Measurement of Lipid Transport in Mitochondria by the MTL Complex

Juliette Jouhet, Valérie Gros, Morgane Michaud

Chapter 7

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Abstract

Membrane biogenesis requires an extensive traffic of lipids between different cell compartments. Two main pathways, the vesicular and non-vesicular pathways, are involved in such process. Whereas the mechanisms involved in the vesicular trafficking are well understood, fewer is known about non-vesicular lipid trafficking, particularly in plants. This pathway involves the direct exchange of lipids at contact sites (MCSs) between organelles. In plants, an extensive traffic of the chloroplast-synthesized digalactosyldiacylglycerol (DGDG) to mitochondria occurs during phosphate starvation. This lipid exchange occurs by non-vesicular trafficking pathways at MCSs between mitochondria and plastids. By a biochemical approach, a mitochondrial lipoproteic super-complex called MTL (Mitochondrial Transmembrane Lipoprotein complex) involved in mitochondria lipid trafficking has been identified in *Arabidopsis thaliana*. This protocol describes the method to isolate the MTL complex and to study the implication of a component of this complex (AtMic60) in mitochondria lipid trafficking.

Key words: mitochondria, lipid transfer, MTL complex, CN-PAGE, mass spectrometry.

1 Introduction

Eukaryotic cells are composed of different organelles that allow the compartmentalization of cellular functions. Each organelle is delineated by at least one membrane composed of a specific assembly of lipids and proteins. This specific composition defines the identity of each organelle and is required to ensure their proper functions. Lipids play multiple roles in the regulation of the architecture and the function of membranes. They are synthesized in different cell compartments and have to be properly distributed to other organelles. Two main lipid trafficking pathways have been described: the vesicular and the non-vesicular pathways. The vesicular pathway is involved in lipid and protein trafficking to the endomembrane system and has been well studied in different organisms [1, 2]. The non-vesicular pathway involves the exchange of lipids directly between two membranes, usually at contact sites (MCSs), when membranes are at a distance $\leq$ 30 nm [3]. This pathway requires the establishment of MCSs, by the so-called tethering proteins or complexes, followed by the transfer of the lipid from one membrane to another.

Identification of proteins involved in non-vesicular lipid trafficking in cells is limited mainly by technical issues. *In vivo* strategies to investigate the role of a protein on lipid trafficking are mostly based 1) on the analysis of the steady state level of lipids in cells or organelles in a mutant or 2) on experiments based on lipid labelling. However, the steady state of a lipid relies on several metabolic processes besides trafficking, such as synthesis, degradation or modification. Lipid labelling experiments can be limited by the availability of labelled (fluorescent, radioactive…) substrates that can be easily uptake by cells and behave like endogenous lipids. In addition, because of the presence of several redundant pathways, the effect of a protein on lipid trafficking can be masked in a mutant. Several *in vitro* assays using liposomes and purified proteins have also been developed to study the ability of a protein to transfer

lipids [4, 5]. However, these assays are adapted for soluble proteins and some specific factors, such as lipid composition of the donor and acceptor liposomes or the presence of partners, might be required to detect and measure the transfer activity of a protein by such assays.

Recently, we took advantage of the massive lipid remodeling triggered by phosphate starvation to identify proteins involved in lipid trafficking to mitochondria in *Arabidopsis thaliana* [6-8]. During phosphate starvation, the phospholipids present in mitochondria membranes are partially degraded to release phosphate [6]. To maintain the integrity of mitochondria membranes, a massive transfer of the non-phosphorous galactoglycerolipid digalactosyldiacylglycerol (DGDG) occurs from chloroplast to mitochondria [6]. DGDG is synthesized in plastids and is mainly retained in this compartment in normal growth condition [9]. During Pi starvation the level of DGDG in mitochondria drastically increase from 2 to 20 % [6, 8]. This transfer occurs at MCSs between chloroplasts and mitochondria, which number increases during Pi starvation [6]. Thus this particular situation constitutes a powerful tool to study lipid trafficking to mitochondria as we can 1) turn on/off the traffic of DGDG to mitochondria by simply modifying the Pi concentration in the media and 2) easily follow the content of this natural non-labeled lipid inside mitochondria by classical methods of lipid analysis such as mass spectrometry or thin layer chromatography. Thus, by looking for mitochondria complexes enriched in DGDG during Pi starvation, we identified on Clear Native Polyacrylamide Gel (CN-PAGE) a super-complex called Mitochondrial Transmembrane Lipoprotein complex (MTL) [8, 10]. The MTL complex is present in cells grown in presence or in absence of Pi. Proteomic analyses have revealed the presence in this complex of proteins located mostly in the mitochondria membranes [8]. However, some components are also located in other cell compartments such as plastids, suggesting the presence of the MTL complex at membrane contact sites. Furthermore, this complex is dynamic and both its lipid and protein composition vary during Pi starvation. Among this complex, a protein localized in the inner membrane of mitochondria, AtMic60, has been further investigated for a putative role in lipid trafficking [8, 10].

This protocol describes the strategy used to reveal the key role of AtMic60 in mitochondria lipid remodeling during Pi starvation [8]. We started from callus cell cultures that allow to easily obtain liquid cultured cell lines from mutant plants. After purification of mitochondria from *atmic60* mutant callus, the involvement of AtMic60 on lipid trafficking is investigated by analyzing 1) the *in vitro* incorporation of radiolabeled DGDG inside the MTL complex and 2) the lipidome of the MTL complex compared to the lipidome of mitochondria membranes (Figure 1).

2 Materials

Prepare all reagents and media with ultrapure water. For cell cultures, always work in sterile condition under a laminar flow hood. For lipid extraction and analysis, always use glass vessels and never plastic with organic solvents. Wash all vessels without detergent but with distilled water, then ethanol. All solvents must be for Analysis grade except Hexane that must be for GC grade. Chloroform should be ethanol stabilized. Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Plant material and culture

1. *Arabidopsis thaliana* Col0 plants are used as wild type (WT). A T-DNA insertion line (SALK_087650c) [11] in AtMic60 gene (AT4G39690) in Col0 background is used as an example to study a defect in lipid trafficking [8].

2. MS +Pi: 4.41 g/L Murashige and Skoog (MS) basal salts, 1X MS Vitamin Solution, 1.5 % (w/v) sucrose, 1.2 $mg.L^{-1}$ 2,4-

dichlorophenoxyacetic acid. The pH is adjusted to 5.7 with NaOH and the media is autoclaved.

