Polyamine metabolism and activation of lipid signalling pathways in Arabidopsis thaliana

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Chapter 3

Analyzing plant signaling phospholipids through $^{32}P_i$-labeling and TLC

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ABSTRACT

Lipidomic analyses through LC-, GC-, and ESI-MS/MS can detect numerous lipid species based on head group and fatty acid compositions but usually miss the minor phospholipids involved in cell signalling because of their low chemical abundancy. Due to their high turnover, these signaling lipids are, however, readily picked up by labeling plant material with ^{32}P-orthophosphate and subsequent analysis of the lipid extracts by thin layer chromatography. Here, protocols are described for suspension-cultured tobacco BY-2 cells, young *Arabidopsis* seedlings, *Vicia faba* roots, and *Arabidopsis* leaf disks, which can easily be modified for other plant species and tissues.

1. INTRODUCTION

The concentration of signaling lipids, such as polyphosphoinositides (PPI), phosphatidic acid (PA), and diacylglycerolpyrophosphate (DGPP), is typically too low to be detected by modern, lipidomic-type analyses such as GC- and LC-MS (1-9). PA is sometimes detectable, as it is also the precursor of phospholipids and galactolipids (1, 2). In addition, it can be massively produced upon PLD hydrolysis of structural phospholipids as an artifact of preparation and, with biological relevance, in response to stresses, such
as wounding and freezing (10-12). The PA pool, however, that would be generated upon PPI hydrolysis by phospholipase C (PLC) and phosphorylation of the resulting diacylglycerol (DAG), is much smaller and easily overlooked (2, 13, 14).

A main difference between structural and signaling lipids is their rate of synthesis and breakdown (i.e., turnover), which in signaling lipids is much higher (6, 13, 15). The benefit of this is that these lipids are therefore faster labeled by inorganic, radioactive- labeled phosphate ($^{32}P_i$ or $^{33}P_i$). This type of label is easily taken up by various plant tissues and is rapidly incorporated into the ATP pools, which are required to phosphorylate the precursors of signaling lipids. Structural lipids also incorporate $^{32}P_i$ but this occurs through a relatively slower pathway involving their de novo biosynthesis (6, 13, 15). Here, a quick and easy protocol is described to analyze signaling lipids like PA, DGPP, and PPIs in different plant tissues, i.e., seedlings, leaves, roots, and suspension-cultured cells.

2. MATERIALS

2.1. Plant media

1. Medium for cell suspensions: For 1 L, 4.4 g Murashige–Skoog (MS) salts with vitamins, 30 g sucrose, 100 µL BAP (6-benzylaminopurine, 10 mM), 100 µl NAA (naphthaleneacetic acid, 54 mM in 70 % EtOH). Adjust pH to 5.8 with KOH. Autoclave 20 min at 120 °C.
2. Medium for *Arabidopsis* seedlings: 0.5x MS medium, supplemented 1 % (w/v) sucrose and 1 % (w/v) agar.
3. Medium for *Vicia* seedlings: 2.72 mM $CaCl_2$, 1.95 mM $MgSO_4$, 2.20 mM KH_2PO_4, 1.26 mM Na_2HPO_4, and 0.08 mM ferric citrate.
4. Phosphate-free labeling medium for *Vicia*: 2.72 mM $CaCl_2$, 1.95 mM $MgSO_4$, 0.08 mM ferric citrate, 10 mM HEPES, pH 6.5.
5. Fertilized pot soil to grow mature plants.

2.2. Plant material and cultivation

1. Suspension-cultured plant cells (tobacco BY-2; 4–5-days old (see Note 1)).

2. Rotary shaker, 125 rpm, 24 °C, in the dark.
3. Seedlings (*Arabidopsis thaliana*, Col-0); ~5-days old.
4. Seedlings *Vicia* (*Vicia sativa* spp. *nigra*); 2–3-days old.
5. Growth chamber, 21 °C, 16 h light, 8 h dark.
6. Mature *Arabidopsis* plants (~3 weeks) for leaf disks.
7. Leaf disk cutter: cork borer (0.5 cm diameter).

