Lyso-Phosphatidic Acid Acyl-Transferases: a link with intracellular protein trafficking in Arabidopsis root cells?

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Highlight

Phosphatidic acid produced by Lyso-Phosphatidic Acid Acyl-Transferases has an impact on the efficiency of the intracellular trafficking of some proteins in *Arabidopsis thaliana* root cells.

Abstract

Phosphatidic acid (PA) and Lysophosphatidic acid acyltransferases (LPAATs) might be critical for the secretory pathway. Four extra-plastidial LPAATs (LPAAT2, 3, 4 and 5) were identified in *A. thaliana.* These AtLPAATs, display a specific enzymatic activity converting lysophosphatidic acid (LPA) to PA and are located in the endomembrane system. We investigate a putative role of the AtLPAATs 3, 4 and 5 in the secretory pathway of root cells through genetical (knock-out mutants), biochemical (activity inhibitor, lipid analyses) and imaging (live and immuno-confocal microscopy) approaches. Treating a *lpaat4;lpaat5* double mutant with the LPAAT inhibitor CI976 showed a significant decrease in primary root growth. The trafficking of the auxin transporter PIN2 was disturbed in this *lpaat4;lpaat5* double mutant treated with CI976, whereas trafficking of H^+-ATPases was unaffected. The *lpaat4;lpaat5* double mutant is sensitive to salt stress and the trafficking of the aquaporin PIP2;7 to the plasma membrane in the *lpaat4;lpaat5* double mutant treated with CI976 was reduced. We measured the amounts of neo-synthesized PA in roots, and found a decrease in PA only in the *lpaat4;lpaat5* double mutant treated with CI976, suggesting that the protein trafficking impairment was due to a critical PA concentration threshold.

Keywords

Arabidopsis, roots, secretory pathway, Lysophosphatidic acid acyltransferases (LPAATs), phosphatidic acid (PA), lysophosphatidic acid (LPA), PIN2, PIP2;7.

Introduction

Lipids are critical for organelle compartmentalization and membrane domain partition in all eukaryotic cells. In plant cells the involvement of lipids and their metabolism in the regulation of the plant secretory pathway is evident (Melser et al., 2011; Boutté and Moreau, 2014). It has been shown that all lipid families such as sterols (Laloi et al., 2007; Men et al., 2008; Boutté et al., 2010), sphingolipids (Melser et al., 2010; Markham et al., 2011; Wattelet-Boyer et al., 2016) and glycerolipids (Pleskot et al., 2012; Boutté and Moreau, 2014) are involved in regulating the secretory pathway . *In vivo* and *in vitro* studies in various eukaryotic models have shown that lipids play critical roles in endomembrane morphodynamics regulation, organelle morphology, trafficking as well as vesicle formation and fusion (Yang et al., 2008, 2011; Ha et al., 2012; Boutté and Moreau, 2014; Melero et al., 2018).

A role for several enzymes in phospholipid metabolism such as phospholipases and lysophospholipid acyltransferases (LPATs) in the secretory/retrograde pathways has been particularly highlighted in animal and yeast cells (Yang et al., 2008, 2011; Melero et al., 2018). In plant cells, several phospholipases have been shown to be required for the functionality of the secretory pathway (Li et al., 2007; Lee et al., 2010; Li et al., 2011; Kim et al., 2011). However, this is not the case for LPATs. Among LPATs, especially lysophosphatidic acid (LPA) acyltransferases (LPAATs) may be crucial for the functionality of the secretory pathway as, in animal cells, phosphatidic acid (PA) and its precursor LPA have been shown to have an

important impact on the functionality of the secretory pathway (Yang et al., 2008, 2011). Therefore, PA and LPA, in addition to their role as precursors for *de novo* phospholipid biosynthesis and their known involvement in many signalling pathways (Pokotylo et al., 2018; Yao and Xue, 2018), are of interest for the secretory pathway in plant cells.

The amount of cellular PA is dependent on the *de novo* synthesis via the Kennedy pathway but can be affected by the activity of multiple enzymes such as phospholipases D, diacylglycerol kinases or LPAATs. In addition, the sequential action of phospholipase C and a diacylglycerol kinase can increase the PA pool(s). In contrast, PA phosphatases or phospholipases A1/A2 cause a decrease in the amount of PA in the cells. A study carried out in *Nicotiana tabacum* indicated that pharmacological inhibition of most of these enzymes leads to very different effects on pollen tube growth (Pleskot et al., 2012), indicating that there could be different PA pools related to different enzyme activities. At present, no studies have been conducted to specifically investigate the putative role of LPAATs in the secretory pathway of plant cells. We hypothesize that LPAATs could, as shown for phospholipases, participate in the regulation of membrane curvature by catalyzing PA production from LPA (two molecules with different physicochemical properties) and therefore contribute to the regulation of membrane trafficking (Yang et al., 2008, 2011; Boutté and Moreau, 2014).

In *Arabidopsis thaliana*, several membrane-bound LPAATs have been identified (Kim and Huang, 2004; Kim et al., 2005; Wang et al., 2013; Körbes et al., 2016; Angkawijaya et al., 2017; Angkawijaya et al., 2019). In higher eukaryotes, these enzymes are named LPAT or LPAAT but here we propose to use the name LPAAT to precisely highlight their lysophosphatidic acid acyltransferase enzymatic activities. It is effectively inconsistent that the first identified isoform was named "LPAAT1" whereas the following isoforms were reported as "LPAT2-5" (Kim et al 2005). AtLPAAT1 has been suggested to be involved in the *de novo* synthesis of PA in plastids (Kim and Huang, 2004; Yu et al., 2004). The ER-located AtLPAAT2 (LPAT2; Kim et al., 2005) has been shown to be critical for female gametophyte development in Arabidopsis (Kim et al., 2005). In addition, the over-expression of AtLPAAT2, which stimulates the *de novo* production of phospholipids, resulted in enhanced primary root growth in phosphate-starved Arabidopsis seedlings (Angkawijaya et al., 2017). This suggests AtLPAAT2 to be a primordial enzyme for the *de novo* synthesis of PA in the ER. Three other potential LPAAT genes, *AtLPAAT3*, *AtLPAAT4* and *AtLPAAT5*, have been identified. Recently, Angkawijaya et al. (2019) have shown that AtLPAAT4 and AtLPAAT5 can be involved in the neo-synthesis of phospholipids and triglycerides in response to nitrogen starvation. Since AtLPAAT2 is probably the main source of PA for the *de novo* synthesis of phospholipids (Kim et al., 2005; Angkawijaya et al., 2017), we have investigated whether other AtLPAATs can be associated with a further role in membrane dynamics linked to the functionality of the secretory pathway.

We first showed that these AtLPAATs have an enzymatic activity specific for producing PA from LPA and that they are located in the endomembrane system (mainly the ER). Through genetic, biochemical and imaging approaches, we show that a *lpaat4;lpaat5* double mutant is sensitive to salt stress and is defective in primary root growth when treated with the LPAT inhibitor CI976 (Brown et al., 2008; Schmidt and Brown, 2009; Yang et al., 2011). In addition, the trafficking of the aquaporin PIP2;7 and the auxin carrier PIN2 is affected in these mutants.

By measuring the amounts of PA in the roots, we were able to link the disturbance of protein trafficking to a critical PA concentration threshold.

Material and Methods

Arabidopsis material and growth conditions

The *Arabidopsis thaliana* ecotype Colombia-0 (Col-0) and the following mutants were used: *lpaat3-1* (SALK_046680), *lpaat4-2* (GK_899A04) and *lpaat5-2* (SALK_020291). Double mutants *lpaat3-1;lpaat4-2, lpaat3-1;lpaat5-2* and *lpaat4-2;lpaat5-2* were obtained crossing the previously listed lines. The triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* was obtained crossing the double mutant *lpaat4-2;lpaat5-2* and SALK_046680. The transgenic fluorescent protein marker line pPIN2::PIN2–GFP (in Col-0; Xu et al., 2005) was crossed with the double mutant *lpaat4-2;lpaat5-2*.

Seeds were sterilized by treatment with 95% (v/v) ethanol for 10 seconds, followed by a bleach solution for 20 minutes, then repeatedly washed with sterile water. Seeds were then sown on 1/2 Murashige and Skoog (MS) agar medium plates (0.8% plant agar (Meridis #P1001,1000), 1% sucrose (Merck # 84100) and 2.5 mM morpholinoethanesulfonic acid (Euromedex # EU0033) pH 5.8 with KOH) left at 4 °C for 2 days and then grown vertically in 16 h light/8 h darkness for 5 days.

