Review

Conservation of PHO pathway in ascomycetes and the role of Pho84

Parul Tomar[†] and Himanshu Sinha*

Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai 400 005, India

Corresponding author (Fax, +91-22-2280-4610; Email, hsinha@tifr.res.in)

[†]*Present address: Max Planck Institute of Immunobiology and Epigenetics, Stübeweg 51, 79108 Freiburg, Germany*

In budding yeast, *Saccharomyces cerevisiae*, the phosphate signalling and response pathway, known as PHO pathway, monitors phosphate cytoplasmic levels by controlling genes involved in scavenging, uptake and utilization of phosphate. Recent attempts to understand the phosphate starvation response in other ascomycetes have suggested the existence of both common and novel components of the budding yeast PHO pathway in these ascomycetes. In this review, we discuss the components of PHO pathway, their roles in maintaining phosphate homeostasis in yeast and their conservation across ascomycetes. The role of high-affinity transporter, Pho84, in sensing and signalling of phosphate has also been discussed

1.   Role of phosphate

Phosphorus as inorganic phosphate (Pi) is an essential component of various biomolecules responsible for cellular structure and storage of cellular energy and is thus indispensable for survival of all living organisms. Hence, it is crucial for living organisms to maintain proper phosphate homeostasis, as any imbalance (hyper- or hypo-phosphatemia) will impact cellular processes, *viz.* cell differentiation and proliferation, metabolic functions, cytoskeletal organization (Dick *et al.* 2011). Non-motile microorganisms, which often encounter limiting conditions of essential nutrients, survive such adverse conditions through evolved complex signal transduction pathways usually involving a sensor, a transporter or a transceptor system. These pathways both by sensing or by limiting the uptake assess nutrient status in the ambient environment and initiate a signal transduction cascade, which leads to the activation of nutrient starvation responses. These responses help the cell in surviving the prevailing adverse conditions and to ensure that a wide variety of physiological activities properly adapt to the

environmental conditions (Wykoff *et al.* 2007). However, if the starvation persists longer, the cells enter into a quiescence phase and can eventually die.

To enable survival in variable and scarce phosphate conditions, budding yeast, *Saccharomyces cerevisiae*, has evolved a phosphate responsive signalling and metabolism pathway, known as PHO pathway, which monitors cytoplasmic levels of phosphate. This pathway regulates genes which are required for maintaining proper phosphate homeostasis such as genes required for phosphate uptake from extracellular environment, mobilization of internal phosphate reserves (polyphosphates) during fluctuations in internal phosphate levels, involved in phosphate sensing and signalling and scavenging phosphate from extracellular sources (Mouillon and Persson 2006; Wykoff *et al.* 2007) (table 1). PHO pathway consists of three important components: (1) a membrane transporter system consisting of low- and high-affinity phosphate transporters, which enables survival over diverse phosphate concentration; (2) polyphosphates (polyP), which are phosphate reserves and are mobilized first during phosphate demands; and (3) the PHO

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Table 1. List of PHO pathway genes and their functions

Gene name	Function in *S. cerevisiae*	*C. glabrata* homologue	*S. pombe* homologue	*N. crassa* homologue
GIT1	Plasma membrane permease; mediates uptake of glycerophosphoinositol and glycerophosphocholine as sources of the nutrients inositol and phosphate; expression and transport rate are regulated by phosphate and inositol availability	*GIT1*	*spbc1271.09*	*ncu04809*
MSN5	Karyopherin; involved in nuclear import and export of proteins, including import of replication protein A and export of Far1 and transcription factors Swi6, Msn2 and Pho4; required for re-export of mature tRNAs after their retrograde import from the cytoplasm	*MSN5*	*spac328.01c*	*ncu02387*
PHO2	Homeobox transcription factor; regulatory targets include genes involved in phosphate metabolism; binds cooperatively with Pho4 to the *PHO5* promoter; phosphorylation of Pho2 facilitates interaction with Pho4	*PHO2*	*phx1*	*kal-1*
PHO4	Basic helix-loop-helix (bHLH) transcription factor of the myc-family; activates transcription cooperatively with Pho2 in response to phosphate limitation; binding to 'CACGTG' motif is regulated by chromatin restriction and cooperation with Pho2; function is regulated by phosphorylation at multiple sites and by phosphate availability	*PHO4*		*nuc-1*
PHO5	Repressible acid phosphatase (1 of 3) that also mediates extracellular nucleotide-derived phosphate hydrolysis; induced by phosphate starvation and coordinately regulated by *PHO4* and *PHO2*		*spbc21H7.03C*	
PHO80	Cyclin, interacts with cyclin-dependent kinase Pho85; regulates the response to nutrient levels and environmental conditions, including the response to phosphate limitation and stress-dependent calcium signalling	*PHO80*		*preg*
PHO81	Cyclin-dependent kinase (CDK) inhibitor; regulates Pho80-Pho85 and Pcl7-Pho85 cyclin-CDK complexes in response to phosphate levels; inhibitory activity for Pho80-Pho85 requires IP_7 generated by Vip1	*PHO81*		*nuc-2*
PHO84	High-affinity Pi transporter; regulated by Pho4 and Spt7; mutation confers resistance to arsenate	*PHO84*	*spac23D3.12*	*pho-5*
PHO85	Cyclin-dependent kinase; has ten cyclin partners; involved in regulating the cellular response to nutrient levels and environmental conditions and progression through the cell cycle	*PHO85*	*pef1*	*pgov*
PHO87	Low-affinity Pi transporter; acts upstream of Pho81 in regulation of the PHO pathway; expression is independent of Pi concentration and Pho4 activity	*PHO87*		
PHO90	Low-affinity phosphate transporter; acts upstream of Pho81 in regulation of the PHO pathway; transcription independent of Pi and Pho4 activity			
PHO91	Low-affinity vacuolar phosphate transporter; exports phosphate from the vacuolar lumen to the cytosol; regulates phosphate and polyphosphate metabolism; acts upstream of Pho81 in regulation of the PHO pathway; transcription independent of Pi and Pho4 activity	*PHO91*		
PPN1	Vacuolar endo-polyphosphatase with a role in phosphate metabolism; functions as a homodimer	*CAGL0K06237g*	*spbc713.07c*	*ncu02996*
PPX1	Exo-polyphosphatase; hydrolyzes inorganic polyphosphate (poly P) into Pi residues; located in the cytosol, plasma membrane and mitochondrial matrix	*CAGL0L08602g*	*spac2F3.11*	*ncu06310*
SPL2	Protein with similarity to cyclin-dependent kinase inhibitors; downregulates low-affinity phosphate transport during phosphate limitation by targeting Pho87 to the vacuole			
VTC1	Subunit of the vacuolar transporter chaperone (VTC) complex	*PHM4*	*nrf1*	*ncu03883*
VTC2	Subunit of vacuolar transporter chaperone (VTC) complex			
VTC3	Subunit of vacuolar transporter chaperone (VTC) complex			