2.3. Labeling components

1. ^{32}P-inorganic phosphate ($^{32}P_i$; carrier-free, 10 μ Ci/ μL).
2. 2 mL "Safe-lock" Eppendorf polypropylene reaction tubes.
3. Three sterile 15 mL tubes.
4. 5 μm, 0.45 μm, and 0.22 μm filters for 10 mL syringes.
5. Labeling buffer for seedlings and leaf disks: 2.5 mM MES-KOH (pH 5.8), 1 mM KCl.
6. Labeling buffer for cell suspensions: CFM (cell-free medium, 0.22 μm filtered).
7. Fume hood.
8. Safety glasses.
9. Safety screen, 1 cm Perspex.
10. Perspex tube holders.
11. Gloves.

2.4. Lipid extraction components (see Note 2)

1. Perchloric acid (PCA): 50 % and 5 % (w/v) in H_2O.
2. CMH: chloroform/methanol/37 % HCl (50:100:1, by vol.).
3. Chloroform.
4. 0.9 % (w/v) NaCl.
5. TUP (Theoretical upper phase) (TUP): chloroform/methanol 1 M HCl (3:48:47, by vol.).
6. Reaction tube microcentrifuge, 13,000 xg.
7. Vortex shaker.
8. Glass Pasteur pipets.
9. *Iso*-propanol.
10. Vacuum centrifuge with cold-trap.
11. Nitrogen (gas).

2.5. Thin layer chromatograph

1. Thin layer chromatograph (TLC) plates: Merck silica 60 TLC plates, 20 x 20 x 0.2 cm.
2. Impregnation solution: 1 % (w/v) potassium oxalate, 2 mM EDTA in MeOH/H_2O (2:3, by vol.).
3. Oven at 115 °C.

4. Graduated cylinders (100 mL; 25 mL; 5 mL).
5. Alkaline TLC solvent: $CHCl_3$/MeOH/25 % NH_4OH/H_2O (90:70:4:16, by vol.).
6. Acidic TLC solvent: $CHCl_3$/CH_3COCH_3/MeOH/HAc/H_2O (40:15:14:13:7.5, by vol.).
7. Filter paper (21 x 21 cm).
8. Two glass rods (~1 cm thick, 2–3 cm long).
9. Clear plastic wrap.
10. TLC Tank.
11. Hair dryer.
12. Autoradiography film (Kodak, X-Omat S).
13. PhosphorImager and screen.
14. Light cassette.

3. METHODS

Described below are the procedures to label and identify signalling lipids in: (1) suspension-cultured plant cells, (2) seedlings, (3) roots, and (4) leaf disks. Once labeling is stopped through PCA, a similar lipid extraction procedure and TLC analysis is performed in all cases. As examples, we used tobacco BY-2 cells, young *Arabidopsis* seedlings, roots of *Vicia* and *Arabidopsis* leaf disks of mature *Arabidopsis* plants, but obviously, other species and materials can be analyzed in a similar fashion.

3.1. Lipid labeling and extraction of cell suspensions

Typically, tobacco BY-2 cells are used but we have also good experience with other cell suspensions, including tomato, potato, *Arabidopsis*, *Medicago*, coffee, or unicellular algae like *Chlamydomonas* (4, 16-19). The protocol also works for pollen tubes and microspores (20) (Parzer and Munnik, unpublished) and *Phytophthora* zoospores (21).

1. Cell suspensions are grown on a rotary shaker (125 rpm) at 24 °C in the dark and subcultured weekly.
2. Pour ~10 mL of 4–5-day-old cell suspensions into a sterile 15 mL test tube.
3. Let the big cell clumps sink for 30 s and pipet 1,200 µL of cell suspension (for 12 samples) from the top into a 2 mL reaction tube (see Note 3). Use residual cell suspension to prepare *Cell-Free Medium (CFM) if required.
4. Preparation of CFM: Let the cells sink to the bottom of the tube. Pipet off the liquid and filter through 5 µm, 0.45 µm and 0.22 µm syringe filters, respectively. This CFM is used for cell treatments as 1:1 dilutions.
5. Add 60 µCi [^{32}P]PO_4^{3-} (~5 µCi/sample) in the fume hood of an isotope lab.
6. Aliquot 12 samples of 80 µL into 2 mL “Safe Lock” reaction tubes in a Perspex rack using large orifice tips (see Note 4). Label for ~3 h; close tubes to prevent water loss

