flooding. J Proteome Res 12:4769–4784
18. Komatsu S, Hiraga S, Nouri MZ (2014) Analysis of flooding-responsive proteins localized in the nucleus of soybean root tips. Mol Biol Rep 41:1127–1139
19. Oh MW, Nanjo Y, Komatsu S (2014) Identification of nuclear proteins in soybean under flooding stress using proteomic technique. Protein Pept Lett 21:458–467
20. Yin X, Komatsu S (2015) Quantitative proteomics of nuclear phosphoproteins in the root tip of soybean during the initial stages of flooding stress. J Proteome 119:183–195
21. Yin X, Komatsu S (2016) Nuclear proteomics reveals the role of protein synthesis and chromatin structure in root tip of soybean during the initial stage of flooding stress. J Proteome Res 15:2283–2298
22. Komatsu S, Nanjo Y, Nishimura M (2013) Proteomic analysis of the flooding tolerance mechanism in mutant soybean. J Proteome 79:231–250
23. Kato M, Shimizu S (1987) Chlorophyll metabolism in higher plants: VII. Chlorophyll degradation in senescing tobacco leaves; phenolic-dependent peroxidative degradation. Can J Bot 65:729–735
24. Huang S, Jacoby RP (1999) Plant mitochondrial proteomics. In: Millar AH, Taylor NL (eds) Plant proteomics. Humana Press, New York, pp 499–524
25. Hasinoff BB (1990) Inhibition and inactivation of NADH-cytochrome c reductase activity of bovine heart submitochondrial particles by the iron (III)-adriamycin complex. Biochem J 265:865–870
26. Yin X, Sakata K, Komatsu S (2014) Phosphoproteomics reveals the effect of ethylene in

soybean root under flooding stress. J Proteome Res 13:5618–5634

27. Olsen JV, de Godoy LMF, Li G, Macek B, Mortensen P, Pesch R, Makarov A, Lange O, Horning S, Mann M (2005) Parts per million mass accuracy on an orbitrap mass spectrometer via lock mass injection into a C-trap. Mol Cell Proteomics 4:2010–2021
28. Brosch M, Yu L, Hubbard T, Choudhary J (2009) Accurate and sensitive peptide identification with Mascot Percolator. J Proteome Res 8:3176–3181
29. Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, Mann M (2005) Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics 4:1265–1272
30. Briesemeister S, Rahnenführer J, Kohlbacher O (2010) YLoc—an interpretable web server for predicting subcellular localization. Nucleic Acids Res 38:497–502
31. Horton P, Park KJ, Obayashi T, Fujita N, Harada H, Adams-Collier CJ, Nakai K (2007) WoLF PSORT: protein localization predictor. Nucleic Acids Res 35:W585–W587
32. Chou KC, Shen HB (2010) Plant-mPLoc: a top-down strategy to augment the power for predicting plant protein subcellular localization. PLoS One 5:e11335
33. Brameier M, Krings A, MacCallum RM (2007) NucPred-predicting nuclear localization of proteins. Bioinformatics 23:1159–1160

Chapter 6

Proteomic Analysis of Rice Golgi Membranes Isolated by Floating Through Discontinuous Sucrose Density Gradient

Kazusato Oikawa, Takuya Inomata, Yoshitoshi Hirao, Tadashi Yamamoto, Marouane Baslam, Kentaro Kaneko, and Toshiaki Mitsui

Abstract

The Golgi apparatus is an endomembrane system organelle and has roles in glycosylation, sorting, and secretion of proteins in the secretory pathway. It has a central function in living organism and is also essential for plant growth. Proteomic approaches to identify the Golgi membrane proteins have been performed in cell suspension cultures and many Golgi membrane-associated proteins were found, whereas it has well established in rice seedling yet. In this chapter, our recent improving published methods for isolated rice Golgi membranes by floating through a discontinuous sucrose density gradient are provided in detail with proteomic analyses.

Key words Golgi membranes, *Oryza sativa*, Proteomic analysis, Floating, Sucrose density gradient

1 Introduction

The Golgi apparatus is a multifunctional organelle that comprises several stacks and associated vesicles, and appears to have an extremely flexible morphology. In higher plant cells, the Golgi apparatus is responsible for the biosynthesis of complex cell-surface polysaccharides, the processing and modification of glycoproteins, and the sorting station of polysaccharides and proteins destined for different locations. The organelle locates the center of the main route of membrane traffic, that is, numerous proteins come in the Golgi from the endoplasmic reticulum (ER), and come out to the plasma membrane and vacuole [1]. Moreover, recent investigations have demonstrated that some glycoproteins are transported to the plastids from the ER-Golgi system through the secretory pathway [2–7].

The proteome analysis is a useful tool for clarifying the dynamic function of the Golgi complex. The Golgi complex is thought to be

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made up of approximately 1000 proteins [8]. A proteomic analysis of *Arabidopsis* Golgi has been carried out using the localization of organelle proteins by an isotope tagging (LOPIT) technique [9]. Eighty-nine proteins assigned by LOPIT to the Golgi apparatus largely belonged to three main classes: predicted glycosyltransferases, endomembrane protein 70 (EMP70), and putative methyltransferases. Previously, we succeeded in separating distinct compartments of Golgi complex from suspension-cultured cells of rice employing both sedimentation and floating through a discontinuous sucrose density gradient centrifugation technique [10, 11]. Our cell fractionation studies revealed that the cis-Golgi membranes contain membrane-bound α-mannosidase activities and several membrane traffic-related proteins including a set of RAB (YPT1) family and ER resident proteins [11].

Needless to say, highly purified Golgi membranes and high performance of mass spectrometry are required for clarifying the plant Golgi proteome in detail. Here, we isolated the Golgi membranes from rice seedlings by using an improved floating method with sucrose density gradient and evaluated the isolated Golgi membranes employing combined LC-MS/MS technology (LTQ Orbitrap XL and Q Exactive mass spectrometer).

2 Materials

Prepare all the solutions using ultrapure water (prepared by purifying deionized water to attain sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents. Prepare and store all the solutions at 4 °C (unless indicated otherwise). Follow all the institutional waste disposal regulations.

2.1 Sample Materials

Rice (*Oryza sativa* L. cv. Nipponbare) seeds were sterilized with 1% sodium hypochlorite solution for 15 min and then rinsed three times with sterile pure water. Sterilized rice seeds were germinated in the dark at 28 °C for 2 weeks [12, 13] (*see* **Note 1**).

2.2 Golgi Isolation Equipment

1. Mill mixer.
2. Ultracentrifuge (*see* **Note 2**).
3. Swinging bucket rotor.
4. Abbe Refractometer.
5. Spectrophotometer.

2.3 Golgi Isolation Buffer

1. Homogenization buffer: 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 M mannitol, adjusted to pH 7.0 with 5 mM potassium hydroxide (KOH) (*see* **Note 3**).

2. Dilution buffer: 25 mM HEPES adjusted to pH 7.0 with 5 mM KOH.
3. Sucrose gradient buffer: 15%, 50%, and 60% (w/w) sucrose containing 25 mM HEPES-KOH (pH 7.0) (*see* **Note 4**).
4. Discontinuous sucrose gradient buffer: 26%, 30%, 34%, and 38% (w/w) sucrose containing 25 mM HEPES-KOH (pH 7.0) (*see* **Note 4**).

2.4 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Stock solutions: 1.5 M Tris–HCl (pH 8.8), 0.5 M Tris–HCl (pH 6.8), acrylamide (30%, w/v)/bisacrylamide (0.8%, w/v) mixture, 10% (w/v) sodium dodecyl sulfate (SDS), 10% (w/v) ammonium persulfate (APS), *N*,*N*,*N*′,*N*′-tetramethylethlenediamine (TEMED).
2. Sample buffer (×10): 50 mM (pH 6.8), 10% SDS, 25% (v/v) 2-mercaptethanol, 50% (v/v) glycerol, and 0.75 mg/mL Bromophenol blue.
3. Separation gel (10%): 10% acrylamide mixture, 30 mM Tris–HCl (pH 8.8), 0.1% SDS, 0.1% APS, and 0.1% TEMED. Before adding APS and TEMED to the separation gel solution, expel any remaining air by reducing the pressure.
4. Stacking gel: 6% acrylamide mixture, 15 mM Tris–HCl (pH 6.8), 0.12% SDS, 0.14% APS, and 0.4% TEMED.
5. Running buffer: dissolve Tris (15.14 g) and glycine (72.07 g) in 500 mL of water to prepare 10× Running buffer. Running buffer containing 0.1% SDS is prepared with 10× Running buffer and 10% SDS, before use.

2.5 Immunoblot

1. Semi-dry electroblotter.
2. Scanner for chemiluminescent Western blots.
3. Western blot analysis software.
4. PVDF membrane.
5. Chemiluminescence reagent.
6. Blotting buffer: Running buffer containing 20% (v/v) methanol prepared with 10× Running buffer and methanol.
7. Blocking buffer: NaCl (40.03 g), KCl (1.01 g), $Na_2HPO_4{\cdot}12H_2O$ (14.50 g), KH_2PO_4 (1.02 g), and Tween 20 (5.0 mL) dissolved in 500 mL of water to prepare 10× TBST (phosphate-buffered saline with Tween 20). Blocking buffer consists of 1× TBST and 2% (w/v) skim milk.
8. Antibodies: anti-UGPase (52 kDa, [14]) for cytoplasmic marker, anti-Rubisco large subunit (53 kDa, [13]) for plastid marker, anti-COXll (30 kDa, Funakoshi Co.) for mitochondrial marker, and anti-ARF (21 kDa, Funakoshi Co.) for Golgi apparatus marker.

2.6 MS Analysis

1. Quadrupole-Orbitrap mass spectrometer (Q Exactive plus, Thermo Scientific) and hybrid Fourier Transform Mass Spectrometer (LTQ Orbitrap XL, Thermo Scientific).
2. Liquid chromatography system: EASY-nLC 1000 and DiNa-A (KYA Tech.) (Thermo Scientific).
3. Peptides preparation

   Procedure 1: 100% (w/v) trichloroacetic acid (TCA), 8 M urea (freshly prepared), 1 M NH_4HCO_3, 45 mM dithiothreitol (DTT), 100 mM iodoacetamide (freshly prepared) endoproteinase Lys-C, trypsin, C18 solid phase extraction columns.
   Procedure 2: 8 M urea, 1 M NH_4HCO_3, 5% (w/v) sodium deoxycholate (freshly prepared), ethyl acetate, endoproteinase Lys-C, trypsin, MonoSpin C18 (GL Sciences).
4. Solvent A: 2% (v/v) acetonitrile (ACN) containing 0.1% formic acid (FA) prepared with ultrapure water in a glass bottle.
5. Solvent B: 80% (v/v) ACN containing 0.1% FA prepared with ultrapure water in a glass bottle.

3 Methods

It is important to keep all buffers on ice before using. Carry out all the subsequent procedures at 4 °C unless otherwise specified. All the centrifugation is performed using a swing-bucket rotor and carefully remove the supernatant and fraction using Pasteur pipet.

3.1 Tissue Disruption

1. Wash 60–80 g of rice seedling (*see* **Note 5**) with pure water and immediately put them on ice.
2. Add 50 mL of Golgi isolation buffer to a stainless steel bath placed on ice. Cut the rice seedling without seed and roots into small pieces (about 3 mm) using dissecting scissors (*see* **Note 6**).
3. The small pieces are further fractured by a mill mixer three times (10 s each) (*see* **Note 7**).

3.2 Filtration

1. Filter the homogenate through two layers of Miracloth (Calbiochem) and collect the filtrate in a flask placed on ice.
2. Filter the first filtrate again through four layers of Miracloth and collect the filtrate (*see* **Note 8**).
3. Centrifuge the filtrate for 20 min, at 2,000 × *g* at 4 °C and collect the supernatant (Sup1) (*see* **Note 9**).
4. Centrifuge the supernatant (Sup1) for 30 min, at 15,000 × *g* at 4 °C and collect the supernatant (Sup2) (*see* Fig. 1 and **Note 10**).

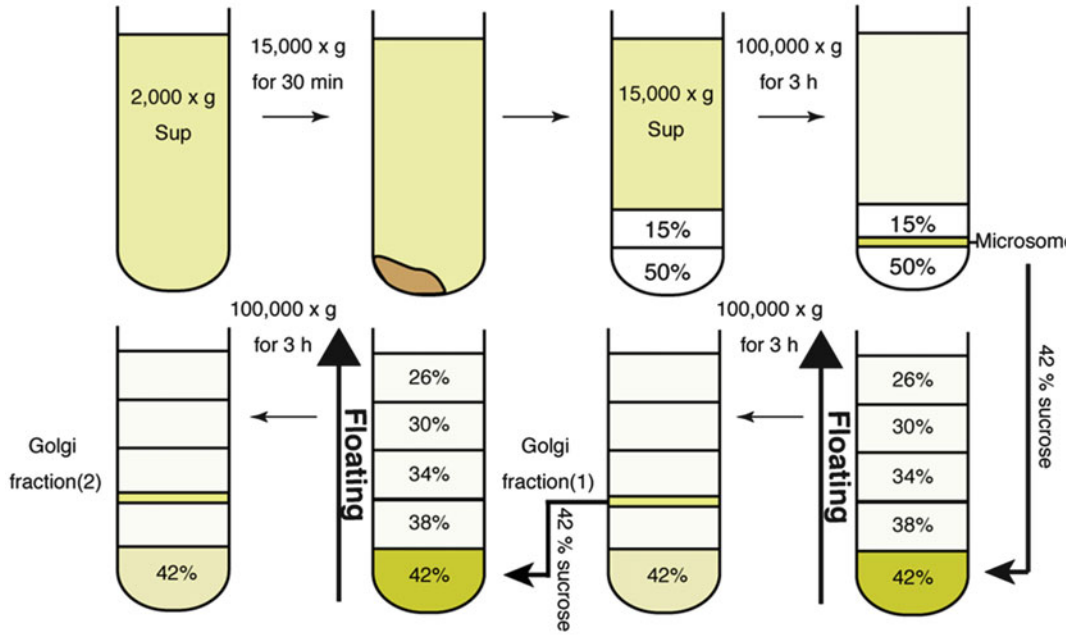

Fig. 1 Flowchart of the procedure for isolating Golgi apparatus by floating with centrifugation through a Discontinuous Sucrose Density Gradient

3.3 Sucrose Density Gradient Ultracentrifugation and Golgi Membrane Isolation

The schematic diagram of the experimental procedure of sucrose density gradient centrifugation for isolating the Golgi membrane is shown in Fig. 1.

1. Slowly layer 1 mL of 15% solution of sucrose over the 50% cushion (1 mL). After that, add carefully 10 mL of Sup2 on the top of the 15% layer (*see* **Note 11**).
2. Centrifuge at 100,000 × *g* for 3 h at 4 °C and collect the membrane fraction trapped on the 50% sucrose cushion (*see* **Note 12**).
3. Adjust the collected fraction to 42% sucrose density with 60% sucrose buffer using a refractometer, and then load on top of another discontinuous sucrose density gradient consisting of 1 mL 26%, 30%, 34%, and 38% sucrose layer each.
4. Centrifuge at 100,000 × *g* for 3 h at 4 °C and collect the Golgi fraction floating as boundary phase between 34% and 38% sucrose layer.
5. Adjust the collected Golgi fraction to 42% sucrose density again, and then apply to the second discontinuous sucrose gradient centrifugation/floating.
6. The Golgi fraction floating as boundary phase between 34% and 38% sucrose layer is recovered (Fig. 2) and subjected to assays and blotting analyses (*see* **Note 13**).

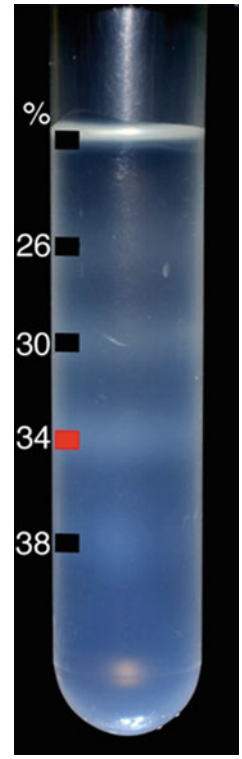

Fig. 2 Subfractionation of rice Golgi apparatus. The *white color band* between 34% and 38% layers (indicated by *red line*) represents the Golgi membrane-rich fraction

3.4 Protein Extraction

1. Dilute the Golgi membranes with an equal volume of 25 mM HEPES-KOH (pH 7.0) (*see* **Note 14**).
2. Centrifuge at 100,000 × *g* for 30 min, and suspend the resulted pellets with 25 mM HEPES-KOH (pH 7.0), and then sonicate for 10 min in ice bath.

3.5 SDS-PAGE and Immunoblot

It is important to examine the quality of the isolated Golgi membranes. SDS-PAGE and immunoblotting were carried out according to Laemmli [15] and Towbin et al. [16], respectively.

1. Measure the protein concentration of the isolated Golgi membranes by Bradford assay at 595 nm with a spectrophotometer.
2. Apply 5 μg of the Golgi membrane proteins to each lane of SDS-PAGE with 10% separation gel and then carry out the electrophoresis at 250 V and 50 mA for 60 min.
3. Transfer the gel to PVDF membranes at 25 V and 180 mA for 65 min using a semi-dry electroblotter.
4. Perform immunoblot analysis using antibodies against organelle marker proteins. The PVDF membranes are incubated in

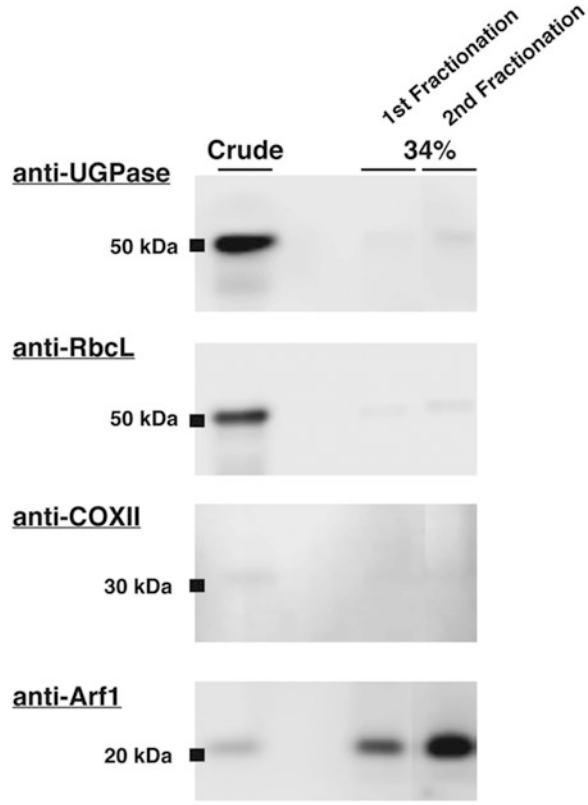

Fig. 3 Immunoblot analysis of subcellular protein markers from rice seedlings from crude mixed organelle and first and second fractionation from Golgi membranes. Antibodies against organelle marker proteins UGPase (cytosol), RbcL (plastid), COXII (mitochondrion), and ARF (Golgi apparatus) are applied to the samples to determine their enrichment of subcellular compartments. The lack of organelle protein contaminants in the second fractionation is clearly seen

blocking buffer consisting of 1× TBST and 2% (w/v) skim milk, and then reacted with specific antibodies against organelle marker proteins: anti-UGPase (rabbit serum; 1:2,000), anti-Rubisco large subunit (rabbit serum; 1:4,000), anti-COXll (rabbit serum; 1:1,000), and anti-ARF (rabbit serum; 1:1,000).

5. Horseradish peroxidase-conjugated Goat anti-rabbit IgG (goat serum; 1:10,000) were used as secondary antibodies. The immunoreactive bands were visualized using a chemiluminescence reagent and analyzed by scanner and quantification software (Fig. 3).

3.6 MS Analysis

Procedure 1 (Quadrupole-Orbitrap mass spectrometer):

1. The Golgi membrane proteins (20 μg) were mixed with 1/10 volume of 100% TCA, incubated on ice for 15 min, and centrifuged at 10,000 × *g* for 15 min at 4 °C.

2. The resulting protein precipitates were washed three times in ice-cold acetone, partially dried, and dissolved in 30 μL of 8 M urea.
3. After centrifuged at 10,000 × *g* for 10 min, 20 μL of the supernatant collected was mixed with 1.25 μL of 1 M NH_4HCO_3 and 3.75 μL of 8 M urea.
4. The proteins were reduced with 0.1 mL of 45 mM DTT at 37 °C for 60 min and then alkalized with 0.1 mL of 100 mM iodoacetamide at 25 °C for 15 min in darkness.
5. The protein samples were digested with 0.5 μg of endoproteinase Lys-C for 4 h at 37 °C.
6. The partially digested protein samples were diluted with 10 volumes of 50 mM of NH_4HCO_3 and 0.5 μg of trypsin overnight at 37 °C were used to further complete the digestion.
7. C18 solid phase extraction columns were washed with 300 μL of 100% ACN and 50% ACN, and then equilibrated with 300 μL of 0.2% TFA. The digested samples were applied to the column and washed with 300 μL of 0.2% TFA. Trapped peptides were eluted by 500 μL of 95% ACN and 5% FA twice. Eluted samples were dried in vacuum and dissolved in 0.1% FA.
8. Liquid chromatography-tandem MS for protein identification was performed by EASY-nLC 1000 interfered to Q Exactive plus. Ionization voltage was set for 2.0 kV and capillary transfer temperature was heated to 200 °C.
9. The peptides solution (500 ng) was loaded onto a trapping column packed with C18 (0.75 × 20 mm) at the flow rate of 10 μL/min in solvent A.
10. Peptides were eluted into a C18 analytical column (0.75 × 120 mm) at the flow rate of 300 μL/min. Peptides were separated using a mobile phase gradient of solvent A and B: 0–30% solvent B in 115 min, 30–90% solvent B in 10 min, and keep 90% solvent B during 5 min.
11. LC-MS/MS data was acquired in data-dependent acquisition (DDA) mode controlled by Xcalibur 2.0 software. All Q Exactive plus experiments were performed using the quadruple mass filter-equipped benchtop Orbitrap mass spectrometry with an isolation width of 4.0 *m/z*. In all experiments, full MS scans were acquired in the Orbitrap mass analyzer over m/z 350–1800 range with resolution 70,000. The 15 most intense precursor ions were selected for higher-energy collisional dissociation with a resolution of 17,500 (AGC target 5×10^4, 50 ms maximum injection time).
12. Identification of proteins was performed by MASCOT version 2.4 (Matrix Science) using the *Oryza sativa* database (62,970 entries) of UniProt (http://www.uniprot.org). The identification of peptide was performed with the following parameters:

enzyme, trypsin and Lys-C; missed cleavages, 2; MS tolerance, 10 ppm; MS/MS tolerance, 0.02 Da; static modification, carbamidomethylation; dynamic modification, oxidation (H, M, W). False discovery rates for peptide identification were less than 1.0%.

13. A normalized label-free quantitative method termed the normalized spectral index (SI_N) analysis was carried out as described by Griffin et al. [17]. As shown in Fig. 4, UDP-glucose

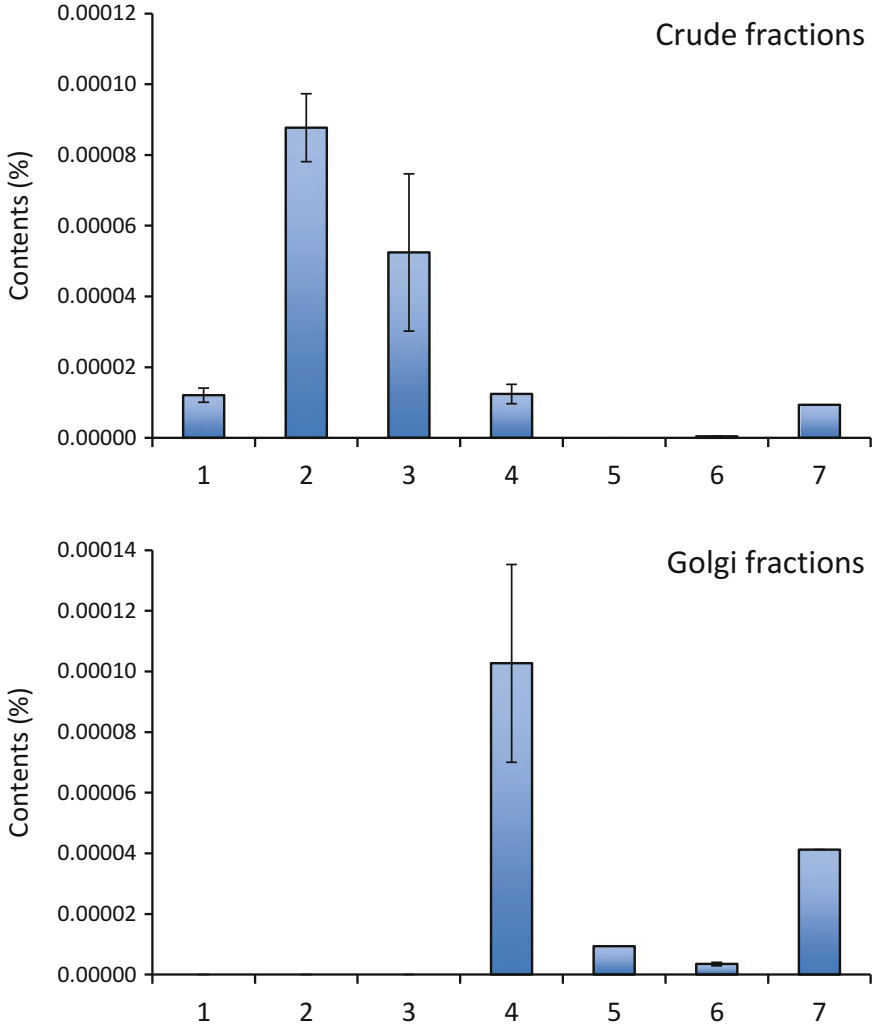

Fig. 4 Label-free normalized quantification analyses of proteins localized in the crude and Golgi membrane fractions. Trypsin/Lys-C digested proteins of the crude extract and isolated Golgi membranes were subjected to mass spectrometry and normalized spectral index (SI_N) analyses. *1*, UDPase (cytoplasm); *2*, RbcL (chloroplast); *3*, ATP synthase subunit alpha (mitochondrion); *4*, Dnak-type molecular chaperone Bip (ER, cis-Golgi); *5*, EMP70 containing protein (Golgi); *6*, Lectin (cell surface glycoprotein); *7*, Arabinogalactan protein-like (cell surface glycoprotein). Content of each protein (%) was estimated by SI_N. Values are represented as mean $\pm$ s.d. ($n = 3$)

pyrophosphorylase (cytoplasm), Ribulose bisphosphate carboxylase large chain (chloroplast), ATP synthase subunit alpha (mitochondria), Dnak-type molecular chaperone Bip (ER, cis-Golgi), lectin and arabinogalactan protein (cell surface glycoproteins) were detected in the crude fractions. Dnak-type molecular chaperone Bip, lectin, and arabinogalactan proteins were significantly concentrated in the Golgi fractions; however, UDP-glucose pyrophosphorylase, Ribulose bisphosphate carboxylase large chain, and ATP synthase subunit alpha were below the detection limit. EMP70 was also detected in the Golgi fractions.

Procedure 2 (hybrid Fourier Transform Mass Spectrometer):

14. The Golgi membrane proteins (20 μg) were solubilized with 50 μL of 8 M urea and 5% sodium deoxycholate (*see* **Note 15**), and centrifuged at 20,000 × *g* for 10 min at 4 °C.
15. The protein samples were diluted with 10 volumes of 50 mM NH_4HCO_3, and digested with 0.5 μg of endoproteinase Lys-C and 0.5 μg of trypsin overnight at 37 °C (*see* **Note 16**).
16. After digestion, the sample solutions were adjusted to 5% FA and mixed with 500 mL ethyl acetate, then spun down to separate ethyl acetate and aqueous phases. The upper aqueous phase was transferred to a new tube (*see* **Note 17**).
17. Stage tip was washed with 20 μL of 80% ACN containing 5% FA, then equilibrated with 20 μL of 5% FA. The digested samples were applied to the Stage tip and washed with 20 μL of 5% FA. Trapped peptides were eluted by 20 μL of 80% ACN containing 5% FA. Eluted samples were dried in vacuum and dissolved in 2% ACN containing 0.1% FA.
18. Liquid chromatography-tandem MS for protein identification was performed on DiNa-A interfered to LTQ Orbitrap XL. Ionization voltage was set to 1.7–2.5 kV and capillary transfer temperature was heated to 200 °C.
19. The peptides solution was loaded onto a trapping column packed with C18 (0.75 × 20 mm) at the flow rate of 10 μL/min in solvent A.
20. Peptides were eluted into a C18 analytical column (0.75 × 2000 mm) at the flow rate of 300 μL/min. Peptides were separated using a mobile phase gradient of solvent A and B: 0–30% solvent B in 300 min, 30–100% solvent B in 10 min, and keep 90% solvent B during 10 min.
21. LC-MS/MS data was acquired in data-dependent acquisition (DDA) mode controlled by Xcalibur 2.0 software. Typical DDA cycle consisted of a survey scan within *m/z* 300–1600 performed at the Orbitrap analyzer under the target mass resolution of 60,000, followed by MS/MS fragmentation of

the three most abundant precursor ions under the normalized collision energy of 35% in the linear ion trap.

22. Identification of proteins was performed in Proteome discoverer 1.4 software with sequest search engine using the *Oryza sativa* database (62,970 entries) of UniProt (http://www.uniprot.org). Peptides were identified with the following parameters: enzyme, trypsin and Lys-C; max missed cleavages, 2; MS tolerance, 5 ppm; MS/MS tolerance, 0.5 Da; dynamic modification, oxidation (H, M, W). False discovery rates for peptide identification were less than 1.0%.
23. The gene ontology (GO) annotation of the group of proteins identified in rice Golgi membrane was analyzed in PANTHER classification system [18]. As shown in Table 1, the enhancement analysis revealed that the Golgi-related proteins were markedly concentrated in the Golgi preparation.

4 Notes

1. Rice seeds (*Oryza sativa* L. cv. Nipponbare) were supplied by the Shiga Agricultural Research Institute (Shiga, Japan). It is important to use etiolated rice seedlings germinated and grown in darkness to prevent greening (i.e., transition from etioplasts to chloroplasts). It is recommended to not use green seedlings due to its hard portions of stems and leaves. Besides, the use of this material can facilitate the contamination of chloroplast proteins from the whole and broken thylakoids.
2. It is important to use swing-out rotor and centrifuge tube during ultracentrifugation.
3. The solutions that contain HEPES must be kept in dark to prevent the formation of hydrogen peroxide. After the preparation of 25 mM HEPES in pure water and mixing well with a stir bar, the KOH (5 mM) can be used to narrow the gap from the starting pH to the required pH (7.0) using the pH meter.
4. All sucrose contents used in discontinuous sucrose density gradient (38%, 34%, 30%, and 26%) are prepared from 50% sucrose using 25 mM HEPES-KOH (pH 7.0). Sucrose gradient buffers are measured and adjusted with a refractometer. The gradient is prepared by layering progressively and carefully less dense sucrose solutions upon one another; therefore, the first (top) solution applied is the 26% sucrose solution.
5. Rice seedlings need to be washed more than five times with pure water to remove any possible contaminants (i.e., bacteria or chemicals). The rice seedlings are placed then on the top of an ice-chilled petri dish.

Table 1
Enrichment analysis of proteins identified in the isolated rice Golgi membranes using GO database

	Oryza sativa (REF)	Isolated Golgi membranes		
GO cellular component complete	**#**	**#**	**Expected**	**Fold Enrichment**
ER membrane protein complex	3	3	0.1	44.5
Endoplasmic reticulum part	318	59	7.2	8.3
Cytoplasmic part	15,599	661	350.9	1.9
Intracellular part	20,438	814	459.7	1.8
Intracellular	20,724	841	466.2	1.8
Cell part	21,870	877	492.0	1.8
Cell	21,918	880	493.0	1.8
Cytoplasm	17,046	762	383.4	2.0
Endoplasmic reticulum	502	90	11.3	8.0
Endomembrane system	1152	181	25.9	7.0
Macromolecular complex	2884	229	64.9	3.5
Oligosaccharyltransferase complex	6	5	0.1	37.1
GPI-anchor transamidase complex	5	3	0.1	26.7
Golgi cisterna membrane	7	4	0.2	25.4
Golgi cisterna	12	5	0.3	18.5
Golgi stack	18	7	0.4	17.3
Golgi subcompartment	82	21	1.8	11.4
Organelle subcompartment	217	34	4.9	7.0
COPI vesicle coat	22	10	0.5	20.2
COPI-coated vesicle membrane	26	11	0.6	18.8
Golgi-associated vesicle membrane	37	15	0.8	18.0
Golgi-associated vesicle	37	15	0.8	18.0
Vesicle	4396	203	98.9	2.1
Endoplasmic reticulum lumen	9	4	0.2	19.8
Retromer complex	9	4	0.2	19.8
Lysosomal membrane	8	3	0.2	16.7
Lysosome	76	7	1.7	4.1
Lytic vacuole	79	7	1.8	3.9
Vacuole	357	42	8.0	5.2

(continued)

Table 1
(continued)

	Oryza sativa (REF)	Isolated Golgi membranes		
GO cellular component complete	**#**	**#**	**Expected**	**Fold Enrichment**
Clathrin vesicle coat	5	2	0.1	17.8
Trans-Golgi network	64	14	1.4	9.7
Plasma membrane protein complex	43	6	1.0	6.2
Plasma membrane part	594	45	13.4	3.4
Plasma membrane	1633	88	36.7	2.4
Cell periphery	2138	99	48.1	2.1
SNARE complex	64	8	1.4	5.6
Plasmodesma	205	12	4.6	2.6
Mitochondrion	4846	87	109.0	0.8
Plastid	4876	153	109.7	1.4
Unclassified	35,940	379	808.5	0.5

6. It is desirable to not crush the tissues strongly to avoid contamination by other organelles and thylakoid membranes. Adding more than the total volume of Golgi isolation buffer may reduce the extraction efficiency of the Golgi membranes.
7. For the same reason as in comment about **Note 6** above, the mixing should not be used more than three times (10 s each).
8. Filter the homogenate through two layers followed by four layers of Miracloth into a glass beaker on ice to eliminate substantial debris. The homogenate may be divided and two times filtered when large volumes are being processed.
9. The aim of this step is to remove nuclei, plastids, and cell fragments.
10. This measure is to rid mitochondria.
11. Add the supernatant to the top of the 15% sucrose layer using Pasteur pipette. Dispense the supernatant very slowly and carefully without disturbing the interface between the sucrose and the supernatant layer.
12. Golgi membranes are layered on the top of 50% sucrose cushion as pale yellow color.
13. As the low amounts of Golgi membrane floated in the boundary phase are difficult to find out very clearly, it is extremely important to recover carefully the white color boundary phase at the bottom of 34% layer.

14. The isolated Golgi membranes should be diluted attentively to prevent any membrane disruption.
15. Sodium deoxycholate was used for the solubilization of membrane proteins and for the activation of proteases [19].
16. The reductive alkylation with iodoacetamide was not carried out to avoid the inhibitory effect of excess iodoacetamide on proteases in the second procedure [20].
17. Ethyl acetate extraction was used to remove detergents from protease digests without loss of peptides [21].

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References

1. Hawes C, Satiat-Jeunemaitre B (1996) Stacks of questions: how does the plant Golgi work? Trends Plant Sci 1:395–401
2. Asatsuma S, Sawada C, Itoh K et al (2005) Involvement of α-amylase I-1 in starch degradation in rice chloroplasts. Plant Cell Physiol 46:858–869
3. Villarejo A, Buren S, Larsson S et al (2005) Evidence for a protein transported through the secretory pathway en route to the higher plant chloroplast. Nat Cell Biol 7:1224–1231
4. Nanjo Y, Oka H, Ikarashi N et al (2006) Rice plastidial *N*-glycosylated nucleotide pyrophosphatase/phosphodiesterase is transported from the ER-Golgi to the chloroplast through the secretory pathway. Plant Cell 18:2582–2592
5. Kitajima A, Asatsuma S, Okada H et al (2009) The rice α-amylase glycoprotein is targeted from the Golgi apparatus through the secretory pathway to the plastids. Plant Cell 21:2844–2858
6. Burén S, Ortega-Villasante C, Blanco-Rivero A et al (2011) Importance of post-translational modifications for functionality of a chloroplast-localized carbonic anhydrase (CAH1) in *Arabidopsis thaliana*. PLoS One 6:1–15
7. Shiraya T, Mori T, Mruyama T et al (2015) Golgi/plastid-type manganese superoxide dismutase involved in heat-stress tolerance during grain filling of rice. Plant Biotechnol J 13:1251–1263
8. Taylor RS, Wu CC, Hays LG et al (2000) Proteomics of rat liver Golgi complex: minor proteins are identified through sequential fractionation. Electrophoresis 21:3441–3459
9. Dunkley TP, Hester S, Shadforth IP et al (2006) Mapping the *Arabidopsis* organelle proteome. Proc Natl Acad Sci U S A 103:6518–6523
10. Mikami S, Hori H, Mitsui T (2001) Separation of distinct compartments of rice Golgi complex by sucrose density gradient centrifugation. Plant Sci 161:665–675
11. Asakura T, Hirose S, Katamine H et al (2006) Isolation and proteomic analysis of rice Golgi membranes: cis-Golgi membranes labeled with GFP-SYP31. Plant Biotechnol 23:475–485
12. Kimura S, Mitsui T, Matsuoka T, Igaue I (1992) Purification, characterization and localization of rice UDP-glucose pyrophosphorylase. Plant Physiol Biochem 30:683–693
13. Kaneko K, Inomata T, Masui T et al (2014) Nucleotide pyrophosphatase/phosphodiesterase 1 exerts a negative effect on starch accumulation and growth in rice seedlings under high temperature and CO_2 concentration conditions. Plant Cell Physiol 55:320–332
14. Nishimura M, Akazawa T (1974) Studies on spinach ribulosebisphosphate carboxylase. Carboxylase and oxygenase reaction examined by immunochemical methods. Biochemistry 13:2277–2281

15. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
16. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76:4350–4354
17. Griffin NM, Yu J, Long F et al (2010) Label-free, normalized quantification of complex mass spectrometry data for proteomic analysis. Nat Biotechnol 28:83–49
18. Mi H, Muruganujan A, Casagrande JT, Thomas PD (2013) Large-scale gene function analysis with the PANTHER classification system. Nat Protoc 8:1551–1566
19. Masuda T, Tomita M, Ishihama Y (2008) Phase transfer surfactant-aided trypsin digestion for membrane proteome analysis. J Proteome Res 7:731–740
20. Crestfield AM, Moore S, Stein WH (1963) The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated proteins. J Biol Chem 238:622–627
21. Yeung Y-G, Stanley ER (2010) Rapid detergent removal from peptide samples with ethyl acetate for mass spectrometry analysis. Curr Protoc Protein Sci, Chapter 16:Unit 16.12

Chapter 7

Analyzing the Vacuolar Membrane (Tonoplast) Proteome

Miwa Ohnishi, Katsuhisa Yoshida, and Tetsuro Mimura

Abstract

A large number of proteins in the vacuolar membrane (VM; tonoplast), including transporters and receptors, support the various functions of the vacuole. Molecular analysis of membrane proteins is an essential step in understanding how the vacuole operates but so far only a small number of tonoplast proteins have been identified at the molecular level. Accordingly, mutant lines with altered level of tonoplast proteins for characterizing their physiological roles have been developed sparsely. Also, detecting activities of tonoplast proteins remains difficult as it requires a certain degree of enrichment of this organelle fraction. In order to extend our understanding of the vacuole, several groups have turned to proteomic analysis of tonoplast membrane proteins. A primary requirement of any organelle analysis by proteomics is that the purity of the isolated organelle needs to be high so that its composition can be accurately analyzed with mass spectrometry. In this chapter, we describe a simple method for the isolation of intact vacuoles and subsequent proteome analysis of the VM fraction of cells from Arabidopsis suspension cultures.

Key words *Arabidopsis*, DRM (detergent-resistant membrane), DSM (detergent-soluble membrane), Microdomain, Plant cell, Proteome, Proteomics, Tonoplast, Vacuole, Vacuolar membrane

1 Introduction

The plant vacuole is the largest organelle that is enveloped with a single lipid bilayer, the vacuolar membrane (VM; tonoplast). Vacuoles play important roles in plant cell function, such as space filling, storage of inorganic ions and metabolites, protein degradation, detoxification and control of ionic homeostasis of the cytoplasm. There are many kinds of transmembrane proteins and peripheral proteins involved in vacuolar activity. Proteomic analyses of the vacuolar membrane (VM) [1–5] have revealed that an excess of 100 proteins are likely to be involved in VM activities. Arabidopsis is usually chosen as the experimental material because its genome has been fully sequenced and it is therefore easier to relate molecular information to protein function. However, the functions of many of VM proteins are still unknown [6]. Furthermore, genomes of many other plants have been reported and availability of

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RNA-seq data makes the analysis of proteome data much easier than previously.

One of the methods used to isolate different membrane fractions for proteomic analysis combines sucrose density gradient centrifugation and liquid chromatographic separation. This method is suitable for large amounts of sample and can generate larger amounts of protein for subsequent analysis than other methods. The drawback is that there is inevitably significant contamination from other membranes. An alternative approach [1, 2, 7] is to first isolate intact vacuoles from protoplasts and then purify membrane proteins by a combination of rupturing the vacuoles and ultracentrifugation, resulting in a much purer vacuolar membrane fraction. The disadvantage of this method is that it is not easy to collect large amounts of protein. Recent improvements in protein analysis by mass spectrometry have increased sensitivity so that less protein is now needed than previously.

The plasma membrane (PM) is composed of liquid-ordered domains enriched in cholesterol/sphingolipids, which co-exist with liquid crystalline domains rich in phospholipids containing unsaturated fatty acids. These membrane domains were termed lipid rafts by Simons and Ikonen [8] and can be isolated as detergent-resistant membranes (DRMs).

Recently, it has been reported that microdomains also exist not only in the plant PM [9–16], but also in the VM [17, 18]. Ozolina et al. [17] described the lipid composition of the DRMs of vacuoles isolated from *Beta vulgaris*. Yoshida et al. [18] showed that the distribution of the vacuolar proton ATPase (v-ATPase) differs from that of the vacuolar proton pyrophosphatase (v-PPase), and that the v-ATPase is mainly located in the detergent-resistant membrane (DRM) fraction. Proteome analysis of DRM and DSM fractions of the VM suggests that proteins in the VM form a more complex functional network than supposed.

In the present article, we describe the methods to isolate protoplasts, to isolate and purify intact vacuoles, and to detect proteins composing the vacuolar membranes using *Arabidopsis* suspension-cultured cells.

2 Materials

2.1 Preparation of Protoplasts

Washing solution: 2 mM $CaSO_4$, 100 mM sorbitol.

Protoplast solution: 10 mM MES (2-(N-Morpholino)ethanesulfonic acid), 1 mM $CaCl_2$, 0.5 M sorbitol, adjusted to pH 6 with Tris (Tris(hydroxymethyl)aminomethane).

Solution A: 1% Cellulase Y-C (1.6 g), 0.1% Pectolyase Y-23 (160 mg), Protoplast solution (160 ml).

2.2 Isolation of Intact Vacuoles from Protoplasts

V solution: 30 mM HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid), 2 mM EGTA (bis(2-aminoethyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid), 30 mM potassium gluconate, 2 mM $MgCl_2$, adjusted to pH 7.2 with Tris, e.g., V (0.4 M sorbitol, 50% Percoll) means V solution containing 0.4 M sorbitol and 50% Percoll, and so forth.

GB instead of sorbitol means Betaine monohydrate.

2.3 Extraction of Membrane Proteins

Lysis buffer: V solution including 1 mM PMSF (phenylmethylsulfonyl fluoride), 50 μg/ml leupeptin.

TNE-buffer: 25 mM Tris, 150 mM NaCl, 5 mM EDTA, adjusted to pH 7.5 with HCl.

Detergent solution: 10% (v/v) Triton X-100 in TNE Buffer.

60% (w/w) Sucrose in TNE-buffer.
35% (w/w) Sucrose in TNE-buffer.
30% (w/w) Sucrose in TNE-buffer.
5% (w/w) Sucrose in TNE-buffer.

2.4 Peptide Preparation for Proteomic Analysis

Sample buffer: 3.4% SDS, 30% glycerol, 2% 2-mercaptoethanol, 0.25 M Tris, adjusted to pH 6.8 with HCl, and 0.012% bromophenol blue.

3 Methods

Carry out all the procedures on ice unless otherwise specified.

3.1 Preparation of Protoplasts

1. Using approximately 12 g cells (fresh weight from 80 ml × 2 suspension-cultured cells in case of *Arabidopsis* suspension cultured cells (Deep line)), remove the culture medium, and wash the cells with Washing solution by aspiration.
2. Incubate the cells in Solution A (80 ml solution per 6 g) for 3 h at 31 °C with shaking at 110 rpm (*see* **Notes 1** and **2**).

3.2 Isolation of Vacuoles and Vacuolar Membrane

1. Transfer all the solution from Subheading 3.1, step 2 to 50 ml plastic tubes (Corning).
2. Underlay with 2 ml V (0.4 M sorbitol, 50% Percoll) by a Pasteur pipette (Fig. 1—step 1).
3. Centrifuge at 190 × *g* for 10 min.
4. Remove the supernatant by careful aspiration.
5. Add V (0.4 M sorbitol, 50% Percoll) up to 15 ml and mix gently by rotating several times.
6. Form a gradient by overlaying with 10 ml V (0.4 M sorbitol, 7.5% Percoll) and 2 ml V (0.4 M sorbitol) (Figs. 1—step 2 and 2a).

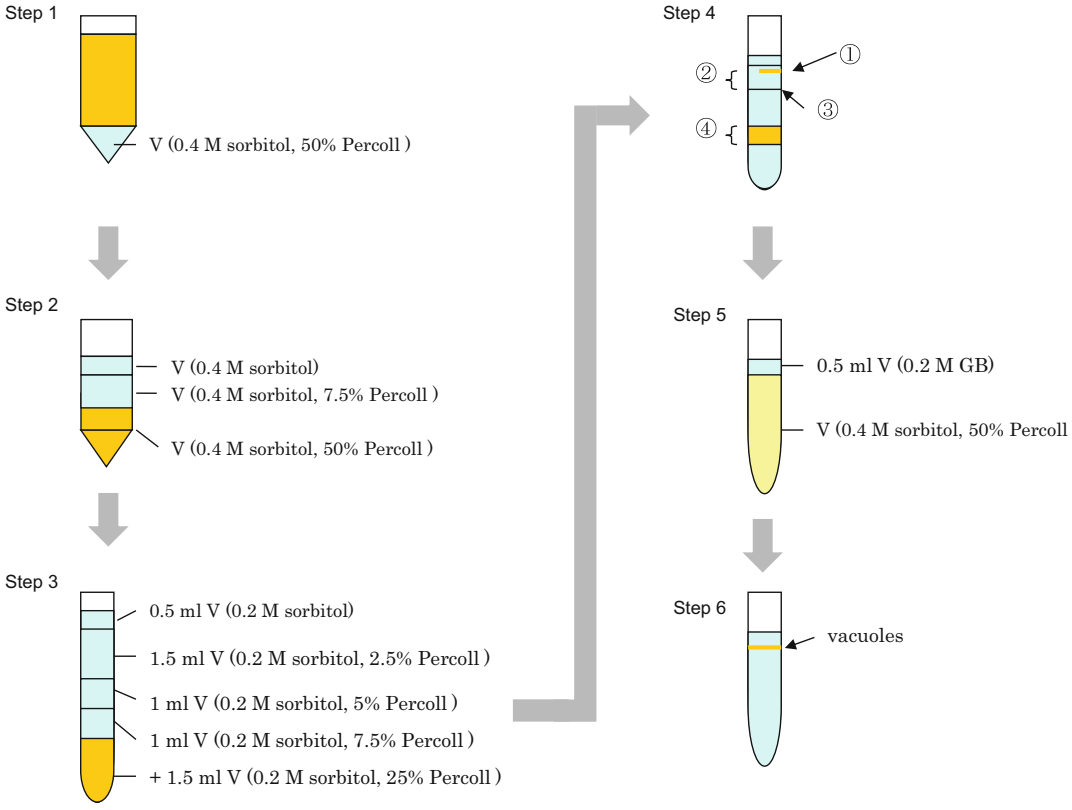

Fig. 1 Schematic steps of isolation of intact vacuoles from protoplasts

7. Centrifuge at 190 × *g* for 2 min, and then 1680 × *g* for 8 min.
8. Remove the solution above the purified protoplasts (Fig. 2b and c, *see* **Note 3**).
9. Transfer the protoplasts to a new tube.
10. Add an equal volume of V solution and gently invert several times to mix.
11. Incubate on ice for 5 min.
12. Divide the solution into eight glass tubes.
13. Add 1.5 ml V (0.2 M sorbitol, 25% Percoll) and mix.
14. Form a gradient by overlaying on the top of the previous one (Figs. 1—step 3 and 2d, e).

    1 ml V (0.2 M sorbitol, 7.5% Percoll),

    1 ml V (0.2 M sorbitol, 5% Percoll),

    1.5 ml V (0.2 M sorbitol, 2.5% Percoll), and

    0.5 ml V (0.2 M sorbitol).
15. Centrifuge at 190 × *g* for 2 min and then at 1680 × *g* for 8 min.

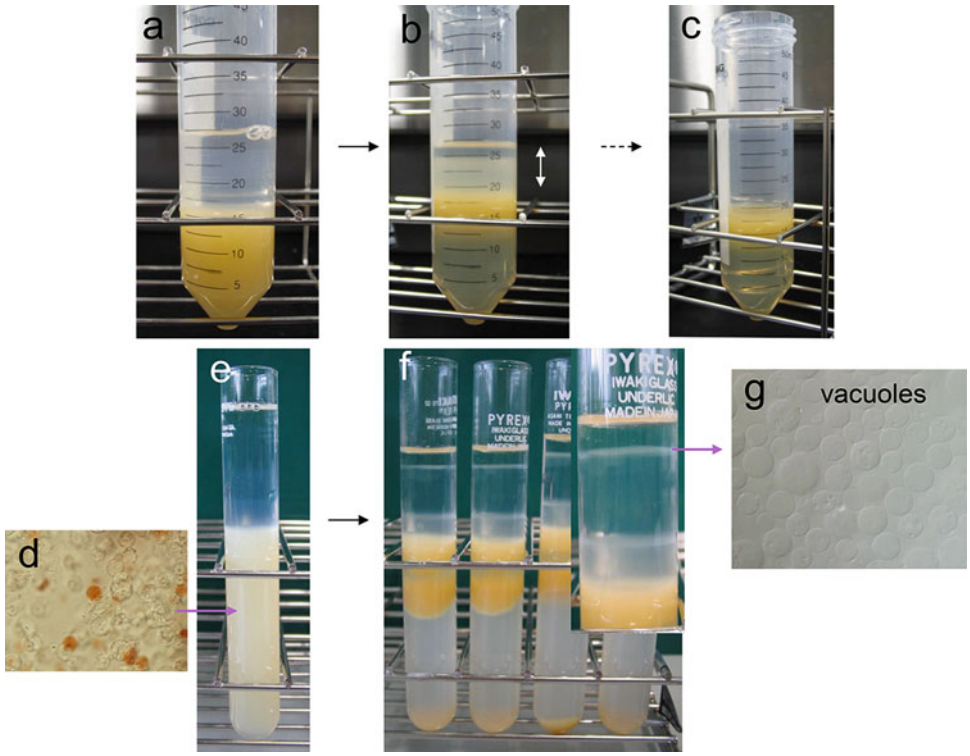

Fig. 2 Photographs of isolation steps of intact vacuoles according to Fig. 1. (**a**) *Step 2*, (**b**, **c**) after centrifugation and removing lighter protoplasts. (**d**) Protoplasts and vacuoles. The vacuoles were stained with neutral *red*. (**e**, **f**) Before (*step 3*) and after (*step 4*) centrifugation to purify vacuoles. (**g**) Isolated intact vacuoles

16. Vacuoles are concentrated in the interphase (Fig. 1—step 4-1) between V (0.2 M sorbitol) and V (0.2 M sorbitol, 2.5% Percoll) (Figs. 1—step 4 and 2f, g).
17. Collect the interphase (Fig. 1—step 4-1), and then confirm the purity of vacuoles in the interphase by microscopy (*see* **Note 4**).
18. Remove the second layer (Fig. 1—step 4-2) and then confirm the purity of vacuoles in the interphase by microscopy.
19. Collect the second interphase (Fig. 1—step 4-3), and then confirm the purity of vacuoles in this interphase by microscopy.
20. If the purity of vacuole is high enough in the second interphase (Fig. 1—step 4-3), collect the interphase. If not, discard this fraction.
21. Remove the solution over the bottom layer (Fig. 1—step 4-4) including remaining intact protoplasts by aspiration.
22. Suck and push the protoplast fraction (Fig. 1—step 4-4) through a syringe equipped with a 19-G needle (1.10 mm × 90 mm) gently.

23. Divide the protoplast fraction into two to four glass tubes.
24. Add 1.5 ml V (0.2 M sorbitol, 25% Percoll) and mix.
25. As described above (**step 14**), form a gradient again.
26. Centrifuge at 190 × *g* for 2 min and then at 1680 × *g* for 8 min.
27. Collect vacuoles as described above (**steps 16** and **17**) and combine the isolated vacuole fractions.
    ↓
    If the density of vacuoles is not high enough, then proceed as follows:
28. To concentrate vacuoles, add V (0.4 M sorbitol, 50% Percoll) to the solution of vacuoles, and overlay with 0.5 ml V (0.2 M GB) (Fig. 1—step 5).
29. Centrifuge at 190 × *g* for 2 min and then at 1680 × *g* for 8 min.
30. Collect concentrated vacuoles (Fig. 1—step 6).

3.3 Extraction of Membrane Proteins

While peripheral proteins may have important functions for vacuolar biological activities, membrane-spanning proteins are likely to have more critical roles in vacuole functions. It is therefore desirable to separate peripheral proteins from membrane intrinsic proteins.

1. Lyse isolated vacuoles by diluting with four times volumes of lysis buffer (v/v).
2. Immediately after diluting, centrifuge lysed vacuoles at 120,000 × *g* for 60 min with an ultracentrifuge.
3. Collect VM as a pellet.
4. Suspend the pellet with 1 ml TNE-buffer using a pipette.
5. Centrifuge again at 120,000 × *g* for 60 min.
6. Recollect VM as a pellet.
7. Suspend the washed VM in 200 μl TNE-buffer.
8. Determine the quantity of protein with Bradford method [19].

3.3.1 Separation of DSM and DRM Fractions of the VM (Tonoplast)

1. Treat this suspended membrane fraction with detergent solution (solution: protein = 5:1) for 30 min at 4 °C (ex: 32.5 μl 10% Triton X-100 (3.25 mg Triton X-100) + 235 μl tonoplast (650 μg protein) + 57.5 μl H_2O, Final concentration of Triton X-100 become 1%).
2. Place the solubilized VMs at the bottom of a centrifuge tube.
3. Mix with 60% sucrose (w/w, dissolved in TNE-buffer) to reach a final concentration of 45–50% (*see* **Note 5**).
4. Overlay discontinuous sucrose gradient (1 ml 35%, 1 ml 30%, 0.5 ml 5% sucrose (w/w), dissolved in TNE-buffer).

