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PSI1 is responsible for the stearic acid enrichment that is characteristic of phosphatidylinositol in yeast

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In yeast, both phosphatidylinositol and phosphatidylserine are synthesized from cytidine diphosphate-diacylglycerol. Because, as in other eukaryotes, phosphatidylinositol contains more saturated fatty acids than phosphatidylserine (and other phospholipids), it has been hypothesized that either phosphatidylinositol is synthesized from distinct cytidine diphosphate-diacylglycerol molecules, or that, after its synthesis, it is modified by a hypothetical acyltransferase that incorporates saturated fatty acid into neo-synthesized molecules of phosphatidylinositol. We used database search methods to identify an acyltransferase that could catalyze such an activity. Among the various proteins that we studied, we found that Psi1p (phosphatidylinositol stearoyl incorporating 1 protein) is required for the incorporation of stearate into phosphatidylinositol because GC and MS analyses of *psi1Δ* lipids revealed an almost complete disappearance of stearic (but not of palmitic acid) at the *sn*-1 position of this phospholipid. Moreover, it was found that, whereas glycerol 3-phosphate, lysophosphatidic acid and 1-acyl lysophosphatidylinositol acyltransferase activities were similar in microsomal membranes isolated from wild-type and *psi1Δ* cells, microsomal membranes isolated from *psi1Δ* cells are devoid of the *sn*-2-acyl-1-lysolysophosphatidylinositol acyltransferase activity that is present in microsomal membranes isolated from wild-type cells. Moreover, after the expression of *PSI1* in transgenic *psi1Δ* cells, the *sn*-2-acyl-1-lysolysophosphatidylinositol acyltransferase activity was recovered, and was accompanied by a strong increase in the stearic acid content of lysophosphatidylinositol. As previously suggested for phosphatidylinositol from animal cells (which contains almost exclusively stearic acid as the saturated fatty acid), the results obtained in the present study demonstrate that the existence of phosphatidylinositol species containing stearic acid in yeast results from a remodeling of neo-synthesized molecules of phosphatidylinositol.

Abbreviations
CDP-DAG, cytidine diphosphate-diacylglycerol; DRM, detergent-resistant membrane; ER, endoplasmic reticulum; FAMES, fatty acid methyl esters; G3PAT, glycerol 3-phosphate acyltransferase; GPI, glycosylphosphatidylinositol; LPAAT, lysophosphatidic acid acyltransferase; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; Psi1p, phosphatidylinositol stearoyl incorporating 1 protein; TAG, triacylglycerol.

Introduction

In *Saccharomyces cerevisiae*, as in other eukaryotic cells, phosphatidylinositol (PI) serves not only as a component of cellular membranes, but also as a precursor for metabolites involved in various important cellular processes, such as glycolipid anchoring of proteins, membrane microdomains, signal transduction, membrane trafficking, protein sorting and cytoskeletal regulation [1–5]. The fatty acid composition of this phospholipid is quite distinctive in living organisms because, in comparison with other phospholipids, PI is by far the most saturated. In bovine heart, bovine liver and rat liver, palmitic and stearic acid represent 5–8% and 32–49% of the total fatty acid esterified to PI, respectively [6–8]. By contrast, PI from plants contains much more palmitic acid than stearic acid as the major (saturated) fatty acid; for example, 48% and 3%, respectively, in *Arabidopsis thaliana* [9]. Similarly, in yeast, PI has a high percentage of saturated fatty acid (52–53%) [10] (see also below) and, as observed in other higher eukaryotes, unsaturated acyl chains are mainly linked to the *sn*-2 position and saturated fatty acid groups to the *sn*-1 position [11] (see also below). Nevertheless, in yeast, the difference between palmitic ($\sim$ 30–40% of total fatty acids) and stearic acid content ($\sim$ 10–15% of total fatty acids) is much less pronounced than in plants and animal tissues [12,13] (see also below).

In *S. cerevisiae*, phospholipids are synthesized via pathways that are largely conserved throughout eukaryotes, and the biosynthetic pathway for PI synthesis is well documented [14]. PI is synthesized from phosphatidic acid (PA), which is further used as substrate by a cytidine diphosphate-diacylglycerol synthase to produce cytidine diphosphate-diacylglycerol (CDP-DAG). CDP-DAG is then converted into PI by a phosphatidylinositol synthase or into phosphatidylserine (PS) by a phosphatidylserine synthase. Nevertheless, as noted above, even though PS and PI are both derived from PA and CDP-DAG, the fatty acid compositions of these glycerophospholipids significantly differ because PS from yeast barely contains any stearic acid [10,12] (see also below). Two main hypotheses have been put forward to explain this difference. One is that a different selectivity of PI and PS synthases for particular molecular species of CDP-DAG (or a distinct channeling of molecular species of CDP-DAG to different subdomains of the endoplasmic reticulum (ER) where the two enzymes may be localized) results in different fatty acid compositions of PS and PI. The other is that, after its synthesis, PI is further modified by a hypothetical acyltransferase that incorporates stearic and/or palmitic acid into neo-synthesized PI molecules [10]. Nevertheless, irrespective of the kingdom, an enzyme of this kind has not been described to date. To identify a protein from *S. cerevisiae* that could catalyze such an activity, we performed a genomic database search, focusing our analysis on proteins belonging to the family of the glycerolipid acyltransferases containing a highly conserved NHX_4D domain. In *S. cerevisiae*, the first characterized members of this family were Slc1p, an lysophosphatidic acid acyltransferase (LPAAT) [15], and Ypr140wp, a lysophosphatidylcholine acyltransferase [16]. It should be noted that other acyltransferases were recently identified in yeast: Yor175cp, a lysophospholipid acyltransferase [17–24], and Gup1p, which adds C26:0 fatty acids into the lysoPI moiety of glycosylphosphatidylinositol (GPI) anchor proteins [25]. These proteins belong to the 'membrane-bound *O*-acyltransferase' family, comprising a family of acyltransferases not yet reported for yeast at the time of our search, and which was therefore not included in our database search. Nevertheless, our analysis of yeast acyltransferases allowed us to identify four uncharacterized proteins, and we further carried out comparative lipidome analyses of wild-type and corresponding deletion strains. As described below, this approach allows us to state confidently that Psi1p (phosphatidylinositol stearoyl incorporating 1 protein, encoded by the *PSI1* gene, alias *YBR042C*) is the yeast protein responsible for the stearic acid enrichment characteristic of phosphatidylinositol. The functional characterization of the other glycerolipid acyltransferase members that we identified is currently under investigation in our laboratory.

