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The *Arabidopsis thaliana* lysophospholipid acyltransferase At1g78690p acylates a variety of lysophospholipids including *bis*(monoacylglycero)phosphate

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ABSTRACT

When the lysoglycerophospholipid (GPL) acyltransferase At1g78690 from *Arabidopsis thaliana* is overexpressed in *Escherichia coli* a headgroup acylated GPL, acyl phosphatidylglycerol (PG), accumulates despite that *in vitro* this enzyme catalyzes the transfer of an acyl chain from acyl-CoA to the *sn*-2 position of 1-acyl phosphatidylethanolamine (PE) or 1-acyl PG to form the *sn*-1, *sn*-2, di acyl PE and PG respectively; it does not acylate PG to form acyl PG. To begin to understand why the overexpression of a lyso GPL acyltransferase leads to the accumulation of a headgroup acylated GPL in *E. coli* we investigated the headgroup specificity of At1g78690. Using membranes prepared from *E. coli* overexpressing At1g78690, we assessed the ability of At1g78690 to catalyze the transfer of acyl chains from acyl-coenzyme A to a variety of lyso GPL acyl acceptors including lyso-phosphatidic acid (PA), -phosphatidylcholine (PC), -phosphatidylserine (PC), -phosphatidylinositol (PI) and three stereoisoforms of *bis*(monoacylglycero)phosphate (BMP). The predicted products were formed when lyso PI and lyso PC were used as the acyl acceptor but not with lyso PC or lyso PA. In addition, At1g78690 robustly acylates two BMP isoforms with *sn*-2 and/or *sn*-2′ hydroxyls in the *R*-stereoconfiguration, but not the BMP isoform with the *sn*-2 and *sn*-2′ hydroxyls in the *S*-stereoconfiguration. This strongly suggests that At1g78690 is stereoselective for hydroxyls with *R*-stereochemistry. In addition, this robust acylation of BMPs by At1g78690, which yields acyl PG like molecules, may explain the mechanism by which At1g78690 so strikingly alters the lipid composition of *E. coli*.

1. Introduction

At1g78690 is a lysoglycerophospholipid found in *Arabidopsis thaliana*. At1g78690 is a member of the 1-acyl-glycerol-3-phosphate O-acyltransferase (AGPAT) family of acyltransferases [1,2]. This class of acyltransferases play important roles in both *de novo* glycerophospholipids (GPL) biosynthesis and in the remodeling of the acyl chain composition of GPLs in eukaryotic cells [3,4]. These lysophospholipid acyl transferases (LPLATs), which utilize acyl-CoAs as the acyl donor, show specificity for both the 1-linked acyl chain length and saturation as well as the headgroup attached to the phosphate. For example, AGPAT1 has preference for lyso phosphatidic acid (PA) but AGPAT9 acylates both lyso phosphatidylcholine (PC) and lyso phosphatidylglycerol (PG). Both of these AGPATs have preference for oleate at the 1-position of the lyso GPL [4]. At1g78690 acylates 1-acyl lyso phosphatidylethanolamine (PE) and 1-acyl lyso phosphatidylglycerol (PG) *in vitro* [5], showing broader substrate specificity than many other AGPATs.

When At1g78690 is overexpressed in *Escherichia coli*, a headgroup acylated form of PG accumulates. Direct acylation of PG is not likely the origin of this acyl PG as At1g78690 does not acylate di-acyl PG [5]. It is unclear how a LPLAT such as At1g78690 overexpression in *E. coli* leads to the accumulation of acyl PG.

Acyl PG is a polyglycerophospholipid classified as a diacylglycerolphosphomonoacylglycerol (DPM) [6,7]. Acyl PG has been identified in a number of organisms including *E. coli* [8] mycoplasma [9] *Corynebacterium* [10] plants [11] and *Trichomonas* [12] yet its function in the cell is not fully understood. There are four different stereoisoforms of DPMs that can be distinguished by

Abbreviations: AGPAT, 1-acyl-glycerol-3-phosphate O-acyltransferase; LPLAT, lysophospholipid acyltransferase; GPL, glycerophospholipid; CoA, coenzyme A; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; cp, cyclopropane; DPM, diacylglycerophosphomonoacylglycerol; BMP, *bis*(monoacylglycero)phosphate; LC–MS, liquid chromatography–mass spectrometry; MS/MS, collision-induced mass spectrometry.

