

## Ectopic Expression of *Arabidopsis* Phospholipase A Genes Elucidates Role of Phospholipase Bs in *S. cerevisiae* Cells

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**Abstract:** In *S. cerevisiae* neither disruption of the phospholipase B triple knockout mutant (*plb1plb2plb3; plb123*) nor over-expression of *phospholipase Bs* (PLBs) result in a phenotype different from wild type. In performing experiments to characterize candidate plant *phospholipase* (PLA) genes, we found, surprisingly, that ectopic expression of either of two different *A. thaliana* PLA2 or PLA1 genes in the yeast *plb123* mutant completely inhibited cell growth. We proposed that while PLBs might not be essential for growth and metabolism of yeast cells, they may play an important role in cell survival by metabolizing excess intracellular *lysophospholipids*. To test our hypothesis, we overexpressed a plant *phospholipase A2* (PLA2) in both WT and *plb123* cells, producing a pool of *lysophosphatidylcholine* (*lysoPtdCho*) in both transformants. In <sup>14</sup>C acetate labeling experiments, WT cells were able to catabolize the resultant labeled *lysoPtdCho*, preventing accumulation, and the cells grew normally. In contrast, in the triple *plb123* mutant PLA2 transformant, *lysoPtdCho* accumulated more than 4-fold to a toxic level, inhibiting cell growth. However, this growth inhibition was complemented by co-expression of either PLB1, PLB2 or PLB3 in the *plb123* triple mutant already expressing the plant PLA2. Furthermore, in labeling experiments, the rescued cells exhibited a 60-75% reduction in <sup>14</sup>C-*lysoPtdCho* build-up compared to *plb123*PLA2 cells. Our data provides conclusive evidence that yeast PLBs can metabolize intracellular *lysoPtdCho* produced by plant PLA2 overexpression in yeast. Our experiments indicate the utility of ectopic plant *phospholipase A* gene expression to characterize poorly-understood phospholipid metabolism mutants in yeast or other organisms.

**Keywords:** Arabidopsis Phospholipase A<sub>2</sub>. Phospholipase B. Phospholipase B mutants, Lysophosphatidylcholine, *Saccharomyces cerevisiae*

### INTRODUCTION

Phospholipids are primary components of all cell membranes. The main glycerolphospholipids in *S. cerevisiae* cells include, from most to least abundant, phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), phosphatidylinositol (PtdIns), phosphatidylserine (PtdSer) and phosphatidic acid (PtdOH). Membrane homeostasis is a dynamic condition wherein lipid synthesis, trafficking and degradation are coordinated to provide and sustain the integrity of different membranous subcellular structures [1]. In *S. cerevisiae*, lipid biosynthesis, its regulation and degradation are quite well understood, but much less is known about the biological function of lipid turnover, as well as the molecular mechanisms that coordinate those processes leading to membrane equilibrium.

As phospholipid turnover products, *lysophospholipids* (*LysoPLs*) have key roles in cell homeostasis. Due to their amphipathic nature, *LysoPLs* have surfactant and detergent-like properties. As a result, control of the *in vivo* levels of *LysoPLs* is essential for the function and survival of cells. In yeast biological membranes, *LysoPLs* are typically found at

very low concentrations, approximately 0.02 to 0.08% of dry cell biomass, as derived from data of Blagovic *et al.* [2] and Summers *et al.* [3], respectively. The total lipid content of yeast varies widely with the species. The majority of yeasts contain 7%-15% total lipids as a proportion of dry weight [4]. Accordingly, the proportion of *LysoPLs* would fall into a range from 0.1%-1.1% of total lipids in different species. At low non-toxic concentrations, *lysophospholipids* which are metabolites of the mitogenically-responsive enzyme phospholipase A<sub>2</sub>, may be a mechanism for modulating the activity of downstream effector molecules. *Lyso*-phospholipids also interact with Ras proteins, regulatory elements for adenylyl cyclase, which catalyze the synthesis of cAMP, a secondary messenger critical for cell cycle progression [5]. However, higher concentrations of *LysoPLs* have been shown to disturb membrane conformation, affecting the activities of many membrane-bound enzymes, and may even lead to cell lysis and death [6]. As reported by Ko *et al.* [7], yeast (1,3)- $\beta$ -glucan synthase was shown to be inhibited by phospholipase A<sub>2</sub> and its reaction products, principally *lysophospholipids*; the inhibition was due, in part, to non-specific detergent-like membrane disruption. This may partially explain the resultant inhibition of cell multiplication. In wild type yeast cells, a typical phospholipid profile analysis does not show any appreciable *lyso*-phospholipid content [8]. In general, elevated *LysoPL* levels are usually

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characteristic of altered membrane phospholipid metabolism and impaired membrane integrity in particular.

In yeast, the de-acylation of PtdCho to produce glycerophosphodiester and FFA is currently known to be catalyzed by the enzymes Phospholipase B1, B2, B3 (Plb1p, Plb2p, Plb3p) and neuropathy target esterase (Nte1p), encoded by *PLB1*, *PLB2*, *PLB3* and *Nte1p* genes, respectively. The *PLB* genes are highly similar (>60% identity at the DNA level). Phospholipase B activity catalyzes the deacylation of glycerophospholipids at both *sn*-1 and *sn*-2 positions. Plbps are located at the plasma membrane and in the periplasmic space with different properties [9]. Plb1p is the main enzyme responsible for PtdCho deacylation with its product, glycerophosphocholine (GroPCho), released into the extracellular medium. At their pH optima, substrate preferences of Plb1p and Plb2p are similar; both appear to accept all major phospholipid classes, whereas Plb3p accepts only PtdIns and PtdSer as substrates. At the physiological pH of yeast growth medium (around pH 5), Plb1p is primarily responsible for the degradation of PtdCho, and to some extent, PtdIns. Interestingly, overexpression of Plb2p does not result in degradation of PtdCho or PtdIns, while Plb3p hydrolyses PtdIns, but not PtdCho, in agreement with the substrate selectivity observed *in vitro* [9, 10]. Nte1p is a phospholipase B which resides in the endoplasmic reticulum, and prefers to deacylate PtdCho produced through the CDP-choline (Kennedy) pathway [11]. PtdCho can also be hydrolyzed by phospholipase D (Spo14p) to produce phosphatidic acid (PA) [12].

Both the triple *PLB* knockout mutant (plb123) and *nte1* null mutant (*nte1*) strains are viable and do not exhibit any detectable phenotype beyond the lack of GroPCho formation [10]. It was reported that while the plb2 mutant and plb1plb2 double mutant grew at slightly reduced rates compared with wild type cells when plated on complete YEPD media [9, 10], the plb3 mutant and plb1plb3 double mutant and the plb1plb2plb3 triple mutant grew the same as wild type on fermentable and non-fermentable carbon sources [9, 10]. Merkel *et al.* [9] suggested that this may be due to the presence of alternative pathways, as was indicated by some residual release of GroPIns and GroPCho from a strain lacking all three phospholipase Bs. In fact, the plb123 mutant grows even better than wild-type cells [9; the current study), prompting questions about the possible physiological role of Plbps.

