Chapter 19

Collection and Analysis of Phloem Lipids

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Abstract

The plant phloem is a long-distance conduit for the transport of assimilates but also of mobile developmental and stress signals. These signals can be sugars, metabolites, amino acids, peptides, proteins, microRNA, or mRNA. Yet small lipophilic molecules such as oxylipins and, more recently, phospholipids have emerged as possible long-distance signals as well. Analysis of phloem (phospho)lipids, however, requires enrichment, purification, and sensitive analysis. This chapter describes the EDTA-facilitated approach of phloem exudate collection, phase partitioning against chloroform–methanol for lipid separation and enrichment, and analysis/identification of phloem lipids using LC-MS with multiplexed collision induced dissociation (CID).

Key words Phloem, Lipids, Long-distance lipid-mediated signaling, Liquid chromatography–mass spectrometry (LC-MS), Multiplexed collision induced dissociation (CID)

1 Introduction

Plants are exposed to a variety of environmental factors of biotic or abiotic nature that influence growth and development. Responses to these conditions can occur locally, where the stress is perceived, or distally, to elicit a systemic response throughout the plant. Unlike in animals, these long-distance signals are not electric but rather chemical and, hence, need specific transport mechanisms. One key conduit for the transport of such mobile signals is the plant phloem. In the past, research largely focused on sugars, small hydrophilic metabolites, proteins, microRNAs, and mRNAs (for a review *see* [1]). Yet, lipophilic compounds, such as oxylipins and some phospholipids have been found in the phloem as well, albeit in small amounts. The importance of oxylipins in the phloem is undisputed. Phospholipids are less well studied but of increasing relevance as phospholipid-binding proteins such as flowering locus T (FT, florigen) have been identified in the phloem [2, 3]. Thus, specific phospholipids could function as components of a lipid-mediated long-distance signaling pathway [3–6]. Compounds

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that require long-distance movements are synthesized in the companion cell, transported into the sieve element and from there throughout the plant (for reviews *see* [1, 7]). As a result, understanding the composition and fluctuations of the lipid content within sieve elements, that is, in the phloem sap, is of increasing importance. Identification of the phloem lipids is hampered by several factors:

1. The phloem consists of three types of cells: phloem parenchyma cells, companion cells, and sieve elements. The latter contain the phloem sap. Because the phloem sap is derived from an internal tissue, it can only be accessed through wounding of other tissues. Hence, many controls are needed to prove purity of the phloem sap. In addition, sieve elements are not easily accessible and the phloem tends to seal itself, potentially resulting in a loss of phloem flow (and hence collection of sap). As a result, a variety of methods for phloem sap collection exists, depending on the plant. Five approaches are the most prevalent:
   - In some species, such as lupine, phloem sap can be harvested by making *shallow incisions or punctures* into the vasculature at the stem or in the petiole without damaging the xylem [8, 9]. After discarding the first few microliters (removing the contents of injured cells) this sap is very pure, yet the amount collected is small. In healthy, well-watered plants, approximately 200 μl can be obtained.
   - In cucurbits, phloem exudates are typically harvested by *cutting the petiole*. The sap pushes from the cut side that is still connected to the stem. After removing the first drop, which contains contaminants from the cut cells, pure phloem sap can be obtained in large amounts. While the amount collected is plentiful, in many cucurbit species, this exudate is diluted with xylem [10] or thickens during extended collection times [11], making it unsuitable for liquid chromatography approaches.
   - Aphids naturally insert their style into the sieve element. During *aphid stylectomy*, the aphids are removed with a laser and pure phloem sap exudes from the remaining style. This method is useful for any plant that can get infected by a sucking insect that pierces the phloem but does require the laser setup [12]. In addition, aphids inject compounds that prevent the phloem from sealing and may be falsely attributed to the plant phloem exudate.
   - As an alternative to stylectomy, researchers have used aphid exudate where the aphid "excrement" or whole aphid analysis is used to identify compounds that were taken up from the plant [13]. While this approach identifies compounds in

the aphid that are derived from the sucking of the phloem, it is difficult to distinguish true phloem components from aphid-generated derivatives.