Results

Aberrant PI-acyl composition in *psi1*Δ mutant

The BLAST algorithm was employed to search the *S. cerevisiae* genome database for putative glycerolipid acyltransferase genes, by using known lysolipid acyltransferase gene sequences from the bacterial, yeast, plant and animal kingdoms as queries. Beside the genes *SLC1* [15] and *YPR140W* [16] that were previously characterized and which encode an acyl-CoA: LPAAT and an acyl-CoA independent lysophosphatidylcholine acyltransferase, respectively, four proteins became evident (not shown). Among them, we decided to focus on Psi1p because of the aberrant PI-acyl composition of the corresponding deletion mutant (see below). This protein contains at least two of the four conserved domains generally associated with

glycerolipid acyltransferases (Fig. S1), and it has been shown that motifs I (NHX_4D) and III (FPEGT) might be the catalytic sites of these enzymes [26,27]. In addition, sequence analysis of Psi1p suggests the presence of a signal anchor at the N-terminus (SIGNALP 3.0 server, www.cbs.dtu.dk/services/SignalP), and the presence of four transmembrane helixes (TMHMM 2.0 server, www.cbs.dtu.dk/services/TMHMM). The use of the PSORT II server (www.genscript.com/psort/psort2.html) did not predict a clear-cut subcellular location of this protein, but large-scale analyses of protein location in *S. cerevisiae* based on the green fluorescent protein-fusion strategy localized Psi1p in ER and/or in lipid particles (http://ypl.uni-graz.at/pages/home.html) [28,29].

As noted above, TLC and GC were used to compare the glycerophospholipid content of wild-type and *psi1Δ* mutant cells (EUROSCARF collection; Frankfurt, Germany, http://web.uni-frankfurt.de/fb15/mikro/euroscarf/col_index.html). These contents were related to that described in a previous study [11], and no differences were found between wild-type and *psi1Δ* mutant cells (Table 1). In other words, the distribution of glycerophospholipid classes was not affected by the deletion. By contrast, as shown in Table 1, analyses of the fatty acid composition of phospholipid classes revealed that the *PSI1* mutation induced a drastic change in PI because PI from mutant cells was practically devoid of stearic acid (1.5 ± 0.2% of total PI fatty acids compared to 10.3 ± 0.1% in wild-type). This decrease in the stearic acid content of PI was mainly compensated for by an increase in the palmitoleic acid content, and was not observed for other phospholipid classes, suggesting a specificity for this particular class of phospholipid.

When the DAG, triacylglycerol (TAG), free fatty acid, steryl ester and total phospholipid contents were analyzed, it was found (Table S1) that the percentage of neutral lipids was slightly higher in the *psi1Δ* mutant (TAG: 6.0 ± 0.4% in wild-type versus 8.8 ± 0.9% in mutant; steryl ester: 2.50 ± 0.42% in wild-type versus 3.8 ± 0.4% in mutant), whereas, correlatively, the percentage of total phospholipids was lower (82 ± 2% in wild-type versus 76 ± 3% in mutant). No significant difference was observed in the fatty acid composition of neutral lipids from *psi1Δ* mutant and from wild-type cells.

The reduction of 18 : 0-containing PI molecular species in mutant cells was further checked by multiple precursor ion scanning analysis [30]. Figure 1 shows the composition of the various PI molecular species purified from wild-type and *psi1Δ* mutant cell cultures. In agreement with the GC analyses, it clearly appears that the percentages of 18 : 0-containing PI molecular species, namely 18 : 0–16 : 1 PI and 18 : 0–18 : 1 PI, were strongly reduced in the mutant: 0.91 ± 0.05% versus 6.25 ± 0.01% in wild-type cells, and 0.83 ± 0.02% versus 6.96 ± 0.07% in wild-type cells, respectively. No significant difference was found between the molecular species of other phospholipids classes [phosphatidylcholine (PC), phosphatidylethanolamine (PE), PA and PS, not shown] of wild-type and *psi1Δ* mutant cells. This is in agreement with the above results obtained by TLC-GC analyses, confirming the specificity of Psi1p for the class of phosphatidylinositol by both an *in vivo* approach and also at the molecular species level.

Aberrant stearate content is specifically associated with the *sn*-1 position

Based on the fatty acid composition of steady-state PI in yeast, it was shown that 77% of the fatty acids are

Table 1. Distribution and fatty acid composition of phospholipids from *psi1Δ* mutant and wild-type cells. Cells were grown in the presence of 2% glucose and harvested at the midlogarithmic phase. Lipids were purified and quantified as described in the Experimental procedures. Results are shown as mol% and represent the mean ± SD of six analyses (independent cultures).

		Fatty acid composition			
Polar lipids	Percentage of total lipids	16 : 0 (%)	16 : 1 (%)	18 : 0 (%)	18 : 1 (%)
PC					
Wild-type	47.24 ± 0.74	11.72 ± 0.33	68.07 ± 0.65	2.04 ± 0.07	18.17 ± 0.75
Mutant	48.86 ± 0.82	16.01 ± 0.51	66.56 ± 0.38	1.62 ± 0.14	15.81 ± 0.34
PS					
Wild-type	9.29 ± 0.56	44.02 ± 1.09	22.49 ± 0.52	3.36 ± 0.32	30.14 ± 1.39
Mutant	9.47 ± 0.95	44.99 ± 0.88	28.44 ± 0.56	2.35 ± 0.86	24.22 ± 1.61
PI					
Wild-type	22.77 ± 0.93	40.75 ± 0.38	18.80 ± 0.29	10.27 ± 0.12	30.18 ± 0.41
Mutant	21.70 ± 0.83	42.17 ± 0.61	28.89 ± 0.88	1.49 ± 0.20	27.45 ± 1.12
PE					
Wild-type	20.70 ± 0.69	18.07 ± 0.40	49.30 ± 0.37	0.71 ± 0.04	31.92 ± 0.34
Mutant	19.97 ± 0.93	17.61 ± 0.52	53.45 ± 0.59	0.59 ± 0.13	28.35 ± 0.64

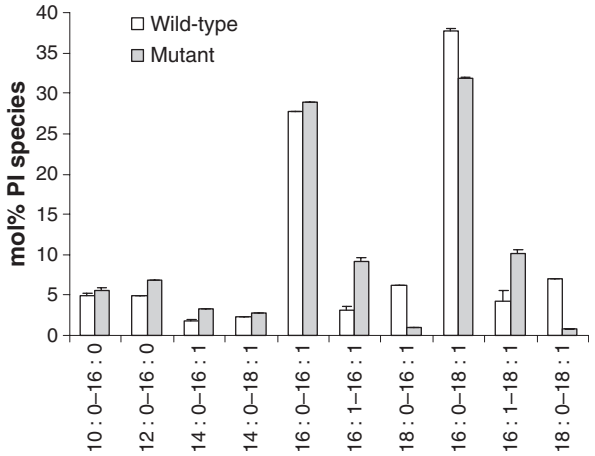

Fig. 1. Molecular composition of PI. PI species were profiled by multiple precursor ion scanning analysis on a quadrupole TOF mass spectrometer as previously described [29]. Error bars indicate ± SD (n = 3 independent experiments).