*S. cerevisiae* has proven to be a valuable experimental system to express and identify the function of plant genes involved in lipid metabolism. Because the plb123 triple mutant cells have a low background phospholipid deacylation activity, we initially tried to use the mutant as a system for screening and expressing some candidate plant *phospholipase A (PLA)* genes. Surprisingly, we found that overexpressing some of the putative *PLA* genes in plb123 completely inhibited the triple mutant cell growth, which suggested an important physiological role for *PLBs*. It had been reported that overexpression of *PLB2* conferred resistance to the toxic effects of exogenous *lysophosphatidylcholine (lysoPtdCho)* supplied in the medium at a concentration of 10 mM (*ca* 0.5% w/v based on the MW of *sn*-1 palmitoyl LPC), which was thought to be consistent with its function as an extracellular *lysophospholipase* [8, 9].

Considering this result, the fact that Plbps are located in plasma membranes, and because of the existence of other PtdCho-hydrolysing pathways (like that involving Nte1p), we proposed that Plbps, while perhaps not essential for metabolism and growth of yeast, may also play an important role in cell survival by catabolizing excess intracellular *lysophospholipids*. In order to test our hypothesis, we decided to reverse conventional approaches and instead of using yeast as a vehicle to characterize plant genes, use a known plant *phospholipase A2 (PLA2)*, to collect relevant metabolic information on the poorly-understood yeast plb123 mutant. Theoretically, overexpressing plant *PLA2* in yeast cells will produce a pool of *lysophospholipids*. If the cells possess the capacity to catabolize these *lysophospholipids*, preventing accumulation, the cells will grow normally. In contrast, if the cells are devoid of this capacity, *lysophospholipids* will accumulate to a toxic concentration. This test system provided direct proof that Plbps, as opposed to other pathways, can scavenge intracellular *lysoPtdCho* produced by overexpression of *PLA2*. Moreover, it indicated the utility of using plant *PLA* genes to screen yeast phospholipid mutants and understand nature of the lesions therein.

## MATERIALS AND METHODOLOGY

### General Molecular Biology Materials, Media, Radiochemicals and Lipid Analysis Supplies

Yeast extract, peptone, and agar were purchased from Difco® Laboratories, Detroit, MI. D-(+)-Glucose and D-(+)-galactose were acquired from Sigma-Aldrich Chemical Co., St. Louis, MO. Yeast nitrogen base without amino acids was a product of Becton, Dickinson and Company Spark, MD. Brent supplement mix (BSM) -URA was purchased from Sunrise Science, while BSM -LEU-URA was purchased from Formedium Ltd. Hunstanton, England. *Escherichia coli* strains Top10 and Topo10F' competent cells, superscript II reverse transcriptase, DNA polymerase, pYES2.1 yeast shuttle vector, pCR®2.1-Topo® TA vector, S.c. EasyComp™ Transformation kit, restriction enzymes were purchased from Invitrogen, Inc., Carlsbad, CA. Pfu Turbo® DNA polymerase, pESC-Leu yeast vector were obtained from Stratagene. RNeasy® plant Mini Kit, QIA prep® Spin Miniprep Kit, QIA EX® II Gel Extraction Kit were products of Qiagen Inc., Mississauga, ON. L- $\alpha$ -1-palmitoyl-2-oleoyl [1-<sup>14</sup>C]-PtdCho was obtained from American Radiolabeled Chemicals, Inc, St. Louis. [1-<sup>14</sup>C]acetate (sodium salt) was purchased from Amersham Biosciences. Silica Gel 60 thin layer chromatography plates were obtained from Whatman Ltd., Maidstone, England. Oligonucleotide primers were synthesized by the Plant Biotechnology Institute. Organic solvents (HPLC grade or higher) were from Fisher Scientific or EMD Chemicals Inc., Darmstadt, Germany.

### Yeast Strains, Growth Conditions and Transformation

All yeast strains used in this study (Table 1) were derivatives of the plb123 (*plb1plb2plb3*) triple mutant and its wild type, W3031A, kindly provided by Dr. Olaf Merkel. Synthetic minimal medium (SC) supplemented with appropriate nutrients (BSM-URA or BSM-LEU-URA plus glucose or galactose) were employed to select colonies containing various different plasmids. Yeast transformation was performed

**Table 1. *S.cerevisiae* Strains Used in This Study**

Strains	Relevant Genotype	Genotype	Source
W3031A	<i>PLB1PLB2PLB3</i>	<i>Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	Olaf Merkel
MF17	<i>plb1plb2plb3</i>	<i>Mata ade2-1 can1-100 his3-11,15 leu2-3,112 ura3-1 plb1::TRP plb2::HIS plb3::kanMX</i>	Olaf Merkel
WTEV <sup>a</sup>	<i>PLB1PLB2PLB3 YESpEV</i>	<i>Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 YESpEV</i>	This study
WTPLA2	<i>PLB1PLB2PLB3 PLA2</i>	<i>Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 YESpPLA2</i>	This study
plbEV	<i>plb1plb2plb3 YESpEV</i>	<i>Mata ade2-1 can1-100 his3-11,15 leu2-3,112 ura3-1 plb1::TRP plb2::HIS plb3::kanMX YESpEV</i>	This study
plbPLA2	<i>plb1plb2plb3 PLA2</i>	<i>Mata ade2-1 can1-100 his3-11,15 leu2-3,112 ura3-1 plb1::TRP plb2::HIS plb3::kanMX YESpPLA2</i>	This study
plbEVEV	<i>plb1plb2plb3</i>	<i>Mata ade2-1 can1-100 his3-11,15 leu2-3,112 ura3-1 plb1::TRP plb2::HIS plb3::kanMX YESpEV ESCpEV</i>	This study
plbPLA2 EV	<i>plb1plb2plb3 PLA2</i>	<i>Mata ade2-1 can1-100 his3-11,15 leu2-3,112 ura3-1 plb1::TRP plb2::HIS plb3::kanMX YESpPLA2 ESCpEV</i>	This study
plbPLA2 PLB1	<i>plb1plb2plb3 PLA2PLB1</i>	<i>Mata ade2-1 can1-100 his3-11,15 leu2-3,112 ura3-1 plb1::TRP plb2::HIS plb3::kanMX YESpPLA2 ESCpPLB1</i>	This study
plbPLA2 PLB2	<i>plb1plb2plb3 PLA2PLB2</i>	<i>Mata ade2-1 can1-100 his3-11,15 leu2-3,112 ura3-1 plb1::TRP plb2::HIS plb3::kanMX YESpPLA2 ESCpPLB2</i>	This study
plbPLA2 PLB3	<i>plb1plb2plb3 PLA2PLB3</i>	<i>Mata ade2-1 can1-100 his3-11,15 leu2-3,112 ura3-1 plb1::TRP plb2::HIS plb3::kanMX YESpPLA2 ESCpPLB3</i>	This study

<sup>a</sup> EV is short for empty vector.

using the *S.c.* EasyComp<sup>™</sup> Transformation kit. Genomic yeast DNA was isolated and prepared for gene cloning by the method of Sambrook and Russell [13]. *Escherichia coli* strains Top10 and Top10F<sup>'</sup> were cultured in Luria broth and used for plasmid transformation by a standard method [14].