and keep the rack in the fume hood to reduce your exposure to radiation.
7. Apply stresses and controls by adding 1:1 volumes (i.e., 80 µL) in CFM* at t = 0 with 15 s intervals (see Note 5); Incubate at RT.
8. Stop reactions by adding 20 µL (=1/10 vol.) of 50 % PCA (5 % final concentration) with 15 s intervals; vortex immediately for 10 s.
9. Shake for 5 min.
10. Spin 10 s (see Note 6).
11. Add 750 µL of CMH [$CHCl_3$/MeOH/HCl (50:100:1)] (see Note 7).
12. Mix for 10 s.
13. Spin 10 s (see Note 6).
14. Add 750 µL of $CHCl_3$ and then 200 µL 0.9 % NaCl.
15. Mix for 10 s; Spin 1 min.
16. Remove the aqueous upper phase (free label) as much as possible with a Pasteur pipet and transfer the organic lower phase to a new 2 mL Safe-lock reaction tube containing 750 µL TUP (see Note 8).
17. Mix for 10 s individually or for 5 min on a shaker; Spin 1 min.
18. Discard the upper phase (see Note 9).
19. Add 20 µ L of iso-propanol (see Note 10).
20. Dry the lipid extract by vacuum centrifugation (45 min, heating at 50 °C).
21. Dissolve into 50 µL $CHCl_3$ and store under N_2 at -20 °C or use immediately for TLC analysis.

3.2. Lipid labeling and extraction of *Arabidopsis* seedlings

1. Grow seedlings on vertical 0.5x MS plates (1 % agar), supplemented with 1 % sucrose, for 5–6 days in a climate room with 16 h light/8 h dark period at 21 °C.
2. Transfer (see Note 11) 3 seedlings/tube in 190 µL labelling buffer (2.5 mM MES-KOH (pH 5.8), 1 mM KCl). Use 2 mL “Safe Lock” reaction tubes (see Note 4). Alternatively, 48-well microtiter plates can be used.
3. Add 10 µL [^{32}P]PO_4^{3-} containing 5–10 µCi per sample (see Note 12).
4. Incubate with radiolabel over night. Leave the lights of the fume hood on to enable photosynthesis and close the tubes to prevent water loss (O_2 will be produced!).
5. Next day, treatments can be applied. If agonists are to be added, e.g., salt stress, then add them in rel. large volumes (e.g., 1:1, i.e., 200 µL) so that one does not have to mix rigorously. For controls, use buffer alone. Start and stop with 15 s intervals.
6. Stop reactions by adding 1/10 vol. of 50 % perchloric acid (PCA) (~5 % final) with 15 s intervals. We use a repeater pipet for this.
7. Shake samples for 5–10 min (green tissue turns brownish) on a Vortex shaker.
8. Spin 10 s (see Note 6).
9. Open tubes and remove all liquid around the seedlings with a Pasteur pipet.
10. Add 400 µL of ‘CMH’ [$CHCl_3$/MeOH/HCl (50:100:1)].
11. Shake for 5 min until leaf material is not colored anymore and the extracts are

yellowish.
12. Spin 10 s (see Note 6).
13. Add 400 µL of $CHCl_3$, followed by 200 µL of 0.9 % NaCl.
14. Mix for 10 s/tube or shake for 5 min on a Vortex shaker.
15. Spin 2 min (see Note 13).
16. Remove the upper phase (aqueous, free label) as much as possible and transfer the lower phase with a Pasteur pipet to a clean tube containing 400 µL 'TUP' (theoretical upper phase; $CHCl_3$/MeOH/1 M HCl, 3:48:47 v/v). Use the two-phases in the Pasteur pipet (see Note 8).
17. Mix for 10 s or shake for 5 min.
18. Spin 2 min.
19. Remove and discard the upper phase completely (see Note 9).
20. Add 20 µL of iso-propanol (see Note 10).
21. Dry the lipid extract by vacuum centrifugation (~45 min, heating 50 °C).
22. Dissolve into 100 µL $CHCl_3$ and store at −20 °C (under N_2 gas) or use immediately for TLC analysis (load 20 µL only!).