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the stereochemistry of the two glycerol moieties. For acyl PG, the *sn*-1 and *sn*-2 hydroxyls are acylated and the phosphate is attached to the *sn*-3 and *sn*-1′ hydroxyls and we designate as 3,1′ DPM. Other DPM isoforms, 1,3′ DPM, 3,3′ DPM and 1,1′ DPM are also possible but because the structure of acyl PG is under characterized it is not known if these isoforms are present in cells in addition to 3,1′DPM.

Another polyglycerophospholipid classified as a monoacylglycerophosphomonoacylglycerol [6,7] is commonly referred to as *bis*(monoacylglycero) phosphate (BMP). Like DPMs, other isoforms of BMP are possible – 3,1′ BMP, 3,3′ BMP, and 1,1′ BMP. The major BMP isoform found in eukaryotic cells is 1,1′ BMP [13,14]. In *E. coli* BMP has been identified but the stereochemical confirmation has not been confirmed [15]. While acyl PG in *E. coli* is formed by the direct acylation of the headgroup of PG using a 2-acyl lyso PG as the acyl donor [16,17]. Acyl PG and other DPM isoforms could also be formed by the acylation of BMP.

To begin to understand the how At1g78690 alters the lipid levels of *E. coli* we assessed the headgroup specificity of At1g78690 using a liquid chromatography mass spectrometry (LC–MS) based *in vitro* assay. Here we show that lyso PS and lyso PI are substrates for At1g78690 as well as two different isoforms of BMP.

2. Materials and methods

2.1. Materials

Solvents for extraction were reagent grade from Sigma. Solvents for high performance liquid chromatography were CHROMASOLV® Plus, HPLC grade from Sigma Aldrich. Other chemicals were purchased from VWR or Sigma–Aldrich. GPLs, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (1-acyl lyso PG), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate (1-acyl lyso PA), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phospho-L-serine (1-acyl lyso PS), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phospho-(1′-myo-inositol) (1-acyl lyso PI), egg lyso PC (1-acyl lyso PC), *sn*-(3-oleoyl-2-hydroxy)-glycerol-1-phospho-*sn*-3′-(1′-oleoyl-2′-hydroxy)-glycerol (3,1′ BMP), *sn*-(3-oleoyl-2-hydroxy)-glycerol-1-phospho-*sn*-1′-(3′-oleoyl-2′-hydroxy)-glycerol (1,1′ BMP), *sn*-(1-oleoyl-2-hydroxy)-glycerol-3-phospho-*sn*-3′-(1′-oleoyl-2′-hydroxy)-glycerol (3,3′ BMP), arachidonyl-CoA (20:4-CoA) and eicosapentaenoyl-CoA (20:5-CoA)were from Avanti Polar Lipids, Alabaster, AL. Oligonucleotides were from Eurofins MWG Operon. Vent DNA Polymerase, NdeI, BamHI, Calf Intestinal Phosphatase (CIP), T4 DNA Ligase were from New England Biolabs.

2.2. Cloning of pHis-At1g78690

The *A. thaliana* At1g78690 was PCR amplified using Vent DNA Polymerase from pAt1g78690p [5] using the following primers: N-terminal primer 5′-CTGGGA*CATATG*GGGAAGATAATGGA ATGG GC-3′ and C-terminal primer 5′-ATGGTA*G GATCC*TCAC AACCGCTT GGCTAAGAG-3′ which introduce a NdeI site (italics) at the N-terminus and a BamHI site (italics) at the C-terminus. Following amplification, the products were purified using the Qiagen QIAquick PCR purification kit, digested with NdeI and BamHI and ligated into similarly digested pET15b (that was also treated with CIP) using T4 DNA ligase. The ligation mixture was transformed into chemically competent DH5α and transformants selected for on lysogeny broth (LB) [18] agar plates containing 100 μg/ml ampicillin (LB-amp plate). Colonies that contained possible clones were grown inoculated into 3 mL of liquid LB media supplemented with 100 μg/ml ampicillin and grown overnight, shaking at 225 rpm, 37 °C. Plasmids were mini-prepped using the Qiagen QIAprep Spin mini plasmid prep kit. The possible clones were digested with NdeI and BamHI to confirm the presence of the insert and then sent for sequencing at Molecular Cloning Laboratories.