- The most broadly applicable and inexpensive method is the *EDTA-facilitated method*. It allows for the collection of phloem exudate from the bottom end of cut petioles. It can be used in many herbaceous (Arabidopsis, Perilla, tomato) and woody (poplar) plants [4]. Instead of collecting sap exuding from the stem-bound connection of the petiole as used in cucurbits, here the exudate is collected from the cut leaves into an EDTA solution. Exposure to EDTA for 1–2 h prevents sealing of the phloem. The leaves can then be moved into water, into which the exudate can be collected for several hours [4, 14] or overnight [15].

In this chapter, I will only refer to the EDTA-facilitated method as it is applicable to most plant species [4, 5, 11]. In the notes, I will list suitable controls to ensure purity of the sap.

2. The phloem is hydrophilic with sugars being its main constituents. Lipids tend to occur in very small amounts. Thus, the phloem has to be phase-partitioned to remove hydrophilic components and concentrated to detect the lipids. Because phloem lipids are very low in abundance, the typical large-scale lipid analysis methods (Chapter 7) [16, 17] that often use infusion as a means of introducing the sample into the mass spectrometer may not be feasible. Instead, LC-ESI-MS with multiplexed CID as described here provides the sensitivity required for lipid analysis in phloem. The general CID-TOF-MS approach is explained in more detail in Chapter 8.

2 Materials

2.1 Phloem Exudate Collection

1. Plants of interest (e.g., 4- to 7-week-old *A. thaliana* plants grown in a 12-h photoperiod).
2. 1.7 ml Eppendorf tubes and rack (for *A. thaliana*) or glass beakers. If a larger plant with longer petioles such as Perilla or tobacco is used, you need twice the number of large beakers than samples, one beaker for incubation in EDTA and a second beaker for sap collection; you need four times the number of tubes if you are working with *A. thaliana*: two tubes to incubate 15 leaves each in EDTA and two tubes to collect sap of these leaves in water.
3. Marker to label all tubes.
4. Glass or plastic petri dish.

5. 20 mM K_2-EDTA solution: make 100 mM stock solution: (20.2 g/500 ml Millipore-purified water); keep in the refrigerator and dilute 1:5 (one part stock, four parts water).
6. 1 ml pipettes or measuring cylinder depending on the plant species.
7. Fresh razor blades.
8. Gloves.
9. Paper towels.
10. Shallow dish with black plastic bag or Plexiglas container.
11. Liquid N_2 and −80 °C freezer to store samples.
12. Lyophilizer: if you work with large plants and need to concentrate your exudate prior to phase partitioning.

2.2 Phase Partitioning

1. Access to a fume hood.
2. Lipid standards. Can be obtained from Avanti Polar Lipids or the Ruth Welti lab (Kansas State University, Manhattan, KS, USA).
3. Chloroform–methanol solution (1:1, v/v).
4. Glass tubes with Teflon caps (twice the number of samples).
5. Glass tube rack.
6. Disposable glass pipettes (ca 2 ml) with pipette bulbs.
7. 10 ml glass pipettes with pipetting tool (pipette bulb or pipette controller).
8. Glass beaker.
9. Tabletop centrifuge.

2.3 LC-ESI-MS

1. HPLC column: C18 column (5 cm × 2.1 mm × 2.7 μm; Supelco, Bellefon, PA, USA).
2. LC-MS system: We use a Waters LCT Premier mass spectrometer (LC-TOF-MS).
3. Solution A: 10 mM ammonium formate.
4. Solution B: acetonitrile/isopropanol (1:2, v/v).
5. Internal standards: We use di14:0 phosphatidic acid (di14:0 PA) and di12:0 phosphatidylcholine (di12:0 PC); each at 0.16 nmol in chloroform/methanol (1:1, v/v).

