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Differential roles for the low-affinity phosphate transporters Pho87 and Pho90 in *Saccharomyces cerevisiae*

Ruben Ghillebert¹, Erwin Swinnen¹, Pepijn De Snijder, Bart Smets and Joris Winderickx*

Functional Biology, Katholieke Universiteit Leuven, Kasteelpark Arenberg 31, Box 2433, B-3001 Heverlee, Belgium

¹ these authors contributed equally and should be considered co-first authors

* for correspondence: joris.winderickx@bio.kuleuven.be; Tel. :+32-16-321516; Fax +32-16-321967

Running title: Regulation of phosphate transporters in yeast

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Summary

When starved for phosphate (Pi), yeast cells activate the PHO-signaling pathway, wherein the Pho4 transcription factor mediates expression of genes involved in Pi-acquisition, such as *PHO84*, encoding the high-affinity H⁺/Pi-symporter. In contrast, transcription of *PHO87* and *PHO90*, encoding the low-affinity H⁺/Pi-transport system, is independent of phosphate status. Here, we reveal that upon Pi-starvation, these low-affinity phosphate transporters are endocytosed and targeted to the vacuole. For Pho87, this process strictly depends on *SPL2*, another Pho4-dependent gene that encodes a protein known to interact with the N-terminal SPX-domain of the transporter. In contrast, the vacuolar targeting of Pho90 upon Pi-starvation is independent of both Pho4 and Spl2, though it still requires its SPX-domain. Furthermore, both Pho87 and Pho90 are also targeted to the vacuole upon glucose starvation or upon treatment with rapamycin, which mimics nitrogen-starvation, but, while these responses are independent of PHO-pathway signaling, they again require the N-terminal SPX-domain of the transporters. These observations suggest that other SPX-interacting proteins must be involved. In addition, we show that Pho90 is the most important Pi-transporter under high Pi conditions in the absence of a high-affinity Pi-transport system. Taken together, our results illustrate that Pho87 and Pho90 represent non-redundant Pi-transporters, which are tuned by the integration of multiple nutrient signaling mechanisms in order to adjust Pi-transport capacity to the general nutritional status of the environment.

Introduction

As inorganic phosphate (Pi) is an essential nutrient for all organisms, maintaining proper Pi homeostasis is critical to ensure survival of cells and organisms. A crucial step in controlling Pi homeostasis encompasses the transport of Pi across the plasma membrane into the cell. Since cellular membranes are impermeable to Pi, specialised integral membrane proteins are required to transport this essential compound. In *Saccharomyces cerevisiae*, two Pi-symport systems exist [1-3]. The low-affinity transport system has a K_m for external Pi of approximately 1 mM and satisfies the cellular need for Pi at normal or high Pi concentrations. This low-affinity Pi-transport system is believed to be composed of the partially redundant Pho87 and Pho90 H⁺/Pi-symporters [4]. A third gene, *PHO91*, was initially considered to be a third component of the low-affinity plasma membrane phosphate transport system [5], but later studies localized the Pho91 protein at the vacuolar membrane, indicating that this protein is not directly involved in the acquisition of phosphate from the external medium [6]. The high-affinity Pi-transport system has a K_m of about 10 μ M and is sustained by the Pho84 and Pho89 high-affinity H⁺/Pi- and Na⁺/Pi-symporters [7, 8]. While expression of the *PHO87*, *PHO90* and *PHO91* genes was shown to be independent of Pi concentrations, transcription of *PHO84* and *PHO89* is induced when Pi becomes limiting, a process controlled by the PHO-pathway [9]. Signal flow through this PHO-pathway is regulated by the cyclin-dependent kinase inhibitor (CKI) Pho81. Under Pi-starving conditions, activation of this CKI leads to inhibition of the Pho85-Pho80 cyclin-dependent kinase complex and nuclear translocation of the dephosphorylated transcription factor Pho4. Nuclear Pho4 then induces a set of genes known as the PHO-genes [10], which includes *PHO84* and *PHO89*. When sufficient amounts of extracellular Pi are available, Pho81 is inactivated and this results in an active Pho85-Pho80 kinase complex that is able to phosphorylate and trigger the nuclear exclusion of Pho4, with as consequence the repression of the PHO-genes [11].

An additional role for the Pho85-Pho80 complex is to regulate the localization and hence the activation of the Rim15 protein kinase in response to fluctuating Pi levels [11]. Rim15 is essential for proper entry into stationary phase as it controls the activity of the transcription factors Msn2,4 and Gis1, which are required for expression of the so-called Stress-Responsive-Element (STRE) genes and the Post-Diauxic-Shift (PDS) genes, respectively [12-14]. Rim15 acts as a nutritional integrator in yeast, since it is regulated by at least four nutrient-regulated kinases: PKA inhibits Rim15 activity [15], while TORC1 and Sch9 promote cytoplasmic retention [16, 17] and the Pho85-Pho80 complex promotes its nuclear exclusion [11, 18]. Under Pi-starving conditions, inhibition of the Pho85-Pho80 kinase by Pho81 results in an active, nuclear Rim15 and proper entry into stationary phase. In contrast, under high Pi conditions, phosphorylation of Rim15 by the Pho85-Pho80 kinase induces its nuclear exclusion thereby leading to its inactivation. In line with this, we already showed that the Pho85-Pho80 complex mediates Pi-dependent activation of typical PKA-targets like activation of trehalase, mobilisation of trehalose and repression of stress-responsive genes and that the regulation of many of these properties occurs via a Pho85-Pho80-dependent inhibition of Rim15 activity [19].

