PHO4 transcription factor regulates triacylglycerol metabolism under low-phosphate conditions in Saccharomyces cerevisiae

Kamlesh Kumar Yadav,1,2 Neelima Singh1,2 and Ram Rajasekharan1,2*

1Lipidomic Centre, Department of Lipid Science, CSIR-Central Food Technological Research Institute (CFTRI), Mysore, Karnataka 570020, India. 2Academy of Scientific and Innovative Research (AcSIR), CSIR-CFTRI Campus

Summary

In Saccharomyces cerevisiae, PHM8 encodes a phosphatase that catalyses the dephosphorylation of lysophosphatidic acids to monoacylglycerol and nucleotide monophosphate to nucleoside and releases free phosphate. In this report, we investigated the role of PHM8 in triacylglycerol metabolism and its transcriptional regulation by a phosphate responsive transcription factor Pho4p under low-phosphate conditions. We found that the wild-type (BY4741) cells accumulate triacylglycerol and the expression of PHM8 was high under low-phosphate conditions. Overexpression of PHM8 in the wild-type, phm8Δ and quadruple phosphatase mutant (pah1Δdpp1Δlpp1Δapp1Δ) caused an increase in the triacylglycerol levels. However, the introduction of the PHM8 deletion into the quadruple phosphatase mutant resulted in a reduction in triacylglycerol levels and LPA phosphatase activity. The transcriptional activator Pho4p binds to the PHM8 promoter under low-phosphate conditions, activating PHM8 expression, which leads to the formation of monoacylglycerol from LPA. The synthesized monoacylglycerol is acylated to diacylglycerol by Dga1p, which is further acylated to triacylglycerol by the same enzyme.

Introduction

Inorganic phosphate (Pi) is an essential nutrient because it is required for the biosynthesis of nucleotides, phospholipids and metabolites in energy metabolism (Tamai et al., 1985). The budding yeast, Saccharomyces cerevisiae, can respond in several different ways to phosphate starvation: the cells can mobilise internal stores of phosphate, such as polyphosphate or they can up-regulate the production of the plasma membrane transporters involved in phosphate uptake (Shirahama et al., 1996; Neef and Kladde, 2003). Additionally, yeast can increase the production of acid and alkaline phosphatases that are secreted into the extracellular environment to liberate Pi (Yoshida et al., 1989; Bun-ya et al., 1991), and can up-regulate genes involved in the catabolism of alternate phosphorous sources such as phospholipids and nucleic acids (Bogan and Brenner, 2010; Smets et al., 2010). These responses are primarily mediated at the level of gene regulation and controlled by a signalling pathway known as the phosphate-responsive signalling (PHO) pathway (Lenburg and Oshea, 1996; Oshima, 1997; Lau et al., 1998). The key components of the PHO signalling pathway include the cyclin/cyclin-dependent kinase complex Pho80p/Pho85p and Pho81p, which regulates the migration of the Pho4p transcription factor in response to Pi conditions (Kaffman et al., 1994; O'Neill et al., 1996). Recently, it has been determined that phosphate starvation-regulated promoters in Candida glabrata do not require Pho2p, a co-activator that is required for the activation of phosphate-responsive genes as well as Pho4p in S. cerevisiae (Kerwin and Wykoff, 2012).

Phosphatases are enzymes that catalyse the hydrolysis of phosphate esters from a variety of phosphorylated substrates, which range from specific Ser/Thr/Tyr residues of proteins to non-protein substrates such as phospholipids and nucleotides. Phosphatase activity is crucial component of signal transduction and cell cycle regulation (Alexander, 1990) and during phosphate starvation (Yin et al., 2007). The observation that low-Pi decreases nucleotide concentration and causes the intracellular accumulation of both nucleosides and bases suggests a positive regulation of nucleotidases under low-Pi conditions (Ashihara et al., 1988). Studies in multiple model organisms have indicated that phosphate starvation induces genes encoding phosphate-liberating enzymes (Goldstein et al., 1988; Duff et al., 1991; Li et al., 2002; Wasaki et al., 2006). In
plants, microarray studies showed the induction of RNases and phosphatases, followed by an increase in hydrolysis of nucleic acids and nucleotides, is a common mechanism in response to phosphate starvation. Phosphate-regulated 5'-nucleotidase activity appears to be conserved in some bacteria and yeast as well as in plants (Hammond et al., 2003; Uhide-Stone et al., 2003; Misson et al., 2005). Nucleotidases catalyse the hydrolysis of ribonucleoside and deoxyribonucleoside monophosphates into the corresponding nucleosides with the release of orthophosphate. In budding yeast, nucleotide hydrolysis by Npp1p, Npp2p and Pho5p is induced under low-Pi condition. Moreover, SdT1 and PHM8 are induced at the transcriptional level by Pi starvation (Kennedy et al., 2005).

In S. cerevisiae, PHM8 encodes a lysophosphatidic acid (LPA) phosphatase and nucleotidase. It is a cytosolic enzyme that catalyses the formation of monacetyl-glycerol (MAG) from LPA and nucleosides from nucleotide monophosphate, accompanied by the release of free Pi (Reddy et al., 2008; Xu et al., 2013). This enzyme is important because its substrate and product are intermediates in the synthesis and turnover of membrane phospholipids and triacylglycerol (TAG). LPA plays an important role in cell signalling. In S. cerevisiae, phosphatases are up-regulated and the level of LPA is drastically decreased under phosphate-starved conditions. phm8Δ cells have shown a decreased LPA-hydrolysing activity under phosphate-limiting conditions, while the overexpression of PHM8 in yeast resulted in an increase in the LPA phosphatase activity in vivo (Reddy et al., 2008). However, very little is known about the transcriptional and biochemical regulation of PHM8-mediated phosphatase activity.

The present study was undertaken to investigate the effects of low-Pi on the regulation of non-polar lipid metabolism in S. cerevisiae, strain BY4741. We found that the level of non-polar lipids increased under low-Pi conditions, which is due to the increased expression level of PHM8. The deletion of PHM8 leads to a decrease in non-polar lipid levels compared with wild-type cells under low-Pi conditions. Overexpression of PHM8 in the wild-type and phm8Δ cells caused an increase in the non-polar lipid levels and the increase was consistent even in quadruple phosphatase mutant (app1Δ::natMX4 pah1Δ::URA3 dpp1Δ::TRP1/Kar1 lpp1Δ::HIS3/Kar1, derivative of W303–1A), but there was no increase in TAG levels when PHM8 is deleted from the quadruple phosphatase mutant under low-Pi conditions. The expression of PHM8 is regulated by the transcription factor Pho4p. In phm8Δ cells, the loss of Pho4p-mediated regulation of PHM8 decreased its expression as well as the LPA phosphatase activity and TAG levels. In this report, we have shown that Dga1p is capable of acylating MAG. This work enhances the understanding of the transcriptional regulation of PHM8, and its role in lipid metabolism.

Results

Low-Pi conditions caused an increase in non-polar lipids

We began our study to see the effects of low-Pi on yeast lipid metabolism. Wild-type (BY4741) cells were grown under varying concentrations of Pi to understand the growth and slow growth was observed under low-Pi conditions (Fig. S1A). Wild-type cells were grown in the presence of [14C]acetate and found that there was an increase in non-polar lipids when Pi concentration was dropped in the medium (Fig. S1B). However, the growth of cells was slow under low-Pi conditions, 1 mM Pi concentration was taken for further experiments to get the enough amounts of cells. A time-dependent incorporation of [14C]acetate in lipids under low-Pi conditions were performed. There was an increase in DAG, TAG and steryl esters was observed in the early log phase and stationary phase (Fig. 1A), but there was no change in the phospholipids levels. Stationary phase grown cells from low-Pi and control Pi were stained with BODIPY™ 493/503 and observed under a confocal microscope. A twofold increase in the number of Lipid droplets (LDs) in the cells grown under low-Pi (LDs 5.91 ± 1.00) and control (LDs 2.82 ± 1.00) conditions were observed (Fig. 1B). The increase in non-polar lipids was further confirmed by the analyses of total cellular fatty acids using gas chromatography (Fig. S1C). These experiments suggested that low-Pi conditions led to the accumulation of non-polar lipids in yeast.

