Isolation of Plasmodesmata membranes for lipidomic and proteomic analysis

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Abstract

Plasmodesmata (PD) are membranous intercellular nanochannels crossing the plant cell wall to connect adjacent cells in plants. Our understanding of PD function heavily relies on the identification of their molecular components, these being proteins or lipids. In that regards, proteomic and lipidomic analyses of purified PD represent a crucial strategy in the field. Here we describe a simple two-step purification procedure that allows isolation of pure PD-derived membranes from *Arabidopsis* suspension cells suitable for "omic" approaches. The first step of this procedure consists on isolating pure cell walls containing intact PD, followed by a second step which involves an enzymatic degradation of the wall matrix to release PD membranes. The PD-enriched fraction can then serve to identify the lipid and protein composition of PD using lipidomic and proteomic approaches, which we also describe in this method article.

Key words Plasmodesmata, Membrane, Cell wall, Cellulase, Suspension cell, Proteomic, Lipidomic, Mass spectrometry, *Arabidopsis thaliana*

1 Introduction

In plants, cell-to-cell communication relies, to a large extent, on plasmodesmata (PD) channels. These are highly specialized structures spanning the plant cell wall creating direct cytosolic bridges between cells. PD are composed of segments of the plasma membrane (PM) and contain a strand of endoplasmic reticulum (ER) in their center. The two forms concentric cylinders tethered together by proteins elements [1]. PD are considered as specialized ER-PM contact sites and contain a specific set of proteins and lipids [2-4]. This unique molecular signature likely defines the functional and structural features organization of PD. The identification of PD molecular components is critical for understanding PD function, and as such, tremendous efforts have been made to purify these structures along with proteomic and lipidomic approaches [5, 6].

Nevertheless, the purification of PD has been a long and tedious task. First, PD are firmly anchored to the cell wall and cannot be easily separated from the cellulose network. Second, because of their nanoscopic size, PD only represent a tiny fraction of the total plant tissue mass. Historically, PD purification has relied on subcellular fractionation and purification of cell wall fractions from various plant tissues. Due to their position embedded into the wall polymers, PD co-purify with the wall fragments. These approaches were successful in getting subcellular fractions enriched for PD and have led to the identification of several PD protein constituents [7-15]. Despite this achievement, the low proportion of PD compared to cell wall mass was a major shortcoming of this method. A major step forward was achieved with the use of cell wall-degrading enzymes which allows the release of the PD membranes from the cell wall matrix [3, 16]. Combined with proteomic analyses, this approach enables a giant step towards the identification of PD associated proteins [3, 7, 8, 12, 25]. Later, through comparative lipidomic analyses, several groups were able to identify the lipid species specifically associated with the channels, highlighting the importance of membrane lipids in functionally defining PD [4, 24].

One crucial aspect of the PD purification protocol is to obtain highly pure cell wall fractions. Contrarily to plant tissues which are complex and often resistant to breakage due to the presence of lignified cell walls, *Arabidopsis thaliana* liquid cultured cells offer a friable population of large cells, from which clean wall fractions can be obtained [3, 4, 16]. The PD population within division walls is relatively homogenous and resemble to PD found in intact tissues. Cultured cells also represent a quick and easy way to generate a large amount of biological material from which large quantities of PD-derived membranes, suitable for biochemical analysis, can be produce.

Here, we will outline a detailed protocol for the isolation of PD from *A. thaliana* liquid cultured cells. Wall fragments containing intact PD are isolated by cell disruption and organelle release by N_2 decompression coupled to grinding in liquid nitrogen. The recovered wall fragments are then digested by cell wall-degrading enzymes enabling the release of PD membranes. PD are

finally recovered by high-speed centrifugation. In this protocol we also detail a procedure to identify the total fatty acid, sterol and phospholipid content of isolated PD and a protocol for label free comparative proteomic analysis of PD fractions.

2. Materials

Prepare all solutions with ultrapure water (sensitivity 18MΩ at 25˚ C) and analytical grade reagents. All buffers and reagents should be kept at 4˚ C. For longer preservation, buffers can be sterilized at 110˚ C for 30 minutes before storage at 4˚ C.

2.1 Plant Material

A. thaliana liquid cultured cells (ecotype *Landsberg erecta*) are grown in Murashige and Skoog medium containing vitamins and supplemented with 2.5 µM α-naphthalen acetic acid (NAA), 0.25 µM kinetin, 87 mM sucrose, and 2.5 mM 2(N-Morpholino)-ethane sulfonic acid (MES). Adjust the pH to 5.8 with 1 M KOH solution and sterilize at 110˚ C for 30 minutes. Subculture the cells once a week (transfer 20 mL of the cultured cells into 200 mL fresh medium) under a laminar flow hood and keep on a shaker set to 220 rpm, at 24˚ C, with constant light.

2.2 Purification of Cell Wall Fragments from Arabidopsis thaliana Suspension Cells

1. Cell wall preparation (CWP) buffer*:* 100 mM Tris-HCl, 100 mM KCl, 10 mM EDTA, 0.45 M D-Mannitol and 10% (v/v) glycerol, pH 8 with KOH 1M. Keep the solution at 4˚ C. Before use, add one tablet of an EDTA-free protease inhibitor cocktail per 50 mL of buffer.