Inhibitor treatment

CI976 was used as a LPAT inhibitor (Merck # C3743). A 10 mM CI976 stock solution was prepared in dimethylsulfoxide (DMSO), stored at -20°C.. Seedlings were grown on 1/2 MS plates containing 10 µM of the inhibitor for all experiments, except to determine the WT, double mutant *lpaat4-2;lpaat5-2* and triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* lines sensitivity to the treatment, where 5 µM of inhibitor were also tested. In all experimental conditions, the final DMSO concentration was the same for the controls without CI976 and at the different concentration of CI976 used.

Phenotypical characterization

Seedlings were grown on 1/2 MS agar medium plate containing varying CI976 concentrations (0, 5 or 10 µM). Root length was measured 5 days after germination using the ImageJ software. To compare experiments, all the measured values were standardized to the WT median value in untreated condition for each experiment.

To assess the different lines sensitivity to salt stress, 50 mM NaCl (Euromedex # 1112) was added to the 1/2 MS agar medium.

Plasmid preparation and transgenic plants

Sequence data of AtLPAATs can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *AtLPAAT2*, At3g57650;

AtLPAAT3, At1g51260; *AtLPAAT4*, At1g75020; *AtLPAAT5*, At3g18850. Coding sequence of *AtLPAAT2*, *AtLPAAT4* were amplified on leaves cDNA, while *AtLPAAT3* and *AtLPAAT5* were amplified on flowers cDNA. We used respectively the primer pairs P1531/P1533, P1539/P1541, P1535/P1537 and P1543/P1545. To generate the following diK mutants: *AtLPAAT2 K387A/K389A*, *AtLPAAT4 K374A/K376A* and *AtLPAAT5 K371A/K375A* the following primer pairs containing the mutation were used: P5294/P5431, P2051/P2052 and P5292/P5432. *LPAAT3* diacidic mutant 1 (D74G/A75/E76G) and 2 (D293G/L294A/E295G) were obtained by overlapping PCR using respectively primers P2578/P2580/P2581/P2582/P66/P67 and P2578/P2580/P2585/P2586/P66/P67. Amplified sequences were cloned by BR reactions in entry vectors pDONR™221 or pENTR-d-TOPO™ (Thermofisher Scientific) using Gateway® recombinational cloning technology (Thermofisher Scientific). For expression in *E.coli*, entry vectors were cut by NcoI (Biolabs #R0193) and XhoI (Biolabs #R0146) restriction endonucleases. The product was cloned into the pET-15b vector (Novagen). For expression in plant, AtLPAAT entry vectors and pK7WGF2 destination vectors were combined by LR recombination using Gateway® recombinational cloning technology (Thermofisher Scientific).

To complement the double mutant *lpaat4-2;lpaat5-2* we generated the construct p*AtLPAAT4*:tagRFP-*AtLPAAT4g*. For that, we amplified the *AtLPAAT4* promoter sequence and the *AtLPAAT4* full length DNA genomic sequence using the primer pairs P5730/P5731 and P5736/5737. Each PCR product was purified and cloned respectively in pDONR^{TM} P4-P1r vector and pDONR^{TM} P2r-P3 vector (Thermofisher Scientific). We used a third vector containing tagRFP in pDONR^{TM}221 backbone (Thermofisher Scientific) and generated the final construct in the pH7m34GW destination vector using the Multisite Gateway® cloning system (Thermofisher Scientific).

All PCR amplifications were performed using Q5^{TM} High-Fidelity DNA polymerase at the annealing temperature and extension times recommended by the manufacturer (Biolabs #M04915). PCR fragments and plasmids were respectively purified with NucleoSpin® Gel and PCR cleanup (Macherey-nagel # 740609) and NucleoSpin® Plasmid (Macherey-Nagel #740499). All the entry vectors were sequenced, and sequences were analyzed with CLC Mainwork Bench 6. Primers used in this study are listed in Supplementary Table S1.

For transient expression in Arabidopsis cotyledons, seeds were sterilized as described above and sown in 6-wells culture plates containing 4 ml 1/2 MS agar medium. Plates were left at 4 °C for 2 days and then grown in 16 h light/8 h darkness for 7 days.

Constructs were transferred into the *Agrobacterium tumefaciens* C58C1Rif^{R} strain harboring the plasmid pMP90. 4 days after germination *Agrobacterium tumefaciens* suspension in MS-Glu liquid medium (0.21% MS (w/v), 2% glucose (w/v), 0.39% MES (w/v), 0.05%Tween, 200mM acetosyringone, pH5.7) was used to transform transiently the cotyledons. For that, seedlings were incubated 40 min at RT with suspension of *Agrobacterium tumefaciens* expressing natives or mutant LPAATs and HDEL marker at 1 OD_{600nm} and 0.2 OD_{600nm}

respectively. Suspension was then removed and plate were left in 16 h light/8 h darkness until the 7th day after germination.

To study the effect of the double mutation *lpaat4-2;lpaat5-2* on PIN2-GFP subcellular localization at the plasma membrane, the double mutant *lpaat4-2;lpaat5-2* and the pPIN2::PIN2–GFP transgenic line (Xu *et al.,* 2005) were crossed. Primer pairs 1905/1906 (LP/RP SALK-020291), 5744/5745 (LP/RP GABI_899A04) and LBa1 were used for genotyping on ammonium glufosinate (10 µg/ml) resistant seedlings.

RNA extraction, RT-PCR and qPCR

Tissues were disrupted using stainless steel beads 5mm (Qiagen#69989) and Tissuelyser II (Qiagen). Total RNA was extracted from roots 5 days after germination using the RNeasy® Plant Mini kit (Qiagen #74904) according to the manufacturer's instructions. First strand cDNA was synthesized using SuperScript® II Reverse Transcriptase (ThermoFisher # 18064014) and OligodT. Then, mRNAs were treated with DNase I using DNa-*free*™ Kit (ThermoFisher # AM1906). Expression analysis of *AtLPAAT2, AtLPAAT3, AtLPAAT4* and *AtLPAAT5* by RT-qPCRs were performed with the Bio-Rad CFX96 real-time system using GoTaq® qPCR Master mix (Promega # A6002). The specific primer pairs used for *AtLPAAT2, AtLPAAT3, AtLPAAT4, AtLPAAT5*, *EF-1α* and *At4g33380* were P5412/P5413, P5414/P5415, P5783/P5784, P5418/P5419, P4833/P4834 and P4847/P4848, respectively.

The transcript abundance in samples was determined using a comparative cycle threshold (C_t) method. The relative abundance of *EF-1α and At4g33380* mRNAs (Czechowski et al. 2005) in each sample was determined and used to normalize for differences of total RNA level as described in Pascal et al. (2013). Semi-quantitative RT-PCR analysis of steady-state At*LPAAT*s transcripts in roots from 5 days old plants was performed to compare *all the* mutant lines with WT plants. The *EF-1α* gene was used as a constitutively expressed control. We used GoTaq® G2 DNA Polymerase (Promega). All primers are listed in Supplementary Table S1, the characterisation of *lpaat* insertion mutant lines and T-DNA positions is shown in Supplementary Fig. S1, and controls for *all the* mutant lines are shown in Supplementary Fig. S2.

Immunocytochemistry, FM4-64 uptake, BFA treatment and confocal laser scanning microscopy

Whole-mount immunolabelling of Arabidopsis roots was performed as described in Boutté and Grebe (2014). In brief, 5 day-old seedlings were fixed in 4% paraformaldehyde dissolved in MTSB (50 mM PIPES, 5 mM EGTA, 5 mM $MgSO_4$ pH 7 with KOH) for one hour at room temperature (RT) and washed three times with MTSB. Roots were cut on superfrost slides (Menzel Gläser, Germany) and dried at RT. Roots were then permeabilized with 2% Driselase (Merck #D9515), dissolved in MTSB for 30 min at RT, rinsed four times with MTSB and treated for one hour at RT with 10% DMSO + 3% Igepal CA-630 (Merck # I3021) dissolved

in MTSB. Unspecific sites were blocked with 5% normal donkey serum (NDS, Merck # D9663) in MTSB for one hour at RT. Primary antibodies, in 5% NDS/MTSB, were incubated overnight at 4 °C and then washed four times with MTSB. Secondary antibodies, in 5% NDS/MTSB, were incubated one hour at RT and then washed four times with MTSB.

Primary antibodies were diluted as follows: rabbit anti-PIP2;7 1:400 (Agrisera, AS09469), rabbit anti-H^+-ATPase 1:1000 (Agrisera AS07260), rabbit anti-echidna 1:600 (Gendre et al., 2011 and Boutté et al., 2013), rabbit anti-Membrine11 1:300 (Marais et al., 2015), rabbit anti-SAR1 1:250 (Agrisera AS08326) . Dilution of secondary antibody donkey anti-rabbit IgG AlexaFluor 488-coupled (Jackson Immunoresearch, 711-605-152) was 1:300.