Expression analysis of lipid metabolizing genes under low-Pi conditions

The expression levels of TAG-metabolizing genes were monitored using Quantitative real time PCR (qRT-PCR). The expression level of PHM8 was the highest among all the lipid genes tested while other TAG biosynthetic genes (GPT2 and DGA1) also showed more than twofold greater expression levels under low-Pi conditions compared with control Pi conditions (Fig. 2A). The expression levels of LRO1, YJU3, ARE1 and ARE2 were slightly high, while the expression of TAG hydrolytic genes such as, TGL3, TGL4 and TGL5 was lower in comparison with the control (base value 1). The increase in the mRNA level was confirmed by the Phm8p levels shown by Western blot (Fig. 2B) using rabbit anti-Phm8 polyclonal antibodies. MAG, the product of LPA phosphatase activity, was threefold higher than control Pi conditions (Fig. 2C). These experiments suggested that the expression of PHM8 is high under low-Pi conditions, causing an increase in LPA phosphatase activity and hence the increased amount of MAG.
Fig. 1. Effect of low-phosphate conditions on non-polar lipid metabolism of BY4741 cells. A. Time-dependent incorporation of [14C]acetate into non-polar lipids during the growth phase. Equal amounts ($A_{600} = 20$) of cells were harvested at different time intervals. The lipids were extracted and separated on a silica-TLC using solvent system A. The lipids were identified by comparison with the known standards. The graph represents the incorporation of radiolabel into various lipids. The lipids were quantified by scraping the spots from the TLC plate and counting using a toluene-based scintillation fluid in a liquid scintillation counter. dpm/$A_{600}$ cells represents the disintegration per minute of radioactivity associated with lipid from 20 OD of cells. Values are the means ± standard deviation (SD) (*$P < 0.05$) of three independent determinations. SE, steryl esters; TAG, triacylglycerol; FFA, free fatty acids; DAG, diacylglycerol; STE, sterol; C, control; LPi, low phosphate.

B. Lipid droplets were stained with BODIPY® 493/503 and viewed under a confocal microscope (bar = 2 μm). Top panel, cells grown under control inorganic phosphate (Pi); bottom panel, cells grown under low-Pi.
Effect of PHM8 deletion and PHM8 overexpression on non-polar lipid metabolism

The wild-type and phm8Δ cells were grown under low-Pi conditions to stationary phase in the presence of [14C]acetate. Equal amounts of cells were harvested and the extracted lipids were separated on silica-TLC using solvent system A. The graph represents the incorporation of radioactivity into sterol esters and TAG.

B. Non-polar lipid profile of wild-type overexpressing PHM8. Transformed cells were induced and labelled with [14C]acetate. Lipids were extracted from the cells (A600 = 20) and analysed by TLC. Data represent the means ± standard deviation (SD) (*P < 0.05) of triplicates from three independent experiments.

Effect of PHM8 deletion and PHM8 overexpression on non-polar lipid metabolism

The wild-type and phm8Δ cells were grown under low-Pi conditions to stationary phase in the presence of [14C]acetate. phm8Δ showed a significant reduction in the incorporation of radioactivity into sterol esters and TAG compared with wild-type cells under low-Pi conditions (Fig. 3A). PHM8 was cloned into a galactose-inducible expression vector, pYES2-NT/C, and overexpressed in wild-type and phm8Δ cells. The level of non-polar lipids in [14C]acetate-labeled stationary phase cells was analysed using one-dimensional Thin Layer Chromatography (TLC). The levels of total non-polar lipids in the cells transformed with pYES2-NT/C-PHM8 were greater than...
those of the cells transformed with the pYES2-NT/C vector. The quantification of individual non-polar lipids showed that \textit{PHM8} overexpression in wild-type cells resulted in a fivefold increase in TAG and threefold increase in steryl esters levels, though no significant difference was observed in other lipids (Fig. 3B). The levels of TAG and steryl esters were also high when pYES2-NT/C-\textit{PHM8} was overexpressed in \textit{phm8}Δ (Fig. S2). These experiments suggested that the deletion of \textit{PHM8} causes a reduction in non-polar lipids under low-Pi conditions and overexpression of \textit{PHM8} caused an increase of non-polar lipids under physiological conditions.

\textbf{Low-Pi conditions increase TAG in the phosphatase quadruple mutant}

To validate that TAG increases in wild-type cells under low-Pi conditions because of the overexpression of \textit{PHM8} and not because of other lipid phosphatase, the quadruple phosphatase mutant (Chae et al., 2012) GHY66 (\textit{app1}Δ::\textit{natMX4} \textit{pah1}Δ::\textit{URA3} \textit{dpp1}Δ::\textit{TRP1}/\textit{Kar1} \textit{lpp1}Δ::\textit{HIS3}/\textit{Kar1}, derivative of W303–1A) was grown in the presence of [14C]acetate under low-Pi conditions. There was an increase in TAG levels under low-Pi conditions (Fig. 4A). An increase in the numbers (LDs in control 3.21 and low-Pi 4.32) and size of the lipid droplets were observed in the GHY66 cells grown under low-Pi conditions (Fig. 4B). The expression profiles of \textit{PHM8}, \textit{YJU3} and \textit{DGA1} were checked in GHY66 cells and found that there was an up-regulation of \textit{PHM8} and \textit{DGA1}, but not \textit{YJU3} (Fig. S3A). \textit{PHM8} was cloned into pRS425-GPD, a constitutive expression vector with a selectable leucine marker and transformed into GHY66 cells. Transformed cells were grown to stationary phase in the presence of [14C]acetate. Non-polar lipids were analysed, and it was found that the cells bearing pRS425-\textit{PHM8} had a threefold higher TAG level and an approximately six-fold increase in free fatty acids as compared vector alone (Fig. 4C). An increase in
Strains and plasmids used in this study.

| Strains and plasmids | Relevant characteristics | Source |
|----------------------|--------------------------|--------|
| Escherichia coli DH5α | F-; glnI·traD38 Dro+ phi80d::Tn10 Tn10 ΔlacZ ΔproZ ΔaraC139 proAB leuB6 phoA supE44 thi-1 endA1 hsdR17 (rK− mK−) recA3 gyrA96 relA1 | Invitrogen |
| BL21(DE3)pLysS | F- ompT hsdSB (r− m−) gal dcm (DE3) pLYsS | Invitrogen |
| BL21-ΔphoA | F- ompT hsdSB (r− m−) gal dcm araB22::Tn10△(lacQZM15 lacZΔ1::Tn10 proA supE44 thi-1 pyrA96 relA1 | Invitrogen |
| Saccharomyces cerevisiae BY4741 | MATα; his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | Euroscarf |
| pAH1 | BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YER037w::kanMX4 | Euroscarf |
| pRK4 -PHM8 | plasmid was also observed (Fig. 4D). The expression of PHM8, DGA1 and YJU3 was checked using qRT-PCR. The up-regulation of PHM8 and DGA1 was observed, but the expression of YJU3 was unchanged (Fig. S3B). The increase in free fatty acids could have been due to the up-regulation of phospholipases. These results suggested that an increase in TAG under low-Pi conditions was due to the up-regulation of PHM8. | This study |
| pHS6 | BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YER037w::kanMX4 | Euroscarf |
| pRK5 | PHM8 coding sequence inserted into pYES2NT/C | This study |
| pRK6 | PHM8 coding sequence inserted into pRS425 | This study |
| pRK7 | PHM8 coding sequence inserted into pRS425 | This study |
| pRK8 | PHM8 coding sequence inserted into pRS425 | This study |
| pRK9 | PHM8 coding sequence inserted into pRS425 | This study |
| pRK10 | PHM8 coding sequence inserted into pRS425 | This study |
| pRK11 | PHM8 coding sequence inserted into pRS425 | This study |
| pRK12 | PHM8 coding sequence inserted into pRS425 | This study |
| pRK13 | PHM8 coding sequence inserted into pRS425 | This study |
| pRK14 | PHM8 coding sequence inserted into pRS425 | This study |
| pYS2NT-C | Yeast expression vector with N-terminal His tag fusion | Invitrogen |
| YEp 357, 358 | Yeast episomal plasmids with lacZ reporter gene | ATCC |
| pRS425 Yeast expression vector with leucine selection | Invitrogen |
| BG1805 Yeast expression vector with uracil selection | Open Biosystem |
| BG1805-PHM8 | PHM8 coding sequence inserted into BG1805 | Open Biosystem |
| BG1805-POH4 | PHO4 coding sequence inserted into BG1805 | Open Biosystem |
| pRK1 | PHM8 coding sequence inserted into pYES2NT/C | This study |
| pRK2 | PHO4 coding sequence inserted into pRSETA | This study |
| pRK3 | P_Nax-lacZ reporter gene containing the PHM8 promoter into YEp357 | This study |
| pRK4 | The PHM8 promoter along with PHM8 (1936bp) into YEp358 | This study |
| pRK5 | Derived from pRK4 in which all three PPHM8-B promoter-binding site mutated | This study |
| pRK6 | Derivative of pRK3 where UAS1 is mutated | This study |
| pRK7 | Derivative of pRK3 where UAS2 is mutated | This study |
| pRK8 | Derivative of pRK3 where UAS3 is mutated | This study |
| pRK9 | Derivative of pRK6 where UAS1 and UAS2 are mutated | This study |
| pRK10 | Derivative of pRK7 where UAS2 and UAS3 are mutated | This study |
| pRK11 | Derivative of pRK8 where UAS1 and UAS3 are mutated | This study |
| pRK12 | Derivative of pRK9 where UAS1, 2 and 3 are mutated | This study |
| pRK13 | DGA1 coding sequence inserted into pYES2NT/C | This study |
| pRK14 | PHM8 coding sequence inserted in pRS425 | This study |