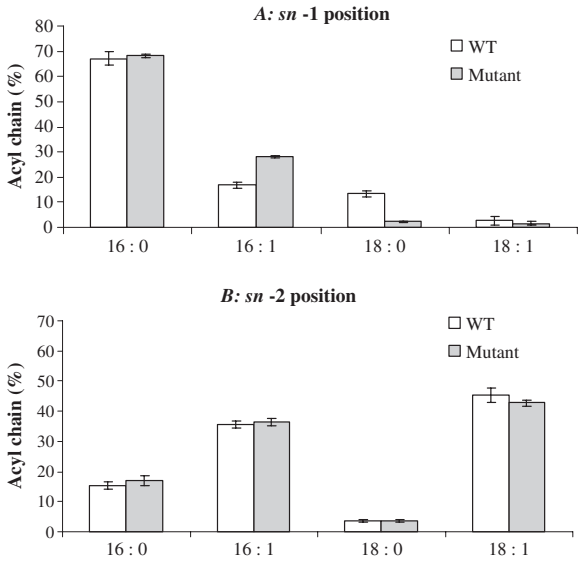

Fig. 2. Fatty acid composition at the *sn*-1 and *sn*-2 positions of PI from wild-type and *psi1Δ* mutant cells. PI was purified from BY4742 wild-type and *psi1Δ* mutant cells grown to midlogarithmic phase on YPD medium and assayed for the positional analysis of fatty acids, as described in the Experimental procedures. Assays were performed in duplicate on three independent cultures.

saturated in the *sn*-1 position, whereas unsaturated fatty acids are predominantly found at the *sn*-2 position [11]. We further determined whether Psi1p is responsible for the incorporation of stearic acid into the *sn*-1 position of PI. To determine the positional distribution of stearic acid, lipid extracts from wild-type and *psi1Δ* mutant cells were purified by TLC, and PI was further subjected to *sn*-2 specific hydrolysis by phospholipase A_2. The reaction products, namely lysophosphatidylinositol and fatty acid, were separated by TLC, and their acyl chain compositions were determined by GC. Figure 2 shows that, as expected [11], the fatty acid composition of PI was characterized by a high degree of saturation associated with the *sn*-1 position in the wild-type (80%; Fig. 2A) and a low degree of saturation associated with the *sn*-2 position (close to 20% for both wild-type and mutant cells; Fig. 2B). These values are in agreement with the percentage of saturated fatty acids detected in PI from wild-type cells (51%; Table 1). In addition, it was clearly apparent (Fig. 2A) that the percentage of stearic acid associated with the *sn*-1 position was strongly reduced in the mutant (13.4 ± 1.0% in wild-type versus 2.2 ± 0.3% in mutant) and that, according to the GC analyses of whole cells (Table 1), this decrease was mainly compensated for by an increase in the percentage of palmitoleic acid. By contrast, the pattern of fatty acid released from *sn*-2 position was similar in wild-type and mutant cells (Fig. 2B). Taken together, these results clearly indicate that the reaction catalyzed by Psi1p exclusively addressed the *sn*-1 position of PI.

Psi1p is associated with microsomal membranes

Next, we examined the phospholipid content of microsomal membranes and mitochondria isolated from wild-type and mutant cells grown on lactate. No significant differences in the phospholipid contents of these fractions were observed between wild-type and mutant cells (Table 2). By contrast, significant differences were observed in the fatty acid composition of phospholipids: the levels of stearic acid in microsomal and mitochondrial PI were drastically reduced in the *psi1Δ* strain. Because, in yeast, PI molecules are synthesized in ER membranes and some are then exported to mitochondria, the results obtained in the present study suggest strongly that PI is remodeled in microsomal membranes before being transferred to mitochondria or, in other words, that Psi1p is located in the microsomal membranes. This finding is in agreement with results of a study based on the green fluorescent protein-fusion strategy (http://ypl.uni-graz.at/pages/home.html) and with the absence of Psi1p among the proteins identified in proteomic studies of *S. cerevisiae* mitochondria [31,32].

Psi1p is involved in PI remodeling

As noted in the Introduction, at least two models can explain the specific enrichment of PI with stearic acid

Table 2. Fatty acid composition of phospholipids purified from microsomes and from mitochondria of *psi1Δ* mutant and wild-type cells. Cells were grown in the presence of 2% lactate and harvested during the midlogarithmic phase. Subcellular fractions were obtained, and lipids were purified and quantified as described in the Experimental procedures. Values represent the mean ± SD (n = 3).