The ability of inorganic Pi to act as a nutrient signal affecting PKA targets is closely correlated to its uptake into the cell, as inactivation of Pi-transport by destruction of the proton gradient across the plasma membrane with the protonophore 2,4-dinitrophenol completely prevented Pi-induced trehalase activation [20]. Although the predominant Pi-transport activity present in Pi-starved cells is represented by the Pho84 transporter [7, 21], which is additionally exemplified by the inability of a *pho84Δ* strain to mediate rapid Pi-signaling [22, 23], we previously observed clear activation of trehalase after addition of Pi to

109 cells deleted for *PHO4* or *PHO81*, in which no *PHO84* transcription can be detected [19].
110 Additionally, both Pho84 and Pho87 have been shown to act as specific Pi sensors for rapid
111 Pi-signaling [20], but the genetic and physiological relevance of Pi-signaling through Pho87
112 was never investigated. Our data reveals that in the absence of Pho4, the low affinity Pi-
113 transporter Pho87 becomes essential for Pi-signaling. We show that in Pi-starved cells, the
114 low-affinity Pi-transporters Pho87 and Pho90 are normally subjected to vacuolar targeting,
115 but in cells devoid of Pho4 activity, this process is specifically hampered for Pho87 due to the
116 lack of expression of *SPL2*, which was previously identified as a negative regulator of low-
117 affinity Pi-transport and was shown to interact with the N-terminal SPX-domain of both
118 Pho87 and Pho90 [4, 24]. However, we now demonstrate that, in contrast to Pho87, the
119 vacuolar targeting of the Pho90 upon Pi-starvation is independent of both Pho4 activity and
120 Spl2. Furthermore, when cells are starved for other essential nutrients or are treated with
121 rapamycin, the vacuolar targeting applies to both low-affinity Pi-transporters and also this
122 process is independent of Pho4 and Spl2. Nonetheless, the vacuolar targeting of both Pho87
123 and Pho90 under these conditions still depends on their N-terminal SPX-domain. This
124 indicates that besides Spl2, other, yet unidentified, proteins must interact with this domain in
125 order to fine-tune the presence of the low-affinity Pi-transporters at the plasma membrane in
126 response to a multitude of nutrient signals.
127

Experimental procedures

Strains

Saccharomyces cerevisiae strains used in this study are listed in Table 1. Deletions were made using polymerase chain reaction-based disruption cassettes as described previously [25]. Yeast was transformed with the Gietz method [26]. Sporulation was performed by spotting diploid cells on plates containing 1% K-acetate, 0.1% KHCO_3 , pH 6.0 for 5 to 6 days at 24°C.

Tetrads were dissected and separated in a raster using a micromanipulator (Singer Instruments). The $P_{\text{TEF}}\text{-EGFP-}PHO87$, $P_{\text{TEF}}\text{-EGFP-}PHO90$, $P_{\text{TEF}}\text{-EGFP-}PHO87\Delta375N$ and $P_{\text{TEF}}\text{-EGFP-}PHO90\Delta375N$ were kindly provided by Hurlimann *et al.* [4, 6].

Culture and starvation conditions

Yeast cells were grown in synthetic complete minimal medium containing 0.5% w/v $(\text{NH}_4)_2\text{SO}_4$, 1.9g/l yeast nitrogen base w/o amino acids, supplemented with all amino acids and 2% w/v glucose (ScD). For monitoring growth on ScD agar with fluctuating extracellular Pi levels, a preculture on ScD was inoculated in fresh ScD medium at an initial OD_{600} of 0.1 and tenfold dilutions were spotted on phosphate starvation medium (5.7 g/l of yeast nitrogen base with ammonium sulphate without phosphate) with 2% glucose, required amino acid/nucleotide supplements and with known Pi concentration. Pictures were taken after 2-3 days. For growth experiments with arsenate, cells were grown in standard rich medium with 2% w/v glucose (YPD) and spotted on YPD or on YPD with an additional 10mM Pi and with or without 5mM AsO_4 . For phosphate signaling and consumption experiments, yeast cells were grown into mid-exponential phase ($\text{OD}_{600} = 1.5\text{-}2$) on ScD medium, harvested, washed

two times and transferred to phosphate starvation medium with 4% glucose and required amino acid/nucleotide supplements. Cells were starved for phosphate for 3 days, during which starvation medium was refreshed daily. Before re-addition of phosphate, the phosphate-starved, glucose-repressed cells were rapidly cooled on ice and harvested by centrifugation. The pellet was washed twice with ice-cold Mes-buffer pH 6.0 and resuspended in fresh phosphate starvation medium with 4% glucose at a cell density of 25mg cells (wet weight) per ml. After 30 min of incubation, KH_2PO_4 was added to the culture and samples were taken at the indicated time points. For monitoring growth recovery after re-addition of phosphate, the Pi-starved cells were resuspended in fresh Pi-starvation medium supplemented with KH_2PO_4 at an initial OD_{600} of 0.1 and growth was followed by measuring OD_{600} at the indicated time points. For the localization studies, cells were grown till mid-exponential phase in ScD and then starved for phosphate and/or glucose or treated with 200nM rapamycin for 6-8 hours unless indicated otherwise. All incubations were done at 30°C under continuous shaking.

Sampling, extraction and determination of trehalase activity

Samples of 50 mg cells (wet weight) were taken at the indicated time-points. Cells were rapidly cooled by addition of ice-cold water, centrifuged and resuspended in 500 μl of 25 mM MES buffer, 50 μM CaCl_2 , pH 7 for extraction. Crude cell extracts were prepared as described previously [20] and dialyzed (BRL microdialysis system) against 25 mM MES buffer pH 7 with 50 μM CaCl_2 at 4°C. Trehalase activity in dialyzed cell extracts was determined as described previously [20]. The specific activity is expressed as nmol glucose liberated per min and per mg protein. Total amount of protein in the samples was determined using a standard method [27].

Phosphate-consumption assay

After addition of 1 mM KH_2PO_4 to Pi-starved cells, 1 ml samples were taken and cells were rapidly pelleted at 4°C. Phosphate levels in the supernatants were determined with the Fiske-Subbarow colorimetric method for the determination of inorganic phosphate [28].