Table 1 (continued)

Gene name	Function in *S. cerevisiae*	*C. glabrata* homologue	*S. pombe* homologue	*N. crassa* homologue
VTC4	Vacuolar membrane polyphosphate polymerase; subunit of the vacuolar transporter chaperone (VTC) complex involved in synthesis and transfer of polyP to the vacuole	*CAGL0G06952g (PHM3)*	*vtc4*	

The main components of PHO pathway genes identified in *S. cerevisiae*. Corresponding functional homologues, if known, of *C. glabrata* (Inglis *et al.* 2012), *S. pombe* (Wood *et al.* 2012) and *N. crassa* (*Neurospora crassa* Sequencing Project 2014) are also listed. *C. glabrata* gene names in brackets are indicated if they differ in name from *S. cerevisiae*.

regulation system, the core signalling cascade, which integrates signals from extra and intracellular environment and depending upon intracellular phosphate status, either up- or down-regulates the PHO responsive genes (Lenburg and O'Shea 1996; Oshima 1997; Persson *et al.* 2003; Mouillon and Persson 2006; Secco *et al.* 2012) (figures 1 and 2).

2. Components of PHO pathway

2.1 *Membrane transporter system*

Plasma membrane of yeast cells is impermeable to phosphate because of its charged nature. Thus, two types of specialized transporters are required to facilitate entry of phosphate inside the cells, which are active over wide range of pH and environmental phosphate concentration (Persson *et al.* 1999).

2.1.1 *Low-affinity transport system:* This consists of two H^+/Pi symporters: Pho87 and Pho90 (Roomans *et al.* 1977; Persson *et al.* 1999) (figure 1). These transporters have 12 transmembrane domains with a long N-terminal SPX domain (Syg1-Pho81-Xpr1), a domain that is common to many PHO genes. Earlier, Pho91 was also considered as a low-affinity transport system but it is localized onto the vacuoles and, thus, not involved in the uptake of phosphate from the extracellular environment (Wykoff and O'Shea 2001). These transporters, Pho87 and Pho90, have a K_m of 1 mM and cater to the cellular phosphate requirement when yeast cells are grown in high-phosphate concentration. When the ambient levels of phosphate drops below their K_m, there occurs PHO-pathway-mediated transporter switching and the high-affinity transporters replace the low-affinity ones. Under low-phosphate conditions, the low-affinity transporters undergo vacuolar degradation. Spl2, a Pho4 target, mediates vacuolar degradation of Pho87; however, the mechanism for Pho90 is not known. GFP-based localization experiments have suggested that for Pho90, the post-transcriptional regulation upon phosphate starvation is independent of both Spl2 and Pho4. However, it has been shown that SPX domain of

Pho90 is required for this vacuolar degradation (Ghillebert *et al.* 2011). It appears that yeast cells use two distinct mechanisms for regulation of low-affinity transporters. This degradation of low-affinity transporters, upon phosphate scarcity, serves as a positive feedback loop for up-regulation of PHO pathway by further decreasing phosphate uptake by low-affinity transporters. However, the exact reason for this down-regulation of low-affinity transporters is not known. These low-affinity transporters have 12 transmembrane domains and, thus, occupy a large space on the membrane. Therefore, it has been proposed that the post-transcriptional down-regulation might create more space on the membrane for the high-affinity transporters.

2.1.2 *High-affinity transport system:* This system consists of two transporters: Pho84, a H^+/Pi- symporter and Pho89, a Na^+/Pi- symporter with a K_m of 10 μM (figure 2). These transporters also have 12 transmembrane domains and have different pH optima: pH 4.5 and 9.5 for Pho84 and Pho89, respectively. This difference in pH optima has been proposed to ensure phosphate uptake over wide pH range. Both these transporters are under tight regulation of PHO pathway and are expressed only in phosphate limiting conditions. However, their maximum activity at their pH optima is quite different, as the activity of Pho84 is 100-fold higher than that of Pho89, making Pho84 the main high-affinity phosphate transporter (Ogawa *et al.* 2000; Persson *et al.* 2003; Mouillon and Persson 2006; Secco *et al.* 2012).