2. Cell wall washing (CWW) buffer: 10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA and 10% (v/v) glycerol, pH 8 with KOH 1M. Keep the solution at 4˚ C.

3. N_2 decompression-based cell disruption: Cell Disruption Device such as, Parr Cell Disruption Device. The total volume of the vessel should be about 900 mL; however, samples to be processed are adjusted to a volume of 50 mL. To reduce the volume capacity of the cell disruption vessel, a zinc cylinder with a hole that can contain a 50 mL falcon tube has to be engineered by a local workshop facility (Figure 1A).

4. Refrigerated centrifuge allowing centrifugation of 50 mL tubes at low speed in a swinging-bucket rotor.

5. 50 mL screw cap tubes.

6. Sterile 25mL pipettes and an electric pipette controller

7. Ice and ice bucket.

8. Rough mortar (diameter 20 cm) and pestle.

9. N_2 liquid and N_2 liquid container.

2.3 Cell wall digestion with cellulase

1. Digestion buffer: 10 mM MES-KOH, 240 mM D-Mannitol, pH 5,5. Immediately before use add 1 mM Phenylmethylsulfonyl fluoride (PMSF) and 1 tablet of complete protease inhibitor cocktail.

2. Cellulase R-10 (Karlan) 1.4% in Digestion buffer (w/v). The solution should be activated 5 minutes at 55°C (*see* **Note 8**) and then filtered using a 0.2 µm filter (*see* **Note 9**). Immediately before use add 1 tablet per 25mL of protease inhibitor cocktail (*see* **Note 10**).

3. Tris-buffered saline buffer (TBS 1X): 20 mM Tris-HCl, 0.14 M NaCl and 2.5 mM KCl, pH 7.4, containing 1 tablet per 25 mL protease inhibitor cocktail before use.

4. 50 mL screw top tubes.

5. 20 mL syringe without needle.

6. Syringe filter (0.2 µm) with support membrane.

7. Waterbath.

8. Orbital shaking incubator (37˚ C).

9. Refrigerated centrifuge for rotation of 30 mL glass centrifuge tubes at 6000 x g.

10. 30 mL glass centrifuge tubes, such as Corex tubes.

11. Ultraspeed refrigerated centrifuge for bucket rotation of 30 mL centrifuge tubes at 100 000 g with fixe angle rotor.

12. 30 mL ultracentrifugation tubes

13. Ultraspeed refrigerated centrifuge for bucket rotation of 4mL centrifuge tubes at 100 000 g with a fixe angle rotor.

14. 4 mL microfuge tubes.

2.4 Purity assessment of the PD fraction

2.4.1 Antibodies

Antibodies against PD proteins PDLP1 [9] and PDCB1 [15] and against potential contaminants, such as BiP (ER marker) [17], Membrin 11 (Golgi marker; we used an antibody provided by A.Hocquellet, L. Maneta-Peyret & P. Moreau, Bordeaux, France), P16 (thylakoid marker) [18], PMA2 (PM marker) [19], Aquaporin PIP2;2 (PM marker) [20], the cellulose synthase subunits CesA3 and CesA6 (PM marker) [21], V-ATPase; e-subunit of tonoplast H+-ATPase (Agrisera) and ECHIDNA (*trans*-Golgi network marker) [22].

2.4.2 Protein Quantification

1. Bicinchoninic acid (BCA) protein assay kit.
2. 1.5 mL microfuge tubes.
3. Spectrophotometer capable of 562 nm.

2.4.3 SDS PAGE Protein Electrophoresis

1. Laemmli buffer 2X: 4% (w/v) sodium dodecyl sulfate (SDS), 16% (w/w) glycerol, 20 mM Tris-HCl, pH 6.8 and 0.02% (w/w) bromophenol blue.
2. Protein electrophoresis device.
3. Power supply capable of an output of 250V, 3.0A and 300 W.
4. Fast cast gel kit, Acrylamide kit 7.5% or 12%.
5. Running buffer, Tris-Glycine-SDS buffer pH 8.5.
6. SDS-PAGE molecular weight standards.

2.4.4 Western Blot Analysis

1. Trans-Blot cell system.
2. Biorad Trans Blot Turbo RTA transfer kit, Nitrocellulose.
3. Washing buffer: 1X TBS 0.1% (v/v) Tween 20.
4. Blocking buffer: 5% (w/v) nonfat dry milk powder in 1X TBS.

5. Secondary antibodies, such as horseradish peroxidase-coupled goat anti rabbit.
6. Enhanced chemiluminescence (ECL) system for the visualization of immunoreactive proteins.
7. Imaging system.

2.5 Lipidomic analysis

2.5.1 Fatty acids profiling by GC-MS

1. Hydrolysis solution: 5% H2SO4 in methanol.
2. Hexane 99%
3. 2.5% NaCl in H2O.
4. 100mM Tris-HCl, 0.09% NaCl, pH 8.
5. BSTFA-TMCS: N,O-Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane.
6. Heptadecanoic acid (C17:0).
7. 2-hydroxytetradecanoic acid (h14:0).