2.3. Growth of E. coli

pET15b and pHis-At1g78690 were transformed into chemically competent *E. coli* BLR(DE3)pLysS and grown as described previously [5]. Cells were harvested by centrifugation for 20 min at 2600*g* and washed with 15 mM Tris, pH 7.4. Cell pellets were frozen at −80 °C until further use.

2.4. Preparation of cell-free extracts and membranes

The cell pellet from a 50 ml culture was re-suspended in 2 ml of 15 mM Tris, pH 7.4 and cells lysed in a French Pressure cell at 18,000 psi. The lysate was centrifuged at 2600*g* to pellet un-lysed cells. The resulting cell-free extract was transferred to a fresh tube and stored at −80 °C.

Washed-membranes were prepared by centrifuging the cell-free extract at 4 °C, 100,000*g* for 1 h. The membrane pellet was re-suspended by homogenization in 2 ml of 15 mM Tris, pH 7.4 and centrifuged again as described above. The final washed membrane pellet was re-suspended by homogenization in 2.0 ml of 15 mM Tris, pH 7.4.

The concentration of all protein samples was determined using the bicinchoninic acid reagent (Thermo Scientific) with bovine serum albumin as the standard. All protein samples were stored at −80 °C.

2.5. Preparation of in vitro enzyme products

In vitro products were generated in a 2 ml reaction that contained 117 μM arachidonyl-CoA or eicosapentaneoyl-CoA, 2 mM acyl-acceptor (1-acyl lyso GPL or BMP isoform), 15 mM Tris, pH 7.4, 0.05% Triton X-100 and 0.46 mg/ml membranes from BLR(DE3)pLysS/pET15b or BLR(DE3)pLysS/pHis-At1g78690. The reaction was incubated for 160 min at 37 °C and terminated by Bligh–Dyer extraction as described previously [5]. The lower phase was dried under N_2 gas and stored at −20 °C until further analysis.

2.6. Mass spectrometry of in vitro products

The products of the *in vitro* reaction was re-suspended in 100 μl of $CHCl_3$:CH_3OH (2:1, v/v) and analyzed using normal phase liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometer as described previously [5,19]. The HPLC effluent (0.4 ml/min) from normal phase chromatography on a Zorbax Rx-SIL column was analyzed using an Agilent 6520 quadrupole time-of-flight mass spectrometer in the negative-ion mode. Mass spectra were obtained scanning from 100 to 2000 at 1 spectra per second with the following instrument parameters: fragmentor voltage – 175 V, drying gas temperature – 325 °C, drying gas flow – 11 l/min, nebulizer pressure – 45 psig, capillary voltage – 4000V. Data were collected in profile mode with the instrument set to 3200 mass range under high resolution conditions at 2 GHz data acquisition rate. The instrument was calibrated using Agilent ESI-L low concentration tuning mix and under normal operating conditions the resolution of the instrument was ∼15,000. The mass accuracy of the instrument was between 1 and 5 ppm, and, therefore measured masses are given to three decimal places. Data acquisition and analysis was performed using Agilent MassHunter Workstation Acquisition Software and Agilent MassHunter Workstation Qualitative Analysis Software (Agilent Technologies, Santa Clara, CA), respectively. Exact masses of lipid species were obtained using PerkinElmer ChemBioDraw Ultra, version 13.0.2.

3. Results

3.1. Headgroup specificity of At1g78690

Because of previous results with similar acyltransferases we assessed the ability of At1g78690 to acylate lyso PI, PC, PS, and PA using a LC-MS based assay. Using 20:4-CoA as the acyl donor we monitored the formation of the distinct di-acyl phospholipid product formed by both its unique *m*/*z* and retention time. The resulting products have a retention time and *m*/*z* that is distinct from other lipids found in *E. coli*. Using this approach we have previously shown that both lyso PG and lyso PE are effective substrates for this enzyme [5].