3.3. Lipid labeling and extraction of *Vicia* roots

1. Germinate *Vicia sativa* spp. *nigra* seeds and grow for 2-3 days in modified Fåhreus slides containing 2.72 mM $CaCl_2$, 1.95 mM $MgSO_4$, 2.20 mM KH_2PO_4, 1.26 mM Na_2HPO_4, and 0.08 mM ferric citrate (15). Each slide contains 1 mL of medium and five seedlings. Grow at 22 °C in a light/dark regime (16 h/8 h) with an average photon flux of 42 µE/m^2s.
2. Before labeling, wash seedling roots twice in phosphate-free labeling medium.
3. Label seedlings in a 200 µL compartment of a Fåhreus slide, modified for $^{32}P_i$-labeling (15) (**Fig. 1**) using 160 µL of phosphate-free labeling medium (2.72 mM $CaCl_2$, 1.95 mM $MgSO_4$, 0.08 mM ferric citrate, 10 mM HEPES, pH 6.5), containing 10 µCi carrier-free $^{32}P_i$.
4. Treat roots by adding 5–40 µL of agonist in labeling buffer.
5. Stop labeling and treatment by transferring each seedling to 1 mL of 5 % PCA.
6. Cut off the root after 5–10 min and add to 400 µL CMH.
7. Proceed further as the *Arabidopsis* seedling labeling protocol, Subheading 3.2, step 11.

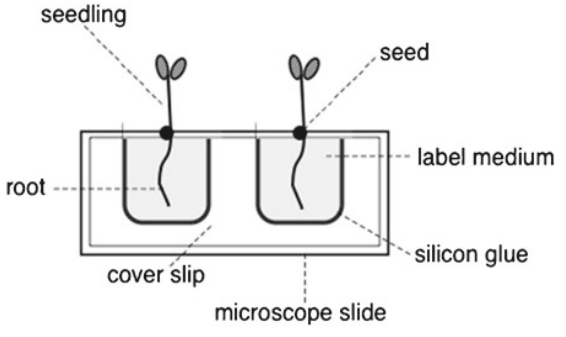

Figure 1. Schematic drawing of a Fåhreus slide modified for $^{32}P_i$-labeling of *V. sativa* seedlings. The slide contains two chambers that were created with silicon glue between a coverslip and a microscope slide. The silicon glue pastes the coverslip to the slide and serves as spacer as well. Adopted from (15).

3.4. Lipid labeling and extraction of *Arabidopsis* leaf disks

The protocol described below is for *Arabidopsis* leaves but can be used for any other plant species, e.g., *Craterostigma plantagineum* (22), sorghum (23) and rice (24), or tissues, e.g., flower petals of carnation or petunia (25, 26) .

1. Punch leaf disks (0.5 cm in diameter) from 2–3-weeks-old *Arabidopsis* plants.
2. Transfer disks to 2 mL Safe Lock reaction tubes containing 190 µL labeling buffer. Use 1 leaf disk per tube and use a yellow tip, with a drop of buffer, for the transfer and guidance into the tube. Make sure all leaf disk orientations are the same (e.g., right sight up).
3. Add 10 µL [^{32}P]PO_4^{3-} (5-10 µCi/sample), diluted in buffer to each sample.
4. Incubate with radiolabel over night. Leave the lights of the fume hood on to enable photosynthesis and close the tubes to prevent water loss (O_2 will be produced!).
5. Next day, treatments can be applied. Apply (e.g., salt stress) in rel. large volumes (1:1) so that one does not have to mix rigorously (e.g., 200 µL); pipet next to the disks in the liquid. For controls, use buffer alone. Start treatments with 15 s intervals.
6. Stop reactions by adding 1/10 vol. of 50 % perchloric acid (PCA ~5 % final) with 15 s intervals (see Note 14).
7. Shake samples for ~15 min (until the whole green tissue turns brownish).
8. Continue as in Subheading 3.2, step 8.