2.5.2 Sterol profiling by GC-MS

1. Chloroform/methanol 2/1 (v/v).
2. 0.09% NaCl solution.
3. Ethanol.
4. 11N KOH.
5. Hexane 99%.
6. BSTFA-TMCS: N,O-Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane.
7. Cholestan-2-ol (also known as α-cholestanol).

2.5.3 Phospholipids relative quantification by LC-MS

1. Internal standard PC 17:0/14:1, PI 17:0/14:1, PS 17:0/17:0, PE 17:0/17:0, PG 17:0/17:0.
2. Eluent A: isopropanol/CH_3OH/H_2O (5/1/4 v/v/v) + 0.2% formic acid + 0.028%

NH_3.

3. Eluent B: isopropanol + 0.2% formic acid + 0.028% NH_3.

2.5.4 Instruments and software for lipidomic analyses

1. Evaporator under N2 flow.
2. GC-MS (Agilent 7890A and MSD 5975 Agilent EI). An HP-5MS capillary column (5% phenyl-methyl-siloxane, 30 m, 250 mm, and 0.25 mm film thickness; Agilent) is used.
3. MassHunter, version B.07 (Agilent).
4. Ultra-high-performance LC 1290 Infinity II LC System (Agilent) with a Luna C8 150×1 mm column, with 100 Å pore size, 5 µm particles (Phenomenex).
5. Qtrap 6500 Mass spectrometer (Sciex).
6. Analyst software, version 1.7.1 (Sciex) and Analyst Device Driver for Agilent 1.3 (Sciex).
7. Multiquant software version 3.0.3 (Sciex).

2.6 Label free proteomic analysis

2.6.1 Gel electrophoresis and staining

1. Laemmli buffer 4X.
2. Coomassie Brilliant Blue G-250.
3. Destaining solution NH_4HCO_3 25 mM / ACN 50%.
4. Proteolysis buffer NH_4HCO_3 50 mM.

2.6.2 In-Gel Trypsin Digestion

1. Dithiothreitol 10 mM in NH_4HCO_3 50 mM.
2. Iodoacetamide 100 mM in NH_4HCO_3 50 mM.

2.6.3 Peptide Extraction

1. Extraction solution Water/ACN/ formic acid 48:48:4 (v:v:v).
2. 100% ACN.
3. 0.1% trifluoroacetic acid (in MS-quality water).

2.6.4 NanoHPLC

1. 0.05% trifluoroacetic acid (in MS-quality water).

2. Solvent A: 0.1% formic acid (in MS-quality water).
3. Solvent B: 80% ACN, 0.1% formic acid (in MS-quality water).

2.6.5 Instruments and softwares for proteomic analysis

1. GS-900 Densitometer (Bio-Rad Laboratories, Inc.).
2. Dionex Ultimate 3000 nanoHPLC system (Thermo Fisher Scientific, San Jose, CA)
3. Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an EasySpray ion Source.
4. Image Lab™.
5. Proteome Discoverer.

3 Methods

PD purification takes place in two main steps (Figure 2): (1) The purification of cell wall fragments containing PD followed by (2) the enzymatic digestion of the cell wall fraction allowing the release of "free" PD. Each step (about 4-5 hours of work for each) can be performed independently during different days as during the first step the pelleted cell wall fraction can be stored at -20°C after section 3.1.1 step 9 or step 6. All steps should be carried out at 4° C unless otherwise specified.

3.1 PD Purification

The procedure starts with the disruption of *A. thaliana* suspension cells using a nitrogen decompression-based cell disruption device and recovery of the cell walls by low speed centrifugation. In order to remove any trapped subcellular debris and organelles, the sample is then ground in liquid nitrogen using a rough mortar and pestle.

3.1.1 Cell Disruption Using a N_2 *Decompression-based Device*

1. Divide 200 mL of 5 to 6 days old cultured cells (*see* **Notes 1 & 2**) into 4 x 50 mL screwtop tubes and centrifuge the suspension cells at 400 x g for 5 minutes, at RT.
2. Meanwhile, place, the cell disruption device (pre-cooled at 4° C) on a magnetic stirrer and open the valve connecting the disruption vessel and the gas nitrogen cylinder so that the device is ready to use (Figure 1B).
3. Discard the supernatant by inverting the tubes and resuspend the cell pellet (about 20 mL) with an equal volume of ice-cold CWP buffer containing protease inhibitors (total volume should be around 50 mL). Gently place a magnetic stirring bar into the screwtop tube.
4. Position the sample inside the cell disruption device; set the head on the vessel with the dip tube extending into the sample and attach the split ring cover clamp (Figure 1A).

Make sure the magnetic bar is stirring the sample before closing (*see* **Note 3**). Close the discharge valve (Figure 1B) and gradually pressurize the vessel to 120 bar (*see* **Note 4**) by opening the charging valve (Figure 1B). When the appropriate pressure is reached, close the charging valve.