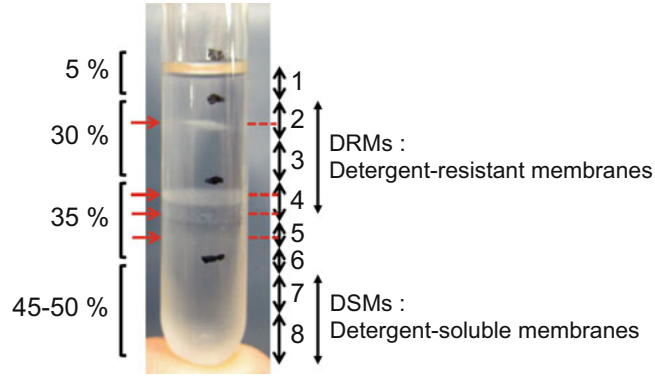

Fig. 3 Density gradient after sucrose gradient centrifugation. *Black marks* show layers of 5%, 30%, 35%, and 45–50% of sucrose concentration (from *top* to *bottom*). *Red arrows* indicate boundaries between layers of 5%, 30%, 35%, and 40–50% of sucrose concentration after centrifugation

5. Centrifuge at 150,000 × *g* for 16 h with an ultracentrifuge.
6. Collect these layers as DRM (namely second to fourth layers from the top in Fig. 3 indicated by black double-headed arrows).
7. Collect the bottom layers as detergent soluble membrane (DSM) (ex. seventh to eighth layers in Fig. 3).

3.4 Electrophoresis of Membrane Proteins

3.4.1 Peptide Preparation for Proteomic Analysis

1. Add equal volumes of 20% TCA to vacuolar DRM and DSM samples.
2. Keep on ice for 30 min.
3. Centrifuge at 20,400 × *g* with 10 min at 4 °C.
4. Collect proteins as pellet.
5. Wash the pellet three times with acetone.
6. Repeat the washing **steps 3–5** three times.
7. Dilute the specimens in 50 μl Sample buffer.
8. Incubate at 55 °C for 5 min.
9. Apply specimens to a precast acrylamide gel (DRC, Perfect NT gel 10% A.A., Japan).
10. Stain the separated proteins with Flamingo (BIO-RAD, Hercules, CA, USA) according to the instruction manual.
11. Slice gel lanes into 15 bands.
12. Wash each slice twice with HPLC-grade water containing 60% (v/v) acetonitrile (Kanto 17 Chemical).
13. Reduce samples with 10 mM dithiothreitol (for 1 h at 56 °C on a shaker) and then alkylate samples with 55 mM iodoacetamide (in the dark for 1 h at room temperature).

14. Wash reduced and alkylated samples twice with HPLC-grade water containing 60% (v/v) acetonitrile.
15. Digest specimens with 10 μl/band (10 μg/ml trypsin, Promega, Tokyo, Japan) in 50 mM ammonium bicarbonate) for 16 h at 37 °C in the dark.
16. Extract digested peptide fragments from the cut gels with 5% (v/v) formic acid and 50% (v/v) acetonitrile for 15 min.
17. Dry the solutions containing the digested peptides in a vacuum concentrator.
18. Dissolve the dried samples in 15 μl 0.1% (v/v) formic acid and 5% (v/v) acetonitrile.

The alternative method is to simply treat the protein sample in buffered solution with trypsin directly. Some of the proteins that cannot be separated by electrophoresis are possibly digested with trypsin and can then be analyzed by LC-MS analysis or direct MS analysis [20].

3.5 Mass Spectrometric (MS) Analysis and Database Searching for Proteome

1. Proteomic analysis is performed by LC-MS/MS using a LTQ-Orbitrap-HTC-PAL system (Thermo Electron, Bremen, Germany) or other available MS instrumentation. The range of MS scan is m/z 200–2000 and the top one to three peaks are subjected to MS/MS analysis [2].
2. Spectra are compared with a protein database from TAIR10 or above using the MASCOT search engine (Matrix Science Inc., Boston, USA; http://www.matrixscience.com). The MASCOT search parameters are dependent on the experimental purpose (*see* **Note 6**).
3. ARAMEMNON database (http://aramemnon.botanik.uni-koeln.de/) is used to predict transmembrane domains in identified proteins. The number of matched peptides can be used as a semi-quantitative reference for the protein abundance.

Proteomic analysis is a powerful tool for determining intracellular protein localization, provided that care is taken to avoid contamination of proteins from other sources. Despite the most careful efforts, we find that there are inevitably some proteins in the vacuolar fraction that are known to be related to activities in other organelles. While they may be true contaminants, it must be remembered that plant vacuoles also have a lytic function, and may therefore contain components of other organelles at various stages of degradation. In order to confirm whether candidate proteins are native to the vacuolar membrane, it is necessary to verify their true location with other methods. The easiest way is to use GFP-fusion proteins, as demonstrated by Endler et al. [7], although there is some suggestion that overexpression and artificial

structure of GFP proteins results in expression in other locations [21]. If it is possible to raise an antibody to a target protein, immunolocalization studies may be also useful.

4 Notes

1. The time needed for the preparation of protoplasts is dependent on the plant material and/or culture conditions. It is important to regularly check that protoplasts have been released (i.e., cell wall has been digested) under a microscope. For microscopic observation of protoplasts and vacuoles, we usually use a slide glass on which two stripes of tape (like a vinyl scotch tape) are parallel pasted. Tapes can protect protoplasts and vacuoles from rupturing on the slide glass by the weight of cover glass.
2. To move protoplasts for checking or to new solutions, plastic pipette tips should be cut to increase the opening in order to avoid rupturing the protoplasts by physical shock.
3. To avoid contamination with lighter protoplasts, which have a similar density to vacuoles, those protoplasts are removed in advance.
4. The accumulation of vacuoles after density gradient centrifugation must be confirmed at every layer. When it is difficult to observe the presence of vacuoles, use a blight contrast of phase-difference microscope or staining with neutral red (about 10 μM) can be used to detect vacuoles (acid compartments).
5. Add 60% sucrose gradually, and then mix well without making foam.
6. In our case, the MASCOT search parameters were set as follows: threshold at 0.05 in the ion-score cutoff, peptide tolerance at 10 ppm, MS/MS tolerance at ±0.8 Da, peptide charge of 2^+ or 3^+, trypsin as enzyme allowing up to one missed cleavage, carbamidomethylation on cysteines as a fixed modification and oxidation on methionine as a variable modification.

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References

1. Carter C, Pan S, Zouhar J, Avila EL, Girke T, Raikhel NV (2004) The vegetative vacuole proteome of *Arabidopsis thaliana* reveals predicted and unexpected proteins. Plant Cell 16:3285–3303
2. Shimaoka T, Ohnishi M, Sazuka T, Mitsuhashi N, Hara-Nishimura I, Shimazaki K, Maeshima M, Yokota A, Tomizawa K, Mimura T (2004) Isolation of intact vacuole and proteomic analysis of tonoplast from suspension-cultured cells

of *Arabidopsis thaliana*. Plant Cell Physiol 45:672–683

3. Jaquinod M, Villiers F, Kieffer-Jaquinod S, Hugouvieux V, Bruley C, Garin J, Bourguignon J (2007) A proteomics dissection of *Arabidopsis thaliana* vacuoles isolated from cell culture. Mol Cell Proteomics 6:394–412
4. Schmidt UG, Endler A, Schelbert S, Brunner A, Schnell M, Neuhaus HE, Marty-Mazars D, Marty F, Baginsky S, Martinoia E (2007) Novel tonoplast transporters identified using a proteomic approach with vacuoles isolated from cauliflower buds. Plant Physiol 145:216–229
5. Whiteman SA, Serazetdinova L, Jones AM, Sanders D, Rathjen J, Peck SC, Frans JM, Maathuis D (2008) Identification of novel proteins and phosphorylation sites in a tonoplast enriched membrane fraction of *Arabidopsis thaliana*. Proteomics 8:3536–3547
6. Martinoia E, Meyer S, De Angeli A, Nagy R (2012) Vacuolar transporters in their physiological context. Annu Rev Plant Biol 63:183–213
7. Endler A, Meyer S, Schelbert S, Schneider T, Weschke W, Peters SW, Keller F, Baginsky S, Martinoia E, Schmidt UG (2006) Identification of a vacuolar sucrose transporter in barley and *Arabidopsis* mesophyll cells by a tonoplast proteomic approach. Plant Physiol 141:196–207
8. Simons K, Ikonen E (1997) Functional raft in cell membranes. Nature 387:569–572
9. Peskan T, Westermann M, Olelmüller R (2000) Identification of low-density Triton X-100-insoluble plasma membranes in higher plants. Eur J Biochem 267:6989–6995
10. Mongrand S, Morel J, Laroche J, Claverol S, Carde JP, Hartmann MA, Bonneu M, Simon-Plas F, Lessire R, Bessoule JJ (2004) Lipid rafts in higher plant cells: purification and characterization of Triton X-100-insoluble microdomains from tobacco plasma membrane. J Biol Chem 279:36277–36286
11. Bhat RA, Panstruga R (2005) Lipid rafts in plants. Planta 223:5–19
12. Borner GH, Sherrier DJ, Weimar T, Michaelson LV, Hawkins ND, Macaskill A, Napier JA, Beale MH, Lilley KS, Dupree P (2005) Analysis of detergent-resistant membrane in *Arabidopsis*. Evidence for plasma membrane lipid rafts. Plant Physiol 137:104–116
13. Laloi M, Perret AM, Chatre L, Melser S, Cantrel C, Vaultier MN, Zachowski A, Bathany K, Schmitter JM, Vallet M, Lessire R, Hartmann MA, Moreau P (2007) Insight into the role of specific lipids in the formation and delivery of lipid microdomains to the plasma membrane of plant cells. Plant Physiol 143:461–472
14. Zappel NF, Panstruga R (2008) Heterogeneity and lateral compartmentalization of plant plasma membranes. Curr Opin Plant Biol 11:632–640
15. Simon-Plas F, Perraki A, Bayer E, Gerbeau-Pissot P, Mongrand S (2011) An update on plant membrane rafts. Curr Opin Plant Biol 14:642–649
16. Cacas JL, Furt F, Le Guédard M, Schmitter JM, Buré C, Gerbeau-Pissot P, Patrick Moreaua P, Bessoulea JJ, Simon-Plasc F, Mongranda S (2012) Lipids of plant membrane rafts. Prog Lipid Res 51:272–299
17. Ozolina NV, Nesterkina IS, Kolesnikova EV, Salyaev RK, Nurminsky VN, Rakevich AL, Martynovich EF, Chernyshov MY (2013) Tonoplast of *Beta vulgaris* L. contains detergent-resistant membrane microdomains. Planta 237:859–871
18. Yoshida K, Ohnishi M, Fukao Y, Okazaki Y, Fujiwara M, Song C, Nakanishi Y, Saito K, Shimmen T, Suzaki T, Hayashi F, Fukaki H, Maeshima M, Mimura T (2013) Studies on vacuolar membrane microdomains isolated from *Arabidopsis* suspension-cultured cells: local distribution of vacuolar membrane proteins. Plant Cell Physiol 54(10):1571–1584
19. He F (2011) Bradford protein assay. Bio-protocol Bio101:e45. http://www.bio-protocol.org/e45
20. Rexroth S, Mullineaux CW, Ellinger D, Sendtko E, Rögner M, Koenig F (2011) The plasma membrane of the cyanobacterium *Gloeobacter violaceus* contains segregated bioenergetic domains. Plant Cell 23(6):2379–2390
21. Segami S, Makino S, Miyake A, Asaoka M, Maeshima M (2014) Dynamics of vacuoles and H^+-pyrophosphatase visualized by monomeric green fluorescent protein in *Arabidopsis*: artifactual bulbs and native intravacuolar spherical structures. Plant Cell 26:3416–3434

Chapter 8

Preparation of Membrane Fractions (Envelope, Thylakoids, Grana, and Stroma Lamellae) from Arabidopsis Chloroplasts for Quantitative Proteomic Investigations and Other Studies

Lucas Moyet, Daniel Salvi, Martino Tomizioli, Daphné Seigneurin-Berny, and Norbert Rolland

Abstract

Chloroplasts are semiautonomous organelles found in plants and protists. They are surrounded by a double membrane system, or envelope. These envelope membranes contain machineries to import nuclear-encoded proteins, and transporters for ions or metabolites, but are also essential for a range of plastid-specific metabolisms. The inner membrane surrounds a stroma, which is the site of the carbon chemistry of photosynthesis. Chloroplasts also contain an internal membrane system, or thylakoids, where the light phase of photosynthesis occurs. The thylakoid membranes themselves have a bipartite structure, consisting of grana stacks interconnected by stroma lamellae. These thylakoid membranes however form a continuous network that encloses a single lumenal space. Chloroplast-encoded or targeted proteins are thus addressed to various sub-compartments that turn out to be flexible systems and whose main functions can be modulated by alterations in the relative levels of their components. This article describes procedures developed to recover highly purified chloroplast membrane fractions (i.e., envelope, crude thylakoid membranes, as well as the two main thylakoid subdomains, grana and stroma lamellae), starting from Percoll-purified *Arabidopsis* chloroplasts. Immunological markers are also listed that can be used to assess the purity of these fractions and reveal specific contaminations by other plastid membrane compartments. The methods described here are compatible with chloroplast proteome dynamic studies relying on targeted quantitative proteomic investigations.

Key words Chloroplast, Chloroplast envelope, Thylakoids, Grana, Stoma lamellae, Cross-contamination, Proteome

1 Introduction

Chloroplasts are semiautonomous organelles universally considered to have originated from endosymbiotic cyanobacteria. Chloroplasts have a specific suborganellar organization (*see* Fig. 1). They are surrounded by a two-membrane system or envelope. This envelope system is composed of the inner and outer membranes and of an intermembrane space located between these two layers.

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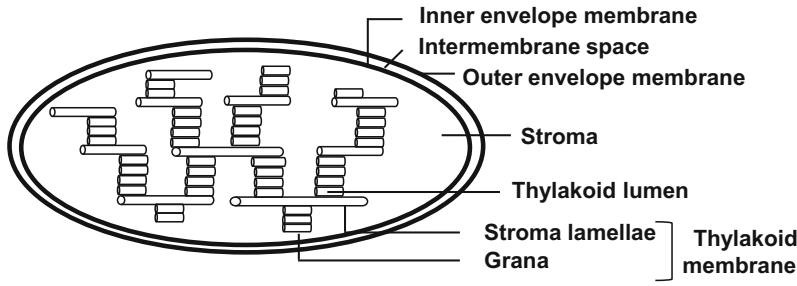

Fig. 1 Chloroplast sub-compartments

Chloroplasts only synthesize less than a hundred proteins, encoded by their own genome, and import two to three thousand nucleus-encoded preproteins synthesized in the cytosol [1, 2]. The envelope is also involved in the controlled exchange of a variety of ions and metabolites between the cytosol and the chloroplast [3, 4], and it is the site of the synthesis of plastid-specific membrane components (glycerolipids, pigments, prenylquinones) or chlorophyll breakdown [5–7].

The chloroplasts also contain a soluble phase, called the stroma, which contains enzymes required for the dark phase of photosynthesis reactions (the Calvin cycle), the synthesis of amino acids or vitamins, and the plastid transcription and translation machineries.

Within the stroma, it is also found an internal membrane system, called the thylakoids where the light phase of photosynthesis takes place. The thylakoid membranes themselves contain two main subdomains: stacked membranes vesicles also called grana, and light membrane vesicles or stroma lamellae [8]. Stacks of thylakoid membranes are enriched in Photosystem II complexes, while stroma lamellae contain most of the Photosystem I, b_6f, NDH, and ATPase complexes [8, 9].

Identifying the subcellular or subplastidial localizations of proteins (and to study their dynamics) has become a critical tool in the functional understanding of plant processes at the molecular level [10, 11]. In order to get access to the protein content of the different plastid sub-compartments, protocols exist that allow the collection of highly pure subplastidial fractions (inner or outer envelope membranes, intermembrane space, stroma, thylakoid grana or stroma lamellae, thylakoid lumen). However, targeted semi-quantitative proteomic investigations have revealed specific cross-contaminations that may occur during the purification of these various subfractions [9, 12, 13].

The purpose of this article is to provide detailed protocols to purify chloroplast membrane subfractions (i.e., envelope, crude thylakoid membranes as well as thylakoids subdomains grana and stroma lamellae) starting from Percoll-purified and intact

Arabidopsis chloroplasts, and to accurately evaluate the cross-contamination of these purified chloroplast subfractions with specific markers upstream quantitative proteomic analyses.

2 Materials

2.1 Growth of Arabidopsis Plants

1. Large (30 cm × 45 cm) plastic cases filled with compost and water.
2. *Arabidopsis thaliana* seeds. These should be sown onto the surface of the compost by scattering them carefully at a high density (around 30 mg of seeds for a whole case).
3. *Arabidopsis* rosette leaves are obtained from 3- to 4-week-old *Arabidopsis thaliana* plantlets (*see* **Note 1**). Four to six cases containing such *Arabidopsis* plantlets are expected to provide 400–500 g of rosette material and are considered enough for the preparation of envelope membranes. For stroma lamellae and grana purifications one case is sufficient.
4. Growth rooms providing a 12-h light cycle, set at 23 °C (day)/18 °C (night) with a light intensity of 150 μmol/m^2/s.

2.2 Purification of Chloroplasts from Arabidopsis Leaves

1. Muslin or cheesecloth, 80 cm large.
2. Nylon blutex (50 μm aperture) (Tripette et Renaud, Sailly Saillisel, France).
3. Beakers (500 mL, 1, and 5 L).
4. Ice and ice buckets.
5. Pipettes (1 and 10 mL).
6. Percoll (GE Healthcare, USA).
7. Motor-driven blender, three speeds, 1 gallon (3.785 L) and 0.325 gallon (1.230 L) capacity (Waring blender).
8. Superspeed refrigerated centrifuge (Sorvall RC5), with the following rotors (and corresponding tubes): fixed-angle rotors GS-3 (6 × 500 mL plastic bottles) and SS34 (8 × 50 mL polypropylene tubes); swinging-bucket rotor HB-6 (6 × 50 mL polycarbonate tubes). Equivalent alternative equipment (e.g., from Beckman) may also be used.
9. Leaf grinding medium: 20 mM Tricine-KOH, pH 8.4, 0.4 M sorbitol, 10 mM ethylenediaminetetraacetic acid (EDTA), 10 mM $NaHCO_3$, 0.1% (w/v) bovine serum albumin (BSA, defatted).
10. Chloroplast washing medium A for envelope and thylakoid purification: 20 mM Tricine-KOH, pH 7.6, 0.4 M sorbitol, 5 mM $MgCl_2$, 2.5 mM EDTA. This solution should also contain the following protease inhibitors when long-term

storage of protein samples is required: 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, and 0.5 mM ε-amino caproic acid.

11. Chloroplast washing medium B for thylakoid subfractions (i.e., grana and stroma lamellae): 20 mM Hepes-KOH, pH 7.6, 0.4 M sorbitol, 5 mM $MgCl_2$, 2.5 mM EDTA, 10 mM $NaHCO_3$, 0.15% (w/v) bovine serum albumin (BSA, defatted).
12. Percoll gradient solution A for envelope and thylakoid purification. Mix 1 vol. of Percoll with 1 vol. of medium containing 40 mM Tricine-KOH, pH 7.6, 0.8 M sorbitol, 10 mM $MgCl_2$, 5 mM EDTA, to obtain a 50% (v/v) Percoll/0.4 M sorbitol solution.
13. Percoll gradient solution B for purification of thylakoid subfractions (i.e., grana and stroma lamellae): Mix 1 vol. of Percoll with 1 vol. of medium containing 40 mM Hepes-KOH, pH 7.6, 0.8 M sorbitol, 10 mM $MgCl_2$, 5 mM EDTA, 20 mM $NaHCO_3$ and 0.3% (p/v) BSA to obtain a 50% (v/v) Percoll/0.4 M sorbitol solution.

2.3 Purification of Envelope and Crude Thylakoids from Arabidopsis Chloroplasts

1. Hypotonic medium for chloroplast lysis: 10 mM 3-(N-morpholino) propane sulfonic acid (MOPS)-NaOH, pH 7.8, 4 mM $MgCl_2$. This solution should also contain the following protease inhibitors when long-term storage of protein samples is required: 1 mM PMSF, 1 mM benzamidine, and 0.5 mM ε-amino caproic acid.
2. Sucrose gradient solutions for chloroplast fractionation: 10 mM MOPS-NaOH, pH 7.8, 4 mM $MgCl_2$, and 0.3, 0.6 or 0.93 M sucrose.
3. Membrane washing medium (to wash chloroplast envelope and thylakoid membranes): 10 mM MOPS-NaOH, pH 7.8, 1 mM PMSF, 1 mM benzamidine, 0.5 mM ε-amino caproic acid.
4. Preparative refrigerated ultracentrifuge (Beckman L7), with a SW 41 Ti rotor (6 × 13.2-mL Ultraclear tubes), or equivalent.
5. Microcentrifuge (Eppendorf 5415D or equivalent) placed in a cold room with 1.5 mL plastic tubes.
6. Branson Sonifier model S-250D (or equivalent), with 3 mm microtip and ice bucket.
7. Nitrogen (or argon) gas supply (from cylinder) with gas pressure regulator connected to a Pasteur pipette via a plastic tube.

2.4 Purification of Stroma Lamellae from Purified Arabidopsis Chloroplasts

1. Hypotonic medium for chloroplast lysis: 20 mM Hepes, 10 mM $MgCl_2$. This solution should also contain the following protease inhibitors when long-term storage of protein samples is required: 0.2 mM PMSF, 0.2 mM benzamidine, and 1 mM ε-amino caproic acid. pH adjustment to 7.8 with KOH.

2. Membrane storing medium used to store stroma lamellae: 0.4 M sorbitol, 10 mM Hepes, 5 mM $MgCl_2$, 0.2 mM PMSF, 0.2 mM benzamidine, 1 mM ε-amino caproic acid. pH adjustment to 7.7 with KOH.
3. 80% (v/v) acetone to determine the chlorophyll concentration [14].
4. 10% (w/v) digitonin (Sigma) to solubilize the lighter fraction of the membranes (*see* **Note 2**).
5. Spectrophotometer.
6. Cell Culture Rotator placed in a cold room for digitonin incubation.
7. Superspeed refrigerated centrifuge (Sorvall RC5), with the rotors SS34 (2 × 50 mL polypropylene tubes).
8. Preparative refrigerated ultracentrifuge (Beckman L7), with a SW 32 Ti rotor (2 × 40-mL Ultraclear Centrifuge tubes), or equivalent.

2.5 Purification of Grana from Purified Arabidopsis Chloroplasts

1. Hypotonic medium for chloroplast lysis: 20 mM Hepes, 10 mM $MgCl_2$. This solution should also contain the following protease inhibitors when long-term storage of protein samples is required: 0.2 mM PMSF, 0.2 mM benzamidine, and 1 mM ε-amino caproic acid. pH adjustment to 7.8 with KOH.
2. Membrane storing medium used to store grana: 0.4 M sorbitol, 10 mM Hepes, 5 mM $MgCl_2$, 0.2 mM PMSF, 0.2 mM benzamidine, 1 mM ε-amino caproic acid. pH adjustment to 7.7 with KOH.
3. 80% (v/v) acetone to determine the chlorophyll concentration [14].
4. Spectrophotometer.
5. Triton X-100.
6. Cell Culture Rotator placed in cold room for Triton X-100 incubation.
7. Superspeed refrigerated centrifuge (Sorvall RC5), with the rotors SS34 (2 × 50 mL polypropylene tubes).
8. Preparative refrigerated ultracentrifuge (Sorvall Discovery M150SE), with a S55 ALrotor (2 × 1.5 mL Microfuge tube pollyallomer), or equivalent.
9. Microcentrifuge (Eppendorf 5415D or equivalent) placed in a cold room with 1.5 mL plastic tubes.

2.6 SDS-PAGE and Protein Transfer to Nitrocellulose

1. Gel electrophoresis apparatus (Bio-Rad Protean 3 or equivalent), with the various accessories needed for protein separation by electrophoresis (combs, plates, and casting apparatus).
2. Acrylamide stock: 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide. Dissolve 300 g of acrylamide and 8 g of bisacrylamide

in H_2O to 1 L. Alternatively, a ready-to-use acrylamide-bisacrylamide solution may be employed.

3. SDS stock solution: 20% (w/v) sodium dodecyl sulfate (SDS). Store at room temperature.
4. 4× Laemmli stacking gel buffer: 0.5 M Tris–HCl, pH 6.8. Dissolve 363 g of Tris in H_2O to 900 mL, adjust to pH 6.8 at 25 °C with concentrated HCl, and make up volume to 1 L. Store at room temperature.
5. 8× Laemmli resolving gel buffer: 3 M Tris–HCl, pH 8.8. Dissolve 60.6 g of Tris in H_2O to 900 mL, adjust to pH 8.8 at 25 °C with concentrated HCl, and make up volume to 1 L. Store at room temperature.
6. Stacking gel (5% acrylamide). Mix 0.83 mL of 30% acrylamide/0.8% bisacrylamide stock solution, 1.25 mL of 4× Laemmli stacking gel buffer, 2.8 mL of H_2O, 25 µL of 20% (w/v) SDS, 5 µL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), and 50 µL of 10% (w/v) ammonium persulfate (dissolve 1 g of ammonium persulfate in H_2O to 10 mL; store at 4 °C and prepare fresh every month). The total volume will be 4.96 mL (sufficient for two 7-cm-long gels).
7. Single acrylamide resolving gels (10%, 12%, or 15% acrylamide). (1) For a 10% acrylamide gel, mix 3.3 mL of 30% acrylamide/0.8% bisacrylamide stock solution, 1.25 mL of 8× Laemmli resolving gel buffer, 5.3 mL of H_2O, 50 µL of 20% (w/v) SDS, 4 µL of TEMED, and 0.1 mL of 10% (w/v) ammonium persulfate. (2) For a 12% acrylamide gel, mix 4 mL of 30% acrylamide/0.8% bisacrylamide stock solution, 1.25 mL of 8× Laemmli resolving gel buffer, 4.6 mL of H_2O, 50 µL of 20% (w/v) SDS, 4 µL of TEMED, and 0.1 mL of 10% (w/v) ammonium persulfate. (3) For a 15% acrylamide gel, mix 5 mL of 30% acrylamide/0.8% bisacrylamide stock solution, 1.25 mL of 8× Laemmli resolving gel buffer, 3.6 mL of H_2O, 50 µL of 20% (w/v) SDS, 4 µL of TEMED, and 0.1 mL of 10% (w/v) ammonium persulfate. In each case, the total volume should be ~10 mL (sufficient for two 7-cm-long gels).
8. 4× Loading buffer for protein solubilization: 200 mM Tris–HCl, pH 6.8, 40% (v/v) glycerol, 4% (w/v) SDS, 0.4% (w/v) bromophenol blue, 100 mM dithiothreitol.
9. Gel reservoir buffer: 38 mM glycine, 50 mM Tris, 0.1% (w/v) SDS. Prepare about 400 mL for each reservoir.
10. Gel staining medium: acetic acid/isopropanol/water, 10/25/65 (v/v/v), supplemented with 2.5 g/L of Coomassie Brilliant Blue R250. Store in clean and closed bottles.
11. Gel destaining medium: acetic acid/ethanol/water, 7/40/53 (v/v/v).

2.7 Western Blots

1. System for protein transfer to nitrocellulose membranes (including central core assembly, holder cassette, filter paper, fiber pads, and cooling unit).
2. Protein transfer medium. To make this solution, dilute Gel reservoir buffer (*see* Subheading 2.4) with ethanol to obtain a 20% (v/v) final ethanol concentration. The final buffer composition is: 30.4 mM glycine, 40 mM Tris, 0.08% (w/v) SDS, 20% (v/v) ethanol. Prepare about 800 mL for each experiment.
3. Nitrocellulose membranes.
4. TBST (Tris Buffered Saline with Triton): 0.15 M NaCl, 50 mM Tris–HCl, pH 7.5, 0.05% (w/v) Triton X-100.
5. Milk-containing TBST. To make this solution, supplement TBST with 50 g/L of fat-free milk powder.
6. Anti-HMA1 antibody [15] raised against a protein from the inner envelope membrane of *Arabidopsis* chloroplasts (used at a 1:1,000 dilution).
7. Anti-ceQORH antibody [16] raised against a protein from the inner envelope membrane of *Arabidopsis* chloroplasts (used at a 1:5,000 dilution).
8. Anti-KARI antibody [12] raised against a soluble protein from the stroma of spinach (*Spinacia oleracea*) chloroplasts (used at a 1:1,000 dilution).
9. Anti-LHCP antibody [17] raised against a thylakoid membrane protein from *Chlamydomonas reinhardtii* chloroplasts (used at a 1:25,000 dilution).
10. Anti-CP43 antibody (Agrisera, Sweden) raised against a synthetic peptide conserved across nearly all known CP43 photosystem II subunits including *Arabidopsis thaliana* (used at a 1:10,000 dilution).
11. Anti-PSAD antibody (Agrisera, Sweden) raised against a synthetic peptide 100% conserved in all known plant PsaD photosystem I subunits including *Arabidopsis thaliana* (used at a 1:3,000 dilution).
12. Solution A: 90 mM P-coumaric acid (14 mg/mL in dimethyl sulfoxide [DMSO]).
13. Solution B: 250 mM luminol (3-aminophalhydrazin) (44 mg/mL in DMSO).
14. 100 mM Tris–HCl, pH 8.5.
15. Chemiluminescence adapted films, and a suitable exposure cassette.
16. Developer and Fixer solutions, for film development under red safe-light in a dark room.

3 Methods (*See* Fig. 2)

3.1 Percoll-Purification of Intact Chloroplasts from Arabidopsis Leaves

All operations should be carried out at 0–5 °C. It is important to note that Fig. 2 and Subheading 2.2 identify independent buffers (Percoll gradient and washing medium) to purify the chloroplasts depending on the targeted chloroplast sub-compartment. In the present section, due to space constraints, we combined the protocol used to purified chloroplasts for further extraction of envelope and thylakoid fractions with the protocol (in brackets) used to purify chloroplasts for further extraction of grana and stroma lamellae.

1. Prior to the experiment, prepare six tubes (each containing 30 mL of a 50% Percoll/0.4 M sorbitol/Tricine pH 7.6 solution A (or two tubes each containing 30 mL of a 50% Percoll/0.4 M sorbitol/Hepes pH 7.6 solution B, *see* Subheading 2.2 and Fig. 2). Preform Percoll gradients for further chloroplast purification by centrifugation at 38,700 × *g* for 55 min (Sorvall SS-34 rotor) (*see* **Note 3**). Store the tubes containing preformed Percoll gradients in the cold room until use.
2. Harvest 400–500 g of rosette leaves (or 100–150 g) (*see* **Note 4**). Wash them with deionized water. Blot the washed leaves on paper tissue, and transfer them into a cold room for the next step.
3. Homogenize the 400–500 g of leaf material with 2 L of Leaf grinding medium (or the 100–150 g of leaf material with 350 mL of Leaf grinding medium) two times, for 2 s each time, in a Waring blender at low speed (*see* **Note 5**). Filter rapidly the homogenate through four to five layers of muslin and one layer of nylon blutex.
4. Distribute equally the filtered suspension into six bottles (or two bottles) for centrifugation and centrifuge them at 2,070 × *g* for 2 min using a Sorvall GS 3 rotor.
5. Suck up the supernatant with a water pump and carefully resuspend each pellet, containing a crude chloroplast fraction, by the addition of a minimal volume to obtain a 36 mL final volume (or 6 mL final volume) of Chloroplast washing medium A (or Chloroplast washing medium B). Use a spatula to gently resuspend the organelles.
6. Load 6 mL (or 3 mL) per tube of the chloroplast suspension on the top of the preformed Percoll gradients (*see* **Note 6**). Centrifuge the gradients at 13,300 × *g* for 10 min using a Sorvall swinging-bucket HB-6 rotor (*see* **Note 7**). At the conclusion of this step, aspirate the upper part of the gradient (*see* **Note 8**), and then recover intact chloroplasts present in the broad, dark-green band (Fig. 2) in the lower part of the gradient with a pipette (*see* **Note 9**).

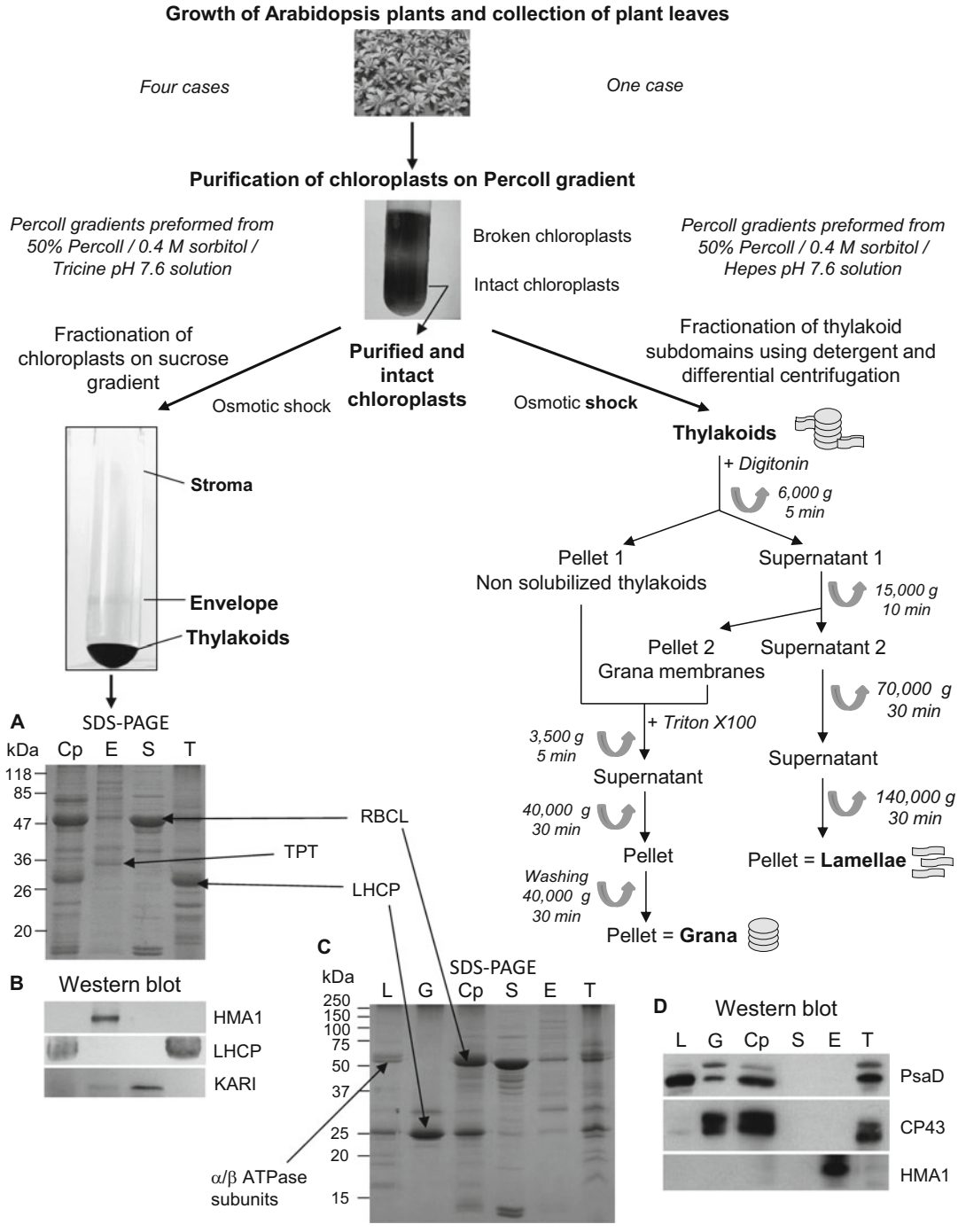

Fig. 2 Scheme of the steps used to purify chloroplast membranes sub-compartments from *Arabidopsis thaliana*, and to evaluate their cross-contamination. *Left*: Purification of envelope and crude thylakoid compartments starting from Percoll-purified *Arabidopsis* chloroplasts. (**a**) SDS-PAGE performed to visualize abundant markers of the purified chloroplast (Cp) subfractions. TPT (30-kDa phosphate/triose-phosphate translocator) is the most abundant protein of the envelope (E). RBCL (45 kDa large subunit of Rubisco) is the

7. Dilute three- to fourfold the intact chloroplast suspension with 200–300 mL of Chloroplast washing medium A (or 50–100 mL of Chloroplast washing medium B, *see* Subheading 2.2). Centrifuge the suspension at 2070 × *g* for 2 min in a Sorvall GS 3 rotor (or in a Sorvall SS-34 rotor).
8. Recover each pellet, containing washed and purified intact chloroplasts. At this stage, the yield of intact chloroplasts is 50–60 mg of protein (or 5–10 mg of protein) (*see* **Note 10**).

3.2 Fractionation of Arabidopsis Chloroplasts to Purify Envelope and Thylakoid Fractions

All operations should be carried out at 0–5 °C.

1. Prior to the experiment, prepare six tubes (13.2 mL, Ultraclear, Beckman) with sucrose gradients each consisting of three layers: 3 mL of 0.93 M, 2.5 mL of 0.6 M, and 2 mL of 0.3 M sucrose. Each layer should be carefully overlaid with a pipette (*see* **Note 11**) on the top of the previous layer, starting with the densest one (0.93 M, at the bottom) and finishing with the lightest one. Store the tubes in the cold room until use.
2. Lyse the purified and washed intact chloroplasts (obtained as described in Subheading 3.1) by adding to the pellets Hypotonic medium containing protease inhibitors (adjust for a final total volume of 21 mL for all six pellets).
3. Load the lysed chloroplasts (3.5 mL per tube) on the top of the sucrose gradients. Centrifuge the tubes at 70,000 × *g* for 1 h (Beckman SW41-Ti rotor) (*see* **Note 12**). After centrifugation, the envelope membranes and the thylakoids are present as a yellow band at the 0.93/0.6 M sucrose interface, and as a dark-green band at the bottom of the tube, respectively. The soluble fraction containing the stroma remains on top of this gradient.
4. To recover the soluble stromal proteins, carefully remove the upper part of the gradient with a pipette (3 mL should be

Fig. 2 (Continued) most abundant protein of the stroma (S), and LHCPs (26 kDa Light Harvesting Complex Proteins) are the most abundant proteins of the thylakoids (T). (**b**) Western blots performed to evaluate the cross-contamination of envelope and thylakoid preparations with marker proteins, from the envelope (HMA1, 80-kDa Cu^{+}-ATPase), the thylakoids (LHCP) or the stroma (KARI, 58-kDa Acetohydroxy-acid reductoisomerase), respectively. *Right*: Purification of grana and stroma lamellae subdomains of thylakoids starting from Percoll-purified *Arabidopsis* chloroplasts. (**c**) SDS-PAGE performed to visualize abundant markers of the thylakoid (T) subdomains. LHCPs are the most abundant proteins of the grana (G). ATPase subunits (55-kDa α/β ATPase subunits) are the most abundant proteins of the stroma lamellae (L). Cp, S and E refer to purified chloroplast, stroma and envelope subfractions, respectively. (**d**) Western blots performed to estimate cross-contaminations of the purified thylakoid subdomains (G and L) with a marker protein (HMA1) from the envelope (E) and the cross contamination of grana (G) and stroma lamellae (L) with marker proteins (CP43, a 43-kDa Photosystem II subunit, and PsaD, a 20-kDa Photosystem I subunit) from the grana and stroma lamellae, respectively

recovered from each gradient tube) and store it in liquid nitrogen (*see* **Note 13**).

5. To purify envelope membranes, recover the yellow band containing the envelope with a pipette, dilute the suspension three- to fourfold in Hypotonic medium (containing protease inhibitors), and concentrate the membranes as a pellet by centrifugation at 110,000 × *g* for 1 h (Beckman SW 41 Ti rotor).
6. Add a minimal volume of Membrane washing medium (containing protease inhibitors) to the envelope pellet. Take an aliquot for protein amount determination (*see* **Note 14**). Store envelope membrane preparations in liquid nitrogen.
7. Thylakoids can be diluted in a minimal volume of Membrane washing medium (containing protease inhibitors) and stored in liquid nitrogen (*see* **Note 15**).

From such preparations, an average of ~30 mg of stroma proteins, ~20 mg of thylakoid proteins, and ~300 μg of envelope proteins can be obtained (*see* **Note 16**).

3.3 Fractionation of Arabidopsis Chloroplasts to Purify the Stroma Lamellae Fraction

All operations should be carried out at 0–5 °C.

1. Lyse the purified and washed intact chloroplasts (obtained as described in Subheading 3.1) by adding to the pellets Hypotonic medium (800 μL max.) containing protease inhibitors. An aliquot of 100–200 μL can be stored as "Thylakoid" for further analyses.
2. Measure chlorophyll concentration by mixing 5 μL of thylakoid suspension in 995 μL of 80% acetone. Centrifuge for 1 min at max speed in a microcentrifuge and measure the OD of the supernatant at 652 nm to determine chlorophyll concentration [14].
3. Dilute the thylakoid suspension with the Hypotonic medium in order to obtain a final chlorophyll concentration equal to 0.5 mg/mL.
4. Add digitonin in order to obtain a final digitonin concentration equal to 0.5% (w/v). Incubate the mixture in the dark for 30 min at 4 °C under agitation on a cell culture rotator.
5. Dilute the sample ten times with the Hypotonic medium and concentrate the membranes as a pellet by centrifugation for 5 min at 6,000 × *g* (Sorvall SS-34 rotor).
6. Store the pellet, consisting of non-solubilized thylakoids, for the following grana extraction (*see* Subheading 3.4) and centrifuge the supernatant for 10 min at 15,000 × *g* (Sorvall SS-34 rotor).
7. Again, store the pellet for the following grana extraction (*see* Subheading 3.4) and centrifuge the supernatant for 30 min at 70,000 × *g* (Beckman SW32Ti rotor) (*see* **Note 17**).

8. Collect the supernatant avoiding the pellet to detach. Waste the pellet and centrifuge the supernatant for 1 h 30 at 140,000 × *g* (Beckman SW32Ti rotor).
9. Remove the supernatant with the aid of the vacuum-pump.
10. The pellet (containing the stroma lamellae fraction) can be diluted in Membrane storing medium (containing protease inhibitors) and stored in liquid nitrogen.

From such preparations, an average of 900 μg of stroma lamellae proteins can be obtained.

3.4 Fractionation of Arabidopsis Chloroplasts to Purify the Grana Fraction

All operations should be carried out at 0–5 °C.

1. Resuspend and wash, with 30 mL of Hypotonic medium each, the pellets stored during the stroma lamellae purification (*see* Subheading 3.3, **steps 6** and **7**). Centrifuge for 10 min at 15,000 × *g* (Sorvall SS-34 rotor).
2. Resuspend the two pellets in 400 μL of Hypotonic medium and measure chlorophyll concentration by mixing 5 μL of thylakoid suspension in 995 μL of 80% acetone. Centrifuge for 1 min at max speed in a microcentrifuge and measure the OD of the supernatant at 652 nm to determine the chlorophyll concentration [14].
3. Dilute the thylakoid suspension with the Hypotonic medium in order to obtain a final chlorophyll concentration equal to 1 mg/mL.
4. Add Triton X-100 to a final concentration of 10 mg/mg chlorophyll. Incubate the mixture in the dark for 30 min at 4 °C under agitation on a cell culture rotator.
5. Centrifuge (Eppendorf Centrifuge) the mixture for 5 min at 3,500 × *g*.
6. Collect the supernatant and centrifuge it at 40,000 × *g* for 30 min (Sorvall S55-AL rotor).
7. Resuspend and wash the pellet, consisting of BBY (i.e., the inner part of the grana stacks) in 1.5 mL of Hypotonic medium (*see* **Note 18**).
8. Ultracentrifuge again at 40,000 × *g* for 30 min.
9. Remove the supernatant with a pipette.
10. Grana fraction can be diluted in Membrane storing medium (containing protease inhibitors) and stored in liquid nitrogen.

From such preparations, an average of 200 μg of grana proteins can be obtained.

3.5 Separation of Membrane Proteins by 1D SDS-PAGE (See Note 19)

1. Prior to the experiment, prepare slab gels for protein electrophoresis (*see* **Note 20**). Assemble the gel apparatus according to the manufacturer's specifications (*see* **Note 21**). Prepare the different gel solutions (stacking gel, and 10%, 12%, or 15% acrylamide separation gel). The volumes to be used are determined by gel dimensions, and therefore by the specifications of the apparatus.
2. Several dilutions (a factor of 3 for every dilution step) of the protein samples might be performed to estimate cross-contaminations during further western blot analyses (*see* **Note 22** and [18]). If western blotting is to be conducted (*see* Subheading 3.6), the Coomassie staining step (*see* **step 5** below) should be omitted.
3. Add 4× Loading buffer to the samples. Heat the protein samples at 95 °C for 5 min to solubilize the proteins. Place protein samples (20 μL) into gel slots by means of a pipette. Load the molecular weight markers in another slot.
4. Set the conditions for the electrophoresis at 150 V (~20 V/cm). Run the gels for 1 h at room temperature (until the bromophenol blue dye reaches the lower part of the gel).
5. After electrophoresis, remove the gels from the apparatus; place them in plastic boxes in the presence of Gel staining medium. Shake the box gently for 30 min. Pour off the staining solution and replace it with the Gel destaining medium. Shake the box gently for 15 min. Repeat the washing step once or twice. Typical results are shown in Fig. 2 (panels a and c).

3.6 Immunological Markers: Western Blot Analyses (See Note 23)

Having access to highly purified chloroplast subfractions is a prerequisite to answer many essential biological questions. However, while they are well established, the protocols used to purify plastid sub-compartments cannot fully exclude cross-contaminations originating from other plastids and cell compartments. An inventory of the origins of the cross-contaminations of Percoll-purified chloroplast, envelope and thylakoid fractions by other cell compartments was performed using semi-quantitative proteomic approaches [12] and discussed previously [9, 18]. Here, we thus concentrate on assays that can be performed to assess marker proteins deriving from the various plastid sub-compartments. The presence of these markers should be tested in purified chloroplast fractions, as well as in crude cell extract and, when available, corresponding preparations of all other chloroplast compartments (*see* Fig. 2), and then compared to assess cross-contamination levels. Western blots should be performed after separation of proteins by SDS-PAGE (*see* Subheading 3.5). After gel migration, transfer the proteins onto a nitrocellulose membrane using a gel transfer apparatus.

1. Prepare the cassette as follows. Add successively: 1× fiber pad, 3× filter papers, the gel, a nitrocellulose membrane, 3× filter papers, 1× fiber pad. Then, insert the sandwich into the holder cassette (the membrane should be placed beside the positive electrode).
2. Insert the cassette in the central core assembly unit (together with the cooling unit).
3. Perform the transfer for 1.5 h at 80 V in Protein transfer medium, and at room temperature.
4. Recover the nitrocellulose membrane. The following incubation and washing steps (**steps 5–10** below) require agitation on a rocking plate.
5. Rinse the nitrocellulose membrane with TBST for 10 min.
6. Saturate the nitrocellulose membrane with milk-containing TBST. Leave it for at least 1 h at room temperature.
7. Add the primary antibody diluted in milk-containing TBST. Leave it for 3 h at room temperature or 12 h at 4 °C (*see* **Note 24**).
8. Wash the nitrocellulose membrane three times, for 10 min each time, with TBST.
9. Add the secondary antibody diluted at 1/10,000 in TBST (*see* **Note 25**). Leave it for 1 h at room temperature.
10. Wash the nitrocellulose membrane three times, for 10 min each time, with TBST. Then, proceed to detect the chemiluminescent signal as described in the following steps.
11. Mix 3 mL of 100 mM Tris–HCl, pH 8.5, with 13.3 µL of Solution A.
12. Mix 3 mL of 100 mM Tris–HCl, pH 8.5, with 30 µL of Solution B.
13. Combine and mix together the two above solutions (prepared in **steps 11** and **12** above) in a dark room.
14. Incubate the nitrocellulose membrane for 1 min in the previously prepared mixture (the chemiluminescence substrate solution).
15. Expose to film for a few seconds and up to several minutes depending on the detected signal. Alternatively, a gel imaging systems can be used for quick visualization and quantification of the signal.
16. Incubate the film successively in the Developer solution (for 1–3 min, depending on the signal-to-noise ratio), in water (for 10 s), and in the Fixer solution (for 2 min). Rinse the film in water and dry it. Typical results are shown in Fig. 2 (panels b and d).

4 Notes

1. The procedures described in this article were applied efficiently to several *Arabidopsis* ecotypes (Landsberg *erecta*, Columbia, and Wassilewskija). Using young *Arabidopsis* leaves (3- to 4-week-old) improves yield, purity, and integrity of the purified chloroplasts. In our hands, isolation of envelope membranes from older leaves was unsuccessful.
2. Solubilization of digitonin is more rapidly and easily obtained using preheated water (90 °C).
3. Vertical rotors can easily be used to obtain preformed Percoll gradients and to subsequently purify chloroplasts [19].
4. Chloroplasts containing large starch grains will generally be broken during centrifugation [19]. Therefore, prior to the experiment (e.g., at the beginning of the afternoon prior to the day of the experiment), the plants can be kept in a dark and cold room (4 °C) to reduce the amount of starch.
5. It is critical to limit the grinding process to 2 s. While longer blending strongly improves the yield of recovered chlorophyll, it also increases the ratio of broken chloroplasts. When large amounts of broken chloroplasts are present in the suspension, this definitively affects the efficiency of the Percoll gradient (*see* Fig. 2) in separating intact and broken organelles.
6. Do not mix preformed Percoll gradients (*see* Subheading 3.1, **step 1**) containing Tricine pH 7.6 solution A (for envelope and crude thylakoid preparation) or Hepes pH 7.6 solution B (for the preparation of thylakoid subdomains). While originally using a medium containing Tricine buffer for envelope purification [20], we now use Hepes instead of Tricine (*see* Fig. 2) for the purification of thylakoid subdomains since Hepes buffer was shown to preserve the photosynthetic activity of the Percoll-purified *Arabidopsis* chloroplasts [21].
7. It is recommended to disconnect the brake or to use the automatic rate controller (if available) to prevent mixing of the gradients at the critical stage of deceleration.
8. It is important to remove carefully the top-content of the tube by aspiration with a water pump. This allows the removing of soluble proteins derived from various cell compartments (*see* [18]). Broken chloroplasts (and some remaining intact mitochondria, *see* [18]) are present in the upper part of the gradient as a broad band (*see* Fig. 2), and must be removed by aspiration. Then recover the intact chloroplasts with a pipette. To limit breakage of chloroplasts, if using the blue tip of an Eppendorf pipette, cut the tip with a razor blade so that the hole will have a 2 mm diameter.

9. A small pellet (containing cell pieces, large debris, starch, DNA, nuclei, etc.) is found at the bottom of the Percoll gradient [18]. It is thus essential to use recovering conditions that will limit aspiration of this pellet with the intact chloroplasts. Note that when some nuclei are taken with the intact chloroplasts at this step, they will later be recovered within thylakoid fractions [18], at the bottom of the sucrose gradient (Fig. 2).
10. Percoll-purified and intact chloroplasts are largely devoid of contamination deriving from other cell compartments (*see* [18] and Table 1). However, considering the high sensitivity of present mass spectrometers, it is not surprising, using proteomic analyses, to detect minute amounts of a few extra-plastidial contaminants, which are major proteins in their respective subcellular compartment. In every case, other complementary approaches are necessary to assert the subcellular localization of a protein (immuno-localization of proteins, expression of GFP fusions in planta coupled to confocal microscopy, etc.).
11. The use of a peristaltic pump to prepare the sucrose gradients is recommended (avoiding mixing of the layers) since some expertise is needed to load the different layers by hand.
12. It is recommended to disconnect the brake to prevent mixing of the gradients at the critical stage of deceleration.
13. The proteins from the stroma will be recovered in the Hypotonic medium used to break the chloroplasts and this fraction will thus contain protease inhibitors (i.e., 1 mM PMSF, 1 mM benzamidine, and 0.5 mM ε-amino caproic acid). Further desalting of these soluble proteins may be performed using G-25 columns (e.g., PD-10 Desalting columns, GE Healthcare Life Sciences) if required. Note that if intact mitochondria are recovered from the Percoll gradient step (due to incomplete removal of the upper part of the Percoll gradient containing broken chloroplasts; *see* [18]), these mitochondria will also be ruptured by the osmotic shock used to fractionate the chloroplasts and soluble proteins from the mitochondrial matrix will copurify with soluble proteins from the stroma [18]. It is however important to note that while the detection of genuine mitochondrial proteins in the purified chloroplast stroma may be the result of cross-contamination, the "dual targeting" of some proteins known to be imported into both plastids and mitochondria might also explain their presence [22].
14. Protein contents of fractions are estimated using the Bio-Rad Protein Assay reagent [23].
15. The major possible contaminations of crude thylakoid membrane preparations derive from envelope membrane proteins [18]. It is estimated that 50% of the envelope membrane vesicles are recovered in the crude thylakoid fraction. However, since the

ratio of envelope to thylakoid membranes is 1/50 in the chloroplast, these contaminations will be limited to less than 1% of the crude thylakoid membrane fraction. Alternatively, heavy components of the cell might be recovered within the thylakoid pellet if originally aspirated with intact chloroplasts from the bottom of the Percoll gradient (*see* **Note 9** and [18]).

16. At this stage, the major possible contaminants of envelope preparations are soluble stroma proteins and light vesicles of thylakoid membranes [12, 20]. Being the most likely source of membrane contamination of the purified envelope fraction, thylakoid cross-contamination needs to be precisely assessed. The yellow color of purified envelope vesicles should first indicate that this membrane system contains almost no chlorophyll and therefore very few contaminating thylakoid vesicles. By Western blot analyses using antibodies raised against LHCP, Ferro et al. [20] demonstrated that several independent Arabidopsis envelope preparations contained an average of 3% thylakoid proteins (Table 1), in agreement with the 6% cross-contamination estimated using spectral counting [12]. Since the envelope membranes are at the interface of two soluble media (i.e., the cytosol and the stroma), and since soluble proteins from both sides of the membrane might be trapped within membrane vesicles during chloroplast rupture (during osmotic shock), envelope contamination with these soluble fractions might be expected. Stroma proteins might represent up to 10% of purified envelope fractions, as estimated from

Table 1
Cross-contaminations of chloroplast subfractions extracted from Percoll-purified *Arabidopsis* chloroplasts

Purified fractions Contaminants	Envelope	Thylakoids	Grana	Stroma lamellae	Stroma
Envelope (WB)	–	~3%[a]	<3%[b]	~10–15%[b]	<1%[a]
Thylakoids (WB)	~3%[a]	–	–	–	<1%[a]
Stroma (WB)	~10%[a]	<1%[a]	<3%[b]	<3%[b]	–
Other cell compartments (SC)	<2%[a]	<2%[a]	<5%[b]	<5%[b]	–

Quantification of the cross-contamination of purified subplastidial fractions according to [a]Ferro et al. [12] and [b]Tomizioli et al. [9]. Western-blot (WB) analyses: contamination of envelope preparations with thylakoid proteins evaluated using light harvesting complex proteins (LHCP) antibodies (1/25,000 dilution). Contamination of envelope preparations with stroma proteins evaluated using acetohydroxy acid isomero-reductase protein (KARI) antibodies (1/1000 dilution). Contamination of thylakoid preparations with envelope proteins evaluated using inner envelope membrane proteins (HMA1 and ceQORH) antibodies (1/1000 and 1/5000 dilution, respectively). Contamination of stroma preparations with envelope proteins evaluated using inner envelope membrane protein (ceQORH) antibodies (1/5000 dilution). Contamination of stroma preparations with thylakoid proteins evaluated using light harvesting complex proteins (LHCP) antibodies (1/50,000 dilution). Contamination of thylakoids with stroma proteins evaluated using acetohydroxy acid isomero-reductase protein (KARI) antibody (1/1000 dilution) and by detection of RuBisCO protein (RBCL) after a Coomassie Blue staining. Spectral counting (SC) analyses: % of spectra (spectral counts) detected for every peptide deriving from non-plastid proteins

western blot analyses (Table 1). On the contrary, cytosolic contaminations are barely detected within purified envelope fractions and mostly result from the detection of cytosolic ribosomal subunits [12].