3. MS –Pi: 4.41 g/L MS basal salts without Pi, 1X MS Vitamin Solution, 1.5 % (w/v) sucrose, 1.2 $mg.L^{-1}$ 2,4-dichlorophenoxyacetic acid. The pH is adjusted to 5.7 with NaOH and the media is autoclaved.

4. MS seedlings: 4.41 g/L MS with basal salts and vitamins, 0.5 % (w/v) sucrose and 0.5 g/L MES. The pH is adjusted to 5.7 with KOH, agar is added at 0.8 % (w/v) and the media is autoclaved.

5. MS callus: 4.41 g/L MS, 3 % (w/v) sucrose and 1.2 $mg.L^{-1}$ of 2,4-dichlorophenoxyacetic acid. The pH is adjusted to 5.7 with KOH, agar is added at 0.8 % (w/v) and the media is autoclaved.

6. Ethanol 70% (v/v).

7. Sterilization solution: bleach 0.4 % active chlorine in water.

8. Agarose 0.2% (w/v) in water. The solution is autoclaved.

9. Sterile ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C).

2.2 Mitochondria purification from callus liquid cultures

1. Grinding buffer: 0.3 M mannitol, 15 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH 8, 2 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) pH 8, 0.6 % (w/v) polyvinylpyrrolidone K25 (PVP25). Just before use, 0.5 % (w/v) bovine serum albumin (BSA), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM aminocaproic acid and 1 mM benzamidine are added.

2. Washing buffer: 0.3 M mannitol, 10 mM MOPS pH 7.4. Just before use, 1 mM PMSF, 5 mM aminocaproic acid and 1 mM benzamidine are added.

3. CX2: 0.6 M mannitol, 20 mM MOPS pH 7.2, 2 mM EGTA. Just before use, 0.2 % (w/v) BSA is added.

4. Percoll 40: 40 % (v/v) Percoll (GE Healthcare Life Sciences), 1X of CX2.

5. Percoll 28: 28 % (v/v) Percoll (GE Healthcare Life Sciences), 1X of CX2.

6. Percoll 23: 23 % (v/v) Percoll (GE Healthcare Life Sciences), 1X of CX2.

7. Mortar and pestle.

8. Sand.

9. 50 mL polycarbonate tubes.

2.3 Galactoglycerolipid labelling and transfer with UDP-[^{14}C]-galactose

1. UDP-[^{14}C]-galactose: 10 mM UDP-[^{14}C]-galactose 167 bq/nmol

2. Washing buffer: 0.3 M mannitol, 10 mM MOPS pH 7.4.

2.4 Mitochondria membranes purification and solubilization

1. Swelling buffer: 10 mM MOPS pH 7.4. Just before use, 1 mM PMSF, 5 mM aminocaproic acid and 1 mM benzamidine are added.

2. Membrane buffer: 50 mM imidazole pH 7, 0.5 M aminocaproic acid, 1 mM EDTA pH 8.

3. DDM 1 %: A stock solution 10 % (w/v) of n-dodecyl β-D-maltoside (DDM) is prepared in water. Then, this solution is diluted 10 times in water to obtain a DDM 1 % (w/v) solution stored at -20ºC.

4. Loading buffer 5X: 300 mM Tris-HCl pH 6.8, 50 % (v/v) glycerol, 0.5 % (w/v) bromophenol blue in water. Store at -20ºC.

2.5 CN-PAGE

Prepare the gel mix just before use.

1. Concentration gel: 3 % acrylamide/bis-acrylamide 37.5/1 (stock solution at 30 %, Bio-Rad), 0.125 M Tris-HCl pH 6.8, 0.01 % (w/v) ammonium persulfate (APS), 0.04 % (v/v) N, N, N', N'-tetramethylethane-1,2-diamine (TEMED). Add the TEMED just before pouring the gel.

2. Separation gel 3.5 %: 3.5 % acrylamide/bis-acrylamide 37.5/1 (stock solution at 30 %, Bio-Rad), 0.125 M Tris-HCl pH 8.8, 0.01 % (w/v) APS, 0.04 % (v/v) TEMED. Add the TEMED just before pouring the gel

3. Separation gel 12 %: 12 % acrylamide/bis-acrylamide 37.5/1 (stock solution at 30 %, Bio-Rad), 0.125 M Tris-HCl pH 8.8, 0.01 % (w/v) APS, 0.04 % (v/v) TEMED. Add the TEMED just before pouring the gel

4. Migration buffer: Prepare a 5X solution composed of 0.25 M Tris, 1.92 M glycine, pH 8.3 with HCl. Store the solution at 4ºC. Dilute 5 times this solution in MilliQ water just before use to obtain the Migration buffer.

5. Gradient Mixer 15 mL.

6. Peristatic pump.

2.6 Fixation and staining of CN-PAGE

1. Fixation solution: 40 % (v/v) ethanol, 10 % (v/v) acetic acid.

2. Staining solution: 34 % (v/v) methanol, 17 % (w/v) ammonium sulfate, 0.5 % (v/v) acetic acid, 0.1 % (w/v) Coomassie blue G250.

3. Ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C).

2.7 Lipid extractions

1. Corex tubes 15 mL high strength.

2. Boiling ethanol.

3. Methanol:chloroform 1:2 (v/v)

4. Quartz wool (*see* **Note** 1).

5. Potters pestles (*see* **Note** 2).

6. Evaporator under argon (*see* **Note** 3).

7. Ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C).

8. NaCl 1 % (w/v) in ultrapure water.

9. Hemolysis glass tube for single use.

2.8 Methanolysis

1. PYREX® tubes of 20 mL (160 mm x 16 mm), tubes in borosilicate glass (high thermal resistance) with screw top. Screw caps in phenolic resin with rubber seal lined with inert PTFE.

2. Standard C15 (fatty acid with 15 carbons) solution: powder of pentadecanoic acid (99%), stored at RT. Prepare a solution at 0.5 mg/mL in chloroform/methanol (1/2, v/v) (*see* **Note** 4).

3. Dry hot bath.

4. Methanolysis buffer: Sulfuric acid (H_2SO_4 24N) 2.5 % in methanol (v/v) (*see* **Note** 5).

5. Ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C).

6. Hexane for GC analysis

7. Hemolysis glass tube for single use.

2.9 Total fatty acid quantification by Gas Chromatography – Flame Ionisation Detector (GC-FID)

1. Vials for automatic sampler with an insert of 250 µL and screw caps 9 mm with PTFE seal.

2. Hexane for GC analysis.

3. Column BPX70 (70% Cyanopropyl Polysilphenylene-siloxane) for GC: length 30 m, internal diameter 0.22 mm, film thickness 0.25 µm.