3 Methods

3.1 Harvest of Phloem Exudates (See Note 1)

1. Prepare a small container that can hold your samples by placing wet paper towels at the bottom to maintain humidity during exudation. This can be a small tray in combination with black plastic bags that were sliced to allow for gas exchange or a Plexiglas chamber.

2. Fill a plastic or glass petri dish (minimum diameter 7 cm) with 20 mM K_2-EDTA. If collecting samples from different genotypes or treatments, prepare separate dishes for each treatment.
3. Label all Eppendorf tubes per sample/replicate and split in two different sets. Fill one set of tubes with 1.4 ml of 20 mM K_2-EDTA solution. Fill an equal number tubes in the second set with 1.4 ml Millipore water or autoclaved deionized water. The latter tubes collect the phloem sap for lipid analysis. Keep in mind that you will need to pool two tubes to obtain sufficient phloem exudate, so technically, you need to prepare four tubes per sample.
4. If you harvest the phloem sap on your bench, place paper towels on the bench to collect any water/dirt from the outside of the pots. Put pots on the paper towel and wear gloves (this is to protect your sample).
5. Harvest rosette leaves from *Arabidopsis* plants by cutting the leaves with the razor blade at the base of the petiole as close to the center of the rosette as possible. Typically, plants that are 4–6 weeks old provide the nicest petioles (*see* **Notes 2** and **3**).
6. Immediately place the leaves in the petri dishes with the K_2-EDTA solution (*see* **Note 4**). The cut end of the petiole has to remain submerged in the solution at all times (Fig. 1a).
7. Once you have collected a sufficient number of leaves, gently stack 15 leaves on top of each other such that the cut petioles are aligned with each other (*see* **Note 5**). It takes about 3–4 plants to obtain 15 leaves. Recut the base of the petioles (ca. 2 mm) while submerged in K_2-EDTA to prevent cavitation, and transfer immediately into one of the Eppendorf tubes containing the 20 mM K_2-EDTA solution. Make sure the cut end of the petiole is well submerged in the solution (*see* **Note 6**).
8. If you work with larger leaves/petioles such as Perilla or tobacco, adjust the container size accordingly. In this case, 200–250 ml glass or Pyrex beakers can be filled with ca. 50 ml of 20 mM K_2-EDTA solution. This will be sufficient to keep the cut end of the petioles submerged. Leaves are again stacked to align the petioles, recut in the EDTA solution and placed into the beaker "like flowers in a vase" (Fig. 1b).
9. Gently cover the samples with a wet paper towel to reduce transpiration. Use a new set of dishes if you are collecting samples from different treatments or genotypes to prevent cross contamination.
10. Once all the samples are harvested, they are placed in the tray with the wet paper towel, and either carefully placed into the black plastic bag (for harvest in the dark) or a plexiglass