FM4-64 uptake was performed on 5 days old seedling that had grown on 1/2 MS agar media containing 10 µM of CI976. Seedlings were dark incubated in 5 µM FM4-64 solution for 5 min. Rapid wash in 1/2 MS solution containing 10 µM of CI976 followed, and FM4-64 uptake was determined each minutes on root epidermal cells by confocal microscopy from 3 to 15 min after seedlings wash. For BFA treatment, 5 day-old seedlings grown on ½ MS agar medium supplemented with 10 µM CI976 were incubated in liquid ½ MS medium with 10 µM CI976 and 50 µM Brefeldin A (BFA; Merck B7651). After 90 mins of incubation, PIN2 accumulation in BFA bodies was determined in roots by confocal microscopy.

Confocal laser scanning microscopy was performed using Zeiss LSM880. For live-cell imaging, seedlings were mounted with MS liquid medium. Laser excitation lines for the different fluorophores were 488 nm for GFP, AlexaFluor 488 and FM4-64, and 561 nm for mCherry. Fluorescence emissions were detected at 490–570 nm for GFP and AlexaFluor 488, 620-695 nm for FM4-64 and 580–660 nm for mCherry. Scanning was performed with a pixel dwell of 3 µs. In multi-labelling acquisitions, detection was in sequential line-scanning mode. An oil-corrected × 63 objective, numerical aperture=1.4 (C Plan Apochromat 63.0x1.40 OIL DIC UV VIS IR-M27) was used in immunolabelling and live-cell imaging experiments, except for FM4-64 uptake where an oil-corrected × 40 objective, numerical aperture=1,3 (Plan Apochromat 40.0x1.3 OIL DIC UV IR-M27) was used. Image analysis was performed using ZEN lite 2.6 2018 (Zeiss) and ImageJ software.

Tobacco leaf infiltration and confocal microscopy

Nicotiana tabacum (SR1 cv Petit Havana) plants were grown in a greenhouse for transient expression of fluorescent constructs according to Sparkes et al. (2006). In brief, each expression vector was introduced into the *Agrobacterium tumefaciens* strain GV3101 by heat shock transformation. Transformed colonies were inoculated into 5 ml of YEB medium (5 g/l beef extract, 1 g/l yeast extract, 5 g/l sucrose and 0.5 g/l $MgSO_4 \cdot 7H_2O$) with 50 µg/ml spectinomycin and rifampicin. The bacterial culture was incubated overnight in a shaker at 180 rpm 25 °C. 1 ml of the bacterial cultures was pelleted by centrifugation at 1800 g at room temperature for 5 minutes. Pellets were washed with infiltration buffer (5mg/ml glucose, 50mM MES, 2mM $Na_3PO_4 \cdot 12H_2O$ and 0.1 mM acetosyringone) and then resuspended in infiltration buffer. The

bacterial suspension was diluted in infiltration buffer to a final OD_{600} of 0.1 for each construct. The bacterial solution was injected into the underside of the tobacco leaf using a 1ml syringe. Infiltrated plants were incubated at 22°C for three days prior to imaging. Leaf epidermal samples were imaged using a Zeiss PlanApo 100×/1.46 NA oil immersion objective on a Zeiss LSM880 confocal equipped with an Airyscan detector. 512 × 512 pixel images were collected in 8-bit with 4-line averaging. For GFP excitation was set at 488 nm and emission at 495-550 nm; for RFP at 561 nm and 570-615 nm, respectively.

AtLPAAT enzymatic activity

The ORF for *AtLPAAT2-5* were amplified by PCR with respectively the sense/antisense primers P1539/1541 and P1543/1545 containing the XhoI and NcoI restriction sites. The PCR products were first subcloned in pGEM®-T Easy vector (Promega, Charbonnieres les Bains, France) before being cloned into the XhoI/NcoI sites of the pET-15 vector (Novagen, Merck Biosciences, Badsoden, Germany). C41 (DE3) *E. coli* bacteria (Avidis, Saint-Beauzire, France) were then transformed with the obtained plasmids. Ectopic expression of the ORF *AtLPAAT2-5* and *E. coli* membranes isolation were performed as described by Testet et al. (2005). *AtLPAAT2-5* were produced in membranes of *E. coli* C41 cell line that was specifically designed for the production of membrane proteins (Miroux and Walker, 1996). Lysophospholipid acyltransferase reactions were carried out in 100 µl of assay mixtures (50 mM Tris-HCl, pH 8) containing 1 nmol of LPA or the other lysophospholipids (either lysophosphatidylcholine, lysophosphatidylethanolamine, lysophsphatidylglycerol, lysophosphatidylinositol or lysophosphatidylserine) , 1 nmol of [^{14}C]oleoyl-CoA and 50 µg of membrane proteins. Reactions were incubated at 30°C and stopped at 30 min by adding 2 ml of chloroform/methanol (2:1, v/v) and 500µl of water. After the isolation of the organic phase, the aqueous phase was re-extracted with 2 ml of chloroform. The lipids were then purified by HPTLC according to Testet et al. (2005) and Ayciriex et al. (2012). The radioactivity incorporated into phospholipids was revealed using a STORM 860 PhosphorImager (GE Healthcare, Waukesha, WI) and quantified with ImageQuant TL software.

LPAAT activities were measured as nmol phospholipids formed.mg proteins^{-1}.30 min^{-1} and the values of the controls (corresponding to purified membranes from *E.coli* transformed with pET-15B empty vector) were normalized to 100 for each lysophospholipid and the corresponding activities for LPAAT2-5 were calculated accordingly for all the phospholipids. Values of the controls were normalized to 100 to take into account the variations observed in the activity levels between experiments, this allowed having the best conditions to compare data and to perform the statistical analysis.

14-C Acetate labeling and lipid analysis

For radiolabel feeding experiments, 20 roots of 5-day-old seedlings were cut with a razor blade and placed in vials containing 2 mL MS. To start the reaction, 200 nmol (10 µCi) [1-14C] acetate (Perkin Elmer Life Sciences) were added to each vial and the reaction was stopped after

4 h with 2 ml preheated isopropanol followed by 20 min incubation at 70°C. After transfer into 6 ml glass tubes, 2ml chloroform/methanol/hydrochloric acid (100:50:1; v/v/v) were added and the mixture was incubated overnight on an orbital rotator (40 rpm) at room temperature. Subsequently, each tube was centrifuged at 1000xg for 10 min. The organic phase was collected in new tubes and the roots were re-extracted with 2 ml chloroform/methanol (2:1, v/v) for 2h. After new centrifugation, the organic phases of each sample were combined, mixed with 1.5 ml 0.9% NaCl and centrifuged at 1000g for 10 min. The organic phases were evaporated to dryness, re-suspended in 40 µl chloroform/methanol (2:1, v/v) and stored at −20 °C. Radiolabeled products were analyzed by thin-layer chromatography using HPTLC Silica Gel 60 plates (Merck) and chloroform/methanol/water/acetic acid (30:17:2:1, v/v/v/v) as solvent to separate phospholipids. They were identified by co-migration with unlabeled standards, and quantification was done by autoradiography using a Storm 860 molecular imager (GE Healthcare).

Statistical analysis

All data analysed were unpaired (samples independent from each other). Normal distribution (Gaussian distribution) of the data set was tested using Shapiro–Wilk normality test. On data normally distributed, sample homoscedasticity was assessed using a Bartlett test before performing parametric tests. On data that were not normally distributed (or on data sets for which n <10), non-parametric tests were performed. To compare two data sets, Welch two sample *t*-tests were performed on normally distributed data sets, whereas Mann–Whitney test was used as non-parametric test. To compare multiple data sets, Kruskal–Wallis tests were used as non-parametric test. Tukey's test was used as a single-step multiple comparison procedure to find means significantly different from each other. All statistical tests were two-tailed (two-sided test). All statistical analyses were performed with the R i386 3.1.0 software. *P*-values were as follows: ^{NS}P-value>0.01 (not significant), $^{*}P$<0.05, $^{**}P$<0.01 and $^{***}P$<0.001.