4. Gas Chromatography – Flame Ionisation Detector

5. Commercial FAME standard solution to calibrate the GC-FID retention time

2.10 Lipid class quantification by liquid chromatography - mass spectrometry (LC-MS)

1. Internal standard solution: prepare 1 mL of stock solution for each internal standard at 1.25 mM in chloroform/methanol [2/1, (v/v)] (*see* **Note** 6). Internal standards were obtained from Avanti Polar Lipids Inc. for PE 18:0–18:0 and PC 18:0-18:0. DGDG 16:0/16:0 was synthesized chemically [12]. As an alternative, DGDG 18:0/18:0 can be obtained from purchased natural extract (Avanti Polar Lipids Inc.) and hydrogenated (*see* **Note** 7).To prepare the internal standard solution, in a volumetric flask of 50 mL, add 50 µL of each individual standard and

complete to 50 mL with chloroform/methanol [2/1, (v/v)]. Aliquot the solution by 1 mL in hermetically sealed vials and store at -20°C.

2. Hemolysis glass tube for single use.

3. Evaporator under argon (*see* **Note** 3).

4. High Pressure Liquide Chromatography (HPLC) coupled to a triple quadripole mass spectrometer (*see* **Note** 8).

3 Methods

3.1 Preparation of callus liquid cultures

1. Put around 20 seeds from WT and mutant plants in a 2 mL Eppendorf tube for sterilization.

2. Add 1 mL of ethanol 70% and incubate the seeds 2 min at room temperature under agitation.

3. Discard ethanol and add 1 mL of Sterilization solution (*see* **Note** 9)

4. Incubate 12 min at room temperature under agitation.

5. Discard the Sterilization solution and wash the seeds 4 times with 1 mL of sterile water.

6. Resuspend the seeds in 100 µL of Agarose 0.2%.

7. Spread the seeds one by one on MS seedling plates with a pipette, tape the plates with a porous adhesive and allow the stratification of the seeds on dark at 4°C for 48H.

8. Incubate the plates under long day condition (16h light/8h dark) at 22°C for 2 weeks.

9. Carefully cut with a sterile razor leaves from seedlings and place them on MS callus plates with the epidermal layer contacting the media.

10. Incubate the plates under continuous light. Callus will be formed after 4 to 5 weeks.

11. To maintain callus, every four weeks, cut small pieces of callus with a sterile razor blade and transfer them on a new MS callus plate.

12. To obtain liquid cultures, add around ten 4 weeks-old callus in 200 mL of MS +Pi and incubate under continuous light at 22ºC on a rotary shaker at 125 rpm. Callus are then sub-cultured every 7 days in 200 mL of MS +Pi (*see* **Note** 10).

13. For one experiment, 5 mL of sedimented callus (*see* **Note** 10) of 7 days old cultures are washed 3 times with MS +Pi or MS –Pi media in a 50 mL sterile tube and used to inoculate 200 mL of MS +Pi or MS –Pi media in a 1L flask. For a typical experiment, 3 flasks of MS +Pi and 4 flasks of MS -Pi are inoculated. To compare the results, experiments from Col0 and *atmic60* grown in presence and absence of phosphate are performed the same day, meaning that 4 mitochondria purifications are performed in the same time.

14. Callus are grown on continuous light under agitation at 125 rpm for 4 days to be in exponential phase of lipid remodeling for mitochondria purification.

3.2 Mitochondria purification from callus liquid cultures

To preserve mitochondria integrity and functions, each step of purification has to be performed at 4ºC with all materials (mortar, tubes, rotors…) and buffers pre-chilled at 4ºC. Mitochondria have to be manipulated carefully. Particularly, during resuspension steps, cut tips have to be used (*see* **Note** 11).

1- Filter each cell flask on round Wattman filter paper using a filtration unit. Cells are weighted and placed on a mortar (*see* **Note** 12). Typically, we obtain around 10 g and 8 g of callus grown for 4 days in MS +Pi or MS –Pi respectively.

2. In a cold room, add 30 mL of Grinding buffer and 15 mL of sand for 64 g of callus in the mortar. Grind the cells with a pestle until obtaining a homogenous suspension. This takes generally 6 to 8 min to obtain a complete grinding.

3. Add Grinding buffer (20 mL) to the lysate to facilitate the transfer into 500 mL centrifuge bottles. Further wash the mortar with 20 mL of Grinding buffer.

4. Centrifuge cell lysates at 700 x *g* 5 min at 4ºC to pellet unbroken cells, heavy material and sand.

5. Centrifuge the supernatant 2 times at 3 000 x *g* 5 min at 4ºC in 50 mL polycarbonate centrifuge tubes.

6. Pellet crude mitochondria by a centrifugation of the supernatant at 20 000 x *g* during 15 min at 4ºC.

7. Carefully resuspend mitochondria in 2 mL of Washing buffer using a paintbrush and completely dissociate the mitochondria in a 5 mL Potter homogenizer by 3 gentle strokes. Use cut tips to manipulate crude mitochondria.

8. Prepare four discontinuous percoll gradients (one for each condition): pour 5 mL of Percoll 40 at the bottom of 32 mL ultracentrifuge tubes for swinging rotor. Then, gently add 20 mL of Percoll 23 and 10 mL of Percoll 18 (*see* **Note** 13). The three layers should be clearly visible.

9. Add carefully 2 mL of crude mitochondria on the top of each gradient with a pipette.

10. Centrifuge at 70 000 x *g* for 45 min at 4ºC in a swinging rotor, with standard acceleration at the beginning of the run but a slow deceleration at the end to preserve the gradient layers.

11. After centrifugation, mitochondria are located at the 32/40 % interface (*see* **Note** 14).

12. Carefully aspirate and discard the upper part of the gradient.

13. Pipette the mitochondria fraction and place them in a 50 mL polycarbonate centrifuge tube (one per gradient). Fill the tube with Washing buffer.

14. Centrifuge at 20 000 x g 15 min at 4ºC.

15. Carefully discard the supernatant (*see* **Note** 15). Fill the tubes with Washing buffer and centrifuge again at 20 000 x *g* 15 min at 4ºC.

16. Discard the supernatant and resuspend carefully the pellet in 2 mL of Washing buffer.

17. Mitochondria are then transferred in 2 mL microfuge tubes and centrifuge at 12 000 x *g* 10 min at 4ºC.

18. Resuspend mitochondria in 100 to 200 µL of Washing buffer and determine the concentration of mitochondria in equivalent protein by Bradford [13].