Table 1. Strains and plasmids used in this study.

the number of lipid droplets in GHY66 cells bearing the pRS425-PHM8 plasmid was also observed (Fig. 4D). The expression of PHM8, DGA1 and YJU3 was checked using qRT-PCR. The up-regulation of PHM8 and DGA1 was observed, but the expression of YJU3 was unchanged (Fig. S3B). The increase in free fatty acids could have been due to the up-regulation of phospholipases. These results suggested that an increase in TAG under low-Pi conditions in GHY66 and wild-type cells was due to the up-regulation of PHM8.

Deletion of all known lipid phosphatases caused a decrease in TAG levels

We constructed the phosphatase quintuple mutant (pah1Δdpp1Δlpp1Δapd1Δ) where the PHM8 deletion was combined with pah1Δdpp1Δlpp1Δapd1Δ mutations (Table 1). The quintuple phosphatase mutant cells were labelled with [14C]acetate and analysed both non-polar and polar lipids. When compared with the control Pi grew cells, low-Pi grown cells did not have a significant effect on the relative amount of sterol esters, DAG, TAG and total phospholipids (Fig. 5A). There was no change in the number and size of lipid droplets in low-Pi grown cells as compared with control Pi grown quintuple mutant cells (Fig. 5B). The LPA phosphatase assay was also performed with the cytosols of wild-type, quintuple and quadruple phosphatase mutants. LPA phosphatase activity from wild-type was considered as 100%. There was a significant reduction in the LPA phosphatase activity in quintuple mutant compared with quadruple mutant (Fig. 5C). However, the quintuple mutant did not show a complete reduction in LPA phosphatase activity. Deletion of PHM8 from quadruple phosphatase mutant led to a twofold reduction in the TAG levels under low-Pi conditions (Fig. 5D). These experiments confirmed that PHM8 has a direct role in TAG metabolism under low-Pi conditions.

Dga1p is capable of acylating MAG

It was clear from the earlier experiments that Phm8p is highly expressed under low-Pi conditions and the enzyme converts LPA to MAG, but we did not observe an accumulation of MAG under the same conditions. Instead, there
was an increase in TAG levels. TAG can be synthesized either by dephosphorylation of PA followed by acylation of DAG or by the successive acylation of MAG. The existence of MAG-dependent TAG biosynthetic pathway in yeast has not been explored, but in other systems it is well established (Saha et al., 2006; Petrie et al., 2012). Recently, it was suggested that Dga1p could perform the acylation of MAG (Heier et al., 2010). In qRT-PCR data of wild-type and quadruple phosphatase mutant grown under low-Pi conditions, the expression of DGA1 was high. DGA1 was cloned into the pYES2-NT/C vector and transformed into wild-type cells. The recombinant protein was purified from the wild-type cells overexpressing DGA1. Microsomal membranes were solubilized with 20 mM n-dodecyl β-D-maltoside (DDM), and the presence of recombinant Dga1p was confirmed by immunoblotting with an anti-His tagged monoclonal antibody (Fig. 6A). The purified fractions were dialysed and assayed for the DAG acyltransferase activity with different fatty acids backbone in DAG as acyl acceptor and [14C]palmitoyl-CoA as an acyl donor. The TAG formation was higher with 1, 2-dioleoyl and 1-palmitoyl, 2-oleoyl-DAG (Fig. S5). The MAG acyltransferase activity of Dga1p was studied using [14C]palmitoyl-CoA as an acyl donor with

---

**Fig. 5.** Effect of all known phosphatases mutations on lipid metabolism.
A. RR55 cells were grown to stationary phase in synthetic medium under control and low-Pi conditions. The graph represents the incorporation of [14C]acetate into various lipids.
B. Confocal microscopy of RR55 cell lipid droplets. Cells were grown to stationary phase under control and low-Pi conditions and stained with BODIPY (493/503).
C. LPA phosphatase activity of phosphatase quadruple and quintuple mutants. Cytosolic fraction was obtained and the activity measured using 100 μg of cytosolic proteins. 4Δ, phosphatase quadruple mutant; 5Δ, phosphatase quintuple mutant. LPA phosphatase activity from wild-type is considered as 100%.

---

**Fig. 6.** Characterization of Dga1p as a MAG acyltransferase.
A. Wild-type (BY4741) yeast cells harbouring pYES2-NT/C–DGA1 were induced with 2% galactose. The upper panel represents the Ni2+-NTA affinity-purified His6-tagged recombinant Dga1p from the solubilized microsomal membranes; the purified fraction was resolved on a 12% SDS-PAGE followed by staining with Coomassie brilliant blue. The lower panel is an immunoblot for Dga1p using an anti-His, monoclonal antibody.
B. The inset represents the phosphorimage of the TLC plate; the assay in which sn-1-oleoyl-MAG, sn-2-oleoyl-MAG and [14C]palmitoyl-CoA were used. The graph represents the total activity of Dga1p. -E, without the enzyme; BE, boiled enzyme. The data shown are the mean ± standard deviation (SD) from three independent experiments.
sn-1 and sn-2-MAG (C18:1) as the acyl acceptor. The formation of TAG and the intermediate 1,3-DAG was observed with a total acyltransferase activity of $15.24 \pm 1.87 \text{ nmol min}^{-1} \text{ mg}^{-1}$ for sn-1-MAG and higher intermediate 1,2-DAG with a total acyltransferase activity of $26.7 \pm 1.87 \text{ nmol min}^{-1} \text{ mg}^{-1}$ was observed for sn-2-MAG (Fig. 6B). These data suggested that Dga1p acylates both sn-1 and sn-2-MAG.

**Pho4p positively regulates PHM8 transcription**

We further attempted to understand the regulation of PHM8 expression at the transcriptional level. We analysed the PHM8 promoter and found that it contains three putative binding sites for Pho4p transcription factor (Lam et al., 2008). Pho4p is the basic helix loop helix (bHLH) protein that activates the transcription of a diverse set of genes involved in phosphate metabolism under low-Pi conditions (Kaffman et al., 1998). The sequence 1000 bp upstream of the PHM8 start site is considered to be the promoter sequence of PHM8, which contains three putative Pho4p-binding sites termed upstream activator sequences (UAS) 1, 2 and 3 (Fig. 7A). UAS1 of the PHM8 promoter was previously identified during a whole-genome binding analysis of Pho4p (Nishizawa et al., 2008). The UAS1 consensus site consists of the sequence CACGTT although some variability exists at the last T position (Zhou and O’Shea, 2011). To examine whether Pho4p binds to the PHM8 promoter, electrophoretic mobility shift assays (EMSA) were performed using the recombinant Pho4p from *Escherichia coli*, and the purified protein was identified by immunoblotting with an anti-His tag monoclonal antibody (Fig. 7B). With increasing amounts of protein, there was an increase in the DNA–protein complex formation (Fig. 7C inset). The binding of the DNA–protein complex was strong for the PHM8 promoter (Fig. 7C). To determine whether Pho4p activates the transcription of PHM8 in vivo, wild-type and pho4Δ cells expressing lacZ under the control of the native PHM8 promoter (pRK3) were grown to the log phase under low and control conditions. The β-galactosidase activity of the pho4Δ strain was substantially lower ($80 \pm 15 \text{ nmol min}^{-1} \text{ mg}^{-1}$) than the wild-type ($1100 \pm 200 \text{ nmol min}^{-1} \text{ mg}^{-1}$) when grown under low-Pi conditions, whereas under normal Pi conditions, the β-galactosidase activity of the pho4Δ strain ($40 \pm 12 \text{ nmol min}^{-1} \text{ mg}^{-1}$) decreased slightly compared with wild-type ($100 \pm 20 \text{ nmol min}^{-1} \text{ mg}^{-1}$) (Fig. 7C). These results suggested that Pho4p positively regulates PHM8 transcription under low-Pi conditions and that the binding of the Pho4p transcription factor to the PHM8 promoter increases PHM8 transcription under low-Pi conditions.