3.5. Thin layer chromatography

1. For good separation of the PPIs, K-oxalate-impregnated plates are required (see Note 15). Impregnate at least 2 days in advance by dipping the TLC plates for 10 s in 1 % (w/v) potassium oxalate, 2 mM EDTA in MeOH/H 2 O (2:3, by vol.).
2. Dry and store at 115 °C (see Note 16).
3. Before loading, mark the TLC plate with a soft pencil where the samples are to be

spotted: draw a line, 2 cm from the bottom. Mark the samples with a small dot, 0.5-1.5 cm apart. The solvent front tends to “smile” so distribute the sample equally and stay at least 2 cm away from the sides of the TLC plate (20 Å~ 20 cm).
4. Return the TLC plate in the oven to keep it heat-activated (see Note 16).
5. Prepare the TLC solvent. For alkaline TLC this is: 90 mL $CHCl_3$, 70 mL MeOH and 20 mL ammonia (25 %, w/v)/H_2O (4:16, by vol.). Mix well and keep 80 mL separate (to run the TLC).
6. Use the remaining solvent to “wash” the tank and to flush the filter paper (~21 x 21 cm) placed at the back of the tank that helps to keep the solvent atmosphere in the chamber saturated.
7. Flush three times and, after the last wash, immediately add two small glass rods that will keep the TLC plate above the solvent when pre-equilibrating. Close the tank quickly with the glass lid and put a weight on top as the solvent pressure my lift the lid and move it to leak. The tank is ready now and can be used three to four runs within a week.
8. Get your lipid samples out of the −20 °C and spin them for 2-4 min in a microcentrifuge to let the samples quickly adjust to room temperature (see Note 17).
9. Meanwhile, take the TLC plate from the oven and put it with the silica-side onto a sheet of plastic wrap, just above the 2 cm line marked with a pencil, and wrap it. The plastic protects the rest of the plate when loading.
10. Place the TLC plate horizontally on the table, behind a 1-cm thick Perspex screen. Put another plate (glass or perspex) on top, but keep the 2-cm line clear to load.
11. Place a hair dryer ~30 cm away from the TLC plate, so that a gentle stream of air is streaming over the plate. This speeds up the evaporation of the chloroform and reduces the size of the loading spots.
12. Place the ^{32}P-lipid samples in a Perspex block behind the TLC.
13. Use a reaction tube filled with chloroform to wash your pipet tip in between the samples.
14. Equilibrate the tip of 20 µL pipet with chloroform by pipetting it up-and down for a number of times (see Note 18).
15. Load the pipet tip with 20 µL lipid extract and spot gently onto the TLC without damaging the silica-coating (see Note 19).
16. Rinse the tip with chloroform to spot the next sample.
17. Repeat until all samples are loaded.
18. After the last sample, wait approx. 1 min to dry the last spot.
19. Remove the plastic wrap and place the TLC plate in the tank on the glass rods so that the plate rests above the alkaline solvent. Quickly replace the lid to limit the loss of solvent vapour.
20. Pre-equilibrate for 30 min.
21. Start the TLC by placing the plate into the solvent. Let the plate stand almost

vertically and quickly close the tank again to prevent the loss of solvent vapour.
22. Run the TLC for 1.5 h (see Note 20).
23. Take the plate out and let it dry in the fume hood for 1 h to eliminate solvent fumes which will affect the PhosphorImager screen or autoradiography film (see Note 21).
24. Wrap the plate in plastic wrap and expose a PhosphorImager screen for ~1 h.
25. For higher resolution images, expose the TLC plate to autoradiography film for ~1 h and over night for a short and long exposure, respectively.
26. A typical alkaline TLC pattern of salt- and heat-stressed *Arabidopsis* seedlings is shown in **Fig. 2**.