### Cloning the PLA2 from Arabidopsis and Expression in Yeast

Total RNA was extracted from Arabidopsis leaves using the RNeasy<sup>®</sup> plant Mini Kit and cDNA was synthesized by superscript II<sup>®</sup> reverse transcription according to the product instructions. The primers PLA2F87 and PLA2R536 (see Table 2) were designed to amplify the coding region of *phospholipase A2* (At2g06925) [14], designated as *PLA2*. Arabidopsis leaf cDNA was used to amplify *PLA2* by Pfu Turbo<sup>®</sup> DNA polymerase. To the resultant blunt end PCR product, an adenine overhang was added at the 3' end using Taq DNA polymerase, and the amplicon cloned into the yeast shuttle vector pYES2.1 in the Top 10F<sup>'</sup> strain. After confirming cloning integrity by sequencing, the vector was transformed into both the WT and the plb123 triple mutant yeast strains using the *S.c.* EasyComp<sup>™</sup> Transformation kit. The self-ligated pYES2.1 was also transformed into the triple mutant and WT strains as controls (see Table 1).

**Table 2. Primers Used in This Study. *NotI* or *SacI* Sites are Underlined**

Primer Name	Primer Sequence
PLA2F87	ACTATGGCGGCTCCGATCA
PLA2R536	TTAGGGTTTCTTGAGGACTTTGC
ACTF	ATGGATTCTGAGGTTGCTGCTT
ACTR	AATGGCGTGAGGTAGAGAGAAAC
PLB1F1	<u>GCGGCCGC</u> ATGAAGTTGCAGAGTTTGTGG
PLB1R1995	<u>GAGCTC</u> TAAATTAGACCGAAGACGGC
PLB2F1	<u>GCGGCCGC</u> ATGCAATTACGGAACATATTACAG
PLB2R2121	<u>GAGCTC</u> TAAATTAGTCCAAATAATGCTGTTAT
PLB3F1	<u>GCGGCCGC</u> ATGATACGTCCATTATGTTCAAAA
PLB3R2061	<u>GAGCTC</u> TTATACTGCTCCGGTAAACATCA

### RT-PCR Confirmation of PLA2 Expression in WT Yeast

Both wild-type *S. cerevisiae* bearing the empty vector and yeast cells bearing *PLA2* were inoculated in YPD

medium containing 2% of dextrose. Cells were cultured at 28°C for 24 hr, followed by culture in YPD medium containing 2%  $\beta$ -galactose at 28°C for 12 hr. Total RNA was isolated from both wild-type *S. cerevisiae* bearing the empty vector and transgenic cells bearing *PLA2* using the standard method of Sambrook and Russell [13]. To examine the transcript levels in transformed yeast, two micrograms of DNase I-treated RNA were used as a template for cDNA synthesis (Invitrogen superscript First-Strand Synthesis kit). To exclude genomic DNA contamination, DNA-free RNAs were tested by PCR using *pLA2* primers (See Table 2). The yeast actin gene *ACT1/YFL039C* was amplified (using primers ACTF and ACTR, Table 2) and used as an endogenous standard to confirm mRNA quality. The cDNA samples were diluted 50-fold for PCR amplification. The PCR program: DNA denaturation at 94°C for 4 min, followed by 35 cycles of PCR at 94°C for 30 seconds; primer annealing at 53°C for 30 seconds and extension and incubation at 72°C for 5.75 min, then cooling at 4°C. PCR products were resolved by electrophoresis on 1% agarose gels. The expected amplified fragment size of yeast *actin* and *pLA2* are 525 bp and 450 bp, respectively. Functional expression of the *PLA2* was confirmed by phospholipase A<sub>2</sub> assay as described below.

### Plate Assay of Strain Growth

All strains were cultured to stationary phase in SC medium with glucose and adjusted to an OD<sub>600</sub> of 3.0 as a starting concentration; cells were also diluted 1:10, 1:100 and 1:1000 with SC medium without saccharide. Ten  $\mu$ l of each was spotted on plates with different media containing either glucose or galactose. After 2, 3 and 5 days, the degree of cell growth was determined.

### Phospholipase A<sub>2</sub> Assay

*PLA2* activity was measured as the release of radioactive free [<sup>14</sup>C] oleic acid from the substrate L- $\alpha$ -1-palmitoyl-2-oleoyl[<sup>14</sup>C]-PtdCho. The substrate was dried under a stream of N<sub>2</sub> before adding the other reaction components. Standard enzyme assay mixtures contained 50 mM Tris-HCl, pH 8.0, 10 mM CaCl<sub>2</sub>, 0.05% (v/v) Triton X-100, 0.1  $\mu$ Ci of the substrate (55mCi/mmol), in a total volume of 200  $\mu$ l. The mixtures were emulsified by sonication at room temperature for 3 min. and reactions started by addition of 60  $\mu$ g of transformant lysate protein. The reactions were incubated in a shaking water bath at 30°C for 30 min and terminated by the addition of 750  $\mu$ l of chloroform: methanol (1:2, v/v). The lipids were extracted and spotted onto a thin layer chromatography plate (Silica Gel G), which was developed with chloroform: methanol: NH<sub>4</sub>OH: water (73:30:4:1). The spots corresponding to the lipid standards FFA and *lyso*PtdCho, were then scraped into vials, 4 ml scintillation fluid added (Aquasol-2, DuPont) and the radioactivity determined by standard scintillation counting.