To understand the physiological effects of PHM8 promoter induction under low-Pi conditions, the native and mutated PHM8 promoters along with the gene (1936 bp) were cloned into YEp358 and named as pRK4 (native promoter) and pRK5 (mutated promoter). In pRK5 plasmid, all of the putative binding sites for Pho4p were randomly mutated to abolish the binding sequence. These constructs were transformed into phm8Δ cells along with the YEp358 control vector. qRT-PCR showed that the levels of PHM8 mRNA in phm8Δ cells expressing pRK5 were six-fold lower than in the cells expressing pRK4 (Fig. S6), although there was no change in the expression of PHM8 in pho4Δ. In addition, the LPA phosphatase activity in phm8Δ cells expressing pRK5 was significant reduced than in the cells expressing pRK4 (Fig. 7D). The Pho4p-binding site mutation also decreased the TAG levels by up to 50% in phm8Δ cells under low-Pi conditions, but the Pho4p-binding site mutations did not cause any change in TAG levels under normal Pi conditions (Fig. 7E). These experiments suggested that under low-Pi conditions, the up-regulation of PHM8 results in an accumulation of TAG.

**UAS1 is the activator of the PHM8 promoter under low-Pi conditions**

To determine which of the three UAS sequences is sufficient for the activation of the PHM8 promoter under low-Pi conditions, mutations in the binding domains of all three UASs of the PHM8 promoter were generated. The mutation in UAS1 resulted in an eightfold decrease in the PHM8 promoter activity. (Fig. 8A). There was a twofold reduction in the promoter activity when UAS2 or UAS3 was mutated (Fig. 8A). The PHM8 promoter activity was also checked when two or all three binding sites were mutated in combination, as in UAS1+UAS2, UAS2+UAS3, UAS1+UAS3 and UAS1+UAS2+UAS3. The promoter was found to be active only with an intact UAS1 site (Fig. 8A and B). Mutation of all three binding sites rendered the promoter completely uninducible (Fig. 8B), and there was no binding of Pho4p to the PHM8 promoter (Fig. 8B inset). These experiments suggested that UAS1 of the PHM8 promoter is responsible for the induction of PHM8 under low-Pi conditions.

**Discussion**

As phosphate is the most common functional group present in the metabolome, yeast have developed mechanisms for sensing and scavenging extracellular phosphate. The budding yeast changes its gene expression profile in response to nutrient availability (Ogawa et al., 2000). The accumulation of lipids is observed during inositol deficiency (Paltauf and Johnston, 1970; Hayashi et al., 1976), low temperatures (Hunter and Rose, 1972) and sporulation (Illiingworth et al., 1973), but
A

B

C

D

E

© 2015 The Authors. Molecular Microbiology published by John Wiley & Sons Ltd., Molecular Microbiology, 98, 456–472
Purification of Pho4p and binding to the PHM8 promoters.
A. A diagrammatic representation of the PHM8 promoter. The location and sequences of the putative binding sites of Pho4p in the PHM8 promoter (Nt). Also shown is the sequence of the mutant (Mt) form of the Pho4p-binding site.
B. Bacterially expressed recombinant Pho4p was affinity purified using Ni²⁺-NTA affinity chromatography and resolved on a 12% SDS-PAGE followed by staining with Coomassie brilliant blue. An immunoblot (lower panel) for Pho4p using an anti-His tagged monoclonal antibody.
C. Interaction of Pho4p with the PHM8 promoter. Inset shows the gel retardation assay. Increasing concentrations of Pho4p (50–500 ng) were added to the PHM8 promoter DNA (4 μg) in a final concentration of 1 × binding buffer. The graph represents the expression of the lacZ reporter gene under the control of the PHM8 promoter in the wild-type and pho4Δ strains. Wild-type and pho4Δ cells bearing pRK4 plasmids were grown to the mid-log phase and switched to low-Pi media for 4 h; cell-free extract was prepared and assayed for β-galactosidase activity.

The specific activity is expressed as units mg⁻¹ (nmol min⁻¹ mg⁻¹). Each value represents the mean of three independent experiments ± standard deviation (SD) (**P < 0.05). D. Cytosol was prepared from phm8Δ cells harbouring the pRK4 and pRK5 plasmids that were grown under low-Pi conditions. The LPA phosphatase activity assay was conducted with 100 μg of cytosol for 15 min with [¹⁴C]LPA. The graph represents the enzyme activity.

An equal absorbance (Aₙₐₜ = 20) of cells harvested from phm8Δ cells harbouring the pRK4 and pRK5 plasmids grown under control and low-Pi conditions in the presence of [¹⁴C]Cetate. The graph represents the incorporation of radiolabel into various lipids. Vec, vector; Nt, native promoter; Mt, mutated promoter. The data represent the mean ± standard deviation (SD) of triplicate from three independent experiments.

there was no report of Pi deficiency and non-polar lipid accumulation. Under low-Pi conditions, the expression of many phosphate transporters (e.g., plasma membrane-associated Pho84p) and phosphatases (e.g., Pho5p) are induced by Pho4p and Pho2p (Moffat et al., 2001; Wykoff and O’Shea, 2001). PHO and PHM genes up-regulated during phosphate starvation (Ogawa et al., 2000). The expression of PHM8 was high in the microarray of wild-type cells grown under low-Pi conditions (Boer et al., 2003). Phm8p is a magnesium-dependent LPA phosphatase which converts LPA to MAG and releases phosphate (Reddy et al., 2008). It was demonstrated earlier that LPA is the important intermediate for the biosynthesis of phospholipids and non-polar lipids (Bell and Coleman, 1980; Bishop and Bell, 1988). Under low-Pi conditions, there was no significant change in the major phospholipids. When cell are grown in the low-Pi medium a decrease in LPA was observed. LPA is hydrolyzed in response to low-Pi conditions in wild-type strain. Phm8p contributes to 22% of the total LPA phosphatase activity in the cytosol under normal growth conditions, whereas, under low-Pi conditions, it contributes to more than 55% of the total LPA phosphatase activity in the cytosol (Reddy et al., 2008).

Lipid biosynthesis is coordinately regulated by transcriptional feedback loops. In yeast, a high proportion of genes involved in phospholipid metabolism are regulated by the same set of three transcription factors. A cis-acting inositol-sensitive upstream activating sequence (UASINS) common to these genes is activated by a pair of transcription factors, Ino2p and Ino4p, which are in turn repressed by Opi1p. Many of the structural genes in the pathway have been shown to be regulated by inositol and choline. In the absence of inositol supplementation, transcriptional activation is mediated by the zinc-sensing and zinc-inducible transcriptional activator Zap1p and the zinc-responsive cis-acting element (UASZRE) in phospholipid biosynthesis genes (White et al., 1991; Greenberg and Lopes, 1996; Henry and Patton-Vogt, 1998; Henry et al.,
the same enzyme (Fig. 9). In yeast, the final acylation of DAG to TAG is carried out by MAG acyltransferase (Dga1p). The conversion of LPA into MAG by the Phm8p. MAG, in turn, is acylated by LPA acyltransferase to form phosphatidic acid (Kennedy, 1961). The first enzyme in this pathway, G3P phosphatase catalyses the dephosphorylation of G3P-1 isomer which can be result of a possible acyl migration (Stimmel and King, 1934). However, we are not certain about any of the possibilities. Under normal physiological conditions, TAG biosynthesis occurs by the sequential acylation of glycerol-3-phosphate (G3P) (Kennedy, 1961). The first enzyme in this pathway, G3P acyltransferase, catalyses the formation of LPA, which is acylated by LPA acyltransferase to form phosphatidic acid (PA). PA phosphatase catalyses the dephosphorylation of PA to form DAG, which is an immediate precursor for the biosynthesis of TAG, phosphatidylcholine and phosphatidylethanolamine. However, MAG-dependent TAG biosynthesis occurs under low-Pi conditions, involving the conversion of LPA into MAG by the Phm8p. MAG, in turn, gets converted to DAG by MAG acyltransferase (Dga1p). In yeast, the final acylation of DAG to TAG is carried out by the same enzyme (Fig. 9).