5. To disrupt the cells, slowly open the releasing valve (Figure 1B) to obtain a drip (*see* **Note 5**) and collect the disrupted cells in a 50 mL screwtop tube. Proceed until only gas exiting the cell disruption device (*see* **Note 6**). Leave the discharging valve opened until the pressure in the vessel is zero.
6. Collect an aliquot (total cell extract) for western blot and proteomic/lipidomic analysis (*see* **section 3.3**). Store at -20˚ C.
7. Divide the solution containing disrupted cells (about 40-50 mL) into two new 50 mL screwtop tubes and adjust the volume to 40 mL with ice-cold CWW buffer. Centrifuge the solution at 400 x g for 5 minutes at 4˚ C. Cell walls will sediment while the cytosolic content stays in the supernatant.
8. Carefully remove the supernatant using 25 mL pipettes and resuspend the sedimented walls in ice-cold CWW buffer. Adjust the volume to 50 mL.
9. Repeat step 4 to 8 two more times (*omit* **step 6**).
10. The cell wall sediment can be stored at -20˚ C.

3.1.2 *Cell Wall Grinding in Liquid Nitrogen*

1. Harvest the frozen pellet of cell walls from section 3.1.1 using a spatula. Place it in a mortar and grind the sample with a pestle in liquid N_2 to a fine powder.
2. Use the spatula to load the frozen powder into a 50 ml screwtop tube and fill with ice-cold CWW buffer. Mix by vortexing.
3. Spin the suspension at 400 x g for 5 minutes at 4˚ C.
4. Carefully remove the supernatant using a 25 mL pipette. Freeze the wall pellet by immersing the 50 mL screwtop tube into liquid N_2.
5. Repeat the grinding steps 1 to 4, two more times.
6. Wash the wall fragments three times with excess ice-cold CWW buffer by centrifugation at 400 x g, for 5 minutes at 4˚ C.
7. The final cell wall fraction can be stored at -20˚ C (*see* **Note 7**).

3.1.3 *Cell Wall Digestion Using Cellulase*

1. Thaw 10 mL of the cell wall fraction from section 3.1.2 and resuspend with excess volume (~ 40mL) of digestion buffer (without protease inhibitors). Centrifuge for 5 minutes at 400 x g at 4˚ C in 50 mL screwtop tubes. Discard the supernatant and keep the 10 mL pellet on ice.

2. Mix one volume of the 1.4% (w/v) cellulase solution with 1 volume of the cell wall fraction from step 1 (10 mL/10 mL). The final concentration of cellulase should be 0.7% (w/v).
3. Incubate the mixture at 37˚ C for 1.5 hours with gentle shaking in a rotating incubator (50-100 rpm).
4. Remove undigested wall fragments by centrifugation at 5850 x g for 5 minutes at 4˚ C in 30 mL glass centrifuge tubes.
5. Collect the supernatant for ultracentrifugation at 110000 x g for 40 minutes at 4˚ C with a swing out rotor in 30 mL ultracentrifuge tubes.
6. Carefully discard the supernatant by inverting the tubes and resuspend the pellet, containing PD membranes, with an excess volume of cold 1X TBS.
7. Sediment the resuspended pellet again at 110000 x g for 40 minutes at 4˚ C.
8. Resuspend the final pellet (the PD fraction) with 100 µL of 1X TBS containing protease inhibitors.
9. Store the PD fraction at -20˚ C.

3.1.4 Quality Assessment of the PD Fraction

The purity level of the PD fraction should be assessed by western blot analysis and compared to total cell extract (Figure 2; *see* **Note 11**).

1. Measure the protein concentration of the final PD fraction using a BCA Assay Kit (*see* **Note 12**).
2. Resuspend 1 volume of PD and total cell extract (*see* **step 6 in section 3.1.1**) with 1 volume of 1X Laemmli buffer and heat the samples for 30 minutes at 50˚ C.
3. Load the same amount of proteins for each sample and separate proteins by 1D-SDS polyacrylamide gel electrophoresis. Subsequently, transfer to nitrocellulose membranes using standard protocols.
4. Perform immunoblotting using the appropriate antisera (*see* **Note 11**) and visualize specific binding using standard techniques.

3.2 Lipidomic analysis

3.2.1 Fatty acids profiling preparation

1. Transfer samples containing around 150 µg of protein equivalent in a screw glass tube, named tube A.
2. Add 1 mL of the hydrolysis solution containing 5 µg/mL of internal standard C17:0 and h14:0 (*see* **Note 13**).