As shown in Fig. 1, membranes derived from *E. coli* containing pET15b or pHis-At1g78690 were tested for the ability to acylate 1-acyl lyso PG, PA, PC, PS, and PI using arachidonyl-CoA (20:4-CoA) as the acyl donor. In panel A, the expected product of 1-oleoyl lyso PG and 20:4-CoA (exact mass, 795.5182) is only formed when extracts containing At1g78690 are used as the enzyme source. When 1-oleoyl lyso PA was used in a similar assay the expected product (exact mass, 721.4814) is formed equally well when At1g78690 is present or absent from the cell extract (Fig. 1, panel

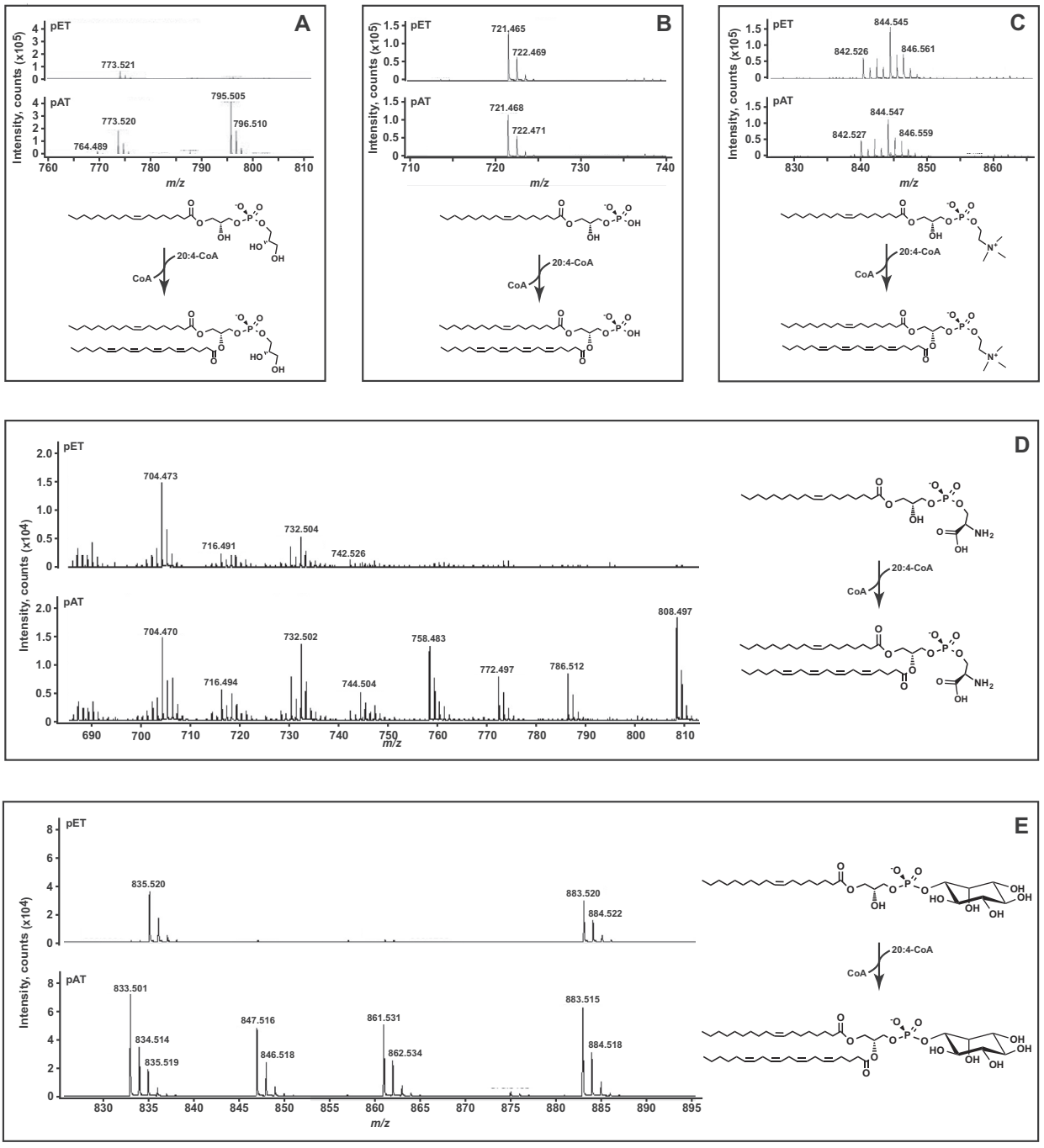

Fig. 1. At1g78690 can acylate lyso-PS and lyso-PI. Each panel shows the negative-ion mass spectrum of the *m*/*z* region for the expected product of the reaction of 18:1 containing lyso GPL with 20:4-CoA. The reaction being examined is also shown with the predicted *m*/*z* for the expected product. The different acyl acceptors are: panel A: 1-acyl lyso PE was used as the acyl acceptor with 20:4-CoA as the acyl donor. Panel B: egg-derived lyso PC. Panel C: 1-acyl lyso PA. Panel D: 1-acyl lyso PS. Panel E: 1-acyl lyso PI. An expanded region of the mass spectrum is shown in panel D and E to show the additional products formed.