RNA extraction and Northern blot analysis

Samples of 120 mg cells (wet weight) were taken at the indicated time-points and rapidly cooled by addition of ice-cold water. The cells were pelleted and washed once with ice-cold water and stored at -80°C. Isolation of total RNA was performed as described previously [20].

Probes were labeled with α - ^{32}P -dCTP using the High Prime kit (Boehringer Mannheim). Northern blots were made by separation of total RNA on gels containing 1% (w/v) agarose in 50 mM boric acid, 1 mM sodium citrate, 5 mM NaOH pH 7.5 and 1% formaldehyde. Subsequently, RNA was transferred by capillary blotting to a Hybond-N membrane (Amersham) using 10× SCC buffer (1.5 M NaCl, 0.15 M Na-citrate, pH 7.0). The filters were hybridized with the ^{32}P -labelled probes consisting of fragments of the desired coding region. The blots were analyzed using PhosphorImager technology (FUJIX, BAS-1000).

Fluorescence microscopy

Cells expressing EGFP-fusion constructs were grown as described above and used directly for microscopy without fixation. Cells were viewed using a 'Leica DM4000B' fluorescence microscope. Pictures were taken with a 'Leica DFC420C' camera using the Leica Application Suite software. For FM4-64 ([N-(3-triethylammoniumpropyl)-4-(p-diethylamino phenyl)hexatrienyl] pyridinium dibromide; Molecular Probes), which specifically stains the vacuolar membrane, strains overexpressing EGFP-fused Pho87 and Pho90 were starved for 6 hours for phosphate. The cells were stained with FM4-64 dye for 20 min at 4°C and incubated at RT for 60 minutes to induce transport of FM4-64 via endocytotic intermediates to the vacuolar membrane and fluorescence of both the FM4-64 and the EGFP-fusions was examined. Overlaying of images was performed with ImageJ.

Reproducibility of the results

All experiments were performed at least three times. For growth curves, trehalase and Pi-consumption experiments, representative figures are shown. The absolute values are somewhat variable between different experiments, but the relative differences observed between separate experiments and strains are highly reproducible. For Northern blot experiments, representative blots from three independent experiments are shown.

Results

The deletion of PHO4 shifts rapid Pi-signaling control from Pho84 to Pho87

Recent papers have demonstrated that Pho84 is the major Pi-transporter in *S. cerevisiae* involved in rapid Pi-signaling [22, 23], as the *pho84Δ* strain is unable to rapidly reverse Pi-starvation responses. However, we previously observed rapid phosphate signaling in strains deleted for *PHO4* or *PHO81* [19], both of which do not display expression of *PHO84*. Thus, either expression of *PHO84* below detection levels is sufficient to sustain phosphate signaling or another phosphate transporter takes over the function of Pho84 in the absence of Pho4 activity. To investigate this in more detail, the individual Pi-transporters were deleted in a *pho4Δ* background. Upon re-addition of KH_2PO_4 to Pi-starved cultures, we observed that additional deletion of *PHO4* not only restored Pi-signaling in *pho84Δ* cells, but it also shifted the Pi-signaling control towards Pho87. The latter is illustrated by the absence of trehalase activation in a *pho4Δ pho87Δ* strain (Fig. 1A), which nicely correlated with an inability to rapidly consume measurable amounts of Pi (Fig. 1B). Rapid Pi-signaling functions in part to quickly restart growth of the yeast cell, which is further highlighted by the rapid induction of ribosomal protein genes and the repression of the stress-responsive genes (Fig. S1A). Consequently, we observed that the *pho84Δ* and the *pho4Δ pho87Δ* mutant display retardation in growth resumption after addition of phosphate to Pi-starved cells when compared to the wild-type strain and the other mutants (Fig. S1B). This nicely correlates with the delayed induction of ribosomal protein genes and the delayed inhibition of stress-responsive genes in these strains (Fig. S1A). Notably, the presence of the other low-affinity Pi-transporter, i.e. Pho90, does not substitute for Pho87 when it comes to Pi-signaling in the *pho4Δ* strain (Fig. 1), indicating that the two low-affinity Pi-transporters are non-redundant. Deletion of *PHO91* in a *pho4Δ* did also not affect rapid Pi-signaling under these conditions (results not shown). Pho91 was not further investigated as this low-affinity Pi-transporter was shown to mediate Pi-transport across the vacuolar membrane [6]. Together, the above described results indicate that specifically the Pho87 Pi-transporter mediates Pi-signaling in the absence of a functional Pho4 transcription factor.

One possibility would be that Pho4 is somehow involved in transcriptional repression of *PHO87*. However, previous studies already indicated that transcription of the low-affinity Pi-transporters is independent of both Pi concentration and Pho4 activity [9]. This was further confirmed in our study, since there was no significant alteration in the expression of either *PHO87* or *PHO90* when wild-type, *pho84Δ* or *pho4Δ* cells were starved for Pi, this in contrast to the expression of *PHO84* and *PHO89*, encoding the high-affinity Pi-transport system (Fig. S1C). Altogether, these results imply that under Pi-limiting conditions, wild-type yeast cells downregulate the low-affinity Pi-transporter Pho87 and turn to the high-affinity Pho84 Pi-transporter to signal and take up the available Pi. Both processes depend on the activation of the Pho4 transcription factor, which directly induces *PHO84* expression and results in the inactivation of Pho87 at a posttranscriptional level.

Spl2 mediates Pi-starvation-induced vacuolar targeting of Pho87, but not of Pho90.