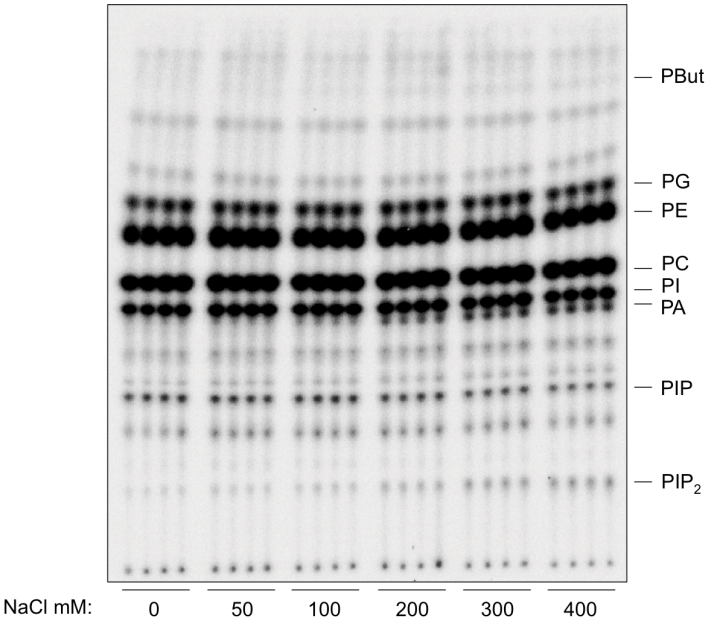

Figure 2. Alkaline-TLC profile of $^{32}P_i$-labeled *Arabidopsis* wild-type seedlings in response to 30 min of increasing concentrations of salt. Five-day-old seedlings of *A. thaliana* (Col-0) were prelabeled overnight with $^{32}P_i$ and then treated with NaCl (0, 50, 100, 200, 300 or 400 mM) for 30 min. Lipids were subsequently extracted, separated by Alkaline TLC and visualized by autoradiography. Treatments were performed in quadruplicates and in the presence of 0.5 % 1-butanol to visualize PLD activity, producing phosphatidylbutanol (PBut) (2, 4, 6, 14, 26, 27). Each sample contained three seedlings.

4. NOTES

1. The protocol works for any cell culture, including *Arabidopsis*, coffee, tomato and *Medicago*, green algae like *Chlamydomonas*, and pollen tubes and microspores (4, 16-20).

2. Use cold solvents. They pipet better (less gas expansion within the pipet) and decrease chemical breakdown.
3. As most cell suspensions tend to clump a bit, the use of large orifice tips is preferred. Alternatively, simply cut off the pipet tips to increase the opening. Note that the latter option is less accurate.
4. The use of Eppendorf Safe Lock reaction tubes is preferred as normal tubes may open due to the gas pressure of the solvent.
5. The CFM is a conditioned medium to which the cells are not, or hardly, responding. This is in huge contrast to using plane water or a buffer, which activates lipid signaling instantly, likely via osmotic stress.
6. A short spin in the microcentrifuge is sufficient to remove the radioactive liquid from the lid to prevent contamination when opening.
7. It is very important that the CMH completely dissolves the aqueous fraction. By using a 3.75 vol. ratio, this will always occur. The MeOH enables to mix the water and chloroform phases. By increasing either chloroform or water fraction, the solution will split into two phases; an aqueous upper phase and an organic lower phase. This effect is used later (Subheading 3.1, step 16) to separate the lipids from other cellular contents, such as proteins, sugars and DNA.
8. Use the two phases in the Pasteur pipet to separate the lipid fraction from the upper aqueous fraction and transfer it to a clean 2 mL Safe-lock Eppendorf tube, already containing 750 µL TUP.
9. It is very important to remove all of the aqueous-upper phase. Water will cause chemical hydrolysis of the lipid extract. First remove the upper phase as much as possible. Then aspire all extract and use the organic phase to “wash” the wall of the tube two to three times. Every time, aspire the complete extract again and let the phases separate (Note that it is important to keep enough “balloon air”). Use the two-phase separation in the Pasteur pipet to remove aqueous fraction. Due to a faster evaporation of the chloroform than the water, two phases may reappear and this cause chemical hydrolysis (breakdown) of the lipids.
10. The iso-propanol keeps the solution in one phase, keeping the water dissolved into the chloroform.
11. First transfer the seedlings to a drop of buffer (2.5 mM MES- KOH, pH 5.8, 1 mM KCl) in an empty petri dish. This discharges the static electricity of the roots and allows a “clean” transfer of all three seedlings in one step into the reaction tube (do not touch the wall!). Use a yellow 20-200 µL tip for the transfers, not tweezers (damage). Alternatively, use a 24 wells microtiter plate containing 400 µL of buffer with 2 or 3 seedlings/ well.
12. Add the ^{32}P-label diluted, i.e., as 10 µL in buffer to lower the variability of labeling. Usually, the label is 10 µCi/ µL, carrier free.
13. The extract now splits into two phases with an “aqueous” upper phase (free label) and an organic lower phase (lipids). At the interphase, solid material (seedling