3.3 Galactoglycerolipid labelling and transfer with UDP-[^{14}C]-galactose (Figure 1.1)

1. The incorporation of UDP-[^{14}C]-galactose in the MTL complex is performed in a final volume of 250 µL of Washing buffer containing 750 µg of mitochondria proteins, 1 mM DTT and 1 mM $MgCl_2$.

2. To start the reaction, add UDP-[^{14}C]-galactose at a final concentration of 1 mM (*see* **Note** 16).

3. Incubate the samples for 30 min at 22ºC.

4. To eliminate unincorporated UDP-[^{14}C]-galactose, pellet the mitochondria by a centrifugation of 10 min at 12 000 x *g* at 4ºC.

5. Discard the supernatant and resuspend the mitochondria in 500 µL of Washing buffer.

3.4 Membrane purification and complex solubilization

1. Mitochondria membranes are broken using osmotic choc. After the last wash, transfer the mitochondria (radiolabeled or not) in a 1.5 mL ultracentrifuge tube and pellet mitochondria 10 min at 12 000 x *g* at 4ºC.

2. Resuspend the mitochondria in 1 mL of Swelling buffer and incubate 5 min on ice.

2. Mitochondria breakage is achieved by mixing the mitochondria for 10 seconds at 500 rpm with a shaker.

3. Pellet the membranes by a centrifugation at 100 000 x *g* 20 min at 4ºC.

4. After centrifugation, discard the supernatant and resuspend the membranes in 30 to 50 µL of Membrane buffer for labeling experiment (Figure 1-1) or in 100 to 300 µL for lipidomic analyses (Figure 1-2).

5. Estimate the concentration of mitochondria membrane proteins by Bradford [13]. We usually obtain around 50-100 µg of mitochondria membrane proteins per g of callus.

6. To isolate and analyze the MTL complex, mitochondria complexes are then solubilized with DDM. Membranes containing 20 µg of proteins are solubilized and loaded in one lane of CN-PAGE (*see* **Note** 17). For lipidomic analysis of the MTL complex, complexes from 4 lanes will be pooled, meaning that 80 µg of membrane proteins have to be solubilized per experiment. For membranes containing 20 µg of proteins, the solubilization is performed in a total volume of 20 µL of

Membrane buffer by the addition of DDM at a final concentration of 1.5 µg / µg of membrane proteins in a 1.5 mL ultracentrifuge tube (*see* **Note** 18).

8. The solubilization is achieved by pipetting slowly the mix 30 times followed by an incubation of 5 min on ice.

9. After incubation, pellet the insoluble materials by a centrifugation of 20 min at 100 000 x *g* at 4ºC.

10. After centrifugation, carefully pipette the supernatant without touching the pellet and mix it with Loading buffer 5X (5 µL for 20 µL of supernatant).

3.5 Isolation of the MTL complex by CN-PAGE

3.5.1 Preparation of the CN-PAGE

1. The native gels are prepared in a Bio-Rad Mini-Protean® gel system with 1 mm glass plates (*see* **Note** 19).

2. A 15-mL gradient mixer and a peristaltic pump are used to cast the gel. Put the chamber on a magnetic stirrer. Link the first chamber of the gradient mixer to the peristaltic pump. Before use, wash the gradient mixer and the pump with 30 mL of ultrapure water. Then, close the connections between the chamber 1 and the pump and between the two chambers and fixe the end of the pump tube on the top of the glass plates.

3. Add a small magnetic stir bar in the first chamber.

4. For one gel, prepare 3 mL of Separation gel 12 % and 3.5 mL of Separation gel 3.5 %.

5. Right away after the addition of TEMED, load 2 mL of Separation gel 12 % and 2.5 mL of Separation gel 3.5 % in the first and second chamber respectively and turn on the stirrer in the first chamber (*see* **Note** 20).

6. Turn on the pump (flux of 3 mL / min), open the connection between the chamber 1 and the pump tube and immediately open the connection between the chambers to allow the formation of the gradient. Wait until all the separation gel fills the glass plates. Normally, with these volumes of separation gels, about 1 cm remains at the top of the gel to allow the addition of the Concentration gel.

7. Carefully add water at the top of the gel to form a straight line of separation gel. Wait about an hour for the polymerization of the separation gel.

8. Eliminate the water by inversion of the gel on a paper.

9. Prepare (around 2 mL) and pour immediately the Concentration gel on top of the separation gel and add a 10 wells comb avoiding air bubble formation. Wait about an hour for the polymerization.

3.5.2 Loading of samples and separation of the complexes

1. Place the gel in the migration tank and add the Migration buffer.

2. Gently clean the wells and add the samples (25 µL/well) (*see* **Note** 21).

3. Operate the migration of the gel at 80 V until the bromophenol blue reach the bottom of the gel. It takes around 2h to 2h30 (*see* **Note** 22).

3.5.3 Fixation and staining of CN-PAGE

1. After the migration of the native gel, remove the gel from the glass plates and cut the concentration gel. Fixe the gel in 25 mL of Fixation solution for at least 30 min at room temperature (*see* **Note** 23).

2. After the fixation, replace the Fixation solution with 25 mL of Staining solution and incubate overnight at room temperature (*see* **Note** 24).

3. Discard the Staining solution and wash the gel with several incubations at room temperature with ultrapure water.

4. The MTL complex appears as a wavy band around 800 KDa (Figure 2, coomassie). In Col0, a decrease of the MTL complex molecular weight is observed in –Pi compared to + Pi. However, a smaller shift of the MTL complex is observed in *atmic60* callus suggesting an impairment in MTL complex remodeling during Pi starvation.

3.6 Analysis of [^{14}C]-DGDG incorporation into the MTL complex

1. If an UDP-[^{14}C]-galactose labelling have been performed (Figure 1-1), dry the gel on a Wattman paper for 2h using a gel dryer.

2. Expose the dried gel with a phosphoimager screen and reveal the radioactive signal with a laser scanner (*see* **Note** 25). Usually, a gel to screen exposure of 3 days is enough to visualize the radiolabeled galactoglycerolipids present in the MTL complex (Figure 2, [^{14}C] labelling). In a typical experiment for Col0, an increase by a factor 2 of the radioactive signal is observed in the MTL complex in –Pi compared to +Pi (Figure 2, [^{14}C] labelling) [8]. In *atmic60* callus, a decrease in the incorporation of [^{14}C]-galactoglycerolipids is observed in the MTL complex in –Pi compared to Col0 callus (Figure 2, [^{14}C] labelling). These results indicate that the rate of incorporation of DGDG into the MTL complex is altered in absence of AtMic60, suggesting an involvement of AtMic60 in lipid trafficking during Pi starvation.