In yeast, several plasma membrane nutrient transporters are inactivated and targeted to the vacuole in response to changes in the availability of nutrients [29-32]. To determine whether the low-affinity Pi-transporters Pho87 and Pho90 are subject to nutrient limitation-induced vacuolar targeting as well and to establish a role of Pho4 in this process, we set out to compare the intracellular localization of the EGFP-tagged Pho87 and Pho90 proteins under different nutritional conditions in the wild-type strain and in the *pho4Δ* mutant. For this, we opted to use the strains previously described [6], where chromosomal N-terminal EGFP-fusion genes of *PHO87* and *PHO90* were expressed by the TEF promoter. As shown in Fig.

2A, Pho87 was localized at the plasma membrane of wild-type cells grown on ScD medium, but this low-affinity transporter is endocytosed and targeted to the vacuole when cells encountered Pi-starvation. This was additionally confirmed by the deletion of *END3*, encoding an essential component for endocytosis, which prevented the internalisation of Pho87 and, in consequence, led to the stabilization of this Pi-transporter at the plasma membrane under Pi-starving conditions. Furthermore, the process could be monitored in wild-type cells when these were stained with FM4-64, a lipophilic dye that is endocytosed and used to mark endosomes and the vacuolar membrane (Fig. S2A). Intriguingly, the Pi-starvation-induced endocytosis and vacuolar targeting of Pho87 was prevented by the deletion of *PHO4* (Fig. 2A) as well as by the deletion of the Pho4-regulated gene *SPL2* (Fig. 2A and S2A). The latter was previously identified to encode a negative regulator of low-affinity Pi-transport [24]. Next, we analysed Pi-starvation-induced endocytosis and vacuolar targeting of Pho90 in the wild-type and the *pho4Δ*, *spl2Δ* and *end3Δ* mutants using the *p_{TEF}-EGFP-PHO90* construct. In contrast to the Pho87 Pi-transporter, endocytosis of Pho90 was only prevented in the mutant strain lacking End3 and not in the strains lacking Pho4 or Spl2, indicating that the Pi-starvation-induced turnover of Pho90 occurred independent of the latter (Fig. 2B and S2B). We then examined a possible involvement of the protein kinase Rim15, another effector of the Pho85-Pho80 kinase complex [19, 33]. However, this effector did not seem to play a role in the control of the low-affinity transporters, since the deletion of *RIM15* did not affect the removal of Pho87 or Pho90 from the plasma membrane during Pi-starvation (Fig. 2A and 2B).

Recently, Spl2 was shown to interact with the N-terminal SPX-domain of Pho87 and Pho90 [4]. This led us to analyse Pi-starvation-induced endocytosis and vacuolar targeting of Pho87Δ375N and Pho90Δ375N, representing Pho87 and Pho90 constructs lacking the N-terminal 375 amino acids. The endocytosis of Pho87 and Pho90 under Pi-starvation conditions was abrogated in the absence of their SPX-domain (Fig 2A and 2B). For Pho87, this observation fits with the proposed role of Spl2 as inhibitor of low-affinity Pi-transport. For Pho90, however, the Pi-starvation-induced turnover occurs independently of Spl2 and, therefore, our data suggest that this process requires the interaction of another factor with the SPX-domain.

The SPX-domain is essential for vacuolar targeting of Pho87 and Pho90 in response to glucose starvation and rapamycin treatment.

Recently, it was reported that the Pi-induced degradation of Pho84 is delayed in strains with reduced PKA activity [22, 34]. As PKA is part of a nutrient-responsive network, which also comprises the protein kinases Sch9, Rim15 and TORC1, we investigated whether other nutritional cues, besides Pi, affected the internalisation and subsequent vacuolar targeting of Pho87 and Pho90. First, to test the effect of carbon source availability, cells were transferred from ScD to Sc medium lacking a C-source for 6-8 hours. Under these conditions Pho87 and Pho90 were also endocytosed and targeted to the vacuole and this process could not be prevented by deletion of *PHO4* or *SPL2* (Fig. 3A and 3B). Interestingly, shifting the cells from ScD medium to Sc containing galactose or glycerol already induced endocytosis of both low-affinity Pi-transporters (results not shown), letting us conclude that a downshift in the quality of the C-source is sufficient to initiate the process. Since Rim15 acts as a nutritional integrator in *S. cerevisiae*, we also analysed the effect of a *RIM15* deletion on endocytosis and vacuolar targeting of both Pi-transporters but, similar as for Pi-starvation, the Rim15 kinase appeared not to be required (Fig. 3A and 3B).

Next, we challenged the cells with the TORC1-inhibiting drug rapamycin, a condition known to mimic amino acid and nitrogen starvation and to induce vacuolar targeting of different permeases in yeast, such as Gap1 and Tat2 [35, 36]. As shown, treatment of yeast cells with rapamycin also triggered endocytosis and vacuolar targeting of Pho87 and Pho90 and this was again independent of Pho4, Spl2 and Rim15 (Fig. 3A and 3B). Similar results were obtained for nitrogen starvation as well (results not shown).

Since the SPX-domain is essential for endocytosis and vacuolar targeting of Pho87 and Pho90 under Pi-starvation conditions, we wondered whether this domain also directs endocytosis upon glucose starvation and rapamycin treatment. Therefore, we analysed the internalisation of the Pho87 Δ 375N and Pho90 Δ 375N constructs, which revealed that the SPX-domain is indeed required to allow endocytosis of the two low-affinity Pi-transporters under both conditions (Fig. 3A and 3B). Thus, despite the difference in the underlying control mechanisms and proteins involved, the internalization of the low-affinity Pi-transporters during Pi-starvation, glucose starvation or rapamycin treatment is based on the same principle, i.e. interactions of endocytosis-inducing proteins with the SPX-domain of the transporter.