remainings, protein precipitates) settles.
14. We use an Eppendorf multistepper/repeater pipet for this.
15. Potassium oxalate chelates cations like Ca^{2+} and Mg^{2+}, which cause PPIs to "smear".
16. Storing the plates above 100 °C prevents the silica from binding the water that is in the air. The latter affects the composition of the TLC solvent and lowers the silica's capacity to bind lipids.
17. When the samples are cold, the water in the air can condensate on top of the extracts causing hydrolysis and smearing of the lipid spots.
18. Chloroform evaporates easily and the gas that is building up inside the pipet tends to push out the liquid within the tip. By equilibrating first the pipet and tip with chloroform, this is strongly reduced and increases the handling and loading of the lipid samples.
19. Because of the rapid evaporation of chloroform, the extract tends to be pushed out. Use that drop to touch the pencil-marked spot on the TLC plate. Keep the pipet vertical at all times. It is also no problem to gently touch the silica with the tip. Prevent the spot from becoming too big, by loading small drops of extract at the time.
20. After 1.5 h, the front will still be a few cm from the top. It has, however, no use to run it completely until the end. The speed of the migration front slows down exponentially, while spots will diffuse in all directions, so there is no gain in resolving power by running the TLC longer.
21. If you do not need to use the lipids any further, one can speed up the solvent evaporation by placing the plate in the 115 °C oven for 5 min.

REFERENCES

1. Welti R, Shah J, Li W, Li M, Chen J, Burke JJ, Fauconnier ML, Chapman K, Chye ML, Wang X (2007) Plant lipidomics: discerning biological function by profiling plant complex lipids using mass spectrometry. *Front Biosci* **12**:2494–2506.

2. Arisz SA, Valianpour F, van Gennip AH, Munnik T (2003) Substrate preference of stress-activated phospholipase D in *Chlamydomonas* and its contribution to PA formation. *Plant J* **34**:595–604.

3. Munnik T, de Vrije T, Irvine RF, Musgrave A (1996) Identification of diacylglycerol pyrophosphate as a novel metabolic product of phosphatidic acid during G-protein activation in plants. *J Biol Chem* **271**:15708–15715.

4. Munnik T, Meijer HJ, Ter Riet B, Hirt H, Frank W, Bartels D, Musgrave A (2000) Hyperosmotic stress stimulates phospholipase D activity and elevates the levels of phosphatidic acid and diacylglycerol pyrophosphate. *Plant J* **22**:147–154.

5. Arisz SA, van Himbergen JA, Musgrave A, van den Ende H, Munnik T (2000) Polar glycerolipids of *Chlamydomonas moewusii*. *Phytochemistry* **53**:265–270.

6. Munnik T, van Himbergen JAJ, ter Riet B, Braun FJ, Irvine RF, van den Ende H, Musgrave AR (1998) Detailed analysis of the turnover of polyphosphoinositides and phosphatidic acid upon activation of phospholipases C and D in *Chlamydomonas* cells treated with non-permeabilizing concentrations of mastoparan. *Planta* **207**:133–145.

7. Meijer HJG, Divecha N, van den Ende H, Musgrave A, Munnik T (1999) Hyperosmotic stress induces rapid synthesis of phosphatidyl- D-inositol 3,5-bisphosphate in plant cells.
Planta **208**:294–298.

8. Meijer HJG, Arisz SA, van Himbergen JAJ, Musgrave A, Munnik T (2001) Hyperosmotic stress rapidly generates lyso-phosphatidic acid in *Chlamydomonas*. *Plant J* **25**:541–548.

9. Meijer HJG, Berrie CP, Iurisci C, Divecha N, Musgrave A, Munnik T (2001) Identification of a new polyphosphoinositide in plants, phosphatidylinositol 5-monophosphate (PtdIns5P), and its accumulation upon osmotic stress. *Biochem J* **360**:491–498.