3. Incubate overnight at 85°C in a dry bath, be sure that the contents of tubes are not evaporating by retightening the screw caps every 5-10 min during the first 30 min. This step aims at releasing the FAs of different lipid classes from their respective backbone.
4. Remove the tubes from the dry bath and let them cool down.
5. Add 1 mL of 2.5% NaCl and then 1 mL of hexane.
6. Mix vigorously.
7. Centrifuge 5 min at 800xg at room temperature to separate the phases.
8. Prepare new screw glass tubes containing 1 mL of 100 mM Tris-HCl, 0.09% NaCl, pH 8, named tube B.
9. Collect the hexane upper phase and transfer it to the tube B prepared in previous step.
10. Add 1 mL of hexane to the initial tube A.
11. Mix vigorously.
12. Centrifuge 5 min at 800 x g at room temperature to separate the phases.
13. Collect the hexane upper phase and add it to tube B.
14. Mix vigorously the content of tube B.
15. Centrifuge 5 min at 800 x g at room temperature to separate the phases.
16. Recover gently the hexane upper phase taking care not to contaminate the sample with the lower aqueous phase and transfer to a new glass tube.
17. Evaporate the hexane through air flow evaporator.
18. Add 200 mL of BSTFA-TMCS.
19. Incubate 15 min at 110 °C in a dry bath.
20. Evaporate the BSTFA-TMCS. through air flux evaporator.
21. Once BSTFA-TMCS. is completely evaporated add 100 µL of hexane and close the lid immediately to avoid hexane evaporation.
22. Mix by vortexing.
23. Transfer into GC vials containing a conical insert.
24. Run the sample in a GC-MS instrument using the FAMES methods.

3.2.2 Sterol profiling preparation

1. Transfer samples containing around 150 µg of protein equivalent in centrifuge glass tube with 1 mL of water.
2. Add 2 mL of chloroform/methanol 2/1 v/v.
3. Sonicate for 30 s at room temperature and mix vigorously.
4. Centrifuge 5 min at 800 x g at room temperature to separate organic and aqueous phases.
5. Collect organic lower phases and transfer to clean glass tubes.
6. Add 2 mL chloroform in the aqueous upper phase.
7. Sonicate for 30 s and mix vigorously.
8. Centrifuge 5 min at 800 x g at room temperature to separate organic and aqueous phases.
9. Collect the organic lower phase and combine with organic phase collected in step 5.
10. Add 2 ml of 0.09% NaCl solution in organic phases.
11. Sonicate for 30 s and mix vigorously.
12. Centrifuge 5 min at 800 x g at room temperature to separate organic and aqueous phases.
13. Collect the organic lower phase.
14. Use the evaporator to dry the combined organic phases.
15. Add 1 mL of ethanol containing 5 µg/mL of cholestan-2-ol and then 100 µL of 11N KOH (*see* Note 14).
16. Incubate 1 h at 80 °C in a dry bath.
17. Let the samples cool.
18. Add 1 mL of hexane.
19. Mix using vortex.
20. Add 2 mL of distillated H_2O.

21. Mix vigorously.
22. Centrifuge 5 min at 800xg at room temperature to separate the phases.
23. Recover gently the hexane upper phase taking care not to contaminate the sample with the lower aqueous phase (*see* **Note 15**).
24. Evaporate the hexane through air flow evaporator.
25. Prepare the sample as describe in steps 18-23 in section 3.2.1
26. Run the sample in GC-MS instrument using the sterol methods.

3.2.3 GC-MS methods for FAMES and Sterol profiling

1. Set the helium carrier gas at 2 mL/min.
2. Use the splitless mode for injection.
3. Set the temperatures of injector to 250 °C and the auxiliary detector to 325°C, the temperature of MS Source is set to 230°C and the MS Quad to 150°C.
4. MS Analyzer is set in scan only with mass range 40-700m/z in positive mode with an electron emission set to 70 eV.
5. For Fames profiling, program the temperature program starts at 50°C for 1 min, then 25°C/min ramp from 50°C to 150°C (2-min hold) and a 10°C/min ramp to 320°C (6-min hold).
6. For Sterol profiling, program the temperature program starts at 200°C for 1 min, then 10°C/min ramp from 200°C to 305°C (2.5-min hold) and a 15°C/min ramp to 320°C.

3.2.4 Mass spectrometry analysis dedicated to phospholipids

1. Phospholipids are extracted as describe in steps 1-14 in section 3.2.2.
2. Resuspend samples in 50 µl of eluent A with 40µg of each internal standard.
3. Transfer samples into glass vial suitable for Injection.
4. Put the vials in the autosampler at 4 °C.
5. Separate phospholipid molecules species on an LC 1200 Infinity II system equipped with a Luna C8 150× 1 mm column with the following gradient:

a. 0 min start at 70% eluent A, 30% eluent B.

b. 0 to 5 min ramp at 50% eluent A, 50% eluent B.

c. 5 to 30 min ramp to 20% eluent A, 80% eluent B.

d. 31 to 41 min hold at 5% eluent A, 95% eluent B.

e. 42 to 52 min hold to 70% eluent A, 30% eluent B.

6. Subject HPLC elutes to coupled electrospray ionization in the negative (PE, PS, PI, and PG) and positive (PC) modes with fast polarity switching (50 ms); use nitrogen for the curtain gas (set to 15), gas 1 (set to 20), and gas 2 (set to 10). Set the needle voltage at -4500 or +5500 V without needle heating; use nitrogen as collision gas. Adjust the declustering potential at -50 V for PE, -35 V for PG, -50 V for PS, -70 V for PI, and +35 V for PC; set collision energy to -43 eV for phosphatidyl-ethanolamine (PE), -50eV for phosphatidyl-glycerol (PG), -55eV for phosphatidyl-serine (PS), -60 eV for phosphatidyl-inositol (PI), and +37 eV for PC. (*see* **Note 16**).
7. Define in Multiquant the integration parameters: Integration Algorithm MQ4; Gaussian Smooth Width 1.0 points and Noise percentage: 40.0%.
8. Integrate all the sample using the Multiquant method and report the area under the peaks for each multiple reaction monitoring (MRM).
9. Sum the transitions corresponding to the same species as for example for PE: PE 32:0 with PE32:0_14:0, PE32:0_16:0 and PE32:0_18:0.
10. Presents the results as a ratio corresponding to the area of the phospholipid species and use the corresponding internal standard for each phospholipids class as reference.