The PHM8-encoded Mg²⁺-dependent LPA phosphatase has emerged as an important lipid metabolic enzyme in S. cerevisiae. This cytosolic enzyme plays an important role in controlling the LPA/MAG balance in the cytosol, which in turn regulates the biosynthesis of lipids and lipid droplet formation. Our data support the conclusion that PHM8 expression is activated by the transcription factor Pho4p through its direct interaction with a Pho4p-binding site in the promoter. PHM8 expression leads to the formation of MAG and the synthesized MAG is sequentially acylated by Dga1p to form TAG.

Experimental procedures

Materials

All chemicals were reagent grade. Yeast nitrogenous base was obtained from Difco (Becton, Dickinson and Company, 1 Becton Drive, Franklin Lakes, New Jersey 07417-1880, 201.847.6800). Restriction endonucleases, modifying enzymes and Phusion high-fidelity DNA polymerase were purchased from New England Biolabs (240 County Road Ipswich, MA 01938-2723). The yeast transformation kit was obtained from Clontech (DSS Takara Bio India Pvt. Ltd. - 5 Mohan Co-op Industrial Estate, New Delhi 110044, India), while radiochemicals were procured from American Radiocalled Chemicals, Inc. (101 ARC Drive St. Louis, MO 63146), Inc. Lipids were obtained from Avanti Polar Lipids, Inc. (700 Industrial Park Drive, Alabaster, Alabama 35077-9105), and silica-TLC plates were from Merck (50 A, 2nd Phase, Ring Road, Peney, Bengaluru 560058, India). The yeast expression vectors, fluorescence-based EMSA kit and phosphatase estimation kit were from Molecular Probes and Life Technologies (Invitrogen BioServices India Pvt. Ltd., Second Floor, First Technology Place, 3EP1P, Whitefield, Bangalore 560 066). The cDNA synthesis kit and Power SYBR Green PCR Master Mix were from Applied Biosystems [Life Technologies Corporation (Headquarters) 5791 Van Allen Way PO Box 6482, Carlsbad, California 92008]. Glass beads, Folin’s reagent, DNA purification kits, oligonucleotides and amino acid mixtures (drop out) were from Sigma-Aldrich (Plot # 12, Bommasandra-Jigani Link Road, Bengaluru - 560 100), India. Anti-poly (His), DDM, phosphoserine, phosphothreonine and phosphotyrosine monoclonal antibodies were raised in mice and were obtained from Sigma-Aldrich. Phm8p polyclonal antibodies were raised in rabbits as described earlier (Reddy et al., 2008) NBT/BCIP (NBT: p-nitroblue tetrazolium chloride/BCIP: 5-bromo-4-chloro-3-indolyl phosphate), substrate of alkaline phosphatase for Western blot analysis was purchased from PerkinElmer PerkinElmer Office (No. 33, ’Swayam Prabhe’ North Park Road, Kumara, Park East, Bangalore, Karnataka 560001, India). Yeast deletion strains were purchased from Euroscarf (Institute for Molecular Biosciences, Johann Wolfgang Goethe-University Frankfurt, Max-von-Laue Str. 9; Building N250, D-60438 Frankfurt, Germany), and the BG1805 vector and Yeast ORF clones were from Biogene India (20A, 2nd Flr, Shivaji Marg, Najafgarh Road, New Delhi, 110 015, India, Fax: 42581260).

Strains, media and culture conditions

The strains used in this work are listed in Table 1. Yeast cells were grown in yeast extract peptone dextrose (YEPD) medium (1% yeast extract, 2% peptone and 2% glucose) or in synthetic...
dropout (SD) medium (with yeast nitrogenous base [YNB]) containing 2% glucose and kanamycin (50 μg ml⁻¹) at 30°C (Rose et al., 1990). For the selection of plasmid-bearing yeast cells, the appropriate amino acids were omitted from the synthetic medium. Phosphate-free medium (SM-Pi) was prepared by replacing KH₂PO₄ (1 gl⁻¹) with KCl (50 mg l⁻¹) in the synthetic complete medium, and Pi was added in the form of KH₂PO₄ whenever required.

Escherichia coli was used for plasmid maintenance/amplifications (strain DH5α) and Pho4p expression (strain BL21-AITM). The bacterial cells were grown in LB medium (1% tryptone, 0.5% yeast extract and 1% NaCl) at 37°C, and ampicillin (100 μg ml⁻¹) was added to select for cells carrying the plasmid. The recombinant protein was expressed in yeast using 2% galactose or glucose. For the growth of yeast and bacteria on solid media, agar plates were prepared with supplementation of 2% agar to the respective broth.

Gene expression analysis

Microarray expression data were retrieved from the Expression Connection available in the Saccharomyces Genome Database (SGD database) and the yeast Microarray Global Viewer (yMGV).

Construction of plasmids and purification of recombinant proteins

The plasmids used in this work are listed in Table 1. Yeast genomic DNA was prepared as previously described (Sambrook et al., 1989). The PHM8 (YER037W) gene was amplified from the genomic DNA of S. cerevisiae using the primers listed in Table 2 and cloned into the yeast expression vector pYES2-NT/C using KpnI and XhoI restriction sites and named pRK1. Clones were confirmed by DNA sequencing. The PHO4 (YFR034C) gene was amplified from the BG1805-PHO4 plasmid purchased from Open Biosystems using the primers listed in Table 2. The gene was subcloned into the bacterial expression vector pRSETA using XhoI and KpnI as restriction sites (pRK2), and the constructs were confirmed by DNA sequencing. Recombinant proteins were purified using Ni²⁺-NTA agarose chromatography, and the protein amounts were determined using Lowry's method (Hartee, 1972). The purified proteins were resolved on 12% SDS-PAGE followed by Coomassie brilliant blue staining, and confirmed by immunoblot analysis. The recombinant Pho4p with the predicted molecular mass of 38-kDa migrated as a 54-kDa protein in a 12% gel (Berben et al., 1990). For promoter cloning, fragments that included the 1000 bp upstream of the PHM8 gene sequences along with 24 nucleotides from the start site were amplified and cloned into the YEp357 vector using the KpnI

Fig. 9. A model depicting TAG biosynthesis and its transcriptional regulation in S. cerevisiae under low-inorganic phosphate (Pi) conditions. G3P, glycerol-3-phosphate; GAT1, glycerol-3-phosphate acyltransferase; GAT2, glycerol-3-phosphate acyltransferase; AYR1, 1-acyl dihydroxyacetone-phosphate reductase; DHAP, dihydroxyacetone-phosphate; LPA, lysophosphatidic acid; PHM8, lysophosphatidic acid phosphatase; MAG, monoacylglycerol; DAG, diacylglycerol; DGA1, diacylglycerol acyltransferase; TAG, triacylglycerol; Pi ↑, low phosphate; Pi ↓, high phosphate; UAS, upstream activator sequence.
Table 2. Primers used in this study.