		Fatty acid composition			
	Percentage of total lipids	16 : 0 (%)	16 : 1 (%)	18 : 0 (%)	18 : 1(%)
Microsomes					
PC					
Wild-type	44.06 ± 0.91	12.38 ± 0.94	55.31 ± 2.61	3.93 ± 0.65	28.38 ± 1.64
Mutant	45.05 ± 1.31	13.87 ± 0.38	58.37 ± 0.23	2.50 ± 0.10	25.27 ± 0.47
PS					
Wild-type	11.00 ± 0.51	22.00 ± 0.72	31.87 ± 5.07	3.96 ± 1.47	42.18 ± 2.94
Mutant	13.16 ± 0.64	24.22 ± 0.97	29.19 ± 3.42	4.74 ± 2.09	41.85 ± 2.25
PI					
Wild-type	17.75 ± 0.40	25.92 ± 0.57	29.92 ± 0.26	7.90 ± 0.36	36.26 ± 0.10
Mutant	17.03 ± 2.55	28.50 ± 1.10	36.91 ± 0.08	2.42 ± 0.24	32.17 ± 0.78
PA + CL					
Wild-type	4.49 ± 1.65	22.58 ± 2.59	25.71 ± 4.37	8.31 ± 0.15	43.40 ± 4.33
Mutant	2.51 ± 0.43	20.51 ± 2.07	33.12 ± 3.08	6.58 ± 1.17	39.79 ± 1.49
PE + PG					
Wild-type	22.70 ± 0.41	9.78 ± 0.92	52.68 ± 2.80	1.46 ± 0.44	36.07 ± 1.73
Mutant	22.60 ± 0.75	9.98 ± 0.30	54.76 ± 0.57	1.65 ± 0.19	33.61 ± 0.08
Mitochondria					
PC					
Wild-type	31.51 ± 8.78	7.82 ± 0.59	63.83 ± 0.69	2.22 ± 0.20	26.13 ± 0.11
Mutant	31.91 ± 0.26	11.14 ± 0.50	64.50 ± 0.66	1.60 ± 0.23	22.76 ± 0.24
PS					
Wild-type	2.37 ± 1.05	15.21 ± 0.62	53.52 ± 1.61	4.41 ± 0.58	26.87 ± 1.65
Mutant	2.95 ± 1.27	17.83 ± 1.10	62.37 ± 6.13	1.89 ± 0.79	17.91 ± 4.23
PI					
Wild-type	10.45 ± 0.39	22.64 ± 0.20	27.53 ± 0.62	8.33 ± 0.19	41.51 ± 0.27
Mutant	13.13 ± 2.05	26.05 ± 0.37	37.34 ± 3.26	1.99 ± 0.50	34.62 ± 3.09
PA + CL					
Wild-type	12.79 ± 1.30	3.63 ± 0.04	46.51 ± 0.72	0.80 ± 0.05	49.06 ± 0.64
Mutant	15.00 ± 0.86	4.84 ± 0.14	50.45 ± 0.04	0.88 ± 0.09	43.83 ± 0.19
PE + PG					
Wild-type	42.89 ± 6.05	8.21 ± 0.18	51.06 ± 0.08	0.31 ± 0.02	40.41 ± 0.16
Mutant	37.00 ± 1.90	10.43 ± 0.15	53.12 ± 0.46	0.31 ± 0.06	36.13 ± 0.55

in yeast. The first hypothesis, previously raised for plant cells [33], involves the synthesis of two kinds of CDP-DAG molecules: the first type containing stearic acid at the *sn*-1 position would be the substrate of the sole PI synthase and the second type would be devoid of the fatty acid at this position. In accordance with this hypothesis, Psi1p would be a glycerol 3-phosphate acyltransferase (G3PAT) synthesizing *sn*-1-stearoyl-2-lysoPA molecules. These molecules would be not synthesized in mutant cells and therefore a decrease in the content of PI in *psi1Δ* cells (and consequently an increase in the percentage of the other phospholipids) could be expected.

This was not observed and, in contrast, it appeared that the phospholipid distribution (and particularly the abundance of PI) was similar in wild-type and mutant cells (Table 1). Therefore, it appears that, whatever the phospholipid taken into consideration (e.g. PI), its *de novo* synthesis was not impaired in *psi1Δ* cells. In agreement, after *in vivo* pulse-labeling experiments using [^{14}C]glycerol, we did not observe any differences in the distribution of the label into the various phospholipids (including PI) in wild-type and mutant cells (Fig. S2). Taken together, these results suggest that the mutation did not induce any specific decrease in the *de novo* synthesis of a given phospholipid, including PI. Hence, the rate of synthesis and the amount of CDP-DAG molecules that were used as substrate for PI synthesis appeared to be the same in mutant and wild-type cells or, in other words, Psi1p does not appear to be a G3PAT specifically involved in the synthesis of phospholipids containing stearic acid at

the *sn*-1 position. To test this assumption, the G3PAT (and LPAAT) activities associated with microsomal membranes isolated from wild-type and *psi1Δ* cells were determined. The results obtained are shown in Fig. 3. As expected (and as a control), the incorporation of oleoyl-CoA into lysoPA and PA was the same when microsomal membranes isolated from wild-type and *psi1Δ* cells were used (synthesis of ~ 80 pmol of PA when 20 μM oleoyl-CoA were used in our experimental conditions). These incorporations were much lower when stearic acid was used as substrate. More importantly, the *in vitro* incorporation of stearic acid into lipids was the same with membranes containing Psi1p as it was in membranes devoid of this protein. In other words, it appears that Psi1p is not a G3PAT (nor a LPAAT) that would specifically incorporate stearic acid into phospholipids.

Although Psi1p does not appear to be involved in PI *de novo* synthesis, it might be involved in the stearic acid incorporation after the *de novo* synthesis of this lipid (i.e. the second hypothesis). According to this hypothesis, previously raised for mammals [34–36], the decrease in the percentage of stearic acid into PI in *psi1Δ* cells would be not accompanied by a change in the phospholipid composition of cells. In addition, because PI and PS are assumed to be synthesized from the same CDP-DAG pool, the absence of Psi1p would lead to a similar fatty acid composition of PI and PS in mutant cells. All these results were observed after the GC analyses: there was no change in the phospholipid composition in mutant cells and the content of various fatty acids within PI class was similar to PS (Table 1). Hence, the results of the GC analyses are in agreement with the possibility that Psi1p catalyzes the incorporation of stearic acid into neo-synthesized PI molecules. To strengthen such an assumption, we designed further experiments to demonstrate that, whereas PI *de novo* synthesis is not affected in the *psi1Δ* deletion mutant, the incorporation of fatty acids associated with PI is decreased in the *psi1Δ* deletion mutant. Hence, we performed *in vivo* labeling experiments using [^{14}C]acetate, a precursor for acyl chain biosynthesis. To carry out such an analysis, the strains were grown to midlogarithmic phase and pulse-labeled with [^{14}C]acetate. After a 40-min pulse, lipids were extracted and the label incorporation into polar and neutral lipids was analyzed by TLC. Under the conditions used, ~ 75% and 25% of the lipid-incorporated label was associated with polar lipids and neutral lipids, respectively (not shown), both in wild-type and *psi1Δ* mutant cells. Within the neutral lipid fraction, the label was mainly associated with TAG, DAG and steryl esters, whereas sterols and free fatty acids were the least labeled species (Fig. S3A). No significant difference was observed between wild-type and *psi1Δ* cells.