Results

***In-vitro* AtLPAAT activities in *E.coli* C41 membranes**

Supplementary Fig. S3 shows the alignment of the amino acid sequences of the AtLPAATs 2-5 from Arabidopsis with the sequence of the human LPAAT3 (Schmidt and Brown, 2009). The boxes indicate conserved sequences which correspond to classical motifs of the LPAAT, DHAPAT, and LPEAT acyltransferase families (Lewin et al., 1999), indicating that AtLPAATs 2-5 are clearly acyltransferases. In *E.coli* and yeast expression systems, AtLPAAT2 and AtLPAAT3 showed lysophosphatidic acid acyltransferase activity but not for AtLPAAT4 and AtLPAAT5 (Kim et al., 2005). Recently, Angkawijaya et al. (2019) showed lysophosphatidic acid acyltransferase activity for AtLPAAT5 expressed in *E.coli* C41 cells but not for AtLPAAT4 using this expression system. They identified AtLPAAT4 activity upon

overexpression in *A. thaliana* plants. However, it is still unknown whether these enzymes only utilize LPA or whether they can also handle other lysophospholipids as substrates. To investigate this, AtLPAAT2-5 were expressed in membranes of an *E.coli* C41 cell line specifically designed for the production of membrane proteins (Miroux and Walker, 1996), and activities were measured *in vitro* as described in the experimental section and according to Testet et al. (2005). Purified membranes of *E.coli* C41 cells were incubated with LPA or the other lysophospholipids (lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylglycerol, lysophosphatidylinositol or lysophosphatidylserine) and [^{14}C]oleoyl-CoA to measure either the formation of [^{14}C]PA or that of the other potentially labeled phospholipids (phosphatidylcholine, phosphatidylethanolamine, phsphatidylglycerol, phosphatidylinositol or phosphatidylserine). Purified membranes from *E.coli* transformed with a pET-15B empty vectorwere used as controls to measure *E.coli* endogenous activities. Lysophospholipid acyltransferase activities were measured as nmol phospholipids formed.mg proteins^{-1}.30 min^{-1} and the values of the controls were normalized to 100 for each lysophospholipid, and the corresponding activities for AtLPAAT2-5 were calculated accordingly for all the phospholipids.

Fig. 1 shows the mean enzymatic activities of AtLPAAT2-5 from three independent experiments. Only LPA acyltransferase activities were detected for the AtLPAAT2-5 and no other lysophospholipid acyltransferase activity was observed, demonstrating that the four AtLPAATs are clearly strict lysophosphatidic acid acyltransferases. It was a very important point to determine in order to investigate the potential role of PA synthesized by these enzymes in the functionality of the secretory pathway.

The activities of PA synthesis were calculated as 1.25 nmol PA.mg proteins^{-1}.30 min^{-1} for AtLPAAT2, 1.08 nmol PA.mg proteins^{-1}.30 min^{-1} for AtLPAAT3, 1.72 nmol PA.mg proteins^{-1}.30 min^{-1} for AtLPAAT4 and 1.75 nmol PA.mg proteins^{-1}.30 min^{-1} for AtLPAAT5. This indicated similar levels of activities in *E. coli* C41 cell membranes, which does not necessarily reflect their level of activities *in planta*.

Subcellular localization of AtLPAATs in Arabidopsis

AtLPAAT2, 4 and 5 have previously been shown to be located in the ER (Kim et al., 2005; Angkawijaya et al., 2019). However, the subcellular localization of the AtLPAAT3 has not been determined. To investigate its membrane localization, roots of an Arabidopsis stable line (5 days after germination) expressing both GFP-LPAAT3 and the ER marker mCherry-HDEL were analysed using high-resolution confocal microscopy (Fig. 2). In contrast to AtLPAAT2, 4 and 5, GFP-AtLPAAT3 was not localized to the ER network but labelled round structures in close proximity to the ER (Fig. 2A-F). We further investigated AtLPAAT3 localization using a heterologous expression system in tobacco leaf epidermal cells in which the ER is more dynamic and accessible than in root cells. Interestingly, here tagRFP-AtLPAAT3 and the ER-export sites (ERES) marker SAR1a-GFP colocalized (Fig. 2G-I). This indicates that

AtLPAAT3 may localise to ERESalso in Arabidopsis roots . The AtLPAAT3 amino acid sequence indeed features di-acidic motifs (D74-X-E76 and D293-X-E295) which could serve as ER export signals and may explain the different localization compared to the other LPAATs. Transient expressionof GFP-LPAAT3 di-acidic mutants 1 (D74G/A75/E76G) and 2 (D293G/L294A/E295G) in Arabidopsis cotyledons together with the ER marker mCherry-HDEL showed significant redistribution of both mutant AtLPAAT3 form to the ER network (Fig. 3)indicating that these motifs may be functional.

AtLPAAT2, AtLPAAT4 and AtLPAAT5 do not cycle between the ER and the Golgi

As shown in Supplementary Fig. S3, AtLPAAT2, AtLPAAT4 and AtLPAAT5, but not AtLPAAT3, feature potential di-lysine motifs at their C-termini (KXK, KXKXX and KXXXK motifs, respectively). Although these motifs are not very typical di-lysine motifs, they may indicate that the enzymes could potentially cycle between the ER and Golgi/post-ER compartments. To investigate this possibility, we mutated the potential cycling motifs (GFP-LPAAT2 *K387A/K389A*, GFP-LPAAT4 *K374A/K376A* and GFP-LPAAT5 *K371A/K375A*) and expressed these mutant fluorescent constructs in Arabidopsis cotyledons together with the ER marker mCherry-HDEL. As shown in Fig. 4, all the mutated versions of the AtLPAAT2, AtLPAAT4, and AtLPAAT5 were still located in the ER and did not label any round structures which could correspond to Golgi/post-ER compartments. Therefore, removing the C-terminal di-lysine motifs did not affect protein localization, indicating that these enzymes do not cycle between the ER and the Golgi or post-ER compartments, excluding any role of these proteins at the level of the Golgi membranes.

Since AtLPAAT2 is most likely the main enzyme responsible for *de novo* synthesis of phospholipids in the ER (Kim et al., 2005; Angkawijaya et al., 2017), AtLPAAT3, 4 and 5 were investigated for involvement in the ER trafficking machinery and their importance for the secretory pathway. Since AtLPAAT3-5 are expressed at similar levels in roots (Supplementary Fig. S4), we chose primary root growth as a phenotypic readout for the functionality of the secretory pathway.

Primary root growth phenotype and sensitivity to CI976 of *lpaat* mutants

Since an *AtLPAAT2* KO mutant is lethal (Kim et al., 2005), we first looked at the primary root growth of the single KO mutants *lpaat3-1* (Angkawijaya et al., 2017), *lpaat4-2 and lpaat5-2* (different alleles from those used by Angkawijaya et al., 2019) 5 days after germination. The characterisation of *lpaat* insertion mutant lines and T-DNA positions is shown in Supplementary Fig. S1. As shown in Fig. 5A, none of these mutants had a decrease in their primary root growth which could be due to complementation by other AtLPAAT proteins (representative images of seedlings are shown in Supplementary Fig. S5). Therefore, we produced the three double mutants *lpaat3-1;lpaat4-2, lpaat3-1;lpaat5-2 and lpaat4-2;lpaat5-2* and analysed the primary root length 5 days after germination (Fig. 5B). A significant but weak

root length phenotype was observed for all the double mutants (see also Supplementary Fig. S5 for representative images). As a consequence, we decided to produce the triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* and analysed again the primary root length in these mutant plants. Surprisingly, primary growth was not inhibited but stimulated in the triple mutant (Supplementary Fig. S6). Since the over-expression of *AtLPAAT2* stimulates the *de novo* synthesis of phospholipids, resulting in an enhanced primary root growth in phosphate-starved Arabidopsis seedlings (Angkawijaya et al., 2017), we wondered whether the expression of *AtLPAAT2* was increased in the triple mutant *lpaat3-1;lpaat4-2;lpaat5-2*. To check this hypothesis, semi-quantitative RT-PCR and real-time RT-PCR analyses of *AtLPAAT2* transcripts were performed in roots 5 days after germination (Supplementary Fig. S7). In comparison to WT plants, the transcription of *AtLPAAT2* was indeed increased in the triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* but not in the double mutant *lpaat4-2;lpaat5-2*. This indicates that *AtLPAAT2* is over-expressed in the triple mutant *lpaat3-1;lpaat4-2;lpaat5-2*, and could compensate for the absence of the three other AtLPAATs resulting in an increase in primary root growth. The triple mutant also contained the same level of PA as WT plants Fig.7A). We attempted an inducible RNAi approach to knock-down the expression of LPAAT2 in the triple mutant background to check whether this could restore the phenotype to the *lpaat4/5* double mutant. Unfortunately, either the drop in the expression was too low to observe a phenotype, or a lethal phenotype was observed, and no lines were obtained with intermediary conditions for such an approach to be possible.