### Labeling Yeast Cells with Radioactive Sodium Acetate and Lyso-Phosphocholine Analysis

Yeast cells were cultured in SC-URA medium with glucose. The cells from stationary stage cultures were diluted to OD<sub>600</sub> 0.1 in a 20 ml volume. 30  $\mu$ Ci of [<sup>14</sup>C]- labeled sodium acetate was diluted in SC medium and the labeled

solution filtered before adding to the cultures. The cells were inoculated and incubated for 10 hours at 28°C. The cultures were then divided in half; One half continued to be incubated in glucose medium, while with the other half, collected cells were washed twice with sterilized water and transferred to galactose medium. After 5 hours incubation, cells were collected and washed twice with PBS (10 mM phosphate, 2.7 mM KCl, 137 mM NaCl, pH7.4), to be analyzed for lipids. Cells were homogenized with an equal volume of glass beads using the BeadBeater at 4°C using 3 bursts of 1 min. Total lipids were extracted by adding 2 ml of chloroform:methanol:KCl/HNO<sub>3</sub>(1:2:0.8,v/v/v) vortexing and then adding 2ml dichloromethane and the total lipids were recovered from the lower organic phase. The labeled lipids were separated by two-dimensional thin layer chromatography on Silica gel 60 plates using chloroform/methanol/acetic acid/water (80:9:12:2) as the first, and chloroform/methanol/acetic acid/water (50:25:10:2) as the second developing solvent, respectively. The TLC plates were screened by a TLC Imaging Scanner (Bioscan, WA, USA). Relevant spots were scraped and quantified by liquid scintillation counting.

### Co-Culture Experiments

The *plb123* mutant was transformed by two empty vectors with URA and LEU selection markers respectively (designated *plbEVEV*). while the wild type cells were transformed by *PLA2* expression vector or its empty vector *pYES2.1* with URA selection marker only and designated as *WTPLA2* and *WTEV*, respectively. The *WTPLA2* was then co-cultured with *plbEVEV* (*WTEV* co-cultured with *plbEVEV* was used as the control). Under the induced condition, we wanted to determine whether *WTPLA2* could release enough *lyso*PC to affect the growth of *plb123* mutant cells. In order to fully reflect the *PLA2* effect, 10-times the number of *WTPLA2* cells and *WTEV* cells were grown against *plbEVEV* cells, which were mixed together for co-culture as follows: After *WTEV*, *WTPLA2* and *plbEVEV* were cultured to OD<sub>600</sub> 3.0, *WTEV* and *WTPLA2* cells were each diluted to OD<sub>600</sub> 1.0 while *plbEVEV* cells were diluted to OD<sub>600</sub> 0.1 and then the preparations co-cultured. Then either before induction or after two days induction in the presence of galactose, cultures were diluted 100-fold and 10  $\mu$ l was spread on plates with SC-URA-LEU medium and colonies counted.

### Cloning the *PLB1*, *PLB2* and *PLB3* Genes from Yeast and Expression in the *plb123* triple Mutant

Yeast DNA was extracted by the method of Sambrook and Russell [13]. The primers *PLB1F1*, *PLB1R1995*, *PLB2F1*, *PLB2R2121*, *PLB3F1*, *PLB3R2061* (see Table 2) with *NotI* or *SacI* sites (underlined) were designed to amplify the coding region of *PLB1*, *PLB2*, and *PLB3*, respectively using Pfu Turbo<sup>®</sup> DNA polymerase. To the resultant blunt end PCR products, adenine overhangs were added at the 3' ends using Taq DNA polymerase and the amplicons cloned into pCR<sup>®</sup>2.1-Topo<sup>®</sup> TA vector. After confirming sequence integrity, the plasmids were digested by *NotI/SacI*, the 1.99kb *PLB1*, 2.12kb *PLB2* and 2.06kb *PLB3* were recovered from the gel and each ligated into the *NotI/SacI* sites in the yeast shuttle vector pESC-LEU. The plasmids and the

pYES2.1PLA2 (described above) were co-transformed into the plb123 triple mutant using the S.c. EasyComp™ Transformation kit and transformant cells selected on SC-LEU-URA medium. The self-ligated pYES2.1 and empty pESC-Leu vector were also used to transform the triple mutant strain as a control. The product strains and their genotypes are shown in Table 1.

## RESULTS

While screening a number of putative plant *PLA* genes by over-expression in the plb123 triple mutant strain, we found that there was generally a complete inhibition of growth. Thus, we considered using a reverse strategy- one of using a known plant *PLA2* gene to determine whether PLBs may play an important role in cell survival, perhaps by scavenging excess lysophospholipids. Although there are reports that *PLA* activity can be measured in yeast, there have been no yeast *PLA* genes cloned thus far [15], making the availability of the plant *PLA* gene even more valuable in probing yeast phospholipid metabolism mutations. A *PLA2* gene from Arabidopsis (At2g06925) encodes a 149-amino acid protein, identified as phospholipase A2, which deacylates phospholipids at the *sn*-2 position to produce lysophospholipids and FFAs [14]. The Arabidopsis *PLA2* was cloned into the yeast shuttle vector pYES2.1, in which *PLA2* was driven by *GALI* promoter (see Materials and methods). The *GALI* promoter can be inhibited by glucose and stimulated by galactose [16].

### Functional Expression of the Arabidopsis *PLA2* in Yeast

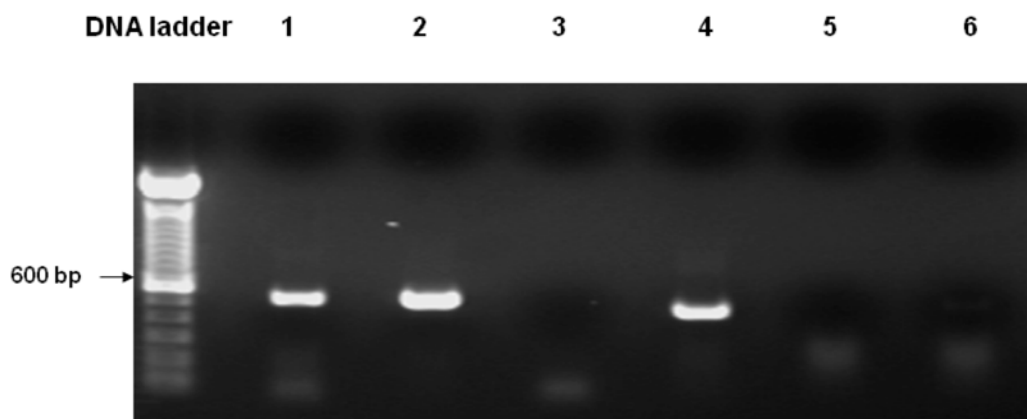
WT yeast strain W3031A was transformed with either pYES*PLA2* plasmid or with empty vector (control). *PLA2* expression was confirmed by RT-PCR (Fig. 1). To assess whether the expressed At*PLA2* had functional activity, cells were collected from exponential stage cultures induced

in galactose medium, lysed and the lysate used in phospholipase A2 assays as described in Materials and methodology. The release of *sn*-2 <sup>14</sup>C-labeled oleic acid from L- $\alpha$ -1-palmitoyl-2-oleoyl[1-<sup>14</sup>C]-PtdCho was 15-fold higher than the low background activity in the WT yeast. This experiment confirmed that the plant *PLA2* gene was functionally expressed in yeast cells and encoded a protein with phospholipase A2 activity. Furthermore, expression of the At*PLA2* was not toxic to WT yeast.