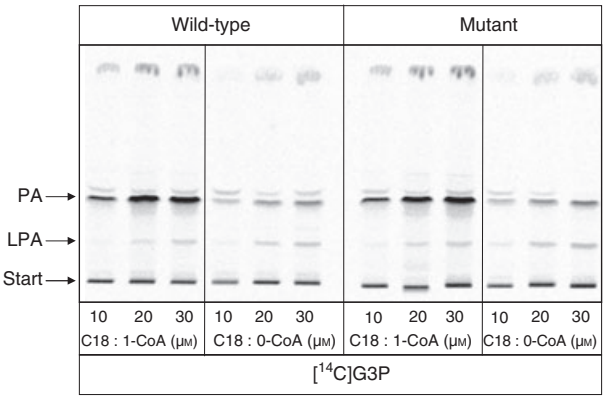

Fig. 3. The microsomal fractions of *psi1Δ* mutant and wild-type cells display similar G3PAT activities. Microsomes from *psi1Δ* mutant and wild-type cells grown on YPL and harvested at midlogarithmic phase were analyzed. G3PAT activity analyzed by TLC using [^{14}C]G3P as a radiolabeled acyl-acceptor and either oleoyl-CoA or stearoyl-CoA as acyl-donor with the indicated concentrations. Similar results were obtained using either [^{14}C]oleoyl-CoA or [^{14}C]stearoyl-CoA as radiolabeled acyl-donor substrates and G3P as acyl-acceptor.

The distribution of the label into the various polar lipids from wild-type and *psi1Δ* cells is shown in Fig. S3B. The [^{14}C]acetate label was mainly incorporated into PI, PC and PS, whereas PA + cardiolipin (CL) and PE + phosphatidylglycerol (PG) were labeled to a lesser extent. Because cells were submitted to a short pulse labeling, it can be hypothesized that the label was mainly incorporated into lipids by acyl exchange rather than by the *de novo* synthesis. In agreement, (a) the distribution of the label into various lipids (Fig. S3) did not reflect the lipid composition at the stationary state (Table 1) and (b) the *de novo* synthesis determined by incorporation of labeled glycerol was not modified in mutant cells (Fig. S2). In other words, differences in the [^{14}C]acetate label incorporation into various lipids between wild-type and mutant cells likely reflect differences in the incorporation by acyl exchange of labeled fatty acids into endogenous lipids. The results shown in Fig. S3 show that the percentages of the [^{14}C]acetate label incorporated into PI differed significantly ($P = 0.03$) in wild-type and in *psi1Δ* cells [37 ± 3% ($n = 7$) and 34 ± 2% ($n = 7$), respectively]. This decrease, which corresponds to ~ 8% of the acetate label associated with the fatty acids esterified to PI in wild-type cells, is in agreement with the difference in the stearic acid content of PI in wild-type and mutant whole cells (~ 8–9%; Table 1). This decrease was compensated by an increase in PC

[31.0 ± 1.5% (n = 7) and 34.2 ± 1.9% (n = 7)]. The label incorporation into other polar lipids was not different in mutant and wild-type cells. Taken together, these results suggest that Psi1p is responsible for the incorporation of stearic acid in neo-synthesized PI molecules.

The above *in vivo* experiments demonstrate that the absence of *PSI1* affects specifically the stearic content of PI. To further confirm these differences *in vitro*, we determined the lysophosphatidylinositol acyltransferase activities associated with microsomal membranes isolated from wild-type and *psi1Δ* cells (Fig. 4). It clearly appeared that membranes from both cell types were able to incorporate stearic acid in the *sn*-2 position of PI (i.e. synthesis of ~ 32 pmol of PI from both in our experimental conditions), but that, unlike microsomes from wild-type cells, microsomes from *psi1Δ* cells were unable to incorporate stearic acid in the *sn*-1 position of PI (i.e. synthesis of 30 pmol of PI for wild-type, traces for mutant). Moreover, because microsomal fractions from wild-type and *psi1Δ* cells showed lysophosphatidylinositol acyltransferase activity when *sn*-1-acyl-2-lysoPI was used as substrate, it appears that *sn*-2-acyl-1-lyso-PI was not significantly converted to the *sn*-1 isomer during the assay procedure (otherwise an activity with microsomes from *psi1Δ* cells would have been observed in the presence of *sn*-2-acyl-1-lyso-PI). Using phospholipase A_2 treatment, we further checked that the labeled stearoyl-CoA was positioned at the *sn*-1 position when integrated into *sn*-2-acyl-1-lysoPI *in vitro* (Fig. S4). After hydrolysis of the resulting PI, lysoPI was the sole labeled product, indicating a direct acylation of stearic acid at the *sn*-1 position of lysoPI and excluding the possibility of a transacylation mechanism from the *sn*-2 to the *sn*-1 position of PI. We further carried out experiments to measure the specificity of the enzyme *in vitro*. The enzyme under study was able to use various long chain acyl-CoAs as substrates