This raises the question how to investigate a putative role for AtLPAATs in the secretory pathway with double mutants showing only a weak growth-inhibition phenotype and a triple mutant showing even an increase in primary root growth? To address this, we took advantage of our experience in combining genetic and biochemical approaches used in investigating the role of sphingolipids in protein trafficking at the Golgi body (Melser et al., 2010; Wattelet-Boyer et al., 2016). CI976 is an inhibitor of LPAT enzyme activities that interferes with both COPII- and COPI-dependent membrane traffic processes (Brown et al., 2008; Schmidt and Brown, 2009; Yang et al., 2011). The effects of this inhibitor on primary root growth were analysed in the double mutant *lpaat4-2;lpaat5-2* (with no increase in the transcription of AtLPAAT2) and the triple mutant *lpaat3;lpaat4;lpaat5*. The double mutant *lpaat4-2;lpaat5-2* was chosen for this study as both AtLPAAT4 and AtLPAAT5 localize to the ER and therefore the inhibitor would affect the ERES-localised AtLPAAT3 . A small but statistically not significant decrease in primary root growth was observed in WT plants. The triple mutant *lpaat3-1;lpaat4-2;lpaat5-* was not affected by the inhibitor which was expected due to overexpression of *AtLPAAT2.* A significant decrease in primary root growth was observed in the double mutant *lpaat4-2;lpaat5-2* treated with 10 µM of CI976 (Fig. 6; representative images of seedlings shown in Supplementary Fig. S5). Higher concentrations of CI976 induced a decrease in primary root growth in WT and mutant lines, justifying 10 µM as the functional concentration of the inhibitor CI976 for these assays. We also checked whether the inhibitor

could enhance the transcription of *AtLPAAT2.* Here transcription was similarly increased in the WT and in the double mutant *lpaat4-2;lpaat5-2* but to a much lesser extent than in the triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* (Supplementary Fig. S7). Therefore, treating the double mutant *lpaat4-2;lpaat5-2* with 10 µM of CI976 permitted to establish primary root growth phenotypic conditions and allowed investigating the efficiency of protein trafficking.

PA biosynthesis in Arabidopsis roots is only affected in the double mutant treated with CI976

Since disturbing AtLPAAT activities led to a decrease in primary root growth, the amount of neo-synthesized PA in Arabidopsis roots of the WT, the double mutant *lpaat4-2;lpaat5-2* and the triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* with or without CI976 treatment was quantified. As shown in Fig. 7A (black columns), in the absence of CI976 the amount of neo-synthesized PA in the double mutant *lpaat4-2;lpaat5-2* was reduced to 60% of that of the WT and the triple mutant *lpaat3-1;lpaat4-2;lpaat5-2*. This was correlated only to a weak primary root growth phenotype as mentioned before (Fig. 5). Treatment of the WT and the triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* with 10µM CI976 (Fig. 7A, red columns) decreased neo-synthesized PA by 60%, reaching a level of neo-synthesized PA similar to that found in the double mutant *lpaat4-2;lpaat5-2* (Fig. 7A, black column). Treating the *lpaat4;lpaat5* double mutant with 10 µM CI976 (Fig. 7A, red column) led to an additional decrease in PA which reached only about 30-35% of WT and triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* levels (Fig. 7A, black columns). In these conditions, we observed a stronger primary root growth phenotype (Fig. 6). This may indicate a concentration threshold for PA with no clear or very weak phenotype at concentrations above this level and a clear primary root growth phenotype at concentrations below (Fig. 6).

A small decrease in the amount of phospholipids was observed in the double mutant *lpat4-1;lpat5-1* in the study of Angkawijaya et al. (2019). Therefore, an additional question was to determine whether the primary root growth phenotype observed in the CI976-treated double mutant *lpaat4-2;lpaat5-2* was only due to a decrease in the neo-synthesis of PA or whether the neo-synthesis of the major phospholipids was also affected by the CI976 treatment and could contribute to the primary root growth phenotype. For this, we measured the level of the neo-synthesis of the two major phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from [^{14}C] acetate in WT roots, the double mutant *lpaat4-2;lpaat5-2* and the triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* treated with CI976. Beside the significant decrease in labeled PA in the CI976-treated double mutant *lpaat4-2;lpaat5-2* compared to the WT and the triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* (Fig. 7A, red columns), we did not observe any significant decrease in the amounts of labeled PC and PE (Fig. 7B). This indicates that the synthesis capability of these major phospholipids is similar in the CI976-treated double mutant *lpaat4-2;lpaat5-2* compared to the CI976-treated WT and triple mutant *lpaat3-1;lpaat4-2;lpaat5-2*. Therefore, the primary root growth phenotype observed in the

CI976-treated double mutant *lpaat4-2;lpaat5-2* (Fig. 6) can be mainly attributed to the disturbance of PA metabolism unrelated to the neo-synthesis of the major phospholipids. By combining genetic and pharmacological approaches, we determined the best conditions, i.e. the *lpaat4;lpaat5* double mutant treated with 10 µM CI976, to investigate the potential role of these LPAATs in the functioning of the secretory pathway.

Efficiency of protein secretion in the CI976 treated double mutant *lpaat4-2;lpaat5-2*

To investigate the efficiency of protein trafficking in the CI976 treated double mutant *lpaat4-2;lpaat5-2*, we decided to look *in situ* at several plasma membrane markers (H^+-ATPases, PIN2 and PIP2,7) with already characterized trafficking to the plasma membrane (Melser et al., 2010; Wattelet-Boyer et al., 2016; Hachez et al., 2014).

We first investigated the trafficking of H^+-ATPases to the plasma membrane using an immunocytochemistry approach. Supplementary Fig. S8 shows that the trafficking to the plasma membrane of H^+-ATPases was not affected in the CI976-treated double mutant *lpaat4-2;lpaat5-2* compared to the WT. As a consequence, the trafficking to the plasma membrane of H^+-ATPases did not seem to require the LPAAT-dependent production of PA.

To investigate the impact of LPAATs on PIN2 trafficking to the plasma membrane, we crossed the stable line pPIN2::PIN2–GFP (Xu and Scheres, 2005) with the WT and the double mutant *lpaat4-2;lpaat5-2* line. Roots were grown with or without 10 µM CI976. Upon CI976 treatment, we observed an increase in intracellular PIN2 in the double mutant (Fig. 8), indicating that the trafficking of PIN2 was disturbed. We also observed that the polarity index of PIN2 was affected with a value of 2.1 for the double mutant compared to 4.3 for the WT (P-value < 0.001). As a consequence, we also tested the effect on gravitropism but could not find a significant effect which could be related to compensation by other factors.

As an approach to try identifying the compartment(s) where PIN2 was retained, we used an immunostaining strategy using antibodies raised against various compartments of the secretory pathway, Echidna (ECH, marker of the SYP61 compartment, Gendre et al., 2011; Boutté et al., 2013), SAR1 (an ERES marker, Hanton et al., 2007) and Membrine11 (Memb11, a *cis*-Golgi marker, Marais et al., 2015). Immunostaining upon CI976 treatment of the double mutant *lpaat4-2;lpaat5-2* expressing PIN2-GFP was performed (Supplementary Fig. S9A-J). A significant co-localization was observed with ECH but not with SAR1 and Memb11 (Supplementary Fig. S9K) indicating that PIN2 was mainly retained at the level of the TGN. We also carried out BFA treatment on both CI976-treated WT and *lpaat4;lpaat5* double mutant plants, and observed the same BFA bodies (Supplementary Fig. S10) with a higher labeling in the case of the double mutant, and without other intracellular structures labeled. This confirmed that PIN2 was effectively present in TGN-derived structures in the *lpaat4;lpaat5* double mutant treated by BFA.