3.7 Analysis of the lipid composition of mitochondria membranes and of the MTL complex

3.7.1 Lipids extraction from mitochondria membranes

1. Start the lipid extraction from a pellet of mitochondria membranes containing at least 200 µg of proteins prepared in part 3.4.5 (*see* **Note** 26).

2. Warm up a water bath with a hot block inside. Experiment can start when the water is above 80°C. In the same time, warm up an

Erlenmeyer flask closed with double aluminum foil containing 25 mL of absolute ethanol until it boils.

3. Lipid extraction: resuspend the mitochondria membranes in 1 mL of boiling ethanol, mix by pipetting and transfer the mitochondria membrane in a hemolysis tube.

4. Close the tube with double aluminum foil and dispose the tube in the hot block in the water bath for 5 min with some shaking with a pestle (*see* **Note** 27). This step will inhibit phospholipase D activity.

5. Remove the tube from the hot block, check the ethanol volume left in the tube and if necessary complete up to 1 ml with boiling ethanol. Add 0.5 mL of methanol to rinse the pestle directly in the tube. Remove the pestle and add 2 mL of chloroform. Blow argon in the solvent mixture to remove oxygen during 1 minute (*see* **Note** 28). Close the tube with foil and leave it for 1 h at room temperature.

6. Filter the liquid in a 15 mL Corex tube with a funnel plugged with ethanol washed glass wool to remove cell debris. Add 0.75 mL of chloroform:methanol (2:1 (v/v)) in the Corex tube containing the cell debris to rinse it and pour the liquid in the funnel.

7. Remove the funnel and add 1.25 mL of NaCl 1% to the filtrate mixture. Blow argon inside the tube to mix up solvents during 1 minute and centrifuge 10 min at 1 000 x *g* to separate the organic and aqueous phase.

8. Collect the lower phase (organic phase) with a Pasteur pipette (*see* **Note** 29) and transfer it in a clean hemolysis glass tube. Dry all the solvents by blowing argon on top of the liquid.

9. Rinse the tube with 200 µL of chloroform and dry lipids by blowing argon on top of the liquid. Close the tube and store the lipid extract at -20°C. Lipid extracts are stable for several months.

3.7.2 Lipids extraction from CN-PAGE

1. To perform a lipidomic analysis of the MTL complex (Figure 1-2), cut 4 bands of MTL complex per samples (equivalent of 80 µg of proteins of solubilized membranes) and pool them in a 5 mL hemolysis glass tube (*see* **Note** 30). As a control, two other bands located in different parts of the gel and a band corresponding to a lane without any protein are also cut for analysis and treated as the MTL complex sample (Figure 3A).

2. The lipids are then extracted with a protocol adapted from Bligh and Dyer [14]. Add 500 µL of H_2O and 1.875 µL of chloroform:methanol 1:2 (v/v) on the acrylamide bands.

3. Vortex and incubate the samples for 1h at room temperature under agitation to extract the lipids from the gel.

4. Add 625 µL of H_2O and 625 µL of chloroform to promote the formation of the biphase.

5. Vortex and centrifuge the tubes 5 min at 1 000 x *g*.

6. Transfer the lower organic phase in a new hemolysis tube and dry it under argon.

7. Perform a second extraction on the aqueous phase with 625 µL of chloroform.

8. After centrifugation 5 min at 1 000 x *g*, pool the second organic phase with the first one and dry the lipids under argon. Lipids can be stored dried at -20ºC before analysis by mass spectrometry.

3.7.3 Production of fatty acid methyl esters (FAMEs) from glycerolipids and free fatty acids by transesterification with methanol (methanolysis)

This step is performed only for lipids extracted from mitochondria membranes to estimate the quantity of lipids that will be used for mass spectrometry analysis. The quantity of lipids extracted from CN-PAGE is too low and all the extracted lipids will be used for mass spectrometry analysis.

1. Turn on the hot block at 100°C and pre-warm an aliquot of standard C15 solution (*see* **Note** 31).

2. Resuspend the lipid extract in 500 µL of chloroform. Uptake 50 µL of the lipid extract and transfer it into the methanolysis tube.

3. With a Hamilton syringe, add 10 µL of C15 solution (5 µg/tube) in the methanolysis tube.

4. Add 3 mL of methanolysis buffer in all the methanolysis tubes. In each tube there are: 10 µL of C15 solution, 50 µL of lipid extract and the methanolysis buffer. Close tightly the glass tube, vortex briefly and incubate 1 h at 100°C for the methanolysis reaction (esterification reaction) to occur.

5. Take the tube out of the hot block 5 min at room temperature to cool down the tube. Stop the reaction by adding 3 mL of water.

6. Add 3 mL of hexane to extract the FAMEs and vortex vigorously. Wait at least 20 min at room temperature to allow the biphase to form correctly. At this step, biphase can be stored a few days at 4°C if necessary. Take the upper phase (hexane phase) containing the FAMEs and transfer it in a hemolysis glass tube, dry it under argon.

7. Repeat step 6 to re-extract FAMEs from the methanol-water phase by adding again 3 mL of hexane in the methanolysis tube. Pour the upper phase in the same hemolysis glass tube than the one used in step 6 and dry it under argon.

8. To concentrate the FAMEs at the bottom of the glass tube, rinse the tube wall with 200 µL of hexane. Allow the liquid to rest at the bottom of the tube and dry it gently under argon.

9. Store the FAMEs at -20°C or proceed with GC analysis.

3.7.4 Quantification of FAMEs by Gas Chromatography – Flame Ionisation Detector (GC-FID)

1. Resuspend the FAMEs in 50 µL of hexane and transfer the FAMEs in an insert vial. Seal the vial tightly to avoid evaporation.

2. Inject 2 µL of the sample in the GC-FID on a BPX70 column. Nitrogen is used as carrier gas with 3.5 mL/min constant flow

compensation, split ratio is 13.3:1, injection temperature of 200ºC, detector temperature of 280°C and the oven temperature range start at 130°C, hold for 7.5 min at 130°C, ramp up to 180°C at 3°C/min, and hold 10 min at 180°C. This will allow FAMEs separation from 12 C up to 24 C in function of the chain length and the number of desaturation.

3. Each FAME is identified by comparison of its retention times with those of standards. FID response is dependent of the mass of the FAME therefore each FAME will be quantified by the surface peak method using C15 surface peak for calibration with the following equation:

$$Quantity\ in\ \mu g\ of\ FAME = \frac{Area\ of\ FAME\ peak \times Quantity\ in\ \mu g\ of\ C15\ (5\mu g)}{Area\ of\ C15\ peak}$$

4. By adding each FAME quantity and taking into account the volume used for the methanolysis, GC-FID analysis gives the total fatty acid content of the lipid extract in µg as well as its fatty acid composition. Fatty acid content in nmol can be established taking into account the molecular mass of each fatty acid.