2.2 *Polyphosphate metabolism*

Polyphosphates (PolyP) are linear phosphate polymers in which individual phosphates are linked together by high-energy phosphoranhydride bonds. The chain length varies from a few to hundreds of phosphate residues (Kornberg *et al.* 1999). These polyP are present in almost all organisms ranging from bacteria, fungi, plants and animals (Kulaev and Kulakovskaya 2000). In yeast, almost all (around 99%) of polyP is present in vacuoles (Kornberg *et al.* 1999) and it is
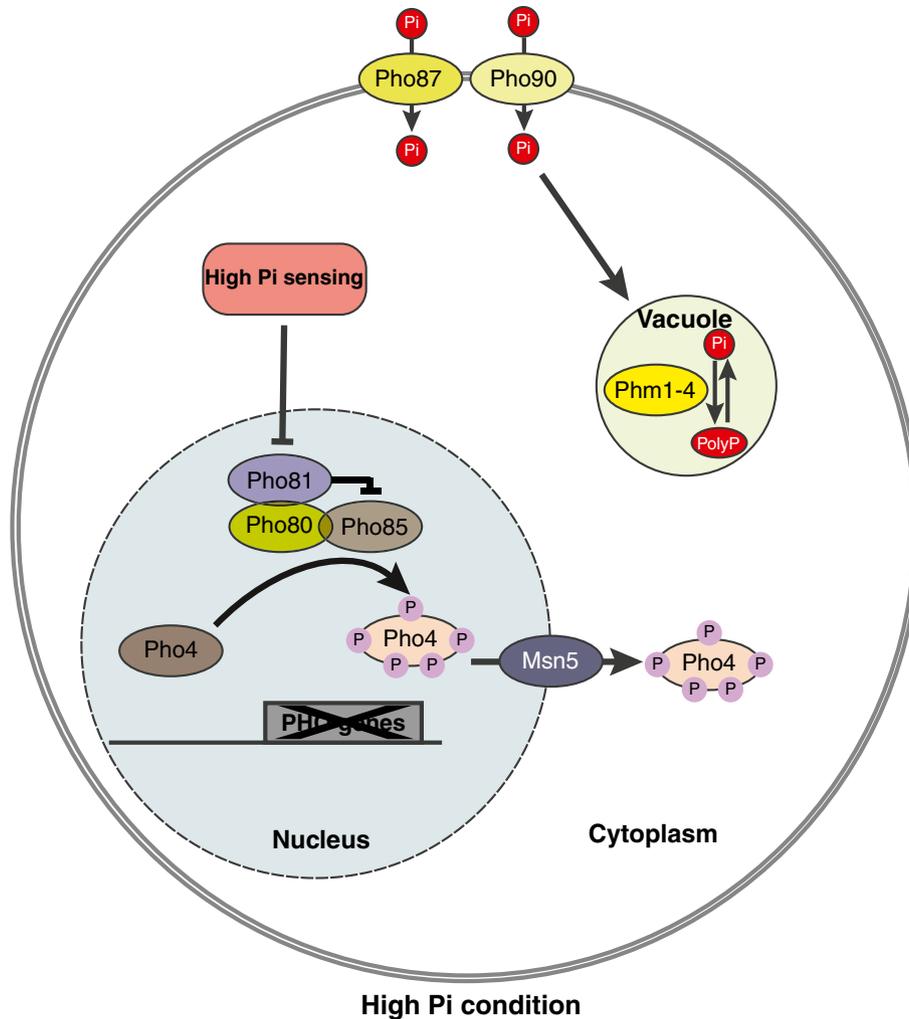
Figure 1. PHO pathway when environmental phosphate concentration is high: In high-Pi conditions, Pho4, transcriptional activator of PHO pathway, is hyper-phosphorylated by Pho80-Pho85 complex, which switches off PHO pathway. Low-affinity phosphate transporters, Pho87 and Pho90, uptake phosphate, excess of which is stored in form of polyP in vacuoles.

this fraction that is mobilized during phosphate limiting conditions (figures 1 and 2). The remaining fraction is present in other subcellular locations like cytosol, internal cell wall layer, mitochondria and nucleus (Secco *et al.* 2012). When a yeast cell is grown in phosphate rich environment, phosphate is taken inside the cells by the low-affinity transporters and is utilized for various cellular functions. The excess of phosphate is stored in the form of polyP and helps the cell meet increased phosphate demands during cell growth and division.

Genome-wide expression analysis, followed by detection of polyP levels by PAGE and enzymatic analysis (Ault-Riché *et al.* 1998), identified genes involved in polyP metabolism which had one or more Pho4 binding sites, CACGTG or CACGTT (Oshima 1997), within the 500 bp promoter region.

The synthesis of polyP is mediated by *PHM1*, *PHM2*, *PHM3* and *PHM4*, as the mutants of these genes showed significant reduction in polyP accumulation (Ogawa *et al.* 2000) (figure 1). There was negligible polyP accumulation in case of *phm3Δ*, *phm4Δ* and *phm1Δphm2Δ* double deletion. Data on measurement of rate of phosphate uptake suggested that polyP levels also help in maintaining the rate at which the cell takes up phosphate. Yeast cells that are defective in polyP synthesis (*phm2Δ*, *phm1Δphm2Δ*, *phm3Δ* and *phm4Δ*) showed a delay in phosphate uptake profile as compared to the wild type (Ogawa *et al.* 2000). It has been also been proposed that polyP synthesis is required to maintain a check on the intracellular Pi levels (Ogawa *et al.* 2000).