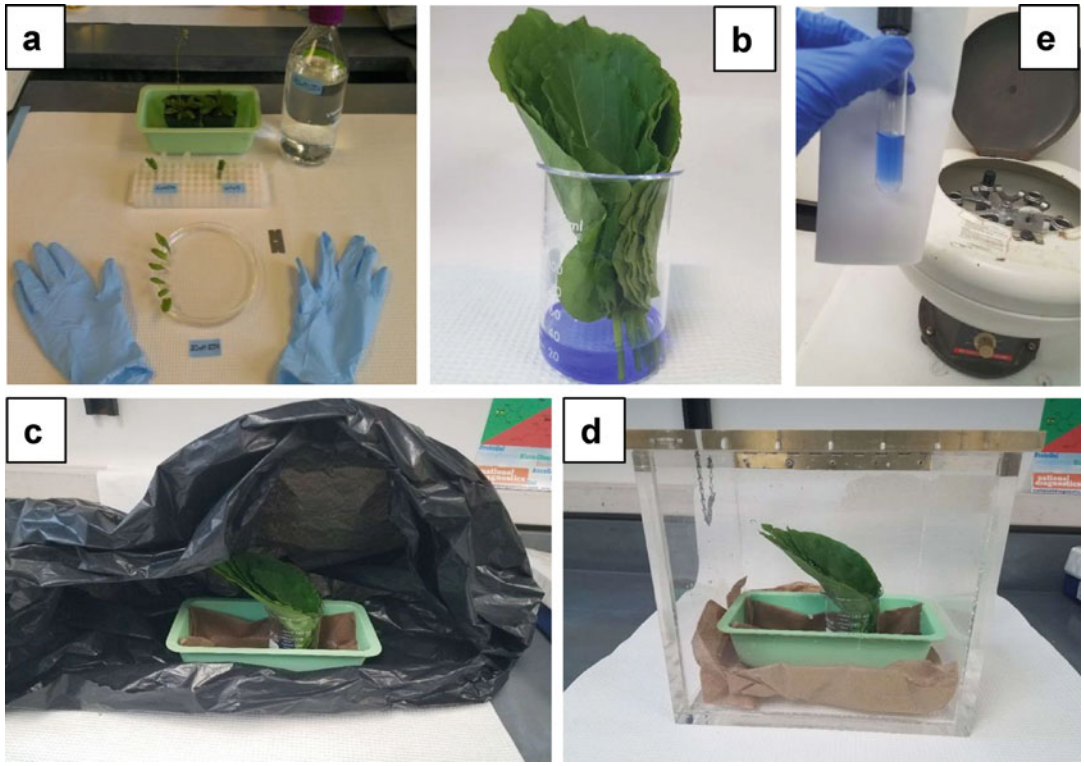

Fig. 1 Setup for phloem exudate collection of *A. thaliana* (**a**) and a larger plant (tobacco, **b**). Possible containers that can be used for exudate collection in the dark (**c**) and light (**d**). Test tube and phase partitioning for lipid purification (**e**). The EDTA solution is dyed blue for better illustration

container (for harvest in the light) and incubated in the K_2-EDTA solution for 1–2 h (longer times for larger plants; Fig. 1c, d).

11. After 1–2 h, the leaves are washed in large volumes of deionized water and moved to the second set of Eppendorf tubes, which only contain Millipore water. This removes the EDTA and any material derived from wounded cells.
12. Again, place the samples carefully in either the plastic bag or the Plexiglass container. For harvest in the darkness, place the entire setup in a cabinet to keep it dark. For Arabidopsis, it takes about 5–8 h to obtain sufficient sap for phloem analysis. For Perilla, 8 h of collection time is advisable (*see* **Note 7**).
13. Once collection is complete, the leaves are pulled out of the containers. Gently collect the drop at the bottom of the petioles. DO NOT SQUEEZE the petioles as this can lead to wounding and contamination. Exudates in Eppendorf tubes can be frozen in liquid nitrogen and stored in a −80 °C freezer (*see* **Note 8**). These samples can be used directly for phase partitioning (*see* **Note 9**).

14. If you collect phloem exudate from larger leaves and in larger volumes, lyophilize the samples to complete dryness and resuspend in a small volume (ca. 4 ml).

3.2 Purification/ Enrichment of Phloem Lipids

1. Perform all steps of the purification/enrichment in the fume hood.
2. Combine the *A. thaliana* phloem exudates of two tubes in a glass screw top tube with a Teflon cap. The volume is approximately 3.5 ml. Internal standards, for example di14:0 PA and di12:0 PC (0.16 nmol each), can be added now if quantification is desired (*see* **Note 10**).
3. Add an equal volume of chloroform–methanol (1:1, v/v), vortex or hand-mix thoroughly and separate phases through centrifugation for 4 min at low speed (400 × *g*) in a tabletop centrifuge. The organic phase containing the lipids will be at the bottom (Fig. 1e).
4. Collect the bottom (organic) phase in a second glass tube with Teflon cap. Repeat phase partitioning with the top phase two more times and collect the bottom phases.
5. Dry the combined organic phases under a stream of N_2 to a volume of 200 μl. At this point, samples will be dissolved in methanol.
6. They are now ready for further analysis or they can be stored at −20 or −80 °C.