The Pho90 low-affinity Pi-transporter is required for proper growth under high Pi conditions in the absence of a high-affinity Pi-transport system

The data described above focussed on the role and turnover of the low-affinity Pi-transporters under starvation conditions. In the next section, the physiological role of the low-affinity Pi-transporters was investigated when sufficient amounts of Pi are present. Therefore, we evaluated the contribution of Pho87 and Pho90 to sustain growth of mutant strains lacking a functional high-affinity Pi-transport system, in relation to increasing levels of extracellular inorganic Pi. In these mutants, the high-affinity transport system was inactivated, either by deletion of *PHO4*, thereby inhibiting the transcription of *PHO84* and *PHO89*, or by the combined deletion of *PHO84* and *PHO89*. The absence of a high-affinity Pi-transport system results in a clear growth defect once the extracellular Pi levels drop below 250 μ M (Fig. 4A). We then additionally deleted either Pho87 or Pho90 in order to determine in more detail the contributions of each of the low-affinity Pi-transporters to sustain growth at different extracellular Pi concentrations. The *pho4* Δ *pho90* Δ and the *pho84* Δ *pho89* Δ *pho90* Δ strains already displayed a clear growth defect once the extracellular Pi concentrations dropped below 5 mM, while the *pho4* Δ *pho87* Δ and the *pho84* Δ *pho89* Δ *pho87* Δ strain displayed a growth defect only at Pi concentrations of 500 μ M and below (Fig. 4A). This indicates that mainly the Pho90 low-affinity Pi-transporter is important when sufficient amounts of extracellular Pi are available.

These results were additionally confirmed when the growth of the different strains was further analyzed on rich YPD media and in the presence of the toxic phosphate analogue arsenate. On YPD medium, specifically the *pho4* Δ *pho90* Δ and the *pho84* Δ *pho89* Δ *pho90* Δ strains exhibit growth defects when compared to the other strains examined. Consistent with the above, these defects can be suppressed by the addition of 10mM Pi, indicating again the importance of Pho90 for Pi-uptake to sustain growth (Fig. 4B). Moreover, when arsenate was added to the medium, an increased resistance to this toxic phosphate analogue was observed for both the *pho4* Δ *pho90* Δ and the *pho84* Δ *pho89* Δ *pho90* Δ strain (Fig. 4B).

Discussion

The low-affinity Pi-transporters Pho87 and Pho90 are controlled by endocytosis in response to nutrient starvation

Our data concerning the rapid phosphate signaling indicated that Pho4 inhibits the function of the low-affinity Pi-transporter Pho87. This inhibition appeared to be independent of *PHO87* transcription, which, in agreement with previous findings [9], is not regulated by Pi concentration or by major components of the PHO pathway, such as Pho4 and Pho84. This indicated that the regulation of Pho87 in function of Pi-availability must occur primarily at a posttranscriptional level and, consistently, we demonstrated that both the Pho87 and Pho90 transporters are targeted to the vacuole when yeast cells are starved for phosphate. It was previously reported that this process was likely to involve ubiquitination-induced endocytosis [37], which is in agreement with our data showing a complete stabilization of Pho87 and Pho90 at the plasma membrane in Pi-starved *end3Δ* cells. Interestingly, the vacuolar targeting is impaired specifically for Pho87 in cells deleted for *PHO4* or *SPL2*, while Pho90 is still routed to the vacuole in the *pho4Δ* strain. As the Pho87Δ375N protein, lacking its SPX-domain, also failed to be targeted to the vacuole in the absence of phosphate, our data suggest a role for Spl2 in mediating endocytosis of Pho87 via interaction with its SPX-domain [4]. Interestingly, the Pho90Δ375N protein displayed a similar localization pattern, indicating that also for Pho90, the SPX-domain is required for its endocytosis and vacuolar targeting under phosphate starvation, although in this case the process is largely independent of the Pho4/Spl2 branch of the PHO-pathway. Hence, it can be concluded that two independent mechanisms regulate the selective vacuolar targeting of the Pho87 and Pho90 low-affinity phosphate transporters in response to depletion of phosphate from the medium, both of which are absolutely dependent on interactions with the N-terminal 375 amino acids of these proteins.

Our data on endocytosis and vacuolar targeting of the low-affinity Pi-transporters clarify the results obtained in the context of phosphate signaling. Since in a wild-type strain both Pho87 and Pho90 are subject to vacuolar targeting during phosphate starvation, rapid phosphate consumption and signaling is largely depending on the high-affinity transporter Pho84 (Fig. 5). However, in a strain lacking Pho4, transcription of *PHO84* is absent, rendering the cells unable to signal phosphate availability via the Pho84 transporter. However, in this strain, specifically Pho87 remains stabilized at the plasma membrane under phosphate starvation, while Pho90 is still undergoing vacuolar targeting, making that rapid phosphate consumption and signaling becomes, indeed, strictly dependent on this low-affinity Pi-transporter (Fig. 5).

We also demonstrate that besides control by Pi-availability, other nutrients are able to regulate the low-affinity Pi-transporter turnover. This was documented for glucose starvation and rapamycin-treatment, the latter being a condition that mimics starvation for nitrogen and amino acids [36]. Both conditions were found to affect both Pho87 and Pho90, and we confirm that this turnover is also driven by endocytosis [22, 37, 38]. Although the vacuolar targeting of both Pi-transporters under these conditions does not involve the Pho4/Spl2, the SPX-domains of Pho87 and Pho90 are again essential for this process. This strengthens our hypothesis that other SPX-binding factors are involved in mediating endocytosis and vacuolar targeting. Furthermore, the data indicate that both glucose starvation and rapamycin treatment induces a general vacuolar sorting response that overrules the Pi-specific feedback loops. This may allow the cell to adjust Pi-uptake not only as a function of the external Pi concentration, but also to balance the Pi-uptake in respect to other metabolic processes, thereby preventing an overflow of internal Pi-pools.