In contrast, not much is known about the genes involved in polyP degradation. Till date, two

Figure 2. PHO pathway when environmental phosphate concentration is low: Under low-Pi conditions, Pho81 represses Pho80-Pho85 complex, which in turn leads to hypo-phosphorylation of Pho4. This Pho4 in turn activates PHO pathway genes mainly including high-affinity transporters (Pho84, Pho89) and secretory phosphatases (Pho5, Pho11 and Pho12).

polyphosphatases are known: *PPX1*, an exopolyphosphatase (Wurst *et al.* 1995), which acts at the terminals of polyP chains and *PHM5* or *PPN1*, an endopolyphosphatase, which acts in between the polyP chains (Ogawa *et al.* 2000; Sethuraman *et al.* 2001) (figure 2). However, in a double deletion mutant of these two genes (*ppx1Δphm5Δ*), polyP utilization is delayed but still occurs, suggesting the presence of other polyphosphatases (Lichko *et al.* 2008). In addition to this, *phm5* mutant also shows growth defect and is unable to survive in minimal medium in the stationary phase. It has been suggested that the excess accumulation of long polyP chains might be the reason for loss of viability of *phm5* mutant (Ogawa *et al.* 2000; Sethuraman *et al.* 2001).

PolyP reserves get utilized first upon a phosphate limitation event and are mobilized immediately, possibly by activation of polyphosphatases independent of PHO pathway. However, if these phosphate reserves are not enough to meet the phosphate requirement, i.e. the starvation is either severe or persists for longer time, yeast cells up-regulate the PHO responsive genes via the PHO regulatory system.

2.3 *PHO regulation system*

The PHO regulation system consists of the core PHO genes including cyclin-dependent kinase inhibitor, Pho81, cyclin-

cyclin-dependent kinase complex, Pho80-Pho85, the transcription factor, Pho4 and the PHO responsive genes involved in acquisition, storage and uptake of phosphate (Lenburg and O'Shea 1996; Oshima 1997; Persson *et al.* 2003; Mouillon and Persson 2006; Secco *et al.* 2012) (figure 2). The activity of this system is dependent on the availability of phosphate and is mediated by the basic helix loop helix transcription factor, Pho4 (Berben *et al.* 1990), which binds as a homodimer (Shao 1998) in association with a co-transcription factor, Pho2 (Vogel *et al.* 1989). Pho4 binds to a specific motif, CACGTG/CACGTT, found in the promoter region of the PHO-responsive genes (Oshima 1997). The activity of Pho4 is regulated by Pho80-Pho85 mediated phosphorylation of its five-serine proline (SP) dipeptides SP1, SP2, SP3, SP4 and SP6, which regulates its subcellular localization – nuclear or cytoplasmic (Komeili 1999). Phosphorylation at sites SP2 and SP3 regulate the nuclear export of Pho4p, at SP4 inhibits the nuclear import and at SP6 blocks the interaction of Pho4p with Pho2p (Komeili 1999). However, the role of phosphorylation at SP1 is not known. The activity of Pho2p is also under the regulation of phosphorylation by Cdc2/Cdc28-type kinase at the SPIK site (amino acid residues 230–233). This phosphorylation improves both the transcriptional activation activity and interaction between Pho4 and Pho2 (Liu 2000). This whole signalling cascade is under the control of CDK inhibitor, Pho81, which negatively regulates the activity of Pho80-Pho85 complex. Pho81 is constitutively localized in the nucleus independent of the Pi levels, where it interacts with Pho80 under low- and high-Pi conditions. This leads to the formation of a ternary complex Pho81-Pho80-Pho85 (Ogawa *et al.* 2000; Mouillon and Persson 2006; Secco *et al.* 2012).

2.3.1 *Yeast cells grown under low-phosphate conditions:* Studies have shown that when the ambient level of phosphate drops below the K_m of low-affinity transporters, yeast cells immediately respond by mobilizing their polyP reserves. However, if a starvation event persists longer or is severe in nature, a complex metabolic network mediated by inositol pyrophosphates is activated and helps the cell in sensing the low-phosphate environment and also in Pho81 mediated inhibition of Pho80-Pho85 kinase activity. Biochemical experiments (involving use of culture extracts from cells grown in high Pi and low Pi and chromatography) performed to identify regulators of Pho80-Pho85-Pho81 activity suggest the role of an evolutionary conserved metabolite, inositol heptakisphosphate (IP_7), in this regulation (Lee *et al.* 2007). IP_7 is produced by sequential phosphorylation reaction of inositol 1,4,5-trisphosphate (IP_3) by inositol polyphosphate multikinase Arg82. This results in the formation of inositol tetrakisphosphate (IP_4) and inositol pentakisphosphate (IP_5), which further undergoes phosphorylation by Ipk1 to form inositol hexakisphosphate

(IP_6). IP_6 then undergo phosphorylation by Kcs1 and Vip1 to form inositol pyrophosphates (IP_7) and double pyrophosphorylated form of IP_8, bis-diphosphoinositol tetrakisphosphate ($PP-IP_4-PP$), respectively (Odom *et al.* 2000; Saiardi *et al.* 2001; Mulugu *et al.* 2007; Saito 2010). Since upon phosphate starvation, the intracellular levels of IP_7 increases, yeast mutants defective in production of IP_7 show failure of inhibition of Pho80-Pho85, even in phosphate-starved conditions (Lee *et al.* 2007). The increased level of IP_7 allosterically modulates Pho81 such that it leads to a conformational change in the ternary complex Pho81-Pho80-Pho85. This conformational change prevents access to the kinase active site by Pho4, which leads to lack of Pho4 phosphorylation (Lee *et al.* 2009; Lee *et al.* 2011). Hypophosphorylated Pho4 remains localized to the nucleus where it interacts with Pho2 to activate phosphate responsive genes, including the high-affinity transporters, *PHO84* and *PHO89*, secretory phosphatase, *PHO5*, and a negative regulator of low-affinity transporter, *SPL2* (figure 2). The high-affinity transporters, as discussed above, have a very low K_m and thus can uptake phosphate even under very low extracellular phosphate concentrations. On the other hand, the secretory phosphatases help in phosphate scavenging by cleaving phosphate from different phosphorylated substrates like nucleic acids, phospho-sugars, phospholipids, *etc.* (Ogawa *et al.* 2000; Persson *et al.* 2003; Mouillon and Persson 2006; Secco *et al.* 2012) (figure 2).