10. Bargmann BO, Laxalt AM, ter Riet B, Testerink C, Merquiol E, Mosblech A, Leon-Reyes A, Pieterse CM, Haring MA, Heilmann I, Bartels D, Munnik T (2009) Reassessing the role of phospholipase D in the *Arabidopsis* wounding response. *Plant Cell Environ* **32**:837–850.

11. Welti R, Li W, Li M, Sang Y, Biesiada H, Zhou HE, Rajashekar CB, Williams TD, Wang X (2002) Profiling membrane lipids in plant stress responses: role of phospholipase D alpha in freezing-induced lipid changes in *Arabidopsis*. *J Biol Chem* **277**:31994–32002.

12. Zien CA, Wang C, Wang X, Welti R (2001) *In vivo* substrates and the contribution of the common phospholipase D, PLDα, to wound induced metabolism of lipids in *Arabidopsis*. *Biochim Biophys Acta* **1530**:236–248.

13. Arisz SA, Testerink C, Munnik T (2009) Plant PA signaling via diacylglycerol kinase. *Biochim Biophys Acta* **1791**:869–875.

14. Arisz SA, Munnik T (2013) Distinguishing phosphatidic acid pools from *de novo* synthesis, PLD and DGK. *Methods Mol Biol* **1009**:55–62.

15. den Hartog M, Musgrave A, Munnik T (2001) Nod factor-induced phosphatidic acid and diacylglycerol pyrophosphate formation: a role for phospholipase C and D in root hair deformation. *Plant J* **25**:55–65.

16. Van der Luit AH, Piatti T, van Doorn A, Musgrave A, Felix G, Boller T, Munnik T (2000) Elicitation of suspension-cultured tomato cells triggers the formation of phosphatidic acid and diacylglycerol pyrophosphate. *Plant Physiol* **123**:1507–1516.
17. den Hartog M, Verhoef N, Munnik T (2003) Nod factor and elicitors activate different

phospholipid signaling pathways in suspension cultured alfalfa cells. *Plant Physiol* **132**:311–317.

18. Ramos-Diaz A, Brito-Argaez L, Munnik T, Hernandez-Sotomayor SM (2006) Aluminum inhibits phosphatidic acid formation by blocking the phospholipase C pathway. *Planta* **225**:393–401.

19. van Leeuwen W, Vermeer JE, Gadella TW Jr, Munnik T (2007) Visualization of phosphatidylinositol 4,5-bisphosphate in the plasma membrane of suspension-cultured tobacco BY-2 cells and whole *Arabidopsis* seedlings. *Plant J* **52**:1014–1026.

20. Zonia L, Munnik T (2004) Osmotically induced cell swelling versus cell shrinking elicits specific changes in phospholipid signals in tobacco pollen tubes. *Plant Physiol* **134**:813–823.

21. Latijnhouwers M, Munnik T, Govers F (2002) Phospholipase D in *Phytophthora infestans* and its role in zoospore encystment. *Mol Plant Microbe Interact* **15**:939–946.

22. Frank W, Munnik T, Kerkmann K, Salamini F, Bartels D (2000) Water deficit triggers phospholipase D activity in the resurrection plant *Craterostigma plantagineum*. *Plant Cell* **12**:111–124.

23. Monreal JA, Lopez-Baena FJ, Vidal J, Echevarria C, Garcia-Maurino S (2010) Involvement of phospholipase D and phosphatidic acid in the light-dependent upregulation of sorghum leaf phosphoenolpyruvate carboxylase-kinase. *J Exp Bot* **61**: 2819–2827.

24. Darwish E, Testerink C, Khaleil M, El-Shihy O, Munnik T (2009) Phospholipid-signaling responses in salt stressed rice leaves. *Plant Cell Physiol* **50**:986–997.

25. Munnik T, Musgrave A, de Vrije T (1994) Rapid turnover of polyphosphoinositides in carnation flower petals. *Planta* **193**:89–98.

26. De Vrije T, Munnik T (1997) Activation of phospholipase D by calmodulin antagonists and mastoparan in carnation petal tissue. *J Exp Bot* **48**:1631–1637.

27. Munnik T, Laxalt AM (2013) Measuring PLD activity in vivo. *Methods Mol Biol* **1009**: 219–231.