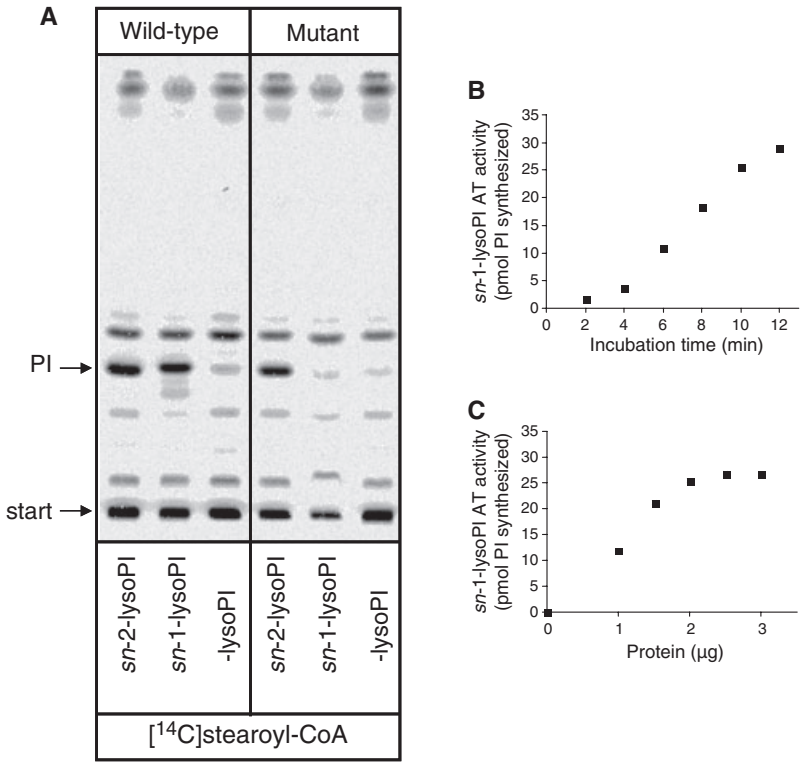

Fig. 4. The microsomal fraction of *psi1Δ* mutant cells lacks *sn*-2-acyl-1-lysoPI acyltransferase activity. (A) Microsomal membrane proteins (2 µg) were incubated with [^{14}C]stearoyl-CoA (1 nmol) in the absence (-lysoPI) or in the presence of *sn*-1-acyl-2-lysoPI or *sn*-2-acyl-1-lysoPI (1 nmol). After 10 min of incubation, lipids were extracted and analyzed by TLC using chloroform/methanol/1-propanol/methyl acetate/0.25% aqueous KCl (10 : 4 : 10 : 10 : 3.6) as solvent followed by radioimaging. Results are from one experiment representative of three experiments performed with independent microsome preparations. Radioactivity located above PI correspond to a contamination of the [^{14}C]stearoyl-CoA (present at t = 0). *sn*-2-lysoPI, *sn*-1-acyl-2-lysoPI; *sn*-1-lysoPI, *sn*-2-acyl-1-lysoPI. (B, C) *sn*-2-acyl-1-lysoPI acyltransferase assays were performed as a function of time using 2 µg of microsomal membrane proteins, 1 nmol [^{14}C]stearoyl-CoA and 1 nmol *sn*-2-acyl-1-lysoPI (B) and with the indicated amounts of proteins using 1 nmol [^{14}C]stearoyl-CoA and 1 nmol *sn*-2-acyl-1-lysoPI, and 10 min of incubation (C).

when these molecules were added to the incubation mixture (not shown). This is not an unexpected result because *in vitro* conditions cannot fully mimic the *in vivo* enzyme environment, and therefore it does not challenge the experimental results in any way with respect to the specificity of the enzyme *in vivo* (Figs 1 and 2; Tables 1 and 2).

As a control, to demonstrate that the absence of *sn*-1-acyl-2-lysoPI acyltransferase activity in *psi1Δ* cells was a result of the absence of *PSI1*, transgenic *psi1Δ* mutant cells overexpressing *PSI1* were generated. As shown in Fig. 5, *sn*-2-acyl-1-lysoPI acyltransferase activity was clearly recovered in cells expressing *PSI1* in the *psi1Δ* mutant background whereas, as expected, this activity was not detected in the homogenates of *psi1Δ* mutant cells grown on the minimal synthetic medium supplemented with 2% glucose. For unknown reasons, the stearic acid contents in PI of *psi1Δ* mutant and wild-type cells grown on this media were slightly higher (3.7 ± 1.4% and 12.2 ± 0.23%, respectively) than the content observed on YPD media (Table 1). However, the main result is that the recovery of *sn*-2-acyl-1-lyso-PI acyltransferase activity *in vitro* that we observed after the expression of *PSI1* in transgenic *psi1Δ* mutant is accompanied by a strong enrichment of stearic acid associated with PI *in vivo* (8.5 ± 0.8%).

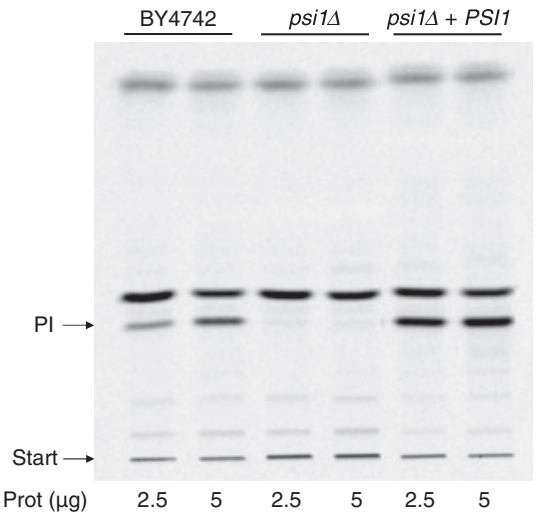

Fig. 5. *PSI1* expression restores the *sn*-2-acyl-1-lysoPI acyltransferase activity in *psi1Δ* mutant cells. Homogenate proteins (2.5 and 5 µg) from BY4742, *psi1Δ* and *psi1Δ* + *PSI1* cells obtained as described in the Experimental procedures were incubated with [^{14}C]stearoyl-CoA in the presence of *sn*-2-acyl-1-lysoPI. After 10 min of incubation, lipids were extracted and analyzed by TLC using chloroform/methanol/1-propanol/methyl acetate/0.25% aqueous KCl (10 : 4 : 10 : 10 : 3.6) as solvent followed by radioimaging. Results are representative of two experiments performed with two transgenic lines.

Discussion

PI is the most saturated phospholipid in plants [9], in mammals [6–8] and in yeast [10,11]. This specificity in the fatty acid composition of PI from various cell types is likely linked to physiological functions. For example, the *A. thaliana* PI species with specific fatty acyl moieties can yield either constitutive or stress-induced physiological pools of polyphosphoinositides [37]. Furthermore, in the epithelial cells of the cockroach rectum, phosphoinositide fatty acids regulate PI5 kinase, phospholipase C and protein kinase activities [38]. In addition, our group recently studied the phosphoinositide content of detergent-resistant membranes (DRM) from plant plasma membranes. We found not only that these microdomains (very likely involved in signaling pathways) are enriched in PI and its derivatives polyphosphoinositides, but also that, in DRM, these lipids contain much fewer polyunsaturated fatty acids than those purified from the total plasma membrane [39].

In Chinese hamster ovary cells, the GPI-anchored proteins that contain two saturated acyl chains in their PI moiety are generated from those bearing an unsaturated chain by fatty acid remodeling. These proteins are typically found within lipid rafts, whereas, very interestingly, the recovery of unremodeled GPI-anchored proteins in the DRM fraction from mutant cells was very low [40].