| No. | Gene   | Forward Primer | Reverse Primer |
|-----|--------|----------------|----------------|
| 1   | GPT2 qRT | GGTCGCCACCATCTGTG | ACTTTCCGGCTGAGTCTTCA |
| 2   | PHM8 qRT | CGACAGACCATCGAGGAA | GACACCCACTTGGCAATTAG |
| 3   | DGA1 qRT | TGATCTCAGCAGCGAGAATG | AAGCAGAAGATCTCTGCTAG |
| 4   | ARE1 qRT | TTGGCCCCTCTCGTGTGA | CGCACACTTCTCCCAACAA |
| 5   | ARE2 qRT | GACACTCCAGCCAAATGAG | ATGCGAGCTTCCGGTTTGA |
| 6   | LPF1 qRT | AGTTGATTGTTGGCTCCTT | GCTAGAAGACTCCAGAGACAA |
| 7   | DPP1 qRT | TCCTCTCCTGTCACACTT | TCACTCCGGATCCCTGAGT |
| 8   | APP1 qRT | CGACGCTCATTCGAAAC | CGTCGTCGATATCAG |
| 9   | PHA1 qRT | CTGTGCTGCGCGGTGATG | ACCCTCTGCTTCCCTTAA |
| 10  | LRO1 qRT | CGTCAACGCCGCGGGAAT | GTCTACGGTTCGGCCTTTT |
| 11  | TGL3 qRT | GTGCTCGCAGAAGAACAATG | ACCCAGAAATGCAACAA |
| 12  | TGL4 qRT | CAAGATTGCGCCGCTAATG | CATTGCAGGTTTGAATCA |
| 13  | TGL5 qRT | CCAAGAATAAACCCGATCCT | GAGCTCGTGAAGTTCCTCC |
| 14  | YU3 qRT | TGAGGTGIGGGAAGTAGTACAA | TAAACGAGCACCCCTTTAG |
| 15  | PHM6 qRT | GATTACAGTAGTGTTGAGCAGG | TCGAGAAACCCCTGAAAG |
| 16  | PHO85 qRT | TTGTCGACCAGTGCTGCAAG | CCGTTATCATTCCGCAGA |
| 17  | PHO80 qRT | TTACGTGTTGTCCTCCTT | TGGGAACCTCTTCTCAC |
| 20  | PHM8 gene | ATATGGTACCATGACTAGCATTACGTTA | ATATCTCGAGTGTAGCTAAG |
| 21  | PHO4 gene | TCCTCACAGGCTGACAACTCTTG | ATGCTACGTGCACTGCTGCTG |
| 22  | PHM8 Pro. | AAGGCAGCATTCCTACGATG | GGCCTCTTCTAGAATCTTTCAGTGATG |
| 23  | PHM8-Yep358 | CCGGGGAAACAGAAGTGTGATTAGTATCACC | GGCGCTGCAAGATGATGATGATGATGATGACTGAA |
| 24  | UA1 | GAAAGAAAGACGGCGAGCTAAGGCTTCACATACAXGA | TGATGGGACCTTATGCTGCTCCCTTTTTTTC |
| 25  | UA2 | AAGGGTCATATTAGGACAGGATGATTJATATATG | ACCTATATATCTCAATCAATGGACAT |
| 26  | UA3 | AGTGTATTAGCTTAAATACGTAGATCGCTAAG | TATAGGCAAGATGCTATGTTATAGGATGTT |
| 27  | DGA1 | GGGGACATCGTCGAGAACATCTCAATGAT | CGTCTAGGATCACCAGATTCAATGAT |
| 28  | PHM8 gene | CTGACGAGTACTTGCGTCAAGAAAGTACACAGA | GCCGCTGCAAGATGATGATGATGATGATGAGT |
| 29  | PHM8 Del (pRS425) | CTGACGAGTACTTGCGTCAAGAAAGTACACAGA | GCCGCTGCAAGATGATGATGATGATGATGAGT |
| 30  | PHM8 Del | GAAAGATTTGATGAGATAAGAAGCATAAG | AATTTCTATCTCTGAGAAGAATCATATGTTTTCCTAAATATC |

qRT, quantitative real-time PCR; Pro, promoter; Del, deletion.

and XbaI sites for PHM8 (pRK3). PHM8 and its promoter were cloned into the YEp358 vector using the Smal and PstI sites and named pRK4. Plasmid pRK5 was derived from plasmid pRK4 by mutating all of the Pho4p-binding sites. This plasmid was constructed by PCR-mediated site-directed mutagenesis using the primers listed in Table 2. Plasmids pRK6, 7 and 8 were derivatives of plasmid pRK3 in which the putative UAS 1, 2 and 3 of the PHM8 promoter were mutated. Plasmids pRK9, 10 and 11 were derived from plasmids pRK6, 7 and 8, respectively, in which the putative UAS was mutated in combinations. Plasmid pRK12 had mutations in all of the Pho4p-binding sites and was a derivative of plasmid pRK9 that was constructed by PCR-mediated site-directed mutagenesis. The DGA1 (YOR243C) gene was cloned into the yeast expression vector pYES2-NT/C using the BamHI and XhoI restriction sites and was a derivative of plasmid pRK9 that was constructed by PCR-mediated site-directed mutagenesis. The PHM8 gene was cloned into the yeast expression vector pYES2-NT/C using the BamHI and XhoI restriction sites and the primers listed in Table 2. The construct was named pRK14, and clones were confirmed by DNA sequencing.

In vivo labelling and lipid extraction from yeast cells

Yeast cells were grown in normal Pi and low-Pi synthetic media to stationary phase (36 h) in the presence of [14C]acetate (0.2 μCi ml−1) for incorporation of the radiolabel into cellular lipids. Cells (A600 = 20) were harvested at different time intervals and washed with cold water. Cells were lysed with acid-washed glass beads and 2% orthophosphoric acid. Total lipids were extracted using a chloroform : methanol mixture (1:1, v/v). Non-polar lipids were separated using solvent system A (petroleum ether : diethyl ether : glacial acetic acid, 70:30:1, v/v). The identities of the labelled lipids on the TLC plates were confirmed by comparison with standards after exposure to iodine vapour. Radiolabelled lipids were visualised under a phosphorimager (Typhoon FLA9500) and quantified in a liquid scintillation counter using a toluene-based scintillation cocktail (Perkin Elmer Micro Beta2 2450 Microplate counter).

Confocal microscopy

Stationary phase cells grown in normal Pi and low-Pi synthetic media were pelleted and washed three times with 1 × PBS. The washed cells were fixed with 3% formaldehyde for 15 min. After fixing, cells were resuspended in 200 μl PBS containing 1 μl of BODIPY™ 493/503 from a 1 mg ml−1 stock solution. The cells were incubated in the dark for 30 min at room temperature. After incubation, the cells were pelleted and washed three times with 1 × PBS. Finally, the cells were resuspended in 20 μl PBS (50% glycerol) and spotted onto slides with an agarose pad and observed under a Leica TCS SP8 STED super-resolution microscope.
**Gas chromatography**

Total lipids were extracted from wild-type yeast cells (BY4741) that were grown in normal Pi and low-Pi synthetic media to stationary phase as described earlier. The extracted lipids were methylated using boron trifluoride-methanol and incubation at 65°C for 15 min. (Araujo et al., 2008). The reaction mixture was immediately cooled on ice for 5 min, and fatty acid methyl esters (FAMEs) were extracted using hexane. The moisture content was removed from the hexane fraction through sodium sulphate and concentrated samples were subjected to gas chromatography. Heptadecanoic acid (100 μg; C17:0) was used as an internal standard for each reaction. Capillary gas chromatography analysis of FAMEs was performed on a DB-Wax-23 column (Agilent Technologies) 60 mm long, 0.25 μm thick and has an inner diameter of 0.25 mm. The method used for separation of FAMEs on the gas chromatography system was as follows: FAMEs were heated up to 50°C for 2 min, the temperature was raised to 180°C at the rate of 5°C min⁻¹ and was thereafter raised to 240°C at the rate of 5°C min⁻¹. The column was further maintained at 240°C for 5 min, and then the temperature was raised to 300°C at the rate of 30°C min⁻¹ and maintained at 300°C for 5 min. The total run time was 34 min.

**RNA isolation and transcript analysis**

Total RNA was isolated using the ‘Nucleospin RNA II’ RNA isolation kit. Complementary DNA (cDNA) was prepared using the high-capacity cDNA reverse transcription kit with 1X RT buffer, 1× random primer, 4 mM dNTP mix, 50 U μg⁻¹ reverse transcriptase and 1 μg total RNA. The primers were designed using the Primer Express® Software 3.0 (Applied Biosystems), and the sequences are listed in Table 2. For the real-time PCR analysis, 1 μl of cDNA diluted in a 1:20 ratio was amplified using Applied Biosystems machine (SDS 2.1) with the Power SYBR Green PCR Master Mix. The samples were analysed in triplicate, and the results were analysed using a relative quantification. The qRT-PCR data are presented as the mean and were analysed using Student’s t-test.