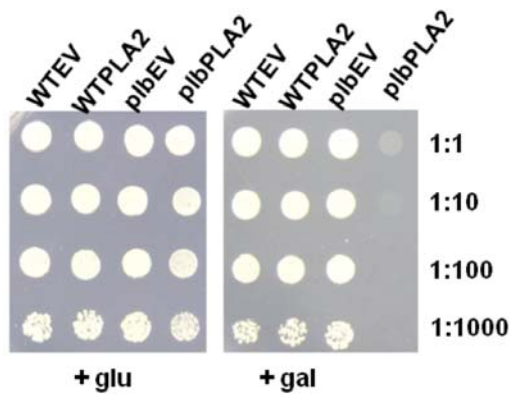
### The Over-Expression of Plant *PLA2*s Completely Inhibits the Growth of Yeast plb123 Triple Mutant Cells but has no Obvious Effect on Wild Type Cells

When the Arabidopsis *PLA2* was introduced into the plb123 triple mutant and maintained on SC-URA medium with glucose, the *PLA2* was not expressed, and there were no obvious changes in cell growth compared to the empty vector controls or the WT*PLA2* transformants. Once cultured in the medium containing galactose, the empty vector control cells and WT*PLA2* cells grew normally, but the growth of plb123 cells harbouring *PLA2* (plb*PLA2*) was completely inhibited (Fig. 2). The results were identical even after 5 days of incubation (data not shown). These results show that the growth inhibition of the plb123 triple mutant was due to the expression of *PLA2*, and that such inhibition acts only in the plb123 triple mutant cells, but not in wild type cells.

To determine whether the cells were dead or growth merely inhibited when *PLA2* was expressed, cells induced for 3 and 6 days in galactose liquid medium were collected and washed, re-suspended in glucose medium and re-plated on glucose plates. The cells were once again able to grow, suggesting that overexpression of At*PLA2* was not lethal to the plb123 cells, but rather, temporarily inhibited cell growth (data not shown).



**Fig. (1).** Expression of Arabidopsis *PLA2* in WT yeast as determined by RT-PCR. Lane 1. Template: WTEV cDNA, Actin primers; Lane 2. Template: WT*PLA2* cDNA, Actin primers; Lane 3. Template: WTEV cDNA, *PLA2* primers; Lane 4. Template: WT*PLA2* cDNA, *PLA2* primers; Lane 5. Template: DNA-free RNA from WTEV, *PLA2* primers; Lane 6. Template: DNA-free RNA from WT*PLA2*, *PLA2* primers. Lanes 1 & 2 show that the cDNA from both empty vector- and *PLA2*- transformed yeast were of good quality, as the yeast actin was constitutively expressed in both samples. Lane 3: The same empty vector-transformed WT yeast as in Lane 1, failed to produce the *PLA2* transcript; Lane 4: In contrast, *PLA2*-transformed WT yeast produced the expected 450 bp *PLA2* transcript. For Lanes 5 & 6, using DNA-free RNA as the template there is no signal with the *PLA2* primers, confirming that there is no possibility that genomic DNA contamination in the cDNA templates could have resulted in the *PLA2* band as observed in Lane 4.



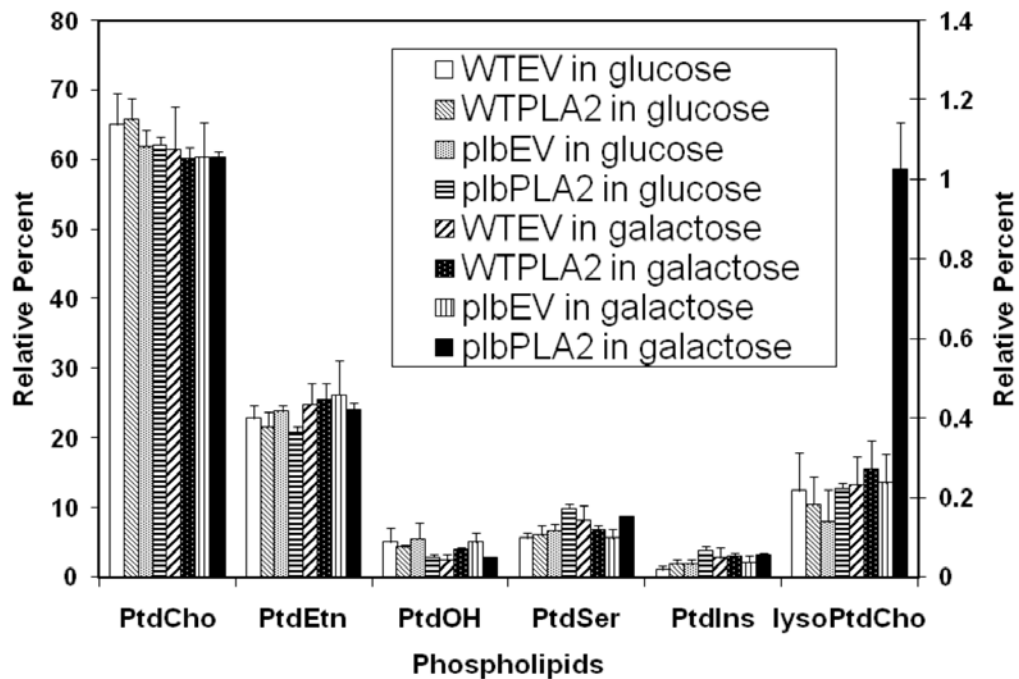
**Fig. (2).** Cell growth of Wild type empty vector (WTEV), wild type *PLA2* (WTPLA2), *plb123* empty vector (plbEV) and *plb123 PLA2* (plbPLA2) transformants (as designated in Table 1) on medium supplemented with glucose or galactose. All strains were cultured to stationary phase in SC medium with glucose and adjusted to OD<sub>600</sub> of 3.0 as a starting cell density; Cells were also diluted 10, 100 and 1000 times and 10  $\mu$ l of each was spotted on a plate with medium as indicated and incubated for three days.

**The Over-Expression of Plant PLA2s Leads to A Toxic Accumulation of lysoPtdCho in plb123 Cells**

Once we confirmed that the plant *PLA2* was functionally expressed (see above), we speculated that its overexpression could be used to stimulate an accumulation of *lysophospho-*

pholipids and/or free fatty acids, which might be toxic to the plb123 cells. No obvious accumulation of free fatty acids was found in growth-inhibited cells (data not shown). Because of the very low level of endogenous *lysophospholipids*, such as *lysoPtdCho*, in yeast cells, it was necessary to label the phospholipids by feeding cells with <sup>14</sup>C acetate to estimate relative polar lipid pool sizes. In preliminary experiments, we found that the phospholipids were well-labeled during the exponential phase but not in later phases (data not shown). This is consistent with the fact that neutral, not polar, lipids accumulate in the late phase of cell growth. Thus labeling was performed during the exponential phase of growth.