3.3 Lipid Analysis of Phloem Exudates

1. The lipids isolated can be analyzed further using thin-layer chromatography (TLC) (Chapter 3) [4] or liquid chromatography–mass spectrometry (LC-MS) with multiplexed collision-induced dissociation (CID) [4]. Typical outcomes are shown in Fig. 2 (TLC) and Fig. 3 (LC-MS). Since the availability of mass spectrometers might vary, programming of the run will need to follow the requirements for the specific mass spectrometer. Here we provide general instructions:
2. Set up the column and solutions and wash the column by running the LC program from 10% B to 99% B with the following LC program:

0–4 min	10% B
4–15 min	80% B
15–20 min	99% B
20–28 min	99% B
28–30 min	10% B

3. Program the instrument to run each sample twice: Once in positive ion mode and once in negative ion mode. This will allow for monitoring of anionic, cationic and neutral lipids as

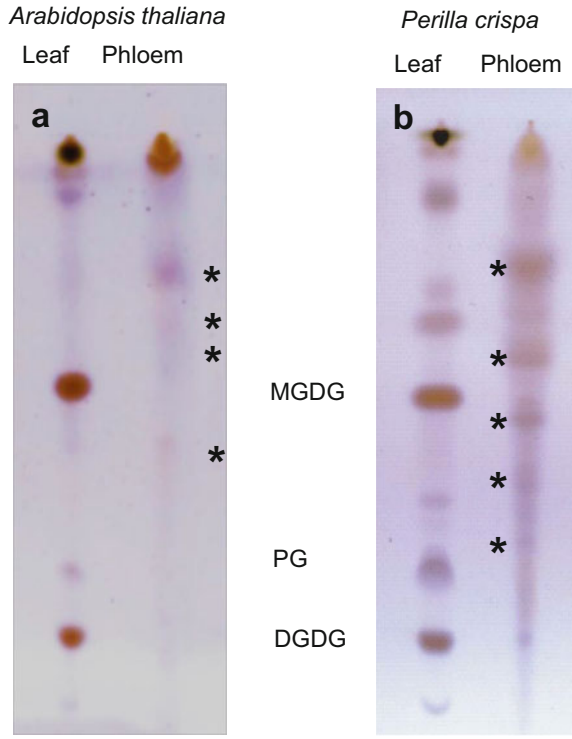

Fig. 2 Analysis of phloem lipids from *A. thaliana* (**a**) and *Perilla* (**b**) using thin-layer chromatography. Leaf lipids serve as control. Asterisks indicate lipids specific for phloem exudates. The chloroplast-specific galactolipids DGDG (digalactosyldiacylglycerol) and MGDG (monogalactosyldiacyglycerol) are absent from phloem samples. PG, phosphatidylglycerol

well as the detection and identification of negatively charged head groups and the two acyl chains after fragmentation. Data acquisition is performed after collision-induced dissociation (CID) using five voltages: 20, 35, 50, 65, and 80 V. This will allow for detection of the parent ion at low CID voltage and of the head group and acyl chains at higher voltages, where the lipid fragments.

4. Insert sample tubes in the autosampler. Injection volume is 10 μl.
5. For subsequent data analysis, chromatograms of the same samples are displayed using the program available with the mass spectrometer. Peaks of the fragment ions and parent ions are aligned at the same retention time and allow for identification of the lipid. Alternatively, public lipid databases can be accessed for identification (Chapter 25). To quantify and compare lipid abundances, the peak areas can be compared and normalized to the known standard that was added prior to phase partitioning.