17. Grana pellet can be stored on ice before ending of the stroma lamellae purification process. Alternatively, to reduce storage time of grana pellets on ice, grana purification can be started simultaneously.
18. Grana fraction is first diluted in a small volume (e.g., 0.3 mL) of Hypotonic medium for chloroplast lysis buffer and membrane aggregates are solubilized using a small plastic Potter in a 1.5 mL Eppendorf tube.
19. Classical proteomic approaches based on the use of two dimensional (2-D) gel electrophoresis proved to be rather inefficient at analyzing highly hydrophobic membrane proteins [24, 25]. Therefore, thylakoid and envelope fractions are preferably analyzed by SDS-PAGE, while further proteomic studies targeted to the stroma (or the thylakoid lumen) can either rely on SDS-PAGE or 2-D-gel electrophoresis.
20. We routinely use the procedure described by Chua [26] to separate membrane proteins by SDS-PAGE. This article describes in detail all stock solutions, and medium for stacking and separation gels.
21. We use a Bio-Rad apparatus, with 7-cm-long gels.
22. As a first step, SDS-PAGE analyses should be used to study the purified envelope fraction to detect known abundant markers associated with this fraction and a lack of markers deriving from other plastidic or cellular compartments (*see* Fig. 2). If cross-contaminations are revealed, additional SDS-PAGE analyses should then include experiments aiming to quantify these cross-contamination levels. For this, it is essential to use dilutions of protein samples deriving from those cell compartments that were shown to cross-contaminate the purified envelope fractions [12, 18].
23. Follow the instructions for saturation (blocking) and incubation of the membrane with primary and secondary antibodies provided by the manufacturer.
24. Several dilutions of the primary antibodies should be tested to determine the best signal/noise ratio.
25. In our case, since the used primary antibodies were obtained from rabbit antisera, the secondary antibody is an anti-rabbit-IgG antibody coupled to the horseradish peroxidase for further detection. However, the secondary antibody has to be adapted to the primary antibody (which may be from rabbit, mouse, goat, guinea pig, etc.) and the detection procedure to be used (alkaline phosphatase or horseradish peroxidase).

References

1. Jarvis P, López-Juez E (2013) Biogenesis and homeostasis of chloroplasts and other plastids. Nat Rev Mol Cell Biol 14:787–802
2. Nakai M (2015) The TIC complex uncovered: the alternative view on the molecular mechanism of protein translocation across the inner envelope membrane of chloroplasts. Biochim Biophys Acta 1847:957–967
3. Linka N, Weber AP (2010) Intracellular metabolite transporters in plants. Mol Plant 3:21–53
4. Rolland N, Curien G, Finazzi G, Kuntz M, Maréchal E, Matringe M, Ravanel S, Seigneurin-Berny D (2012) The biosynthetic capacities of the plastids and integration between cytoplasmic and chloroplast processes. Annu Rev Genet 46:233–264
5. Block MA, Douce R, Joyard J, Rolland N (2007) Chloroplast envelope membranes: a dynamic interface between plastids and the cytosol. Photosynth Res 92:225–244
6. Joyard J, Ferro M, Masselon C, Seigneurin-Berny D, Salvi D, Garin J, Rolland N (2009) Chloroplast proteomics and the compartmentation of plastidial isoprenoid biosynthetic pathways. Mol Plant 2:1154–1180
7. Joyard J, Ferro M, Masselon C, Seigneurin-Berny D, Salvi D, Garin J, Rolland N (2010) Chloroplast proteomics highlights the subcellular compartmentation of lipid metabolism. Prog Lipid Res 49:128–158
8. Pribil M, Labs M, Leister D (2014) Structure and dynamics of thylakoids in land plants. J Exp Bot 65:1965–1972
9. Tomizioli M, Lazar C, Brugière S, Burger T, Salvi D, Gatto L, Moyet L, Breckels LM, Hesse AM, Lilley KS, Seigneurin-Berny D, Finazzi G, Rolland N, Ferro M (2014) Deciphering thylakoid sub-compartments using a mass spectrometry-based approach. Mol Cell Proteomics 13:2147–2167
10. Agrawal GK, Bourguignon J, Rolland N, Ephritikhine G, Ferro M, Jaquinod M, Alexiou KG, Chardot T, Chakraborty N, Jolivet P, Doonan JH, Rakwal R (2011) Plant organelle proteomics: collaborating for optimal cell function. Mass Spectrom Rev 30:772–853
11. Joshi HJ, Hirsch-Hoffmann M, Baerenfaller K, Gruissem W, Baginsky S, Schmidt R, Schulze WX, Sun Q, van Wijk KJ, Egelhofer V, Wienkoop S, Weckwerth W, Bruley C, Rolland N, Toyoda T, Nakagami H, Jones AM, Briggs SP, Castleden I, Tanz SK, Millar AH, Heazlewood JL (2011) MASCP Gator: an aggregation portal for the visualization of *Arabidopsis* proteomics data. Plant Physiol 155:259–270
12. Ferro M, Brugière S, Salvi D, Seigneurin-Berny D, Court M, Moyet L, Ramus C, Miras S, Mellal M, Le Gall S, Kieffer-Jaquinod S, Bruley C, Garin J, Joyard J, Masselon C, Rolland N (2010) AT_CHLORO, a comprehensive chloroplast proteome database with subplastidial localization and curated information on envelope proteins. Mol Cell Proteomics 9:1063–1084
13. Bruley C, Dupierris V, Salvi D, Rolland N, Ferro M (2012) AT_CHLORO: a chloroplast protein database dedicated to sub-plastidial localization. Front Plant Sci 3:205
14. Porra RJ, Thomson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted wth four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim Biophys Acta 975:384–394
15. Seigneurin-Berny D, Gravot A, Auroy P, Mazard C, Kraut A, Finazzi G, Grunwald D, Rappaport F, Vavasseur A, Joyard J, Richaud P, Rolland N (2006) HMA1, a new Cu-ATPase of the chloroplast envelope, is essential for growth under adverse light conditions. J Biol Chem 281:2882–2892
16. Miras S, Salvi D, Piette L, Seigneurin-Berny D, Grunwald D, Garin J, Reinbothe C, Joyard J, Reinbothe S, Rolland N (2007) Toc159- and Toc75-independent import of a transit sequence-less precursor into the inner envelope of chloroplasts. J Biol Chem 282:29482–29492
17. Vallon O, Bulte L, Dainese P, Olive J, Bassi R, Wollman FA (1991) Lateral redistribution of cytochrome b6/f complexes along thylakoid membranes upon state transitions. Proc Natl Acad Sci U S A 88:8262–8266
18. Salvi D, Moyet L, Seigneurin-Berny D, Ferro M, Joyard J, Rolland N (2011) Preparation of envelope membrane fractions from *Arabidopsis* chloroplasts for proteomic analysis and other studies. Methods Mol Biol 775:189–206
19. Douce R, Joyard J (1982) Purification of the chloroplast envelope. In: Edelman M, Hallick R, Chua NH (eds) Methods in chloroplast molecular biology. Elsevier/North-Holland, Amsterdam, pp 139–256
20. Ferro M, Salvi D, Brugière S, Miras S, Kowalski S, Louwagie M, Garin J, Joyard J, Rolland N (2003) Proteomics of the chloroplast envelope

membranes from *Arabidopsis thaliana*. Mol Cell Proteomics 2:325–345

21. Seigneurin-Berny D, Salvi D, Dorne A-J, Joyard J, Rolland N (2008) Percoll-purified and photosynthetically active chloroplasts from *Arabidopsis thaliana* leaves. Plant Physiol Biochem 46:951–955
22. Carrie C, Giraud E, Whelan J (2009) Protein transport in organelles: dual targeting of proteins to mitochondria and chloroplasts. FEBS J 276:1187–1195
23. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
24. Ferro M, Seigneurin-Berny D, Rolland N, Chapel A, Salvi D, Garin J, Joyard J (2000) Organic solvent extraction as a versatile procedure to identify hydrophobic chloroplast membrane proteins. Electrophoresis 21:3517–3526
25. Santoni V, Molloy M, Rabilloud T (2000) Membrane proteins and proteomics: un amour impossible? Electrophoresis 21:1054–1070
26. Chua NH (1980) Electrophoretic analysis of chloroplast proteins. Methods Enzymol 69:434–436

Chapter 9

Isolation of Intact Thylakoid Membranes from Heterocysts of Filamentous, Nitrogen-Fixing Cyanobacteria

Ann Magnuson and Tanai Cardona

Abstract

The isolation of thylakoid membranes, including intact membrane protein complexes, from heterocysts of filamentous cyanobacteria such as *Nostoc punctiforme*, is described. Protocols for BN-PAGE/SDS-PAGE 2-D electrophoresis are not included. However, the chapter ends with advisory notes on sample preparation for blue-native PAGE of thylakoid membrane proteins, which can then be used together with any standard protocol.

Key words Cyanobacteria, Heterocyst, Membrane proteins, Cell disruption, Sonication

1 Introduction

The study of photosynthesis and bioenergetics of cyanobacteria has become widely recognized as one of the cornerstones in developing alternative energy sources. Several filamentous, nitrogen-fixing cyanobacteria, such as the model organism *Nostoc punctiforme* ATCC 29133 (identical to PCC73102, and from here on referred to as *N. punctiforme*), develop specialized cells called heterocysts in response to nitrogen starvation [1]. The heterocysts provide a microoxic environment for the enzyme nitrogenase, and differ from the vegetative cells in several other ways. One important difference is the absence of oxygenic photosynthesis inside the heterocysts, which makes heterocysts dependent on the vegetative cells for electron sources in the form of sugars [2, 3].

Despite not performing standard photosynthesis, the heterocysts are still very much dependent on their thylakoid (photosynthetic) membranes. The nitrogenase reaction has an extremely high demand for ATP which is generated by ATP synthase in the thylakoid membrane. This is a light-driven process aided by Photosystem-I, which remains active in the heterocysts [4].

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Thylakoid membranes can be isolated from heterocysts with the membrane protein complexes intact and functioning [5]. However, first the heterocysts need to be separated from the rest of the filament, broken, and membranes separated from the broken cells with a minimum of protein degradation. In early preparation protocols the cells could undergo lysozyme treatment for up to 24 h at 35–38 °C, which in mesophilic cyanobacteria will lead to denaturation or inactivation of most enzyme complexes. The thylakoid membranes, being primarily the host of photosynthetic reaction centers, are also very sensitive to light-induced damage. Most laboratories, which are not specialized in photosynthesis research, may carry out biochemical preparations under ambient light and possibly at room temperature. This treatment will inevitably lead to degradation of photosynthetic proteins, leading to photobleaching and loss of the green color from chlorophyll. Thus, unintentional denaturation by harsh isolation procedures is probably the reason for what seems to be a persistent misconception that heterocysts are brownish rather than green in color.

We developed an isolation method for heterocyst thylakoid membranes which avoids prolonged high temperature treatment, and where most manipulations are performed at 4 °C and under dim, green light to maintain the integrity of pigment-binding proteins. This ensures maximum yield of active membrane-bound proteins, including Photosystem-I and Photosystem-II. This isolation method, if used correctly, should lead to a heterocyst preparation with no visible vegetative cells.

2 Materials

Prepare all solutions for both the heterocyst isolation and the ensuing thylakoid isolation, using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at 4 °C.

2.1 Components for Cell Harvesting and Heterocyst Isolation

1. Heterocyst isolation buffer: 50 mM HEPES/NaOH, pH 7.5, 10% (w/v) glycerol, 10 mM NaCl, 10 mM EDTA.
2. Lysozyme: 50–60 mg/mL chicken egg lysozyme in dH_2O (*see* **Note 1**).
3. A floor centrifuge, and centrifuge tubes and bottles.
4. Shaker-incubator, keeping 35 °C.
5. Sonicator (sonication horn).
6. Microscope.
7. Plastic container, approx. 500 mL.

8. Plastic test tubes, 15–20 mL.
9. Aluminum foil.

2.2 Components for Determination of Chlorophyll Concentration

1. UV/VIS spectrophotometer.
2. Dry methanol, analytical grade.
3. 1.5 mL microcentrifuge tubes.

2.3 Components for Heterocyst Breakage and Thylakoid Isolation

1. Disruption buffer: 50 mM HEPES/NaOH, pH 7.5, 10% (w/v) glycerol, 5 mM $MgCl_2$, 5 mM $CaCl_2$.
2. Protease inhibitor cocktail (*see* **Note 2**).
3. Bovine DNAse-I (optional, *see* **Note 3**).
4. A nitrogen cavitation chamber for cell disruption, for example a "Cell Bomb" (Parr Instruments, Fig. 1), and N_2 gas for pressurization (*see* **Note 4**).
5. An ultracentrifuge.
6. A small (150 mL) Erlenmeyer flask.

Fig. 1 A cell disruption chamber for nitrogen decavitation (Parr® Instruments). The cell suspension is placed inside the chamber, which is closed and filled with N_2. When the suspension, still under pressure, is slowly let out through a valve at the bottom of the chamber, the cells will disrupt due to rapid decompression

3 Methods

The protocol is optimized for approx. 3 L N_2-fixing culture of *Nostoc punctiforme* (*see* **Note 5**).

Important: After cell harvesting, all manipulations must be performed under dim, green light and at 4 °C, ideally in a refrigerated cold room. Some steps require that the samples are kept on ice.

The same precautions should be taken during blue-native gel electrophoresis, in the event that this is used for analysis.

Time considerations: Cell harvesting and isolation of heterocysts typically takes 4–5 h, not including preparation of buffer solutions. After that, breakage of the heterocysts and isolation of the thylakoid membranes takes an additional 2.5–3 h. All the steps can be done on the same day, or, alternatively, the intact heterocysts may be frozen at −80 °C until later.

3.1 Cell Harvesting and Heterocyst Isolation

1. Collect the culture in centrifuge bottles and sediment the cells by centrifugation at 5,000 × *g* for 10 min at 4 °C.
2. Resuspend the cells in chilled Heterocyst isolation buffer.
3. Measure the Chl-*a* concentration according to Subheading 3.2. Adjust the volume so that the final Chl-*a* concentration in the cell suspension is 100–150 μg/mL.
4. Keep the cells on ice in the dark for 30 min (*see* **Note 6**).
5. Pour the cell suspension in plastic test tubes and add 1ysozyme from the stock solution to a final concentration of 1 mg/mL.
6. Wrap the test tubes in aluminum foil and place in a shaker at 35 °C for 1 h (*see* **Note 7**).
7. Cool down the test tubes on ice, and sonicate each tube one at a time. Sonicate at 20 W amplitude in intervals of 10 s, for a total duration of 1 min (i.e., 6 × 10 s) (*see* **Note 8**).
8. Inspect the contents of each tube using a light microscope. If unbroken filaments of vegetative cells can still be observed, continue sonication for a few more cycles (*see* **Note 9**).
9. Pool the contents of all tubes and centrifuge in 35–50 mL tubes at 1,500 × *g* for 5 min at 4 °C.
10. Discard the supernatant and resuspend the pellet in a small volume of Heterocyst isolation buffer, using a Potter homogenizer pestle (*see* **Note 10**).
11. Add Heterocyst isolation buffer up to the volume that can be contained in a centrifuge tube. Centrifuge at 1,000 × *g* for 5 min at 4 °C.
12. Repeat **steps 10** and **11** twice, so that the heterocyst isolate is washed in total three times (*see* **Note 11**).

13. After the final centrifugation, discard the supernatant and resuspend the pellet in a small volume of Heterocyst isolation buffer, using a Potter homogenizer pestle.
14. Inspect the contents by a light microscope, looking for cell debris. When the sample consists only of heterocysts and is free of cell debris, continue with thylakoid membrane isolation (Subheading 3.3) (*see* **Note 12**).

Alternatively, the heterocysts can be stored at −80 °C. In this case, resuspend the heterocysts in a minimal volume of buffer only (*see* **Note 13**).

3.2 Determination of Chlorophyll Concentration

1. Add 10 μL cell suspension to 90 μL Heterocyst isolation buffer and 900 μL dry methanol in a 1.5 mL microcentrifuge tube.
2. Mix well.
3. Keep the tube on ice in the dark for 5 min, to extract the chlorophyll.
4. Precipitate insoluble materials in a precooled microcentrifuge for 3 min at maximum speed.
5. Measure the absorbance of the supernatant at 665 nm, and calculate the Chl-*a* concentration by using the following formula: $[\text{Chl-}a] = (A_{665}) \times 12.7 \times 100$ (*see* **Note 14**).

3.3 Heterocyst Breakage and Thylakoid Membrane Isolation

1. Adjust the sample volume to fit in the cell disruption chamber, for the Parr cell bomb this is typically 30–40 mL, by adding Heterocyst isolation buffer.
2. Add protease inhibitor cocktail according to the manufacturer's specifications (*see* **Note 2**).
3. Add a "spatula tip" of DNAse-I (optional, *see* **Note 3**).
4. Pour the cell suspension into the chamber, close, and raise the N_2 pressure to 170 bar.
5. Wait 5 min for the gas pressure to equilibrate in the heterocysts.
6. Slowly release the suspension dropwise through the bottom valve. Collect the suspension in an Erlenmeyer flask (*see* **Note 15**).
7. Repeat **steps 4–6** four times, i.e., so that the cell suspension has been pressurized and decompressed five times in total.
8. Precipitate heavy particles and cell debris by centrifugation at 6,000 × *g* for 15 min at 4 °C.
9. Keep the supernatant and discard the pellet.
10. Double the volume of the suspension by adding Heterocyst isolation buffer.
11. Precipitate the thylakoid membranes by ultracentrifugation at 150,000 × *g* for 30 min at 4 °C.

12. Discard the supernatant and resuspend the thylakoid membranes in a small amount of Heterocyst isolation buffer, using a glass rod as a homogenizer (*see* **Note 16**).
13. Store the thylakoid membranes at −80 °C (*see* **Note 13**).

4 Notes

1. We have found that it is best to prepare this solution fresh directly before use.
2. We recommend using commercial premade mixes of protease inhibitors for consistent results. A typical such cocktail, optimized for bacterial cells, contains inhibitors of serine proteases (e.g., AEBSF; 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride), cysteine proteases (e.g., the epoxide E-64), aspartic proteases (e.g., Pepstatin), and aminopeptidases (e.g., Bestatin).

   Alternatively, inhibitors can be added individually. For an affordable mix, add to a final concentration of 1 mM of PMSF or AEBSF, 1 mM benzamidine, 1 μg/mL Pepstatin, and 10 μg/mL Leupeptin. We do not recommend using EDTA for this step.
3. A few μg (i.e., a "spatula tip") of DNAse-I can be added as powder directly to the cell suspension immediately after cell disruption. This is not essential for the stability of the proteins, but improves the band resolution on BN acrylamide gels, as DNA can cause smearing.
4. We find that nitrogen cavitation (nitrogen decompression), such as in the Parr Instruments Cell Bomb, is the best and most affordable cell disruption method for retaining membrane structure and enzyme activity [6]. Alternatively, a temperature-controlled cell disruptor from Constant Systems Ltd. will give a high yield of active membranes. A "bead beater" may also be used, but the results thereof are less reliable. We advise against older instruments methods using mechanical pressure, such as in a "French press," due to the heat that develops during this treatment.
5. *N. punctiforme* is grown in $BG11_0$-medium [7] for 6–7 days: Two 1.5 L batch cultures are grown under constant stirring and illumination at 50 $\mu E/m^2$ s, sparged with air enriched with 3% CO_2.
6. We find that this increases the efficiency of the lysozyme treatment.
7. Shorter times than 1 h may be insufficient to weaken the vegetative cells completely. After a successful lysozyme treatment the cell suspension turns from slightly viscous and dark

green, to more watery and reddish in color due to the release of phycobilisomes.

8. Be careful to keep test tubes in a plastic container filled with ice at all time during sonication. The period between sonication intervals should be 10 s to allow the sample to cool.
9. The sonication will break vegetative cells and leave heterocysts intact.
10. Under normal white light, the heterocyst pellet should appear light green. If intact filaments are present, these will be at the bottom of the centrifuge tube (under the heterocysts) and dark green to black in color (Fig. 2).
11. After the final washing step the supernatant should be completely colorless, and the isolated heterocysts should be light green.
12. To further assess the purity of the isolated heterocysts, confocal microscopy may be used to discern between heterocysts and intact vegetative cells [5]. Quantitative immunoblot analysis, using antibodies against rubisco and nitrogenase subunits, may be used to quantify the purity of isolated heterocysts, in comparison to whole filaments.

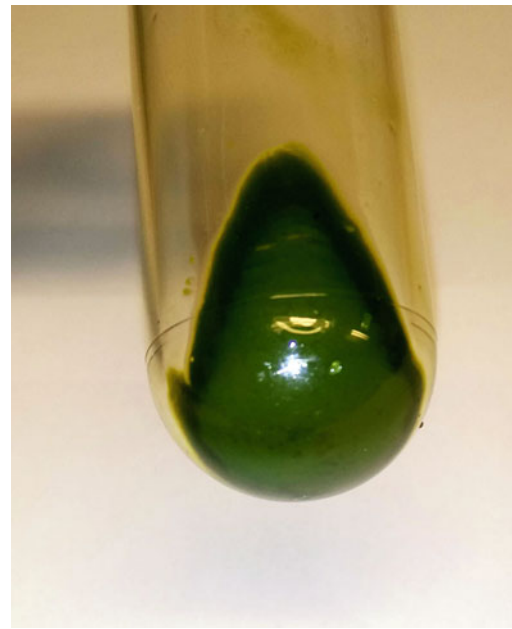

Fig. 2 A centrifuge tube containing precipitated heterocysts and vegetative cells, after sonication of the culture. The heterocysts constitute the *lighter green* fraction at the bottom of the tube, which can be distinguished from the *darker green* vegetative cell fraction that remains due to incomplete cell breakage

13. When the samples are to be frozen at −80 °C, it is better to keep the concentration as high as possible: around 0.5–1 mg of Chl-*a*/mL is preferable.
14. The mass extinction coefficient at 665 nm is $\varepsilon = 0.07874$ mL/mg cm, as described in [8]. Multiplication by 100 is made to correct for dilution of the sample.
15. Open the valve slowly to avoid spurting.
16. If the culture volume was 3 L initially, the pellet should be resuspended in around 250–500 μL to keep a good concentration. *See* **Note 11** above.

5 Advise on Blue-Native Gel Electrophoresis of Thylakoid Membrane Proteins

We have utilized blue-native (BN) gel electrophoresis, together with SDS-PAGE, to separate membrane proteins in two dimensions, to prepare for protein analysis by mass spectrometry. Well-written protocols for these can be found elsewhere [9–11].

However, when preparing samples for the BN gel, it is important to use the right type and amount of detergent, in order to get good separation of protein complexes. In several papers, the detergent that has yielded the most consistent results is *n*-dodecyl-β-D-maltoside (DDM).

Since a large portion of the thylakoid membrane proteome consists of photosynthetic proteins, the concentration of Chlorophyll-*a* may be used as a point of reference when setting the sample concentration. Thus, a "typical" BN-PAGE protocol for thylakoid membranes includes solubilization at a chlorophyll concentration of 0.5 mg/mL, and a DDM concentration of 1–2% [5, 12, 13]. However, we do recommend trying a range of DDM concentrations. The different protein complexes in the thylakoid membrane will be solubilized to varying degrees at a given detergent concentration. Depending on which protein complex is of interest, the amount of DDM will therefore have to be fine-tuned to fit the purpose [14].

As with the thylakoid isolation procedures, it is important to perform all the steps, from sample preparation to running the BN-PAGE, at low temperature (0–4 °C) and under dim green light. SDS-PAGE (the second dimension) may be performed at room temperature and ambient light, since this leads to denaturation of the proteins.

References

1. Muro-Pastor AM, Hess WR (2012) Heterocyst differentiation: from single mutants to global approaches. Trends Microbiol 20:548–557
2. Curatti L, Flores E, Salerno G (2002) Sucrose is involved in the diazotrophic metabolism of the heterocyst-forming cyanobacterium *Anabaena* sp. FEBS Lett 513:175–178
3. López-Igual R, Flores E, Herrero A (2010) Inactivation of a heterocyst specific invertase indicates a principal role of sucrose catabolism in the heterocysts of *Anabaena* sp. J Bacteriol 192:5526–5533
4. Magnuson A, Cardona T (2016) Thylakoid membrane function in heterocysts. BBA-Bioenergetics 1857:309–319
5. Cardona T, Battchikova N, Zhang PP, Stensjö K, Aro EM, Lindblad P, Magnuson A (2009) Electron transfer protein complexes in the thylakoid membranes of heterocysts from the cyanobacterium *Nostoc punctiforme*. BBA-Bioenergetics 1787:252–263
6. Simpson RJ (2010) Disruption of cultured cells by nitrogen cavitation, Cold Spring Harb Protoc 2010. doi:10.1101/pdb.prot5513
7. Rippka R, Deruelles J, Waterbury JB, Herdman M, Stonier RY (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 111:1–61
8. Meeks J, Castenholtz R (1971) Growth and photosynthesis in an extreme thermophile, *Synechococcus lividus* (Cyanophyta). Arch Mikrobiol 78:25–41
9. Schägger H, vonJagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166:368–379
10. Schägger H, vonJagow G (1991) Blue native electrophoresis for isolation of membrane protein complexes in ezymatically active form. Anal Biochem 199:223–231
11. Wittig I, Braun HP, Schägger H (2007) Blue native PAGE. Nat Protoc 1:418–428
12. Prommeenate P, Lennon AM, Markert C, Hippler M, Nixon PJ (2004) Subunit composition of NDH-1 complexes of *Synechocystis* sp. PCC 6803. J Biol Chem 279:28165–28173
13. Danielsson R, Suorsa M, Paakkarinen V, Albertsson PA, Styring S, Aro EM, Mamedov M (2006) Dimeric and monomeric organization of photosystem II – distribution of five distinct complexes in the different domains of the thylakoid membrane. J Biol Chem 281:14241–14249
14. Watanabe M, Kubota H, Wada H, Narikawa R, Ikeuchi M (2010) Novel supercomplex organization of photosystem I in *Anabaena* and *Cyanophora paradoxa*. Plant Cell Physiol 52:162–168

Chapter 10

Targeted Quantification of Isoforms of a Thylakoid-Bound Protein: MRM Method Development

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Abstract

Targeted mass spectrometric methods such as selected/multiple reaction monitoring (SRM/MRM) have found intense application in protein detection and quantification which competes with classical immunoaffinity techniques. It provides a universal procedure to develop a fast, highly specific, sensitive, accurate, and cheap methodology for targeted detection and quantification of proteins based on the direct analysis of their surrogate peptides typically generated by tryptic digestion. This methodology can be advantageously applied in the field of plant proteomics and particularly for non-model species since immunoreagents are scarcely available. Here, we describe the issues to take into consideration in order to develop a MRM method to detect and quantify isoforms of the thylakoid-bound protein polyphenol oxidase from the non-model and database underrepresented species *Eriobotrya japonica* Lindl.

Key words MRM, Polyphenol oxidase, Protein isoform, Thylakoid-bound, Loquat

1 Introduction

Targeted protein detection and quantification is an essential tool in all areas of life science research and biochemical analysis. Immunoaffinity-based techniques, such as dot blot, Western blot, and ELISA, have been classically used to carry out this task in complex samples. While these techniques can be highly sensitive and quantitatively accurate, the two major hurdles in their implementation are the limited specificity and availability of antibodies. These drawbacks can be a difficult issue to resolve when the target protein belongs to a protein family encoded by several paralogs, which compromises the specificity, or when the species under study is not a model organism, thus antibody availability may be nil.

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Opposite to the time consuming and highly expensive development of monoclonal antibodies against individual target protein isoforms, targeted mass spectrometric methods such as selected/multiple reaction monitoring (SRM/MRM) provide a universal procedure to develop a fast, highly specific, sensitive, accurate, and cheap methodology for targeted detection and quantification of proteins, whatever is its biological origin and up to isoform resolution level [1].

SRM/MRM experiments are run on triple quadrupole (TQ) mass spectrometers, which have three quadrupole mass analyzers serially arranged, used as a detector of a liquid chromatography system. The first (Q1) and third (Q3) analyzers are set as filters to transmit a narrow window of fixed m/z values, whereas the second (Q2) works as a collision cell. An SRM experiment refers to monitoring the signal generated by a single-fragment ion transmitted by Q3 which results from the fragmentation in Q2 of a single-parent ion transmitted by Q1 along a chromatographic run, thus serving to detect the presence of a particular compound, i.e., a peptide, eluting from the chromatography column. The pair of m/z values which let the fragment ion reach the detector is called a transition. In complex samples of protein tryptic digests, there is a chance that two different peptide sequences produce parent and fragment ions detectable with the same transition, thus monitoring several transitions per peptide provides specificity to the experiment as all daughter fragments show co-eluting peaks in the chromatographic run. The monitoring of several transitions in the same analysis is referred to as MRM experiment.

In this chapter, we describe the issues to take into consideration in order to develop a MRM method in general and, in particular, to specifically detect and quantify isoforms of the thylakoid-bound protein polyphenol oxidase from the non-model and database underrepresented species *Eriobotrya japonica*, Lindl [2]., for which commercial immunoreagents are unavailable.

2 Materials

2.1 Sample Preparation

1. 25 mM ammonium bicarbonate (ABC).
2. 16 μg/mL sequencing grade trypsin in ABC.
3. 100 mM Iodoacetamide (IAM) in ABC.
4. 10 mM dithiotreitol (DTT) in ABC.
5. SDS-PAGE reagents and device for 10 × 10 cm gels electrophoresis [3].
6. CBB, Coomasie brilliant blue staining reagent (Sigma-Aldrich).
7. C18 Spin cartridges (Thermo-Fischer).

8. Protein standards: purified recombinant fragments of three loquat (*Eriobotrya japonica*) polyphenol oxidase (PPO) isoforms [2].
9. In-gel protein digestion automatic system (Progest-Protein Digestion Station, Genomic Solutions).

2.2 MS Instrumentation

1. Triple quadrupole mass spectrometer operated in SRM mode: Agilent 6490 Triple Quadrupole equipped with the JetStream ion source and iFunnel technology.

2.3 Liquid Chromatography Separation

1. Liquid chromatography system: Agilent 1290 Infinity UHPLC.
2. Reversed-phase C18 column: Zorbax Eclipse Plus C18 2.1 × 50 mm, 1.8 μm analytical column, operated at a 0.4 mL/min flow rate.
3. HPLC buffer A: 0.1% formic acid.
4. HPLC buffer B: 95% acetonitrile, 0.1% formic acid.

There are several triple quadrupole mass spectrometer vendors that couple their instruments to either nano-HPLC or UHPLC chromatographic systems. The data presented here were generated on an Agilent 6490 Triple Quadrupole coupled to an Agilent 1290 Infinity UHPLC system equipped with a Zorbax Eclipse Plus C18 2.1 × 50 mm, 1.8 μm analytical column, operated at a 0.4 mL/min flow rate. The mass spectrometer is equipped with JetStream ion source and iFunnel technology. In this way, the lower sensitivity delivered by the standard flow versus nanoflow chromatography is greatly compensated by the higher sample load allowed and the narrower peaks obtained with ultrahigh pressure and smaller particle diameter packed standard columns. As an advantage, LC runs are shortened several folds as compared with nano-HPLC.

3 Methods

TQ instruments carry out MRM experiments through monitoring sequentially a number of transition signals during a duty cycle, coming from just one or from many different peptides. When UHPLC separation is used, peak width can be approximately 0.1 min at baseline, thus acquiring 12 data points per each transition across a peak, which is recommended to accurately define the peak shape and thus the peak area [4], the duty cycle will last for around half second. The larger the transition list the shorter the dwell time, i.e., the time the instrument spends on acquiring each transition signal, and thus the lower the accuracy of the signal intensity measurement. Consequently, the need for an accurate quantitation constricts the number of monitored transitions per

experiment quite below the capacity of the instruments. Typically, during a standard 500 ms duty cycle at 10 ms dwell time, 50 transitions can be accurately monitored. Longer duty cycle and/or shorter dwell time allow extending the transition list at the cost of accuracy. Complete fragmentation of the parent ion in Q2 will be achieved if enough collision energy is applied without over-fragmenting, i.e., multipoint fragmentation [5]. Fortunately, optimal collision energy for peptide fragmentation follows on average a linear relationship with the m/z of the parent ion, the equation parameters being charge state- and instrument-specific, thus it can be easily calculated according to the instrument to be used [5–7].

3.1 Sample Preparation

Sample preparation for MRM needs particular considerations as compared with other MS-based protein analyses. Since peptides are the target analytes for MRM, the protein sample has to be cut down into peptides as for any other typical proteomic experiment, trypsin digestion being a good option [8], but in contrast with protein identification by mass spectrometry, MRM is essentially a quantitative technique and consequently protein extractability and digestibility are two major issues to correlate measured peptide abundance with protein abundance in the sample. Moreover, if the sample undergoes some processing prior to extraction, protein stability should also be considered or controlled. To overcome these problems spiked standards, similar to the target proteins, can be mixed with the sample as early as possible. Although ideal standards are pure isotopically labeled proteins [9], these are rarely accessible, and other strategies such as artificial proteins of concatenated tryptic peptides [10] or synthetic isotopically labeled peptides [11] are commonly used, but in the latter case information about protein losses due to extraction, digestion, and stability is missing, which have to be taken into account when interpreting quantitative results. Trypsin itself has been shown to often incur in missed cleavages due to incomplete denaturation of the protein substrates. Double digestion Lys C-trypsin can be a good solution as the former endures harsher denaturing conditions, and after dilution trypsin can cleave the resulting peptides at Arg positions [12].

Another issue with sample preparation is target protein abundance in relation to MRM detection limits. Lower abundance proteins may undergo ion suppression when present in a complex mixture that precludes its ionization and thus its detection [13]. To overcome these matrix effects, the complex protein sample may be submitted to an enrichment process such as SDS-PAGE [14] or other fractionation techniques [15, 16], prior to digestion and MRM analysis.

3.1.1 Protein Extraction

Conventional techniques for protein extraction from either original biological material or from recombinant bacterial cultures can be applied (*see* **Note 1**). In the case studied here, loquat fruit protein

extracts were prepared as described [17–19]. In parallel, PPO isoform standards were prepared by overexpressing and purifying a corresponding His-tagged protein fragment in *E. coli* [2].

3.1.2 Protein Enrichment (If Necessary)

Whole protein extracts represent a highly complex matrix for the analysis of target proteins present in low or very low abundance. Thus, enrichment of these target proteins may be necessary and gel electrophoresis is a convenient option. Here, one-dimensional SDS-PAGE was sufficient to reduce sample complexity of loquat fruit protein extracts [2]. So to have the recombinant protein standards processed in the same way as the sample, these were also electrophoresed. Gel protein bands stained with CBB of the expected Mw from both protein extracts and standards were excised with a scalpel (*see* **Note 2**).

3.1.3 Protein Digestion

In-gel tryptic digestion of excised bands was performed in a Progest automatic protein in-gel digestion station system according to the manufacturer's instructions (*see* **Note 2**). The digestion protocol [8] includes cysteine reduction with DTT and alkylation with IAM steps. The recovered peptides were vacuum evaporated and resuspended in HPLC buffer A just prior to use.

3.2 Liquid Chromatography Separation

General chromatographic conditions for peptide separation are used, i.e., 3–45% solvent B linear gradient, but the gradient length can be varied according to the column geometry. The higher resolution of UHPLC allows for minimal gradient lengths with suitable separation. For 50, 100, and 150 mm × 2.1 mm id UHPLC columns perform well with gradients from 7, 15, and 25 min respectively. The sample complexity is a major factor to choose the length of the column and the gradient so as to minimize the analysis time. For pure proteins and SDS-PAGE bands used in this case, a 7 min gradient was run in 50 × 2.1 mm column with a 1.8 μm particle size at a 0.4 mL/min flow rate.

3.3 MRM Method Setup

The design of the MRM experiment very much depends on the scope of the study. If precise and absolute quantitation of a protein is the main goal, then a limited number of their surrogate peptides can only be included monitored through few highly specific transitions in each duty cycle for the reasons given above; in addition, peptide standards have to be included in the experimental design. One option is to spike in their heavy isotopically labeled peptide version in the sample that has to be detected and monitored for quantitative reference [9–11]. The other option is to make a calibration curve with non-labeled standards that can be synthetic or be obtained by digesting the purified protein of interest, e.g., a recombinant protein [2]. If the goal is to screen large sets of peptides, either as an initial selection or to carry out relative

quantitation across multiple samples, the transition list can be largely extended and there is no need for reference peptides.

In the case shown here, absolute quantitation of three target PPO isoforms in the loquat samples is performed using external calibration curves of the surrogate peptides generated by in-gel digestion of the recombinant version of each isoform.

3.3.1 Selection of Surrogate Peptides

As a targeted hypothesis-driven quantitative analysis, the first task to set up an MRM method is to define the set of target proteins which can serve to test the hypothesis (i.e., validation/analysis of biomarkers, spatio-temporal expression of isoforms, expression of biochemical pathways or protein networks, PTM status change, etc. [1]). Then, a set of surrogate peptides of each protein has to be selected for monitoring. This and the subsequent tasks can be facilitated through the use of software packages commercialized by MS instruments vendors, although the open-source programs such as Skyline have become a reference tool in MRM method development [20] even for instrument vendors. Although every proteolytic peptide may potentially be monitored by MRM, only those that fulfill a number of requirements should be included in the method [6]. For any peptide the presence of methionine, tryptophan, cysteine, asparagine, glutamine and well as N-terminal glutamic acid should be avoided for their chemical instability either during sample preparation or MS analysis, while presence of proline is desirable for its fragmentation easiness. Peptides that are unique to a protein of interest in the sample can provide unambiguous information about the presence and abundance of such protein. These peptides were called proteotypic [21] and can be in silico predicted [22].

Here, we focus on three loquat PPO isoforms that were previously cloned and sequenced [23] as a first stage on the search for tissue, development, and shelf-life differential expression. Based on sequence alignment [24] an initial number of potential proteotypic tryptic peptides were selected. Final selection of peptides was accomplished using an empirical approach; after preparing each pure recombinant PPO isoform the tryptic peptides were analyzed by LC-MS/MS in data-dependent acquisition mode and identified in a custom-made database. Only those identified peptides that fulfill the following requirements were selected: amino acid length from 8 to 23, MS/MS spectrum acquired repeatedly, stable charge state and short retention time, in addition to being isoform-specific. As a result, a final set of seven unique peptides were selected. As one of them was repeatedly detected in two charge states, +2 and +3, both parent ions were included. The alignment of the regions containing such peptides in the three isoforms shows the sequence divergence that warrants the typicity of such peptides (Fig. 1a).

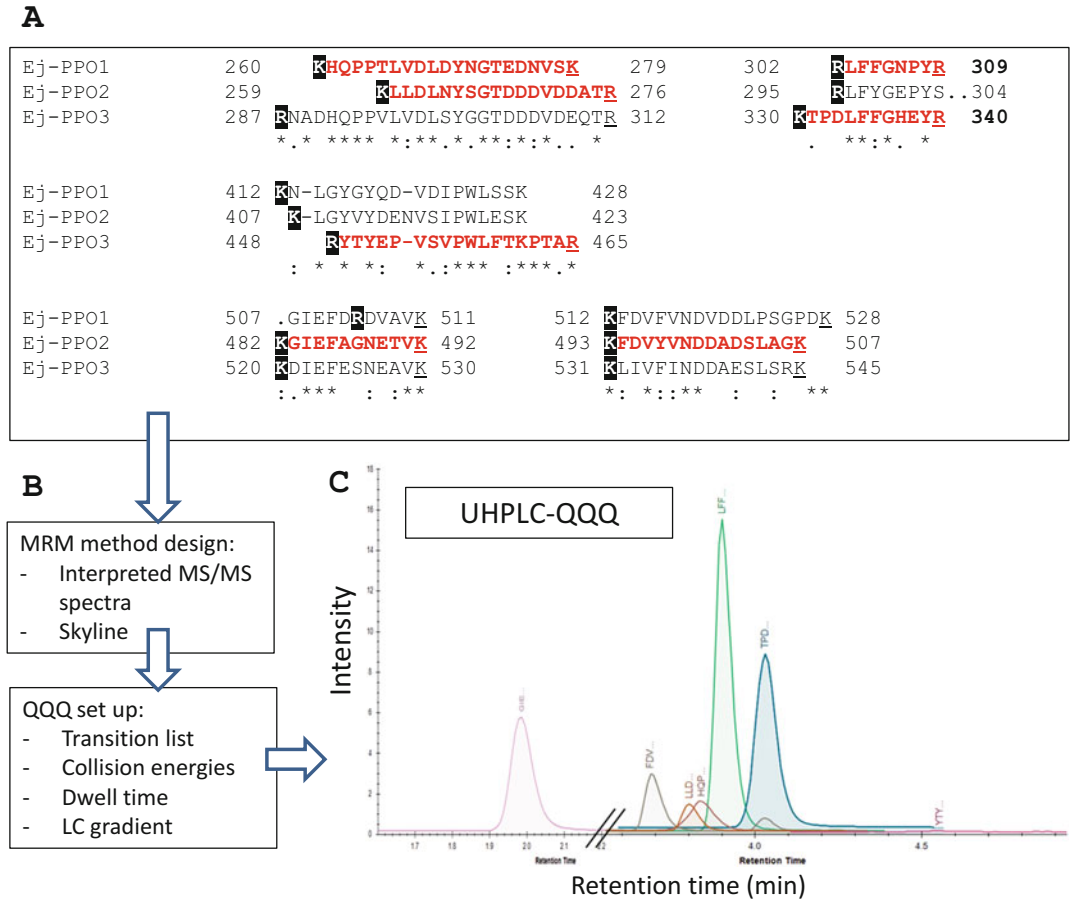

Fig. 1 Overview of the method development to quantify isoforms of polyphenol oxidase from loquat (*Eriobotrya japonica*). (**a**) Surrogate tryptic peptide selection: each paralog gene was cloned and sequenced [23] and candidate tryptic peptides were selected guided by proteotypicity, divergence of aligned sequences, general rules for amino acid composition peptide exclusion-inclusion (*see* the main text) and experimental features (MS detection frequency, charge state stability, retention time). (**b**) Transition list creation and instrument setup: using previously acquired interpreted MS/MS spectra information, transitions were selected using Skyline [20] which in turn generated the formatted list and calculated other parameters to run the method in an Agilent QQQ instrument. (**c**) Results were back imported into Skyline to review transition chromatograms for method refinement

However, depending on the quality of the signal, only a subset of proteotypic peptides become useful to provide accurate and unambiguous quantitative information. Those proteotypic peptides that are formally and quantitatively representative of a unique protein serving to carry out a robust and reproducible quantitation, have been named quantotypic peptides [25], but their finding is still a largely empirical task [26]. Data from two proteotypic peptides are considered to be the minimal information to carry out quantitative analysis of a given protein of interest [7]. Being a low

threshold, finding two proteotypic peptides may become an almost impossible mission in the case of large protein families whose paralogs have a low extent of divergence.

3.3.2 Selection of Transitions and Data Acquisition Parameters from Surrogate Peptides

Software packages may help to find transitions for each selected peptide in several ways (*see* **Note 3**).

- *"De novo" transition selection*: every possible transition is calculated for each precursor ion charge state, typically +2 and +3. This option should only be used when no experimental MS/MS information is available and can be difficult to obtain, i.e., low abundance proteins, non-model species, etc. In such a case, a number of rules to select fragment ions can be followed. The precursor and fragment *m/z* must be within the mass range of the instrument, fragment *m/z* preferably above that of the parent ion, y-type preferred over b-type fragment ions. The list of transitions per peptide will be rather long, i.e., from 7 to 10, but after a round of analysis it may reduce to the best 2 to 4.
- *MS/MS spectra-based transition selection*. Here, experimental information from MS/MS data-dependent analysis of either complex mixtures or pure protein samples is produced. As a result, interpreted MS/MS spectra are available. Ideally, these MS/MS spectra should have been acquired with the same instrument the MRM will be carried out or with an instrument whose collision cell has a similar geometry, such as a QTOF. Data from other instruments such as ion traps can be quite useful although the fragmentation pattern may change significantly. Experimentally detected proteotypic peptides are then selected and the best transitions can be either exported automatically from the search engine formatted for the particular TQ model, or selected from an already generated "de novo" transition list and then exported. This selection strategy has been followed in the case shown here.
- *Spectral library-based transition selection*. In this case interpreted MS/MS spectra deployed in public repositories and formatted as spectral libraries (*see* **Note 4**) are mined. Such experimental information is used to predict the best transitions and thus to select for including in the transition list. This is quite a good option to design transition lists for human and model-species protein samples, which benefit from the extensive worldwide research efforts on these species that have enriched the public repositories and used to create spectral libraries. Using in-house obtained interpreted MS/MS spectra a custom spectral library can be created as well and used to predict the best transitions set (*see* **Note 5**).

The transition list has to be completed with other acquisition parameters that include:

- *Transmission window resolution for Q1 and Q3*: it determines the mass range that is transmitted through the quadrupoles. *High* resolution transmits the narrowest window which lets only the monoisotopic peak pass; *Unit* resolution lets the two first peaks of the isotopic envelope pass; and *Low* resolution sets the broadest window, thus letting more isotopic peaks to pass. As resolution decreases sensitivity of the scan increases as stronger signals from more isotopic peaks are detected, but if two different ions have very close m/z values the higher sensitivity will negatively affect scan specificity. *Unit* resolution is used as default.
- *The collision energy (CE)*: at a fixed CE fragmentation will be affected by amino acid sequence, m/z and charge state, thus each transition CE needs to be optimized. So, from a starting CE value, it is increased and decreased so as to empirically select the optimum. Software packages include a tool to calculate the starting CE using linear equations whose parameters are adjusted for the instrument used and for the precursor ion charge state. CE optimization for peptides may result in modest increase of sensitivity, so CE optimization is not generally a critical issue.
- *The dwell time*: as explained above, is the time the instrument spends measuring each transition of the list during a duty cycle, and it determines the accuracy of the signal intensity measurement. Measured intensity approaches exponentially the real value as dwell time increases, thus increasing dwell times beyond a threshold value may not contribute significantly to the measurement accuracy. This compromised time is around 10 ms in our instrument that allows for accurately measuring 50 transitions per duty cycle. When the transition list is significantly larger, it is recommended to split it into several lists that will be tested in successive sample injections, thus enough sample supply has to be assured.

Table 1 shows the final result of the designed MRM method. Once the transition list and its corresponding parameters are entered in the equipment control system, the MRM experiment can be started (Fig. 1b). As seen in Fig. 1c, the overlapping of peptide-specific transitions traces provides evidence of the presence of each of the seven peptides analyzed. Likewise, for a particular PPO isoform a peptide stands over other in terms of signal intensity, thus being a candidate for quantotypic peptide.

3.4 Optimizing/ Validating the MRM Method

The set of transitions selected for each peptide has to be validated for specificity and sensitivity, which eventually may need the optimization of CE.

A major evidence for transition specificity is the co-elution if they come from the same precursor ion and thus from the same

Table 1
MRM method for *Eriobotrya japonica* Lindl. PPO isoform detection and quantification

Q1	Q3	Ion name	Isoform	Sequence	Dwell time	CE	RT
748.03	849.39	y8	PPO1	HQPPTLVDLDYNGTEDNVSK	10	22.1	3.84
748.03	562.28	y5	PPO1	HQPPTLVDLDYNGTEDNVSK	10	22.1	3.84
748.03	501.27	b9	PPO1	HQPPTLVDLDYNGTEDNVSK	10	22.1	3.84
748.03	640.32	b11	PPO1	HQPPTLVDLDYNGTEDNVSK	10	22.1	3.84
507.26	753.37	y6	PPO1	LFFGNPYR	10	16.7	3.9
507.26	606.30	y5	PPO1	LFFGNPYR	10	16.7	3.9
507.26	549.28	y4	PPO1	LFFGNPYR	10	16.7	3.9
507.26	435.24	y3	PPO1	LFFGNPYR	10	16.7	3.9
582.80	865.44	y8	PPO2	GIEFAGNETVK	10	19.1	1.99
582.80	647.34	y6	PPO2	GIEFAGNETVK	10	19.1	1.99
582.80	347.23	y3	PPO2	GIEFAGNETVK	10	19.1	1.99
582.80	518.26	b5	PPO2	GIEFAGNETVK	10	19.1	1.99
814.88	1104.52	y11	PPO2	FDVYVNDDADSLAGK	10	26.3	3.7
814.88	1005.45	y10	PPO2	FDVYVNDDADSLAGK	10	26.3	3.7
814.88	776.38	y8	PPO2	FDVYVNDDADSLAGK	10	26.3	3.7
814.88	590.31	y6	PPO2	FDVYVNDDADSLAGK	10	26.3	3.7
999.45	1266.51	y12	PPO2	LLDLNYSGTDDDVDDATR	10	32	3.81
999.45	1179.48	y11	PPO2	LLDLNYSGTDDDVDDATR	10	32	3.81
999.45	906.38	y8	PPO2	LLDLNYSGTDDDVDDATR	10	32	3.81
999.45	791.35	y7	PPO2	LLDLNYSGTDDDVDDATR	10	32	3.81
691.33	955.44	y7	PPO3	TPDLFFGHEYR	10	22.4	4.03
691.33	808.37	y6	PPO3	TPDLFFGHEYR	10	22.4	4.03
691.33	661.31	y5	PPO3	TPDLFFGHEYR	10	22.4	4.03
691.33	1044.48	b9	PPO3	TPDLFFGHEYR	10	22.4	4.03
461.22	808.37	y6	PPO3	TPDLFFGHEYR	10	11.8	4.03
461.22	640.81	y10	PPO3	TPDLFFGHEYR	10	11.8	4.03
461.22	592.28	y9	PPO3	TPDLFFGHEYR	10	11.8	4.03
461.22	534.77	y8	PPO3	TPDLFFGHEYR	10	11.8	4.03
1078.06	1402.78	y12	PPO3	YTYEPVSVPWLFTKPTAR	10	34.4	4.55
1078.06	1216.68	y10	PPO3	YTYEPVSVPWLFTKPTAR	10	34.4	4.55

(continued)

Table 1
(continued)

Q1	Q3	Ion name	Isoform	Sequence	Dwell time	CE	RT
1078.06	820.47	y7	PPO3	YTYEPVSVPWLFTKPTAR	10	34.4	4.55
1078.06	673.40	y6	PPO3	YTYEPVSVPWLFTKPTAR	10	34.4	4.55

The method was generated using Skyline [20]. Collision energies are calculated for an Agilent triple quadrupole. Retention times were experimentally determined using a 7 min linear gradient of 3–45% acetonitrile containing 0.1% formic acid at 0.4 mL/min flow rate in a 50 × 2.1 mm Zorbax Eclipse Plus C18 column with a 1.8 μm particle size

peptide. As seen in Fig. 2, two left columns, a number of fragments coming from the same precursor m/z display a co-eluting peak. In the central row chromatograms, two additional signals are seen, but these are produced by individual transitions that occur at different retention times by chance, and thus can be discarded as true signals of the target peptide. Also, the bottom row shows the co-elution of transitions from different parent ions that actually are different charge states of the same peptide, which is a further evidence of transition specificity. The review of overlapped transition chromatograms leads to the removal of bad quality transitions and validation of those transitions displaying high specificity and intensity.

The transition sensitivity is particularly useful for peptide and in turn, protein quantitation. Dose-response curves using standards lead to check for concentration ranges of linear response useful for quantitation in real samples. As seen in Fig. 2, two right columns, different peptides from the same protein have different response factors, but along the concentration range analyzed, all of them display an excellent linearity as judged by the correlation coefficient R^2. Per each PPO isoform the one with the highest response factor would be selected as quantotypic peptide. A comparison between the quantotypic peptides shows that the different PPO isoforms would be detected with quite a similar sensitivity, being the largest difference of 35%.

3.5 Running the MRM Method

Once the transition list has been validated, the method is ready to run samples. Since real samples can be highly complex, new unspecific signals may appear along the chromatograms that aesthetically distort the analysis result and produce undesired automatic peak selection that needs manual correction. As these signals are useless for the analysis, they can be avoided using transition retention times (RT) in several ways:

– *Scheduling the transition list*: instead of monitoring a transition list along the whole chromatographic run, sublists of transitions are monitored only during a preset, i.e., scheduled, window of RT. Knowing in advance or predicting the peptide RT [27, 28], transitions can be arranged in their corresponding window.