2.3.2 *Yeast cells grown under high- or normal-phosphate conditions:* When the ambient phosphate concentration is either high or normal, phosphate is taken in the cell by low-affinity transporters and is utilized for various cellular processes. The excess of cytoplasmic phosphate is stored in the vacuoles in the form of polyP. During such conditions, there is no accumulation of IP_7. This lack of IP_7 prevents the activation of Pho81 and thus the conformational change in the ternary complex (Lee *et al.* 2009, Lee *et al.* 2011). As a result, the kinase domain of Pho85 remains available to the transcription factor Pho4 (figure 1). The hyperphosphorylated Pho4 is a preferred substrate for the nuclear export receptor, Msn5 and is exported to the cytoplasm (Kaffman *et al.* 1998) (figure 1). The absence of hypophosphorylated nuclear Pho4, thus, prevents the upregulation of phosphate responsive genes under high-phosphate conditions (Ogawa *et al.* 2000; Persson *et al.* 2003; Mouillon and Persson 2006; Secco *et al.* 2012) (figure 1).

A complex feedback mechanism exists, which enables the yeast cells to switch from the low- to high-affinity transporter system and vice versa, depending upon the environmental Pi levels (Wykoff *et al.* 2007). During high-phosphate conditions, cells use low-affinity transport system to cater the phosphate requirements. However, as the phosphate

conditions become limiting, PHO pathway gets activated and it causes the up-regulation of high-affinity transporters and consequently, down-regulation of the low-affinity transporters. A recent study highlighted the advantages of having a dual transport system consisting of low- and high-affinity transporters (Levy *et al.* 2011). In case of a single transport system, when the nutrients in the ambient environment start depleting initially, there is no effect on cellular nutrient pool as the transporters uptake nutrients. However, as the external nutrient level approaches K_m of the transporters, the uptake decreases and a starvation response is initiated. Soon after the starvation response, the cell growth is limited. Thus, a single transporter system gives cells less time to prepare for future starvation events (Levy *et al.* 2011). In contrast, it has been seen that the dual transport system helps the cell in prolonging the time period between starvation response and growth limitation. In this case, the two low- and high-affinity transporters have different dissociation constants. When the ambient nutrient levels are high, low-affinity transporters transport nutrients inside the cell. As the levels of the nutrient drop below K_m of the low-affinity transporters, the uptake of nutrients via these transporters decreases and a starvation response is initiated, which includes the up-regulation of high-affinity transporters. These high-affinity transporters then uptake nutrients until the nutrient levels reach K_m of high-affinity transporters, following which the cell growth is inhibited. Thus, the existence of PHO dual transport system increases the time period between the initiation of starvation response and limitation of growth and this foreseeing of a possible future PHO limitation might help the cell to adapt better (Levy *et al.* 2011).

3. Comparative analysis of PHO pathway in other ascomycetes

3.1 *PHO pathway in Schizosaccharomyces pombe*

Recent attempts to understand the phosphate starvation response in other ascomycetes have suggested the existence of both common and novel components of the budding yeast PHO pathway in other ascomycetes (Henry *et al.* 2011). Studies in *Schizosaccharomyces pombe* (Sp) have suggested the occurrence of a phosphate starvation response consisting of high-affinity transporter *SpPHO84* and acid phosphatase *SpPHO1* (orthologues of *Saccharomyces cerevisiae* (Sc): *PHO84* and *PHO5*, respectively) (Maundrell *et al.* 1985; Schwaninger *et al.* 1990; Schweingruber *et al.* 1992; Henry *et al.* 2011; Carter-O'Connell *et al.* 2012) (figure 3b). However, homology searches have failed to identify the orthologues of the core PHO regulon (*ScPHO4*, *ScPHO2* and *ScPHO81*) (Henry *et al.* 2011). Further, acid phosphatase activity in low-phosphate conditions did not confirm roles of putative orthologues of cyclin-CDK complex

SpPHO80-SpPHO85 in phosphate starvation response (Henry *et al.* 2011). However, for upstream pathways like IP_7 and polyphosphate metabolism, similar mutant phenotypes were seen in both the yeasts. It is, therefore, possible that these two fungi have evolved a similar phosphate starvation response by two different mechanisms, suggesting the existence of common ancestry (Henry *et al.* 2011). Role for a zinc transcription factor, *SpPHO7*, and few upstream proteins like IP_7 and SpCsk1 in regulation of phosphate transcriptional stress response has been identified. Like *ScPHO4*, *SpPHO7* also binds to an upstream region of *SpPHO1* (orthologue of *ScPHO5*). However, unlike *ScPHO4*, which is tightly under the control of phosphate status of the cell and binds to PHO pathway genes only during low-phosphate conditions, *SpPHO7* is more of a general stress response transcription factor (involved in iron, copper, osmotic and alternative carbon utilization stress) and is always bound to its targets. In the phosphate-enriched condition, SpCsk1 prevents the full activation of the starvation response. During low-phosphate conditions, an unknown mechanism prevents this repression leading to the full activation of phosphate starvation response (Henry *et al.* 2011; Carter-O'Connell *et al.* 2012).