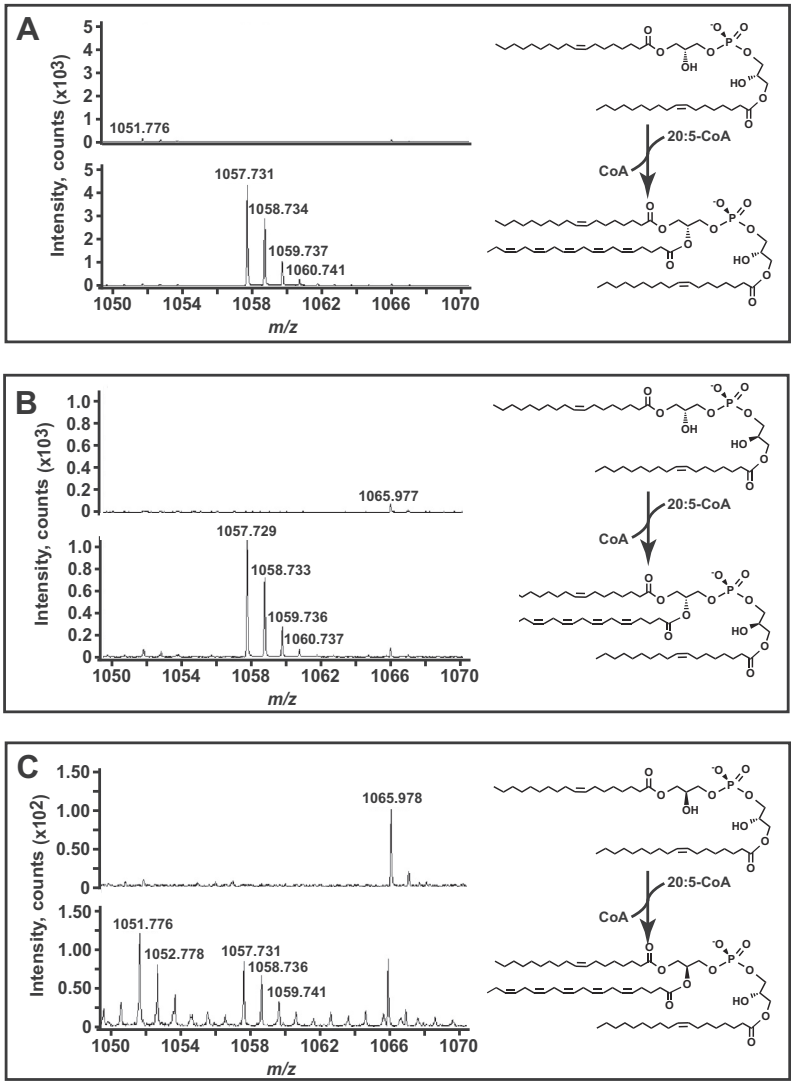

Fig. 2. 3,1′ BMP and 3,3′ BMP but not 1,1′ BMP are acylated by At1g78690. Three different isoforms of BMP containing two oleates were tested as acyl-acceptors for At1g78690 with 20:5-CoA as the acyl-donor. The BMP stereoisoforms are distinguished in name by the carbons attached to the phosphate. For 3,1′ BMP, the phosphate is attached to the *sn*-3 position of one glycerol and the *sn*-1′ of the second glycerol. This yields three BMP stereoisoforms with different stereochemistry at the *sn*-2 and *sn*-2′ carbon of the glycerols. The *m*/*z* of the expected product is 1057.7478. Panel A: 3,1′ BMP. Panel B: 3,3′ BMP. Panel C: 1,1′ BMP.

B). Egg lyso PC was used as the acyl acceptor and, as with 1-acyl lyso PS, there was no difference in the acylation of the lyso PC between the pET and pHis-At1g78690 reactions. These results indicate that lyso PA and lyso PC are not effective acyl-acceptors for the reaction catalyzed by At1g78690.