As our study indicates that both Spl2 and the SPX-domain of Pho87 are required for Pi-starvation-induced endocytosis of Pho87, these results fit nicely with the previously reported interaction of Spl2 with the SPX-domain of this Pi-transporter [4]. In the same study, however, a similar interaction of Spl2 was observed with the SPX-domain of Pho90, while we did not observe a necessity for Spl2 in the endocytosis of Pho90 upon Pi-starvation, although the SPX-domain of the latter still seemed absolutely required. These results seem contradictory, but one has to keep in mind the differences in experimental approaches in our study and the previous one. The negative interaction between Spl2 and Pho90, as described by [4], was seen in strains overexpressing *PHO90* and/or *SPL2* on rich YPD medium, which contains intermediate levels of Pi. In contrast, effects in our study were observed upon deletion of *SPL2* in Pi-starvation medium. In addition, our results obtained under different nutritional conditions, as described above, indicate that other factors, besides Spl2, exist that mediate endocytosis of both Pho90 and Pho87. Consequently, overexpression of *SPL2* may generate dominant effects overruling the regulation of the Pi-transporters by other factors, while deletion of *SPL2* makes the regulation of Pho87 and Pho90 exclusively dependent on these factors. It is therefore very likely that Spl2 mediates the regulation of Pho90 activity under certain physiological conditions not addressed in this study. In this respect, we note that although a transcriptional regulation of *SPL2* in response to Pi-levels is already documented [10, 24], other modes of regulation of Spl2 function may exist which respond to different environmental cues.

Non-redundant functions for the Pho87 and Pho90 low-affinity Pi-transporters

In this study, we unveiled different situations in which either the Pho87 or the Pho90 Pi-transporter is required for signaling and/or optimal growth. Indeed, we showed that in a *pho4Δ* background, phosphate signaling and rapid growth recovery from Pi-starvation depends specifically on the Pho87 transporter, while under conditions where sufficient amount of Pi are available, Pho90 is the most important low-affinity Pi-transporter. These transporter-specific requirements signify that Pho87 and Pho90 represent non-redundant Pi-transporters which are tuned by the integration of multiple nutrient signaling mechanisms in order to adjust Pi-transport capacity to the general nutritional status of the environment.

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Table 1: Strains used in this study

Strain	Genotype	Reference
WT (BY4741)	Mata <i>his3Δ1 leu2Δ0 ura3Δ0 met15Δ0</i>	Y.K.O. (Invitrogen)
<i>pho84Δ</i>	<i>pho84::KANMX4</i>	Y.K.O. (Invitrogen)
<i>pho4Δ</i>	<i>pho4::KANMX4</i>	Y.K.O. (Invitrogen)
<i>pho4Δ pho84Δ</i>	<i>pho4::KANMX4 pho84::KANMX4</i>	This study
<i>pho4Δ pho87Δ</i>	<i>pho4::KANMX4 pho87::KANMX4</i>	This study
<i>pho4Δ pho90Δ</i>	<i>pho4::KANMX4 pho90::KANMX4</i>	This study
<i>pho84Δ pho89Δ</i>	<i>pho84::KANMX4 pho89::LEU2</i>	This study
<i>pho84Δ pho89Δ pho90Δ</i>	<i>pho84::KANMX4 pho89::LEU2 pho90::KANMX4</i>	This study
<i>pho84Δ pho87Δ pho89Δ</i>	<i>pho84::KANMX4 pho87::KANMX4 pho89::LEU2</i>	This study
<i>P_{TEF}-EGFP-PHO87</i>	Mata NatMx- <i>P_{TEF}-EGFP-PHO87</i>	Hürlimann <i>et al.</i> , 2007
<i>P_{TEF}-EGFP-PHO87Δ375N</i>	Mata NatMx- <i>P_{TEF}-EGFP-PHO87Δ375N</i>	Hürlimann <i>et al.</i> , 2009
<i>pho4Δ P_{TEF}-EGFP-PHO87</i>	<i>pho4::KANMX4</i> NatMx- <i>P_{TEF}-EGFP-PHO87</i>	This study
<i>spl2Δ P_{TEF}-EGFP-PHO87</i>	<i>spl2::KANMX4</i> NatMx- <i>P_{TEF}-EGFP-PHO87</i>	This study
<i>end3Δ P_{TEF}-EGFP-PHO87</i>	<i>end3::KANMX4</i> NatMx- <i>P_{TEF}-EGFP-PHO87</i>	This study
<i>rim15Δ P_{TEF}-EGFP-PHO87</i>	<i>rim15::KANMX4</i> NatMx- <i>P_{TEF}-EGFP-PHO87</i>	This study
<i>P_{TEF}-EGFP-PHO90</i>	Mata NatMx- <i>P_{TEF}-EGFP-PHO90</i>	Hürlimann <i>et al.</i> , 2007
<i>P_{TEF}-EGFP-PHO90Δ375N</i>	Mata NatMx- <i>P_{TEF}-EGFP-PHO90Δ375N</i>	Hürlimann <i>et al.</i> , 2009
<i>pho4Δ P_{TEF}-EGFP-PHO90</i>	<i>pho4::KANMX4</i> NatMx- <i>P_{TEF}-EGFP-PHO90</i>	This study
<i>spl2Δ P_{TEF}-EGFP-PHO90</i>	<i>spl2::KANMX4</i> NatMx- <i>P_{TEF}-EGFP-PHO90</i>	This study
<i>rim15Δ P_{TEF}-EGFP-PHO90</i>	<i>rim15::KANMX4</i> NatMx- <i>P_{TEF}-EGFP-PHO90</i>	This study
<i>end3Δ P_{TEF}-EGFP-PHO90</i>	<i>end3::KANMX4</i> NatMx- <i>P_{TEF}-EGFP-PHO90</i>	This study

Figure legends

Figure 1: The low-affinity Pi-transporter Pho87 is essential for rapid Pi-signaling in cells lacking the transcriptional regulator Pho4

The wild-type (WT) strain (\square) and the mutants *pho84Δ* (\triangle), *pho4Δ* (\blacktriangle), *pho4Δ pho84Δ* (\blacklozenge), *pho4Δ pho87Δ* (\blacksquare) and *pho4Δ pho90Δ* (\blacklozenge) are grown to mid-exponential phase in ScD medium after which they are transferred to Pi-starvation medium for 3 days. The cells were then analysed for (A) activation of trehalase after addition of 10 mM KH_2PO_4 and (B) Pi consumption after re-addition of 1 mM KH_2PO_4 to the Pi-starved cells.