To investigate the impact of AtLPAATs on PIP2;7 trafficking to the plasma membrane, the subcellular location of PIP2;7 in the WT, the double mutant *lpaat4;lpaat5* and the triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* treated with 10 µM CI976 was analysed. For this, an

immunocytochemistry approach to reveal the *in situ* localization of PIP2;7 was used. Whole-mount immunolabelling of Arabidopsis roots was performed as described previously (Boutté and Grebe, 2014). As shown in Fig. 9, a decrease in the mean fluorescence ratio of plasma membrane to cytoplasm was observed for the double mutant *lpaat4-2;lpaat5-2* compared to the WT and the triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* which corresponded to both a decrease in PIP2;7 in the plasma membrane and an increase in the protein amount in the cytoplasm. It was reported that PIP2;7 is highly upregulated under salt stress (Pou et al., 2016) which also increases *AtLPAAT4* gene transcription in roots (Supplementary Fig. S11). We first checked for a sensitivity of the double mutant *lpaat4-2;lpaat5-2* to salt stress. The double mutant *lpaat4-2;lpaat5-2* was effectively more sensitive to salt stress than the WT and the triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* at 50 mM NaCl (Fig. 10). Higher salt concentrations up to 150 mM resulted in stronger phenotypes but without significant differences between the WT plants and the mutant lines. Interestingly, looking at the sensitivity of the different lines to salt stress in the presence of CI976 (Fig. 10), we observed that the double mutant *lpaat4-2;lpaat5-2* became less sensitive in the presence of the drug, and reached the value observed with the WT and the triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* (Fig. 10). This is most likely due to less PIP2;7 localizing to the plasma membrane in double mutant *lpaat4-2;lpaat5-2* root cells under CI976 treatment than in WT and triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* (Fig. 9). Complementation of the double mutant *lpaat4-2;lpaat5-2* by overexpression of AtLPAAT4 (*AtLPAAT4* relative transcript abundance in the double mutant *lpaat4-2;lpaat5-2* is shown in the Supplementary Fig. S12), resulted in a partial restoration of the localization of PIP2;7 at the plasma membrane (Fig.9). These results together with the fact that salt stress increases *AtLPAAT4* gene transcription in roots (Pou et al., 2016) suggest that PA formed by some LPAATs is involved in both the correct functionality of the secretory pathway and lipid signaling processes.

The increase in the amounts of PIP2;7 and PIN2 in intracellular membranes was interpreted as a consequence of a decrease in the trafficking of these proteins to the plasma membrane. However, the same result could have been the consequence of an increase in their internalization. To test whether this might have been the case, we measured endocytosis of the marker FM4-64 in both WT and double mutant *lpaat4-2;lpaat5-2* lines treated with CI976. As shown in the Supplementary Fig. S13, it was found that the internalization of the marker FM4-64 did not increase but was rather slightly decreased. As a consequence, it is unlikely that the increase of PIP2;7 and PIN2 was due to an increase in endocytosis. Therefore, our results strongly argue that a decrease in the trafficking efficiency of PIP2;7 and PIN2 to the plasma membrane explains the increase of these protein amounts in intracellular compartments.

In addition, our results show that the disruption of the AtLPAAT activities affects to some extent the efficiency of the secretory pathway followed by PIP2;7 and PIN2 but not H^+-ATPases, suggesting either different sensitivities to PA for their trafficking process or different requirements for it.

Discussion

PA is a central phospholipid metabolic intermediate and essential for the *de novo* synthesis of membrane lipids. It is also a key second messenger and source of other signalling lipids for numerous signalling pathways activated during stress conditions (Pokotylo et al., 2018; Yao and Xue, 2018). PA, like phosphoinositide and phosphatidylserine, is involved in the differentiation of various electrostatic compartments in the cell (Platre et al., 2018) and has been shown to interact more or less specifically with numerous proteins involved in a large variety of cell functions (Pokotylo et al., 2018). PA may also contribute to the function of the plant secretory pathway through its physico-chemical properties (Furt and Moreau, 2009; Boutté and Moreau, 2014). From a mechanical point of view, PA is a cone-shaped lipid favouring negative membrane curvature, and its precursor lysophosphatidic acid has the tendency to favour positive membrane curvature due to its inverted cone-shape. Both of these lipids can stimulate physico-chemical mechanisms linked to membrane morphodynamics depending on the membrane leaflet they are produced on (Boutté and Moreau, 2014). Yang et al. (2011) have demonstrated the interplay between phospholipase A2 and LPAAT in regulating COPI vesicles versus tubule formation from Golgi membranes in mammalian reconstituted systems. More recently, lysophospholipids have been shown to be critical in the formation of COPII vesicles by inducing the required membrane deformation (Melero et al., 2018). Lysophospholipids are also involved in PIN intracellular trafficking (Lee et al., 2010) as well as pollen germination and development (Kim et al., 2011). In addition to enzymes such as phospholipases which have been shown to be involved in membrane trafficking in plant cells (Li et al., 2007; Lee et al., 2010; Li et al., 2011; Kim et al., 2011), acyltransferases which are potentially involved in lipid metabolism in the Land's cycle may have an impact on membrane morphodynamics in plant cells (Boutté and Moreau, 2014).

Interestingly, Pleskot et al. (2012) have shown different roles for PA produced by either phospholipases D or diacylglycerol kinases in pollen tube growth. Their results strongly suggest that several pools of PA may exist according to the biosynthetic pathway followed by PA and the cellular process concerned. Similarly, different LPAATs could produce different pools of PA linked to various cellular processes (*de novo* lipid synthesis for membrane formation, lipid synthesis for stress-related responses, lipid synthesis for mechanical processes in membrane trafficking etc…). The aim of this study was therefore to investigate the possibility that some LPAATs might have a role in the secretory pathway through PA neo-synthesis not related to the bulk neo-synthesis of membrane phospholipids.

First, we determined that the four extra-plastidial AtLPAAT proteins (AtLPAAT2-5) are strict LPA acyltransferases which was a prerequisite for such a study. Then, we confirmed that AtLPAAT2, 4 and 5 are ER-localised and that they do not cycle between the ER and Golgi bodies. The localization of AtLPAAT3 was unknown but we showed that AtLPAAT3 is located in round structures corresponding to ERES.

Since AtLPAAT2 is likely the primary enzyme for the *de novo* synthesis of PA sustaining the overall *de novo* synthesis of phospholipids in the ER (Angkawijaya et al., 2017), we focused our attention to AtLPAAT3-5. Given the results obtained on root growth phenotypes with the single, double and triple mutants, the strategy was to create a combined genetic and biochemical approach as already managed successfully for the study of sphingolipids in the plant secretory pathway (Melser et al., 2010; Wattelet-Boyer et al., 2016). Treating the double mutant *lpaat4-2;lpaat5-2* with the LPAT inhibitor CI976 (Brown et al., 2008; Schmidt and Brown, 2009; Yang et al., 2011) defined experimental conditions in which a significant decrease in neo-synthesized PA without any decrease in the neo-synthesis of the major phospholipids PC and PE was obtained.

This suggests that under these experimental conditions other types of LPAT (LPCAT and LPEAT which synthesize new PC and PE from lysophosphatidylcholine and lysophosphatidylethanolamine), that are present in all lines (double mutant *lpaat4-2;lpaat5-2* as well as the WT and the triple mutant *lpaat3-1;lpaat4-2;lpaat5-2*), are not greatly affected. The effect of the drug was therefore likely related to its action on the LPAATs. Since the major phospholipids PC and PE were not decreased, it is likely that the amount of PA synthesized was still sufficient for sustaining phospholipid synthesis but not sufficient for its role related to the trafficking of PIP2;7 and PIN2. Therefore, the effects observed on primary root growth and protein trafficking could likely be attributed to the inhibition of "specific" pool(s) of PA and that a threshold concentration of PA was required for the trafficking of some proteins.

The fact that PIN2 was partially retained at the TGN but not significantly at the ERES is intriguing compared to the potential localization of AtLPAAT3. A first possibility could be that some of these AtLPAATs are present in the TGN but at a concentration that was not detected/detectable in our approach. In addition, AtLPAAT3 localization seemed to depend on active ER export motifs supporting its presence in a post-ER compartment. However, none of the AtLPAAT2-5 have so far been identified in Golgi body/TGN proteomes (Drakakaki et al., 2012; Parsons et al., 2012; Groen et al., 2014; Heard et al., 2015). Another possibility would be that ER-TGN connections may support the feeding of PA to the TGN but no clear relationship has been demonstrated between these two compartments in plant cells as shown in mammalian cells (Mesmin et al., 2017). In addition, Li and Xue (2007) have shown that PA (produced by PLDζ2) is required for the normal cycling of PIN2-containing vesicles and stimulates the gravitropic response. In our conditions, the gravitopic response was not significantly affected. Moreover, Li and Xue (2007) have observed that, in *pldζ2* and PLDζ2 -deficient plants, endocytosis in root cells was strongly decreased, leading to smaller BFA compartments. This was likely due to a decrease in the formation of PA at the plasma membrane. In our case, endocytosis was not significantly reduced, and therefore, BFA compartments could still be alimented by both reduced exocytosis at the TGN and endocytosis. Therefore, the effects of PA decrease did not have the same consequences when comparing PLDζ2 -deficient plants and the CI976-treated *lpaat4;lpaat5* double mutant plants, suggesting that different PA pools are used.