3.2 *PHO pathway in Candida species*

In case of *Candida glabrata* (Cg), a phosphate starvation response similar to *S. cerevisiae* has been proposed (figure 3c). Comparative genomic analysis between *S. cerevisiae* (figure 3a) and *C. glabrata* (figure 3c) for PHO pathway genes has identified orthologues of most of the genes. Analyses of deletion mutants of these orthologues for acid phosphatase activity show similar phenotypes in *C. glabrata* as *S. cerevisiae*, suggesting similar functions in phosphate starvation response (Kerwin and Wykoff 2009). Upon phosphate starvation, *Candida* up-regulates a starvation response including secretion of phosphatases and up-regulation of high-affinity transporter (*ScPHO84*). However, homology searches have failed to identify orthologues of acid phosphatases (*ScPHO5*, *ScPHO11*, *ScPHO12*) and the high-affinity transporter *ScPHO89* in *Candida* species (Kerwin and Wykoff 2009). Instead, *CgPMU2*, a gene containing a phosphomutase-like domain, identified by complementation of *Scpho5Δ* strain with *C. glabrata* genomic DNA library, encodes for phosphatases secreted in the periplasmic space during low-phosphate conditions (Orkwis *et al.* 2010). This starvation response is expected to be under the control of *CgPHO4*, which is regulated by its nuclear or cytoplasmic localization. *CgPHO4* is regulated by cyclin/CDK/CDK-inhibitor complex, *ScPHO80-ScPHO85-ScPHO81*. However, *CgPHO2* (a co-activator of *CgPHO4*) is not required for up-regulation of phosphate starvation response in *Candida* (Kerwin and Wykoff 2009; 2012).
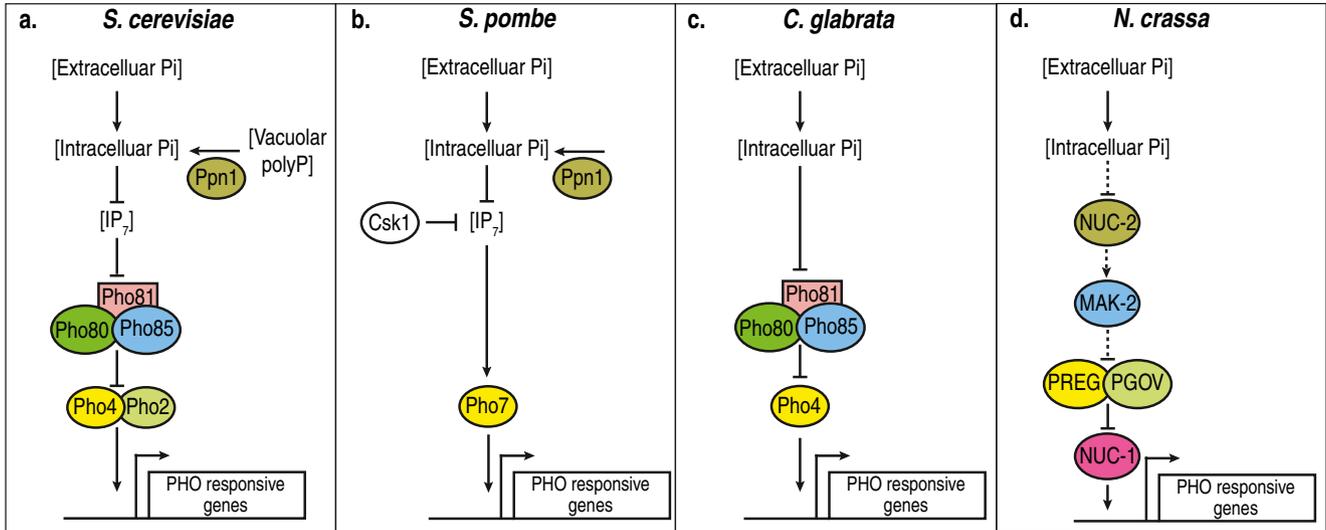
Figure 3. Schematic representation of comparison of PHO pathway in *S. cerevisiae*, *S. pombe*, *C. glabrata* and *N. crassa*: (**a**) In *S. cerevisiae*, levels of IP$_7$ increase in response to limited phosphate levels. IP$_7$ allosterically modulates Pho81 and thus the ternary complex Pho81-Pho80 and Pho85. This in turn leads to hypo-phosphorylated Pho4 and thus activation of phosphate starvation response. (**b**) In *S. pombe*, phosphate depletion has been proposed to increase levels of IP$_7$, which in turn directly or indirectly by an unknown mechanism, regulates the transcription factor Pho7 that is usually under Csk1 repression. However, during phosphate starvation the Csk1 mediated repression of Pho7 is removed and phosphate starvation response is induced (adapted from Henry *et al.* 2011). (**c**) In *C. glabrata*, a phosphate starvation response similar to *S. cerevisiae* is present. Low phosphate levels cause increase in IP$_7$ concentration, which in turn inactivates the ternary complex Pho81-Pho80-Pho85. This leads to the activation of *C. glabrata* homologue of transcription factor Pho4. However, unlike *S. cerevisiae*, Pho2 is not required for up-regulation of phosphate starvation response (adapted from Kerwin and Wykoff 2009). (**d**) In *N. crassa*, PHO pathway is similar to *S. cerevisiae*. The core pathway consists of NUC-2, MAK-2, PREG, PGOV and NUC-1 (Gras *et al.* 2013). The dotted line indicate probable interactions.

Further, the motif CACGTG/CACGTT found in the promoter region of all *ScPHO4*-regulated genes (Oshima 1997) is absent in *C. glabrata* (Kerwin and Wykoff 2009). The inability to identify *SPL2* orthologues indicates that either the positive feedback loop of PHO pathway is absent or *C. glabrata* has other analogs. Therefore, although the general response to phosphate starvation seems similar in the two ascomycetes, there are some differences in the manner the pathway is regulated which might have arisen due to different ecological niches and the type of the phosphate resources available to them (Kerwin and Wykoff 2009) (see figure 3).