**Preparation of cell extracts**

Cell extracts were prepared by lysing the cells with glass beads and lysis buffer. Cells were vortexed for 30 s, each time keeping the cells on ice for 1 min between vortexing for 30 cycles. The lysis buffer contained 50 mM Tris-HCl (pH 8.0), 1 mM MgCl₂, 10% glycerol and 1 mM phenylmethylsulfonyl fluoride (PMSF). Unbroken cells were removed by centrifugation at 3600 × g for 5 min at 4°C. The cytosolic fraction was prepared by centrifuging the cell extract at 100,000 × g for 1 h at 4°C (Beckman ultracentrifuge [Beckman Coulter, Inc., Atlanta Vision Center 3353 Peach Tree N.E. Suite 200 Atlanta GA 30326, (800) 468-0305, (404) 504-8411]).

**Radiometric LPA phosphatase assay**

[14C]LPA (0.1 μCi ml⁻¹) was suspended in 5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and sonicated for 5 min in a sonic bath. The LPA phosphatase assay mixture consisted of 50 mM Tris-HCl (pH 8.0), 40 μM [14C]LPA, 5 mM MgCl₂ and 100 μg of yeast cytosolic proteins in a total volume of 100 μl. The incubation was carried out at 30°C for 15 min and stopped by the addition of 200 μl of 2% (v/v) orthophosphoric acid, 200 μl chloroform and 400 μl methanol (1:1:2), followed by lipid extraction. Lipids were resolved on a silica-TLC plate using solvent system B (chloroform : methanol : acetic acid, 98:2:5, v/v).

**In vivo labelling of the phosphatase quadruple mutant**

(app1Δ::natMX4 pah1Δ::URA3 dpp1Δ::TRP1/Kar′ lpp1Δ::HIS3/Kar′, derivative of W303–1A)

The quadruple phosphatase mutant GHY66 strain (app1Δ::natMX4 pah1Δ::URA3 dpp1Δ::TRP1/Kar′ lpp1Δ::HIS3/Kar′, derivative of W303–1A) was a kind gift from George M. Carman. GHY66 cells were grown in normal Pi and low-Pi synthetic media to stationary phase in the presence of [14C]acetate (0.2 μCi ml⁻¹) for uniform incorporation into cellular lipids. Cells (A600 = 20) were harvested, and total lipids were extracted and run in solvent system A.

**Knock out of PHM8 in phosphatase quadruple mutant**

(app1Δ::natMX4 pah1Δ::URA3 dpp1Δ::TRP1/Kar′ lpp1Δ::HIS3/Kar′, derivative of W303–1A)

The PHM8 deletion in the quadruple phosphatase deletion strain was generated by one-step gene replacement (Rothstein, 1991). The strain was transformed with the 1800 bp disruption cassette (pHM8Δ::LEU2) that was amplified from YEp351 with primers listed in Table 1. Yeast transformants were selected on synthetic medium without histidine, tryptophan, uracil and leucine medium and the PHM8 deletion in the cells was confirmed by PCR amplification of the (1000 bp) fragment from genomic DNA with the 5′UTR of PHM8 forward, 5′-GCAGCAACGTGCTTCTCATACACACTGTTGTCAAATTTGCCG-3′ primers and also with the amplification of 700 bp with the 5′UTR of PHM8 forward, 5′-GCAGCAACGTGCTTCTCATACACACTGTTGTCAAATTTGCCG-3′; PHM8 ORF reverse, 5′-TTGTAACACGCTAGTTTGTCAAAATTTGCCG-3′ primers and also with the amplification of 700 bp with the 5′UTR of PHM8 forward, 5′-CCAATGCGGTGTTCCTGGCG-3′. PHM8 deletion was confirmed by sequencing.

**Purification of recombinant Dga1p from wild-type cells**

DGA1 was overexpressed with a His tag in wild-type (BY4741) yeast cells, which were then harvested by centrifugation at 3600 × g for 5 min, washed with water, and either used directly or frozen at −8°C. All subsequent steps were carried out on ice or at 4°C. Microsomal membranes were prepared by centrifugation of the cell-free lysate at 100,000 × g for 1 h. The pellet was resuspended by vortexing in lysis buffer containing 20 mM DDM, followed by incubating the mixture for 1 h on ice. After solubilising, the mixture was centrifuged at 71,500 × g for 1 h. Dga1p was purified by loading the supernatant onto Ni²⁺-NTA agarose beads following the manufacturer’s instructions (Qiagen, Samyak Towers, Property No. 39, 2nd Floor, Pusa Road, Karol Bagh, New Delhi – 110005).
**Immunoblot analysis**

Immunoblotting was performed by transferring the SDS-PAGE gels to a nitrocellulose membrane. The blots were incubated for 2 h with anti-His monoclonal primary antibodies (1:500, v/v) raised in mice, followed by incubation with anti-immunoglobulin G secondary antibodies (1:5000, v/v) tagged with alkaline phosphatase enzyme for 1 h. Finally, the blots were developed in the dark using alkaline phosphate substrate.

**Spectrophotometric phosphatase assay**

Cell lysate (100 μg) used in a 100 μl reaction mixture containing Tris, pH 8.0, and 5 mM MgCl₂ and 500.0 μM LPA. The reaction mixture was incubated at 30°C for 15 min, followed by heating at 95°C for 5 min to stop the reaction. Release of phosphate was measured using the EnChek phosphate assay kit from Life Technologies (E6646). The assay had consisted of 1× reaction buffer, substrate MESG, MgCl₂, 5 mM of phosphatase, and 2-amino-6-mercapto-7-methylpurine when incubated at 22°C for 30 min. A shift in the absorbance maximum from 330 nm of the substrate to 360 nm of the product allowed for the quantitative determination of the phosphatase activity.

**MAG and DAG acyltransferase assays**

A working stock (1 mM) of sn-1 and 2-oleoyl MAG (Sigma-Aldrich) and different DAGs (Avanti Polar lipids), was prepared by dissolving in 5 mM CHAPS, followed by a 5 min sonication in sonic bath. [14C]Palmitoyl-CoA (0.1 μCi) was used in the final 100 μl reaction volume. The MAG acyltransferase assay mixture consisted of 100 mM Tris (pH 8.0), 20 μM palmitoyl-CoA, 50 μM of MAG or DAG, 1 mM PMSF, 1 mM MgCl₂, 10 mM KCl and 1 μg of purified recombinant Dga1p. The incubation was carried out at 30°C for 15 min and the reaction stopped by the addition of 200 μl of 2% orthophosphoric acid, 200 μl chloroform and 400 μl methanol. Lipids were then extracted and separated using solvent system A.

**EMSA**

The YEp357 constructs containing the PHM8 promoter was used as templates to amplify the promoters with the primers listed in Table 2. The amplified fragments were gel purified. DNA (4 μg) was incubated for 1 h at 30°C with the increasing amounts of purified recombinant Pho4p (50–500 ng) in the presence of 1× binding buffer (750 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 50 mM Tris, pH 7.4). A non-denaturing polyacrylamide gel (6%) with 0.5× Tris-borate-EDTA buffer was used to separate the DNA–protein complexes. The gel was stained with SYBR Green and visualised under a UVP transilluminator at 300 nm.

**β-Galactosidase assay**

Cells (A₆₀₀ = 0.8–1.0) were harvested and washed with water, and the cell extracts were prepared as described earlier. The β-galactosidase activity was measured (Rose and Botstein, 1983) and the specific activity was expressed as nmol min⁻¹ mg⁻¹ protein.

**Site-directed mutagenesis**

Point mutations were introduced into the Pho4p-binding sites in the PHM8 promoter alone and the PHM8 promoter with the PHM8 gene by PCR-based amplification of the entire plasmid using the primers listed in Table 2. The underlined bases represent the sites of mutations. The entire amplified plasmid was treated with 10 units of DpnI at 37°C for 1 h to digest the methylated parent template. The newly amplified unmethylated plasmid was transformed into DH5α competent cells. The presence of a mutation in the binding sites was confirmed by DNA sequencing using specific primers.

**Data analysis**

All quantitative data are analysed using Student’s t-tests, and P < 0.05 was considered statistically significant. Each experiment was repeated at least three times, data are presented as the average ± standard deviation. Statistical analyses, wherever required, were performed using the SigmaStat™ software (Sigma Software Solutions Pvt. Ltd. 7th Floor, Brigade Software Park, 27 Cross Banashankari Stage 2, Bangalore 560027 India).