3.3 Label free proteomic analysis

3.3.1 Gel electrophoresis and staining

Samples are solubilized in Laemmli buffer and deposited onto a 10% acrylamide SDS–PAGE gel for concentration and cleaning purposes.

1. Depending of sample concentrations, take the volume V corresponding to 10 µg of proteins.
2. Add V/3 µL of Laemmli buffer 4X to the samples.
3. Heat samples at 99°C for 2 min.

4. Load samples onto a 10% acrylamide SDS-PAGE.
5. Migrate the gel (150 V. max, 20 mA) until the migration front progress by 5 mm.
6. Stop the migration, unmold the gel from the plate and rinse it twice in ultrapure water for 3 min.
7. Incubate the gel overnight in 100 ml of Coomassie Brilliant Blue G-250.
8. Wash the gel in water until complete destaining of the background.

3.3.2 In-Gel Trypsin Digestion

1. Scan the gel on a densitometer and estimate protein quantities.
2. Cut each lane with a scalpel. Subsequently cut the gel pieces in 1 x 1mm smaller cubes and transfer into a tube.
3. Wash with 300 µL ultrapure water for 15 min.
4. Wash with 300 µL (v:v) 25mM NH_4HCO_3: 100% ACN for 4 h or until complete destaining.
5. Dehydrate with 300 µL 100% ACN for 10 min. and remove the solution.
6. Dry the gel pieces at room temperature for 15 min.
7. Add 300 µL Dithiothreitol 10M and incubate for 30 min. at 56°C.
8. Remove the solution and add Iodoacetamide 100 mM for 30 min at room temperature and in total darkness.
9. Remove solution and dehydrate with 300 µL 100% ACN for 10 min. Remove the solution.
10. Dry the gel pieces at room temperature for 15 min.
11. Rehydrate with 50 µL of 10 ng/µL trypsin in 50 mM NH_4HCO_3 for 10 min. at 4°C.
12. Add sufficient 50 mM NH_4HCO_3 to cover gel pieces.
13. Incubate overnight at 37 °C.

3.3.3 Peptide Extraction

1. Transfer supernatant to a new sample tube and add 150 µL of 50 mM NH_4HCO_3 and mix 15 min.
2. Transfer supernatant to sample tube and add 150 µL extraction buffer water:ACN:formic acid 48:48:4 (v:v:v) and mix 15 min.
3. Repeat step 2.
4. Lyophilize pooled sample in a vacuum to complete dryness.
5. Suspend the pellet in 100 µL 0.1% trifluoroacetic acid (*see* **Note 17**).

3.3.4 High Performance Liquid Chromatography (HPLC) in line with mass Spectrometer

1. Trypsin-digested peptides are separated using a Dionex Ultimate 3000 nanoHPLC system.
2. Load 1 µg of the sample in 1% formic acid (v:v) on a 300-µm-inner diameter × 5-mm C18 PepMap™ trap column (LC Packings) for 3 min at a constant flow rate of 10 µL/min. (*see* **Note 17**).
3. After trapping and desalting on the C18 cartridge, peptides are eluted on an analytical PepMap C18 nanocolumn (75 µm x 50 cm) with a linear gradient of 4% to 40% solvent B over 108 min. with a flow rate of 200 nL/min.

3.3.5 Mass Spectrometry Analysis

1. The mass spectrometer operates in positive ion mode at a 2-kV needle voltage.
2. Acquire data using Xcalibur software in a data-dependent mode. Record MS scans (at 375 to 1,500 m/z) at a resolution of R = 120,000 (at 200 m/z) and collect AGC target of 4.10^5 within 50 msec. Set the dynamic exclusion to 60 sec and perform top speed fragmentation in HCD mode over a 3 sec cycle. Collect the MS/MS scans with a target value of 2.10^3 ions in the ion trap with a maximum fill time of 35 msec. Additionally select only +2 to +7 charged ions for fragmentation. Additional settings include no sheath nor auxiliary gas flow, a 275°C heated capillary temperature, a 35% normalized HCD collision energy and a 1.6 m/z isolation width. Monoisotopic precursor selection (MIPS) is set to Peptide and an intensity threshold is set to 5.10^3 to trigger MS/MS.