In animal cells, PI contains more saturated fatty acids than its precursor (CDP-DAG and PA) because neo-synthesized PI is rapidly remodeled by a deacylation/reacylation process that incorporates stearic acid predominantly at the *sn*-1 position [34–36]. The reason for the presence of saturated fatty acids associated with PI appears to be different in the plant kingdom because a recent study showed that *A. thaliana* contain two PI synthases (PIS1 and PIS2) differing in their substrate specificity *in vitro*: PIS1 prefers CDP-DAG species containing palmitic and oleic acids, whereas PIS2 prefers CDP-DAG species containing linoleic and linolenic acids [33]. The existence of a PI synthase using CDP-DAG species containing palmitic acid could explain why PI from *A. thaliana* contains more saturated (palmitic) fatty acids than other phospholipids. By contrast with plants, *S. cerevisiae* contains a unique PI synthase that is located in endoplasmic reticulum membranes [13,41]. Until the results of the present study were obtained, one hypothesis to explain the higher amount of stearic acid associated with PI in *S. cerevisiae* than with PS was that the PI synthase (but not the PS synthase) could use CDP-DAG containing stearic acid as substrate. A similar hypothesis

was put forward by Ferreira *et al*. [12] who showed that the increase in the amount of saturated fatty acids observed under conditions of impaired unsaturated fatty acid synthesis (i.e. heme depletion) is specifically associated with phosphatidylinositol. Such a hypothesis was also raised by Kaliszewski *et al*. [13] who observed a similar phenomenon in *rsp5Δ* mutant cells overexpressing PI synthase (a mutation in *rsp5*, a ubiquitin ligase gene, tends to indirectly induce the accumulation of saturated fatty acids). Moreover, the results of the present study provide strong evidence that the stearic acid content of PI from yeast is controlled by Psi1p, a specific acyltransferase that catalyzes the incorporation of this fatty acid in the *sn*-1 position of neo-synthesized PI. Nevertheless, the results obtained in the present study are not in disagreement with those obtained by Kaliszewski *et al*. [13] because the overexpression of the PI synthase in *rsp5Δ* mutant cells induced an increase only in the palmitic (but not in the stearic) acid content of PI. In other words, after PI synthase overexpression, the specific 'rerouting of CDP-DAG with saturated fatty acids towards PI' has an impact only on the palmitic (but not the stearic) acid content of PI. By contrast, even in the absence of PI synthase overexpression, the stearic (but not the palmitic) acid content was increased in PI from *rsp5Δ* mutant cells. Hence, taken together, these results clearly indicate that, in *S. cerevisiae*, the stearic and palmitic acid contents of PI are controlled by distinct mechanisms and that the stearic acid content is mediated by Psi1.

Experimental procedures

Materials

TLC plates were HPTLC silica gel 60 F 254 10 × 10 cm or TLC silica gel 60 F 254 20 × 20 cm (Merck, Darmstadt, Germany). Phospholipase A_2 from porcine pancreas was purchased from Sigma-Aldrich (St Louis, MO, USA). [1-^{14}C]acetic acid, sodium salt and [U-^{14}C]glycerol were obtained from GE Healthcare (Milwaukee, WI, USA); [^{14}C]glycerol 3-phosphate was obtained from Perkin Elmer Life Sciences (Boston, MA, USA); and [1-^{14}C]stearoyl-CoA was obtained from American Radiolabeled Chemicals (St Louis, MO, USA). Phosphatidylinositol, stearoyl-CoA, oleoyl-CoA, *sn*-1-acyl-2-lysoPI from soybean and *Rhizopus arrhizus* lipase were obtained from Sigma-Aldrich.

Yeast strains, growth media and preparation of homogenates, microsomes and mitochondria

The strains used in the present study were obtained from the European *S. cerevisiae* Archive for Functional Analysis (EUROSCARF) library. BY4742 (MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0) is a wild-type strain and *psi1Δ* (MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; *YBR042C*::kanMX4) is the deletion mutant. The cells were grown in a shaking incubator at 30 °C, in 250 mL Erlenmeyer flasks containing 50 mL of liquid medium YP (1% yeast extract, 1% peptone, 0.1% potassium phosphate and 0.12% ammonium sulfate) supplemented with 2% glucose (YPD) or 2% lactate (YPL) or 3% glycerol plus 1% ethanol (YPGE) or 1% ethanol (YPE) as the carbon substrate. The pH was set at 5.5. The cells were harvested at midlogarithmic grown phase (D_{600} in the range 3–4). Microsomes and mitochondria were prepared as described previously [16]. For rescue experiments, the ORF of *PSI1* was inserted into pVT-U-GW vector containing a GAL1 promoter using the Gateway® system (Invitrogen, Carlsbad, CA, USA). The plasmids constructed were transformed into BY4742 or *psi1Δ* strains. The cells were selected and grown on a minimal synthetic medium [0.67% yeast nitrogen base without amino acid, with ammonium sulfate (Invitrogen), 0.192% Yeast Synthetic Drop-out Medium Supplement without Uracil (Sigma)] supplemented with 2% glucose as a carbon source. Cells from 50 mL of culture were harvested by centrifugation at mid-logarithmic phase. Homogenates were prepared by disrupting pelleted cells with glass beads in 0.4 M mannitol, 25 mM Tris–HCl pH7 at 4 °C, using a Mini-beadbeater (BioSpec Products, Inc., Bartlesville, OK, USA). Cell lysates were centrifuged at 550 ***g*** for 20 min at 4 °C. The supernatant was used as source of enzyme.

Lipid fatty acid composition

Cells from 50 mL of culture were harvested by centrifugation at D_{600} of 3–4 (midlogarithmic growth phase). The resulting pellets were then washed once with 50 mL of water and resuspended in 3 mL of water. To extract yeast lipids from whole cells, 500 μL of the cell suspensions were vigorously shaken with glass beads (six times for 30 s with intermittent cooling on ice). Two milliliters of chloroform/methanol (2 : 1) were added and the cell suspensions containing beads were vigorously shaken for 30 s. After centrifugation, the organic phase was isolated and the remaining lipids were further extracted by the addition of 2 mL of chloroform to the aqueous phase and by shaking (in the presence of the glass beads). The organic phases were then pooled and evaporated to dryness. Next, the lipids were redissolved in 70 μL of chloroform/methanol (2 : 1).

Neutral and polar lipids were purified from the extracts by one-dimensional TLC on silica gel plates (20 × 20 cm; Merck) using hexane/diethylether/acetic acid (90 : 15 : 2), and chloroform/methanol/1-propanol/methyl acetate/0.25% aqueous KCl (10 : 4 : 10 : 10 : 3.6) as solvent, respectively [42,43]. The lipids were then visualized by spraying the plates with a solution of 0.001% (w/v) primuline in

80% acetone, followed by exposure of plates under UV light. The silica gel zones corresponding to the various lipids were then scraped from the plates and added to 1 mL of methanol/2.5% H_2SO_4 containing 5 μg of heptadecanoic acid methyl ester. After 1 h at 80 °C, 1.5 mL of H_2O was added and fatty acid methyl esters (FAMES) were extracted by 0.75 mL of hexane. Separation of FAMES was performed by GC (Hewlett Packard 5890 series II; Hewlett-Packard, Palo Alto, CA, USA) as described previously [16]. Alternatively, to determine the fatty acid label incorporated into PI during a pulse experiment carried out with [^{14}C]stearate, FAMES prepared from this glycerophospholipid were separated on a TLC plate previously immersed in a 10% solution of $AgNO_3$ in ethanol/H_2O (3 : 1), dried overnight at room temperature and activated for 30 min at 110 °C. Plates were developed in hexane/diethyl ether (60 : 40) [44]. The label was located and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale CA, USA).