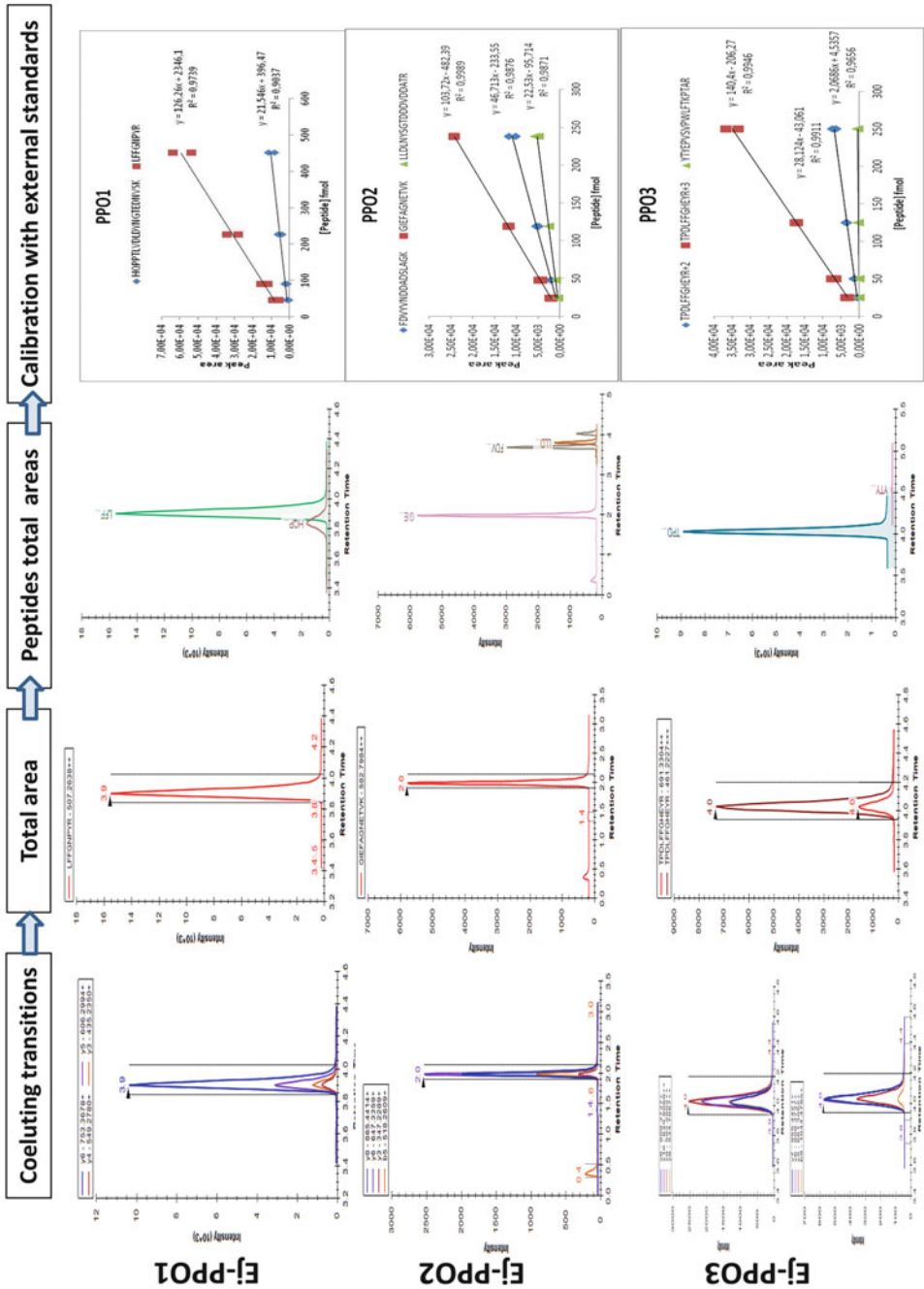

Fig. 2 Validation of the MRM method. Target peptide standards were prepared by in-gel tryptic digestion of the gel bands of His-tagged purified recombinant PPO isoforms [2]. The transitions recorded per each peptide were assessed for co-elution as an evidence of peptide detection specificity (*fist column*, which shows one peptide per protein) and manually edited if necessary to correct erroneous automatic peak assignment. Peptide quantification is based on total area of the recorded transitions (*second column*) and areas of peptides from the same protein are put together to assess for potential quantotypic peptides (*third column*). Several concentration levels and replicates of standards are injected to assess for linearity and determine response factors (*fourth column*). Each row corresponds to data from a different PPO isoform

– *Changing the transition list dynamically*: the peptide's RTs are used in a smarter way as each transition is monitored only around its expected RT. In this way, the transition list is continuously changing along the chromatographic run in a RT-dependent manner.

Both the strategies are aimed at increasing the list of transitions per MRM analysis without a detrimental effect on accuracy associated with a longer duty cycle and/or a shorter dwell time. However, these functionalities are not implemented in every instrument. In the case shown here, the acquisition was eventually done in dynamic mode so as to optimize the accuracy of the analysis.

Figure 3 shows the total intensity recorded for peptides through their corresponding transitions across samples and four levels of standard concentration. Both the samples and standards were submitted to SDS-PAGE and the bands of interest excised and in-gel digested before analysis with the MRM optimized method. As seen, PPO2 isoform is highly abundant in samples of loquat fruit in different developmental and stress conditions, whereas PPO1 and PPO3 isoforms were detected only at trace level in a few samples, as can be seen in the two selected chromatograms.

4 Notes

1. Whole protein extracts from plant material must be free from pigments and polyphenolics. It is generally achieved by previously cleaning extensively the tissue. Detailed protocols derive mainly from the original description in Refs. 17, 18.
2. In order to produce quantitative information by MRM representative of the protein amount in the biological sample, whole protein bands have to be excised and digested. The band is then cut in smaller pieces to perform digestion.
3. Detailed explanation on how to determine and select transitions is provided through Manuals and Tutorials in the corresponding software websites. For the case of Skyline used here, these can be found in https://skyline.gs.washington.edu/labkey/project/home/software/Skyline/begin.view.
4. Some public repositories of large spectral libraries: Peptide Atlas http://www.peptideatlas.org/speclib/; NIST http://peptide.nist.gov/; Global Proteome Machine ftp://ftp.thegmp.org/projects/xhunter/libs/.
5. This can be done using the tool Bibliospec (https://skyline.gs.washington.edu/labkey/project/home/software/BiblioSpec/begin.view) as an operation separated or within Skyline.

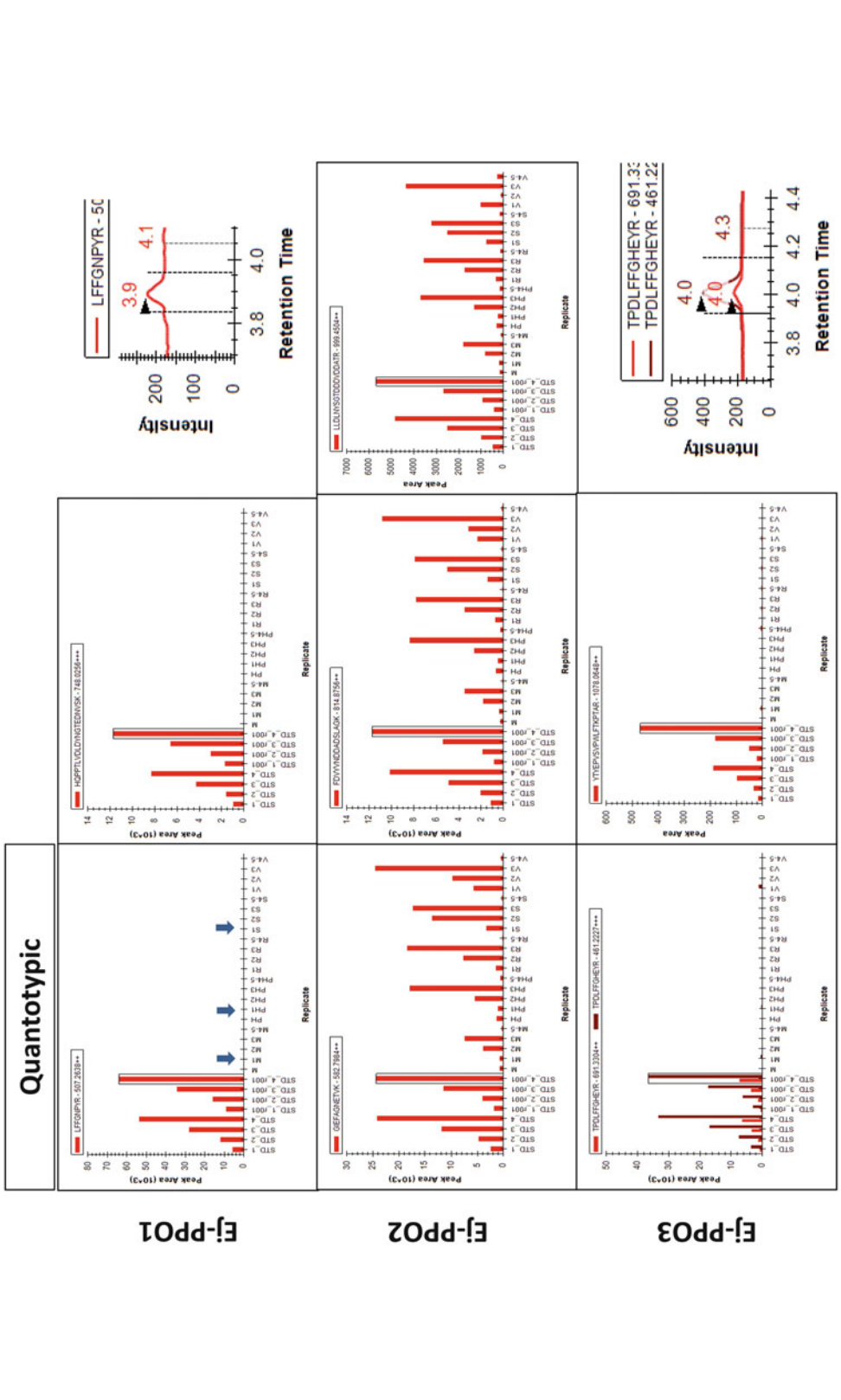

Fig. 3 Application of the MRM method to real samples. Total protein was extracted from loquat fruit samples in different conditions of developmental stage (V, R, S), post-harvest (PH) and bruising (M). After SDS-PAGE several bands of each sample covering a range of Mw from 59 to 66 kDa were excised for MRM analysis. PPO2 is present in all samples and in different gel bands at a level that can be quantified using the standards (STD). PPO1 and PPO3 were only detected as traces in some samples, as seen in the selected chromatograms

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References

1. Picotti P, Aebersold R (2012) Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions. Nat Methods 9(6):555–566
2. Martínez-Márquez A, Morante-Carriel JA, Sellés-Marchart S, Martínez-Esteso MJ, Pineda-Lucas JL, Luque I, Bru-Martínez R (2013) Development and validation of MRM methods to quantify protein isoforms of polyphenol oxidase in loquat fruits. J Proteome Res 12:5709–5722
3. Simpson RJ (2003) One-dimensional polyacrylamide gel electrophoresis. In: Simpson RJ (ed) Proteins and proteomics, a laboratory manual. CSHL Press, Cold Spring Harbor, New York, pp 39–91
4. Heftmann E (2004) Chromatography: fundamentals and applications of chromatography and related differential migration methods – Part A: Fundamentals and techniques, 6th edn. Elsevier Science, The Netherlands
5. Lange V, Picotti P, Domon B, Aebersold R (2008) Selected reaction monitoring for quantitative proteomics: a tutorial. Mol Syst Biol 4:222
6. James A, Jorgensen C (2010) Basic design of MRM assays for peptide quantification. Methods Mol Biol 658:167–185
7. Kiyonami R, Domon B (2010) Selected reaction monitoring applied to quantitative proteomics. Methods Mol Biol 658:155–166
8. Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 68:850–858
9. Brun V, Dupuis A, Adrait A, Marcellin M, Thomas D, Court M et al (2007) Isotope-labeled protein standards: toward absolute quantitative proteomics. Mol Cell Proteomics 6:2139–2149
10. Beynon RJ, Doherty MK, Pratt JM, Gaskell SJ (2005) Multiplexed absolute quantification in proteomics using artificial QCAT proteins of concatenated signature peptides. Nat Methods 2:587–589
11. Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. Proc Natl Acad Sci U S A 100(12):6940–6945
12. Saveliev S, Bratz M, Zubarev R, Szapacs M, Budamgunta H, Urh M (2013) Trypsin/Lys-C protease mix for enhanced protein mass spectrometry analysis. Nat Methods 10:i–ii
13. Jessome LL, Volmer DA (2006) Ion suppression: a major concern in mass spectrometry. LCGC N Am 24:498–510
14. Lehmann U, Wienkoop S, Tschoep H, Weckwerth W (2008) If the antibody fails – a mass western approach. Plant J 55:1039–1046
15. Halvey PJ, Ferrone CR, Liebler DC (2012) GeLC-MRM quantitation of mutant KRAS oncoprotein in complex biological samples. J Proteome Res 11:3908–3913
16. Song J, Du L, Li L, Palmer LC, Forney CF, Fillmore S, Zhang Z, Li X (2015) Targeted quantitative proteomic investigation employing multiple reaction monitoring on quantitative changes in proteins that regulate volatile biosynthesis of strawberry fruit at different ripening stages. J Proteome 126:288–295
17. Sellés-Marchart S, Luque I, Casado-Vela J, Martínez-Esteso MJ, Bru-Martínez R (2008) Proteomics of multigenic families from species underrepresented in databases: the case of loquat (Eriobotrya Japonica Lindl.) polyphenol oxidases. J Proteome Res 7:4095–4106
18. Wang W, Scali M, Vignani R, Spadafora A, Sensi E, Mazzuca S, Cresti M (2003) Protein extraction for two-dimensional electrophoresis from olive leaf, a plant tissue containing high levels of interfering compounds. Electrophoresis 24:2369–2375
19. Hurkman WJ, Tanaka CK (1986) Solubilization of plant membrane proteins for analysis by

two-dimensional gel electrophoresis. Plant Physiol 81(3):802–806

20. MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, Kern R, Tabb DL, Liebler DC, MacCoss MJ (2010) Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics 26:966–968
21. Kuster B, Schirle M, Mallick P, Aebersold R (2005) Scoring proteomes with proteotypic peptide probes. Nat Rev Mol Cell Biol 6 (7):577–583
22. Mallick P, Schirle M, Chen SS, Flory MR, Lee H, Martin D, Ranish J, Raught B, Schmitt R, Werner T, Kuster B, Aebersold R (2007) Computational prediction of proteotypic peptides for quantitative proteomics. Nat Biotechnol 25:125–131
23. Morante-Carriel JA (2012) Ph.D. thesis, Universidad de Alicante
24. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948
25. Brownridge P, Holman SW, Gaskell SJ, Grant CM, Harman VM, Hubbard SJ, Lanthaler K, Lawless C, O'Cualain R, Sims P, Watkins R, Beynon RJ (2011) Global absolute quantification of a proteome: challenges in the deployment of a QconCAT strategy. Proteomics 11 (15):2957–2970
26. Worboys JD, Sinclair J, Yuan Y, Jørgensen C (2014) Systematic evaluation of quantotypic peptides for targeted analysis of the human kinome. Nat Methods 11(10):1041–1044
27. Krokhin OV, Craig R, Spicer V, Ens W, Standing KG, Beavis RC, Wilkins JA (2004) An improved model for prediction of retention times of tryptic peptides in ion pair reversed-phase HPLC: its application to protein peptide mapping by off-line HPLC-MALDI MS. Mol Cell Proteomics 3:908–919
28. Escher C, Reiter L, MacLean B, Ossola R, Herzog F, Chilton J, MacCoss MJ, Rinner O (2012) Using iRT, a normalized retention time for more targeted measurement of peptides. Proteomics 12(8):1111–1121

Chapter 11

Sample Preparation for Analysis of the Plant Mitochondrial Membrane Proteome

Christine Schikowsky, Beate Thal, Hans-Peter Braun, and Holger Eubel

Abstract

Containing plastids and vacuoles in addition to those organelles also found in other (heterotrophic) cells, the plant cell displays an extraordinary level of compartmentalization, largely obtained by the utilization of membranes. These membranes not only confine reaction spaces but must also facilitate cross-talk between organelles and other cell compartments. They also host important components of the plant energy metabolism, i.e., the electron transport chains of mitochondria and chloroplasts. Characterization of the proteomes of these membranes requires isolation of pure and intact organelles from plant tissues followed by subsequent purification of their respective membranes. Membrane fractions are then amenable for further analyses using gel electrophoresis procedures or gel-free proteomic approaches. Here, we describe the preparation of intact mitochondria from *Arabidopsis thaliana* cell-culture, the isolation of outer and inner mitochondrial membranes and downstream proteomic applications for analyzing their membrane protein content.

Key words Mitochondria, Isolation, Subfractionation, BN-PAGE, Trypsin digestion, Mass spectrometry

1 Introduction

Mitochondria are coated by two distinct membranes, the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). The OMM surrounds the organelle and mainly functions in controlling the transport of proteins into the organelle. The folding of the IMM into cristae leads to an increase of membrane area and facilitates accommodation of higher amounts of the protein complexes involved in oxidative phosphorylation (OXPHOS). A substantial proportion of the adenosine triphosphate (ATP) consumed by the plant cell is produced by the action of these protein complexes. Recently, new results on the protein-protein interactions of OXPHOS subunits provided interesting insights into the functioning of the branched electron transport

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chain of plants [1–3]. Analyzing the protein content especially of the inner mitochondrial membrane and the association patterns therefore is a promising approach to investigate processes related to key mitochondrial functions.

The first crucial step is the isolation of pure mitochondrial fractions, a task that is particularly challenging from leaf material due to the strong presence of chloroplasts in these organs. For the analysis of leaf mitochondria, it is advisable to use young rosette leaves that are easier to disrupt and allow a better extraction of intact mitochondria. *Arabidopsis* plants grown hydroponically on wire mesh [4] may also be used for this. These systems offer the potential to harvest green shoots and non-green roots at the same time and allow a direct comparison of their mitochondria. Non-green organs, such as roots, usually yield isolates of higher purity. However, due to the absence of photosynthesis and photorespiration in these organs, their mitochondria have different functions that are expressed in their protein content [5]. As an alternative to roots, cell or callus cultures grown in darkness can be used. An advantage of the cell culture is the ease with which cells can be manipulated by effector molecules or subjected to other treatments. In addition, these cells also possess softer cell walls making them less resistant to mechanical disruption. The selection of plant material therefore has a profound impact on mitochondrial yield and purity and is an important factor to be considered for the experimental setup. In terms of organelle isolation we here focus on the procedure used in our laboratory for the preparation of mitochondria from cell suspension cultures. While this method may require modification for other plant material, the downstream procedures described in this chapter (subfractionation, BN/SDS-PAGE, MS sample preparation) are transferable.

The procedure of isolating mitochondria can be broken up into three parts: cell disruption, differential centrifugation (to gain crude mitochondria), and density gradient centrifugation (to further eliminate organellar contaminants) [6, 7]. For analyzing the total mitochondrial membrane proteome (IMM and OMM) subpartitioning into membrane and soluble fraction can be performed. In-depth analyses of OMM and IMM proteomes require removal of the OMM from intact mitochondria by hypo-osmotic conditions causing swelling and OMM rupture [7]. At the same time, the cristae membranes connected to the inner boundary membrane (collectively denominated as IMM in the following) grant a higher degree of elasticity to this membrane system, thereby retaining its integrity under the conditions applied. OMM fragments and the remaining mitoplasts (IMM and matrix) can now be separated by centrifugation [7]. To disrupt the mitoplasts and isolate the IMM, a combination of swelling and sonication is applied, followed by centrifugation to pelletize the IMM. It should be noted that contact sites between OMM and IMM [8] prevent quantitative

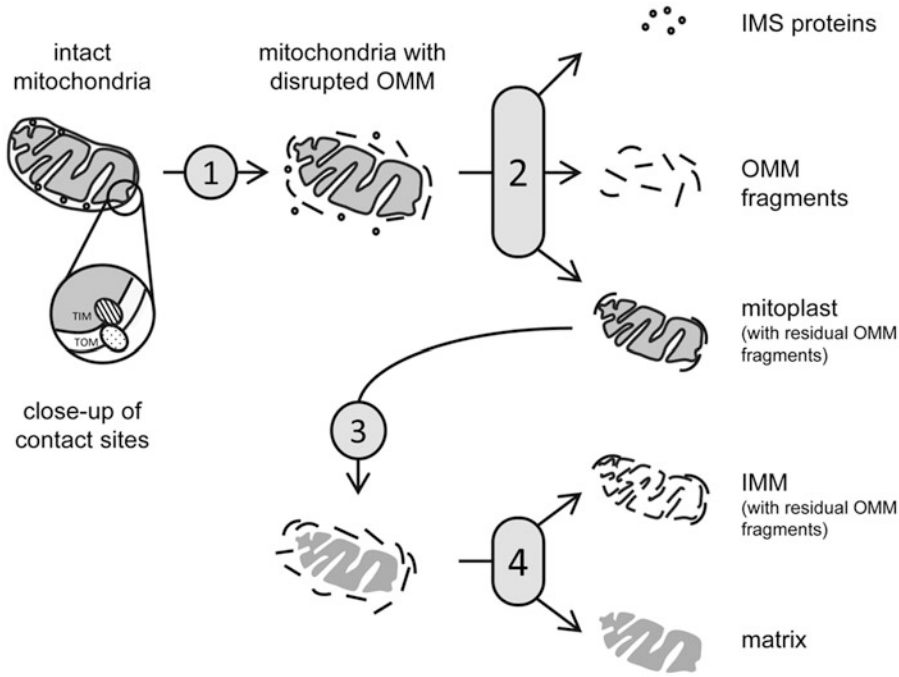

Fig. 1 Subfractionation of isolated mitochondria. Rupture of the outer mitochondrial membrane (OMM) caused by osmotic swelling (*1*) releases OMM fragments as well as proteins of the intermembrane space (IMS, small circles). However, the removal of the OMM is not quantitative since contact sites between the inner mitochondrial membrane (IMM) and the OMM tether OMM fragments to the IMM, which is mediated by the protein import pore consisting of the translocases of the inner and outer mitochondrial membranes (TIM and TOM). OMM fragments (most likely forming vesicles) can be removed from mitoplasts by centrifugation in a sucrose density gradient (*2*), which also produces a top phase enriched in IMS. Mitoplasts (mitochondrial matrix surrounded by IMM) can be subfractionated by further swelling and sonication (*3*) and centrifugation (*4*). IMM fractions will, however, be contaminated with OMM to some degree

removal of the outer membrane from the mitoplasts. Hence, while it is possible to obtain OMM fractions largely devoid of IMM, this does not apply vice versa.

Proteomic analyses can therefore be performed either focusing on a total mitochondrial membrane fraction, the OMM or the (OMM-contaminated) IMM (Fig. 1). Two approaches aiming at either the general identification of mitochondrial membrane proteomes or the analysis of protein-protein interactions with IMM and OMM will be presented here. Shot-gun mass spectrometry enables the identification of thousands of proteins in a given sample and is therefore an appropriate tool for investigating entire proteomes, including those of the mitochondrial membranes [9, 10]. The key for successful identification of membrane proteins is the sample preparation. Sodium dodecyl sulfate (SDS) is widely used for solubilization of membrane proteins prior to MS analysis. However, SDS is not compatible with enzymatic digestion of proteins due to its highly denaturing properties and must be depleted prior

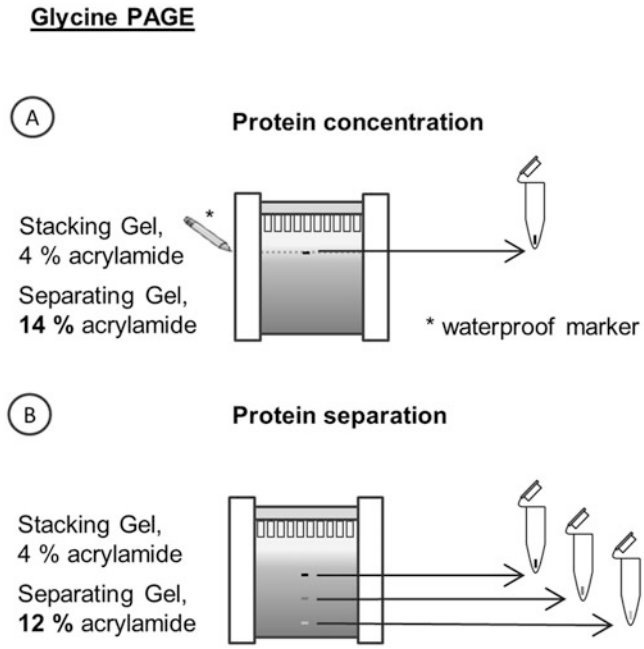

Fig. 2 Utilization of glycine PAGE for preparation of shot-gun mass spectrometry samples. Glycine gels consisting of the separating gel (*dark gray*) and the overlying stacking gel (*light gray*) can be used to deplete detergent form SDS-solubilized proteins and prepare them for mass spectrometry in two ways. Proteins can be concentrated at the border between stacking and separation gel (**a**). For this, a separation gel with a high acrylamide concentration is used (14% instead of 12%). It is recommended to highlight the border between both gel phases with a waterproof marker on the glass plate. When the running front reaches this line the gel run is stopped and a gel slab containing both, stacking and separating gel is cut from the gel and submitted to in gel tryptic digestion. For pre-fractionation prior to MS analysis proteins are separated according to size by means of a standard glycine/SDS PAGE procedure (**b**). The gel lane is then cut out and sliced. Each gel slab is then dried, digested, and analyzed individually

to this step. Filter-aided sample preparation [11] has been successfully applied for this. Alternatively, free SDS can be depleted from samples by SDS-PAGE that may additionally be used for pre-fractionation of the sample prior to MS. If no fractionation is required, the gel run is aborted as soon as the running front reaches the separation gel (Fig. 2a). Proteins concentrate at this position and, after Coomassie staining, can be cut en bloc from the gel to be subjected to standard in-gel digestion procedures [12]. For pre-fractionation of the sample the gel is run until the desired degree of separation is achieved. The gel lane will then be cut into fine slices, each of these to be treated separately in respect to digestion and peptide extraction (Fig. 2b, [12]).

Since the proteomes of OMM and IMM are organized in multi-protein complexes to a high degree, it may be desirable to analyze the subunit compositions of these complexes. For this, a gel-based approach combining a Blue Native polyacrylamide

gelelectrophoresis (BN-PAGE) with a denaturing SDS–PAGE is a highly valuable tool [13, 14]. BN/SDS-PAGE allows the separation of native protein complexes in the first dimension [15, 16] followed by the separation of their subunits in the second dimension. It thus enables the assignment of proteins to multiprotein complexes.

2 Materials

All buffers are prepared with analytical grade chemicals and with ultra-pure deionized water (0.055 μS/cm). Stock solutions can be stored at 4 °C for several weeks. All other solutions are prepared freshly on the day of use or the day before use.

2.1 Mitochondria Isolation from Arabidopsis Cell Cultures

1. 1 M 3-(N-morpholino)propanesulfonic acid (MOPS, stock).
2. 100 mM ethylene glycol tetra-acetic acid (EGTA, pH 7,2, stock).
3. 200 mM phenylmethanesulfonylfluoride (PMSF, in EtOH, stock).
4. 10% (w/v) bovine serum albumin (BSA, stock).
5. Disruption buffer: 450 mM sucrose, 15 mM MOPS, 1.5 mM EGTA, 6 g/l PVP40, 0.2% (w/v) BSA, 10 mM sodium ascorbate, 10 mM cysteine, 0.2 mM PMSF, pH 7.4 (adjusted at 4 °C with KOH).
6. 5× Gradient buffer: 1.5 M sucrose, 50 mM MOPS, pH 7.2 (adjusted at 4 °C with KOH).
7. Percoll (GE Healthcare Life Sciences, Solingen, Germany).
8. Percoll solutions (for 3/4/6 gradients *see* Table 1).
9. Washing buffer: 300 mM sucrose, 10 mM MOPS, 1 mM EGTA, 0.2 mM PMSF, pH 7.2 (adjusted at 4 °C with KOH).
10. Resuspension buffer: 400 mM mannitol, 1 mM EGTA, 10 mM tricine, 0.2 mM PMSF, pH 7.2 (adjusted at 4 °C with KOH).

Table 1
Casting Percoll gradients (3/4/6 gradients) for mitochondria isolation

	Three gradients			Four gradients			Six gradients		
	18%	23%	40%	18%	23%	40%	18%	23%	40%
Percoll (ml)	5.4	6.9	12	8.3	10.5	18	10.8	13.8	24
H_2O (ml)	18.6	17.1	12	27.8	25.5	18	37.2	34.2	24
5× gradient buffer (ml)	6	6	6	9	9	9	12	12	12

11. Muslin cotton gauze, filter (~26 × 26 cm, mesh size: 180 μm), Miracloth (Merck Millipore, Darmstadt, Germany).
12. Waring Laboratory Blender.
13. Fine paintbrush.
14. Dounce homogenizer (15 ml).
15. Glass pipettes (1–5 ml), Pasteur pipettes with long neck (230 mm; Brand, Wertheim, Germany).
16. Ultracentrifuge tubes: clear, 25 × 89 mm (e.g., Beckman 1 × 3 ½ UC tubes; Beckman Coulter, Krefeld, Germany).
17. Swing-out rotor (e.g., Surespin 630; Thermo Fisher Scientific, Waltham, MA, USA).
18. Ultracentrifuge (e.g., Thermo Sorvall WX Ultra 80; Thermo Fisher Scientific, Waltham, MA, USA).
19. Large (approximately 500 ml) and small (approximately 50 ml) volume centrifuge tubes.
20. Fixed-angle rotors (e.g., F12-6 × 500 LEX, A27-8 × 50; Thermo Fisher Scientific, Waltham, MA, USA).
21. Refrigerated centrifuge: (e.g., Thermo Sorvall LYNX 6000; Thermo Fisher Scientific, Waltham, MA, USA).

2.2 Subfractionation into Mitochondrial Membranes and Matrix

1. 100 mM ethylene glycol tetra-acetic acid (EGTA, pH 7,2, stock).
2. 200 mM phenylmethanesulfonylfluoride (PMSF, in EtOH, stock).
3. Resuspension buffer without mannitol: 1 mM EGTA, 10 mM tricine, 0.2 mM PMSF, pH 7.2 (adjusted at 4 °C with KOH).
4. Sonicator (probe with 3–5 mm diameter).
5. Ultracentrifuge.

2.3 Subfractionation into Outer Mitochondrial Membrane and Mitoplasts

1. 100 mM ethylene glycol tetra-acetic acid (EGTA, pH 7,2, stock).
2. 200 mM phenylmethanesulfonylfluoride (PMSF, in EtOH, stock).
3. 100 mM potassium dihydrogen phosphate (KH_2PO_4, pH 7.2 by titration with 100 mM potassium hydrogen phosphate, K_2HPO_4, stock).
4. Swelling buffer: 5 mM KH_2PO_4 (pH-adjusted stock), 0.2 mM PMSF.
5. 10× Gradient buffer: 10 mM EDTA, 100 mM MOPS, 1 mM PMSF, pH 7.2 (adjusted at 4 °C with KOH).
6. Solutions for sucrose gradients (*see* Table 2).

Table 2
Composition of sucrose gradient solutions for OMM isolation

Sucrose concentration (%)	Sucrose (g)	10× gradient buffer (ml)	PMSF (200 mM) (μl)	Total volume (ml)
0	0	10	100	100
15	7.5	5	50	50
32	32.0	10	100	100
60	30.0	5	50	50
70	35.0	5	50	50

7. Ultracentrifuge: Thermo Sorvall WX Ultra 80 (Thermo Fisher Scientific, Waltham, MA, USA).
8. Swinging bucket rotor: Surespin 630 (Thermo Fisher Scientific, Waltham, MA, USA).
9. Ultracentifuge tubes: Ultra-Clear Centrifuge Tubes, 14 × 95 mm (Beckman Coulter, Krefeld, Germany).

2.4 Subfractionation into Inner Mitochondrial Membrane and Matrix

1. 100 mM ethylene glycol tetra-acetic acid (EGTA, pH 7.2, stock).
2. 200 mM phenylmethanesulfonylfluoride (PMSF, in EtOH, stock).
3. Swelling buffer: 10 mM tricine, 1 mM EGTA, 1 mM PMSF, pH 7.3 (adjusted at 4 °C with NaOH).
4. 10× Gradient buffer: 10 mM EDTA, 100 mM MOPS, 1 mM PMSF, pH 7.2 (adjusted at 4 °C with KOH).
5. Storage buffer: 10% (v/v) 10× gradient buffer, 10% (v/v) glycerol, 0.4 mM PMSF.

2.5 Gel-Based Shot-Gun-MS

1. 0.5 M Tris–HCl, pH 6.8 (stock).
2. 1.5 M Tris–HCl, pH 8.8 (stock).
3. 10% (w/v) sodium dodecyl sulfate (SDS, stock).
4. 10% (w/v) ammonium persulfate (APS, stock).
5. Tetramethylethylenediamine (TEMED).
6. 2× sample buffer: 4% (w/v) SDS, 125 mM Tris–HCl pH 6.8, 20% (v/v) glycerol.
7. 10× Tris-glycine SDS buffer: 248 mM Tris, 1.92 M glycine, 1% (w/v) SDS.
8. 1 mg/ml bromophenol blue (in 2-mercaptoethanol).
9. BioRad Protean II gel unit (BioRad, Richmond, CA, USA).

2.6 2-D BN/SDS-PAGE

1. 5× BN—cathode buffer: 250 mM tricine, 75 mM Bis-Tris, 0.1% (w/v) Coomassie blue 250 G, pH 7.0 (adjusted at 4 °C with HCl).
2. 6× BN—anode buffer: 300 mM Bis-Tris, pH 7.0 (adjusted at 4 °C with HCl).
3. 6× BN—gel buffer: 1.5 M aminocaproic acid (ACA), 150 mM Bis-Tris, pH 7.0 (adjusted at 4 °C with HCl).
4. 5% Serva blue G: 750 mM ACA, 5% (w/v) Coomassie blue 250 G.
5. Solubilization buffer without digitonin: 30 mM HEPES, 150 mM potassium acetate, 10% (v/v) glycerol, pH 7.4 (adjusted at 4 °C with HCl).
6. Digitonin (Sigma Aldrich, St. Louis, MO, USA).
7. Solubilization buffer with digitonin: 30 mM HEPES, 150 mM potassium acetate, 10% (v/v) glycerol, 5% (w/v) digitonin, pH 7.4 (adjusted at 4 °C with HCl).
8. Incubation buffer: 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol.
9. Tricine gel buffer: 3 M Tris, 0.3% (w/v) SDS, pH 8.45.
10. Tricine anode buffer: 200 mM Tris, pH 8.9.
11. Tricine cathode buffer: 100 mM Tris, 100 mM Tricine, 0.1% (w/v) SDS, pH 8.25.
12. Overlay solution: 1 M Tris, 0.1% (w/v) SDS, pH 8.45.
13. 10% (w/v) ammonium persulfate (APS).
14. Tetramethylethylenediamine (TEMED).
15. Gradient former: BioRad 485 Gradient Former (BioRad, Richmond, CA, USA).
16. Peristaltic pump: BioRad EP-1 Econo Pump (BioRad, Richmond, CA, USA).
17. Hose attached to hypodermic needle (18 G/1.2 mm diameter, length 40 mm).
18. BioRad Protean II gel unit (BioRad, Richmond, CA, USA).

2.7 Tryptic Digestion of Samples from Gel-Based Proteomics for MS

1. 100 mM ammonium bicarbonate (Ambic, NH_4HCO_3).
2. Trypsin solution: Promega Sequencing Grade Modified Trypsin (Promega, Madison, MI, USA), prepared according to the manufacturer (for example, Promega Sequencing Grade Modified Trypsin, Porcine, 20 μg, reconstituted in 100 μl Promega Trypsin Resuspension Buffer and kept at RT for 30 min). Before use the buffer is diluted in 900 μl 100 mM Ambic to yield a final concentration of 0.02 μg/μl.

3. Low-binding reaction tubes: Eppendorf Protein LoBind Tube 1.5 ml (Eppendorf, Hamburg, Germany).
4. Vacuum centrifuge: Eppendorf Concentrator plus (Eppendorf, Hamburg, Germany).

3 Methods

3.1 Mitochondria Isolation from Arabidopsis Cell Suspension Cultures

For the in-depth analysis of mitochondrial membrane proteomes organelles have to be isolated first. From these, either total membranes (Subheading 3.2) or the OMM (Subheading 3.3) and IMM (Subheading 3.4) can be prepared. For the isolation of OMM and IMM it is mandatory to use freshly prepared mitochondria.

1. For each 50 g fresh weight used produce one discontinuous Percoll gradient (*see* **Note 1**). Casting the gradients into ultracentrifuge tubes is done as follows: underlay the lightest Percoll solution with the two heavier solutions by the help of a 20 ml glass syringe attached to a big-diameter (~3 mm) injection needle. First, 10 ml of the 18% Percoll solution are transferred into a centrifuge tube. Then, 10 ml of the 23% Percoll solution are injected below the 18% solution. Finally, 10 ml of the 40% Percoll solution are placed below the 23% Percoll solution.
2. Harvest the cells by filtration through muslin cotton gauze and determine the fresh weight.

   The following steps must be performed at 4 °C or on ice.
3. Add chilled disruption buffer to the cells at a ratio of 2 ml of buffer per gram fresh weight (min. 100 ml). Homogenize the cells in a blender. The first homogenization step is done at high speed (15 s), followed by two steps at low speed (2 × 15 s). Leave 30 s intervals in between the three blending steps (*see* **Note 2**).
4. Filter the homogenate through 2–4 layers of Miracloth.
5. Centrifuge the filtrate for 5 min at 2700 × *g* and 4 °C.
6. The supernatant is centrifuged at 4 °C three times (*see* **Note 3**):
   (a) 5 min at 2,700 × *g* (discard the pellet).
   (b) 5 min at 8,300 × *g* (discard the pellet).
   (c) 10 min at 17,000 × *g* (retain the pellet, discard the supernatant).
7. Mitochondria (and other organelles) are pelletized in the last centrifugation step. Carefully suspend the mitochondria pellet in 3–5 ml of washing buffer using a fine, soft paintbrush. Transfer the mitochondria solution into a Dounce homogenizer and adjust the volume [ml] with washing buffer to a maximum of 3 ml per Percoll gradient. Homogenize the solution performing two careful strokes.

8. Carefully lay the homogenized suspension on the top of the Percoll gradients and ultra-centrifuge for 90 min at 70,000 × *g* and 4 °C.
9. Mitochondria are located in the area of the interphase between the 23% and 40% Percoll phase. Remove the 18% phase and part of the 23% phase with a vacuum water pump without disturbing the mitochondrial band. Carefully collect the mitochondria using a Pasteur pipette (*see* **Note 4**).
10. Remove Percoll by diluting the mitochondria in resuspension buffer and centrifuge for 10 min at 14,500 × *g* and 4 °C. Repeat until the mitochondrial pellet becomes stable (usually three to four times). Before each centrifugation step gently suspend organelles using a pipette.
11. Determine the weight of the mitochondria pellet after the last wash step by determining the weight of the dry tube before the last centrifugation step and after pelletizing the mitochondria and removing the supernatant.
12. Resuspend the mitochondria in 0.2–1.0 ml of resuspension buffer (depending on yield) using a pipette. Adjust final mitochondria concentration to 0.1 g mitochondria per ml (*see* **Note 5**). Use a chilled glass pipette to measure the volume of the mitochondria solution and to fill up with resuspension buffer.
13. Split the mitochondria suspension into 100 µl aliquots or continue with Subheading 3.2 or 3.3.
14. Centrifuge aliquots for 10 min at 14,300 × *g* and 4 °C. Discard the supernatant and store the pellets at −80 °C.

3.2 Subfractionation of Intact Mitochondria into Mitochondrial Membranes and Matrix

1. Using a pipette resuspend the mitochondrial pellet (from Subheading 3.1) in resuspension buffer without mannitol by adding 1 ml of resuspension buffer to 100 mg of mitochondria.
2. Sonicate the solution four times using a probe-type instrument with 1 min intervals in between.
3. Centrifuge the suspension for 7 min at 5,000 × *g* and 4 °C. Retain the supernatant and store the pellet at −80 °C (*see* **Note 6**).
4. Ultra-centrifuge the supernatant for 90 min at 150,000 × *g* and 4 °C. The supernatant includes the matrix fraction and the pellet represents the membrane fraction. Store the pellet at −80 °C.

3.3 Subfractionation of Intact Mitochondria into Outer Mitochondrial Membrane and Mitoplasts

1. Calculate the number of required gradients by first estimating the final volume of the mitochondria suspension to be loaded on the gradients as outlined in **steps 3** and **4**. Each gradient will be loaded with 3 ml of this suspension.
2. Prepare the appropriate number of discontinuous sucrose gradients (15/32/60%) sucrose by transferring 1 ml of the 60%

Table 3
Swelling buffer volume for OMM isolation

Mitochondria pellet (g)	Total volume (ml)
<0.3	6
<0.45	9
<0.6	12
<0.8	15
≥0.8	18

sucrose solution into a centrifugation tube. Overlay this solution with 4 ml of the 32% sucrose solution, then overlay with 1.5 ml of the 15% sucrose solution.

3. Suspend the freshly isolated mitochondria (from Subheading 3.1) in 6 ml of swelling buffer and incubate for 6 min (*see* **Note 7**).
4. Add the equivalent volume of swelling buffer by referring to the pellet weight as determined in **step 11** of Subheading 3.1 and Table 3.
5. Incubate on ice for additional 4 min.
6. Transfer the solution to a Dounce homogenizer and release OMM fragments by 20 careful strokes.
7. Layer 3 ml of the solution on top of each sucrose gradient and ultra-centrifuge for 60 min at 92,000 × *g* and 4 °C.
8. The outer mitochondrial membranes are located at the interphase between 15% and 32% sucrose. Mitoplasts form a band at the interphase between 32% and 60% sucrose. Collect the OMM and mitoplasts using a Pasteur pipette (*see* **Note 8**). Store mitoplast fraction at −80 °C.
9. Continue with the OMM fraction and determine the total volume. The concentration of sucrose is approximately 25%. Adjust to a final sucrose concentration of 50% by adding the 70% sucrose solution (Table 2). Determine the volume of the solution. Below 5 ml one gradient is prepared in the next step; between 5 and 10 ml two of them are required.
10. Prepare the discontinuous sucrose gradients (0%/32%/50%) by first transferring 5 ml of the 50% sucrose solution into a centrifugation tube. Overlay the solution with 5 ml of the 32% sucrose solution and then overlay with 1.5 ml of the 0% sucrose solution (*see* **Note 9**).

11. Transfer the OMM fraction to the top of the sucrose gradient and ultra-centrifuge for 5 h at 170,000 × *g* and 4 °C (overnight).
12. To sharpen the bands ultra-centrifuge for 30 min at 170,000 × *g* at 4 °C next morning.
13. The outer mitochondrial membranes are located at the interphase between 0% and 32% sucrose. Collect the OMM using a Pasteur pipette. Dilute OMM in gradient buffer containing 0% sucrose by factor 4 and ultra-centrifuge for 90 min at 140,000 × *g* and 4 °C to pelletize the OMM.
14. Remove the supernatant and store the pellet at −80 °C.

3.4 Subfractionation into Inner Mitochondrial Membrane and Matrix

1. Carefully thaw the frozen mitoplasts (from Subheading 3.3) and determine the total volume (about 2.5 ml). Transfer the mitoplasts into a fresh (>20 ml) centrifugation tube.
2. Add swelling buffer in the intervals described in Table 4. The added volume is proportional to the initial volume; values given in Table 4 are for 2.5 ml of mitoplast fraction.
3. Centrifuge for 15 min at 12,000 × *g* and 4 °C.
4. Carefully remove the supernatant (*see* **Note 10**).
5. Resuspend the pellet in 10 ml swelling buffer and sonicate for 3 s. Repeat sonication step twice leaving 2 min intervals between each repetition.
6. Centrifuge for 7 min at 5,100 × *g* and 4 °C.
7. Intact mitoplasts are found in the pellet. The inner membrane remains in the supernatant.
8. Ultra-centrifuge the supernatant for 90 min at 140,000 × *g* and 4 °C to pelletize the IMM.
9. Remove the supernatant and store the IMM pellet at −80 °C.

Table 4
Addition of swelling buffer to Mitoplast

t (min)	*V* (ml)
0	0.5
2	1
4	2
6	4
8	8
15	Centrifugation

3.5 Sample Preparation for Shot-Gun-MS

Important: Tris-glycine SDS gel must be ready before commencing with the sample preparation. For best quality this should be done 1 day in advance.

3.5.1 Sample Preparation

1. Add 2× sample buffer to your mitochondrial membranes (both membranes: 100 µl, OMM, or IMM pellet: 50 µl). Incubate for 5 min at 60 °C under mild shaking. If necessary, resuspend pellet with pipette and incubate for another 5 min.
2. Add equal amount of H_2O.
3. Centrifuge samples for 10 min at maximum speed in your benchtop centrifuge (>18,000 × *g*).
4. Determine protein concentration using a SDS-compatible protein assay (for example the BCA protein assay kit by Pierce). Produce aliquots equivalent to 50 µg of protein (*see* **Note 11**).
5. If more than one sample is to be loaded on a gel, adjust sample volumes taking the highest one as the reference. Keep final volume below 150 µl.
6. Add bromophenol blue in 2-mercaptoethanol mix to a final concentration of 5% 2-mercaptoethanol (*see* **Note 12**).

3.5.2 Tris-Glycine SDS-PAGE

1. Set up gel caster and prepare solution for the separating gel (14% acrylamide, 1.5 mm × 200 mm × 200 mm, *see* Table 5).
2. Cast the separating gel and overlay with iso-butanol (under the fume hood). To minimize disturbance of the gel phase when applying the iso-butanol tilt the assembly backwards to a near horizontal orientation and let the iso-butanol slowly run down on the inside edge of the spacer.
3. Remove the iso-butanol soon after polymerization, shortly rinse gel with H_2O, and indicate the top of the separation gel

Table 5
Composition of Tris-glycine SDS-PAGE (1.5 mm × 200 mm × 200 mm)

	Separation gel (14%)	Stacking gel (4%)
1.5 M Tris–HCl, pH 8.8	12.5 ml	–
0.5 M Tris–HCl, pH 6.8	–	4 ml
10% SDS (w/v)	0.5 ml	0.16 ml
40% acrylamide	17.5 ml	1.6 ml
H_2O	19.5 ml	10.2 ml
Σ	50 ml	16 ml
10% APS[a]	171.5 µl	100 µl
TEMED[a]	17.1 µl	10 µl

[a]Add prior to use

with a waterproof marker pen on the outside of the larger gel plate.

4. Prepare solution for the stacking gel (4% acrylamide, *see* Table 5).
5. For proper polymerization and well-formed pockets warm gel stand, solution, comb, and pipette to 37 °C, cast the gel and leave it at 37 °C until setting is complete (*see* **Note 13**). Cover the gel with cling film and store at RT until further use.
6. Prepare 1× Tris-glycine SDS buffer from 10× buffer and load sample.
7. Perform the gel run at 30 mA (max. 500 V) at room temperature (*see* **Note 14**). Gel run is finished when the bromophenol blue front reaches the separation gel (as indicated by the marker).
8. Stain the gel (for Coomassie colloidal *see* [17]) and cut bands. Dice bands to yield cubes with edge lengths of approximately 1.5–2 mm. Dry cubes in a vacuum centrifuge and store at −20 °C until further use.

3.6 2-D BN/SDS-PAGE

3.6.1 First Dimension: Blue Native PAGE

1. Prepare the gradient mixer (close all valves) as well as 4.5% and 16% acrylamide gel solutions (*see* Table 6). Precool the solutions in the gradient mixer for 10 min at −20 °C.
2. Prepare gel casting assembly using 1.5 mm spacers. Precool the gel caster at 4 °C.
3. Insert injection needle (attached to pump hose) in between gel glass plates of the gel assembly by piercing the lower rubber gasket (Fig. 3).

Table 6
Composition of gel solutions for BN-PAGE (1.5 mm × 200 mm × 200 mm)

	Chamber one 4.5%	Chamber two 16%
H_2O	15.1 ml	4.6 ml
6× BN—Gel buffer	3.5 ml	3 ml
Acrylamide (40%)	2.4 ml	7.4 ml
Glycerol (100%)	–	3.5 ml
Σ	21 ml	18.5 ml
APS (10%)[a]	95 µl	61 µl
TEMED[a]	9.5 µl	6.1 µl

[a]Add prior to use

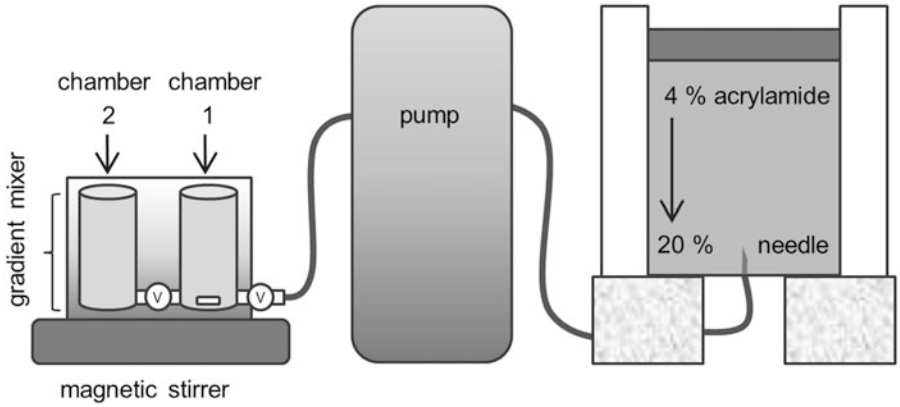

Fig. 3 Casting a Blue Native electrophoresis gel. The gradient mixer consists of two chambers linked via a valve (v). Chamber one contains a magnetic stirring bar (*white bar*). Chambers are filled with the gel solutions given in Table 6. A flexible hose (*dark gray line*) connects the gradient mixer via the pump with the gel casting device. The hose ends in a hypodermic needle that is pierced between the glass plates from below. For this purpose the gel casting device is placed on two Styrofoam blocks (or similar). Because of the pump continuously removing the light phase (low acrylamide concentration) from the first chamber, the difference in hydrostatic pressure between the chambers is forcing heavy solution (high acrylamide concentration containing glycerol) from the second chamber into chamber 1 where it mixes with the light solution. As such, acrylamide and glycerol concentrations are increasing constantly in the first chamber and the light solution initially pumped between the glass plates is continuously underlaid with heavier gel solution

4. Inject a small volume of water (~5 ml) using the pump. This will act as an overlay solution.
5. Let the pump draw some air to create diffusion barrier between the water and the gel solutions.
6. Connect gradient mixer to pump tubing. Add APS and TEMED to both chambers and mix thoroughly while keeping both the valves of the gradient mixer closed. Remove the stirring rod in the second chamber and open the outlet valve of the first chamber. Start the pump with a speed of ~1.3 ml/min until the 4.5% solution reaches the gel caster. Lower the speed to ~0.6 ml/min and scan the needle tip for small residual air bubbles. If these persist stop pump and carefully tilt the gel assembly sideways until the needle tip is not covered in fluid anymore, then slowly tilt back to horizontal position. Continue pumping until volumes in both the chambers are equal. Open the tap connecting both chambers and ensure that no air bubbles are trapped in the connection of the two chambers by sealing the top of the first chamber with Parafilm, then applying light pressure on the middle of the sealed opening with a fingertip (wear gloves!).
7. Slowly increase pump speed to ~1.1 ml/min in 0.1 ml/min intervals of 30 s each (*see* **Note 15**).

8. Remove the overlay solution (H_2O) and add comb (for 10 or 15 sample pockets).
9. Cast the stacking gel: 11 ml H_2O, 2.5 ml 6× BN—gel buffer, 1.5 ml 40% (w/v) acrylamide solution, 65 µl 10% (w/v) APS, 6.5 µl TEMED (*see* **Note 16**).
10. Prepare 1× BN—cathode and 1× BN—anode buffer. Store at 4 °C until further use.

3.6.2 Sample Preparation

The protocol below gives directions for the analysis of mitochondrial fractions. Sub-mitochondrial fractions may be subjected to BN-PAGE as well but the solubilization procedure may benefit from optimization.

Important: BN gel must be ready before commencing with sample preparation. For best quality this should be done 1 day in advance.

1. Suspend mitochondria pellet in solubilization buffer containing 5% digitonin and incubate 10–20 min on ice. Pellet originating from 100 µl of mitochondria solution (corresponding to 500 µg protein, please refer to **step 12** of Subheading 3.1 and **Note 5**) is resolved in 100 µl of solubilization buffer (*see* **Notes 17** and **18**).
2. Centrifuge for 10 min at 18,300 × *g* and 4 °C.
3. Solubilized proteins and protein complexes are in the supernatant. Transfer the supernatant to a new tube and add 5% Serva blue G (1 µl buffer/20 µl the supernatant).
4. Load samples, perform a two-step gel run at 4 °C: first step at constant voltage (100 V for 45 min) and second step at constant current (15 mA for 11 h).

3.6.3 Second Dimension: Tricine SDS-PAGE

1. Cut a lane from the BN-PAGE, remove the stacking gel, and incubate for 30 min in incubation buffer (1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol) and rinse in H_2O afterward.
2. Place the gel strip horizontally on a glass plate at the height of a stacking gel. Make sure to leave a gap of approximately 1 cm to both spacers. If the gel strip is too long, cut it on the least important end (usually the low molecular weight end) and continue assembling the gel caster.
3. Prepare solutions for a Tricine-SDS-PAGE (1.0 mm × 200 mm × 200 mm, *see* Table 7).
4. Transfer 25 ml of separation gel solution in the gel caster by using a pipette and letting the solution run down on the inner side of the spacer without it touching the BN gel strip. To overlay the spacer gel, tilt the gel caster backward to a near-horizontal position, and let 5 ml of spacer gel solution slowly run down on the inner edge of the gel spacers. Make sure that the spacer gel does not touch the BN gel strip. Repeat the

Table 7
Composition of Tricine SDS gel (1.0 mm × 200 mm × 200 mm)

	Separation gel (16.5%)	Spacer gel (10%)	Stacking gel (10%)
H_2O	3.6 ml	4.1 ml	2.9 ml
Tricine—Gel buffer	10 ml	3.4 ml	–
6× BN—Gel buffer	–	–	3.4 ml
SDS (10%)	–	–	100 µl
Glycerol	4 ml (87%)	–	1 ml (100%)
Acrylamide (40%)	12.4 ml	2.5 ml	2.5 ml
Σ	30 ml	10 ml	10 ml
APS (10%)[a]	100 µl	34 µl	83 µl
TEMED[a]	10 µl	3.4 µl	8.3 µl

[a]Add prior to use

procedure with 1 ml of overlay solution. Polymerization takes up to 45 min and setting of the gel is indicated by a sharp phase border between spacer gel and overlay solution.

5. Decant the overlay solution and rinse with H_2O. Remove as much H_2O as possible by inserting strip of suitably thick Whatman paper between the glass plates.
6. Cast the stacking gel by tilting the gel caster sideways left by approximately 30°. Cast the gel by pouring the solution along the inner edge of the lower spacer and slowly move the gel caster back to its horizontal position while continuing to add the gel solution. This will avoid air bubbles getting trapped under the gel strip (*see* **Note 19**). The stacking gel should now fully enclose the BN gel strip. Polymerization takes up to 60 min.
7. Run the gel at 30 mA (max. 500 V) at room temperature for 18 to 20 h.
8. Stain the gel (for Coomassie colloidal *see* [17]) and cut out spots. Dry in vacuum centrifuge.

3.7 Tryptic Digestion of Samples for MS

Stated volumes are for gel spots. For bands volumes are given in brackets.

1. Reduce cysteine residues by adding 40 µl (150 µl) of reduction solution (20 mM DTT [3.1 mg/ml] in 0.1 M NH_4HCO_3) to the dried gel piece(s).
2. Incubate for 30 min at 56 °C. Discard the supernatant.
3. Dehydrate by adding 200 µl ACN. Discard the supernatant.