3.3.6 Data Processing for Protein Identification and Quantitation

1. Search the data using SEQUEST HT through Proteome Discoverer interface against *Arabidopsis thaliana* protein database (https://www.arabidopsis.org/index.jsp).
2. Set parameters as follows:
    a. Reject spectra from peptides higher than 5,000 Da or lower than 350 Da.
    b. Set up the mass accuracy of the monoisotopic peptide precursor and peptide fragments to 10 ppm and 0.6 Da, respectively.
    c. Consider only b- and y-ions for mass calculation.
    d. Consider Oxidation of methionine (+16 Da) and protein N-terminal modifications (Acetylation +42 Da, Met-loss -131 Da, Met-loss+Acetyl -89 Da) as variable modifications and carbamidomethylation of cysteines (+57 Da) as fixed modifications.

e. Allow two missed trypsin cleavages.

f. Perform peptide validation using Percolator algorithm [23] and consider only "high confidence" peptides corresponding to a 1% False Positive Rate at peptide level.

g. Use the Minora algorithm embedded in Proteome Discoverer to detect and integrate the peaks.

h. Quantify the proteins on unique peptides intensities. Normalize on total protein amount and calculate protein ratio as the median of all possible pairwise peptide ratios. Calculate a t-test based on background population of peptides or proteins. Then consider as quantitative data the proteins quantified by a minimum of two unique peptides and an adjusted p-value lower than 0.05.

3. Using a reference list of previously identified PD proteins determine cut-off enrichment ratios. Select different cut-off scores for PD/PM, PD/microsome, 30 PD/TP and PDCW to filter out the false positives.

4 Notes

1. *Arabidopsis* cells grow in clumps of about 30-40 cells. Optimal culture condition with the appropriate hormonal balance is important for cell wall purification. In the case of hormonal overdose cells often divide faster, forming large clumps (liquid culture looks grainier). These clumps are more resilient to disruption with the cell disruption device and could result in a wall fraction of insufficient quality.
2. In *Arabidopsis thaliana* suspension cells, PD are mostly present on the division walls. Cell walls are prepared from cultured cells in the middle of the linear growth phase (about 5 days after sub culturing) in order to maximize the proportion of dividing walls containing PD.
3. Stirring will help to maintain cells in a uniform suspension in the N_2 cell disruption vessel. This will guarantee maximum cell disruption and accelerate the equilibrium step.
4. A pressure lower than 10000 KPa inside the vessel will reduce the efficiency of the disruption process and may lead to a contaminated cell wall fraction.
5. The actual disruption process does not take place while the cells are pressurized within the vessel. Cell disruption occurs at the instant of decompression as the sample goes from high-pressure environment to atmospheric pressure. Shearing of the cells as they pass through the discharging valve also participates in cell disruption. The valve is

therefore opened gradually to leave as little space as possible for the liquid suspension cell to pass through.

6. Beware that the last 5 mL of solution may come at once and produce a splash.
7. The purity of the final cell wall fraction can be visually estimated, by looking at the color of the pelleted walls. The final pellet should be bright white and not yellowish. If it is not the case, additional grinding and washing steps may be necessary.
8. Do not exceed 5 minutes or the enzyme activity will diminish.
9. This step helps to remove any residual debris contained in the cellulase solution. It is recommended for electron microscopy studies.
10. It is necessary to use both PMSF and a complete inhibitor cocktail to avoid protein degradation during the incubation of the wall fragment with cellulase. PMSF is rapidly degraded upon light and water exposure. The stock solution should therefore be kept in a tube opaque to light. PMSF is very toxic and should be handled with appropriate care.
11. As PD membranes are continuous with the ER and the PM, these two compartments are likely to be a source of contamination and should therefore be tested for (using for instance PMA2 and BiP antisera; *see* **section 2.4.1**). Contamination from any other subcellular compartments can also be investigated (for instance chloroplast and Golgi). Enrichment in PD membranes is monitored through the use of intrinsic PD protein markers (PDCB and PDLP; *see* **section 2.4.1**).
12. The PD purification yield (assessed by protein quantification) can be affected by a decrease of the cellulase activity. This effect has been observed with the use of "old" cellulase (older than a year).

13. The internal standard chosen for lipidomic classes are absent from plants membranes. The C17:0 standard, for GC analyse, is used to quantify the most common fatty acids (FAs) coming from the glycerolipid and glycerophospholipid pool. The h14:0 standard is used to quantify the α-hydroxylated (2-hydroxy)-FAs coming exclusively from the sphingolipid pool. For LC-MS analyses, all internal standard contains a fatty acid with an odd number of carbons, i.e. 17 carbons.
14. This method based on saponification will allow the quantification of the sterol backbone from sterols, sterylesters, sterylglycosides or acetylated sterylglycosides.
15. It is important to not collect any of the lower aqueous phase as this will results in misleading chromatogram results and may destroy the GC column. If some of the aqueous phase was accidentally collected, a white residue coming from the hydrolysis of trimethylsilyl (TMS) molecules that release silica atoms

16. The MRM mode on the Qtrap 6500 is based on the loss of the fatty acid part for the negative analysis (PE, PS, PI, and PG), and on the loss of the polar head in positive

mode for phosphatidyl-choline (PC) analysis. The PC class can be analyzed in negative ionization mode, but the intensity will be lower than in positive ionization mode.

17. Sample injection should be randomized in order to reduce experimental bias induced by instrument performance variation over the period of analysis.