Lipid analysis by MS

Lipid extracts were analyzed in negative ion mode on a QSTAR Pulsar-*i* instrument (MDS Analytical Technologies, Concord, Canada) equipped with the robotic nanoflow ion source NanoMate (Advion Biosciences, Inc., Ithaca, NY, USA) as previously described [30]. Glycerophospholipid species were identified and quantified by LIPID PROFILER software (MDS Analytical Technologies) [30].

Positional analysis of fatty acids

Lipids of wild-type and *psi1Δ* strains were extracted and separated by TLC plates (20 × 20 cm) as described above for polar lipids. The area of silica gel corresponding to PI was scraped off the plates into vials. Two hundred microliters of 5 mM $CaCl_2$, 50 mM Tris–HCl (pH 8.9) and 200 μL of diethyl ether were added. After 15 min of sonication, 15 units of porcine pancreatic phospholipase A_2 were added for 30 min at room temperature with vigorous stirring. After incubation, diethyl ether was evaporated. The reaction products were extracted twice with 200 μL of 1-butanol. After phase separations, the resulting 1-butanol phases were pooled and evaporated to dryness. The reaction products were redissolved in 100 μL of methanol (containing 1% water) and were then purified by TLC as described above. Spots corresponding to *sn*-1-acyl-2-lysoPI and to free fatty acids were scraped off the plates and corresponding FAMES were analyzed as described above.

In vivo [^{14}C]acetate incorporation

To analyze newly synthesized lipids, cells grown at 30 °C in YPD medium to the midlogarithmic growth phase were pulse-labeled with [^{14}C]acetate (50 μCi per 5 mL of cell culture) for 40 min. The incorporation was stopped by 1 mL 10% trichloroacetic acid. Cells were pelleted by centrifugation and washed once with water. Lipids were extracted and separated as described above, except that we used 10 × 10 cm HPTLC plates. The label was located and quantified using a PhosphorImager (Molecular Dynamics). Incorporation of [^{14}C]label into individual lipids was expressed as the percentage of radioactivity incorporated into total neutral lipids or total phospholipids.

In vivo [^{14}C]glycerol incorporation

The strains were grown in YPGE to midlogarithmic phase, and 5 mL of cell culture were washed twice with sterile water and resuspended in 5 mL of YPE with [^{14}C]glycerol (5 μCi) for 40 min. Phospholipids were analyzed as reported above.

Preparation of *sn*-2-acyl-1-lysoPI

sn-2-acyl-1-lysoPI was prepared as described previously [45], with slight modifications. PI (0.2–0.4 μmol) from soybean was purified by TLC on silica gel plate (10 × 10 cm) as described above for polar lipids. The silica gel zone corresponding to PI was then scraped off the plates into vials. Four hundred microliters of diethylether and 280 μL of 50 mM Tris–maleate 10 mM $CaCl_2$ (pH 5.8) were added. After 15 min of sonication, 160 μL of enzyme solution containing 160 units of *R. arrhizus* lipase were added for 15 min at room temperature with vigorous stirring. After incubation, diethyl ether was evaporated. The reaction product was extracted twice with 400 μL of 1-butanol. After phase separations, the resulting 1-butanol phases were pooled and the concentration of the corresponding FAMES was determined as described above. The *sn*-2-acyl-1-lysoPI was immediately used for acyltransferase assays.

Acyltransferase activity assays

G3PAT assays were performed as described previously [46]. The assays were conducted at 30 °C in 100 μL of assay mixture (1 mM dithiothreitol, 2 mM $MgCl_2$, 75 mM Tris–HCl, pH7.5) with 70 μM [^{14}C]glycerol 3-phosphate (148 Ci·mol^{-1}) and 10–30 μM oleoyl-CoA or stearoyl-CoA as substrates. The reaction was initiated by adding 8 μg of microsomal membrane proteins to the assay mix. After 10 min of incubation at 30 °C, the reaction was stopped by adding 2 mL of chloroform/methanol (2 : 1, v/v) and 500 μL of 1% perchloric acid, 1 M KCl aqueous solution. The organic phase was isolated and the aqueous phase was re-extracted with 2 mL of chloroform. These combined lipid extracts were dried, redissolved in 50 μL of chloroform/methanol (2 : 1, v/v), and the lipids were separated

by HPTLC as described above. The radioactivity incorporated into phospholipids was detected and quantified using a PhosphorImager (Molecular Dynamics).

LysoPI acyltransferase reactions were conducted in 100 μL of assay mixtures (0.4 M mannitol, 25 mM Tris–HCl, pH7) containing 1 nmol of [^{14}C]stearoyl-CoA, 2 μg of microsomal membrane proteins and, when added, 1 nmol of *sn*-1-acyl-2-lysoPI or *sn*-2-acyl-1-lysoPI. Alternatively, 2.5 or 5 μg of homogenate proteins from transgenic *psi1Δ* mutant cells overexpressing *PSI1* were added to 1 nmol of *sn*-2-acyl-1-lysoPI and 1 nmol of [^{14}C]stearoyl-CoA. Incubations were performed at 30 °C for 10 min. Reactions were stopped by the addition of 2 mL of chloroform/methanol (2 : 1, v/v) and 500 μL of water. The formed products were extracted and analyzed as described above.

Statistical analysis

The level of significance (P) of the difference between means ± SD was calculated by Student's t-test.

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Supporting information

The following supplementary material is available:

Fig. S1. Conserved acyltransferase motifs in Psi1p and in characterized acyltransferases.

Fig. S2. Label distribution in phospholipids from wild-type and *psi1Δ* mutant cells after a pulse with [^{14}C]glycerol.

Fig. S3. Label distribution in polar and neutral lipids from wild-type and *psi1Δ* mutant cells following a pulse with [^{14}C]acetate.

Fig. S4. Label position in PI generated *in vitro*.

Table S1. Distribution and fatty acid composition of neutral lipids from *psi1Δ* mutant and wild-type cells.

This supplementary material can be found in the online version of this article.

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