Figure 2: Spl2 mediates the vacuolar targeting of Pho87 but not of Pho90 under Pi-starving conditions

Cells carrying a chromosomal P_{TEF} -EGFP-*PHO87* (A), P_{TEF} -EGFP-*PHO87Δ375N* (A), P_{TEF} -EGFP-*PHO90* (B) or a P_{TEF} -EGFP-*PHO90Δ375N* (B) construct were used to monitor the localization of the low-affinity transporters. Cells were grown exponentially (Exp) and transferred for 6-8 hours to Pi-starvation medium (-Pi). As indicated, the wild-type cells or mutants carrying a *PHO4*, *SPL2*, *RIM15* or *END3* deletion were used. Over more than 300 fluorescent cells were counted and >90% of the cells exhibit the phenotypes as visualised in the picture.

Figure 3: The SPX-domain of Pho87 and Pho90 mediates the vacuolar targeting of both Pi-transporters during glucose starvation conditions and after rapamycin treatment

Cells carrying a chromosomal P_{TEF} -EGFP-*PHO87* (A), P_{TEF} -EGFP-*PHO87Δ375N* (A), P_{TEF} -EGFP-*PHO90* (B) or a P_{TEF} -EGFP-*PHO90Δ375N* (B) construct were used to monitor the localization of the low-affinity transporters. Cells were grown exponentially (Exp) and transferred for 6-8 hours to medium without glucose (-D). Also, the localization of both Pi-transporters was monitored after treatment of exponentially grown cells for 6-8 hours with 200nM rapamycin (Rap). As indicated, the wild-type cells or mutants carrying a *PHO4*, *SPL2*, *RIM15* or *END3* deletion were used. Over more than 300 fluorescent cells were counted and >90% of the cells exhibit the phenotypes as visualised in the picture.

Figure 4: The Pho90 Pi-transporter is required under high Pi conditions in absence of a high-affinity Pi-transport system

(A) To evaluate the contribution of the Pho87 and the Pho90 low-affinity Pi-transporters to strains lacking a functional high-affinity Pi-transport system, the growth of strains containing only Pho87 (the *pho4Δ pho90Δ* and the *pho84Δ pho89Δ pho90Δ* strain) or Pho90 (the *pho4Δ pho87Δ* and *pho84Δ pho89Δ pho87Δ* strain) at the plasma membrane, was compared with the growth of strains only containing both low-affinity Pi-transporters (the *pho4Δ* and the *pho84Δ pho89Δ* strain). Cells were grown in ScD medium and tenfold serial dilutions were spotted on Pi-starvation medium containing a specific amount of inorganic phosphate. Pictures were taken after 2-3 days. (B) To evaluate the growth of these strains in the presence of the toxic phosphate analogue arsenate, cells were grown in YPD medium and tenfold serial dilutions were spotted on YPD, YPD containing an additional 10mM inorganic phosphate and/or 5mM AsO_4 . Pictures were taken after 3-5 days.

Figure 5: Regulation of yeast Pi-transporters during phosphate starvation

When Pi becomes limiting, the activity of the Pho85-Pho80 kinase is inhibited by Pho81, leading to nuclear translocation of the transcription factor Pho4. In the nucleus, Pho4 activates expression of typical PHO genes such as *PHO84* and *SPL2*. Induction of Spl2 activity stimulates vacuolar targeting of Pho87, while Pho90 is targeted to the vacuole in a Spl2-independent manner. Although not depicted here, we also demonstrated that under acute

632 glucose starvation and after the addition of rapamycin, the low-affinity Pi-transporters Pho87
633 and Pho90 are both targeted to the vacuole in a Spl2-independent way.