Acknowledgements

This work was supported by the National Agency for Research (Grant ANR-18-CE13-0016 to E.M.B). Lipidomic analyses were performed on the Bordeaux Metabolome Facility supported by the grant MetaboHUB (ANR-11-INBS-0010).

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Figure Legends

Figure 1. Description of the N_2 decompression-based cell disruption device: A) The parts of the disassembled cell disruption device. B) Cell disruption device ready for use with loaded sample.Vessel (1), Zinc cylinder which goes inside the vessel (2), Split ring (3), Cover ring (4), Exit sample collection (5), Discharging valve (6), Charging valve (7), Valve connecting the cell disruption vessel with the gas cylinder (8).

Figure 2. Workflow of the PD purification protocol. “Store -20°C” indicates steps where the purification process can be interrupted.

Fig.1: Description of the N_2 decompression-based cell disruption device

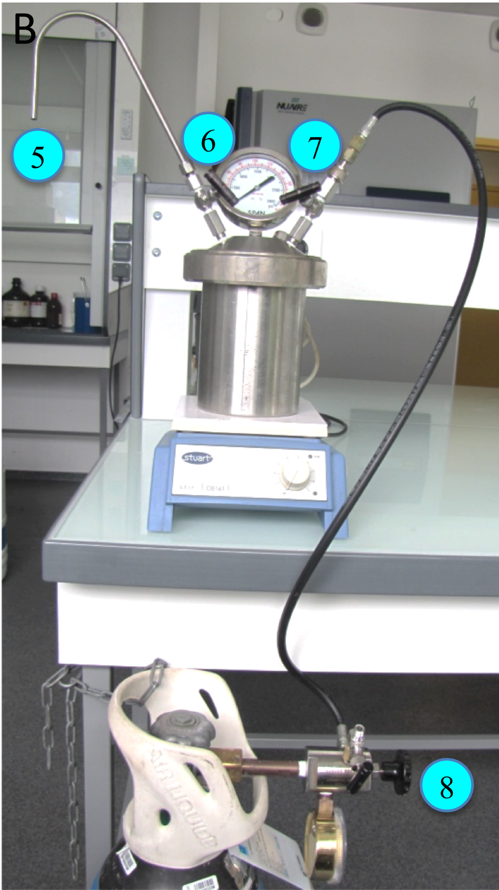
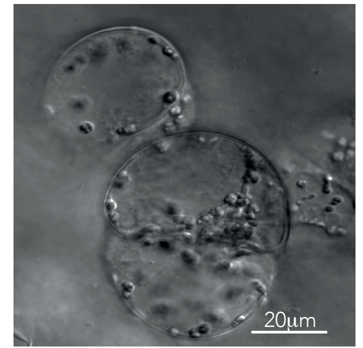


Fig.2: PD purification procedure

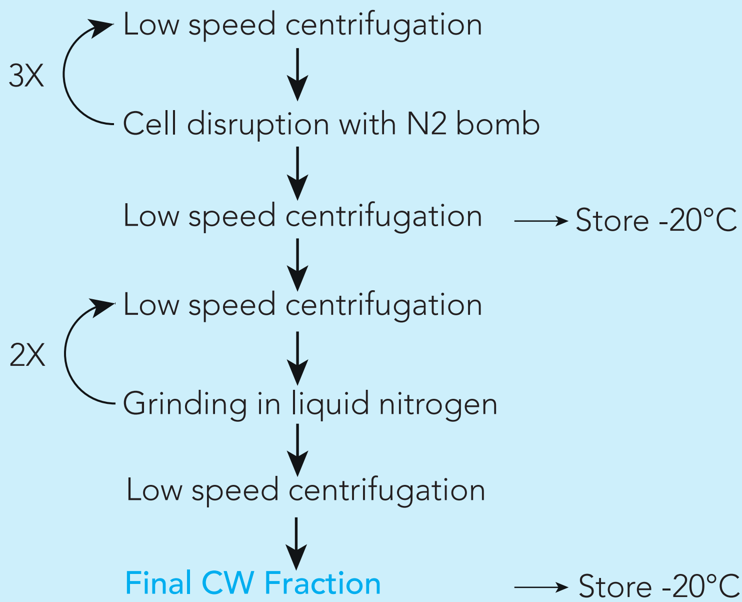
Starting material:
Arabidopsis thaliana cultured cells

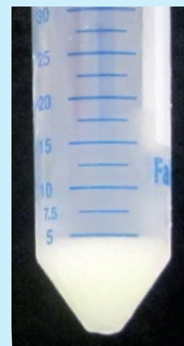
pelleted
intact cells

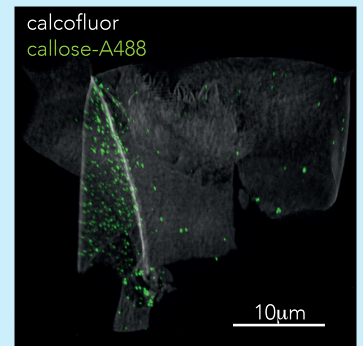


Arabidopsis supension cells
light microscopy

Fisrt Step: Cell Wall purification




CW Fraction



wall fragments
confocal microscopy

Second Step: PD membrane purification