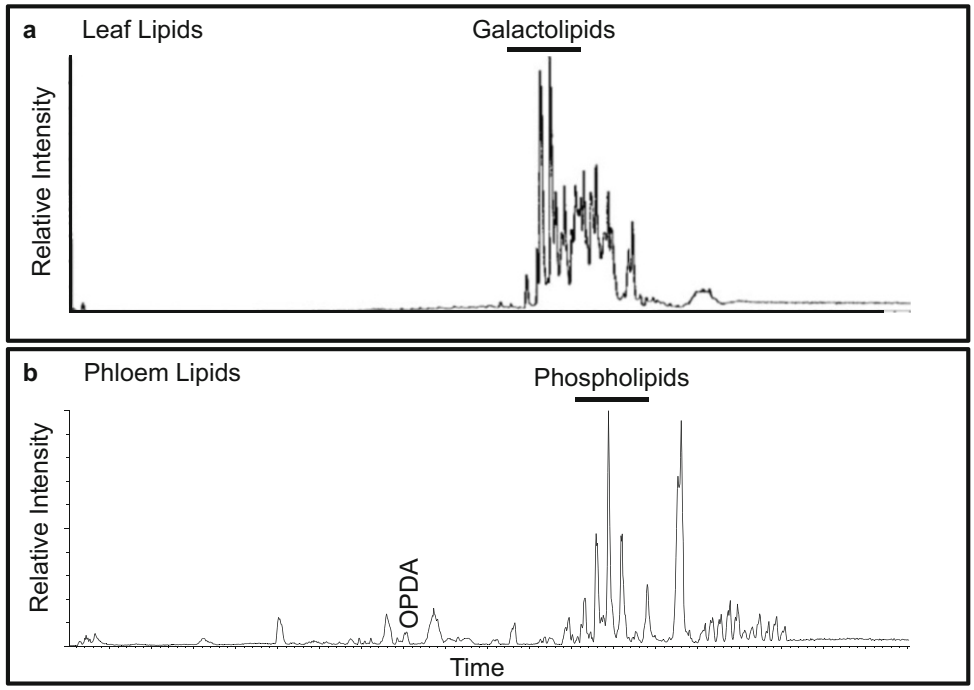

Fig. 3 Determination of leaf (**a**) and phloem (**b**) lipids from *A. thaliana* using LC-CID-MS. The phloem lipid extract contains only minor amounts of galactolipids, which are found in chloroplasts, but instead specific phospholipids and oxylipins (e.g., 12-oxo-phytodienoic acid, OPDA) are detected in the phloem

4 Notes

1. This method was published for *A. thaliana* as an open-access video and text ([11]; https://www.jove.com/video/51111/collection-analysis-arabidopsis-phloem-exudates-using-edta).
2. Grow *Arabidopsis thaliana* plants in a 12 h photoperiod—this gives longer petioles and bigger, healthier leaves. Overall, the healthier your plants, the more exudate you obtain.
3. Move plants out of the growth chamber ahead of time so they are not drought stressed.
4. K_2 EDTA should be used as it provides higher yields than Na_2-EDTA.
5. The leaves fit easiest into Eppendorf tubes if they are slightly rolled up. Avoid squeezing the leaves since this might cause injury of cells and, thus, leads to the collection of leaf cell content.
6. When collecting exudate from leaves, make sure the cut part of the petiole is sufficiently submerged in water and kept in a humid environment. Otherwise all water will be transpired and no phloem is collected.

7. If you start ca. 9:00 am in the morning, you can be ready to collect/freeze your sap at as early as 5:00–6:00 pm. Some labs collect overnight or for multiple days. Just keep in mind that the phloem has a high sugar content and provides nutrients for microorganisms that could contaminate your sample. Also, many phloem enzymes retain their activity at least for a few hours and could start degrading your sample (*see* [4] and the text to [11]).
8. If you want to store samples for an extended period of time, lyophilize them to complete dryness and store at −80 °C.
9. If you are interested in aqueous metabolites or proteins, you do not need to phase partition but can use collected exudate after concentrating to a small volume (200—500 μl) or you can use the upper phase from the phase partitioning.
10. Because there are only very small amounts of lipids in phloem exudates, you need to combine two collection tubes from Arabidopsis samples prior to phase partitioning. In case of larger plants like Perilla or tobacco, exudate is collected into larger volumes and can simply be concentrated through lyophilization.

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