While most major phospholipids of WTEV, WTPLA2, plbEV and plbPLA2 strains were all labeled, among *lyso* phospholipids only *lysoPtdCho* (and not *lysoPtdEtn*, *lysoPtdIns*, *lysoPtdSer*) was significantly labeled. The level of *lysoPtdCho* in induced plbPLA2 increased more than 4-fold over the low endogenous level consistently observed in control cells (Fig. 3). In WT cells expressing *PLA2* (WTPLA2) there was no enhanced accumulation of *lysoPtdCho* and cell growth was unaffected. Collectively, these results mean that the over-expression of plant *PLA2* can be used to produce *lysophospholipids*, mainly *lysoPtdCho*, which can be catabolized by wild type yeast, but accumulates in the plb triple mutant. This suggests that phospholipase Bs play an important role in scavenging excessive *lysoPtdCho* in yeast cells and that the plb123 mutant has lost this ability.



**Fig. (3).** Relative <sup>14</sup>C labeling of various phospholipids isolated from transformed strains fed with <sup>14</sup>C acetate. Starting from a cell density of 0.1 (OD<sub>600</sub>) in 20 ml of glucose medium, cells of WTEV, WTPLA2, plbEV and plbPLA2 (as designated in Table 1) were labeled with 30  $\mu$ Ci <sup>14</sup>C-sodium acetate for 10 hours. Half of culture remained in glucose medium while the other half was induced in galactose medium for 5 hours. The total lipids were extracted and resolved by two dimensional thin layer chromatography and each of the phospholipids was quantified (see Materials and methodology). The proportions of <sup>14</sup>C-labeled *LysoPtdCho* (relative to the total <sup>14</sup>C phospholipids) are depicted using the Y axis scale on the right side, while the proportions of other <sup>14</sup>C-labeled phospholipids are expressed using the Y axis scale on the left. The results are the means  $\pm$ S.E of three experiments.

### The Growth Inhibition of the plb123 Mutant Upon AtPLA2 Expression is Principally the Result of Intracellular Accumulation of lysoPC

Because in *Arabidopsis*, PLA2, has been observed in both intracellular and extracellular spaces [14], the possibility remained that the expressed *AtPLA2* is producing *lysoPC* extracellularly at a level high enough to also inhibit the growth of the plb123 mutant. In that case, using *PLA2* expression to produce *lysoPC* would not be a qualitatively different experiment than providing *lysoPC* to the culture media. To demonstrate that it was the ectopic expression of the plant PLA2 giving rise to increased intracellular *lysoPC* that was primarily responsible for the growth inhibition, we designed the following co-culture experiment: The plb123 mutant was transformed by two empty vectors with URA and LEU selection markers, respectively (designated plbEVEV), while the wild type cells were transformed with the PLA2 expression vector or with its empty vector pYES2.1, with URA selection marker only, and designated as WTPLA2 and WTEV, respectively. We then co-cultured WTPLA2 with plbEVEV and, as a control, we co-cultured WTEV with plbEVEV. Under induced (+ gal) conditions, we wondered whether WTPLA2 might release some amount of extracellular *lysoPC*, which might affect the growth of the plb123 mutant cells. After two days of induction, the co-cultures were diluted and spread on plates with SC-URA-LEU medium. Due to the presence of the both selection nutrients, only plbEVEV cells were able to grow, while WTEV or WTPLA2 could not. By counting colony numbers it was possible to evaluate the effect of *lysophospholipids* released by WTPLA2 on plbEVEV cells. Our results showed that there is no difference in growth between the two co-cultures (Table 3). Combining these results with those from  $^{14}\text{C}$  acetate labeling experiments (above), it is clear that the intracellular accumulation of *lysoPC* by *AtPLA2* expression was the principle reason for the growth inhibition of the plb123 mutant.

### The Expression of PLB1, or PLB2 or PLB3 can Eliminate the Inhibition of PLA2s on plb123 Triple Mutant Cell Growth

In order to determine whether one or other of the phospholipase Bs could rescue plb123 cell growth which had been inhibited by the over-expression of *AtPLA2*, each *PLB* gene was co-expressed separately with *PLA2* in plb123. All three *PLB* genes could rescue cell growth (Fig. 4). Under our experimental conditions, there was no difference in the rate of growth in the rescued cells. Most importantly,  $^{14}\text{C}$  acetate labeling experiments showed that those cells rescued by co-expression of any of the three *PLB* genes exhibited a 60-75% reduction in the  $^{14}\text{C}$  *lysoPtdCho* which otherwise accumulated to a toxic level in the case of the plb123PLA2 transformant (Fig. 5). Thus, it appears that *lysoPtdCho* threshold proportions of 0.5% or higher are highly toxic to the plb123 mutant, which is supported by the earlier findings of Merkel *et al.* [9] and Fyrst *et al.* [8] in studies of PLPB2 as a putative extra-cellular *lysophospholipase*. We also co-expressed in plb123, one of *PLB1*, *PLB2* or *PLB3* with another *Arabidopsis PLA* gene, *PLA1* (At2g44810), and found that the growth inhibition caused by this *PLA1* could also be rescued by each of the *PLBs* (data not shown). These results demonstrate that *PLBs* are redundant in scavenging excess *lysoPtdCho*, and that such mechanisms are generally induced by plant *PLA* expression in plb123 cells.