**Acknowledgements**

This work was supported by the Council of Scientific and Industrial Research (CSIR), New Delhi under the 12th 5-year plan project LIPIC. K.K.Y. and N.S. received fellowship from CSIR, New Delhi. The corresponding author is a recipient of the JC Bose fellowship. We thank George M. Carman for his kind gift of the phosphatase quadruple deletion strain (GHY66). We are grateful to Dr. Malathi Srinivasan, Lipidomic Center, Bangalore for the use of their microscopic facility and the Department of Biochemistry, Indian Institute of Science, Bangalore for their help with the radioactive work. We also thank Director, Central Institute of Medicinal and Aromatic Plants, Lucknow for his valuable support. The authors have declared no conflicts of interest.

**References**

Alexander, D.R. (1990) The role of phosphatases in signal transduction. *New Biology* 2: 1049–1062.

Araujo, P., Nguyen, T.T., Fryland, L., Wang, J., and Kang, J.X. (2008) Evaluation of a rapid method for the quantitative analysis of fatty acids in various matrices. *J Chromatogr A* 1212: 106–113.

Ashihara, H., Li, X.N., and Ukaji, T. (1988) Effect of inorganic phosphate on the biosynthesis of purine and pyrimidine nucleotides in suspension-cultured cells of *Catharanthus roseus*. *Ann Bot (Oxford, U. K.)* 61: 225–232.

Bell, R.M., and Coleman, R.A. (1980) Enzymes of glycerolipid synthesis in eukaryotes. *Ann Rev of Biochem* 49: 459–487.
Bun-ya, M., Nishimura, M., Harashima, S., and Ohima, Y. (1991) The \textit{PHO84} gene of \textit{Saccharomyces cerevisiae} encodes an inorganic phosphate transporter. \textit{Mol Cell Biol} \textbf{11}: 3229–3239.

Chae, M., Han, G.-S., and Carman, G.M. (2012) The mitochondria of \textit{Saccharomyces cerevisiae} express a \textit{PHO84}-dependent phosphate transporter.

Duff, S.M., Plaxton, W.C., and Lefebvre, D.D. (1991) \textit{Saccharomyces cerevisiae} encodes an inorganic phosphate transporter. \textit{Mol Cell Biol} \textbf{11}: 3229–3239.

Chae, M., Han, G.-S., and Carman, G.M. (2012) The \textit{Saccharomyces cerevisiae} actin patch protein App1p is a phosphatidate phosphatase enzyme.

Duff, S.M., Plaxton, W.C., and Lefebvre, D.D. (1991) Phosphate-starvation response in plant cells: de novo synthesis and degradation of acid phosphatases.

Goldstein, A.H., Baertlein, D.A., and McDaniel, R.G. (1988) Phosphatidate phosphatase enzyme.

Hartee, E.F. (1972) Signaling phosphate starvation during phosphate starvation: the potential for developing smart plants.

Greenberg, M.L., and Lopes, J.M. (1996) Genetic regulation of phospholipid biosynthesis in \textit{Saccharomyces cerevisiae}. \textit{Microbiol Rev} \textbf{60}: 1–20.

Hammond, J.P., Bennett, M.J., Bowen, H.C., Broadley, M.R., Eastwood, D.C., May, S.T., \textit{et al}. (2003) Changes in gene expression in \textit{Arabidopsis} shoots during phosphate starvation and the potential for developing smart plants.

Han, G.-S., Wu, W.-I., and Carman, G.M. (2006) The \textit{Saccharomyces cerevisiae} lipin homolog is a Mg\textsuperscript{2+}-dependent phosphatidate phosphatase enzyme.

Hartee, E.F. (1972) Determination of protein: a modification of the lowry method that gives a linear photometric response.

Hayashi, E., Hasegawa, R., and Tomita, T. (1976) Accumulation of neutral lipids in \textit{Saccharomyces carlsbergensis} by myo-inositol deficiency and its mechanism. Reciprocal expression and the potential for developing smart plants.

Heier, C., Taschler, U., Rengachari, S., Oberer, M., Wolinski, H., Natter, K., \textit{et al}. (2010) Identification of Yju3p as functional orthologue of mammalian monoglyceride lipase in the yeast \textit{Saccharomyces cerevisiae}. \textit{Biochim Biophys Acta} \textbf{1801}: 1061–1071.

Henry, S.A., and Patton-Vogt, J.L. (1998) Genetic regulation of phospholipid metabolism: yeast as a model eukaryote.

© 2015 The Authors. \textit{Molecular Microbiology} published by John Wiley \& Sons Ltd., \textit{Molecular Microbiology}, \textbf{98}, 456–472
Oneill, E.M., Kaffman, A., Jolly, E.R., and Oshea, E.K. (1996) Regulation of PHO4 nuclear localization by the PHO80–PHO85 cyclin–CDK complex. *Science* **271**: 209–212.

Oshima, Y. (1997) The phosphatase system in *Saccharomyces cerevisiae*. *Genes & Genet Sys* **72**: 323–334.

Paltauf, F., and Johnston, J.M. (1970) Lipid metabolism in inositol-deficient yeast, *Saccharomyces carlsbergensis*: I. Influence of different carbon sources on the lipid composition of deficient cells. *Biochim Biophys Acta* **218**: 424–430.

Petrie, J.R., Vanhercke, T., Shrestha, P., Tahchy, A.E., White, A., Zhou, X.-R., et al. (2012) Recruiting a new substrate for triacylglycerol synthesis in plants: the monoacylglycerol acyltransferase pathway. *PLoS ONE* **7**: e35214.

Reddy, V.S., Singh, A.K., and Rajasekharan, R. (2008) The *Saccharomyces cerevisiae* PHM8 gene encodes a soluble magnesium-dependent lysophosphatidic acid phosphatase. *J Biol Chem* **283**: 8846–8854.

Rose, M., and Botstein, D. (1983) Construction and use of gene fusions to lacZ (β-galactosidase) that are expressed in yeast. *Methods in Enzymol.* **101**: 167–180.

Rose, M.D., Winston, F., and Heiter, P. (1990) *Methods in Yeast Genetics: A Laboratory Course Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Rothstein, R. (1991) Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol* **194**: 281–301.

Stimmel, B.F., and King, C.G. (1934) Preparation and properties of β-monoglycerides. *J Am Chem Soc* **56**: 1724–1725.

Tamai, Y., Toh-e, A., and Oshima, Y. (1985) Regulation of inorganic phosphate transport systems in *Saccharomyces cerevisiae*. *J Bacteriol* **164**: 964–968.

Uhde-Stone, C., Zinn, K.E., Ramirez-Yanez, M., Li, A., Vance, C.P., and Allan, D.L. (2003) Nylon filter arrays reveal differential gene expression in proteoid roots of white lupin in response to phosphorus deficiency. *Plant Physiol* **131**: 1064–1079.

Wasaki, J., Shinano, T., Onishi, K., Yonetani, R., Yazaki, J., Fujii, F., et al. (2006) Transcriptomic analysis indicates putative metabolic changes caused by manipulation of phosphorus availability in rice leaves. *J Exp Bot* **57**: 2049–2059.

White, M.J., Hirsch, J.P., and Henry, S.A. (1991) The OPI1 gene of *Saccharomyces cerevisiae*, a negative regulator of phospholipid biosynthesis, encodes a protein containing polyglutamine tracts and a leucine zipper. *J Biol Chem* **266**: 863–872.

Wykoff, D.D., and O’Shea, E.K. (2001) Phosphate transport and sensing in *Saccharomyces cerevisiae*. *Genetics* **159**: 1491–1499.

Xu, Y.-F., Letisse, F., Absalan, F., Lu, W., Kuznetsova, E., Brown, G., et al. (2013) Nucleotide degradation and ribose salvage in yeast. *Mol Sys Biol* **9**: 665.

Yin, Y., Shimano, F., and Ashihara, J.H. (2007) Involvement of rapid nucleotide synthesis in recovery from phosphate starvation of *Catharanthus roseus* cells. *J Exp Bot* **58**: 1025–1033.

Zhou, X., and O’Shea, E.K. (2011) Integrated approaches reveal determinants of genome-wide binding and function of the transcription factor Pho4. *Mol Cell* **42**: 826–836.

**Supporting information**

Additional supporting information may be found in the online version of this article at the publisher’s web-site.