When 1-oleoyl lyso phosphatidylserine (PS) or 1-oleoyl lyso phosphatidylinositol (PI) were used as the acyl acceptor with 20:4-CoA as the acyl donor, At1g78690 promoted the formation of the expected products are formed as well as several others (Fig. 1, panel D and E). At1g78690 promoted the formation of PS

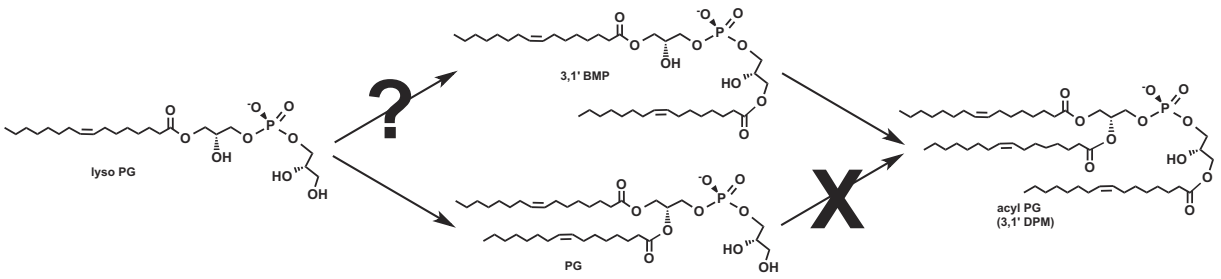

Fig. 3. Proposed mechanism for acyl PG accumulation *in vivo* when At1g78690 is overexpressed. At1g78690 acylates BMP but not PG to form acyl PG [5]. If At1g78690 can acylate 1-acyl lyso PG to BMP then the resulting BMP may be acylated to acyl PG or other DPM stereoisoform. While here we show that At1g7869 can acylate BMP to form acyl PG it remains to be determined if At1g78690 can acylate 1-acyl-lyso PG to form BMP.

species at *m*/*z* 744.504, 758.483, 772.497, and 786.512 and 808.497. The ion with *m*/*z* 808.497 is the expected *m*/*z* for PS with 38 carbons and 5 unsaturations (38:5) in the acyl chains, consistent with oleate and arachidonate as the acyl chains. The other ions correspond to PS with 33:2, 34:2, 35:2, and 36:2. A similar result was obtained when lyso PI was used as the acyl acceptor. The expected product of 1-oleoyl lyso PI and 20:4 CoA has an expected *m*/*z* of 883.5342 is detected when At1g78690 at slightly higher levels than when it is not present. When extracts containing At1g78690 are used as the enzyme source additional PI species are detected at *m*/*z* 833.501, 834.516, and 861.531 which correspond to PI with 34:2, 35:2 and 36:2 carbons in the acyl chains. These lipid molecular species may represent transacylation activity of At1g78690 or efficient usage of endogenous acyl-CoAs with the 1-oleoyl lyso PS or 1-oleoyl lyso PI acyl acceptor.

3.2. Acylation of the PG isoform bis(monoacylglycero) phosphate by At1g78690

Because acyl PG accumulates in cells that overexpress At1g78690 yet this enzyme does not acylate *sn*-1,2-diacyl PG [5] we hypothesized that At1g78690 may have the ability to acylate BMP, an isoform of PG. Again, using a mass spectrometry based assay, we asked whether At1g78690 was able to acylate three stereoisoforms of BMP that differ in the stereochemistry of the glycerols, 3,1′ BMP, 3,3′ BMP, and 1,1′ BMP. When the BMPs, acylated with oleate but with differing stereochemistry at the 2 and 2′ positions, are used as the acyl acceptor with eicosapentaneoyl-CoA (20:5-CoA) as the acyl donor the expected product has an exact *m*/*z* of 1057. 7478. As shown in Figs. 2 and 3, 1′ BMP and 3,3′ BMP are efficiently acylated only in the presence of At1g78690; 1,1′ BMP is not acylated. This strongly indicates that At1g78690 has stereoselectivity; it only acylates the *sn*-2 or 2′ position when it has *R* stereochemistry.