4. Alkylate by adding 40 µl (150 µl) 55 mM iodoacetamide (10.2 mg/ml) in 0.1 M NH_4HCO_3.
5. Incubate for 30 min at RT in the dark. Discard the supernatant.
6. Dehydrate by adding 200 µl ACN. Discard the supernatant.
7. Wash gel pieces in 200 µl (500 µl) 0.1 M NH_4HCO_3.
8. Incubate for 15 min at RT. Discard the supernatant.
9. Dehydrate by adding 200 µl ACN. Discard the supernatant.
10. Dry gel pieces using a vacuum centrifuge for 5 min.
11. Add 20 µl (70 µl) of trypsin solution equaling 0.4 µg (1.4 µg).
12. Incubate for 10 min, then check that the gel pieces are fully rehydrated and that there are only minute amounts of liquid left. If all liquid has been taken up by the gel pieces add more trypsin solution (*see* **Note 20**).
13. Incubate overnight at 37 °C.
14. For peptide extraction add 20 µl (70 µl) 5% (v/v) formic acid (FA) in 50% (v/v) ACN.
15. Incubate for 20 min at 37 °C under mild shaking.
16. Collect the supernatant in new Eppendorf tubes. The supernatant already contains extracted peptides. For the following steps it is advisable to start drying down the peptides in a vacuum centrifuge. This will reduce final fluid level in the tubes therefore reducing unspecific binding of peptides to the tube wall.
17. Repeat **steps 14–16** twice with the exception of using 1% (v/v) FA, 50% (v/v) ACN. Add the supernatant to the corresponding Eppendorf tubes already containing the first extracts.
18. Add 20 µl (70 µl) of ACN, incubate for 20 min. Gel pieces will turn white.
19. Add the supernatants to the corresponding Eppendorf tubes containing the previous extracts.
20. Dry extracts in a vacuum centrifuge and store at −20 °C until further use.

4 Notes

1. For maximal yield use about 300 g of fresh material and six Percoll gradients.
2. The homogenization steps in the blender should be interrupted to prevent the solution from heating and to allow the undisrupted material to sediment again.

3. For all centrifugation steps it is advantageous to choose a low brake setting to avoid perturbations of the pellets or the gradients.
4. To collect mitochondrial bands from an interphase it is important to expel the air from the pipette before it is immersed in gradient solution and carefully collect the mitochondria by moving the pipette tip in smooth circles at the interphase.
5. The protein content of this mitochondria diluted to 0.1 g/ml typically is about 5 μg/μl (according to the Bradford protein concentration determination). If further fractionation of mitochondria is required, stop here and continue with Subheading 3.2 or 3.3.
6. Disruption of mitochondria is usually not quantitative and the pellet often contains residual intact mitochondria. To increase the yield of membrane and matrix fraction, sonication and centrifugation can be repeated using the pellets of **step 3** in Subheading 3.2.
7. For isolation of outer membranes it is mandatory to use fresh mitochondria directly after isolation. Rapid freezing of mitochondria in liquid nitrogen is not sufficient to keep the outer membrane intact.
8. For higher purity OMM fractions continue the protocol, if the purity at this step is deemed sufficient and higher yields are desired skip further steps.
9. When using ultra-centrifuge tubes with thin walls (e.g., Beckman Coulter Ultra-Clear Centrifuge Tubes, 14 × 95 mm) it is important to fill them up completely to prevent the upper tube walls from collapsing into the solution that results in the complete loss of the sample. For the tubes described here at least 10 ml must be used.
10. The mitoplast pellet is typically very soft so handle carefully.
11. It is important that loading volume is equal for all samples. To adjust volumes use the final volume of the sample with least protein concentration.
12. Premixing 2-mercaptoethanol with bromophenol blue results in an orange color of the solution. Make sure you add a large amount of bromophenol blue (1 mg/ml) because it will be highly diluted being added to the sample.
13. Heating all components increases the polymerization and results in well-defined pockets. This increases the maximum loading volume and sharpens the lanes. The gel should be prepared the day before usage to ensure complete polymerization and absence of residual persulfate radicals.

14. The gel run has to be stopped as soon as the bromophenol blue running front reaches the interphase between stacking and separation gel. Initially, check every 15 min, more often during the final stages.
15. The gradient gel is cast from the bottom. With increasing amount of solution from the second chamber the amount of glycerol (and acrylamide) concentration increases. This increases density of the solution in the first chamber and allows layering the new (heavier) solution underneath the lighter gel solution. To circumvent mixing, injection speed is slow at the beginning and raised only moderately. Gel casting takes 20–25 min and polymerization of the acrylamide (at 37 °C) additionally requires 45–60 min.
16. Prepare the gel the day before usage to guarantee optimal polymerization and reduce the presence of residual persulfate radicals. Store at 4 °C overnight.
17. If protein concentration is checked using a standard assay (e.g., Bradford), it should be done prior to the addition of digitonin because it will disturb quantification. The protein amount in mitochondria isolated from cell culture (0.1 g mitochondria per ml) is approximately 5 μg/μl.
18. Preparing the solubilization buffer with digitonin requires heating close to the boiling point to dissolve the digitonin. Cool down to 4 °C before use.
19. It is important to carefully cast the stacking gel avoiding any air becoming trapped underneath the gel strip since migration of proteins into the second dimension gel is disturbed at these sites. Avoid creating sharp edges on the gel strip when cutting it from the first dimension gel because air bubbles preferentially get stuck in these places.
20. It is important to add just enough trypsin solution to ensure full rehydration. Adding more trypsin than necessary may compromise analytical sensitivity.

References

1. Eubel H, Jänsch L, Braun HP (2003) New insights into the respiratory chain of plant mitochondria. Supercomplexes and a unique composition of complex II. Plant Physiol 133:274–286
2. Sunderhaus S, Dudkina NV, Jänsch L et al (2006) Carbonic anhydrase subunits form a matrix-exposed domain attached to the membrane arm of mitochondrial complex I in plants. J Biol Chem 281:6482–6488
3. Jacoby RP, Li L, Huang S et al (2012) Mitochondrial composition, function and stress response in plants. J Integr Plant Biol 54:887–906
4. Schlesier B, Bréton F, Mock HP (2003) A hydroponic culture system for growing *Arabidopsis thaliana* plantlets under sterile conditions. Plant Mol Biol Report 21:449–456
5. Lee CP, Eubel H, O'Toole N et al (2011) Combining proteomics of root and shoot mitochondria and transcript analysis to define constitutive and variable components in plant mitochondria. Phytochemistry 72:1092–1108
6. Klein M, Binder S, Brennicke A (1998) Purification of mitochondria from *Arabidopsis*. In: Martinez-Zapater J, Salinas J (eds) Methods

in molecular biology, vol 82. Humana Press Inc., Totowa, pp 49–53

7. Werhahn W, Niemeyer A, Jänsch L et al (2001) Purification and characterization of the preprotein translocase of the outer membrane membrane from *Arabidopsis*. Identification of multiple forms of TOM20. Plant Physiol 125:943–954
8. Murcha MW, Elhafez D, Millar AH et al (2005) The C-terminal region of TIM17 links the outer and inner mitochondrial membranes in *Arabidopsis* and is essential for protein import. J Biol Chem 280:16476–16483
9. Baerenfaller K, Grossmann J, Grobei MA et al (2008) Genome-scale proteomics reveals *Arabidopsis thaliana* gene models and proteome dynamics. Science 320:938–941
10. Duncan O, Taylor NL, Carrie C et al (2011) Multiple lines of evidence localize signaling, morphology, and lipid biosynthesis machinery to the mitochondrial outer membrane of *Arabidopsis*. Plant Physiol 157:1093–1113
11. Wisniewski JR, Zougman A, Nagaraj N et al (2009) Universal sample preparation method for proteome analysis. Nat Methods 6:359–363
12. Klodmann J, Sunderhaus S, Nimtz M et al (2010) Internal architecture of mitochondrial complex I from *Arabidopsis thaliana*. Plant Cell 22:797–810
13. Klodmann J, Senkler M, Rode C et al (2011) Defining the protein complex proteome of plant mitochondria. Plant Physiol 157:587–598
14. Kiirika LM, Behrens C, Braun HP et al (2013) The mitochondrial complexome of medicago truncatula. Front Plant Sci 4:1–7
15. Schägger H, von Jagow G (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal Biochem 199:223–231
16. Wittig I, Braun HP, Schägger H (2006) Blue native PAGE. Nat Protoc 1:418–428
17. Neuhoff V, Arold N, Taube D et al (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogramm sensitivity using Coomassie brilliant blue G-250 and R-250. Electrophoresis 6:255–262

Chapter 12

Plasma Membrane Proteomics of *Arabidopsis* Suspension-Cultured Cells Associated with Growth Phase Using Nano-LC-MS/MS

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Abstract

Arabidopsis thaliana suspension-cultured cells (T87 line) are important model system for studies of responses to biotic and abiotic stresses at the cellular level in vitro since the cells have certain advantages compared with the whole plant system. However, the physiological and morphological characteristics of the cells are influenced by the progress of the growth phase of cells, which may result in different stress tolerance. To obtain comprehensive proteome profiles of the plasma membrane of *Arabidopsis thaliana* T87 suspension-cultured cells at the lag, log, or stationary growth phase, a shotgun proteomics method using nano-LC-MS/MS is used. The results obtained indicate that proteome profiles of the plasma membrane with the progress of the growth phase of cells dynamically changed, which may be associated with the physiological and morphological characteristics of the plasma membrane of the suspension-cultured cells. The proteomics results are further applied to explain different responsive patterns in the plasma membrane to cold acclimation and ABA treatment, which lead to understanding of different freezing tolerance associated with the growth phase of the cells.

Key words Plasma membrane, Culture cells, Growth phase, Nano-LC-MS/MS, Proteomics

1 Introduction

The physiological and morphological characteristics of plant-cultured cells are influenced by nutrient availability, cell density, temperature, and hormonal concentration [1]. *Arabidopsis* suspension-cultured cells (T87 line) have different adhesion strengths of cell walls, which are associated with their growth phases [2]. In addition, differential freezing tolerance was observed in cultured cells (such as bromegrass and *Arabidopsis* suspension-cultured cells), which is apparently growth phase dependent [3, 4].

In recent years, *Arabidopsis* suspension-cultured cells have been chosen for plasma membrane proteome analysis. Protein profiles in the plasma membrane [5–9] or lipid rafts of the plasma membrane [10], and determination of GPI-anchored proteins [11–13] or

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phosphorylated membrane proteins [14] have been reported. With *Arabidopsis* whole plant system, on the other hand, a few plasma membrane proteome studies associated with biotic and abiotic stress responses have been reported. The alteration of plasma membrane proteins was demonstrated that the dramatic modifications to cell physiology occur during execution of immune responses [15]. Cold-responsive plasma membrane proteins were identified in *Arabidopsis* leaves using two-dimensional gel electrophoresis [16]. Thus, these studies indicate that the plasma membrane is restructured and/or reorganized to withstand several stresses that occur during a freeze/thaw cycle [17]. The plasma membrane is probably the most diverse form of cellular membranes with a complex protein composition that varies with cell types, developmental stages, and environments [18].

In this chapter, we introduce the procedures for characterizing plasma membrane proteome profiles of *Arabidopsis* suspension-cultured cells (T87 line) at three different growth phases (lag, log, and stationary phase) [19–21]. The nano-LC-MS/MS-based shotgun proteomics of the plasma membrane are applicable to any other plants for studies of time-course proteomics of the plasma membrane during changing environment.

2 Materials

Prepare all solutions with ultrapure water (prepared by purifying deionized water to attain a resistance of 18.2 MΩ cm at 24 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Carefully follow all waste disposal regulations determined by local authorities when disposing of waste materials.

2.1 Jouanneau and Péaud-Lenoel (JPL) Medium [4, 22]

1. JPL stock A (500 mL): 32.75 g KNO_3, 2.20 g $CaCl_2{\cdot}2H_2O$, 1.85 g $MgSO_4{\cdot}7H_2O$, 0.85 g KH_2PO_4.
2. JPL stock B (100 mL): 0.62 g H_3BO_3, 2.41 g $MnSO_4{\cdot}5H_2O$, 1.06 g $ZnSO_4{\cdot}7H_2O$, 83.00 mg KI, $Na_2MoO_4{\cdot}2H_2O$. 2.50 mg $CoCl_2{\cdot}6H_2O$, 2.50 mg $CuSO_4{\cdot}5H_2O$.
3. JPL stock C (100 mL): 0.75 g Na_2EDTA, 0.56 g $FeSO_4{\cdot}7H_2O$.
4. JPL stock D (100 mL): 1 g *myo*-inositol, 20 mg glycine.
5. JPL stock VT (100 mL): 50 mg nicotinic acid, 50 mg pyridoxine·HCl, 40 mg thiamine·HCl.
6. Na_2HPO_4/KH_2PO_4 buffer solution (100 mM, pH 7.0, 100 mL): 30.5 mL 0.2 M Na_2HPO_4, 19.5 mL 0.2 M KH_2PO_4.
7. NAA solution (1 mM, 100 mL): 18.6 mg α-naphthaleneacetic acid (NAA), potassium salt.

8. Sucrose.
9. Casamino acids.
10. JPL medium (1000 mL): 30 mL Stock A, 0.3 mL Stock B, 2 mL Stock C, 10 mL Stock D, 1 mL Stock VT, 1 mL Na_2HPO_4/KH_2PO_4 buffer solution, 1 mL NAA solution, 15 g sucrose, 0.1 g casamino acids.

2.2 Plasma Membrane Purification

Several items, including 2 L of ultrapure water, a homogenizer, centrifuge rotors, and ultracentrifuge rotors, should be chilled at 4 °C.

1. Homogenizing medium: 0.5 M sorbitol, 50 mM Mops-KOH (pH 7.6), 5 mM EGTA (pH 8.0), 5 mM EDTA (pH 8.0), 1.5% (w/v) polyvinylpyrrolidone 40 (molecular weight 40,000), 0.5%(w/v) BSA, 2 mM phenylmethanesulfonyl fluoride (PMSF), 4 mM salicylhydroxamic acid (SHAM), 2.5 mM 1,4-dithiothreitol (DTT). Store at 4 °C (*see* **Note 1**).
2. Microsome (MS)-suspension medium: 10 mM KH_2PO_4/K_2HPO_4 (phosphate buffer) buffer (pH 7.8), 0.3 M sucrose. Store at 4 °C in a refrigerator (*see* **Note 2**).
3. NaCl medium: 100 mM NaCl in MS-suspension medium. Store at 4 °C in a refrigerator.
4. Plasma membrane (PM)-suspension medium: 10 mM Mops-KOH (pH 7.3), 2 mM EGTA (pH 8.0), 0.25 M sucrose. Store at 4 °C (*see* **Note 3**).
5. Two-phase medium: weigh 1.45 g of polyethylene glycol 3350 and 1.4 g dextran in a 40 mL centrifuge tube (5.6% [w/w] polymers in final solution with microsomal suspensions). Add 9.4 mL MS-suspension medium and 7.3 mL NaCl medium (30 mM NaCl in final solution) to the centrifuge tube and mix well by shaking. Prepare three tubes per sample. Store in a refrigerator overnight to completely dissolve the polymers.
6. Bradford Protein Assay Kit: store in a refrigerator.

2.3 In-Solution Tryptic Digestion

To avoid contamination by keratin, dust, and other exogenous proteinaceous materials, all of these processes must be carefully performed at a clean bench with gloves and a clean lab coat throughout.

1. MPEX PTS reagents kit (GL Science, Inc., Tokyo, Japan): Make solution B according to the manufacturer's instruction manual. Only solution B can be stored in a refrigerator. Prepare DTT, indole-3-acetic acid (IAA), and trypsin solutions according to the manufacturer's instruction manual freshly immediately before use.

2. 5% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid (TFA): Add 50 μL of acetonitrile and 1 μL of TFA into 949 μL of water and mix well (*see* **Note 4**).
3. BCA Protein Assay Kit: Store at room temperature.

2.4 Peptide Purification

1. Desalting column (e.g., SPE C-TIP T-300, Nikkyo Technos Co., Ltd., Tokyo, Japan).
2. 1.5 mL microtubes: Make a hole of 3 mm in diameter on the cap with a soldering iron. Prepare two tubes per sample.
3. Solution A: Add 800 μL of acetonitrile and 5 μL of TFA into 195 μL of water and mix well (*see* **Note 5**).
4. Solution B: Add 40 μL of acetonitrile and 5 μL of TFA into 955 μL of water and mix well (*see* **Note 5**).
5. 0.1% (v/v) TFA solution: Quickly add 1 μL of TFA into 999 μL of water and mix well (*see* **Note 5**).

3 Methods

3.1 T87 Cells Culture and Growth Curve Determination

1. *Arabidopsis thaliana* T87 suspension-cultured cells, derived from seedlings of the ecotype Columbia, were subcultured at 2 week intervals in JPL medium at 23 °C on a rotary shaker (120 rpm) under continuous light (100 μmol/m^2/s).
2. Growth curve is determined by fresh weight of *Arabidopsis thaliana* T87 suspension-cultured cells harvested daily for 21 days. After cells collected from a unit volume are dried on a paper towel, fresh weight is determined. The lag, log, or stationary growth phases are 8-, 12-, or 16-day-old cells, respectively.

3.2 Plasma Membrane Purification

Wear gloves and a clean lab coat throughout the processes to avoid contamination by keratin, dust, and other exogenous proteinaceous materials. It is preferable to use low-protein-absorption microtubes at all stages. Perform all the steps on crushed ice. Schematic outline of the procedure is described in Fig. 1.

1. Harvest T87 cells at lag, log, or stationary growth phases (i.e., 8-, 12-, or 16-day-old cells, respectively). Put the harvested cells in a plastic container and wash with chilled distilled water three times. Drain the harvested cells on paper towels on crushed ice.
2. Put the samples into four volumes of chilled homogenizing medium and mix well with spatula. Cool on crushed ice.
3. Homogenize with a chilled homogenizer until the samples are broken (80–100 s). Filter the homogenates through four layers

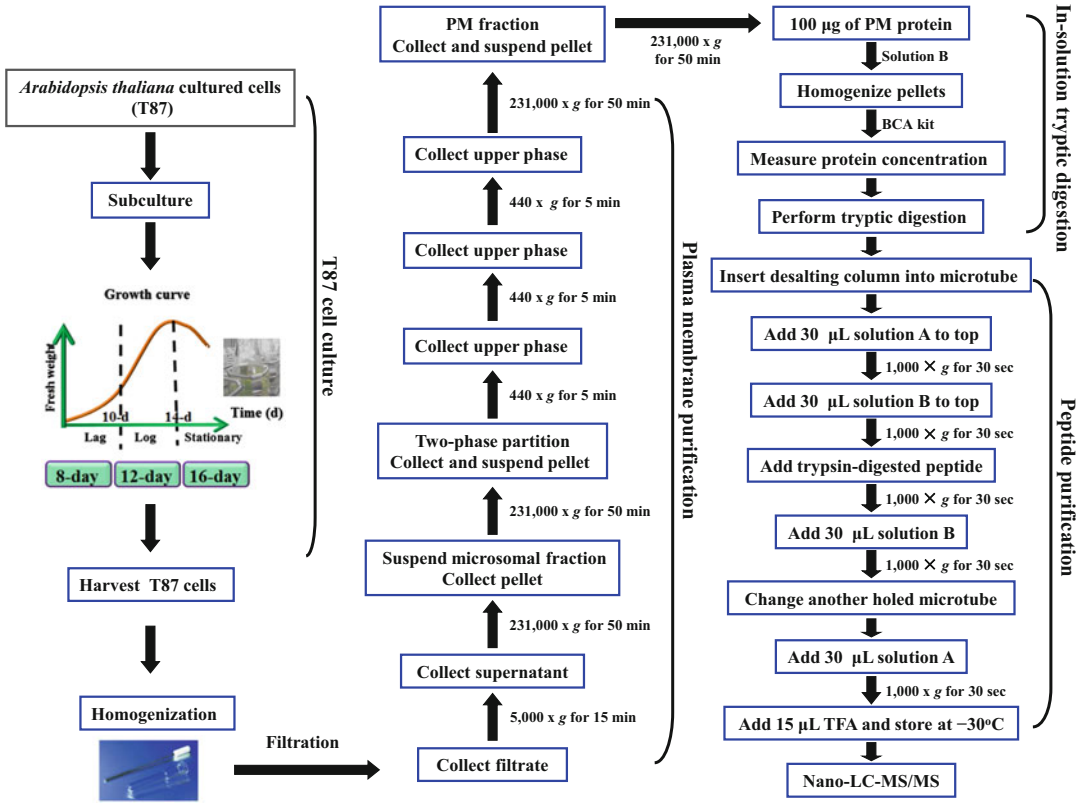

Fig. 1 A representative workflow for plasma membrane proteomics of *Arabidopsis* suspension-cultured cells associated with growth phase using Nano-LC-MS/MS. It consists of four parts; *Arabidopsis* suspension-cultured cells cultivation, Plasma membrane purification, In-Solution tryptic digestion and Peptide purification. *Arabidopsis* suspension-cultured cells are harvested at the lag, log, or stationary growth phase (i.e., 8-, 12-, or 16-day-old cells, respectively). Plasma membrane (PM) fractions are isolated using a two-phase partition system, and proteins in the plasma membrane fractions are digested with trypsin. Subsequently, peptides obtained are purified and concentrated with SPE-C-TIP. Peptides are then subjected to nano-LC-MS/MS spectrometry and then analyzed with a software for label-free identification and semiquantification of plasma membrane proteins

of gauze and squeeze thoroughly. Move the filtrate into 40 mL centrifuge tubes.

4. Centrifuge at 5,000 × *g* for 15 min with a chilled rotor to remove debris and heavy membrane fractions. Transfer the supernatants into ultracentrifuge tubes by decantation and discard precipitates.
5. Centrifuge at 231,000 × *g* for 50 min with a chilled ultracentrifuge rotor to precipitate the microsome fractions and discard the supernatants by decantation.
6. Add appropriate volume of MS-suspension medium to each tube (usually 2–3 mL per tube) and homogenize the pellets with a Teflon-glass homogenizer. Collect the microsomal

suspensions with a large-aperture Pasteur pipette into ultracentrifuge tubes. Balance ultracentrifuge tubes in pairs with MS-suspension medium.

7. Ultracentrifuge at 231,000 × *g* for 50 min as described in **step 5**. Put 5 mL of MS-suspension medium in a Teflon-glass homogenizer and mark the solution surface on the glass homogenizer. After centrifugation, discard the supernatant with an aspirator.
8. Put 2 mL of MS-suspension medium into microsomal pellets in the ultracentrifuge tubes and break up the precipitated pellets with a glass rod. Transfer into a Teflon-glass homogenizer using a large-aperture Pasteur pipette. Put 2 mL of MS-suspension medium into the same ultracentrifuge tubes and break up the remaining pellets by pipetting. Transfer into the Teflon-glass homogenizer already containing the first part of the resuspended pellet and add MS-suspension medium up to 5 mL. Homogenize well with an electric Teflon-glass homogenizer (moving up and down five times) on ice with cooling (*see* **Note 6**).
9. Put all of the homogenate in a centrifuge tube containing two-phase partition medium (tube A). Add 5 mL of MS-suspension medium to other two-phase partition systems (tubes B and C). Chill on crushed ice for 10 min. During this time, mix well every 1–2 min.
10. Centrifuge tubes A and B at 440 × *g* for 5 min in a chilled rotor. Two phases should be observed to have settled in tubes A and B. Discard the upper phase of tube B with a Pasteur pipette and transfer the upper phase of tube A into tube B. Chill on crushed ice for 10 min. Mix well every 2 min during this time (*see* **Note 7**).
11. Centrifuge tubes B and C at 440 × *g* for 5 min in a chilled rotor. Discard the upper phase of tube C with a Pasteur pipette and transfer the upper phase of tube B into tube C. Balance tube C with another centrifuge tube filled with water. Chill on crushed ice for 10 min. During this time, mix well every 2 min (*see* **Note 7**).
12. Centrifuge at 440 × *g* for 5 min and split the resultant upper phase of tube C into two ultracentrifuge tubes. Fill up the tubes with PM-suspension medium and balance them.
13. Ultracentrifuge at 231,000 × *g* for 50 min, as described in **step 4** (*see* **Note 7**).
14. Discard the supernatant with an aspirator. Add an appropriate quantity of PM-suspension medium to each tube and homogenize the pellets with a Teflon-glass homogenizer. Collect the plasma membrane suspensions with a large Pasteur pipette into

ultracentrifuge tubes. Balance ultracentrifuge tubes in pairs with PM-suspension medium. Ultracentrifuge again at 231,000 × *g* for 35 min.

15. Discard the supernatant with an aspirator. Add a minimal quantity of PM-suspension medium to the plasma membrane pellets. Homogenize the pellets with a glass rod. Transfer into a Teflon-glass homogenizer and homogenize well using an electric Teflon-glass homogenizer (moving up and down five times) with cooling on ice. Transfer into a 1.5 mL microtube.
16. Measure the protein content using the Bradford assay. Use 10 μg of protein for tryptic digestion and LC-MS/MS analysis. The remaining PM fractions should be frozen in liquid nitrogen immediately and stored at −80 °C.

3.3 In-Solution Tryptic Digestion

All of these procedures must be performed at a clean bench whenever possible and at room temperature unless otherwise specified (Fig. 1).

1. Precipitate 100 μg of PM protein using an ultracentrifuge (231,000 × *g*, 4 °C, 50 min).
2. Discard the supernatant by decantation. Add solution B to the PM pellets. Homogenize the pellets with a glass rod. Transfer into an electric Teflon-glass homogenizer and homogenize well (moving up and down five times) with cooling on ice. Transfer to 1.5 mL microtubes.
3. Solubilize samples and measure the protein concentration with a BCA protein assay kit according to the instruction manual from the manufacturer.
4. Transfer 5 μg of PM protein to another 1.5 mL microtube. Make up to 20 μL with solution A.
5. Perform reductive alkylation and tryptic digestion according to the instruction manual, and store at −30 °C (*see* **Note 8**).

3.4 Peptide Purification

All of these procedures must be at room temperature unless otherwise specified and be performed at a clean bench whenever possible (Fig. 1).

1. Insert a desalting column into the 3 mm hole in the top of a microtube.
2. Add 30 μL of solution A to the upper side of the desalting column for preconditioning. Centrifuge at 1,000 × *g* for 30 s to get solution A through the tip column.
3. Add 30 μL of solution B to the upper side of the desalting column for preconditioning. Centrifuge at 1,000 × *g* for 30 s to get solution B through the tip column.
4. After confirming that the desalting column is moist, add the entire trypsin-digested peptide sample to the upper side of the

column for absorption. Centrifuge at 1,000 × *g* for 30 s to get the sample solution through the tip column.

5. Add 30 μL of solution B from the upper side of the desalting column for cleaning. Centrifuge at 1,000 × *g* for 30 s to get solution B through the tip column.
6. Put a vial insert for each LC-MS/MS sampler into another holed microtube. Transfer the desalting column into the microtube.
7. Add 30 μL of solution A to the upper side of the desalting column for elution. Centrifuge at 1,000 × *g* for 30 s to get solution A through the tip column. Discard the SPE C-TIP.
8. Dry out the eluted samples using a centrifugal concentrator for 15 min. Add 15 μL of 0.1% (v/v) TFA. Put the vial insert into the vial and close the lid. Store at −30 °C.

3.5 Nano-LC-MS/MS Analysis

1. Mobile phase for peptide elution from trap column: 0.1% (v/v) formic acid in acetonitrile.
2. Mobile phase for peptide separation: linear gradient of acetonitrile from 5% (v/v) to 45% (v/v).
3. Flow rate and analysis time: 500 nL/min for 120 min.
4. Spray voltage for peptide ionization: 1.8 kV.
5. Mass spectrometer control settings: scan range, 400–1,800 *m/z*; resolution, 30,000; Collision induced dissociation, five most intense ions with a threshold above 500.
6. Parameters for conversion from raw files to mgf files (Proteome Discoverer software 0; the minimum total intensity of a spectrum, 0; and the mini-): precursor mass range, *m/z* 350–5,000; highest and lowest charge state, 0; lower and upper RT limit, 0; the minimum total intensity of a spectrum, 0; and the mini-mum number of peaks in a spectrum, 1.
7. Parameters for identification of proteins (Mascot search engine): database, Arabidopsis TAIR 10 protein database; allowance of missed cleavage, 1; fixed modification, carbamidomethylation (C); variable modification, oxidation (M); peptide mass tolerance, 5 ppm; MS/MS tolerance, 0.6 Da; peptide charges, +1, +2, +3.

4 Notes

1. Mops-KOH (pH 7.6), EGTA (pH 8.0), and EDTA (pH 8.0) should be prepared as 0.5 M stock solutions and stored at 4 °C. The pH of EGTA and EDTA should be adjusted using NaOH. When BSA is dissolved, BSA powder should be preset at room temperature. PMSF and SHAM should be prepared as 1 and

1.6 M stock solutions in DMSO, respectively, and stored at 4 °C. DTT should be stored at −30 °C as a 1 M stock solution. PMSF, SHAM and DTT should be diluted only as needed just before use.

2. Phosphate buffer (pH 7.8) should be prepared as a 0.5 M stock solution and diluted to make the MS-suspension medium. First, 200 mL of 0.5 M K_2HPO_4 and 30 mL of 0.5 M KH_2PO_4 are prepared. The pH of the 0.5 M K_2HPO_4 is adjusted to 7.8 by adding 0.5 M KH_2PO_4.
3. Mops-KOH (pH 7.3) and EGTA (pH 8.0) should be prepared as a 0.5 M stock solution and stored at 4 °C.
4. DTT and IAA can be easily dissolved in solution for a short period and TFA evaporates quickly. Solutions including DTT, IAA, and TFA should be freshly prepared just immediately before use.
5. TFA evaporates quickly. Thus, solutions containing TFA should be freshly prepared just immediately before use.
6. In this step, homogenization should not be too long or too vigorous because harsh homogenization can severely disrupt membrane integrity.
7. Two-phase partitioning is the most important step for preparing highly purified plasma membrane. When the upper phase of the two-phase partition medium is removed, the Pasteur pipette should be moved from left to right near the boundary of the two phases to prevent taking lower phase.
8. Digested and purified peptides should be analyzed by nano-LC-MS/MS within 1 week.

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References

1. Zottini M, Barizza E, Bastianelli F et al (2006) Growth and senescence of *Medicago truncatula* cultured cells are associated with characteristic mitochondrial morphology. New Phytol 172:239–247
2. Leboeuf E, Thoiron S, Lahaye M (2004) Physico-chemical characteristics of cell walls from *Arabidopsis thaliana* microcalli showing different adhesion strengths. J Exp Bot 55:2087–2097
3. Ishikawa M, Suzuki M, Nakamura T et al (2006) Effect of growth phase on survival of bromegrass suspension cells following cryopreservation and abiotic stresses. Ann Bot 97:453–459
4. Sasaki Y, Takahashi K, Oono Y et al (2008) Characterization of growth-phase-specific responses to cold in *Arabidopsis thaliana* suspension-cultured cells. Plant Cell Environ 31:354–365

5. Dunkley TP, Hester S, Shadforth IP et al (2006) Mapping the *Arabidopsis* organelle proteome. Proc Natl Acad Sci U S A 103:6518–6523
6. Lanquar V, Kuhn L, Khafif M et al (2007) ^{15}N-metabolic labeling for comparative plasma membrane proteomics in *Arabidopsis* cells. Proteomics 7:750–754
7. Marmagne A, Rouet MA, Ferro M et al (2004) Identification of new intrinsic proteins in *Arabidopsis* plasma membrane proteome. Mol Cell Proteomics 3:675–691
8. Marmagne A, Ferro M, Meinnel T et al (2007) A high content in lipid-modified peripheral proteins and integral receptor kinases features in the *Arabidopsis* plasma membrane proteome. Mol Cell Proteomics 6:1980–1996
9. Nelson CJ, Hegeman AD, Harms AC et al (2006) A quantitative analysis of *Arabidopsis* plasma membrane using trypsin-catalyzed $^{(18)}$O labeling. Mol Cell Proteomics 5:1382–1395
10. Borner GH, Sherrier DJ, Weimar T et al (2005) Analysis of detergent-resistant membranes in *Arabidopsis*: evidence for plasma membrane lipid rafts. Plant Physiol 137:104–116
11. Borner GH, Lilley KS, Stevens TJ et al (2003) Identification of glycosylphosphatidylinositol-anchored proteins in *Arabidopsis*: a proteomic and genomic analysis. Plant Physiol 132:568–577
12. Elortza F, Nühse TS, Foster LJ et al (2003) Proteomic analysis of glycosylphosphatidylinositol-anchored membrane proteins. Mol Cell Proteomics 2:1261–1270
13. Elortza F, Mohammed S, Bunkenborq J et al (2006) Modification-specific proteomics of plasma membrane proteins: identification and characterization of glycosylphosphatidylinositol-anchored proteins released upon phospholipase D treatment. J Proteome Res 5:935–943
14. Nuhse TS, Stensballe A, Jensen ON et al (2004) Phosphoproteomics of the *Arabidopsis* plasma membrane and a new phosphorylation site database. Plant Cell 16:2394–2405
15. Elmore JM, Liu J, Smith B et al (2012) Quantitative proteomics reveals dynamic changes in the plasma membrane proteome during *Arabidopsis* immune signaling. Mol Cell Proteomics 11:M111.014555
16. Kawamura Y, Uemura M (2003) Mass spectrometric approach for identifying putative plasma membrane proteins of *Arabidopsis* leaves associated with cold acclimation. Plant J 36:141–154
17. Steponkus PL (1984) Role of the plasma membrane in freezing injury and cold acclimation. Annu Rev Plant Physiol 35:543–584
18. Alexandersson E, Saalbach G, Larsson C et al (2004) *Arabidopsis* plasma membrane proteomics identifies components of transport, signal transduction and membrane trafficking. Plant Cell Physiol 45:1543–1556
19. Li B, Takahashi D, Kawamura Y et al (2012) Comparison of plasma membrane pro teomic changes of Arabidopsis suspension cells (T87 line) after cold and abscisic acid treatment in association with freezing tolerance development. Plant Cell Physiol 53:542–554
20. Takahashi D, Li B, Nakayama T et al (2013) Shotgun proteomics of plant plasma membrane and microdomain proteins using nano-LC-MS/MS. In: Novo JVJ, Komatsu S, Weckwerth W (eds) Plant proteomics: methods and protocols, 2nd edn. Springer Science+Business Media, LLC, New York, pp 481–498
21. Takahashi D, Li B, Nakayama T et al (2013) Plant plasma membrane proteomics for improving cold tolerance. Front Plant Sci 4:90
22. Axelos M, Curie C, Mazzolini L et al (1992) A protocol for transient gene expression in *Arabidopsis thaliana* protoplasts isolated from cell suspension cultures. Plant Physiol Biochem 30:123–128

Chapter 13

Mini-Scale Isolation and Preparation of Plasma Membrane Proteins from Potato Roots for LC/MS Analysis

Anna M. Jozefowicz, Andrea Matros, Katja Witzel, and Hans-Peter Mock

Abstract

Plasma membrane (PM) proteins are of special interest due to their function in exchanging material and information with the external environment as well as their role in cellular regulation. In quantitative proteomic studies PM proteins are underrepresented mostly because they constitute only small percent of all membrane proteins. Strong demand is placed on plasma membrane enrichment methods. For decades two-phase partitioning Dextran T500/PEG 3350 isolation protocols were applied for many different animal and plant species and also a variety of tissue types. The typical quantity of material used in the enrichment protocols is 10–30 g of fresh weight. The main difficulty of working with in vitro cultivated plants is the low amount of material, especially when roots are examined. In addition, roots are frequently characterized by low protein concentrations. Our protocol established for roots of in vitro cultivated potato plants is adjusted to amounts of fresh weight not exceeding 7.5 g and allows studying the plasma membrane proteome by LC-MS.

Key words Plasma membrane, Two-phase partitioning, Plants, Potato, Roots, Enrichment, Digestion, Mass spectrometry

1 Introduction

The plasma membrane has two very important roles, first it acts as a barrier separating cell from its environment and second, it is a sensor for the environmental conditions, which transduces the signal to the inside of the cell [1, 2]. Plasma membrane proteins fulfill many different cellular functions including metabolite and ion transport, endocytosis and cell differentiation [3]. The plasma membrane is likely to be the most diverse cell membrane as its composition is not only organ and tissue specific but also strongly depends on developmental stage and environmental conditions [4]. In plants, signals related to biotic and abiotic stresses are processed in the plasma membrane causing significant alteration in protein composition and abundance. Better understanding of changes

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occurring in the proteomic profile of the PM in response to environmental stimuli will help to derive novel concepts not only to generate more resistant plants but also to develop stress and disease biomarkers [5, 6].

In quantitative proteomic studies PM proteins are underrepresented mostly because the PM constitutes only 5–20% of the total membranes [7]. The aqueous two-phase partitioning method, which separates membranes according to their affinity to two immiscible polymers, was developed and improved over decades for efficient isolation of plasma membrane vesicles [8]. Together with the Brij-58 treatment this method results in the highly enriched plasma membrane fraction free of contaminating soluble and loosely bound proteins [9]. The majority of the current available protocols require large quantities of starting material ranging from 30 to 125 g to obtain a decent yield [7, 9–11]. This is especially difficult to obtain when working with plants cultivated in vitro. In our protocol established for potato roots steps were adjusted to allow working with amounts not exceeding 7.5 g.

Successful identification of plasma membrane proteins requires not only efficient isolation method. The difficulty of solubilization and mass spectrometry analysis lies in the nature of integral membrane proteins containing transmembrane domains composed of highly hydrophobic amino acids [10]. Commonly used in the gel-based approaches detergents that allow overcoming insolubility problem interfere with LC-MS by suppressing spectral signal and lowering chromatographic performance [12]. In our study proteins are solubilized in the buffer commonly used for the 2-D electrophoresis composed of urea, thiourea, and CHAPS. Quantification is performed using modified version of 2-D Quant Kit protocol, where the amount of protein is reduced to a minimum. Proteins are further digested using the in-filter-based method [13] allowing removal of undesirable compounds prior to trypsination. Peptides are further analyzed using nanoflow liquid chromatography coupled to a tandem mass spectrometer.

2 Materials

2.1 Enrichment of Plasma Membrane

1. Homogenization Buffer: 50 mM MOPS, 5 mM EDTA, 330 mM sucrose, 10% (w/v) glycerol, 5 mM DTT, 5 mM ascorbate, 0.6% (w/v) PVPP, cOmplete Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Germany), adjust to pH 7.5 with KOH. Prepare the buffer 1 day before and cool it overnight in the fridge. PVPP, DTT, ascorbate, and protease inhibitors should be added right before homogenization.
2. Plasma membrane Buffer: 330 mM sucrose, 0.1 mM DTT, 0.1 mM EDTA, 5 mM potassium phosphate buffer pH 7.8.

Table 1
Two-phase system composed of 6.2% Dextran T500, PEG 3350, 8 mM KCl, 5 mM potassium phosphate and 300 mM sucrose

Component	Stock concentration	Mixture
Dextran T500	20% (w/w)	3.72 g
PEG 3350	40% (w/w)	1.86 g
Phosphate buffer pH 7.8	200 mM	300 µl
KCl	2 M	48 µl
Sucrose	1.6 M	2.25 ml
Water		To 9 g

Prepare aliquots and store it at −20 °C. Add DTT and EDTA before use.

3. 20% (w/w) Dextran T500 stock solution (Pharmacosmos; Denmark). Prepare aliquots and store it at −20 °C (*see* **Note 1**).
4. 40% (w/w) PEG 3350. Prepare aliquots and store it at −20 °C.
5. 1.6 M sucrose.
6. 2 M KCl.
7. 200 mM potassium phosphate buffer pH 7.8.
8. Two-phase system: 6.2% Dextran T500, 6.2% PEG 3350, 300 mM sucrose, 8 mM KCl, 5 mM potassium phosphate buffer pH 7.8. For one sample four systems 9 g each should be prepared. One day before PM enrichment mix all components in a glass beaker on a balance according to Table 1 and stir it for 5 min. You can mix each system separately but more reproducible results are obtained if a batch is prepared. To prevent phase separation divide the mixture into falcon tubes (9 g each) under constant stirring. Store the two-phase system overnight in the fridge (*see* **Note 2**).
9. Brij-58 Buffer: 330 mM sucrose, 200 mM KCl, 5 mM phosphate buffer pH 7.8, 0.2% (w/v) Brij-58. Prepare 2% stock solution of Brij-58 and 2 M KCl. Mix the components shortly before use.

2.2 Protein Precipitation

1. Ice-cold methanol.
2. Ice-cold chloroform.
3. Ultrapure water, 4 °C.

2.3 Protein Resuspension and Quantification

1. Resuspension Buffer: 7 M urea, 2 M thiourea, 5 mM DTT, 2% CHAPS, adjust to pH 8.0.
2. 2-D Quant Kit (GE Healthcare).

2.4 Solutions for Protein Digestion

1. Microcon-10 kDa Centrifugal Filter Unit with Ultracel-10 membrane (Merck Millipore; USA).
2. Urea Buffer: 7 M urea, 100 mM Tris–HCl, 5 mM EDTA, adjust to pH 8.0. Buffer can be stored at room temperature.
3. Solution 1: 20 mM DTT in urea buffer. Prepare 1 M stock solutions, aliquot and store at −20 °C.
4. Solution 2: 80 mM Iodoacetamide in urea buffer. Prepare 1 M stock solutions, aliquot and store at −20 °C. Iodoacetamide is light sensitive and should be kept in the dark.
5. Solution 3: 50 mM ammonium bicarbonate.
6. Trypsin Gold, Mass Spectrometry Grade (Promega, USA). Prepare 200 ng/μl stock solution, aliquot and store at −20 °C.

3 Methods

3.1 Enrichment of the Plasma Membrane from Potato Roots

1. Homogenize 5–7.5 g of roots with a kitchen blender in 100 ml of ice-cold homogenization buffer. Use 20 s pulses to avoid overheating of sample (*see* **Note 3**).
2. Filter the slurry through two layers of Miracloth and centrifuge it for 15 min at 10,000 × *g* at 4 °C.
3. Ultracentrifuge the supernatant for 50 min at 50,000 × *g* at 4 °C (e.g., Beckman, Fixed angle rotor type 70 Ti).
4. Resuspend the pellet with a glass tissue homogenizer in about 2.8 ml of plasma membrane buffer. The end mass of the microsomal fraction should not exceed 3 g. The membrane homogenate can be directly loaded onto the two-phase system or frozen in liquid N_2 and stored at −80 °C.
5. Load exactly 3 g of the microsomal suspension onto the top of the Dextran T500/PEG 3350 two-phase system prepared the day before (Fig. 1). Mix by inversion 30 times (Do not vortex!). Mix the second two-phase system with 3 g of plasma membrane buffer (*see* **Note 4**).
6. Spin both the tubes at 1,000 × *g*, 4 °C for 7 min.
7. On a balance, remove about 90% of upper phase from the second system and replace it with exactly the same amount of upper phase one. Mix it by inversion 30 times. Pretreat a third prepared two-phase system with 3 g of plasma membrane buffer.
8. Spin both the tubes at 1,000 × *g*, 4 °C for 7 min.

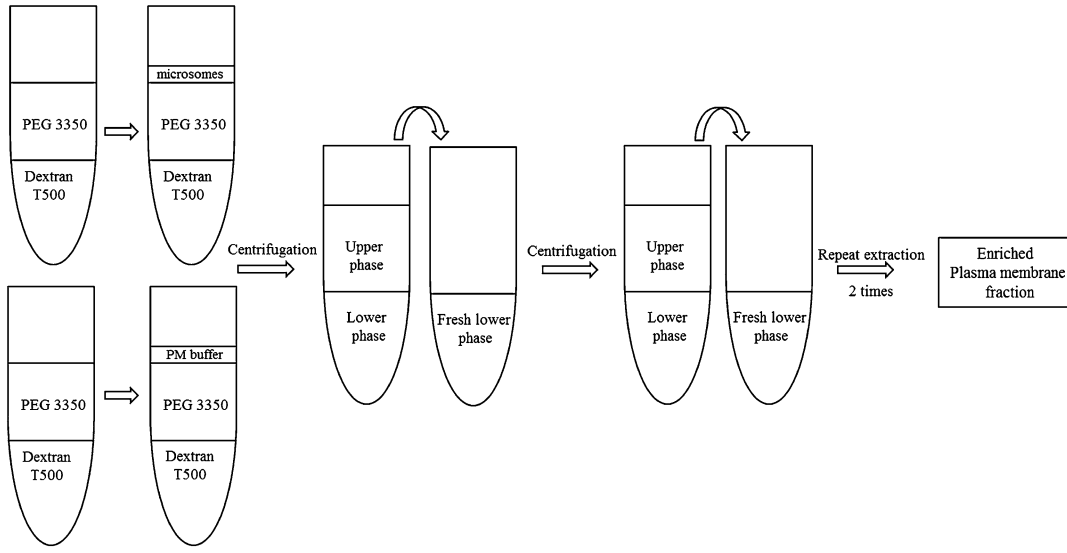

Fig. 1 Plasma membrane enrichment workflow

9. Repeat these steps with the third and fourth systems. The final fourth upper phase contains the enriched plasma membrane fraction.
10. Dilute the PM fraction at least 1:4 with plasma membrane buffer. For recovering the endomembranes dilute the first lower phase 1:10 with plasma membrane buffer.
11. Spin it at 100,000 × *g*, 4 °C for 1 h (Beckman, Fixed angle rotor type 70 Ti).
12. Discard the supernatant. In order to remove contaminating soluble and loosely bound proteins resuspend the pellet with a glass tissue homogenizer in 8 ml Brij-58 buffer and incubate it for 10 min at RT.
13. Spin the tube at 100,000 × *g*, 4 °C for 1.5 h (e.g., Beckman, Fixed angle rotor type 70.1 Ti).
14. Resuspend the pellet in 150 µl of plasma membrane buffer and freeze it in liquid N_2 (*see* **Note 5**).

3.2 Delipidation of Membrane Proteins

1. Mix 100 µl of sample with 200 µl of ice-cold chloroform.
2. Add 400 µl of ice-cold methanol and vortex shortly.
3. Add 1000 µl of cold water, vortex and incubate on ice for 5 min.
4. Centrifuge at 9,000 × *g*, 4 °C, for 2 min.
5. Remove the upper phase carefully without disturbing the middle phase.

Table 2
Preparation of standard curve

Tube number	0	1	2	3	4	5
Volume of BSA standard solution (2 mg/ml)	0 µl	1.25 µl	2.5 µl	3.75 µl	5 µl	6.25 µl
Protein quantity	0 µg	2.5 µg	5 µg	7.5 µg	10 µg	12.5 µg

Note that each sample is prepared in duplicate

6. Add 500 µl of methanol and vortex.
7. Centrifuge at maximal speed, 4 °C, for 5 min.
8. Discard the supernatant and dry the pellet on ice for 15 min (*see* **Note 6**).
9. Resuspend the pellet in 50 µl of resuspension buffer, vortex and sonicate it for 5 min.
10. Incubate the samples at 30 °C with gentle shaking for 1 h.
11. Centrifuge the sample for 15 min at maximal speed and move the supernatant to a new reaction tube.

3.3 Protein Quantification with 2-D Quant Kit

1. Prepare the Color Reagent by mixing 100 parts of Color reagent A and one part of Color reagent B. For each single measurement 250 µl is needed. Each sample should be measured in duplicate.
2. Use the bovine serum albumin (BSA) 2 mg/ml solution provided by the manufacturer for the standard curve. Set up 1.5 ml reaction tubes and pipette solution according to Table 2 (*see* **Note 7**).
3. Prepare tubes containing 1–2.5 µl of sample. A test assay for any new sample type is necessary to ensure that the protein amount is sufficient to fall within the range of the standard curve.
4. Add 125 µl of precipitant to each tube. Use a multipipette to assure accuracy of the assay. Vortex briefly and incubate at RT for 2–3 min.
5. Add 125 µl of co-precipitant to each tube. Vortex shortly and centrifuge for 5 min at maximal speed.
6. To avoid resuspension of the pellet rapidly aspire the supernatant (*see* **Note 8**).
7. Add 25 µl of cooper solution and 100 µl of water. Vortex each sample about 10–20 s (*see* **Note 9**).
8. Centrifuge briefly to collect all liquid at the bottom of the tube.
9. Add 250 µl of Color reagent to each tube, mix it by inversion and centrifuge shortly.

10. Incubate at room temperature for 20 min.
11. Shortly before the end of incubation time move 200 µl of each sample to a 96-well plate.
12. Read the absorbance at 480 nm using water as a blank.

3.4 In-Filter-Based Digestion of Proteins

1. Fill the YM-10 filter with 200 µl of ultrapure water and centrifuge it at 14,000 × *g*, 5 min, at RT. Carefully remove remaining water (*see* **Note 10**).
2. Mix 5–10 µg of PM protein with resuspension buffer to obtain 200 µl. Fill the filter with protein solution.
3. Centrifuge until there is no more than 10–15 µl of sample on the filter (about 45–60 min) (*see* **Note 11**).
4. Apply 100 µl of urea buffer and centrifuge it for about 20–30 min. Repeat this step three times to remove detergents from the sample.
5. Fill the filter with 200 µl of Solution 1. Incubate for 60 min with gentle shaking at 60 °C.
6. Cool down the sample at RT. Add 100 µl of Solution 2. Incubate at 37 °C, 30 min in the darkness with gentle shaking (*see* **Note 12**).
7. Centrifuge until no liquid remains (about 40 min).
8. Apply 200 µl of Solution 3 on the filter and centrifuge it for about 20 min. Repeat this step three times (*see* **Note 13**).
9. Mix 200 µl of Solution 3 with Trypsin stock solution. The recommended ratio of Trypsin to protein is 1:50, so for 10 µg of protein 1 µl of Trypsin stock should be used.
10. Fill the filter with Trypsin solution and incubate it overnight at 37 °C (without shaking) (*see* **Note 14**).
11. Elute peptides into fresh Eppendorf tube by centrifuging for about 30 min.
12. Apply 50 µl of Solution 3 on the filter and centrifuge it for about 10 min. Repeat this step three times to elute remaining peptides.
13. Dry the sample in the vacuum centrifuge for about 2 h.
14. Prior to mass spectrometric analysis resuspend the pellet in 1% Acetonitrile/0.1% formic acid/water (LC/MS grade) to obtain a concentration suitable for loading onto a reverse-phase column. Vortex and centrifuge for 5 min with maximal speed. Peptides can be analyzed using a nanoflow liquid chromatography coupled to a tandem mass spectrometer.

4 Notes

1. Dextran T500 contains 5–10% water, so the end concentration of your stock might not be 20%. By drying or lyophilizing small amounts (10–20 mg) of the stock solution in several replicates the exact concentration can be determined. For reproducible results use the same stock solutions for all the samples.
2. Given concentrations of PEG 3350, Dextran T500, and KCl were optimized for in vitro cultivated potato root tissue. In the literature concentrations used for various tissues and species vary from 6.2% to 6.5% Dextran and PEG and from 2 to 8 mM KCl. A series of test experiments covering concentration range of polymer from 6.0% to 6.5% and KCl from 1 to 8 mM is advised to provide appropriate yield and purity of the PM for specific tissues.
3. All the steps should be performed at 4 °C. All the beakers and centrifugal tubes should be precooled in the fridge before starting the experiment.
4. Phase separation is strongly dependent on temperature. Keep all two-phase systems on ice. Do not overheat the samples during mixing. Every ten inversion cool the tubes down on ice or work in the cold room.
5. Efficiency of the plasma membrane enrichment can be tested by Western blot with marker antibodies for cellular compartments (for example H^+-ATPase as PM marker, Fig. 2). Due to the low concentration of proteins in obtained membrane fraction a highly sensitive fluorescent detection method is advised.
6. The pellet can be dried in the vacuum centrifuge, but the time should be kept to a minimum. An overdried pellet is hard to resuspend and the protein yield is lower.

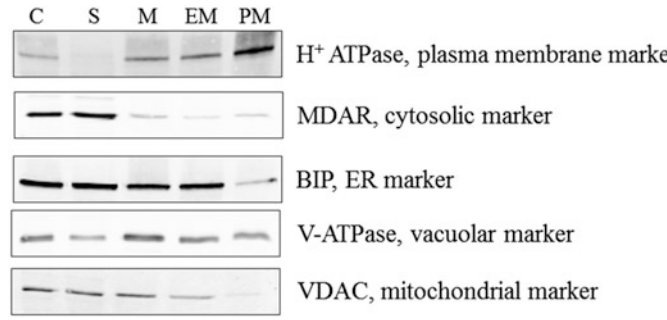

Fig. 2 Purity of plasma membrane fraction was evaluated using Western blot with antibodies against compartment markers. *C* crude, *S* soluble, *M* microsomal, *EM* endomembrane, *PM* plasma membrane fraction

7. To assure better accuracy of the assay and reproducible results using Microman (Gilson) pipettes is recommended.
8. A peristaltic pump with a cut gel loading tip attached to the end of the tubing can be used to remove the supernatant. There should be no remaining liquid in the tube.
9. As the colorimetric reagent reacts with unbound cupric ions it is very important to add exactly the same amount of copper solution to each tube. Use a Microman (Gilson) pipette if available.
10. All steps requiring centrifugation should be performed at maximal 14,000 × *g*. High speed might destroy the filter and lead to samples loss. As the solubility of urea drops with temperature do not centrifuge at 4 °C.
11. In the protocol putative times of centrifugation are given. It is recommended to check the level of liquid every 10–15 min. Avoid overdrying of the filter.
12. Ioadoacetamide is light sensitive. Brownish coloration of IAA indicates decomposition and such solutions should not be used. To provide proper conditions for sample alkylation cover your Thermomixer with aluminum foil.
13. Urea in high concentration is known to suppress ionization in the ESI source. It is important to remove it from the sample prior to trypsination.
14. Trypsin is usually first choice enzyme for protein digestion. Positively charged amino acids arginine and lysine are underrepresented and not equally distributed in the transmembrane helices [14]. To improve membrane protein analysis unspecific proteases like elastase can be additionally used.

References

1. Tang W (2012) Quantitative analysis of plasma membrane proteome using two-dimensional difference gel electrophoresis. Methods Mol Biol 876:67–82
2. Barkla JB, Pantoja O (2010) Plasma membrane and abiotic stress. Plant Cell Monogr 19:457–470
3. Santoni V, Molloy M, Rabilloud T (2000) Membrane proteins and proteomics: un amour impossible? Electrophoresis 21:1054–1070
4. Bernfur K, Larsson O, Larsson C, Gustavsson N (2013) Relative abundance of integral plasma membrane proteins in *Arabidopsis* leaf and root tissue determined by metabolic labeling and mass spectrometry. PLoS One 8:e71206
5. Komatsu S (2008) Plasma membrane proteome in Arabidopsis and rice. Proteomics 8:4137–4145
6. Cordwell SJ, Thingholm TE (2010) Technologies for plasma membrane proteomics. Proteomics 10:611–627
7. Larsson C, Widell S (2000) Isolation of plant plasma membranes and production of inside-out vesicles. Methods Enzymol 11:159–166
8. Schindler J, Nothwang HG (2006) Aqueous polymer two-phase systems: effective tools for plasma membrane proteomics. Proteomics 6:5409–5417
9. Alexandersson E, Saalbach G, Larsson C, Kjellbom P (2004) *Arabidopsis* plasma membrane proteomics identifies components of transport, signal transduction and membrane trafficking. Plant Cell Physiol 45:1543–1556
10. Mitra SK, Clouse SD, Goshe MB (2009) Enrichment and preparation of plasma membrane proteins from *Arabidopsis thaliana* for

global proteomic analysis using liquid chromatography-tandem mass spectrometry. Methods Mol Biol 564:341–355

11. Nouri MZ, Komatsu S (2010) Comparative analysis of soybean plasma membrane proteins under osmotic stress using gel-based and LC MS/MS-based proteomics approaches. Proteomics 10:1930–1945
12. Mirza SP, Halligan BD, Greene AS, Olivier M (2007) Improved method for the analysis of membrane proteins by mass spectrometry. Physiol Genomics 30:89–94
13. Distler U, Kuharev J, Navarro P, Levin Y, Schild H, Tenzer S (2014) Drift time-specific collision energies enable deep-coverage data-independent acquisition proteomics. Nat Methods 11:167–170
14. Fischer F, Poetsch A (2006) Protein cleavage strategies for an improved analysis of the membrane proteome. Proteome Sci 4:2

Chapter 14

Assay of Plasma Membrane H^+-ATPase in Plant Tissues under Abiotic Stresses

Małgorzata Janicka, Anna Wdowikowska, and Grażyna Kłobus

Abstract

Plasma membrane (PM) H^+-ATPase, which generates the proton gradient across the outer membrane of plant cells, plays a fundamental role in the regulation of many physiological processes fundamental for growth and development of plants. It is involved in the uptake of nutrients from external solutions, their loading into phloem and long-distance transport, stomata aperture and gas exchange, pH homeostasis in cytosol, cell wall loosening, and cell expansion. The crucial role of the enzyme in resistance of plants to abiotic and biotic stress factors has also been well documented. Such great diversity of physiological functions linked to the activity of one enzyme requires a suitable and complex regulation of H^+-ATPase. This regulation comprises the transcriptional as well as post-transcriptional levels. Herein, we describe the techniques that can be useful for the analysis of the plasma membrane proton pump modifications at genetic and protein levels under environmental factors.