Early observation in *C. albicans* pathogenic isolates by Cassone *et al.* (1983) gave indirect evidence of relationship between external Pi and hyphal growth, which was reduced in absence of external Pi. Several candidate PHO pathway genes have been shown to influence hyphal formation and filamentation (Nobile *et al.* 2012). However, it is surprising that even with 24 genes associated with PHO pathway in *C. albicans* genome, not much is known about the function of most genes and their role in fugal pathogenicity. Phosphatase activity, which is a phosphate limitation response, was previously shown to be very low or delayed in *C. albicans* (Smith *et al.* 1973). However, recently Romanowski *et al.* (2012) showed that the phosphate limitation had a dramatic effect on phosphatase activity, which

corroborates with *S. cerevisiae* results. This phosphate limitation leads to extensive filamentation observed in *C. albicans* and could represent a mechanism by which it invades tissues to obtain phosphate. This extensive filamentation was found to be dependent on *CaPHO4* (Romanowski *et al.* 2012). *CaPHO4*, the homologue of *ScPHO4*, is required for growth in phosphate-depleted medium (Homann *et al.* 2009). Up-regulation of *CaPHO4* in response to phosphate limitation lead to an increased expression of the secreted phosphatase (Romanowski *et al.* 2012). Negative regulator of PHO pathway, *CaPHO85*, the homologue of *ScPHO85*, was shown to complement *Scpho85Δ* (Miyakawa 2000) and was involved in temperature-dependent filamentation under control of CaHsp90 (Shapiro *et al.* 2012). In contrast, *CaGRF1*, the homologue of *ScPHO2*, is not required for phosphate limitation response (Homann *et al.* 2009). Thus, it is possible that pathogenicity and hyper-filamentation (a fungal virulence factor) in *C. albicans* is PHO pathway dependent.

3.3   *PHO pathway in Neurospora crassa*

The regulation of phosphate homeostasis in *Neurospora crassa* is similar to the regulation in *S. cerevisiae*

(figure 3d). *Neurospora* also has a core phosphate regulatory unit consisting of NUC-1, PREG, PGOV and NUC-2, which have homology to ScPho4, ScPho80, ScPho85 and ScPho81, respectively (Metzenberg 1979; Gras *et al.* 2007) (figure 3d). NUC-1 is a basic helix-loop-helix transcriptional regulator whose activity is under the control of PREG, a cyclin-like protein kinase and PGOV, which is a cyclin-dependent protein kinase (Peleg and Metzenberg 1994; Kang and Metzenberg 1990; Peleg *et al.* 1996). The PREG-PGOV complex is in turn regulated by the ankyrin repeat protein, NUC-2 (Poleg *et al.* 1996). When *Neurospora* is grown under adequate supply of phosphate, PREG-PGOV complex is active and it antagonizes the function of NUC-1, preventing the up-regulation of phosphate starvation response genes (Poleg *et al.* 1996). An immunofluorescence study to understand the effect of ambient phosphate concentration on posttranslational regulation of NUC-1, suggests that under such high-Pi conditions, NUC-1 predominantly localizes to the cytoplasm (Peleg *et al.* 1996). When the ambient phosphate levels becomes limiting, NUC-2 inhibits the PREG-PGOV complex. Under such conditions, there occurs a 5-fold increase in the levels of NUC-1 and it accumulates in the nucleus (Peleg *et al.* 1996), where it binds to a 6 bp motif CACGTG in the promoter region of phosphate responsive genes. This includes genes encoding for phosphate permeases and repressible alkaline phosphatase that help the cell to survive in the prevailing low-phosphate environment (Metzenberg 1979; Lenburg and O'Shea 1996; Gras *et al.* 2007; Leal *et al.* 2007). In order to provide insight into molecular events involved in phosphate sensing, a suppression subtractive hybridization study was performed by Gras *et al.* (2007). This study identified genes that might play a role in phosphate sensing and signalling. Interestingly, as compared to a wild-type strain, in a *nuc-2* deletion strain, genes involved in initiation of mRNA translation were found to be significantly up-regulated. The other genes that showed a differential expression covered diverse cellular processes like amino acid metabolism, protein synthesis, protein fate, extracellular matrix protein precursor signal transduction mechanisms (MAP kinase) and transport facilitation (Gras *et al.* 2007). In another recent study by Gras *et al.* (2013), the role of MAP kinase MAK-2 in phosphate signalling pathway has been further explored. Comparison of transcriptional profile of a *mak-2* deletion strain, grown in low phosphate with a wild-type grown in low- and high-phosphate conditions revealed over 900 differentially expressed genes. Detailed analysis of the differentially expressed gene set showed the inability of the *mak-2* deletion strain to sense the ambient phosphate conditions. Irrespective of the external Pi concentration, the *mak-2* deletion strain showed a profile similar to wild-type strain grown in high-Pi conditions. Several genes like those encoding for acetamidase, chitinase, acid and alkaline

phosphatases, carboxipeptidase S1, glucan 1,3-β-glucosidase and NUC-2 and PHO-4 proteins were down-regulated in a *mak-2* deletion mutant when grown both in low- and high-Pi conditions. Interestingly, NUC-2 and NUC-1 mutants also show a similar phenotypic profile. *In silico* protein interactions have suggested that MAK-2 interacts with PREG and PGOV and not with MUC-2 and MUC. Based on all these results, it has been proposed that under adequate supply of phosphate, MAK-2 is inactive. In such a case, PREG-PGOV complex interacts with the transcriptional regulator NUC-1, preventing its nuclear localization and thereby prevent up-regulation of phosphate starvation response genes. However, when the phosphate concentration becomes limiting, MAK-2 inhibits the PREG-PGOV complex ultimately leading to nuclear localization of NUC-1 and up-regulation of phosphate responsive genes (Gras *et al.* 2013).