3.7.5 Quantification of lipid molecules by LC/MS/MS

1. All the lipid extracted from CN-PAGE or 25 nmol of fatty acids from mitochondria membranes lipid extract are dissolved in 100 µL of Internal standard solution.

3. Lipids are then separated by HPLC and quantified by ESI-MS/MS. Lipid classes are separated using an HPLC system on a 150 mm×3 mm (length × internal diameter) 5 µm diol column at 40°C. The mobile phases consisted of hexane/isopropanol/water/ammonium acetate 1M, pH5.3 [625/350/24/1, (v/v/v/v)] (A) and isopropanol/water/ammonium acetate 1M, pH5.3 [850/149/1, (v/v/v)] (B). The injection volume is 20 µL, corresponding to 5 nmol of total fatty acid (*see* **Note** 32), and each sample is injected 3 times as technical replicates. After 5 min, the percentage of B was increased linearly from 0% to 100% in 30 min and stayed at 100% for 15 min. This elution sequence was followed by a return to 100% A in 5 min and an equilibration for 20 min with 100% A before the next injection, leading to a total runtime of 70 min. The flow rate of the mobile phase is 200 µL/min (*see* **Note** 33).

4. Mass spectrometric analysis is done on a triple quadripole mass spectrometer (*see* **Note** 34). The quadrupoles Q1 and Q3 were operated at widest and unit resolution respectively. Specific MRM (Multiple Reaction Monitoring) scans used to define molecular species need to be known (*see* **Note** 35). As MTL complex contains a small amount of lipids, only the main lipids (PC, PE and DGDG) are detected and quantified (*see* **Note** 36). Negligible amounts of lipids are detected in control bands (Figure 3A, crt1 and crt 2) showing that the MTL complex is truly enriched in lipids [8]. The Table 1 describes the acquisition settings for the glycerolipids analyzed.

5. To quantify each molecule of lipid, each chromatographic peak is integrated by the mass spectrometer software. The area of the peak is proportional to the quantity of the molecule. Because mass spectrometry detection efficiency is always molecule dependent, the

internal standard is used to correct molecule bias quantification. Each molecular species (ms_LIP) of a lipid class (lc_LIP) is corrected by its corresponding internal standard (lc_is) to neglect the matrix effect by applying the following formula with Q corresponding to the quantity in pmol:

$$Q(ms\_LIP) = \frac{Area\ (ms\_LIP)}{Area\ (lc\_is)} * Q(lc\_is).$$

Then all molecules of the same lipid class are summed to obtain the quantity of one lipid class:

$$Q\ (lc\_LIP) = \sum Q\ (ms\_LIP)$$

Data are then expressed in mol% of PC, PE and DGDG in the MTL complex or in mitochondria membranes (Figure 3B). Typically, in Col0 callus, we observe an increase in the DGDG content and a decrease in PE and PC in both mitochondria membranes and MTL complex. However, in absence of AtMic60, DGDG barely increase during Pi starvation. Interestingly, whereas the level of PE slightly decreases in mitochondria membranes, it increases in the *atmic60* MTL complex during Pi starvation, suggesting that PE accumulates inside the MTL complex. These results show that the traffic of both DGDG and PE is altered in *atmic60* mitochondria during Pi starvation.

4 Notes

1. Quartz wool corresponds to pure SiO_2 wool with fiber thickness from 4 to 12 µm. It does not react with solvent and do not contain lipids.

2. Rinse potter pestles with distilled water, then ethanol.

3. If the evaporator bath could warm: put the set temperature at 40°C maximum. It will decrease the evaporation time.

4. To prepare the standard solution at 0.5 mg/mL: put 25 mg of C15 in a 1.5 mL tube. Add 1 mL of chloroform/methanol 1/2 (v/v), make hand agitation. Transfer this solution in a graduated flask of 50 mL. Complete until 50 mL with chloroform/methanol 1/2 (v/v). Put a glass cap and parafilm around. Make some inversions. Put some milliliters of this C15 solution in a little beaker (to avoid evaporation) and realize aliquots of 500 µL and store them at -20°C.

5. Prepare the solution at 4°C to avoid overheating of the solution. Put 400 mL of methanol in a glass bottle, under agitation. Then, add slowly 10 mL of sulfuric acid. Put a glass cap and store at RT.

6. All the stock standard solutions need to be quantified by GC-FID to be sure the concentrations are accurate. If necessary volume will be adjusted to be sure the standard solution will be at 1.25 µM for each standard.

7. Solubilize 5 mg of DGDG in 1 mL of chloroform in a glass vial HPLC type. Add a small spatula of PtO_2 powder (catalyser). Add a small bar magnet (4 mm). Label the level of liquid on the vial. Close the vial, add at the center of the lid 2 thin needles to allow hydrogen to

get in and out. Needles need to be above the liquid. Prepare an Erlen with a lid insensitive to chloroform with 2 holes, one for the entrance of hydrogen and the other one for a "safety valve" constituted of a Pasteur pipette with a finger of cut latex glove. Put the HPLC vial into the Erlen with the gaz entrance of the Erlen connected to the first needle of the vial (use silicon tube and junctions). Put the Erlen above a magnet stirrer under the fume hood. Open the hydrogen bottle or generator to deliver 3.5 bars maximum to fill the Erlen with hydrogen then reduce the pressure to 1 bar. Leave overnight under small stirring. The following day recover the lipid by eventually adding chloroform and collecting the liquid. Quantify and check the DGDG composition by methanolysis and GC-FID (methods 3.7.3 and 3.7.4)

8. Here, settings are described for an HPLC Agilent 1200 and a triple quadripole Agilent 6460. Parameters might vary for other instruments.

9. To avoid pipetting of seeds, centrifuge the tubes few seconds at low speed and pipette carefully the solution without touching the seeds.

10. To obtain stable culture, transfer 50 mL of the 7 day old culture into a sterile 50 mL tube. Wait until the callus sediment (the volume of callus should not exceed 5 mL) and throw away the excess of media. Recover the callus by resuspending them with fresh media and transfer them into the culture flask. Complete the media to 200 mL.

11. Materials for mitochondria preparations (tubes, glassware…) have to be free of detergent. It is recommended to dedicate a batch of material to mitochondria preparation and to wash the material with distillated water only.