Key words Abiotic stresses, Plasma membranes, Isolation, H^+-ATPase, Proton transport, Protein phosphorylation, *CsHAs* transcript level

1 Introduction

A characteristic feature of H^+-ATPase associated with plasma membrane is the ability to combine the hydrolysis of ATP with proton pumping across the membrane. Thus, this activity can be measured as the rate of ATP hydrolysis (enzymatic, colorimetric method) or as a proton transport (determination of H^+ movement coupled with the changes in acridine orange absorbance). It is generally apparent that a critical factor determining the credible measurements of both activities is the quality and quantity of membranes. Among methods that have been developed for the separation of plasma membranes from plant tissues, the partitioning of the microsomal fraction in the dextran-polyethylene glycol two-phase system is the most preferable [1]. This method guarantees not only the substantial amounts of isolated plasma membranes but also their high purity and determined, right-side out orientation of

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vesicles (exposing out the membrane apoplastic side), which can be easily turned out (exposing out the membrane cytoplasmic side). Right-side out vesicles are excellent objectives for the determination of the hydrolytic activity of H^+-ATPase, while in-side out membranes are the most adequate system for the study of ATP-dependent proton pumping activity. Both the activities of PM H^+-ATPase can be modulated by environmental factors transcriptionally and post-transcriptionally. Many studies have revealed that H^+-ATPase regulation at the protein level involves reversible phosphorylation of the penultimate Thr residue in the C-terminal regulatory domain. It triggers subsequent binding of 14-3-3 protein, which presumably displaces the regulatory domain from the core part of the enzyme leading to its activation [2, 3]. The stress-induced differences in phosphorylation state of enzyme protein could be easily measured with the antibody against phosphothreonine residue. To verify the specificity of the antibody against phosphorylated threonine, a control with antibody against PM H^+-ATPase is mandatory. The transcriptional regulations of the plasma membrane H^+-ATPase in response to environmental impacts engage the gene expression level [4]. It is well known that the H^+-ATPase in many species is encoded by a multigene family. In *Cucumis sativus* we identified ten genes (*CsHA1–10*), differently expressed under various environmental conditions [5, 6].

2 Materials

2.1 Plant Material

Eight-days old cucumber (*Cucumis sativus*, L) seedlings treated with salt (NaCl) or heavy metals (Cd or Cu).

2.2 Reagents

1. Basic nutrient solution: 5 mM $Ca(NO_3)_2{\cdot}4H_2O$, 5 mM KNO_3, 1 mM KH_2PO_4, 1 mM $MgSO_4{\cdot}7H_2O$, 75 μM ferric citrate, 10 μM $MnSO_4{\cdot}5H_2O$, 5 μM H_3BO_4, μM $CuSO_4{\cdot}5H_2O$, 0.01 μM $ZnSO_4{\cdot}7H_2O$, 0.05 μM $Na_2MoO_4{\cdot}2H_2O$, pH 7.5.
2. NaCl-enriched nutrient solution: 5 mM $Ca(NO_3)_2{\cdot}4H_2O$, 5 mM KNO_3, 1 mM KH_2PO_4, 1 mM $MgSO_4{\cdot}7H_2O$, 75 μM ferric citrate, 10 μM $MnSO_4{\cdot}5H_2O$, 5 μM H_3BO_4, μM $CuSO_4{\cdot}5H_2O$, 0.01 μM $ZnSO_4{\cdot}7H_2O$, 0.05 μM $Na_2MoO_4{\cdot}2H_2O$, and 100 mM NaCl, pH 7.5.
3. Heavy metal-enriched nutrient solutions: 5 mM Ca $(NO_3)_2{\cdot}4H_2O$, 5 mM KNO_3, 1 mM KH_2PO_4, 1 mM $MgSO_4{\cdot}7H_2O$, 75 μM ferric citrate, 10 μM $MnSO_4{\cdot}5H_2O$, 5 μM H_3BO_4, μM $CuSO_4{\cdot}5H_2O$, 0.01 μM $ZnSO_4{\cdot}7H_2O$, 0.05 μM $Na_2MoO_4{\cdot}2H_2O$, and 10 μM $CdCl_2$ or 10 μM $CuSO_4$, pH 5.5.
4. Extraction buffer: 25 mM BTP-MES (pH 7.5), 330 mM sorbitol, 5 mM KCl, 5 mM EDTA, 5 mM DTT, 0.5 mM PMSF

(*see* **Note 1**), 0.2% BSA. First make 25 mM BTP-MES pH 7.5. Weigh 1.764 g BTP and resolve in 250 mL of water. Resolve 1.22 g MES in 250 mL H_2O. Add BTP to MES. Mix for pH adjustment to 7.5 adding an appropriate amount of BTP or MES solutions. Store at 4 °C. Fresh, on the day of plasma membrane isolation, add to 100 mL of buffer (25 mM BTP-MES pH 7.5) 6 g sorbitol, 186 mg EDTA, 77 mg DTT, 200 mg BSA, 37 mg KCl, and 0.5 mL 0.5 mM PMSF.

5. Basic buffer: 5 mM BTP-MES, pH 7.5 containing 5 mM KCl, and 0.1 mM EDTA. Weigh 0.353 g BTP and resolve in 250 mL water. Resolve 0.244 g MES in 250 mL water. Using BTP solution adjust pH of MES to a value 7.5. Resolve 37 mg KCl and 4 mg EDTA in 100 mL BTP-MES.
6. Resuspending buffer: To 100 mL of basic buffer add 6 g sorbitol.
7. 6.2% two-phase system of dextran T500 (DEX) and polyethylene glycol 3350 (PEG): e.g., 8.0 g phase system with final composition 6.2% (w/w) each of DEX (2.48 g of 20% DEX) and PEG (1.24 g of 40% PEG), 1 mL of 330 mM sorbitol (*see* **Note 2**), 2 mL of microsomal fraction. The whole of phase system is complemented to 8 g with basic buffer.
8. Incubation buffer 1: 165 mM TRIS-MES, pH 7.5, with 250 mM KCl and 12.5 mM $MgSO_4$. Weigh 2 g TRIS and resolve it in 100 mL H_2O and 3.22 g MES and resolve it in 100 mL H_2O. Add MES into TRIS solution until pH 7.5. Resolve 308 mg $MgSO_4{\cdot}7H_2O$ and 1.86 g KCl in 90 mL of TRIS-MES and supplement the volume to 100 mL with buffer.
9. Ames reagent: Mix one part of 10% (w/v) ascorbic acid with six parts of 0.42% (w/v) ammonium molybdate in 1 N H_2SO_4. Add the sodium dodecyl sulfate (SDS) to the final concentration of 2%.
10. Incubation buffer 2: 250 mM BTP-MES, pH 7.5, with 330 mM sorbitol, 50 mM KCl, 3.75 mM $MgSO_4$, 0.1% BSA, 10 uM acridine orange, and 0.05% Brij 58. Prepare solutions of 7.08 g BTP and 4.88 g MES in 100 mL of water. Using BTP solution adjust pH of MES to 7.5. Resolve 6.01 g sorbitol in 100 mL H_2O, 373 mg KCl in 100,100 mL H_2O, 92 mg $MgSO_4{\cdot}7H_2O$ in 100 mL H_2O, 100 mg BSA in 100 mL H_2O, 500 mg Brij 58 in 100 mL H_2O, and 3 mg acridine orange in 100 mL H_2O. Finally, mix 0.1 mL BTP-MES (pH 7.5), 0.1 mL sorbitol, 0.1 mL KCL, 0.1 mL $MgSO_4$, 0.1 mL BSA, 0.1 mL acridine orange, 0.1 mL Brij 58, and 0.1 mL H_2O (*see* **Note 3**).
11. Running buffer: 25 mM TRIS (pH 8.3) with 192 mM glycine and 0.2% SDS. Weigh 3.03 g TRIS, 14.41 g glycine, 2.0 g SDS and resolve them in 900 mL of water. Mix and adjust pH to 8.3

using small amounts of TRIS or glycine. Make up to 1 L with water. Store at 4 °C.

12. 7.5% SDS-polyacrylamide gel: Mix 3 mL of 30% acrylamide/bisacrylamide solutions (29:1), commercially available, with 9 mL of running buffer and 72 μL of AP (ammonium persulfate 10%, solution in water, make fresh each time) and 24 μL TEMED.
13. SDS sample loading buffer (4×): 8% SDS, 40% glycerol, 40 mM TRIS, 4 mM EDTA, 320 mM DTT, and 0.2% bromophenol blue. Weigh 0.8 g SDS, 48.4 mg TRIS, 14.9 mg EDTA, 493 mg DTT, 0.02 g bromophenol blue, and 4 mL glycerol. Resolve in 10 mL of water and mix. Divide into 100 μL portions and store at −20 °C (*see* **Note 4**).
14. 5 mM PMSF: 174 mg PMSF dissolve in 10 mL ethanol.
15. Transfer buffer: 25 mM TRIS (pH 8.3) with 150 mM glycine and 10% methanol. Weigh 3.03 g TRIS and 11.0 g of glycine and resolve in 800 mL H_2O. Mix and adjust pH to 8.3 using small amounts of TRIS or glycine. Add 100 mL of methanol. Make up to 1 L with water and store at 4 °C.
16. TBS: 50 mM TRIS (pH 7.5) with 138 mM NaCl, 2.7 mM KCl. 5.1 Weigh 6 g TRIS, 8 g NaCl, and 0.2 g KCl. Add water to volume of 900 mL. Mix and adjust pH to 7.5 with HCl. Make up to 1 L with water.
17. Blotting buffer: Roti®-Block, ready to use, 10× concentrated, protein-free blocking solution.
18. DAB solution: 4 mg 3,3′-Diaminobenzidine (DAB) and 4 μL H_2O_2 dissolve in 5 mL TBS.
19. TRI Reagent (Sigma).
20. 0.1% diethylpyrocarbonate in milli-Q water (DEPC-treated water), autoclaved and stored at room temperature.
21. Chloroform.
22. Isopropanol.
23. 75% ethanol.
24. DNase I (RNase-free) and reaction buffer with $MgCl_2$.
25. cDNA synthesis kit.
26. SYBR Green DNA dye.
27. 10 μM-specific forward and reverse primers in DEPC-treated water.

3 Methods

3.1 Plant Cultivation

1. Germinate the cucumber seeds (*Cucumis sativus* L.) 48 h in darkness at 25 °C.
2. Transfer the seedlings to the basic nutrient solution for 6 days (control plants) or to the NaCl or heavy metal-enriched nutrient solution (stressed plants).
3. Put the seedlings in growth chamber under 16 h photoperiod (180 μmol/m^2/s) at 25 °C during the day and 22 °C during the night at the relative humidity 70%.

After 6 days of growing cut the roots of control plants and plants treated with NaCl, or with heavy metals, rinse them twice with distilled water, blot and froze in −80 °C until used.

3.2 Isolation of Plasma Membrane from Plant Roots

Carry out all the procedures at ice.

1. 30 g of plant roots homogenize with 60 mL of extraction buffer in cold mortar.
2. Filter the homogenate through four layers of gauze.
3. Centrifuge homogenate at 18,000 × *g* for 10 min (4 °C) and remove the pellet.
4. Centrifuge the supernatant at 120,000 × *g* for 30 min (4 °C) (BECKMAN L7-35 ultracentrifuge).
5. Suspend the pellet (microsomal fraction) in 2 mL of resuspending buffer.
6. Add the microsoms into the centrifuge tube containing 6.2% two-phase system of dextran T500 (DEX) and polyethylene glycol 3350 (PEG).
7. Mix thoroughly the contents by around 20–25 inversions of the tube.
8. Centrifuge the tube at 500 × *g* for 5 min, (4 °C) to phase separation.
9. Remove carefully with Pasteur pipette about 90–95% of the PEG-upper phase enriched in plasma membranes, without disturbing the interphase (Fig. 1).
10. Discard the interphase and DEX-lower phase containing the mixture of different intracellular membranes.
11. Dilute the upper phase about fivefold with suspending buffer and pellet it by centrifugation at 120,000 × *g* for 30 min (4 °C).
12. Suspend pellet enriched in plasma membranes in suspending buffer to a protein concentration of about 2–4 mg/mL.

Fig. 1 Two-phase system after centrifugation

The plasma membrane vesicles obtained by this procedure are mainly right-side-out oriented [7] and are useful for the determination of the hydrolytic ATPase activity as well as for Western blot analysis.

To turn the orientation of vesicles, add Brij 58 to the final concentration 0.05% into plasma membranes suspension [8]. Use vesicles to the measurements of ATP-dependent H^+ transport across the plasma membrane.

3.3 Determination of H^+-ATPase Activities

3.3.1 The Hydrolytic Activity of H^+-ATPase [9, 10]

1. Mix 0.2 mL of incubation buffer 1 with 0.1 mL 1 mM Na_2MoO_4 (inhibits the activity of acid phosphatases), 0.1 mL 10 mM NaN_3 (inhibitor of the mitochondrial ATPase), 0.1 mL 500 mM $NaNO_3$ (inhibitor of tonoplast ATPase), 0.1 mL 2 mM Na_3VO_4 (inhibitor of plasma membrane ATPase), 0.1 mL 30 mM ATP, 0.1 mL 0.2% (v/v) Triton X-100, and 0.1 mL of plasma membrane vesicles (20–50 μg protein). Supplement a volume to 1 mL with water. To control samples add 0.1 mL H_2O instead of Na_3VO_4.
2. Place the samples for 30 min in a water bath at 37 °C.
3. To terminate the reaction add 1 mL of 10% trichloroacetic acid (TCA) to the reaction mixture.
4. Centrifuge the samples for 10 min at 10,000 × *g*.
5. To 1 mL of the supernatant add 2.6 mL of Ames' reagent containing 0.1% (w/v) sodium dodecyl sulfate (SDS) to determine Pi released during enzymatic reactions [11].

6. After 20 min at room temperature read absorbance of samples at 700 nm.
7. The amount of inorganic phosphate read from the standard curve (0.1–2.0 μP_i).
8. Calculate the differences between P_i amount released in assays without and with Na_3VO_4 per 1 g of fresh weight during 1 h.

3.3.2 The Proton Transport Activity of H+ ATPase [7]

1. Mix 0.8 mL of incubation buffer 2 with 0.1 mL plasma membrane vesicles (20–50 μg protein).
2. Incubate the samples 3 min at room temperature before the reaction is started.
3. Add 0.1 mL 37.5 mM ATP and read the absorbance (A_{495}) in every 30″ during 3 min (*see* **Note 3**).
4. To measure the passive proton movement through the membrane, determine A_{495} changes in samples without ATP.
5. Express the proton transport as the ΔA_{495} per 1 min and 1 mg of protein.

3.4 Western Blot Analysis

1. Mix 15 μL of plasma membranes (10–30 μg) with 5 μL SDS sample loading buffer and 1 μL 5 mM PMSF. Incubate the mixture for 30 min at room temperature and load it on 7.5% SDS-polyacrylamide gel.
2. After 1 h of electrophoresis at room temperature (25 mA), electro-transfer proteins to nitrocellulose membrane at room temperature (60 V, 200 mA) for 1.5 h using transfer buffer by semi-dry blotting (*see* **Note 4**).
3. Block the blot by soaking in blotting buffer for 1 h with shaking.
4. To identify the plasma membrane H+-ATPase, incubate the blots overnight (8 °C) with antibody against plasma membrane H+-ATPase diluted 2,000-fold (*see* **Note 5**).
5. Wash the nitrocellulose membrane two times and incubate it at room temperature for 1 h with 1:4,000 diluted secondary antibodies (anti-mouse, conjugated to horseradish peroxidase).
6. Visualize bands by staining with DAB.
7. To identify the phosphorylation of plasma membrane H+-ATPase repeat above procedure skipping **steps 4** and **5**.
8. Incubate the membrane overnight (8 °C) with antibody against phosphothreonine (rabbit polyclonal to phosphothreonine) at a concentration of 2 μg/mL.
9. Wash the nitrocellulose membrane twice and incubate it at room temperature for 1 h with 1:5,000 diluted secondary antibody (anti-rabbit, conjugated to horseradish peroxidase) and visualize bands by staining with DAB.

3.5 Extraction of Total RNA from Cucumber Roots

Clean the equipment with an RNase decontamination solution. Carry out all procedure at ice unless otherwise specified.

1. Homogenize frozen plant roots in cold mortal (*see* **Note 6**) and add TRI Reagent (1 mL per 50–100 mg of plant tissue).
2. Transfer homogenate into a 1.5 mL tube and keep 5 min on ice.
3. Add 0.2 mL of chloroform per mL of TRI Reagent used, cover the sample and shake vigorously by hand for 15 s.
4. Incubate the sample for 10 min on ice and centrifuge then at 12,000 × *g* for 15 min at 4 °C.
5. Transfer the colorless aqueous phase to a new plastic tube and add 0.5 mL of isopropanol per mL of TRI Reagent used in **step 1**; mix gently by repeated inversion.
6. Incubate the sample for 10 min on ice.
7. Centrifuge the mixture at 12,000 × *g* for 10 min at 4 °C to pellet precipitate.
8. Decant and discard the supernatant (*see* **Note 7**).
9. Add 1 mL of 75% ethanol per mL of TRI Reagent used in **step 1** to rinse the pellet and mix by gently inversion.
10. Re-collect the precipitate by centrifugation at 7,500 × *g* for 5 min at 4 °C.
11. Decant ethanol and air-dry the pellet at room temperatures for about 10 min (*see* **Note 8**).
12. Resuspend the pellet in 20–50 μL of DEPC-treated water and mix by repeated, carefully pipetting with a micropipette for 5–10 min (*see* **Note 9**).
13. Store at −80 °C until used.

3.6 Removal of DNA from Total RNA and Synthesis of Single-Strand cDNA

1. Add in the following order and mix well: 1 μg of RNA, 1 μL of 10× reaction buffer with $MgCl_2$, 1 U of DNase I, RNase-free, and DEPC-treated water to volume 10 μL.
2. Incubate the mixture at 37 °C for 30 min.
3. Add 1 μL 50 mM EDTA and incubate at 65 °C for 10 min to stop the reaction.

   To convert total RNA into cDNA, High Capacity cDNA synthesis Kit (Applied Biosystems) was used.
4. Prepare 10 μL of DNA-free total RNA (2 μg) and add to the mixture, placed on ice: 2 μL of 10× RT Buffer, 0.8 μL of 25× dNTP Mix (100 mM), 2 μL of 10× RT Random Primers, 1 μL of MultiScribeTM Reverse (50 U/μL), 1 μL of Transcriptase RNase Inhibitor (20 U/μL), and 3.2 μL of Nuclease-free H_2O.

5. Perform reverse transcription in a thermal cycler according to the program:

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	∞

6. cDNA is ready to use for real-time PCR, as eightfold-diluted in nuclease-free water.

3.7 Specific Primer

The specific primers used for amplifying of fragments of *CsHA2*, *CsHA3*, *CsHA4*, *CsHA8*, *CsHA9*, and-*CsHA10* that are expressed in cucumber roots, as well as for *TIP4*, were designed using LightCycler Probe Design Software 2:

5′-ACCCGAGTCGACAAACATCT-3′ (forward) and 5′-CTTGGCACAGCAAAGTGAAA-3′ (reverse) for *CsHA2*, 5′-AAGTTTCTGGGGTTCATGTGGAAT-3′ (forward) and 5′-GTAACAGGAAGTGACTCTCCAGTC-3′ (reverse) for *CsHA3*, 5′-CTACAGCTTGGTAACATACATTC-3′ (forward) and 5′-GTTGTAGTCCATGTAATGTCCTC-3′ (reverse) for *CsHA4*, 5′-CTCATGCGCAAAGAACATTAC-3′ (forward) and 5′-CTGAATTGTGTCAATGTCAAGTC-3′ (reverse) for *CsHA8*, 5′-AAACCAGAAGTGCTGGAG-3′ (forward) and 5′-CTCAGCACCCTCACTAGTAA-3′ (reverse) for *CsHA9*, 5′-GACATAATCAAGTTTGCAATCAGATA-3′ (forward) and 5′-TTCTGTATAAGTTGTGCGGT-3′ (reverse) for *CsHA10*,

and 5′-CAACAGGTGATATTGGATTATGATTATAC-3′ (forward) and 5′-GCCAGCTCATCCTCATATAAG-3′ (reverse) for *TIP41*-like protein (used as an internal control in Real-time PCR).

3.8 Real-Time Quantitative PCR Analyses

To examine alterations in *CsHAs* expression we recommend real-time RT quantitative PCR analysis of cDNA from the roots.

1. Place 0.2 mL tube on ice and add 2× PCR Master Mix SYBR in the following order, according to the A&A Biotechnology instruction: 5 μL of 2× Master SYBR Green I B, 1 μL of *CsHA*-specific primer forward (10 μM), 1 μL of *CsHA*-specific primer reverse (10 μM), 2 μL of Nuclease-free water, and 1 μL of eightfold-diluted cDNA template.
2. Mix gently and avoid air bubbles.
3. Place the tube in a real-time PCRcycler according to the program:

Steps	Temperature (°C)	Time (s)	Number of cycles
Initial denaturation	95	30	1
Denaturation	95	10	
Anealing	68	10	45
Extention	72	15	
Final extention	65	30	1
Hold	40	30	1

4. The gene encoding TIP41 was used as an internal control.

See also **Notes 11–15**.

4 Notes

1. Phenylomethylsulfonyl fluoride dissolves in 96% ethanol and is stored at 4 °C.
2. Prepare all stock solutions used in two-phase system: 20% (w/w) DEX, 40% (w/w) PEG, and 330 mM sorbitol in the basic buffer.
3. When proton transport is weak, increase (e.g., twice) amount of plasma membrane protein in the reaction mixture.
4. Cut a nitrocellulose membrane to the size of gel and soak it in transfer buffer for 10 min.
5. We use monoclonal antibody against plasma membrane H^+-ATPase, (46E5B11D) kindly provided by W. Michalke (Universität Freiburg, Germany) or antibody made by Agrisera (cat. nr AS07 260).
6. We use clean mortal and grinder cooled in the fridge the day before RNA preparation.
7. If the sample has a high content of fat, protein, or parts of plants after homogenization, centrifuge the sample one more time at 12,000 × *g* for 10 min at 4 °C and remove the insoluble materials.
8. The RNA pellet is on a side of bottom tube and looks like a gel.
9. Do not dry the RNA pellet completely, because this can decrease RNA solubility.
10. The quantity of RNA can be determined using a spectrophotometer. Final preparation of RNA should be free of DNA and proteins and have an A260/A280 ratio between 1.7 and 2.0.
11. Successive dilutions of the sample with the lowest Cp are used as a standard curve.

12. A negative control without cDNA template should be included in the same qPCR run for each primer pair.
13. Amplification efficiency for each primer pair should be in the range 1.9–2.0.
14. The data are analyzed by the ΔΔCT method using LightCycler Software 4.1 (Roche).
15. To determine the statistical significance of the quantitative PCR data, analysis is carried out using standard *t*-test and $P < 0.05$ is accepted as significant.

References

1. Larsson C (1985) Plasma membranes. In: Jackson JF, Linskens HF (eds) Modern methods of plant analysis. Cell components, vol 1. Springer Verlag, Berlin, Heidelberg, New York, pp 85–104
2. Axelsen KB, Venema K, Jahn T, Baunsgaard L, Palmgren MG (1999) Molecular dissection of the C-terminal regulatory domain of the plant plasma membrane H^+-ATPase AHA2: mapping of residues that when altered give rise to an activated enzyme. Biochemistry 38 (22):7227–7234
3. Morsomme P, Boutry M (2000) The plant plasma membrane H^+-ATPase: structure, function and regulation. Biomembranes 1465:1–16
4. Duby G, Boutry M (2009) The plant plasma membrane proton pump ATPase: a highly regulated P-type ATPase with multiple physiological roles. Pflugers Arch 457:645–655
5. Janicka-Russak M, Kabała K, Wdowikowska A, Kłobus G (2012) Response of plasma membrane H^+-ATPase to low temperature in cucumber roots. J Plant Res 125:291–300
6. Janicka-Russak M, Kabala K, Wdowikowska A, Kłobus G (2013) Modification of plasma membrane proton pumps in cucumber roots as an adaptation mechanism to salt stress. J Plant Physiol 170:915–922
7. Kłobus G, Buczek J (1995) The role of plasma membrane oxidoreductase activity in proton transport. J Plant Physiol 146:103–107
8. Johansson F, Olbe M, Sommarin M, Larsson C (1995) Brij 58, a polyoxyethylene acyl ether, creates membrane vesicles of uniform sidedness. A new tool to obtain inside-out (cytoplasmic side-out) plasma membrane vesicles. Plant J 7:165–173
9. Gallagher SR, Leonard RT (1982) Effect of vanadate, molybdate and azide on membrane-associated ATPase and soluble phosphatase activities of corn roots. Plant Physiol 70:1335–1340
10. Sze H (1985) H^+-translocating ATPase: advances using membrane vesicles. Annu Rev Plant Physiol 36:175–208
11. Ames BN (1966) Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol 8:115–118

Chapter 15

Absolute Quantitation of In Vitro Expressed Plant Membrane Proteins by Targeted Proteomics (MRM) for the Determination of Kinetic Parameters

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Abstract

The purification of a functional soluble protein from biological or in vitro expression systems can be problematic and the enrichment of a functional membrane protein for biochemical analyses can be a serious technical challenge. Recently we have been characterizing plant endomembrane nucleotide sugar transporters using a yeast expression system. However, rather than enriching these in vitro expressed proteins to homogeneity, we have been conducting biochemical characterization of these transport proteins in yeast microsomal fractions. While this approach has enabled us to estimate a variety of kinetic parameters, the accurate determination of the turnover number of an enzyme-substrate complex (k_{cat}) requires that the catalytic site concentration (amount of protein) in the total reaction volume is known. As a result, we have been employing targeted proteomics (multiple reaction monitoring) with peptide standards and a triple quadrupole mass spectrometer to estimate the absolute amount of protein in a mixed protein microsomal fraction. The following method details the steps required to define the absolute quantitation of an in vitro expressed membrane protein to define complete kinetic parameters.

Key words Membrane proteins, Multiple reaction monitoring, Enzyme kinetics

1 Introduction

The development of the Michaelis–Menten model over 100 years ago [1, 2], in which the rate of the enzyme is related to the substrate concentration, now represents one of the most widely used equations for the examination of enzyme kinetics. While the original derivation of the equation was based on a number of assumptions, some of which were subsequently addressed [3], the equation is still incredibly useful in understanding an enzymes function as it provides a kinetic description of its activity. Significantly, both the Michaelis constant (K_m) providing a measure of substrate affinity and the maximal rate or maximum velocity of the

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enzyme (V_{max}) can be empirically derived by measuring catalytic rates at various substrate concentrations. However, while the determination of both K_m and V_{max} does not require any knowledge of the enzyme concentration, this information is necessary to derive the turnover number (k_{cat}) of the enzyme. In general, the turnover number (k_{cat}) and specificity constant (k_{cat}/K_m) are now regarded as the principal steady-state kinetic parameters necessary to adequately describe an enzyme [4].

The determination of an enzyme's kinetic parameters is commonly accomplished after the enrichment and purification of the enzyme to near homogeneity. This enrichment process was traditionally accomplished directly from complex biological material [5], but is now more commonly achieved after in vitro expression in a heterologous system such as *Escherichia coli* [6]. However, the purification and enrichment of membrane proteins to near homogeneity using the above approaches can be extremely challenging. While there are documented successes using such approaches with membrane proteins [7], these likely hide the numerous failures. We recently faced similar problems when attempting to biochemically characterize the members of the Nucleotide Sugar Transporter family from *Arabidopsis* [8, 9]. Although we had developed a sensitive assay [10] using microsomal fractions from *Saccharomyces cerevisiae* (yeast) expressing our transporters of interest, the nature of these membrane proteins (8–10 predicted transmembrane domains), incentivised us to develop an alternative approach over further enrichment and purification. The determination of both K_m and V_{max} was possible; however, an estimation on the amount of protein in this mixed microsomal fraction was required to determine k_{cat}. As a result, we developed an MRM-based assay for the absolute quantitation of our expressed transporter in yeast microsomal preparations using protein-specific synthetic peptides and triple quadrupole mass spectrometry. This approach has now been successfully applied on numerous occasions [8, 9] to determine the concentration of a protein and to determine complete kinetic parameters. The method details the in vitro expression of a membrane protein in yeast, the preparation of a mixed membrane fraction containing the membrane protein of interest, and the absolute quantitation of the expressed protein by triple quadrupole mass spectrometry (MRM) and peptide standards for the estimation of k_{cat}.

The accurate characterization of enzymes will be a vital aspect in our capacity to develop next-generation agricultural crops. Future bioengineering programs will rely on large-scale systems biology approaches to develop models that predict engineered outcomes and thus the accuracy of these models will directly rely on the quality of data and the contributions of well-characterized parts [11, 12].

2 Materials

Prepare solutions using ultrapure water (18 MΩ cm at 25 °C) and analytical grade reagents. Utilize LC-MS grade reagents for solutions and buffers used in conjunction with mass spectrometry. Prepare all reagents at room temperature, unless otherwise indicated.

2.1 In Vitro Expression in Saccharomyces cerevisiae

1. *Saccharomyces cerevisiae* (yeast) strain INVSc1 (*see* **Note 1**).
2. Yeast inducible expression vector, such as pYES-DEST52 (*see* **Note 2**).
3. Yeast transformation kit, such as, S.c. EasyComp Transformation Kit (Thermo Fisher Scientific) (*see* **Note 3**).
4. Agar.
5. Yeast Nitrogen Base without amino acids (*see* **Note 4**).
6. Yeast Synthetic Drop-out Medium, lacking the appropriate selectable marker, such as uracil (*see* **Note 5**).
7. 50 mL tubes.
8. 250 mL baffled flask (*see* **Note 6**).
9. Bench top centrifuge with rotor for 50 mL tubes capable of 3,000 × *g*.
10. 1000 mL baffled flask (*see* **Note 6**).
11. Preparative centrifuge for 250 mL tubes and capable of 3,000 × *g*.
12. 20% dextrose (filter sterilized).
13. 20% galactose (filter sterilized) (*see* **Note 7**).
14. 1 M sodium azide solution (*see* **Note 8**).

2.2 Isolation of Yeast Microsomes

1. Yeast Resuspension Buffer: 50 mM potassium phosphate pH 7.1, 1.4 M sorbitol, 10 mM NaN_3, and 40 mM 2-mercaptoethanol.
2. Lyticase from *Arthrobacter luteus*.
3. Yeast Lysis Buffer: 0.8 M sorbitol, 10 mM triethanolamine/acetic acid pH 7.2, 1 mM EDTA.
4. Protease inhibitors, such as Protease Inhibitor Cocktail for general use (Sigma-Aldrich).
5. 100 mM phenylmethanesulfonyl fluoride (PMSF).
6. Microsome Resuspension Buffer: 10 mM N-(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine (Tricine-KOH) pH 7.5, 50 mM potassium D-gluconate, 20% glycerol (*see* **Note 9**).
7. Bench top centrifuge with rotor for 15 and 50 mL tubes capable of up to 8,000 × *g*.

8. Ultracentrifuge with rotor for 30 mL tubes and capable of 100,000 × *g* for pelleting microsomes.
9. Glass beads acid-washed.
10. Colorimetric protein quantification assay.

2.3 Analysis of Microsomes by Immunoblotting

1. Mini electrophoresis chamber for protein separation.
2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) precast gels compatible with the electrophoresis chamber (*see* **Note 10**).
3. Electrophoresis Buffer: 248 mM Tris-base, 1.92 M glycine, 1% (w/v) SDS.
4. Sample Buffer: 40 mM Tris–HCl pH 6.8, 5% (w/v) SDS, 0.4% (w/v) bromophenol blue, 1% (v/v) 2-mercaptoethanol, 1× protease inhibitor, 5 mM PMSF, 8 M urea, 0.1 mM EDTA.
5. 2-mercaptoethanol solution.
6. SDS-PAGE protein markers.
7. Immunoblot apparatus.
8. Western Transfer Buffer: 25 mM Tris-base, 192 mM glycine, 10% (v/v) methanol.
9. 100% (v/v) methanol.
10. Filter paper for immunoblotting.
11. Nitrocellulose membrane.
12. TBST Buffer: 50 mM Tris–HCl pH 7.6, 150 mM NaCl, and 0.1% (v/v) Tween-20.
13. Skim milk powder.
14. Protein tag antibody, such as Anti-V5 antibody (Invitrogen) (*see* **Note 11**).
15. Secondary antibody, Anti-Mouse- or Anti-Rabbit- IgG Peroxidase (*see* **Note 12**).
16. Chemiluminescent substrates for horseradish peroxidase.
17. Chemiluminescence imaging system.

2.4 Determination of Peptides for MRM Quantitation

1. Tandem mass spectrometer (MS/MS) with liquid chromatography (LC) delivery system capable of data-dependent acquisition (DDA)/independent data acquisition (IDA) (*see* **Note 13**).
2. Reverse phase column for peptide separation, such as a C_{18} HPLC column.
3. Digestion Buffer: 1 M urea and 10 mM tris(hydroxymethyl) aminomethane (Tris–HCl) pH 8.5 solution (*see* **Note 14**).
4. 1 M dithiothreitol (DTT) (*see* **Note 15**).
5. 1 M iodoacetamide (IAA) (*see* **Note 16**).

6. Sequencing grade trypsin.
7. Solid phase extraction (SPE) for peptides, such as Micro Spin-Columns with C_{18} (Harvard Apparatus, MA, USA).
8. SPE Buffer 1: 80% acetonitrile (v/v) with 0.1% trifluoroacetic acid (v/v).
9. SPE Buffer 2: 2% acetonitrile (v/v) with 0.1% trifluoroacetic acid (v/v).
10. MS Buffer A: 2% acetonitrile, 0.1% formic acid.
11. SpeedVac concentrator.
12. Access to search engine to identify proteins from data generated by tandem mass spectrometry (*see* **Note 17**).

2.5 Quantitation of Induced Membrane Protein by MRM

1. Triple quadrupole mass spectrometry (MS) system coupled to a liquid chromatography (LC) delivery system capable of MRM mode (*see* **Note 13**).
2. Synthetic peptide standards >95% purity (*see* **Note 18**).
3. Infusion Buffer: 50% acetonitrile and 0.1% formic acid.
4. Syringe pump.
5. Gastight Hamilton syringe 500 μL.
6. MS Buffer A: 2% acetonitrile, 0.1% formic acid.
7. Reverse phase column for peptide separation, such as a C_{18} HPLC column.
8. Software for MRM method development, profiling, and quantitation, such as Skyline (https://skyline.gs.washington.edu/labkey/project/home/software/Skyline/begin.view) (*see* **Note 19**).

3 Methods

3.1 Yeast Transformation and Protein Induction

1. Transform the expression vector containing the sequence encoding your gene of interest (*see* **Note 20**) into *Saccharomyces cerevisiae* (yeast) using a commercial yeast transformation kit (*see* **Note 21**).
2. Make dropout media using Yeast Synthetic Drop-out Medium and Yeast Nitrogen Base according to the product description. For plates add agar (20 g/L). After autoclaving, add dextrose to a final concentration of 2%.
3. Plate transformed yeast onto agar plates and incubate for 3–4 days at 30 °C.
4. Pick a colony and inoculate a 50 mL starter culture (dropout media, *see* **Note 5**) in a 250 mL flask.
5. Incubate with shaking for approximately 24 h at 30 °C.

6. Transfer culture to a 50 mL tube and centrifuge at 2,500 × *g* for 5 min at room temperature.
7. Take yeast pellet and inoculate 200 mL of dropout media, replacing the 2% (w/v) dextrose with 2% (w/v) galactose, in a 1000 mL flask (*see* **Note 22**).
8. Incubate with shaking overnight at 30 °C (*see* **Note 23**).
9. Harvest yeast cells from the induced culture by centrifugation at 2,500 × *g* for 5 min at room temperature.
10. Wash the pellet with 30 mL of 10 mM sodium azide (*see* **Note 8**) and repeat centrifugation.
11. Decant the supernatant and freeze pellet at −80 °C.

3.2 Enrichment of Microsomes from Yeast Cultures

1. Weigh the yeast pellet to estimate the amount of cells (fresh weight), should be around 2.5 g.
2. Resuspend the yeast pellet in 10 mL of Yeast Resuspension Buffer.
3. Add the appropriate amount of Lyticase to the yeast solution (*see* **Note 24**).
4. Incubate at 37 °C for 1 h to generate spheroplasts (*see* **Note 25**).
5. Harvest spheroplasts by centrifugation at 2,500 × *g* for 5 min.
6. Wash the spheroplasts pellet with Yeast Lysis Buffer.
7. Resuspend the washed spheroplasts in 5 mL Yeast Lysis Buffer containing protease inhibitor cocktail and 1 mM PMSF.
8. Add 5 mL acid-washed glass beads and disrupt spheroplasts by vortexing for 1 min, place on ice for 1 min, and repeat twice.
9. Centrifuge spheroplast lysate at 3,000 × *g* for 5 min at 4 °C, keep the supernatant on ice.
10. Resuspend the un-ruptured spheroplast pellet in 5 mL Yeast Lysis Buffer containing protease inhibitor cocktail and 1 mM PMSF; repeat the vortexing step.
11. Collect the supernatant by centrifugation at 3,000 × *g* for 5 min at 4 °C; repeat this step two more times to produce a 20 mL lysate.
12. A microsomal fraction is obtained by sequential centrifugation.
13. Centrifuge the 20 mL lysate at 8,000 × *g* for 10 min and take the supernatant.
14. Centrifuge the supernatant at 100,000 × *g* for 75 min and discard the supernatant.
15. Resuspend the microsomal pellet in Microsome Resuspension Buffer (*see* **Note 9**).
16. Estimate the protein concentration in the microsomal fraction using a protein quantification assay and then store fractions at −80 °C.

3.3 Assessment of Protein Induction by Immunoblotting

1. To ensure the protein has been induced and is present in the microsomal fraction, immunoblotting can be undertaken using a protein-specific antibody or with an antibody against a tag, such as the V5-tag or His-tag (Fig. 1a).
2. If using a mini-gel system, around 2 μg of protein is suitable. Add Sample Buffer (~5 μL) and then incubate at 65 °C for 15 min.
3. Load samples and protein standards onto a preassembled pre-cast gel (*see* **Note 26**).
4. Separate the protein samples on the gel at 200 V for 45–60 min or until the dye front reaches the bottom of the gel.
5. Remove the gel from the plates and place into Western Transfer Buffer.
6. Cut nitrocellulose membrane to the size of the gel and briefly rinse in water, and then place in Western Transfer Buffer (*see* **Note 27**).
7. Cut two pieces of filter paper to the size of the gel and soak in Western Transfer Buffer.
8. Assemble transfer stack according to the manufacturer's instructions, normally in the following order from the cathode,

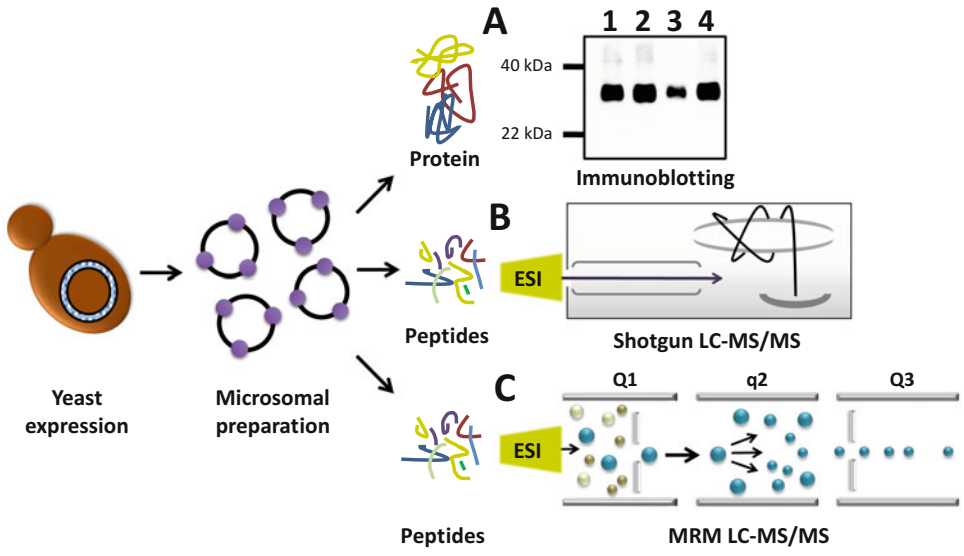

Fig. 1 Workflow outlining the basic procedure for quantification of expressed membrane proteins in yeast. (**A**) Immunoblot analyzing the expression of an induced membrane protein in yeast microsomal fractions using the Anti-V5-tag antibody (*see* Subheading 3.3). (**B**) Shotgun LC-MS/MS analysis of yeast microsomal preparations containing the induced membrane protein to identify unique peptides for MRM (*see* Subheading 3.4). (**C**) Analysis of yeast microsomal preparations containing the induced membrane protein using MRM with a triple quadrupole mass spectrometer (*see* Subheading 2.5, **item 2**)

blotting pad (2), filter paper, gel, nitrocellulose membrane, filter paper, and blotting pad (2).

9. Place transfer stack into the Western apparatus, assemble, add Western Transfer Buffer and connect to an electrophoretic power source.
10. Transfer at the recommended current for 1 h (*see* **Note 28**).
11. Disassemble transfer stack, remove membrane, and place in TBST Buffer.
12. Incubate the membrane with gentle shaking in TBST Buffer with 5% skim milk powder for 1 h at room temperature.
13. Dilute primary antibody in TBST Buffer with 1% skim milk powder (*see* **Note 29**).
14. Incubate the membrane with primary antibody diluted in TBST Buffer with 1% skim milk powder with gentle shaking at room temperature for 1 h (*see* **Note 30**).
15. Wash the membrane with TBST Buffer for 10 min with shaking. Repeat the wash in TBST Buffer two more times.
16. Dilute HRP-conjugated secondary antibody in TBST Buffer with 1% skim milk powder (usually around 10,000-fold dilution) and incubate the washed membrane with gentle shaking at room temperature for 1 h (*see* **Note 31**).
17. Wash the membrane with TBST Buffer for 10 min with shaking. Repeat the wash in TBST Buffer two more times.
18. Prepare the HRP substrate (about 1 mL) by mixing the two reagents (*see* **Note 32**).
19. Drain excess liquid from the membrane and add HRP substrate and incubate for 5 min.
20. Image using a chemiluminescence imager.

3.4 Determining Optimal Peptides for Quantification by MRM

1. Resuspend 50 μg of microsomal protein in about 100 μL Digestion Buffer.
2. Add DTT to the diluted protein extract to a final concentration of 25 mM and incubate for 30 min (room temperature).
3. Add IAA to a final concentration of 50 mM and incubate for 30 min (room temperature) in the dark.
4. Add trypsin at a 1:10 trypsin:protein ratio (*see* **Note 33**) and incubate overnight (37 °C) (*see* **Note 34**).
5. Remove urea and concentrate samples with a Micro SpinColumn (25–75 μL capacity). Initially, hydrate the C_{18} matrix with ultrapure water (75 μL) for 10 min and centrifuge (1,000 × *g*, 2 min) as per the manufacturer's instructions.

6. Wash the SpinColumn with the 50 μL SPE Buffer 1 and centrifuge (1,000 × *g*, 2 min) and prime twice with 50 μL SPE Buffer 2, centrifuging (1,000 × *g*, 2 min) after each step.
7. Add digested peptides in urea solution to the SpinColumn and centrifuge (1,000 × *g*, 2 min), wash twice with 50 μL SPE Buffer 2, centrifuging (1,000 × *g*, 2 min) after each step.
8. Finally, elute into a new tube with 25–50 μL SPE Buffer 1 by centrifuging 1,000 × *g* for 2 min. Concentrate and remove acetonitrile with a SpeedVac concentrator until 1–5 μL of peptide solution remains in the tube.
9. Analyze about 1 μg (approximately 0.1 μL) of the trypsin digested microsomal preparation by nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS) using an automated data-dependent acquisition method optimized for the analysis of complex protein samples (*see* **Note 35**) (Fig. 1b).
10. Data produced after LC-MS/MS analysis can be interrogated with search algorithms, such as the software package Mascot (Matrix Science), to identify peptides from the induced membrane protein within the microsomal fraction suitable for MRM (*see* **Note 36**).
11. Select unique peptides specific for the expressed protein based on precursor (optimally 500–700 m/z), charge state (optimally $[M+2H]^{+2}$), and peptide match score, as it provides an indication of a MS compatibility factor.
12. Obtain synthesized selected peptides (2–3) from a commercial supplier specific for the induced membrane protein to use as standards for MRM.

3.5 Estimating Abundance of Induced Membrane Protein by MRM

3.5.1 Develop the MRM Method and Assess Standard Peptides

1. Dilute synthesized peptide standards to around 1 pmol/μL with Infusion Buffer. Then using the syringe pump and the Hamilton syringe, infuse the mixture into the triple quadrupole mass spectrometer at about 50 μL/min (or a similar flow rate to be used for MRM analysis) and monitor peptide using a Q1 scan mode (*see* **Note 37**).
2. Optimize conditions for the peptides, for example declustering potential (DP), gas flow rate, gas temperature (*see* **Note 38**) to maximize their signal-to-noise ratio.
3. Optimize the fragmentation conditions for each peptide in turn using Product Ion mode (MS2) or equivalent. Take note of the value used for the collision energy (CE) that resulted in the absence of the precursor ion (peptide) and also produced product ions with an optimal signal-to-noise ratio. Take note of two or three product ions corresponding to valid *y*- or *b*-series ions for each peptide.

	Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	CE (volts)
1	712.400	1113.600	100.0	42
2	712.400	1000.500	100.0	42
3	712.400	528.300	100.0	42

Fig. 2 Basic parameters to be included in the MS method necessary to conduct MRM analyses. The "Q1 Mass" corresponds to the observed *m/z* of the peptide. The "Q3 Mass" represents the *m/z* of dominant product ions. The "Time" represents the time spent collecting data on each transition. The "CE" indicates the collision energy. Both, the dominant product ions and the collision energy can be obtained empirically by initially infusing the synthesized peptide

4. Create MRM method using the experimentally optimized values for each transition including Q1 mass (precursor ion), Q3 masses for each precursor ion (product ions), Collision Energy (CE) and a time (ms) for each transition (*see* **Note 39**) (Fig. 2).
5. Create a simple LC elution method (30–50 min) with a 10–20 min acetonitrile gradient (2% acetonitrile to 80% acetonitrile).
6. Dilute synthesized peptides in MS Buffer A, load about 5 pmol and analyze using the MRM and LC methods outlined above.
7. View and assess the subsequent results using the MS software provided with the mass spectrometer, making note of the elution window for each peptide, elution times, and signal intensities for different transitions (product ions) from the same peptide. At this stage, transitions (product ions) resulting in poor signals could be eliminated from the method (retain at least two), peptides could be eliminated if they are not retained on the column, elute as a split peak or elution occurs over a wide window (*see* **Note 40**).
8. Add the peptide sequences to the Skyline software package, then select the precursor mass using "pick children" with the corresponding charge state as observed in the mass spectrometer (e.g., ++ or +++ or ++++). Select the product ions using the "pick children" option for the appropriate charge state empirically determined and analyzed above (e.g., *y* 6, *b*3). Save the target list as a Skyline file (Fig. 3a).
9. Import the raw MS data file from the 5 pmol standard peptide analysis into Skyline and check to ensure that all peptides and transitions have imported correctly by selecting each peptide and transition and comparing them to the results observed on the MS software provided with the mass spectrometer (Fig. 3b).

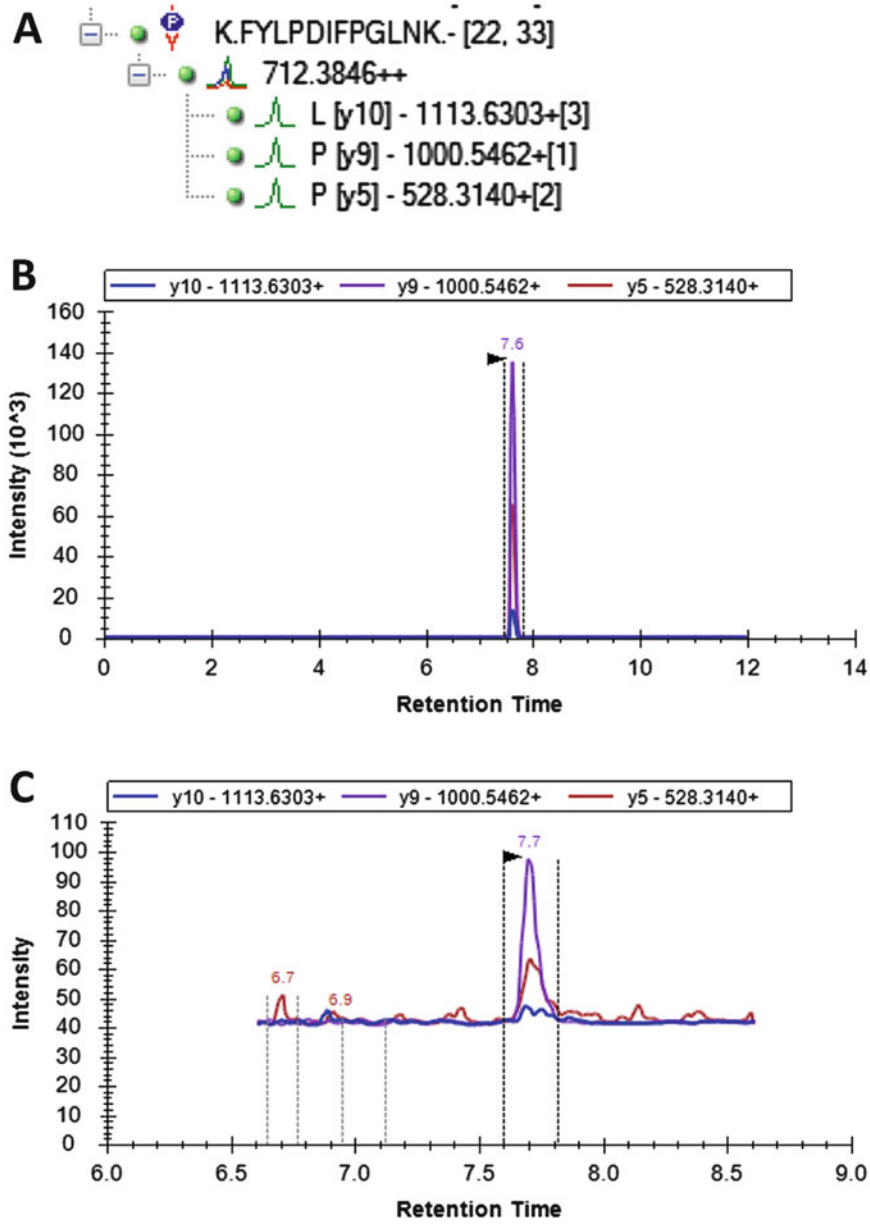

Fig. 3 Overview of the main features of Skyline. (**A**) An example peptide entry in Skyline with the charge state for the peptide selected (++) and the three dominant product ions [y10], [y9], and [y5] shown. The *green circles* indicate that all three transitions were detected in the sample. (**B**) MRM raw data for a synthesized peptide (FYLPDIFPGLNK) imported into Skyline. Note the differences in transition intensity, with [y9] and [y5] producing more intense signals under these condition compared with the [y10] fragment ion. (**C**) MRM data from a complex lysate with a protein containing the tryptic peptide (FYLPDIFPGLNK). Data from complex biological samples result in background and reduced signal. Note that the retention time matches the one of the standard peptide and the profile of transitions also corresponds, albeit the signal for the [y10] fragment ion is low

3.5.2 Absolute Quantification of the Induced Protein in the Microsomal Membrane Fraction

1. Utilize approximately 10 μg (1 μL) of the trypsin digested microsomal preparation (*see* Subheading 3.4) in MS Buffer A and analyze using the method that was developed and validated with the standard peptides (*see* Subheading 3.5.1).
2. View and assess the results using the MS software provided with the mass spectrometer. Check the elution times and the signal intensities for different transitions and compare with the results from the 5 pmol peptide standard analysis.
3. If the peptides are clearly detectable in the microsomal preparation, a rough estimation of induced protein content can be made by comparing the intensity from the 5 pmol standard sample to the microsomal sample.
4. Based on comparisons between the signals from the 5 pmol standard peptide and the 10 μg microsomal preparation, estimate a range of concentrations required to generate a standard curve from the peptide standards. Based on numerous examples, we have found the following standard curve seems to cover most variations in expression when conducting MRM analysis at flow rates around 400 μL/min: 0.1, 0.25, 0.5 fmol, 1, 5, 20 pmol.
5. Create a sample sequence with the MRM method that analyzes the range of concentrations for the standard curve (**step 4** above), with the digested 10 μg microsomal preparation interspersed in the sequence (Fig. 1c). The objective is to generate a minimum of three replicates for each concentration of the standard peptide solution and a similar number for the microsomal preparation (10 μg each).
6. Open the previously saved peptide target list in Skyline and import the raw MS data files for the replicates of the microsomal preparation and the standard curve (Fig. 3c).
7. Inspect each peptide from these samples to ensure that the correct peaks have been assigned. Compare the retention time with that of the synthetic standard peptides. The correct peak can be manually selected in Skyline using the mouse. The retention times for all the samples and all the peptides can be easily checked using View > Retention Times > Replicate Comparison option. Also check that each fragment transition is present and that each one has a similar proportion as the fragment transitions found for the synthetic standard peptides.
8. Export the results (File > Export > Report) as a .csv (a report) file with data corresponding to precursor 'Total Area" (*see* **Note 41**).
9. Open exported data in Microsoft Excel (or similar) and generate a standard curve using data from the synthetic standard

peptides, e.g., amount loaded (pmol) against the precursor 'Total Area" values for each peptide.