Since PIN2 labeling was high in the *lpaat4;lpaat5* double mutant treated with CI976 and BFA (Supplementary Fig. S10) with similar BFA bodies as compared to the WT, it is likely that PA decrease through inhibition of ER LPAAT activities resulted in a disturbance of ER-Golgi-TGN trafficking of PIN2 to the plasma membrane, and we do not exclude that PIN2 recycling back to the plasma membrane could have also been affected. As shown in Fig. 9, the potential impact of LPAATs on PIP2;7 trafficking to the plasma membrane was evidenced through both a decrease in PIP2;7 on the plasma membrane and its increase in the cytoplasm. With PIP2;7 being highly regulated under salt stress (Pou et al., 2016) and *AtLPAAT4* gene transcription being enhanced in roots under these conditions (Supplementary Fig. S11), we could perform critical experiments supporting our conclusion that PIP2;7 trafficking to the plasma membrane is linked to LPAAT activities: (i) the double mutant *lpaat4-2;lpaat5-2* was more sensitive to salt stress than the WT and the triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* (Fig. 10), (ii) the double mutant *lpaat4-2;lpaat5-2* became less sensitive to salt stress in the presence of CI976 (Fig. 10), (iii) PIP2;7 localized less to the plasma membrane of double mutant *lpaat4-2;lpaat5-2* root cells under CI976 treatment (Fig. 9), (iv) complementation of the CI976-treated double mutant *lpaat4-2;lpaat5-2* by overexpression of AtLPAAT4 partially restored the localization of PIP2;7 at the plasma membrane (Fig.9). Therefore, we were able to correlate the efficiency of PIP2;7 trafficking to the plasma membrane with the functionality of LPAATs. By using a PA-specific optogenetic biosensor which determines the precise spatio-temporal dynamics of PA at the plasma membrane, Li et al. (2019) showed that salt stress triggers an accumulation of PA via the activity of a phospholipase Dα1 (PLDα1). Investigating a pldα1 mutant indicated that PA signalling integrates with cellular pH dynamics to mediate plant responses to salt stress (Li et al., 2019). Therefore, it is likely that PA is involved in both signalling and mechanistic processes in regulating various fundamental biological functions in plants.

Unfortunately, because the immunostaining strategy was not possible with PIP2;7 (primary antibodies being from rabbit as those used for the markers), we could not address the question of the nature of the compartments where PIP2;7 was retained. Hachez et al. (2014) found that PIP2;7 interacts with the SNAREs SYP61 and SYP121 to reach the plasma membrane, and we determined that the sorting of PIN2 at the TGN occurs at a SYP61 TGN sub-domain (Wattelet-Boyer et al., 2016) where PIN2 was partially retained. We may hypothesize that PIP2;7 was to some extent retained in the same compartment as PIN2. In addition, it has been shown that under salt stress, loss of phospholipase D (PLD) function impairs auxin redistribution and this resulted in decreased primary root growth (Wang et al., 2019). Therefore, these plasma membrane proteins may have similar dependencies on some aspects of the trafficking machinery, and may also be similarly dependent on a specific formation of PA. Moreover, the data of Wang et al. (2019) indicate a role of PA in coupling extracellular salt signaling to PA-regulated PINOID kinase dependent PIN2 phosphorylation and polar auxin transport. In conclusion, PA produced by different enzymes (PLDs, LPAATs…) at different intracellular sites (early secretory pathway, late secretory pathway, plasma membrane) may regulate diverse

715 aspects of protein trafficking, dynamics and lipid signaling functions. The recent implication of
716 AtLPAAT4 and AtLPAAT5 in nitrogen-starvation response (Angkawijaya et al., 2019) also
717 illustrates how the same lipid metabolizing enzymes can be engaged in multiple different
718 cellular functions (Pokotylo et al., 2018).
719 Phospholipases and therefore lysophospholipids are involved in membrane trafficking in roots
720 and pollen (Lee et al., 2010; Kim et al., 2011). and AtLPAATs have a role in the trafficking of
721 PIP2;7 and PIN2 and primary root growth. Hence an interplay between phospholipases and
722 LPAATs is likely to occur in plant cells as already demonstrated in mammalian cells (Yang et
723 al., 2011). Pagliuso et al. (2016) identified a key component (CtBP1-S/BARS) of a protein
724 complex that is required for fission of several endomembranes in mammalian cells which binds
725 to and activates a *trans*-Golgi LPAAT protein. This interaction is essential for fission of
726 transport vesicles. Interconversion of LPA and PA probably facilitates the fission process either
727 directly or indirectly (through binding of protein(s) of the machinery to PA). In addition, the
728 production of PA by phospholipase(s) D can also be critical for membrane trafficking in plant
729 cells (Pleskot et al., 2012) as evidenced in mammalian cells for vesicle fission (Yang et al.,
730 2011), suggesting that PA produced by different enzymes (phospholipase D, LPAATs) can be
731 involved at different steps or pathways. We must also consider another potential complexity
732 since some enzymes (cytosolic or membranous) may be active both as phospholipase
733 (producing lysophospholipids from phospholipids) and acyltransferase (to reform a
734 phospholipid) in order to contribute to membrane deformation/re-arrangements involved in the
735 fusion/fission processes via lipid remodeling (Ghosh et al., 2009; Jasieniecka-Gazarkiewicz et
736 al., 2016).
737 In addition, since the trafficking of H^+-ATPases to the plasma membrane was not affected, this
738 suggests either different sensitivities of the trafficking process of different proteins to PA
739 concentration or different molecular requirements for their trafficking. Such a difference could
740 be related to what has been observed in the role of PA in pollen tube growth (Pleskot et al.,
741 2012). Hence several different mechanisms/pathways need to be considered in the complexity
742 of the regulation of protein trafficking.
743 Finally, since other lipid families (sterols, sphingolipids…) are also critical in the functioning
744 and regulation of the plant secretory pathway (Laloi et al., 2007; Men et al., 2008; Boutté et al.,
745 2010; Melser et al., 2010; Markham et al., 2011; Wattelet-Boyer et al., 2016), we must consider
746 that a huge interplay between lipids, lipid-synthesizing/modifying enzymes and lipid-binding
747 proteins is at work to govern and regulate the plant secretory pathway.
748 In conclusion, we have designed an experimental set up which allowed investigating the
749 potential involvement of AtLPAATs and PA in the functioning of the plant root secretory
750 pathway. The double mutant *lpaat4-2;lpaat5-2* treated with the LPAT inhibitor CI976 was
751 significantly affected in primary root growth and the trafficking of PIP2;7 and PIN2 was found
752 to be disturbed. Our results support a critical PA concentration threshold involved in the
753 trafficking of some proteins through the plant root secretory pathway. Since phospholipases

and therefore lysophospholipids are involved in protein membrane trafficking in roots (Lee et al., 2010), the implication of AtLPAATs in the trafficking of PIP2;7 and PIN2 in roots also suggests an interplay between phospholipases and LPAATs in root cells as shown in mammalian cells (Yang et al., 2011).

Supplementary data

Supplementary Table 1. Primers used in the study

Supplementary Fig. S1 Caracterization of *lpaat* insertion mutant lines and T-DNA positions

Supplementary Fig. S2 AtLPAAT3, AtLPAAT4 and AtLPAAT5 expression levels in the different mutant genetic backgrounds

Supplementary Fig. S3

Alignment of the Human LPAAT3 with the AtLPAATs 2-5 from Arabidopsis

Supplementary Fig. S4 *AtLPAAT3*, *AtLPAAT4* and *AtLPAAT5* are expressed at similar levels in *Arabidospis* roots

Supplementary Fig. S5 Images of seedlings from the different lines

Supplementary Fig. S6 Primary root length of the triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* is stimulated

Supplementary Fig. S7 *AtLPAAT2* expression level in primary roots

Supplementary Fig. S8 Trafficking of PM H^+ATPases is not altered upon CI976 treatment

Supplementary Fig. S9 PIN2-GFP accumulates in punctuated structures that colocalise with a TGN marker in CI976-treated *lpaat4-2,lpaat5-2* mutant

Supplementary Fig. S10 Effect of BFA treatment on CI976-treated seedlings

Supplementary Fig. S11 Salt stress (NaCl 150 mM) impact on *LPAAT4* gene expression in roots

Supplementary Fig. S12 Expression of *AtLPAAT4* in 5-days old *Arabidopsis* roots

Supplementary Fig. S13 Endocytosis is not accelerated in the double mutant *lpaat4-2;lpaat5-2* line treated with 10µM of CI-976

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Author contributions

V.W-B., M.L.G., Y.B., J-J.B and P.M. conceived the different parts of the study; V.W-B. performed most of the experiments; M.L.G. performed the *in vitro* LPAAT activities in *E.coli*; F.D-D. and L.M-P. performed the assays on the effect of CI976 on phospholipid *de-novo* synthesis; V.K. performed the colocalization of LPAAT3 with SAR1. V.W-B., Y.B. and P.M. analyzed the microscopy data; V.W-B., J-J.B and P.M. analyzed all the data and P.M.

supervised the experiments; P.M. wrote the article with contributions of all the authors; P.M. is the author responsible for contact and ensures communication.