4. Discussion

Understanding the mechanisms of substrate specificity is necessary to understand how AGPATs such as At1g78690 influence the lipid composition of cells and cellular function. Here, using a sensitive LC–MS based assay, we present a preliminary survey of the head group specificity of At1g78690. Our data shows that lyso PE, lyso PG, lyso PS, and lyso PI were substrates for At1g78690 but not lyso PA or lyso PC.

At1g78690 displays headgroup preference, as not all of the lyso GPLs were substrates. The enzyme seems to prefer substrates with a head group; PA was not utilized as a substrate. Lyso PC was also not a substrate for At1g78690. Perhaps the positive charge of the choline headgroup prevents effective binding to the active site of At1g78690. The size of the headgroup may also play a role however since lyso PI was an effective substrate for At1g78690 bulkiness may not be the primary explanation for this result.

When lyso PE [5] and lyso PG (this work and [5]) were used as acyl acceptors, the only prominent product is that expected by the combination of 18:1 lyso GPL acyl acceptor and the 20:4-CoA acyl donor. When lyso PS and lyso PI are used as substrates, the expected product is formed, strongly indicating that these are substrates for At1g78690. Interestingly, additional products are also formed that correspond to products with acyl chains more typical of *E. coli* GPLs. When lyso PS is used as the acyl acceptor, PS molecular species that correspond by exact *m*/*z* to PS containing 33:2, 34:2, 35:2, and 36:2 are detected. These products may be the result of the 1-oleoyl lyso PS being acylated by At1g78690 using acyl-CoAs found in *E. coli*. Specifically, the 34:2 PS and 36:2 PS could be formed using 16:1-CoA or 18:1-CoA as the acyl donor; 33:2 PS and 35:2 PS could be formed using 15cp-CoA (cp indicates cyclopropane in the fatty acyl group [20]) or 17cp-CoA as the acyl donor.

An alternative is that these additional PS and PI products may have arisen from transacylase activity of At1g78690. This enzyme has high homology to tafazzin [21–24], a transacylase implicated in cardiolipin (CL) remodeling. Tafazzin is reported to catalyze the transfer of an acyl chain from di-acyl GPLs (usually PC) to mono- or di-acyl CL [23,24]. It remains to be investigated whether At1g78690 can utilize di-acyl GPLs as an acyl donor similar to tafazzin.

Glycerol-3-phosphate O-acyltransferases (GPATs) and AGPATs have been assumed to be stereospecific based on the structure of the resulting GPLs. In this work, using different stereoisoforms of BMP, we show direct enzymatic evidence for the *R*-stereospecificity of At1g78690, an AGPAT. Efficient acylation of BMP only occurred with 3,1′ BMP and 3,3′ BMP. These BMPs have the 2 and/or 2′ hydroxyl in the *R* stereochemistry. 1,1′ BMP, where both the 2 and 2′ hydroxyls are in the *S* stereochemical configuration, was not efficiently used as a substrate by At1g789690 (Fig. 2).

Previously we showed that At1g78690 acylated the 2-position of 1-acyl-lyso PE; no *N*-acylation was detected. When 1-acyl lyso PG was acylated by At1g78690 and the resulting product analyzed by collision-induced dissociation mass spectrometry (MS/MS), the product ions formed were consistent with the 2-position being acylated to form PG. However, the product ions that are formed by MS/MS of BMP, which is isobaric with PG, are similar to major product ions detected by MS/MS of PG. Further work needs to be done to determine if At1g78690 can acylate the head-group of 1-acyl lyso PG to form BMP.

The acylation of BMP by At1g78690 begins to explain the accumulation of DPMs in cells in which it is overexpressed. Acylation of BMP leads to the formation of acyl PG or a DPM isoform depending on the stereochemistry of the BMP used as the acyl acceptor. At1g78690 may be efficiently acylating the low levels of BMP found in *E. coli* [15]. In addition, if At1g78690 is capable of acylating 1-acyl lyso PG to BMP then the levels of BMP available for acylation is increased and may further explain the accumulation of DPMs *in vivo* (Fig. 3).

The formation of DPMs by acylation of BMP has not been previously observed and represents a new pathway for the biosynthesis of DPMs. In eukaryotic cells BMPs are found in the late endosome and is considered a biomarker of phospholipidosis [25]. The ability of an AGPAT such as At1g78690 to acylate BMPs has implications for the role of BMPs and potentially DPMs in the phospholipid degradative pathways of the late endosome.

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