10. Using the standard curve and the 'Total Area" values, estimate the average amount (pmol) of peptide in the microsomal fractions. This value corresponds to the number of moles of the expressed membrane protein. Estimate the amount (g) of protein in the total analyzed microsomal fraction (10 μg) using the calculated molecular weight of the expressed fusion protein. Then estimate the percentage of expressed protein in the microsomal fraction (*see* **Note 42**).
11. Estimate the amount of expressed protein used in the assay and in combination with the previously determined value for V_{max} to calculate k_{cat}.

4 Notes

1. The yeast strain, INVSc1, (Thermo Fisher Scientific) is a fast-growing strain and ideal for protein expression. INVSc1 genotype: MATa his3D1 leu2 trp1-289 ura3-52 MAT his3D1 leu2 trp1-289 ura3-52.
2. Any suitable yeast expression vector should suffice. The pYES-DEST52 expression vector is galactose inducible with a uracil (URA3) selectable marker for yeast growth and with C-terminal V5 epitope and 6×His tags. The vector is a Gateway destination vector with antibiotic resistance (ampicillin) for initial cloning in *Escherichia coli*. We have used this vector successfully to express over 50 plant, fungi, and mammalian membrane proteins in yeast.
3. Transformation of yeast can be readily undertaken without a commercial kit; however, these systems are a cost-effective and efficient means to accomplish a successful transformation, especially if being undertaken for the first time.
4. It contains a nitrogen source, vitamins, trace elements, and salts for optimal yeast growth and is used in conjunction with the yeast drop-out media. The ratio required for yeast media will depend on the supplier.
5. It contains amino acids and other nutrients (e.g., adenine, inositol, etc.) absent from the Yeast Nitrogen Base. The pYES-DEST52 vector contains the auxotrophic selection marker URA3 (orotidine 5′-phosphate decarboxylase) that converts orotidine monophosphate (OMP) to uridine monophosphate (UMP). Depending on the yeast expression vector, the appropriate drop-out media is required.
6. Baffled flasks create a turbulent flow and increase gas exchange at the surface of the liquid to increase oxygen intake.

7. The pYES-DEST52 vector contains the promoter sequence of the GAL1 gene upstream of the *att*R region for the induction of the gene of interest. The appropriate induction method is dependent on the yeast expression vector utilized.
8. The addition of sodium azide (often referred to as STOP buffer) will inhibit further activity and arrest metabolism. Take adequate precautions as sodium azide is a potent inhibitor of mitochondrial respiration.
9. The type of buffer and volume used will greatly depend on the downstream enzyme assay that will be conducted.
10. A 12% polyacrylamide gel is a good compromise for the generic separation of proteins. If purchasing precast polyacrylamide gels, consider a gradient gel employing 8–16% acrylamide.
11. The selection of the primary antibody will be determined by the protein tag (if any) in the expression vector.
12. The type of secondary antibody will depend on the origin of the primary antibody, but is commonly either Anti-Mouse (usually monoclonal antibodies) or Anti-Rabbit (usually polyclonal antibodies).
13. In recent times, access to tandem mass spectrometers has become relatively cost-effective with a variety of service-based facilities at many institutions.
14. Urea can be unstable and degrade in solution when exposed to elevated temperatures (>25 °C) or over time. Degradation products such as isocyanic acid can react with the amino terminus of proteins and sidechains of lysine and arginine residues. Consequently, it is recommended that urea solutions be made fresh as required.
15. A stock solution of DTT can be stored in aliquots at −20 °C.
16. A stock solution of IAA can be stored in aliquots at −20 °C. IAA is used to alkylate thiol group on cysteine residues after reduction with DTT. It is virtually impossible to detect cysteine containing peptides unless controlled alkylation is undertaken. IAA is light and heat sensitive and should be stored in the dark.
17. Tandem mass spectrometers designed for proteomic workflows will usually ship with software for the interrogation of tandem spectra for the identification of proteins and it is likely that any MS service facility will have a variety of packages. However, there are a number of open-source software packages available as well as a range of third-party products that can be accessed online. The algorithms are generally similar in their approach, although we use Mascot (a third-party software package from Matrix Science) as it provides quality results when high confidence cutoffs are employed. Access to the software is free if submitting less than 1200 spectra in a single submission (http://www.matrixscience.com/).

18. The selection of the synthetic peptides to use as standards for MRM-based quantitation will depend on a number of factors after inspecting the results from the LC-MS/MS analysis of the microsomal fraction. If a yeast expression vector with a protein tag has been employed, e.g. the V5 epitope, it is often cost-effective to select a tryptic peptide from this region that can be used in future experiments with different proteins. If no tag was employed or if no suitable tag-derived peptides were identified after shotgun analysis of the microsomal fraction, select protein-specific tryptic peptides. The objective is to select MS-compatible tryptic peptides with no miscleavage and no modifications (avoid methionine and cysteine containing peptides). Optimally, 2–3 doubly charged $[M+2H]^{2+}$ peptides in the range of 500–700 m/z appear to produce optimal results with regard to signal and fragmentation. If no peptides were identified after shotgun analysis of the microsomal fraction, first ensure adequate expression and if necessary optimize protein expression. If there are still no candidates, there are a number of utilities that can predict peptides suitable for MRM from the amino acid sequence, such as the Arabidopsis Proteotypic Predictor [13].
19. The Skyline software package is a freely available, open-source application for developing MRM methods and analyzing the resultant data. The software can be used to select candidate peptides and fragment ions for an MRM experiment from a protein sequence. It can also be used to optimize the declustering potential and collision energy for a set of peptides. The Skyline website has an extensive collection of tutorials and help pages.
20. In parallel, ensure that an empty vector control is also transformed and resultant microsomes are employed throughout the entire assay.
21. While there are numerous protocols for yeast transformation, we have found that using a commercial kit is cost and time effective.
22. The pYES-DEST52 vector harbors a galactose inducible promoter for the induction of the transgene. This step will be dependent on the expression vector being employed.
23. If there are difficulties obtaining a stably expressed protein, check expression by immunoblotting at earlier time points, such as after 6 h.
24. Depending on the source and batch of Lyticase, the appropriate proportion to add to the solution, based on mg fresh weight of yeast, will need to be calculated.
25. The use of 37 °C is optimal for digestion of yeast cell walls; however, the expressed protein may be heat sensitive.

Consequently, this step may require assessment depending on protein expression levels. A lower temperature for a longer incubation period can be used.

26. Using a precast gel system is the simplest and most convenient way to analyze multiple fractions. Assemble the apparatus with the precast gel and compatible buffers following the manufacturer's instructions.
27. Ensure that the methanol has been added to commercially sourced transfer buffers.
28. A range of conditions can be used including overnight transfers; however, voltage adjustments are required. Check the instructions of the specific transfer apparatus for advice on specific conditions.
29. The dilution of a primary antibody will vary depending on its specificity. A range will be provided by the supplier; however, some testing may be required to obtain an optimal signal. A dilution range between 500 and 5000 is common for polyclonal antibodies.
30. To minimize the amount of primary antibody employed, only make enough of this solution so as to cover the membrane and employ a small container.
31. A variety of conjugations are available for the detection of secondary antibodies; however, HRP (horse radish peroxidase) is one of the most common as it is a robust enzyme that enables detection by a variety of techniques.
32. When mixed, the HRP substrate is light sensitive. Therefore keep away from light (wrapped) and use immediately.
33. The recommended ratio of trypsin to protein (w/w) is generally 1:20 or 1:50. However, this will depend on the type of trypsin being employed. If using modified trypsin (not subject to autolysis), then the recommended ratios can be used; however, if unmodified trypsin is used, we recommend using it at higher ratios. The advantage of unmodified trypsin is that autolytic products (peptides) can act as internal controls for sample handling and mass spectrometry.
34. An overnight digestion is likely excessive; however, the timing usually suits the standard sample processing workflow.
35. The analysis of 1 μg of a complex peptide lysate by nano-LC-MS/MS should result in the high-confidence identification of between 1000 and 2000 proteins for species with well-curated genomes. In this instance, up to 1000 yeast proteins are likely to be detected using this approach.
36. To ensure that only high-confidence proteins and peptides are identified from the fragmentation spectra, employ

recommended protein and peptide cutoffs to ensure spectral match probabilities are <0.05 or false discovery rates are <1%. If using Mascot, an ions score value is provided for peptide matches and needs to be entered manually in the "Ions score or expect cut-off" box. Using such an approach, only high-confidence peptide matches are used to define the resultant identified proteins. This means that even proteins identified by a single peptide can be regarded as high-confidence matches, although a replicate experiment will be required to independently identify and confirm its existence in the sample.

37. As indicated above, the Skyline software package can be used to create the MRM method and to optimize collision energy and fragment ions (File > Export > Method) after entering peptide information into the software.
38. Check that you have selected the most abundant charge state for the peptide, often peptides >1600 Da will be found in two charge states $[M+2H]^{+2}$ and/or $[M+3H]^{+3}$.
39. Newer instruments can employ quite short dwell times to monitor hundreds of MRM transitions, however given that only a handful of peptides will be analyzed, dwell times of 50–100 ms can be readily employed.
40. The peptide elution window will depend on a number of factors. Using a traditional setup employing nanoflow or capflow LC delivery rates, peak widths of 1–2 min would be expected. However, it is now more common to conduct MRM-type analyses using Ultra Performance Liquid Chromatography (UPLC) and depending on the flowrate employed, peptide elution windows can be less than 10 s.
41. Skyline has a collection of report templates; however none provides 'Total Area," i.e., the area of all transitions of a given peptide. Simply edit a report template, e.g., Peptide RT Results, and add 'Total Area" to the report. The 'Total Area" option is found under Proteins > Precursors > Precursor Results.
42. For proteins exhibiting good expression in yeast, after MRM quantitation, we have calculated that the expressed protein comprises around 1% of total microsomal protein.

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References

1. Michaelis L, Menten ML (1913) The kenetics of the inversion effect. Biochem Z 49:333–369
2. Henri V (1903) Lois générales de l'action des diastases. Hermann, Paris
3. Briggs GE, Haldane JBS (1925) A note on the kinetics of enzyme action. Biochem J 19:338–339
4. Johnson KA, Goody RS (2011) The original Michaelis constant: translation of the 1913 Michaelis-Menten paper. Biochemistry 50:8264–8269. doi:10.1021/bi201284u
5. Matern U, Grisebach H (1977) UDP-apiose-UDP-xylose synthase – subunit composition and binding studies. Eur J Biochem 74: 303–312. doi:10.1111/j.1432-1033.1977.tb11394.x
6. Rautengarten C, Ebert B, Ouellet M et al (2012) *Arabidopsis* deficient in cutin ferulate encodes a transferase required for feruloylation of omega-hydroxy fatty acids in cutin polyester. Plant Physiol 158:654–665. doi:10.1104/pp.111.187187
7. Lim L, Linka M, Mullin KA et al (2010) The carbon and energy sources of the non-photosynthetic plastid in the malaria parasite. FEBS Lett 584:549–554. doi:10.1016/j.febslet.2009.11.097
8. Ebert B, Rautengarten C, Guo X et al (2015) Identification and characterization of a Golgi localized UDP-xylose transporter family from *Arabidopsis*. Plant Cell 27:1218–1227. doi:10.1105/tpc.114.133827
9. Rautengarten C, Ebert B, Moreno I et al (2014) The Golgi localized bifunctional UDP-rhamnose/UDP-galactose transporter family of *Arabidopsis*. Proc Natl Acad Sci U S A 111: 11563–11568. doi:10.1073/pnas.1406073111
10. Ito J, Herter T, Baidoo EE et al (2014) Analysis of plant nucleotide sugars by hydrophilic interaction liquid chromatography and tandem mass spectrometry. Anal Biochem 448:14–22. doi:10.1016/j.ab.2013.11.026
11. Aderem A (2005) Systems biology: its practice and challenges. Cell 121:511–513. doi:10.1016/j.cell.2005.04.020
12. Arkin AP (2013) A wise consistency: engineering biology for conformity, reliability, predictability. Curr Opin Chem Biol 17:893–901. doi:10.1016/j.cbpa.2013.09.012
13. Taylor NL, Fenske R, Castleden I et al (2014) Selected reaction monitoring to determine protein abundance in *Arabidopsis* using the *Arabidopsis* proteotypic predictor. Plant Physiol 164:525–536. doi:10.1104/pp.113.225524

Chapter 16

MSE for Label-Free Absolute Protein Quantification in Complex Proteomes

Stefan Helm and Sacha Baginsky

Abstract

Label-free peptide quantification is a promising approach for the large-scale characterization of proteome dynamics at low cost. Here, we describe a method for absolute label-free quantification using an untargeted approach for peptide fragmentation referred to as MSE. We show that spiked external standards provide sufficient accuracy for the quantification of proteins in complex samples resulting in similar protein quantification results as spectral counting. As an advantage, label-free quantification also works for small numbers of samples whereas spectral counting requires large datasets to result in a similar robustness. The sensitivity of protein identification increases significantly when ion mobility separation is included in addition to the standard LC-MS setup in the analysis workflow. Ion mobility decreases sample complexity and serves as an additional separation criterion to align a parent ion with its product ions after MSE fragmentation. As a drawback, quantification of high abundance proteins becomes inaccurate because of detector saturation. We describe here a suitable workflow to achieve good sensitivity for protein quantification and give initial guidance on data interpretation. To achieve good identification and quantification accuracy, the protein amount loaded onto the column should not exceed 400–600 ng. In a dynamic range window of 3–4 orders of magnitude, robust quantification can be obtained with complex samples comprising up to 2000–3000 proteins.

Key words Proteome analysis, Label-free quantification, MSE, Ion mobility separation, In solution digest

1 Introduction

Quantitative proteomics comes in various flavors and many methods and approaches were developed that enable experimentalists to adapt protein quantification to their particular purpose [1]. *Relative quantification* is based on the comparison of peptide extracted ion chromatograms (XIC) between different samples, both in label-free and labeling methods. *Absolute quantification* is more difficult because peptide ionization properties vary and it is difficult to accurately quantify this variability. Thus, an external standard is usually spiked into the sample at known concentration and the abundance of a native peptide is inferred by the XIC ratio compared

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with the spiked standard. This approach is often coupled with targeted mass spectrometric analyses such as *multiple reaction monitoring* enabling accurate peptide and protein quantification at very high sensitivity [2]. Because the synthesis of standard peptides is cost intensive, normalized spectral counting has developed into a popular alternative. This method is based on the observation that the number of spectra identifying a protein correlates with protein abundance, provided that suitable normalization procedures are applied to correct for variations in the quality of the mass spectrometric analysis [3].

In recent years, a new mass spectrometric acquisition technique has been developed that is referred to as MS^E. In MS^E, the mass spectrometer switches between low and high collision energy with quadrupole settings that allow all precursor ions within a wide molecular mass window to pass through [4]. Thus, all peptides falling into the chosen mass window will be detected and fragmented. The downside of this approach is the lost connection between precursor and fragment ions, and algorithms are required to re-establish the connection between precursor and product ions based on their identical elution profiles in the chromatography (Fig. 1). On the other hand, the rapid cycling between low and high-

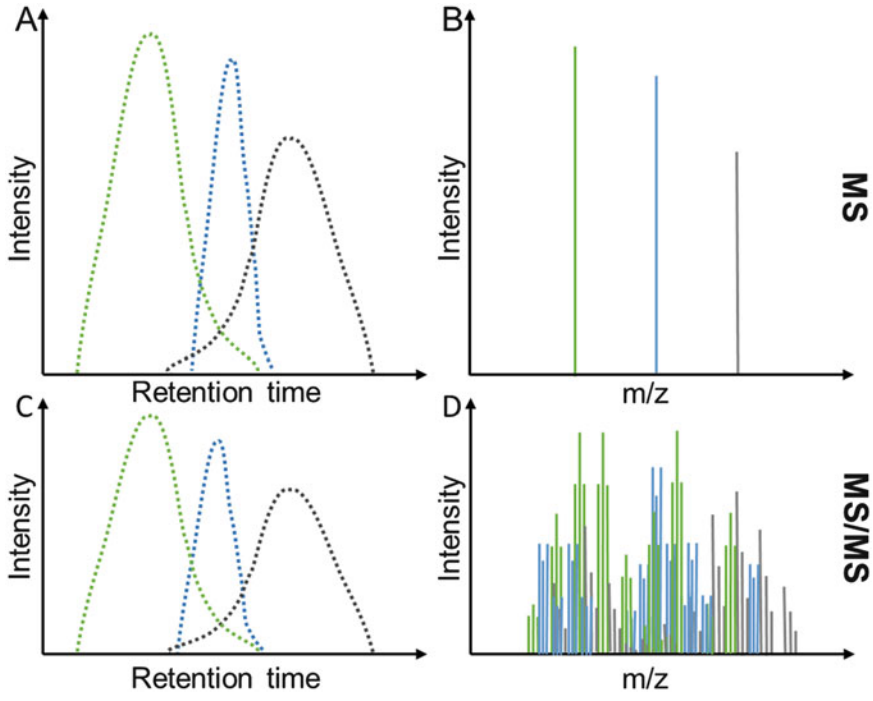

Fig. 1 MSE workflow with precursor and fragment ion alignment. Elution profile (**a**) of precursor ions with a certain *m/z* value (**b**) in MS mode. Elution profile of fragment ions in MS/MS mode. Based on the elution profile of the fragment ions, an alignment with the precursor ions is performed. *Identical peak shapes* are used to sort fragment ions to a certain precursor (**d**) as indicated by the *colors*

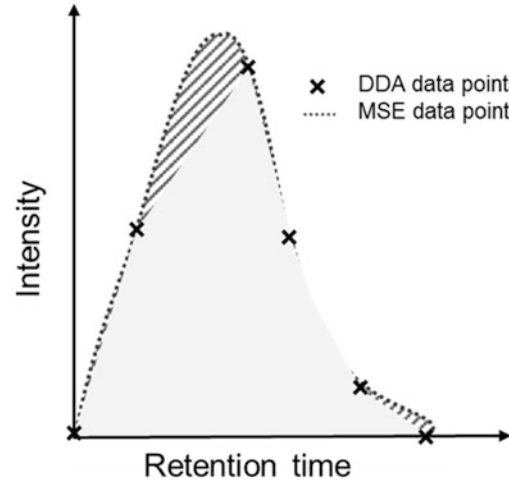

Fig. 2 Advantage of MSE over DDA acquisition for quantification. MSE harbors the advantage that the switch from low-collision energy to high-collision energy occurs at high frequency such resulting in a good resolution of the precursor peak shape (*dotted line*), which is necessary for accurate quantification. In DDA, a significant amount of information is lost at the MS level in times the mass spectrometer is acquiring MS/MS data from different precursors (up to 20 in contemporary studies). The fragmentary nature of the information is illustrated by the difference between the area under curve for DDA (data points indicated as $\times$) and MSE resolved precursor ions (*hatched area*)

collision energy and the fact that potentially all peptides are fragmented preserves more accurate quantitative information compared with the standard data-dependent acquisition mode (DDA). Thus, MS^E is perfectly suitable for label-free quantification based on peptide XIC (Fig. 2).

An elegant modification on MS^E approaches allows absolute quantification of protein in the sample based on spiked peptides. Here, the correlation between signal intensity of the three most intense peptide ions and protein concentration is used to infer the abundance of native proteins in comparison with the external standard. Silva and colleagues have shown that the response factor, i.e., the count of measured signal intensity per amount of protein is constant for all proteins tested [5], provided that the three peptides with the highest XIC readout are used for response factor calculation. We have furthermore shown that this approach also works for complex samples [6]. This approach works because selecting the three "best" ions causes an optimization for efficient ionization with peptides that are sufficiently different in their composition so that different ionization properties are averaged out. More recent analyses showed that this quantification strategy that was originally developed for MS^E in principle also works with DDA, using the N most intense ions [7].

In combination with ion mobility separation, MSE achieves very high sensitivity because ion mobility serves as additional dimension for precursor and fragment ion alignment. Thus, two different modification of the MSE workflow can be employed for quantification, on the one hand employing ion mobility separation in a workflow that is referred to as high-definition (HD)-MSE, and on the other hand omitting the ion mobility separation. HD-MSE has the advantage of a much higher sensitivity compared with standard approaches, but has the disadvantage of underestimating high abundance proteins because of detector saturation. MSE achieves lower sensitivity but provides more accurate results on high abundance proteins [8]. Thus, a combination of both the methods should be employed for large-scale proteome quantification. We report here on a workflow to employ MSE-based quantification in the characterization of complex proteome samples, such as plant leaf extracts or isolated organelles.

2 Materials

All chemicals and solvents should be MS- or at least HPLC grade.

2.1 Protein Precipitation

1. Acetone and methanol precooled to −20 °C.
2. Wash solution: 10% ddH_2O[v/v], 10% methanol [v/v], 80% acetone [v/v].

2.2 In-Solution Digest

1. Working solution: 25 mM ammonium bicarbonate (AmBic, pH not adjusted).
2. 0.1% RapiGest SF (w/v), commercialized by Waters, prepared according to the producer protocol.
3. Solution for disulfide reduction: 10 mM dithiothreitol (DTT), solved in 25 mM AmBic.
4. Solution for cysteine methylation: 30 mM iodoacetamide (IA), solved in 25 mM AmBic, keep in the dark.
5. Enzyme solution: 0.25 μg/μL trypsin (Trypsin Gold, Mass spectrometry Grade; Promega), buffer according to the producer protocol.

2.3 Sample Preparation for Mass Spectrometric Analysis

1. Dilution buffer: 2% acetonitrile 0.1% formic acid (v/v).
2. For quantification, a peptide solution from a single protein of a foreign organism is required at known concentration (*see* **Note 1**).

2.4 Solvents for the Nano Liquid Chromatography Separation

1. Solvent A: water with 0.1% formic acid (v/v).
2. Solvent B: acetonitrile with 0.1% formic acid (v/v).
3. Solvent C: water with 0.1% trifluoroacetic acid.

3 Methods

3.1 Determination of Protein Concentration

The method for protein concentration determination is up to your own preference and has no influence on the analysis.

3.2 Protein Precipitation

We strongly recommend protein precipitation to make the MS analysis compatible with different isolation methods including detergent solubilization. Different precipitation methods are available and the optimal choice depends on the protein sample and the properties of the proteins of interest. In our hands, acetone-methanol precipitation works well with the subsequent MS analysis.

1. Determine the protein concentration of the sample.
2. Make 100 µg aliquots (*see* **Note 2**).
3. Add one part methanol and eight parts acetone to the protein mixture (10% [v/v] sample and 90% [v/v] organic solvent).
4. Store the solution overnight at −80 °C.
5. Centrifuge the sample at 16,000 × *g*, 4 °C for 10 min.
6. Discard the supernatant.
7. Add 1 mL wash solution and vortex the sample.
8. Centrifuge the sample at 16,000 × *g*, 4 °C for 10 min.
9. Discard the supernatant.
10. Repeat the washing step once.
11. Dry the pellets, e.g., in a vacuum concentrator.

3.3 In Solution Digest

1. Dissolve the 100 µg protein pellet in 221 µL AmBic (*see* **Note 3**).
2. Add 12.5 µL 0.1% RapiGest SF solution and incubate for 10 min at 80 °C, vortex shortly after 5 min.
3. Add 6.2 µL DTT solution to reduce disulfide bridges, vortex and incubate for 10 min at 60 °C.
4. Cool the sample at room temperature and centrifuge the condensate down.
5. To alkylate the cysteine residues, add 6.2 µL IA solution, vortex, and incubate for 30 min in the dark.
6. Add 4 µL trypsin solution (0.25 mg/mL, final concentration: 1:100 (w/w) corresponding to 1 µg protease per vial (i.e., per 100 µg protein) and incubate overnight at room temperature or for 4 h at 37 °C (*see* **Note 4**).
7. Lower the pH to a value <2 to stop the protease activity. For very small volumes we recommend testing with pH paper optimal for the target pH range (pH 1–5).
8. Centrifuge 30 min at 4 °C and 10,000 × *g* and transfer the supernatant (containing the tryptic peptides) into a new vial (*see* **Note 5**).

3.4 Sample Preparation for Analysis

Based on the above protocol, the protein concentration in the aliquot should be around 0.4 μg/μL, which is the optimal concentration for the here-described experiments However, the optimal concentration for the analysis depends on some mass spectrometer-specific features, most importantly detector saturation. Since the analysis should be quantitative, it is mandatory that detector signal intensity correlates with peptide abundance. This correlation is lost under conditions of full detector saturation. As described earlier, when ion mobility separation is used as an additional separation step for the analysis, detector saturation occurs more rapidly with the consequence that high abundance proteins are inaccurately quantified. This can be circumvented by omitting the ion mobility separation step and using MSE instead of HD-MSE. This problem is partially circumvented by an algorithm that recognizes detector saturation and changes ion optics settings to defocus the ion beam before the ions reach the TOF analyzer. This avoids detector saturation on the fly. The changes in ion optics settings are considered for the reporting of the authentic ion intensity values in the raw data. While this algorithm improves the dynamic range of HD-MSE analyses, it comes to its limits when highly abundant proteins are quantified. For absolute quantification, a peptide mixture should be injected at known concentration to correlate the XIC of the spiked standard with those of the internal peptides. There are different peptide spikes available that serve different needs. We describe here our standard workflow using glycogen phosphorylase B (GPB) at a spiked concentration of 10 fmol on column.

1. Pipet 2 μL spike solution (100 fmol/μL) into the vial.
2. Pipet 18 μL of the protein sample to be analyzed directly into the provided spike solution. This allows better mixing of the two solutions. Of this mixture, 1 μL (about 400 ng peptides) is injected for MS analysis.

3.5 Setup of the Online Liquid Chromatography Prior to MS

The common method for proteome analysis uses standard nano-LC coupled online to a mass spectrometer. Depending on the aim of the study and the sample complexity, the LC separation could be adapted individually. It is possible to adjust the length of the separation column and the number of columns (*see* **Note 6**), but it is usually sufficient to adjust the characteristics of the mobile phase, i.e., the length and the properties of the gradient, depending on sample complexity. Common gradients are 30, 60, 90, 120, and 180 min runtime. Standard gradients start with low concentration [3–8%] organic (Solvent B) and increase this up to 35–40% over the time. Under these conditions, all tryptic peptides should be eluting from the column. Subsequent to the gradient, a column wash step is employed, in which the ACN concentration increases up to 85% (Solvent B) for 1 min followed by a re-equilibration of the column to the initial conditions. We are using the following gradient for

acquisition of data on full proteome samples, providing a good tradeoff between sensitivity and quantification robustness:

1. 1 μL peptide solution is loaded onto an ACQUITY UPLC System (Waters, Eschborn, Germany) equipped with a 200 mm × 180 μm fused silica trap column packed with 5 μm Symmetry C18 (Waters, Eschborn, Germany) as well as a 250 mm × 75 μm fused silica separation column packed with 1.8 μm HSS T3 C18 (Waters, Eschborn, Germany).
2. Peptides are trapped for 5 min at 5 μL/min at 99% Solvent C and 1% Solvent B.
3. For chromatographic separation we use a linear gradient of 7–35% Solvent B within 140 min at a flow-rate of 300 nL/min.

3.6 MS Data Acquisition

MSE data acquisition can be either in HD-MSE mode using ion mobility separation as an additional separation step for the peptide mixture or by omitting the ion mobility in normal MSE runs. Most of the parameters are rather device specific and it is usually not necessary to adjust them to a specific peptide sample. Bear in mind that most of the machine settings are optimized for a standard proteome analysis using tryptic digests of complex proteome samples. It is necessary to adjust instrument parameters to a different protease treatment and to specific molecular masses of interest, e.g., very small (<100) or very high (>1800) m/z values. For standard chloroplast proteome quantification, we are using a range from 100 Da up to 2000 Da. For the fragmentation type different choices are available, i.e., *collision-induced dissociation* or the gentle fragmentation method *electron transfer dissociation* which is more suitable for the analysis of posttranslational modifications like phosphorylation. Precursor peptide information and fragmentation spectra are acquired on a SYNAPT G2-S mass spectrometer (Waters, Eschborn, Germany) in vendor defined "resolution-"and "positive ionization-mode." We use the following parameter and settings for data acquisition:

1. Eluting peptides are ionized at 2.1 kV and 80 °C in a nanoESI source using a Pre-cut PicoTip Emitter; 360 μm OD × 20 μm ID, 10 μm tip; 2.5″ long (Waters, Eschborn, Germany).
2. Cone voltage is set to 40 V.
3. Nitrogen is used for the cone, nano flow, and purge gases with 10 L/h, 0.4 bar, and 450 L/h.
4. For ion mobility separation, ions are accumulated in the trap cell with a release time of 500 μs and afterward "cooled" for 1000 μs (mobility separation delay after trap release) in the helium cell (helium pressure 4.7 mbar) that is located between trap and ion mobility separation (IMS) cell.
5. IMS cell is filled with nitrogen at a pressure of 2.87 mbar.

6. For optimal ion mobility separation, wave height is set to 38 V and wave velocity is ramped from 1200 to 400 m/s.
7. Scan time is set to 1 s.
8. *Collision-induced dissociation* is achieved by Argon (collision gas) and a ramp transfer collision energy of 25–55 V is established.
9. Glu-1-Fibrinopeptide B (Glu-Fib), 250 fmol/μL, 0.3 μL/min is used as lock mass ($m/z = 785.8426$, $z = 2$) every 90 s.
10. Instrument calibration is based on the MS/MS masses obtained from the Glu-Fib peptide.
11. After establishing the different parameters for the Synapt G2S, place your prepared sample in the auto sampler and note the position.
12. Define sample name for the data acquisition. The name should include the responsible researcher, the date of measurement, and a short but distinct description of the sample (*see* **Note 7**).
13. Choose the MS acquisition method as well as the method for the LC system and the position in the auto sampler in your operating software.
14. Double check all settings and start the acquisition.

3.7 Data Processing

Following data acquisition, the MS file must be processed to prepare the dataset for database matching. Data processing results in a list with mass and charge state of the precursor ion and its aligned fragment ions (*see* **Note 8**). In case of using MSE or HDMS^E, Waters software is required for data processing. Here, we use ProteinLynx Global Server 3.0.1 (PLGS) (*see* **Note 9**). Some of the processing parameters must be defined as follows:

1. Chromatographic peak width and MS TOF resolution, both values are reported from the mass spectrometer and are automatically defined.
2. Lock mass for charge state 1 and 2. This will depend on the properties of the internal lock spray used during the analysis.
3. Lock mass window should be set to 0.25 Da.
4. Low energy threshold should be set to 180 counts.
5. Elevated energy threshold should be set to 15 counts.
6. Intensity threshold should be at least 750 counts (*see* **Note 10**).

3.8 Data Analysis and Interpretation

After this initial step, the dataset is ready for database searches. It is important to use a database that comprises all theoretical constituents of the sample, which is the case for fully sequenced organisms such as *Arabidopsis thaliana*. The following parameters need to be defined:

1. Select a suitable protein database that comprises all proteins that are potentially in the sample, if available. It is important that the database contains the reference protein (external standard) and common contaminations such as, e.g., keratin.
2. Set the parameters for peptide and fragment mass tolerance. PLGS is able to determine both settings automatically. These parameters correspond directly to instrument accuracy and resolution. In the standard setting these values equal 10 ppm for precursor ion tolerance and 0.5 Da for fragment ion tolerance.
3. The characteristics of the peptide and protein matching must be defined next. We are using the following settings: Minimal number of fragment ion matches for each peptide match =2, minimal number of fragment matches to a protein =5, as well as the minimal number of matched peptides per identified protein =2 (*see* **Note 11**).
4. Define the maximum protein mass, we are using 250,000 as default.
5. Choose your primary and, if used, secondary digesting enzyme. For a common proteomics approach its only one enzyme, trypsin.
6. Set the number of allowed missed cleavages, 1 is recommended.
7. Define fixed modifications. This is also dependent on your protocol, if you followed the instructions here, this setting can be restricted to carbamidomethylation on cysteine residues.
8. Define variable modifications. Oxidation on methionine is only recommended. If you search for others like phosphorylation or acetylation, you should also include them in the database search, but be aware of the larger search space and the additional degrees of freedom for peptide matching (*see* **Note 12**).
9. Set the allowed false discovery/false positive rate at the protein level. Recommended is 4% (*see* **Note 13**).
10. For absolute quantification, define the calibration settings (i.e., what protein was used at what concentration) per injection. For relative quantification, define a "manual response factor" in counts per fmol.
11. Usually, you should acquire at least three technical replicates per sample to make the analysis more robust. Combine the technical samples in your results file.
12. It is advisable to export on the desired information in the datafile, e.g., protein amount, peptide spectrum matches (PSM), unique peptides, modifications, mass error, and score

details for the match are the minimum requirements for data reporting (*see* **Note 14**).

13. We recommend averaging the quantitative data from the technical replicates. Usually, only protein identified in at least two out of three replicates is considered further (*see* **Note 15**).
14. Bear in mind the possibility of false identifications when interpreting the data. With PGLS, it is possible to define a false discovery rate at the protein level. Chose a setting as stringent as possible.
15. For quantitative data interpretation it may be necessary to check the quantitative assignment individually for every peptide of a quantified protein. The three peptides used for protein quantification should be unique and the abundance values, i.e., the XIC counts should be relatively similar. We have previously quantified the difference between the highest peptide intensity and the second highest intensity in a "coefficient of peptide intensity difference," but the quality control can be performed in different ways [6].
16. In case three biological replicates were performed, a statistical analysis should follow, e.g., by a standard t-test in case the data are normally distributed or by some nonparametric test if this is not the case. Please remember that a quantitative proteome contains many protein hits and a standard threshold for significance, e.g., $p < 0.05$ may be insufficient because of the multiple testing problem. We recommend adjusting the p-value by some of the numerous multiple testing correction procedures available.

4 Notes

1. It is important that the concentration of the standard digest is accurate so we recommend using a company-offered solution such as glycogen phosphorylase B used in our analyses [6]. Additionally, the standard peptides should cover a broad range in the LC chromatogram, because ionization properties differ at different acetonitrile concentrations. Depending on the goal of the study, labeled peptides that are specifically tailored for the quantification of one or several proteins are the gold standard for quantification, because their elution properties are identical to the native peptide, such eliminating quantification errors due to differences in ionization properties.
2. It is possible to start the sample preparation with less than 100 μg but the volumes should be adjusted accordingly to assure optimal peptide concentration during the analysis.

3. In case the sample contains many membrane proteins, the pellet is difficult to resolve in AmBic solution alone. In this case, adding RapiGest SF directly to the AmBic solution will help in resolving the pellet. The solution with RapiGest SF should be carefully pipetted up and down to avoid foaming.
4. Trypsin is the protease of choice because it cleaves the peptide bond C-terminal to lysine and arginine, thus generating peptides of a convenient size and with at least two protonated primary amines. Bear in mind that it could be necessary to use a different protease, e.g., if the protein sample of interest does not contain sufficient lysine or arginine residues.
5. In solution digest is easy and quick and the few steps necessary for such a digest make it more robust for protein quantification. However, other protocols are available, e.g., operating via *in gel* digests. This could be necessary for some samples to get rid of any contaminating ingredients.
6. Here, it is certainly possible to include a multidimensional peptide separation step (MudPIT) in the analysis. This is advisable for highly complex samples.
7. Trivial to mention but very important: generate a suitable name for the acquisition files that allows the identification based on date, organism, and name.
8. Alignment of precursor and fragment ions is a critical step in the processing of MSE data. Because of the data-independent nature of fragment ion acquisition, the connection between precursor and fragment ions is lost and needs to be reestablished. Computer algorithms are in place that deliver reliable alignments taking into account abundance distribution of precursor and fragment ions over time (*see* Fig. 1). The alignment works surprisingly well especially when ion mobility is included in the workflow as additional characteristic feature for precursor and fragment ion alignment. The algorithm is implemented in the Waters data processing software PLGS. We recommend using the standard setting for the alignment as implemented in PLGS 3.0.1.
9. For the interpretation of MSE data, MSE PLGS is required. Following the PLGS-based processing of the data, spectra can be exported in a peak list file format (pkl.txt) and proceeded for data analysis with alternative software such as, e.g., Mascot, ProteomeDiscoverer, MaxQuant, or PEAKS that can be used for data validation [9].
10. The different thresholds required are defined as follows: "Low energy threshold"—low-collision energy resulting in almost no precursor fragmentation; "Elevated energy threshold"—high-collision energy resulting in precursor fragmentation. To distinguish fragment ion spectra from noise, a minimum

threshold of 15 is set as a default value, but this can be raised in case higher stringency is required; "Intensity threshold"—the summed intensities of precursor and the corresponding fragment ions; i.e., in case the summed intensities of precursor (low energy scan) and the associated fragment ion scans (elevated energy scan) is below 750 counts, the signals will be ignored in the generated peak list.

11. Other settings are conceivable, e.g., increasing the minimal number of assigned peptides required for protein identification. The setting 2-5-2 as mentioned in the protocol above is recommended by the vendor and we have good experience with the reliability of the assignments. This judgement comes from comparative analyses between MSE and DDA analyses performed in our laboratory (data not shown).
12. Adding additional posttranslational modifications is possible but harbors the risk of increasing false assignments due to more degrees of freedom for peptide matching (larger search space). Apply the concept "as low/few as reasonably achievable."
13. Different programs determine false discovery rates (FDRs) at different levels. In PLGS, the FDR is calculated at the protein level and 4% is a reasonably good value. FDRs at the protein level are preferably over FDRs at the spectrum level because in large-scale studies, even FDRs of 0.1% at the spectrum level, can result in many false protein assignments, depending on the filtering criteria.
14. For practical reason of data interpretation, it is usually not necessary to export all the setting and data points theoretically available from the data. Make a smart selection allowing you to demonstrate the reliability of the peptide spectrum match (scores, fragments, FDRs, error tolerance) and in case of post-translational modification, the reliability of the assignment of the site of modification to your peers. There are some reporting guidelines published as MIAPE standards that you should refer to [10].
15. Before you combine the data from the technical replicates take a decision on what selection criteria should be applied for peptide identification. For example, it is almost certain that many peptides will not be identified in all three technical replicates. We suggest using peptides identified in at least two out of three technical replicates. Since standard workflows usually quantify three biological replicates, we usually consider peptides that were identified at least in two out of three biological replicates, but with a minimum of five detection out of nine replicates (three technical time three biological replicates) [6].

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References

1. Nikolov M, Schmidt C, Urlaub H (2012) Quantitative mass spectrometry-based proteomics: an overview. Methods Mol Biol 893:85–100
2. Picotti P et al (2013) A complete mass-spectrometric map of the yeast proteome applied to quantitative trait analysis. Nature 494:266–270
3. Baginsky S (2009) Plant proteomics: concepts, applications, and novel strategies for data interpretation. Mass Spectrom Rev 28:93–120
4. Distler U et al (2014) Biomedical applications of ion mobility-enhanced data-independent acquisition-based label-free quantitative proteomics. Expert Rev Proteomics 11:675–684
5. Silva JC et al (2006) Absolute quantification of proteins by LCMSE – a virtue of parallel MS acquisition. Mol Cell Proteomics 5:144–156
6. Helm S et al (2014) Protein identification and quantification by data-independent acquisition and multi-parallel collision-induced dissociation mass spectrometry (MS(E)) in the chloroplast stroma proteome. J Proteome 98:79–89
7. Grossmann J et al (2010) Implementation and evaluation of relative and absolute quantification in shotgun proteomics with label-free methods. J Proteome 73:1740–1746
8. Bond NJ et al (2013) Improving qualitative and quantitative performance for MS(E)-based label-free proteomics. J Proteome Res 12:2340–2353
9. Lu Q et al (2015) On the extent of tyrosine phosphorylation in chloroplasts. Plant Physiol 169:996–1000
10. Taylor CF et al (2007) The minimum information about a proteomics experiment (MIAPE). Nat Biotechnol 25:887–893

Chapter 17

Identification and Characterization of Plant Membrane Proteins Using ARAMEMNON

Rainer Schwacke and Ulf-Ingo Flügge

Abstract

Membrane proteins are estimated to constitute a quarter of all proteins encoded in plant genomes, yet only a limited number have been experimentally characterized. This is mainly due to the large variation in particular physical properties coupled with purification difficulties. Computational methods are therefore very helpful for the initial characterization of a candidate membrane protein. Individual prediction tools can, with varying levels of success, predict the occurrence of transmembrane spans, the subcellular location, and lipid posttranslational modifications. Since it can be tedious to consult each prediction tool separately, ARAMEMNON has been designed to compile various computational predictions for plant membrane proteins and to present the results via a user-friendly web interface. This protocol describes how to use ARAMEMNON to identify and characterize plant membrane proteins.

Key words Plant membrane protein, Integral membrane protein, Lipid-anchored membrane protein, Subcellular location, Plant permeome, Membrane protein database, Computational prediction tool, Prediction data visualization

1 Introduction

One of the main goals in proteomics is to functionally characterize proteins found in various cell compartments. Membranes separate different compartments with membrane proteins playing an important role for the interdependency of two adjacent compartments, for example as transporters or signal propagation factors.

Membrane proteins can be categorized as falling into one of three groups: integral membrane proteins, lipid-anchored membrane proteins, and peripheral membrane proteins. Integral membrane proteins consist of hydrophobic domains, hydrophilic loops, and termini that extend into the cytoplasm, an organelle or the extracellular space. The secondary structure of the hydrophobic domain can either be of the α-helical or β-barrel type. In plants, β-barrel type membrane proteins only occur in outer membranes of mitochondria and plastids. Four main classes of integral membrane

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proteins are distinguished according to the number and organization of the transmembrane (TM) stretches [1]. Type I and II integral membrane proteins have a single TM domain with the N-terminus on the non-cytoplasmic side (type I) or on the cytoplasmic side (type II) of the membrane. Type III and IV integral membrane proteins have multiple TM regions either in a single protein (type III) or in homologous components organized as aggregates (type IV) [1]. Lipid-anchored membrane proteins are anchored to the membrane by lipid moieties attached in a post-translational process. The lipid moiety does not pass through the membrane but is embedded in the hydrophobic membrane lipid bilayer. Common lipid modifications are myristoylation, prenylation, S-acetylation (also known as palmitoylation), and the glycosylphosphatidylinositol (GPI) anchoring [2]. Peripheral membrane proteins interact with integral membrane proteins or with the peripheral regions of the lipid bilayer [3].

Information on whether a protein can be assigned as a membrane protein, how its topology looks like, and where it is located at a subcellular level can provide useful hints for revealing its function. It is often difficult and time-consuming to acquire this information experimentally. Many computational methods are available to generate predictions for most types of membrane proteins. Although the accuracy of each prediction method is limited [4–6], they can nonetheless be useful. It is possible to increase the reliability of a prediction by testing several tools on a single candidate membrane protein, or to apply a single prediction tool also on several orthologous protein sequences of the candidate membrane protein. For a substantiation of the overall prediction, both the approaches can be combined. However, manually compiling and combining all the required data can be a tedious and time-consuming task.

The online plant membrane protein database ARAMEMNON alleviates this task greatly by providing a single established resource for a comprehensive set of data about sequences, topology predictions, and subcellular localization predictions of membrane proteins from several plant species (among them are *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, *Solanum lycopersicum,* and others) [7]. Here, we describe how to identify and characterize plant membrane proteins using the ARAMEMNON database.

2 Materials

2.1 ARAMEMNON

The online plant membrane protein database is publicly available at "http://aramemnon.uni-koeln.de". The version at the time this article was written is ARAMEMNON 8.1. An overview of the main navigation including window names as used in Subheading 3 is displayed in Fig. 1.

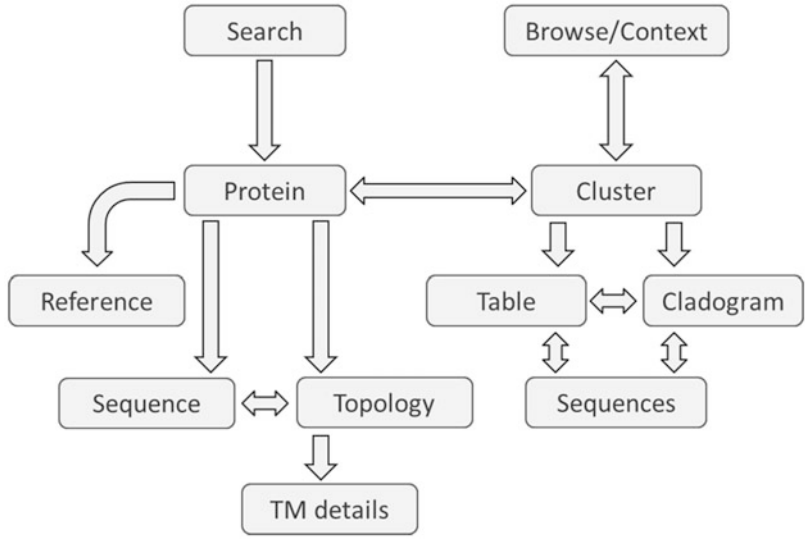

Fig. 1 Overview of the main navigation in ARAMEMNON. The window names are referred and further described in the text

2.2 Computational Prediction Tools

ARAMEMNON maintains and updates lists of all the tools, together with their URLs, used for the generation of its data. These can be found by navigating to <Resource> in the main menu of the database.

3 Methods

3.1 Search by Name or Sequence

ARAMEMNON provides the ability to search for an individual membrane protein using either its name or the protein/nucleotide sequence. Common names such as those assigned during publication (e.g., TPT, *see* **Note 1**) or the locus name of the gene that encodes the protein (e.g., At5g46110, LOC_Os01g13770, *see* **Note 2**) may be used. To carry out this search, the user should click the <Search> button in the main menu, choose "Search by keyword or protein name" and enter the name of the protein (*see* **Note 3**). To search using the protein or nucleotide sequence, the user should instead select "Search by sequence," then indicate the sequence type and enter the sequence. For a nucleotide sequence, it is also necessary to select the plant species, while protein searches are performed on all available species. The nucleotide sequence search can optionally be extended to the 5′- and 3′-untranslated regions that will include 1800 bases upstream and 300 bases downstream of the open reading frame. The sequence search is based on a BLAST local alignment [8].

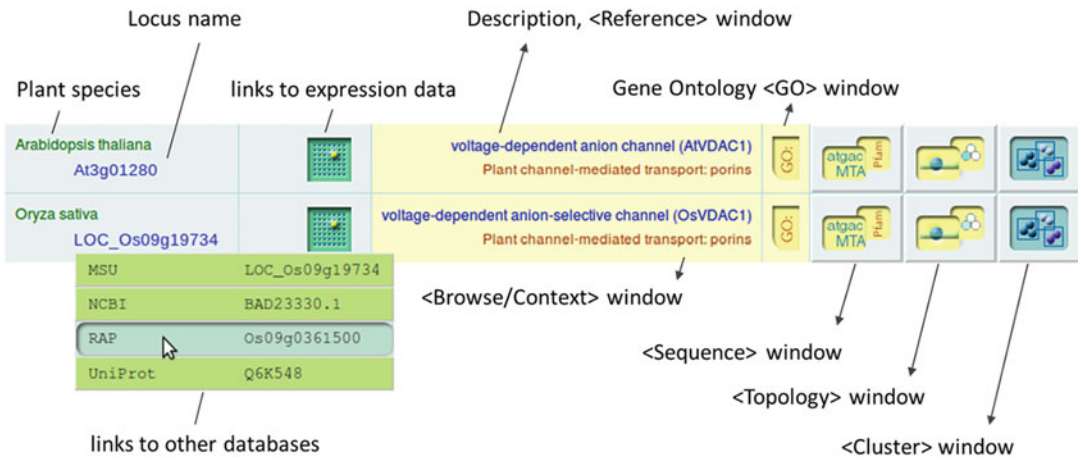

Fig. 2 Screenshot of a <Protein> window as an example for detail information about an individual protein. The *arrows* indicate the windows that are launched upon clicking the various buttons/text/icons

3.2 Search by Keywords or Features

If neither the protein name nor the sequence is known, the search can be performed using keywords or protein features. Click the <Search> button in the main menu, go to the "Search by keyword or protein name" section and enter a keyword (*see* **Note 3**). It is recommended to start by entering a single search keyword, then proceeding to add additional keywords to narrow down the results (*see* **Note 4**).

It is also possible to search by specifying a membrane protein property or a combination of several properties. Navigate to <Search> and "Search by predicted protein properties" and specify a combination of plant species, membrane association, subcellular localization, and optionally an additional keyword. It should be noted that this search is based on predictions and not on experimental data.

3.3 Protein Search Results

Regardless of which search option has been chosen, a <Protein> window containing all protein query results arranged as rows in a table is shown to the user. Each protein entry contains data and links to detailed information about the protein (Fig. 2).

1. The first column shows the plant species and the gene locus name. Other protein names and links to corresponding entries in other databases open when the mouse is moved over the locus name.
2. The next column contains an icon that will list, if available, links to expression data when the mouse is moved over the icon.
3. The third column contains a short protein description (*see* **Note 5**) which includes common names (mostly acronyms) used for the protein in publications. If the description has a

blue font color, it will launch a <Reference> window (*see* Subheading 3.4) when clicked.

4. The <GO> button links to a list of Gene Ontology (GO) terms if any are available for the protein.
5. The <Sequence> and <Topology> buttons allow the user to launch windows (*see* Subheadings 3.5 and 3.6) which present comprehensive information about the sequence and topology of the chosen protein (*see* **Note 6**).
6. The <Cluster> image button within each protein row opens the <Cluster> window (*see* Subheading 3.8) which will search for orthologous proteins and their relationships to each other (*see* **Notes 7** and **8**).

3.4 Bibliographic References

The <Reference> window lists respective publications about the protein. This list is in a reverse chronological order. Clicking on the author's name displays other articles by that author contained in the database. Direct links to the abstract and to the full article are also included (*see* **Note 9**).

3.5 Sequence Data

The <Sequence> window provides access to the genomic details for the gene, providing sequences for the protein, cDNA, and genomic DNA. In addition, it includes details such as the calculated molecular weight (MW) and the isoelectric point (IEP) of the protein as well as the positions and sequences of the coding exons of the gene (*see* **Note 10**). It also indicates the type and position of conserved domains as specified by the PFAM database ([9] and *see* **Note 11**).

All splice variants are presented as tabs (e.g., <At3g55740.1>, <At3g55740.2>), with each tab providing specific information about that splice variant (*see* **Note 12**). The different protein sequences from alternative splicing can be compared by clicking on the <Alignment> button in the "Protein sequence alignment" section.

The sequences are formatted in blocks of ten amino acids/nucleotides to aid in reading. Clicking on the <FASTA> button transforms the format into FASTA format that allows more convenient copy and paste functionality.

Clicking on the <Search> button in the "Non-plant related proteins" section evokes a local alignment-based search for related protein sequences in selected non-plant organisms. The non-plant organism can be chosen from a list.

3.6 Topology Data

The <Topology> window, which also contains information on each splice-variant, consists of the sections: "transmembrane spans," "subcellular location," and "lipid modification."

1. The "transmembrane spans" section visualizes the positions of α-helical transmembrane spans on the protein sequence as predicted by several tools (*see* **Note 13**). The name of the prediction program is shown on the left side. Each line represents a single prediction for the protein sequence with rectangles symbolizing the transmembrane stretches (*see* **Note 14**). There are two built-in consensus predictions, AramTmCon and AramTmMultiCon, generated by naive Bayesian approaches that combine the individual predictions. The consensus prediction AramTmCon combines the individual predictions for the protein, while AramTmMultiCon also takes into account the predictions from orthologous protein sequences. The third consensus prediction, ConPred, uses an approach based on a subset of the individual predictions [10]. The image is a clickable map—clicking on a prediction opens an extra window (<TM details>) with details about the positions, hydrophobicities, and amphiphilicities of the predicted α-helical transmembrane stretches. The extra window also includes a hydropathy profile of the protein sequence.
2. The "subcellular location" section of the <Topology> window contains an image that shows several predictions for the subcellular localization of the protein. The subcellular prediction tools are listed on the left side with links to its relevant publications. The result of an individual prediction is visualized as a circle with the intensity of the color indicating the strength of the prediction. The consensus prediction AramLocCon is based on a naive Bayesian approach [11] and summarizes the individual prediction results (*see* **Note 15**).
3. A table in the "lipid modification" section of the <Topology> window contains predictions for posttranslational lipid modifications of the protein, i.e., prenylation, myristoylation, S-acylation (palmitoylation), and GPI-anchoring of the protein. Each field shows an individual prediction, with the intensity of the background-color indicating the strength of the prediction.

3.7 Browse Protein Clusters

This feature is available by clicking on the <Browse> button in the main menu and displays groups of plant protein clusters. One of the groups is the plant permeome, divided into the main groups of channel-mediated, carrier-mediated, and primary active transport. The categories are subdivided hierarchically down to transport protein families. Expandable and collapsible lists allow convenient navigation through the individual plant protein families. Clicking on the <Cluster> image button next to a protein family opens it in the <Cluster> window (*see* Subheading 3.8).

3.8 Cluster of Related Proteins

The <Cluster> window shows all proteins of a chosen protein cluster as clustered by the MCL algorithm [12], grouped by plant species (Fig. 3). Clicking on the context description displays the

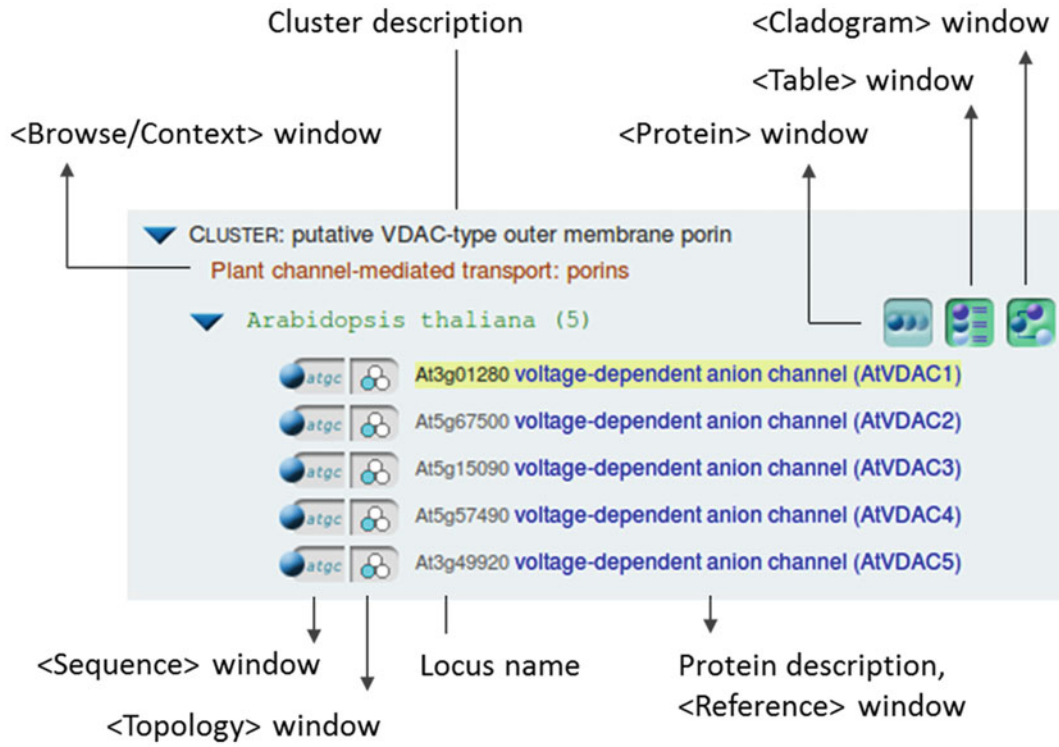

Fig. 3 Screenshot of a <Cluster> window as an example for detail information about a protein cluster. The *arrows* indicate the windows that are launched upon clicking the buttons/text/icons

cluster context in the appropriate <Browse/Context> window (*see* **Note 16**). Each group entry provides access to a protein list, a cluster table, and a cladogram via icon buttons. While the blue <Protein> button simply opens all protein entries from the group in the <Protein> window (*see* Subheading 3.3), the functionality of the other two requires further explanation (*see* Subheadings 3.9 and 3.10).