Data availability statement

The data supporting the findings of this study are available within the paper and within its supplementary data.

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Figure legends

Fig. 1 *In-vitro* AtLPAAT2-5 LPA acyltransferase activities in *E.coli* C41 membranes

AtLPAAT2-5 were produced in membranes of *E. coli* C41 cell line that was specifically designed for the production of membrane proteins (Miroux and Walker, 1996), and activities were measured *in vitro* as described in the experimental section and according to Testet et al. (2005). Activities were tested with lysophosphatidic acid (LPA) and the other lysophospholipids (LPL: either lysophosphatidylcholine, lysophosphatidylethanolamine, lysophsphatidylglycerol, lysophosphatidylinositol or lysophosphatidylserine). Lysophospholipid acyltransferase activities were measured as nmol phospholipids formed/mg proteins/30min and the values of the controls (corresponding to purified membranes from *E.coli* transformed with pET-15B empty vector) were normalized to 100 for each lysophospholipid and the corresponding activities for AtLPAAT2-5 were calculated accordingly for all the phospholipids. Only LPA acyltransferase activities were detected for the AtLPAAT2-5 and no other lysophospholipid acyltransferase activity was detected, determining that AtLPAAT2-5 are strict LPA acyltransferases.

Fig. 2 Subcellular localization of AtLPAAT3 in various plant models

Roots, 5 days after germination, of an Arabidopsis stable line expressing both GFP-LPAAT3 (a,d) and the ER marker mCherry-HDEL (b,e). AtLPAAT3 was found in punctate structures in close proximity to the ER (c,f).

A transient expression of tagRFP-LPAAT3 (g) and the ERES marker SAR1a-GFP (h)in *Nicotiana tabacum* leaf epidermis cells suggested that the punctate structures observed for AtLPAAT3 in roots could potentially correspond to ERES (i).Scale bars 1µm.

Fig. 3 Subcellular localization of AtLPAAT3 depends on active ER export motifs

Transient expression of LPAAT3 GFP fused native forms (a), GFP-LPAAT3 di-acidic mutant 1 (d), GFP-LPAAT3 di-acidic mutant 2 (g) and the ER marker mCherry-HDEL (b,e,h) in Arabidopsis cotyledons. The mutation of each diacidic motif induces redistribution of LPAAT3 into the ER network (f,i). Scale bars 5µm.

Fig. 4 AtLPAAT2, AtLPAAT4 and AtLPAAT5 do not cycle between the ER and the Golgi
Transient expression of GFP-LPAAT2 diK mutant (b), GFP-LPAAT4 diK mutant (f), GFP-LPAAT5 diK mutant (j) and the ER marker mCherry-HDEL (c,g,k) in Arabidopsis cotyledons. Results are compared to transient co-expression of GFP-fused native forms of AtLPAAT2 (a), AtLPAAT4 (e) and AtLPAAT5 (i) with the ER marker mCherry-HDEL.
Mutations of AtLPAAT2, AtLPAAT4 and AtLPAAT5 diK motifs do not impact their location to the ER network (d,h,l). Scale bars 5µm.

Fig. 5 Primary root growth of *lpaat* KO mutants (A) is not impaired and that of *lpaat* KO double mutants (B) is significantly but only slightly impaired
Primary root length was measured 5 days after germination. Data are mean values ± SE, n = 40 (*lpaat* KO mutants), n = 80 (*lpaat* KO double mutants). Statistics were done by Kruskal-Wallis rank sum test, NS = not significant, **P-value <0.01, ***P-value < 0.001.

Fig. 6 Sensitivity of WT, double mutant *lpaat4-2;lpaat5-2* and triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* lines to CI976 treatment
Seedlings were grown on MS agar medium plates containing different concentrations of CI976 (0, 5, 10µM). Primary root length was measured 5 days after germination and standardized to the untreated condition for each line. Data are mean values ± SE from three biological replicates, n = 60. The asterisks indicate significant difference between untreated condition (black bar) and CI976 treated conditions (red bars). Statistics were done by non-parametric Kruskal-Wallis rank sum test, ****P*-value < 0.001, NS = not significant.

Fig. 7 CI976 treatment affects the neo-synthesis of PA but not that of the major phospholipids in Arabidopsis roots
Seedlings were grown on MS agar medium plates or MS agar medium plates complemented with 10 µM CI976 or not. Primary roots from 20 seedlings were collected 5 days after germination for both conditions and incubated 4 hours with [14C] acetate +/- 10 µMCI976. Lipids were extracted and separated by HPTLC.
A. Amounts of neo-synthesized [14C] PA, [14C] PC and [14C] PE were quantified for the WT line and the mutant lines *lpaat4-2;lpaat5-2* and *lpaat3-1;lpaat4-2;lpaat5-2*. The amounts of [14C] PA, produced were calculated for each line without treatment (black bar) or under treatment (red bar) by taking as 100 the amounts of [14C] PA produced in the WT line without treatment. Data are mean values ± SD from three biological replicates. Statistics were carried

out using the non-parametric Kruskal-Wallis test, similar letters above bars indicate that dataset are not significantly different, b/a and c/b: P-values < 0.001.
B. [14C] PC and [14C] PE produced were quantified in the WT line, the double mutant line *lpaat4-2;lpaat5-2* and the triple mutant line *lpaat3-1;lpaat4-2;lpaat5-2* upon CI976 treatment, and compared to the amounts measured for each line without treatment. The % of labeling of PC and PE in the presence of CI976 was expressed as compared to the untreated conditions taken as equal to 100. Data are mean values ± SD from three biological replicates. Statistical analysis used the non-parametric Kruskal-Wallis test and the p values for PC (0.393) and PE (0.288) show no significant differences.

Fig. 8 Auxin carrier PIN2-GFP trafficking is altered in double mutant *lpaat4-2;lpaat5-2* upon CI976 treatment

Localization of PIN2-GFP in WT (a) and *lpaat4-2,lpaat5-2* (b) background upon 10 µM CI976 treatment. Sum of fluorescense intensity per μm^2 was calculated in the cytoplasm for each line upon inhibitor treatment. The intracellular increase in PIN2-GFP in *lpaat4-2,lpaat5-2* upon CI976 treatment is shown (c) (n=27 cells quantified over 8 independent roots). All the data were represented for each line (black dots) with the median of each dataset (red bar). Statistics were done by non-parametric Kruskal-Wallis rank sum test, ***P-value < 0.001.

Fig. 9 Double mutant *lpaat4-2;lpaat5-2* is highly sensitive to salt stress as compared to WT, and triple mutant *lpaat3-1;lpaat4-2;lpaat5-2*

Seedlings were grown on MS agar medium plates supplemented or not with NaCl 50 mM. The same experiment was performed with (a) or without (b) CI976 treatment. Primary root length was measured 5 days after germination. Values were standardized to WT for each condition. Results indicate a higher sensitivity from the double mutant *lpaat4-2;lpaat5-2* to salt stress in comparison to the WT and triple mutant *lpaat3-1;lpaat4-2;lpaat5-2* lines (a), while this sensitivity is lost upon CI976 treatment (b).
Data are mean values ± SE from three biological replicates (n = 150). Statistics were done by non-parametric Kruskal-Wallis rank sum test, ***P-value < 0.001.

Fig. 10 Aquaporin PIP2;7 trafficking is altered in double mutant *lpaat4-2;lpaat5-2* upon CI976 treatment

Immunolocalization of the Aquaporin PIP2;7 in root epithelial cells from *Arabidopsis* WT (a), double mutant *lpaat4-2,lpaat5-2* (b), *triple mutant lpaat3-1;lpaat4-2,lpaa5-2* (c) and double mutant *lpaat4-2,lpaat5-2* over-expressing LPAAT4 (d) lines upon CI976 treatment. Scale bar represents 5µm. Mean fluorescence intensity was measured at the plasma membrane and in the cytosol for each line upon treatment. The ratio of fluorescence intensity between the plasma membrane and the cytosol was calculated. (e) All the ratios were represented for each line

(black dots) with the median of each dataset (red bar). Statistics were done by non-parametric Kruskal-Wallis rank sum test, ***P-value <0.001, NS: not significant.