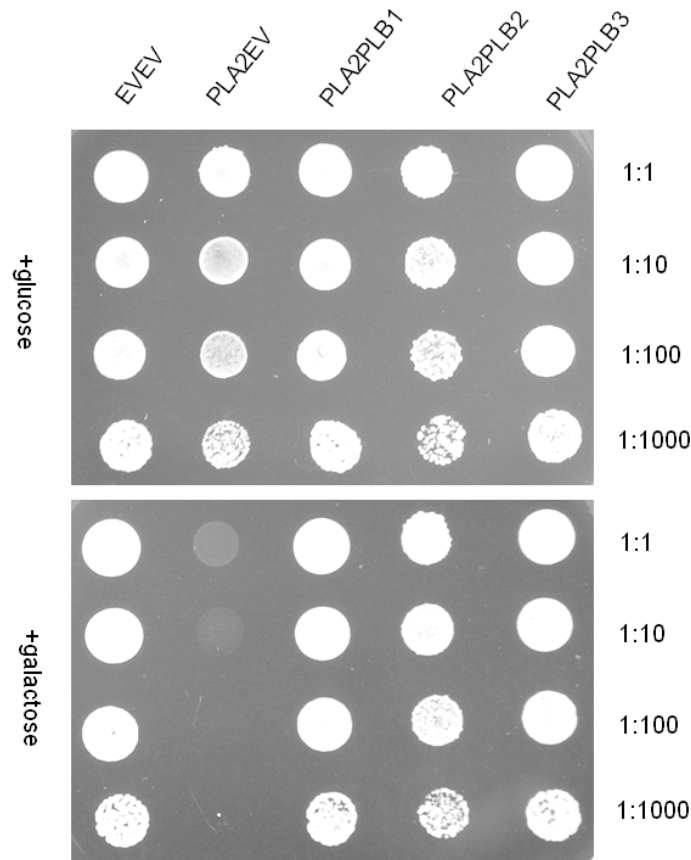
### DISCUSSION

*LysoPtdCho* is an important *lysophospholipid* in cells; its potential generation and degradation in yeast cells is summarized in the simplified model shown Fig. (6). The production of *lysoPtdCho* occurs *via* different pathways: One is *via* the deacylation of *PtdCho* by *Plb1p*, *Plb2p* and *Nte1p* [8, 9, 11, 17]; In the other pathway, *lysoPtdCho* is taken up into the intracellular compartment by the action of *Dnf2p* [18, 19]. When *GroPcho* is taken up into yeast by *Git1p*, *lysoPtdCho* can be produced by *GPCAT* [20, 21]. *LysoPtdCho* can be

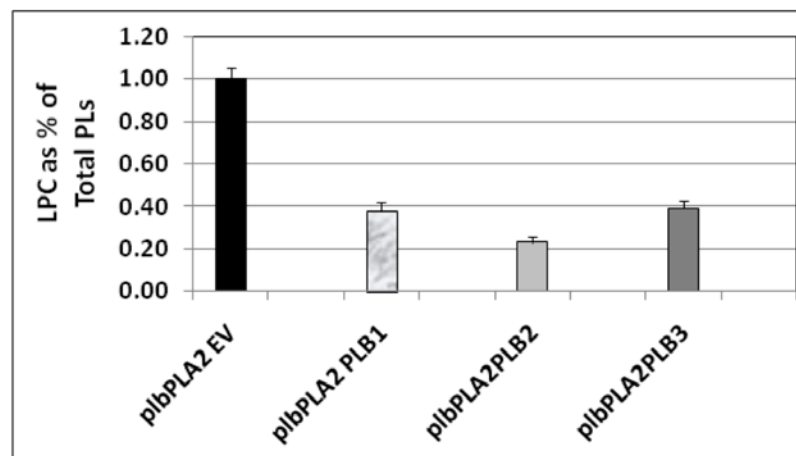
**Table 3. The Growth Inhibition of the plb123 Mutant Upon AtPLA2 Expression is Principally the Result of Intracellular Accumulation of lysoPC. In Co-Cultivation Experiments, the plb123 Mutant was Transformed by Empty Vectors pYES2.1 and pESC with URA and LEU Selection Markers (Called plbEVEV), WT was Transformed by the Empty Vector (Called WTEV) or by PLA2 (Called WTPLA2) with the URA Selection Marker. The WTPLA2 was Then Co-Cultured with plbEVEV (WTEV Co-Cultured with plbEVEV was Used as the Control). Cells were Induced to Determine Whether WTPLA2 Could Release Enough lysoPC to Affect the Growth of plb123 Mutant Cells. In Order to Fully Reflect the PLA2 Effect, 10-Times the Number of WTPLA2 Cells and WTEV Cells were Grown Against plbEVEV Cells, which were Mixed Together for Co-Culture as Follows: After WTEV, WTPLA2 and plbEVEV were Cultured to OD<sub>600</sub> 3.0, WTEV and WTPLA2 Cells were Each Diluted to OD<sub>600</sub> 1.0 while plbEVEV Cells were Diluted to OD<sub>600</sub> 0.1 and Then the Preparations Co-Cultured. Then Either Before Induction or After Two Days Induction in the Presence of Galactose, Cultures were Diluted 100-fold and 10  $\mu\text{l}$  was Spread on Plates with SC-URA-LEU Medium and Colonies Counted.**

Co-Culture Strain Combination	Induction Time	# of Cells / mL Culture
WTEV + plbEVEV	0 hr	2.77E+05 $\pm$ 1.533E+04
WTPLA2 + plbEVEV	0 hr	2.83E+05 $\pm$ 5.774E+03
WTEV + plbEVEV	48 hr	1.40E+06 $\pm$ 1.447E+05
WTPLA2 + plbEVEV	48 hr	1.42E+06 $\pm$ 1.422E+05

Values are reported as # of colonies per mL of culture and are the average of three replicates  $\pm$  SD.

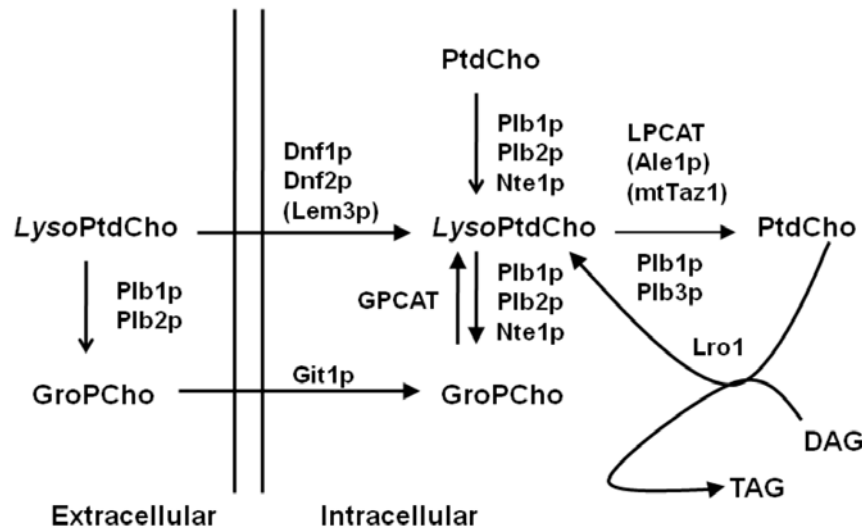


**Fig. (4).** Co-expression of either *PLB1* or *PLB2* or *PLB3* with *PLA2*, respectively, in the *plb123* triple mutant. All strains are designated by those plasmids expressed in the *plb* strain as designated in Table 1 (e.g. *plbPLA2PLB1* for the *plb* triple mutant co-expressing the Arabidopsis *PLA2* and the yeast *PLB1*). Transformants were cultured to the stationary phase in SC medium with glucose and adjusted to an  $OD_{600}$  of 3.0 and also diluted by 10, 100 and 1000-fold; 10  $\mu$ l of each was spotted on plates with different media. The photograph was taken after 3 days of growth.