4. Role of Pho84 in phosphate signalling, sensing and transport

Pho84, a high-affinity transporter, is up-regulated upon a phosphate starvation event. A *pho84Δ* deletion strain shows a number of phenotypes: arsenate resistance, poor growth on low-phosphate medium, constitutive up-regulation of PHO pathway as measured by Pho5 activity and negligible levels of polyP (Wykoff *et al.* 2007). These all phenotypes are based on the existence of a feedback mechanism in PHO pathway. It has been proposed that because of low polyP levels, a *pho84Δ* deletion strain lacks the phosphate buffering system (Wykoff *et al.* 2007; Ogawa *et al.* 2000). As a consequence, yeast cells up-regulate their PHO pathway every time the cellular phosphate demand increases, *e.g.* during cell growth and division. Due to this up-regulation, the low-affinity transporters get down-regulated, further lowering the phosphate uptake. Since the major high-affinity transporter, Pho84 is deleted, this cell enters a state in which PHO pathway is constitutively on but the phosphate uptake is negligible (Wykoff *et al.* 2007). While the reason for the depleted polyP reserves in a *pho84Δ* strain (Ogawa *et al.* 2000) is not known, it is possible that there might be some alternate pathway regulating this step.

In the common lab strain S288c, PHO pathway has been found to be constitutively up-regulated with 15 of 32 PHO pathway genes up-regulated (Gagneur *et al.* 2009). Since, *pho84Δ* leads to constitutive up-regulation of PHO pathway even in a phosphate-rich environment and S288c shows such an up-regulation (even in high Pi), it has been hypothesized that Pho84 might be non-functional in S288c. Comparison of protein sequence of Pho84 in 26 yeast strains (*http://www.yeastgenome.org/cgi-bin/FUNGI/alignment.pl?locus=YML123C*) showed that a non-conservative amino acid substitution, P259L, is a rare

mutation found only in lab strains including S288c and isogenic strains like BY4741 and BY4742. It has been suggested that this substitution of proline by leucine in Pho84 transmembrane domain may prevent kinks, which reduces protein flexibility and therefore, might affect its transporter function (Cordes *et al.* 2002).

Studies done to understand the effect of phosphate on growth, metabolism and other regulatory functions in *S. cerevisiae* have identified links between phosphate signalling and Protein Kinase A (PKA) pathway. Re-addition of phosphate to a phosphate-starved yeast cell, in the presence of glucose, has been shown to up-regulate PKA pathway and causes trehalase activation, mobilization of trehalose, repression of STRE genes, *etc.* Analysis of yeast strains containing strong constitutive overexpression of phosphate transporters (Pho87, Pho90, Pho84, Pho89 and Pho91) revealed the contribution of each of the phosphate transporters in transport and signalling property. Yeast strains containing constitutive overexpression of Pho87 and Pho84 were able to sustain phosphate induced trehalase activation, whereas the others could not. Also, this phosphate-induced trehalase activation in phosphate-starved cells was maximum in strain containing a constitutive expression of Pho84, suggesting a key role of Pho84 in PKA signalling as well as phosphate transport (Giots *et al.* 2003). The existence of these two features in Pho84: a transporter and a receptor, identified it as a 'transceptor'. Furthermore, it has also been shown that this phosphate-mediated induction of PKA pathway in phosphate-limited cells can cause down-regulation of Pho84 via a negative feedback loop. This down-regulation is caused by phosphorylation of Pho84 followed by ubiquitination which marks the protein for endocytosis and eventually causes degradation in vacuoles (Lundh *et al.* 2009). Both phosphorylation and ubiquitination occur at amino acid residues in the loop connecting the two helical transmembrane domains, as the Pho84 central loop mutant (with 304-372 amino acid deletion) shows no ubiquitination and also reduced phosphorylation. Interestingly, non-transported organic phosphate esters like glycerol-3-phosphate can trigger PKA signalling (Popova *et al.* 2010). The existence of this signalling function without any transport strongly suggests the role of Pho84 as a receptor. Furthermore, other competitive inhibitors of phosphate transport via Pho84 like phosphonoacetic acid could not trigger PKA signalling. This suggests that simple binding of a substrate at the binding site is not enough to trigger signalling function of Pho84. Binding of only specific compounds at these sites trigger conformational changes, which in turn activates the signalling cascade, and the complete transport cycle is not required to trigger. It has been seen that same binding sites of Pho84 are responsible for both signalling and transport. Using Substituted Cysteine Accessibility Method (SCAM), Popova *et al.* (2010) identified F160 in transmembrane-IV

and V392 in transmembrane-VIII as residues whose side chains are exposed in the phosphate binding site of Pho84. Covalent modification of these residues to cysteine, coupled with addition of MTSEA (a thiol-reactive reagent for labeling proteins at cysteine residues), causes inhibition of both signalling and transport function, suggesting that same residues might be responsible for both the functions. In a later study Samyn *et al.* (2012), based on 3-dimensional structure of Pho84 (generated using bacterial GlpT permease as a template and using multiple sequence alignment of phosphate transporters from plants, bacteria and fungi), two amino acid residues (D358 in transmembrane-VII and K492 in transmembrane-XI) were identified to play an important role in phosphate transport.

Phosphate metabolism is an important pathway to understand the sensing and signalling of essential nutrient, phosphate. Since phosphate is an essential component, an accurate and robust sensing of the nutrient is critical for an organism. Thus, a dual-affinity transporter system, consisting of low- and high-affinity transporters, gives an evolutionary advantage to the organism to respond to a wider concentration of phosphate in the environment and time to adapt to changes in phosphate availability. Within ascomycetes, there are several molecular components that are conserved in sequence and function, while other components have diversified functions and responses. Thus, comparative analysis of PHO pathway will help in understanding the diversification of this pathway and its relationship with specific environmental responses and evolutionary history of a species especially fungal species.

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