3.9 Table of Cluster Member Proteins

The <Table> window displays all the pairwise sequence identities between the reference protein, on the top of the list, and its orthologous proteins ([13], *see* **Note 17**). The selected reference protein can be replaced by any protein in the table by clicking on its <exchange> icon button. The list updates with the selected protein reordered as the new reference protein. All protein entries in the table include the plant species name, the gene locus name, the protein description, and direct links to the <Sequence> and <Topology> windows (*see* **Note 6**).

Optional functionality is available to select individual protein sequences from the table for a multiple sequence alignment. The required proteins are selected by ticking the corresponding checkboxes, then the alignment is initiated by clicking the <Alignment>

icon button. The window that opens shows the alignments for both the protein sequences and the consensus predictions of α-helix transmembrane stretches.

3.10 Cladogram of Cluster Member Proteins

The <Cladogram> window shows the cluster member proteins as a cladogram based on a neighbor-joining clustering of the sequences performed after multiple alignment and pairwise distance calculations ([14–16], *see* **Note 17**). Within the cladogram image, the same functionalities are available as for the <Table> window, i.e., protein specifications, links to <Sequence> and <Topology> windows and selectable proteins for a multiple alignment.

4 Notes

1. Many proteins are referenced by diverse names in different publications. ARAMEMNON perseveres to include all of them, thereby allowing users to find their protein. In addition, many proteins have been assigned additional names from major databases such as GenBank and UniProt, e.g., for the protein of the gene locus "LOC_Os01g13770" the names are "Q9FTT3" (UniProt) and "BAB17213" (GenBank).
2. In general, the gene locus name is clearly defined. There are, however, exceptions: for rice (*Oryza sativa*) two nomenclature systems exist. The Rice Genome Annotation Project (MSU) named the genes in a different way than the Rice Annotation Project Database (RAP-DB), e.g., "LOC_Os01g13770" (MSU) and "Os01g0239200" (RAP-DB) both refer to the same gene.
3. Several windows of the ARAMEMNON user interface (including the start window) provide an input field for a quick search. This works only for searches using a common protein name (e.g., "ProT1"), a gene locus name ("At2g39890"), or a keyword ("transporter"). The search is case insensitive, i.e., both "At2g39890" and "AT2G39890" are treated as identical in text searches.
4. When a search returns many protein hits from different plant species, a window opens that optionally enables the user to continue just with the protein hits for a single selectable plant species.
5. The ARAMEMNON database maintains and updates its own functional descriptions of proteins based on publications. Therefore, some membrane proteins that may be poorly annotated in other databases could be better functionally annotated in ARAMEMNON and vice versa.

6. The icon image button that launches the <Topology> window also enables a preview, thereby allowing a broad comparison of subcellular locations for several proteins at a glance. The individual icons contain colored circles with each of them symbolizing a possible location of the protein (green for chloroplast, blue for mitochondrion, brown for nucleus, and purple for various secretory pathways). The intensity of the circle color broadly indicates the strength of a positive prediction.
7. By default, the hierarchical list of a cluster opens in a collapsed form. When an individual protein is opened in the <Cluster> window, the lists expand to the extent that the entry for the query protein is visible and highlighted within the hierarchy. This helps to keep track of the query protein.
8. Sometimes, it can be useful to look up other clusters that share the same conserved domains. All shared conserved domains (as specified by PFAM) are listed within an extra section of the window. Click on the <Cluster> icon button of the conserved domain to see all clusters sharing that domain.
9. Depending on the journal, you may not be allowed to access the full article without a valid subscription to the journal.
10. The displayed genomic DNA ("gDNA" section) sequence uses gray lowercase letters for untranslated regions and blue uppercase letters for exons. If the sequence is copied and pasted into an application of your choice, the different font color (although useful for web pages) is not retained, but the case sensitivity still enables the identification of the exons.
11. The PFAM database—and other databases designed to store conserved protein domains such as PANTHER or SMART—defines and includes many thousands of domains. If none of the domains has been assigned to a protein sequence, it does not necessarily mean that the protein has no conserved domain. A putative conserved domain signature may simply not yet be defined.
12. The <Sequence> window shows all predicted splice variants of an annotated gene. Alternative splicing represents different combinations of exons and results in different mRNAs and therefore different protein sequences. The gene annotation software had predicted the different splice variants based on experimentally available mRNA sequences.

    It should be noted that any computational prediction process includes the possibility of false positive predictions, i.e., not every of the predicted splice variants actually exists.
13. The database also contains predictions for β-barrel transmembrane proteins of the outer mitochondrial and plastidial membranes. As the vast majority of all membrane proteins are of the

α-helical type, the predictions for β-barrel transmembrane regions are only shown where appropriate.

14. In general, transmembrane (TM) prediction tools are prone to misinterpret a cleavable signal peptide as an N-terminal TM domain and vice versa. Some tools (e.g., PHOBIUS) perform predictions for both and balance one interpretation against the other. A predicted cleavable signal peptide is symbolized by a small triangle at a plausible cleavage site (which may not necessarily be the exact cleavage site).
15. Empirically, a score for AramLocCon greater than 10 has been found to be reasonably reliable while a score greater than 20 is considered a very strong indication. Many chloroplast-localized proteins tend to show higher prediction scores for a mitochondrial location. On the other hand, mitochondrial proteins are less conspicuously predicted as being chloroplast localized.
16. The context description of a cluster is only visible (and clickable) if the cluster belongs to a group of clusters for which a context is defined (e.g., channel-mediated transport). *See* also Subheading 3.7 to find out which contexts are specified.
17. The multiple sequence alignment takes the longest protein sequence as a representative sequence (encoded by the longest available splice variant). If you wish to include other splice variants, or all of them, navigate to the top of the window and click the <Sequences> button. The window that opens provides all available splice-variant sequences of the orthologous proteins in a FASTA format. Copy the sequences and paste them into an external multiple alignment tool of your choice.

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References

1. Singer SJ (1990) The structure and insertion of integral proteins in membranes. Annu Rev Cell Biol 6:247–296
2. Hemsley PA (2015) The importance of lipid modified proteins in plants. New Phytol 205 (2):476–489
3. Moravcevic K, Oxley CL, Lemmon MA (2012) Conditional peripheral membrane proteins: facing up to limited specificity. Structure 20 (1):15–27
4. Möller S, Croning MD, Apweiler R (2001) Evaluation of methods for the prediction of membrane spanning regions. Bioinformatics 17(7):646–653
5. Tusnády GE, Simon I (2010) Topology prediction of helical transmembrane proteins: how far have we reached? Curr Protein Pept Sci 11 (7):550–561
6. Reeb J, Kloppmann E, Bernhofer M, Rost B (2015) Evaluation of transmembrane helix predictions in 2014. Proteins 83(3):473–484

7. Schwacke R, Schneider A, van der Graaff E, Fischer K, Catoni E, Desimone M, Frommer WB, Flügge UI, Kunze R (2003) ARAMEMNON, a novel database for *Arabidopsis* integral membrane proteins. Plant Physiol 131(1):16–26
8. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215(3):403–410
9. Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, Heger A, Hetherington K, Holm L, Mistry J, Sonnhammer EL, Tate J, Punta M (2014) Pfam: the protein families database. Nucleic Acids Res 42(Database issue):222–230
10. Arai M, Mitsuke H, Ikeda M, Xia JX, Kikuchi T, Satake M, Shimizu T (2004) ConPred II: a consensus prediction method for obtaining transmembrane topology models with high reliability. Nucleic Acids Res 32(Web Server issue):390–393
11. Schwacke R, Fischer K, Ketelsen B, Krupinska K, Krause K (2007) Comparative survey of plastid and mitochondrial targeting properties of transcription factors in *Arabidopsis* and rice. Mol Gen Genomics 277(6):631–646
12. Enright AJ, Van Dongen S, Ouzounis CA (2002) An efficient algorithm for large-scale detection of protein families. Nucleic Acids Res 30(7):1575–1584
13. Pearson WR (2000) Flexible sequence similarity searching with the FASTA3 program package. Methods Mol Biol 132:185–219
14. Edgar RC (2004) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5:113
15. Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol 17 (4):540–552
16. Gascuel O (1997) BioNJ: an improved version of the NJ algorithm based on a simple model of sequence data. Mol Biol Evol 14(7):685–695

Chapter 18

VANTED: A Tool for Integrative Visualization and Analysis of -Omics Data

Anja Hartmann and Anna Maria Jozefowicz

Abstract

The investigation of biological systems from different perspectives leads, due to novel -omics technologies, to large-scale, heterogeneous, and complex datasets. To elucidate molecular programs that control biological systems growth and development the integration and analysis of these -omics data remains challenging. Network-integrated visualizations based on graphical standards support intuitive exploration and interpretation of -omics data within the functional context. This integrated vision of the biological system to be studied tries to extract all hidden information for deepening our understanding and reveals new biological insights.

The method described here gives detailed instructions on the generation of such an integrative visualization of -omics data in the context of networks presented in the Systems Biology Graphical Notation (SBGN) using VANTED; a software tool for systems biology applications. An example illustrates the application of the method for metabolomics and proteomics data integration and analysis using a primary metabolic pathway, for the model crop potato.

Key words Systems biology, -Omics data, Metabolomics, Proteomics, Networks, Pathways, Integration, Analysis, Visualization, SBGN

1 Introduction

For deepening our understanding of complex biological systems high-throughput technologies are utilized to generate large-scale -omics data at different biological levels, for instance genomics, transcriptomics, proteomics, and metabolomics data. In systems biology these -omics data are combined to enhance information and unravel the underlying mechanisms that control systems growth and development. Thus, software tools for integrative data visualizations allow exploring and understanding the comprehensive -omics data representing a network of genes, proteins, metabolites, and environmental signals that lead to different and complex phenotypes.

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As stated by Fukushima et al. [1] in case of plant systems biology the major effective and efficient approaches to analyze -omics data are network and pathway analysis. It provides the opportunity to evaluate cellular behaviors from a multi-level perspective and enhance our understanding of plant systems. In different applications integrative -omics visualizations and analysis are used to elucidate previously unknown functional relations (for details the reader is referred to [2–9]).

The overall challenge is to create clear, meaningful, and integrated visualizations that give biological insight, without being overwhelmed by the intrinsic complexity of the data [10] to generate testable hypotheses about biological processes that govern system behavior [11]. Therefore, biological processes are modeled as networks by generating graphical maps that consist of nodes and edges representing the individual system components and their relations, respectively. Dependent on the used system components different biological networks arise for instance gene regulatory networks, signaling networks, and metabolic networks.

The biological networks used here represent metabolic networks or pathways where the individual components are metabolites that are converted by transport or enzymatic reactions. The connection between metabolic pathways and -omics data is based on the flow of information from genes over transcripts to proteins and metabolites. Proteins catalyze as enzymes the conversion of metabolites in reactions or transport as transport proteins metabolites within and between cells. Hence, -omics data can intuitively be integrated into different network elements: genomics, transcriptomics, and proteomics data can be integrated into reaction nodes whereas metabolomics data is designated for the integration into metabolite nodes.

To enhance information exchange and mutual understanding system biology standards are required. A detailed overview of major standards in systems biology is given in [12] in which one of the most common standard formats used for data exchange is SBML (Systems Biology Markup Language) [13]. SBML allows for sharing and reusing biological networks and enables their interpretation in terms of several software tools. To interpret biological networks more easily without the need of extensive descriptions, the Systems Biology Graphical Notation (SBGN) [14] has been developed. SBGN is a standard for the visual representation of biological networks and consists of the three languages Process Description (PD) [15], Entity Relationship (ER) [16], and Activity Flow (AF) [17]. The Process Description language used here shows the temporal dependencies of biological interactions in detail, and is thus suited for the visualization of metabolic pathways.

Databases and software tools supporting these standards are available from http://sbml.org, http://sbgn.org and the OMICtools database [18]. Both are necessary to store, manage, analyze,

and support the large amount of complex -omics data and biological networks as well as integrated visualizations. One of the software tools available from all the above-mentioned repositories is VANTED (*V*isualization and *A*nalysis of *Ne*tworks containing *E*xperimental *D*ata) [19]. VANTED is an open source software tool that comprises a comprehensive set of tasks for systems biology applications and provides direct access to several databases, for instance MetaCrop [20]. MetaCrop is a hand-curated database of high-quality data about plant metabolism on different levels of detail; e.g., from overview maps to detailed clickable plant-specific pathways visualized in SBGN. Together with VANTED' s extension SBGN-ED [21] for editing, translation, and validation of SBGN maps, plant-specific pathways from MetaCrop can be enhanced with -omics data.

In the following, a method for integrative -omics data visualization and analysis using VANTED is described (Subheading 2) and applied in a case study for the exploration of potato -omics data in the context of the tricarboxylic acid (TCA) cycle (Subheading 3).

2 Method for Integrative -Omics Data Visualization and Analysis

In this section, a detailed protocol is provided for the integrative visualization analysis of -omics data by means of the VANTED software tool. First, VANTED and the SBGN-ED addon have to be downloaded and installed (Subheading 2.1). Then a detailed description on the generation of integrative -omics visualizations using VANTED is given. First, a template file for the generated -omics datasets is created and imported into VANTED (Subheading 2.2). Dependent on the -omics dataset a suitable biological network is created or imported into VANTED (Subheading 2.3). Subsequently, a mapping procedure is conducted to result in an integrated visualization of different -omics data levels (Subheading 2.4). According to the individual requirements for data visualization the integrated -omics dataset can be customized (Subheading 2.5). Finally, examples for the integrative network analysis are provided (Subheading 2.6).

2.1 Download and Install VANTED and SBGN-ED

VANTED is a platform-independent Java open source software tool that requires Java SE 7 or higher.

1. Download and install the latest Java version according to the instructions given here http://www.oracle.com/technetwork/java/javase/downloads/ (*see* **Note 1**).
2. Download and install the latest version of VANTED for your operating system (Windows, Linux, or Mac) from http://www.vanted.org/ (*see* **Note 2**).

3. Download and install the latest version of SBGN-ED as follows (*see* **Note 3**):
   - Start VANTED.
   - Choose the main menu entry **Edit** → **Preferences**.
   - Select **Addon Manager**.
   - Press **Install/Configure Addons**.
   - Press **Find Addons/Updates**.
   - Choose **SBGN-ED** and press **Install Addon.**
   - Press **OK**.

2.2 Create a Template File for the Input of -Omics Data

Dependent on the amount of substances (genes, proteins, metabolites) measured in the -omics dataset you can choose between two types of input-templates (MS Excel format) for structured import together with meta-data; (1) a template for up to 250 substances, or (2) a transposed template for more than 250 substances.

1. To create a template file choose the side panel **Experiments** and expand **Data Input Templates**. Press either **Experiment Data** or **Experiment Data (Transposed)** to save the template file onto your desktop (*see* **Note 4**).
2. Enter meta-data and the generated -omics data of your experiment into the template file (*see* **Note 5**).
3. Save the filled template file after editing (*see* **Note 6**).

2.3 Create or Import a Suitable Biological Network

VANTED supports different options to create a suitable biological network with respect to SBGN standard for graphical representations:

1. Manual drawing using SBGN-ED addon (*see* **Note 7**) which additionally supports to validate the created biological network (SBGN map) according to the SBGN specifications.
2. Direct access and import from different repositories, e.g., metabolic pathways of the MetaCrop database:
   - Choose the side panel **Pathways** → **MetaCrop**.
   - Press **Get List of Pathways** to get a tree view of available pathways in MetaCrop.
   - Select a category your desired pathway belongs to, e.g., the category **Carbohydrate Metabolism** for **TCA cycle** (*see* **Note 8**).
   - Double-click the desired pathway for download and visualization.
3. Import any biological network defined in, e.g., SBML file format and translate it into SBGN (*see* **Note 9**).

- Choose the side panel **SBGN-ED** → **Tools** sub-tab.
- Press **Translate SBML to SBGN** for visualization in SBGN.

Once the biological network is created you can safe it in a suitable file format for further use, e.g., in the Graph Modelling Language (GML) [22] file format. GML supports the storage of networks together with all related attributes such as layout information and the full set of integrated -omics data including the corresponding visualization options (*see* **Note 10)**.

1. Choose the main menu entry **File** → **Save as**.
2. Select **Datatype** GML (*.gml).
3. Press **Save as** ....

2.4 Integrate -Omics Data into Biological Networks

Descriptive -omics data (e.g., intensity values) are integrated into biological networks by the visualization of corresponding charts inside the network nodes. To connect the data with network elements common identifiers are needed. A mapping table is used to unify the identifiers in the -omics data contained in the template file and the biological network. This is a simple Table (MS Excel format) which lists the identifiers as used in the template file in the **first** column and corresponding (alternative) identifiers as used in the biological network in the **second** column (*see* **Note 11**). For the integration of -omics data into network nodes:

1. Load your biological network as described in Subheading 2.3.
2. To load your -omics data choose the side panel **Experiments** → Press **Load Dataset** and choose your -omics data template created in Subheading 2.2.
3. For identifier mapping select **Identifier Annotation** in the side panel **Experiments** → Press **Add Alternative IDs** and choose your mapping table (*see* **Note 12**). To check for the correct identifier mapping press the link **Alternative Identifiers**. Here, you will already see the number of used alternative identifiers.
4. To integrate the -omics data into the biological network press **Perform Data Mapping** (*see* **Note 13**). If your -omics data template contains more substances than present in the biological network, they cannot be integrated. Therefore, you can choose to allow for the creation of new network elements for these substances (default: checked checkbox) or avoid it (unchecked checkbox) in the following dialogue **Integrate Data into Network** (*see* **Note 14**) and press **OK**. Investigate the **Data Mapping Results** in the next dialogue and press **OK** (*see* **Note 15**).

2.5 Customize the Visualization

VANTED supports a fully customizable visual appearance of the integrated -omics data providing adaptations on the (A) network (e.g., layout), (B) node (e.g., shape, charting style**),** and (C) edge-level (e.g., directionality, thickness). To customize the charting style according to your individual needs the following adaptations are preferentially applied.

(A) Network-level

1. Choose the side panel **Attributes** → **Network** → **Charting (General Settings)**:
   - Adapt the colors for different genotypes or conditions choosing **Condition Colors**:
     - Press **P** to use predefined color ranges, **or**
     - Choose an individual color for each condition:
       Press the upper condition (**Line/Bar–Color**).

       Select your favorite **Color**.

       Press **OK**.

       Continue with the next condition.
   - To add axis to the bar charts check both checkboxes **Show Category Labels** for *x*-axis and **Show Range Labels** for *y*-axis. The axis titles were defined previously in the template file for the -omics data (units for substance measurements and time).
2. Press **Apply Changes** to pass the adaptations to network.
3. Add a legend to your network.
   - Choose **Mapping** → **Create Legend** in the main menu.
   - Select a suitable type of legend, e.g., **All Condition Attributes except Condition ID** which results in a combination of species, genotype, and treatment description edited previously in the template file for the -omics data.
   - Press **OK** for this and for the next dialog that informs about the legend placement.

(B) Node-level

1. Select all nodes with integrated -omics data in the biological network (*see* **Note 16**).
2. Adjust the label positions, select a suitable chart style and adapt the axis dimensions:
   - Choose the side panel **Attributes** → **Node**.
     - Adjust the label position **Label** → **Position** and select **Inside, Top** alignment position in the label field panel.
     - Choose a suitable chart style, e.g., **Charting (Selected Elements)** → **Diagram** and select **Bar Charts (Flat)** to

investigate the mean and standard deviation between replicates of genotypes.

- Adapt the **Range Axis: Maximum** and the **Range Axis: Minimum** according to your needs and check checkbox **Range Axis: Custom Min/Max** to ensure your preferred axis dimensions.

– Press **Apply Changes** to pass the adjustments to the selected nodes (*see* **Note 17**).

Safe your customized integrated -omics data visualization as described in Subheading 2.3.

2.6 Perform Integrated Network Analysis

To perform integrated network analysis VANTED offers several methods, e.g., analysis at the network-level (centralities, shortest path, cycle detection, motifs), statistics at the data-level (correlation, clustering, t-test), or enrichment analysis (for more details the reader is referred to *(*19*)*). To identify similarities of the integrated -omics data to one of the nodes in the network correlation analysis is performed.

1. Select one node to correlate all others with (target parameter).
2. Choose the side panel **Analysis** → **Data** → **Statistics** → **Correlate 1:n**.
3. Use default settings and press **Calculate and Visualize Correlations** (*see* **Note 18**).

The target parameter is colored in yellow while red and blue-colored nodes indicate the positive and negative correlation coefficients, respectively. The darker or more intense a color the stronger the correlation is.

Safe your analyzed integrated -omics data visualization as described in Subheading 2.3. Thereafter, you can generate (A) an image file and (B) export the results of the correlation analysis.

(A) Image file generation

1. Choose the main menu entry **File** → **Export** → **Network as Image**.
2. Select an image file format, e.g., PNG (*.png) press **Create PNG Image**.
3. Use default settings and press **OK** (*see* **Note 19**).
4. Press **Create Image File**.

(B) Export of correlation analysis results

– Choose the main menu entry **File** → **Export** → **Calculated Data**.

– To export the correlation results check both checkboxes **Label** and **Correlation**.

– Press **OK**.
– To save the text file give a certain name, e.g., **Correlation.txt** and press **Save**.

In the following, a case study for potato illustrates as an example the application of the method to elucidate previously unknown functional relations of the -omics data in the context of a metabolic pathway.

3 Case Study: Exploration of Potato Nitrogen Use Efficiency

Nitrogen (N), as an essential element in amino acids, nucleotides, and proteins, is the major macronutrient limiting crop yield worldwide [3]. Therefore, breeding of crops with higher nitrogen use efficiency (NUE) is of great importance for future agronomical performance. To investigate N-limitation responses and adaptation mechanisms of the crop potato (*Solanum tuberosum*) a combined -omics approach was performed over time for two genotypes with contrasting NUE.

Due to its role as potential novel target for the enhancement of NUE and as a central metabolic hub for the interacting pathways of respiration, nitrogen assimilation, and photorespiration [23], the tricarboxylic acid (TCA) cycle is used for the integration, exploration, and analysis of metabolomics and proteomics data using the described method.

3.1 Template Creation for Metabolomics and Proteomics Data

The -omics dataset comprises measurements from two genotypes (six replicates each) subjected to N-limitation (-N) or control conditions (+N) within four time points to study early and late responses, and represents ratios between -N and +N treatments for each replicate of the two genotypes. With the objective to integrate -omics data into the TCA cycle it is sufficient to use a subset of the measured metabolomics and proteomics data. Therefore, the non-transposed input-template is completed with data together with meta-data as depicted in Fig. 1 (*see* **Note 20**, Subheading 2.2).

3.2 TCA Cycle Import from MetaCrop

The TCA cycle is directly imported from the MetaCrop database and visualized in SBGN using VANTED and SBGN-ED (*see* Fig. 2, Subheading 2.3).

3.3 Metabolomics and Proteomics Data Integration into the TCA Cycle

For the integration of -omics data first a mapping table is generated to unify the identifiers of the metabolomics and proteomics data contained in the template file and the TCA cycle (*see* Fig. 3).

Following the steps in Subheading 2.4 (load the TCA cycle, import the template file, add the mapping table) the metabolomics and proteomics data are subsequently integrated into nodes representing metabolites and enzymes, respectively.

	A	B	C	D	E	F	G	H	I	J
1	**Experiment-Data – Database**									
2										
3	**Experiment**				**Help**					**Internal Info**
4	Start of Experiment (Date)	28.10.15			- Fields with a * are optional					**V1.2**
5	Remark*				- Yellow cells allow input					
6	Experiment Name (ID)	Potato NUE			** These cells must contain numbers as 1, 2, 3, ...					
7	Coordinator	A.M. Jozefowicz			*** These cells must correlate to the numbers in **					
8	Sequence-Name*									
9										
10										
11	**Plants/Genotypes****	1								
12	Species	Potato								
13	Variety*									
14	Genotype	N inefficient								
15	Growth conditions*									
16	Treatment*	(-N/+N)								
17										
18										
19										
20	**Measurements**				**Substance**	Citric	Fumaric	Malic	Oxoglutarate	PGSC0003DMT400058223
21					**Meas.-Tool***	GCMS	GCMS	GCMS	GCMS	QTOF
22	**Plant/Genotype*****	**Replicate #**	**Time**	**Unit (Time)**	**Unit**	log2 FC (-N/+N)	log2 FC (-N/+N)	log2 FC (-N/+N)	log2 FC (-N/+N)	log2 FC (-N/+N)
23	1	1	0	Hour		0,895887464	0,961827957	0,825647517	0,620287868	0,951089694
24	1	2	0	Hour		0,755331919	0,798503166	0,670703455	0,700526452	0,990874258
25	1	3	0	Hour		1,643245517	1,047333765	1,016456374	1,420016634	0,961563829
26	1	4	0	Hour		1,987150722	1,131578947	1,136916924	1,146126372	0,850112248
27	1	5	0	Hour		0,619666617	0,099514312	0,597185892	0,563673917	1,083153228
28	1	6	0	Hour		1,217587769	0,908840566	1,05671851	1,171709851	0,889622033
29	1	1	1	Hour		1,038857867	1,140505585	1,021756202	1,05624771	1,299888997
30	1	2	1	Hour		0,722092932	0,987007925	0,789700145	0,811165367	1,34435415
31	1	3	1	Hour		0,826772855	0,739961113	0,844184007	1,426441483	1,005401648
32	1	4	1	Hour		0,817701861	0,717479439	0,96662314	1,271542642	1,089343594
33	1	5	1	Hour		1,7426898	1,250245752	1,390327211	1,553732401	0,907370452
34	1	6	1	Hour		1,102572923	1,220937986	1,088749951	1,173566327	1,263117492
35	1	1	24	Hour			0,216433462	0,10803473	0,176176176	1,05626196
36	1	2	24	Hour		0,346876972	0,649776286	0,315325417	0,678346028	0,356126007
37	1	3	24	Hour		0,974533135	0,361641977	0,424828684	1,02183541	1,095751931
38	1	4	24	Hour		0,532529874	0,705047872	0,491844001	1,019649536	0,684369357
39	1	5	24	Hour		0,41919607	0,724266667	0,392776481	1,268855883	1,015721105
40	1	6	24	Hour		0,503845382	0,766085856	0,466397699	1,391683917	1,143772697
41	1	1	72	Hour		0,415487768	0,69171906	0,607332978	2,812649623	0,92045587
42	1	2	72	Hour		0,478072511	0,047503383	0,477934095		0,689239508
43	1	3	72	Hour		0,782882202	0,247799726	0,510024424	1,795835387	1,32949081
44	1	4	72	Hour		0,803845715	0,693439233	0,619931868	1,868680215	1,043733564
45	1	5	72	Hour		0,501394143	0,420892361	0,61747692	0,974451712	0,973295639
46	1	6	72	Hour		0,51925188	0,471096407	0,452472038	0,870203341	1,068997317

Fig. 1 Screenshot of a template file (MS Excel format) filled with metabolomics and proteomics data of potato. Only for visual purpose *different colors* indicate the distinction between time points

3.4 Visualization of the -Omics Enriched TCA Cycle

To customize the charting style bar charts are utilized to represent the time course of -omics data (Subheading 2.5). In Fig. 4 each bar represents the mean ratios and standard deviation between -N and +N treatments for each replicate of the two genotypes that are depicted in different colors. The N-inefficient genotype is visualized in the left chart colored in orange and the N-efficient genotype is depicted in the right chart colored in green. Additionally, nodes containing bar charts are adapted in size.

The investigated metabolomics and proteomics data clearly show an upregulation of TCA cycle enzymes and correlating metabolite levels under N-limitation already as early stress response in both genotypes. There are slide differences with respect to both genotypes. For example, the metabolite succinate is upregulated after short-term treatment and during long-term treatment it decreases for the N-inefficient genotype. For the N-efficient genotype this difference in regulation between short- and long-term treatments for succinate is missing.

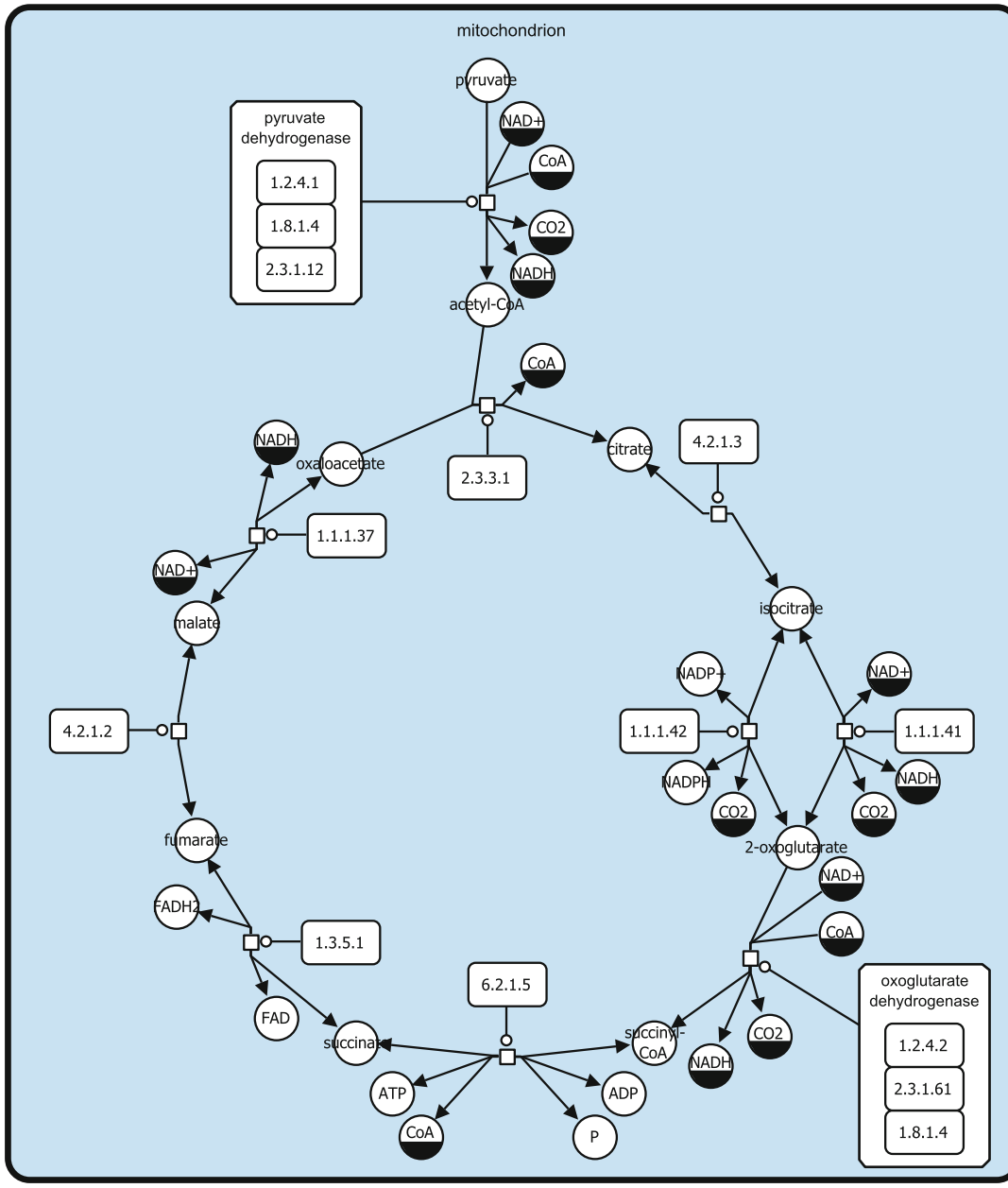

Fig. 2 The TCA cycle metabolic network located in the mitochondria represented in SBGN from the MetaCrop database. Reactions are catalyzed by enzymes (represented by macromolecules and labeled with EC numbers as unique identifiers, *rectangles*) converting metabolites (represented by the simple chemicals, *circles* and a *clone marker* depicts the repetition of metabolites (e.g., NAD+)). Pyruvate dehydrogenase and oxoglutarate dehydrogenase are multienzyme complexes composed of several enzymes catalyzing successive steps in a metabolic pathway

	A	B
1	**Data**	**Map**
2	Citric	citrate
3	Fumaric	fumarate
4	Malic	malate
5	Oxoglutarate	2-oxoglutarate
6	Succinic	succinate
7	PGSC0003DMT400058223	1.2.4.1
8	PGSC0003DMT400022547	4.2.1.3
9	PGSC0003DMT400050235	1.1.1.37
10	PGSC0003DMT400042326	6.2.1.5
11	PGSC0003DMT400067503	1.1.1.41
12	PGSC0003DMT400074559	2.3.3.1
13	PGSC0003DMT400081790	1.1.1.42

Fig. 3 Screenshot of the mapping Table (MS Excel format) which lists the identifiers as used in the template file in the **first** column (data) and corresponding (alternative) identifiers as used in the TCA cycle in the **second** column (map)

3.5 Integrated Network Analysis for the -Omics Enriched TCA Cycle

To identify similarities between -omics data succinate is chosen as the target parameter and correlations are calculated to all other metabolic parameters in the TCA cycle (Subheading 2.6). The result of the correlation analysis is visualized in Fig. 5. The target parameter succinate is colored in yellow. Nodes are colored according to the correlation coefficients where positive correlations are displayed in red and negative correlations in blue. The color intensity is proportional to the correlation coefficients and the thickness of the node border depicts the correlation significance levels (or *p*-value). Here, a thick border indicates a *p*-value less than 0.05.

Additionally, a table is exported that summarizes the results of the correlation analysis including node labels, correlation coefficients, and *p*-values (*see* Fig. 6).

The correlation analysis revealed a significant positive correlation between succinate and the metabolites fumarate, malate, citrate, and the enzyme 1.1.1.42 -isocitrate dehydrogenase. This observation indicates a coregulation between the five members of the metabolomics and proteomics data in the functional context of the TCA cycle. During N-deficiency the N-inefficient genotype reflects early response while the N-efficient genotype shows upregulation over the whole period.

3.6 Conclusion

The described method addresses the challenge to create a clear, meaningful, and integrated visualization from large-scale, heterogeneous, and complex -omics datasets using networks for deepening our understanding of biological systems and resolves biological insights. Detailed instructions are given to utilize VANTED for integrative -omics data visualizations and analysis within the

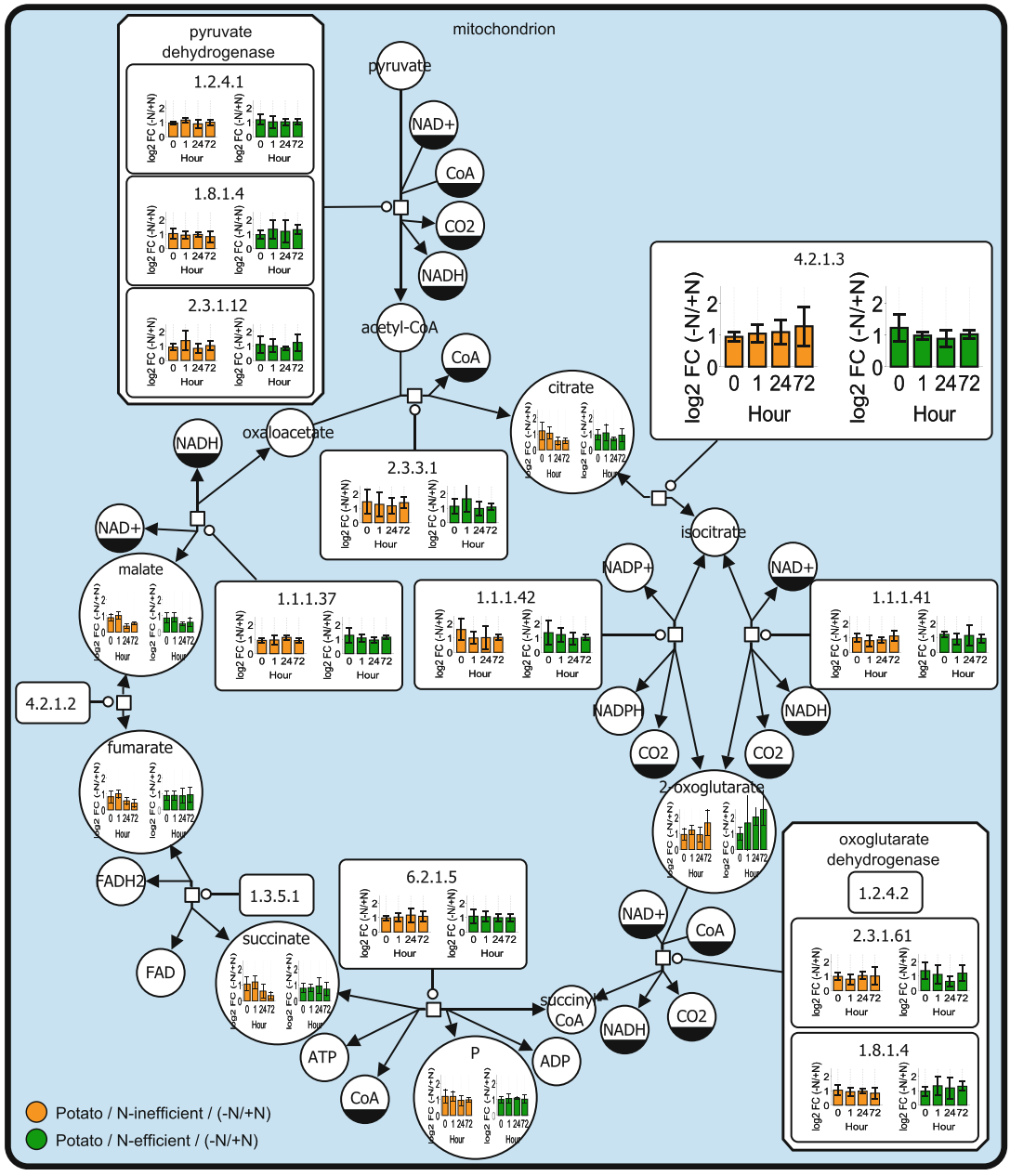

Fig. 4 The TCA cycle metabolic network enhanced with metabolomics and proteomics data of potato. *Bar charts* represent mean ratios and standard deviation between -N and +N treatments for each replicate of the two genotypes (*left*: N-inefficient genotype colored in *orange*, *right*: N-efficient genotype colored in *green*) in a time course. The bar chart for enzyme 4.2.1.3 is enlarged to depict the axes in more detail

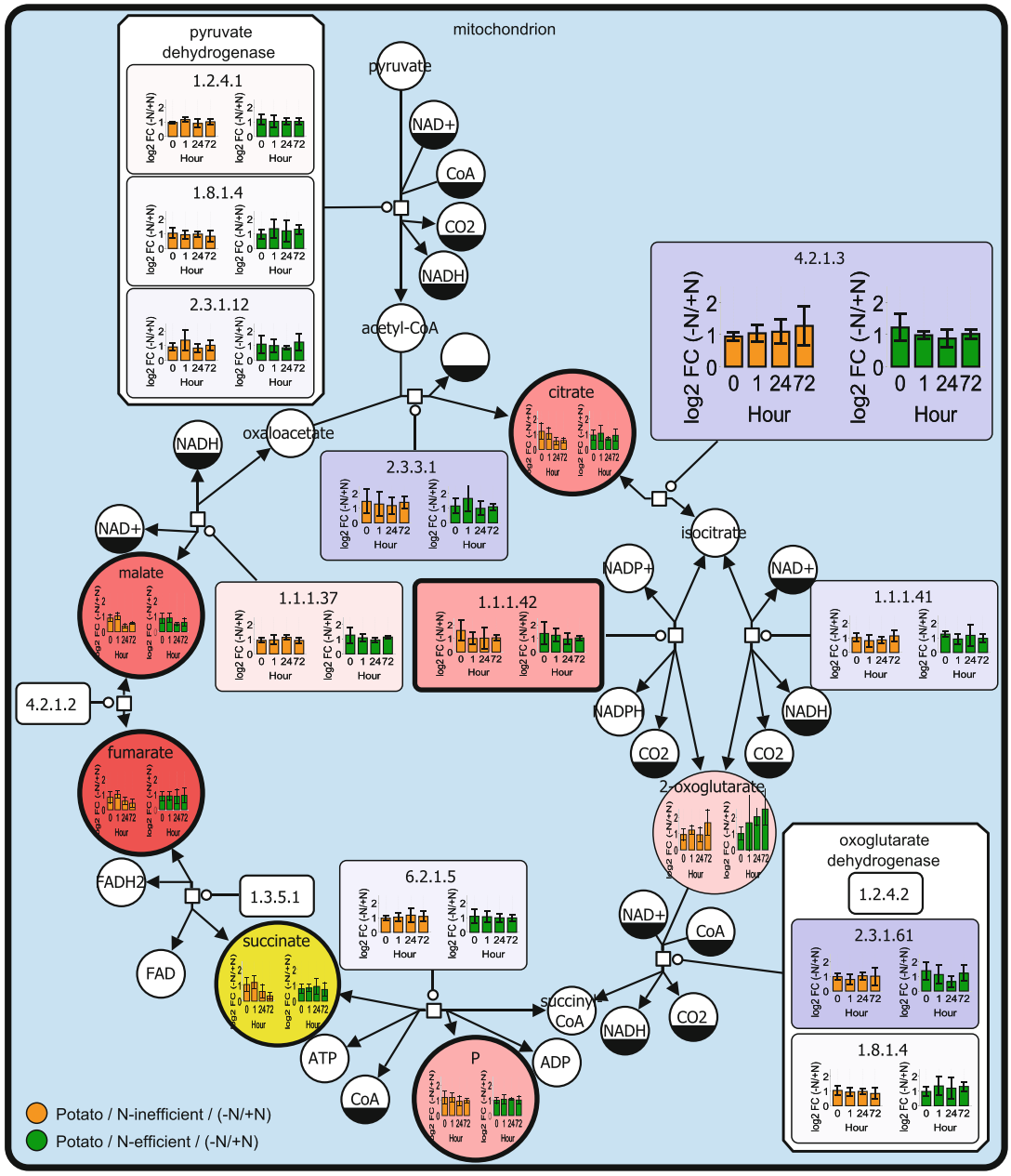

Fig. 5 The TCA cycle metabolic network enhanced with metabolomics and proteomics data of potato including correlation analysis results. Nodes are colored according to the correlation coefficients (*red* positive and *blue* negative correlations) and the target parameter succinate is colored in *yellow*. The color intensity is proportional to the correlation coefficients and a thick border indicates a *p*-value less than 0.05

	A	B	C
1	**Label**	**1:n Correlation**	**p-Value**
2	fumarate	0,70	0,00
3	malate	0,56	0,00
4	citrate	0,43	0,00
5	1.1.1.42	0,34	0,02
6	P	0,32	0,04
7	2-oxoglutarate	0,17	0,27
8	1.1.1.37	0,08	0,60
9	1.2.4.1	0,03	0,85
10	1.8.1.4	-0,02	0,89
11	2.3.1.12	-0,04	0,79
12	6.2.1.5	-0,05	0,74
13	1.1.1.41	-0,10	0,52
14	4.2.1.3	-0,18	0,22
15	2.3.3.1	-0,19	0,22
16	2.3.1.61	-0,21	0,16

Fig. 6 Screenshot of the correlation analysis results Table (MS Excel format) which lists for each node in the **first** column the name (label), in the **second** column the correlation coefficient (1:*n* correlation), and in the **third** column the corresponding *p*-value

functional context of biological networks represented in SBGN. An example illustrated the application of the method for potato metabolomics and proteomics data integration, analysis and exploration in the context of the tricarboxylic acid (TCA) cycle and revealed different regulation patterns between short- and long-term treatments, especially for the N-inefficient genotype.

Here, a single-metabolic pathway was used for an illustrative presentation of the integrative -omics data visualization and analysis. In principle, the method is capable of examining more than one pathway of primary and secondary metabolism at once or a metabolic model including compartmentalization and transport proteins for the investigation of the whole -omics dataset. In combination with additional -omics data, e.g., at the level of genomics or transcriptomics, this systems biology method is capable of elucidating programs that control systems growth and development.

4 Notes

1. It is strongly advised to uninstall a previously installed VANTED version, since the installation process has changed.
2. VANTED does not contain any logging functionality or hidden features. It connects to the internet only for network-related functionality on user's request. During startup the Java

Web Start system will automatically look for updated system files. Configure proxy settings if needed:

- Choose the main menu entry **Edit** → **Preference**.
- Expand **General** and select **VANTED Preferences**.
- Edit the Name or IP of the proxy host in field **Proxy Host**.
- Edit the Port number of the proxy in field **Proxy Port**.
- Press **Apply**.
- Press **Ok**.

3. Please check for version numbers of VANTED and SBGN-ED for compatibility. In case that SBGN-ED could not be found directly through the **Find Addons/Updates** option of the **Addon Manager**:
   - Download SBGN-ED manually from http://www.sbgn-ed.org/.
   - Start VANTED.
   - Choose the main menu entry **Edit** → **Preferences**.
   - Select **Addon Manager**.
   - Press **Install/Configure Addons**.
   - Press **Install Addon**.
   - Choose the downloaded **SBGN-ED** addon (sbgn-ed.jar).
   - Press **Install.**
   - Press **OK**.
   - Press **OK**.
4. The template file will automatically be loaded using your MS Excel application. While editing your -omics data do not fill 0 when there is a missing value, leave it blank.
5. As an example how to fill in the template with -omics data both template files contain example data. Detailed descriptions of the VANTED template files can be found at http://www.vanted.org/ choose **Input Formats**.
6. It is important not to insert rows or columns at places where it is not supported by the system. Also the data format of the input needs to be valid, fields that are not marked to be optional, need to contain data.
7. For a tutorial and a protocol demonstrating how to use SBGN-ED to draw SBGN, please refer to http://www.sbgn-ed.org/ and Junker et al. [24] *Step 9: Creation of SBGN maps*, respectively.
8. For download and visualization of more than one pathway VANTED supports to select the categories itself containing several pathways or different pathways of one or different

categories at once: Select all desired pathways and use the **Download Selected Pathways** option that offers you additionally the possibility of combining the different pathways within one file.

In case that the connection to MetaCrop is out of VANTED not possible, download the TCA cycle manually from MetaCrop http://metacrop.ipk-gatersleben.de:

- In MetaCrop choose **Pathways**.
- Search for **TCA cycle**.
- Press the link for **Download Map.**
- Save the **TCA cycle.gml** file onto your desktop.
- Start VANTED.
- Choose the main menu entry **File** → **Open**.
- Choose the downloaded **TCA cycle.gml** file.
- Press **Open**.

Due to the downloaded GML file format of the TCA cycle of MetaCrop it is automatically visualized in SBGN.

9. VANTED supports additional file formats to import biological networks, e.g., KGML, BioPax, SIF, DOT. Beside KGML VANTED does not support to transform them into SBGN maps.
10. For exchange of the graphical representation with the systems biology community biological networks can be additionally exported as SBGN-ML (no additional data included).
11. The mapping table can contain more than one corresponding identifier in the subsequent columns to allow several alternative identifiers.
12. Press **OK** for the next two dialogs to use the default settings.
13. Please note that only selected nodes are used for mapping.
14. If the alternative identifiers for the biological network and the -omics data template are not common, e.g., due to spelling mismatches, no -omics data will be integrated. Please check all identifiers (node labels in the biological network, substance names in the template) and the corresponding mapping table carefully.
15. Due to the annotation of, e.g., proteomics data (isoforms of proteins could be annotated with one and the same enzyme), it is possible that more than one chart will be integrated in the nodes of your network.
16. After the -omics data integration into the biological network (as described in Subheading 2.4) nodes containing data are automatically selected. Otherwise, hold **Control Key** while

selecting the desired nodes with the mouse pressing **Left Mouse Button**.

17. Further useful adaptations to customize nodes are according to the **Label** (Color, Font, Font-Size, Font-Style), **Node Attributes** (Size) and **Shape** (Boarder-Width, Fill-Color, Frame-Color).
18. Calculation and visualization settings are adjustable, e.g., you can choose between **Pearson's** or **Spearman's Correlation**, set thresholds for **Significance** and the correlation coefficient **|r|** as well as to adapt the **Color-Code for |r|**.
19. You can additionally adjust for, e.g., the image resolution or image border.
20. Two template files are used, one for each genotype, to generate two distinct diagrams in each node. Using one template for both genotypes would result in one diagram containing data from both.

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References

1. Fukushima A, Kanaya S, Nishida K (2014) Integrated network analysis and effective tools in plant systems biology. Front Plant Sci 5:598–607
2. Hirai MY, Yano M, Goodenowe DB, Kanaya S, Kimura T, Awazuhara M, Arita M, Fujiwara T, Saito S (2004) Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in *Arabidopsis thaliana*. Proc Natl Acad Sci 101:10205–10210
3. Fukushima A, Kusano M (2014) A network perspective on nitrogen metabolism from model to crop plants using integrated 'omics' approaches. J Exp Bot 65:5619–5630
4. Hossain MS, Joshi T, Stacey G (2015) System approaches to study root hairs as a single cell plant model: current status and future perspectives. Front Plant Sci 6:363–370
5. Soni P, Nutan KK, Soda N, Nongpiur RC, Roy S, Singla-Pareek SL, Pareek A (2015) Towards understanding abiotic stress signaling in plants: convergence of genomic, transcriptomic, proteomic, and metabolomic approaches. In: Pandey GK (ed) Elucidation of abiotic stress signaling in plants. Springer, New York
6. Yoshida T, Mogami J, Yamaguchi-Shinozaki K (2015) Omics approaches toward defining the comprehensive abscisic acid signaling network in plants. Plant Cell Physiol 56:1043–1052
7. Carneiro JMT, Madrid KC, Maciel BCM, Arruda MAZ (2015) *Arabidopsis thaliana* and omics approaches: a review. J Integr OMICS 5:1–16
8. Komatsu S, Shirasaka N, Sakata K (2013) 'Omics' techniques and their use to identify how soybean responds to flooding. J Proteome 93:169–178
9. Guerriero G, Sergeant K, Hausman JF (2013) Integrated -omics: a powerful approach to understanding the heterogeneous lignification of fibre crops. Int J Mol Sci 14:10958–10978
10. Gehlenborg N, O'Donoghue SI, Baliga NS, Goesmann A, Hibbs MA, Kitano H, Kohlbacher O, Neuweger H, Schneider R, Tenenbaum D, Gavin A-C (2010) Visualization of

omics data for systems biology. Nat Methods 7: S56–S68

11. Moyano TC, Vidal EA, Contreras-López O, Gutiérrez RA (2015) Constructing simple biological networks for understanding complex high-throughput data in plants. Methods Mol Biol 1284:503–526
12. Hucka M, Nickerson DP, Bader GD, Bergmann FT, Cooper J, Demir E, Garny A, Golebiewski M, Myers CJ, Schreiber F, Waltemath D, Le Novère N (2015) Promoting coordinated development of community-based information standards for modeling in biology: the COMBINE initiative. Front Bioeng Biotechnol 3:19–25
13. Hucka M, Finney A, Sauro HM, Bolouri H, Doyle JC, Kitano H, Arkin AP, Bornstein BJ, Bray D, Cornish-Bowden A, Cuellar AA, Dronov S, Gilles ED, Ginkel M, Gor V, Goryanin I, Hedley WJ, Hodgman TC, Hofmeyr JH, Hunter PJ, Juty NS, Kasberger JL, Kremling A, Kummer U, Le Novère N, Loew LM, Lucio D, Mendes P, Minch E, Mjolsness ED, Nakayama Y, Nelson MR, Nielsen PF, Sakurada T, Scha JC, Shapiro BE, Shimizu TS, Spence HD, Stelling J, Takahashi K, Tomita M, Wagner J, Wang J (2003) The Systems Biology Markup Language (SBML): a medium for representation and exchange of biochemical network models. Bioinformatics 19:524–531
14. Le Novère N, Hucka M, Mi H, Moodie S, Schreiber F, Sorokin A, Demir E, Wegner K, Aladjem M, Wimalaratne SM, Bergman FT, Gauges R, Ghazal P, Kawaji H, Li L, Matsuoka Y, Villéger A, Boyd SE, Calzone L, Courtot M, Dogrusoz U, Freeman T, Funahashi A, Ghosh S, Jouraku A, Kim S, Kolpakov F, Luna A, Sahle S, Schmidt E, Watterson S, Wu G, Goryanin I, Kell DB, Sander C, Sauro H, Snoep JL, Kohn K, Kitano H (2009) The Systems Biology Graphical Notation. Nat Biotechnol 27:735–741
15. Moodie S, Le Novère N, Demir E, Mi H, Villéger A (2015) Systems Biology Graphical Notation: Process Description language level 1, version 1.3. J Integr Bioinform 12:263
16. Sorokin A, Le Novère N, Luna A, Czauderna T, Demir E, Haw R, Mi H, Moodie S, Schreiber F, Villéger A (2015) Systems Biology Graphical Notation: Entity Relationship language level 1, version 2. J Integr Bioinform 12:264
17. Mi H, Schreiber F, Moodie S, Czauderna T, Demir E, Haw R, Luna A, Le Novère N, Sorokin A, Villéger A (2015) Systems Biology Graphical Notation: Activity Flow language level 1, version 1.2. J Integr Bioinform 12:265
18. Henry VJ, Bandrowski AE, Pepin A-S, Gonzalez BJ, Desfeux A (2014) OMICtools: an informative directory for multi-omic data analysis. Database 2014:1–5
19. Rohn H, Junker A, Czauderna T, Hartmann A, Klapperstück M, Treutler H, Grafahrend-Belau E, Klukas C, Schreiber F (2012) VANTED v2: a framework for systems biology applications. BMC Syst Biol 6:139–152
20. Schreiber F, Colmsee C, Czauderna T, Grafahrend-Belau E, Hartmann A, Junker A, Junker BH, Klapperstück M, Scholz U, Weise S (2012) MetaCrop 2.0: managing and exploring information about crop plant metabolism. Nucleic Acids Res 40:D1173–D1177
21. Czauderna T, Klukas C, Schreiber F (2010) Editing, validating, and translating of SBGN maps. Bioinformatics 26:2340–2341
22. Himsolt M (1996) GML: a portable graph file format. Technical Report, University of Passau
23. Foyer CH, Noctor G, Hodges M (2011) Respiration and nitrogen assimilation: targeting mitochondria-associated metabolism as a means to enhance nitrogen use efficiency. J Exp Bot 62:1467–1482
24. Junker A, Rohn H, Czauderna T, Klukas C, Hartmann A, Schreiber F (2012) Creating interactive,web-basedanddata-enrichedmaps using the Systems Biology Graphical Notation. Nat Protoc 7:579–593

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