12. After filtration, cells have to be maintained at 4ºC before grinding. Thus, a pre-chilled mortar is stored on ice close to the balance. Cells are immediately deposited inside after weighting.

13. To add the 23 % and 18 % Percoll layers above the Percoll 40 % layer, we use a peristaltic pump. The tubes containing the 5 mL of Percoll 40 % are slightly inclined on ice and the exit tube of the pump is fixed at the top of the 32 mL tube. The entry tube of the pump is dived on a falcon containing 20 mL of Percoll 23 % and the pump is turned on. Usually, we use a flow rate of 1.5 mL/min to layer the Percoll 23 % and 18 % solutions. During the distribution with the pump, the solution has to slide on the surface of the tube. If a drop falls directly on the gradient, it can create perturbation of the gradient.

14. Mitochondria purified from MS –Pi are more contaminated by the plastid fraction at this step. Thus, some green material could be visible with the mitochondria at the 23/40 % interface.

15. At this step, the mitochondria pellet is not stable. Discard the supernatant by carefully pipetting the upper part of the tube. Leave around 5 mL in the tube to not loose mitochondria.

16. If labelling is performed, from this step, all samples will be radioactive and all materials and wastes in the subsequent steps have to be treated in consequence in appropriate waste disposals.

17. We usually load 20 µg of membranes per lane of gel. It is possible to load a higher quantity if necessary but we recommend to do not load more than 30 µg per lane to do not disturb the migration of the MTL complex.

18. For 20 µg of membranes, 30 µg of DDM is added. Thus, we add 3 µL of DDM 1 % (w/v) to 20 µg of membrane fraction and adjust the volume to 20 µL with Membrane buffer. Higher concentration of DDM can also be used but 1.5 % is the reference concentration we used to analyze the MTL complex.

19. The native gel can be casted the day before the experiment and stored at 4ºC. Do not use a gel that have been stored more than 48h at 4ºC.

20. At this step, it is important to go as fast as possible to avoid the polymerization of the gel in the gradient chamber or in the pump system. If more than one gel is required, it is better to cast one gel then, prepare a new mix of 12 % and 3.5 % separation gel and finally cast the second gel.

21. Do not vortex or heat the samples before loading in the gel.

22. Do not perform the migration at a voltage higher than 80 V to avoid the formation of a smear by the MTL complex.

23. The fixation can also be performed overnight.

24. This process of fixation and staining is compatible with a subsequent analysis by mass spectrometry

25. Add a thin plastic layer that do not absorb the radiation between the dried gel and the screen.

26. Mitochondria membranes can be stored at -80°C if long storage is required.

27. Be careful: samples should not dry. If there is almost no liquid left, take out the tube from the bath before 5 min of incubation and continue the protocol. If several extractions are done in the same time, it is recommended to treat each sample one by one at this step.

28. Open the argon very slowly after putting Pasteur pipette into the liquid to avoid any spilling of the solvent.

29. To avoid any contamination with the upper phase, use a propipette on top of the Pasteur pipette. Suck a little bit of air into the pipette, plunge it at the bottom of the tube, eject one or 2 bubbles that will reject any upper phase that might have penetrate into the pipette and suck up the lower phase.

30. The bands can be stored at -80°C if long storage is required.

31. Put the aliquot of standard C15 at least 20 min at room temperature before use. No overthrow but gently agitation.

32. We have verified that the method is within a linear range up to 15 nmol of total fatty acid.

33. The distinct glycerophospholipid classes were eluted successively as a function of the polar head group. Under these conditions, they were eluted in the following order: DGDG, PE and PC.

34. For a 6460 triple quadrupole mass spectrometer (Agilent) equipped with a Jet stream electrospray ion source the source parameters are the following settings: Drying gas heater: 260°C, Drying gas flow 13 L/min, Sheath gas heater: 300°C, Sheath gas flow: 11L/min, Nebulizer pressure: 25 psi, Capillary voltage: ± 5000 V, Nozzle voltage ± 1000. Nitrogen is used as collision gas.

35. To use this method, the studied organism glycerolipidome needs to be known. Only lipid transitions that are entered in the method will be measured. It is a targeted method.

36. After an initial analysis, only three lipids (PC, DGDG and PE) were detected in the MTL complex. If all the lipids need to be analyzed, you can find all the transitions and standards we usually use in our lab in [15].

Table 1. Transition and mass spectrometer parameters used to detect and quantify Arabidopsis lipids. Prec Ion: precursor ion; Res: resolution; Prod Ion: product ion; DGDG: digalactosyldiacylglycerol; PE: phosphatidylethanolamine; PC: phosphatidylcholine.