**Fig. (5).** Relative  $^{14}C$  *lysoPtdCho* content in  $^{14}C$  acetate labeling experiments conducted following co-expression of either *PLB1* or *PLB2* or *PLB3* with *PLA2*, respectively, in the *plb123* triple mutant. All strains are designated by those plasmids expressed in the *plb* strain as explained for Table 3 and indicated in Table 1. Starting from a cell density of 0.1 ( $OD_{600}$ ) in 20 ml of glucose medium, cells of *plbPLA2EV*, *plbPLA2PLB1*, *plbPLA2PLB2* and *plbPLA2PLB3* were labeled with 30  $\mu$ Ci  $^{14}C$ -sodium acetate for 10 hours, then induced in galactose medium for 5 hours. The total lipids were extracted and resolved by two dimensional thin layer chromatography and the  $^{14}C$  *lysoPtdCho* was quantified (see Materials and methods). The *plb123* triple mutant expressing only the *PLA2*, designated as *plbPLA2EV*, accumulated  $^{14}C$  *lysoPtdCho* to 1.0 % of the total phospholipid (PL) fraction, as was typical in previous experiments (See Fig. 3). The *plbPLA2PLB1*, *plbPLA2PLB2* and *plbPLA2PLB3* co-transformants exhibited 60-75% reductions in the relative  $^{14}C$  *lysoPtdCho* content. Values are the average of 2 replicates.





**Fig. (6).** Simplified scheme of *lysoPtdCho* metabolism in *Saccharomyces cerevisiae*. **Abbreviations are:** *LysoPtdCho* (lysophosphatidylcholine), *GroPCho* (glycerophosphocholine), *PtdCho* (phosphatidylcholine), *LPCAT* (lysophospholipase:acyl-CoA acyltransferase), *mtTaz1* (mitochondrial acyl-CoA-independent LPCAT; [25]); *Plb1p*, *Plb2p*, *Plb3p* (phospholipases B1, B2 and B3), *Nte1p* (neuropathy target esterase); *Dnf1p* and *Dnf2p* (aminophospholipid translocating P-type ATPases 1 and 2, respectively); *Lem3p* (noncatalytic  $\beta$  subunit of *Dnf1p* and *Dnf2p*); *GPCAT* (glycerophosphocholine acyltransferase); *LRO1* (phospholipid:diacylglycerol acyltransferase; PDAT; [19, 26]); *Git1p* (glycerophosphoinositol/glycerophosphocholine transporter; [27]). Not shown in schematic: PLBs are also involved in the hydrolysis of PC and PI to produce extracellular glycerophosphocholine and glycerophosphoinositol, respectively [28].

produced *in vitro* in yeast lysates *via* *Lro1*, transferring an acyl group from PC to diacylglycerol [22].

There are several ways to eliminate excess *lysoPtdCho*. *LysoPtdCho* can be converted to *PtdCho* in the presence of acyl-CoA by lysophospholipase:acyl-CoA acyltransferase (*LPCAT*; EC 2.3.1.23) and there are several recent reports to support this [23-27]. Over-expression of *LPCAT* can enhance the cell's resistance to *lysoPtdCho* [27]. In yeast, there is also a mitochondrial acyl-CoA-independent *LPCAT* activity, mediated by *Taz1* [28]. Finally, *lysoPtdCho* can also be catabolized *via* *Plb1p*, *Plb2p*, *Plb3p* or *Nte1p*. Over-expression of *PLBs* was known to enhance cell resistance to *lysoPtdCho* and the *plb123* triple mutant cells showed more sensitivity to *LysoPtdCho* [8, 9]. At the time, extracellular *PLB* activity was known but the existence of the exogenous *lysolipid* metabolism (*ELM*) pathway, reported only recently [19], was not. Thus it was thought that *PLBs* could degrade exogenous *lysophospholipids* and increase the resistance to the occurrence of such lipids in the medium; the degradation of *LysoPtdCho* by *PLBs* was considered to occur only extracellularly.

In our hands, and consistent with these previous studies, when cultured on synthetic minimal medium (*SC*) supplemented with appropriate nutrients (*BSM-URA* or *BSM-LEU-URA* plus glucose or galactose), we did not observe any apparent difference in growth rate between the wild type and *plb1plb2plb3* triple mutant. In this study, by means of expressing an *AtPLA2* in *plb123* and *WT* cells and comparing their growth with un-induced cells, we found that expression of *AtPLA2* could completely inhibit growth of the *plb123* triple mutant cells, but not that of wild type cells. Further investigation revealed that excessive *lysoPtdCho* accumulation occurred only in *plb123* cells expressing the *AtPLA2*. Co-cultivation experiments supported the hypothe-

sis that this effect was due primarily to *intracellular*, rather than extracellular, *lysoPtdCho* accumulation.

Complementation experiments showed that all three *PLBs* could rescue the growth inhibition of *plb123* triple mutant cells caused by *AtPLA2* expression. Finally, in addition to relief from growth inhibition, the cells of *plb123PLA2* complemented with *PLBs* also exhibited a dramatic 60-75% reduction in *lysoPtdCho* accumulation. Collectively, these findings directly support our hypothesis that *PLBs* play a very important role in catabolizing excess intracellular *lysoPtdCho*. Phospholipase B exhibits three activities *in vitro*: 1) phospholipase A activity; 2) *lysophospholipase* activity; 3) *transacylase* activity. The *lysophospholipase* activity of *Plb1p* and *Plb2p* is about 100 fold higher than its *PLPase A* activity; even its *transacylase* activity is higher than that of *PLPase A*. In this regard, it is probably more suitable to refer to *PLBs* as *lysophospholipases* or *transacylases*, rather than phospholipase Bs. As the exception, no *lysophospholipase* activity has been detected in *Plb3p*, but it shows some *transacylase* activity [12]. Thus, our observation that *Plb3p* can rescue the growth inhibition of the *plb123* triple mutant expressing the *AtPLA2*, might be due to *Plb3p* acting as a *transacylase*.

## CONCLUSION

In conclusion, our results show that *PLBs* can efficiently eliminate excess *lysoPtdCho* produced by *PLA2* to maintain healthy cell growth. In the absence of any experimental evidence, it cannot be excluded that extrusion and processing by transporters such as *Nte1p*, also contribute to the processing of *lysoPC* to maintain homeostasis. It is, however, interesting to note that, even though *NTE1* and *LPCAT* are still present in the *plb123* mutant, our results suggest that the intracellular *lysoPtdCho* which accumulates in the presence of over-

expressed Arabidopsis PLAs is more reliant on PLBs to maintain yeast membrane homeostasis through lipid catabolism. Our results do not preclude other roles for PLBs. Given that the common reaction product of PLAs acting on PtdCho is *lyso*PtdCho, and that we were able only to observe increases in this specific *lyso*-phospholipid upon expression of PLA2, it is reasonable to assume that the over-expression product in this case is solely *lyso*PtdCho. However, this does not *a priori*, rule out other unknown lipid products being produced.

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