Segment 1: from 0 to 7 min										
Compound Group	**Compound Name**	**Prec Ion**	**MS1 Res**	**Prod Ion**	**MS2 Res**	**Dwell**	**Fragmentor**	**Collision Energy**	**Cell Accelerator Voltage**	**Polarity**
DGDG	DGDG-36-1	964	Widest	623	Unit	30	135	8	7	Positive
DGDG	DGDG-36-2	962	Widest	621	Unit	30	135	8	7	Positive
DGDG	DGDG-36-3	960	Widest	619	Unit	30	135	8	7	Positive
DGDG	DGDG-36-4	958	Widest	617	Unit	30	135	8	7	Positive
DGDG	DGDG-36-5	956	Widest	615	Unit	30	135	8	7	Positive
DGDG	DGDG-36-6	954	Widest	613	Unit	30	135	8	7	Positive
DGDG	DGDG-34-0	938	Widest	597	Unit	30	135	8	7	Positive
DGDG	DGDG-34-1	936	Widest	595	Unit	30	135	8	7	Positive
DGDG	DGDG-34-2	934	Widest	593	Unit	30	135	8	7	Positive
DGDG	DGDG-34-3	932	Widest	591	Unit	30	135	8	7	Positive
DGDG	DGDG-34-4	930	Widest	589	Unit	30	135	8	7	Positive
DGDG	DGDG-34-5	928	Widest	587	Unit	30	135	8	7	Positive
DGDG	DGDG-34-6	926	Widest	585	Unit	30	135	8	7	Positive
DGDG	DGDG-32-1	908	Widest	567	Unit	30	135	8	7	Positive
DGDG	DGDG-32-2	906	Widest	565	Unit	30	135	8	7	Positive
DGDG	DGDG-32-3	904	Widest	563	Unit	30	135	8	7	Positive
Segment 2: from 7 to 16 min										
Compound Group	**Compound Name**	**Precursor Ion**	**MS1 Res**	**Product Ion**	**MS2 Res**	**Dwell**	**Fragmentor**	**Collision Energy**	**Cell Accelerator Voltage**	**Polarity**
DGDG	DGDG-36-0	966	Widest	625	Unit	30	135	8	7	Positive
DGDG	DGDG-36-1	964	Widest	623	Unit	30	135	8	7	Positive
DGDG	DGDG-36-2	962	Widest	621	Unit	30	135	8	7	Positive
DGDG	DGDG-36-3	960	Widest	619	Unit	30	135	8	7	Positive
DGDG	DGDG-36-4	958	Widest	617	Unit	30	135	8	7	Positive
DGDG	DGDG-36-5	956	Widest	615	Unit	30	135	8	7	Positive
DGDG	DGDG-36-6	954	Widest	613	Unit	30	135	8	7	Positive
DGDG	DGDG-34-0	938	Widest	597	Unit	30	135	8	7	Positive
DGDG	DGDG-34-1	936	Widest	595	Unit	30	135	8	7	Positive
DGDG	DGDG-34-2	934	Widest	593	Unit	30	135	8	7	Positive
DGDG	DGDG-34-3	932	Widest	591	Unit	30	135	8	7	Positive
DGDG	DGDG-34-4	930	Widest	589	Unit	30	135	8	7	Positive
DGDG	DGDG-34-5	928	Widest	587	Unit	30	135	8	7	Positive
DGDG	DGDG-34-6	926	Widest	585	Unit	30	135	8	7	Positive
DGDG	DGDG-32-1	908	Widest	567	Unit	30	135	8	7	Positive
DGDG	DGDG-32-2	906	Widest	565	Unit	30	135	8	7	Positive
DGDG	DGDG-32-3	904	Widest	563	Unit	30	135	8	7	Positive
Std DGDG	DGDG-32-0	910	Widest	569	Unit	30	135	8	7	Positive
PE	PE-36-1	746	Widest	605	Unit	30	135	20	7	Positive
PE	PE-36-2	744	Widest	603	Unit	30	135	20	7	Positive
PE	PE-36-3	742	Widest	601	Unit	30	135	20	7	Positive
PE	PE-36-4	740	Widest	599	Unit	30	135	20	7	Positive
PE	PE-36-5	738	Widest	597	Unit	30	135	20	7	Positive
PE	PE-36-6	736	Widest	595	Unit	30	135	20	7	Positive
PE	PE-34-1	718	Widest	577	Unit	30	135	20	7	Positive
PE	PE-34-2	716	Widest	575	Unit	30	135	20	7	Positive
PE	PE-34-3	714	Widest	573	Unit	30	135	20	7	Positive
PE	PE-34-4	712	Widest	571	Unit	30	135	20	7	Positive
PE	PE-32-1	690	Widest	549	Unit	30	135	20	7	Positive
PE	PE-32-2	688	Widest	547	Unit	30	135	20	7	Positive
std PE	PE-36-0	748	Widest	607	Unit	30	135	20	7	Positive
Segment 3: from 16 to 40 min										
Compound Group	**Compound Name**	**Precursor Ion**	**MS1 Res**	**Product Ion**	**MS2 Res**	**Dwell**	**Fragmentor**	**Collision Energy**	**Cell Accelerator Voltage**	**Polarity**

PC	PC-36-1	788	Widest	184	Unit	30	135	34	7	Positive
PC	PC-36-2	786	Widest	184	Unit	30	135	34	7	Positive
PC	PC-36-3	784	Widest	184	Unit	30	135	34	7	Positive
PC	PC-36-4	782	Widest	184	Unit	30	135	34	7	Positive
PC	PC-36-5	780	Widest	184	Unit	30	135	34	7	Positive
PC	PC-36-6	778	Widest	184	Unit	30	135	34	7	Positive
PC	PC-34-0	762	Widest	184	Unit	30	135	34	7	Positive
PC	PC-34-1	760	Widest	184	Unit	30	135	34	7	Positive
PC	PC-34-2	758	Widest	184	Unit	30	135	34	7	Positive
PC	PC-34-3	756	Widest	184	Unit	30	135	34	7	Positive
PC	PC-34-4	754	Widest	184	Unit	30	135	34	7	Positive
PC	PC-32-0	734	Widest	184	Unit	30	135	34	7	Positive
PC	PC-32-1	732	Widest	184	Unit	30	135	34	7	Positive
PC	PC-32-2	730	Widest	184	Unit	30	135	34	7	Positive
Std PC	PC-36-0	790	Widest	184	Unit	30	135	34	7	Positive

Figure 1: Overview of the methods used to analyse defects in lipid trafficking in the MTL complex of *atmic60* mutant callus. The dashed boxes indicate the two strategies used: 1) study of the *in vitro* synthesis and transfer of radiolabelled galactoglycerolipids in the MTL complex; 2) study of the mitochondria membranes and MTL complex lipidome. DGDG: digalactosyldiacylglycerol, DGD: DGDG synthase, MGDG: monogalactosyldiacylglycerol, OE: outer envelope of plastids, Pi, phosphate, UDP-Gal*: UDP-[^{14}C]-galactose.

Figure 2: *In vitro* synthesis and transfer of radiolabelled galactoglycerolipids in the MTL complex of Col0 and *atmic60* callus grown in presence (+) or absence (-) of phosphate (Pi). After labelling, the MTL complex have been isolated by CN-PAGE. A coomassie staining of the CN-PAGE and its exposure to a phosphorimager plate are shown. The MTL complex is labelled by an asterisk. C: Col0, m: *atmic60*, MW: molecular weight (in KDa).

Figure 3: Lipidomic analysis by mass spectrometry of the mitochondria membranes and MTL complex of Col0 and *atmic60* callus grown in presence (+) or absence (-) of phosphate (Pi). A. CN-PAGE bands analysed by mass spectrometry. The MTL complex and two others complexes located at different parts of the gel were analysed (crt 1 and crt 2). A control corresponding to a band cut in a lane without proteins was also included. B. Lipid content of mitochondria membranes and MTL complex of callus grown in + or –Pi. No lipids were detected in the control bands. Only DGDG, PC and PE were detected in the MTL complex. Consequently, only these three lipids have been analysed in mitochondria membranes. The results are expressed in mol%. DGDG: digalactosyldiacylglycerol; PE: phosphatidylethanolamine; PC: phosphatidylcholine, C: Col0, m: *atmic60*, MW: molecular weight (in KDa). Statistical significance of the data was evaluated with two-tailed unpaired t-test using GraphPad software. *$p < 0.05$, **$p < 0.01$, n=3.

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