Figure 1

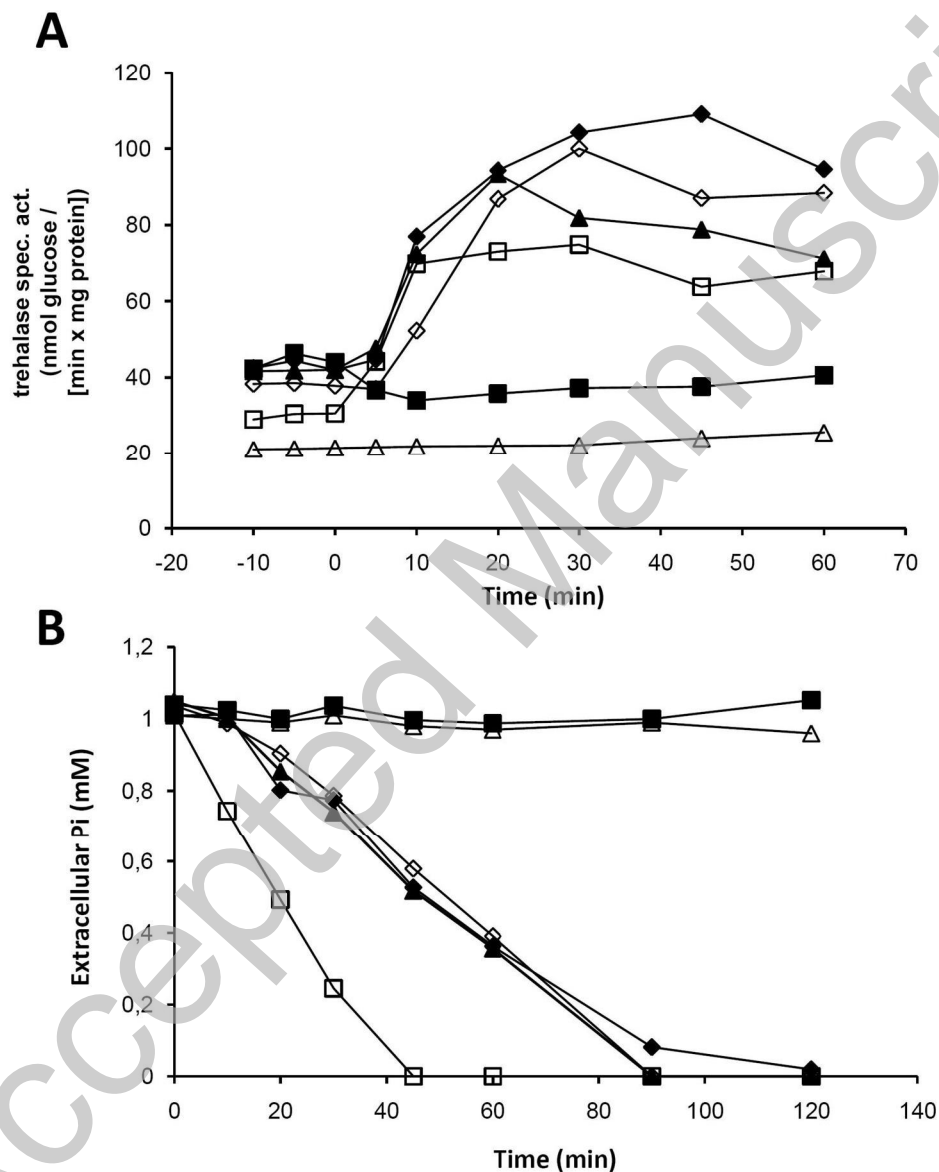


Figure 2

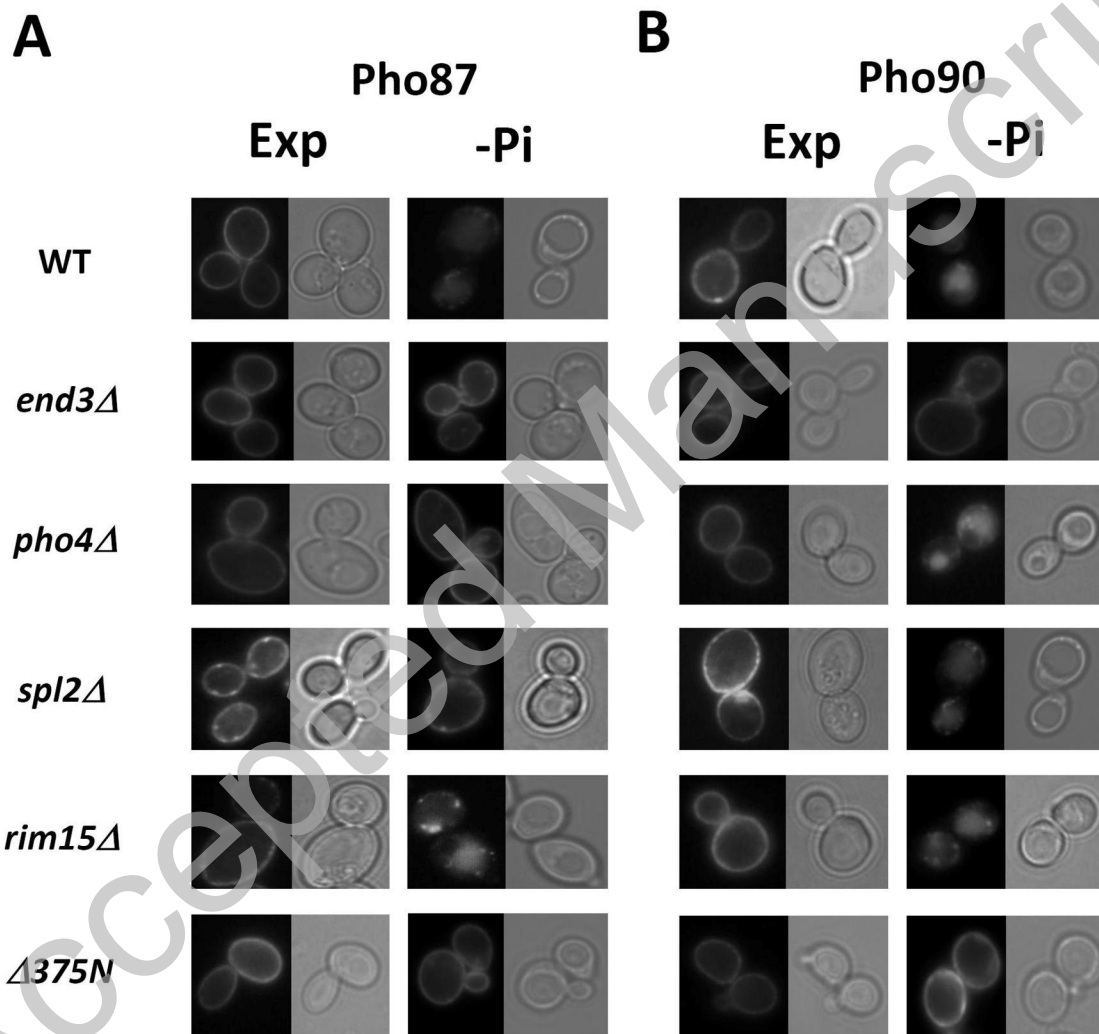


Figure 3

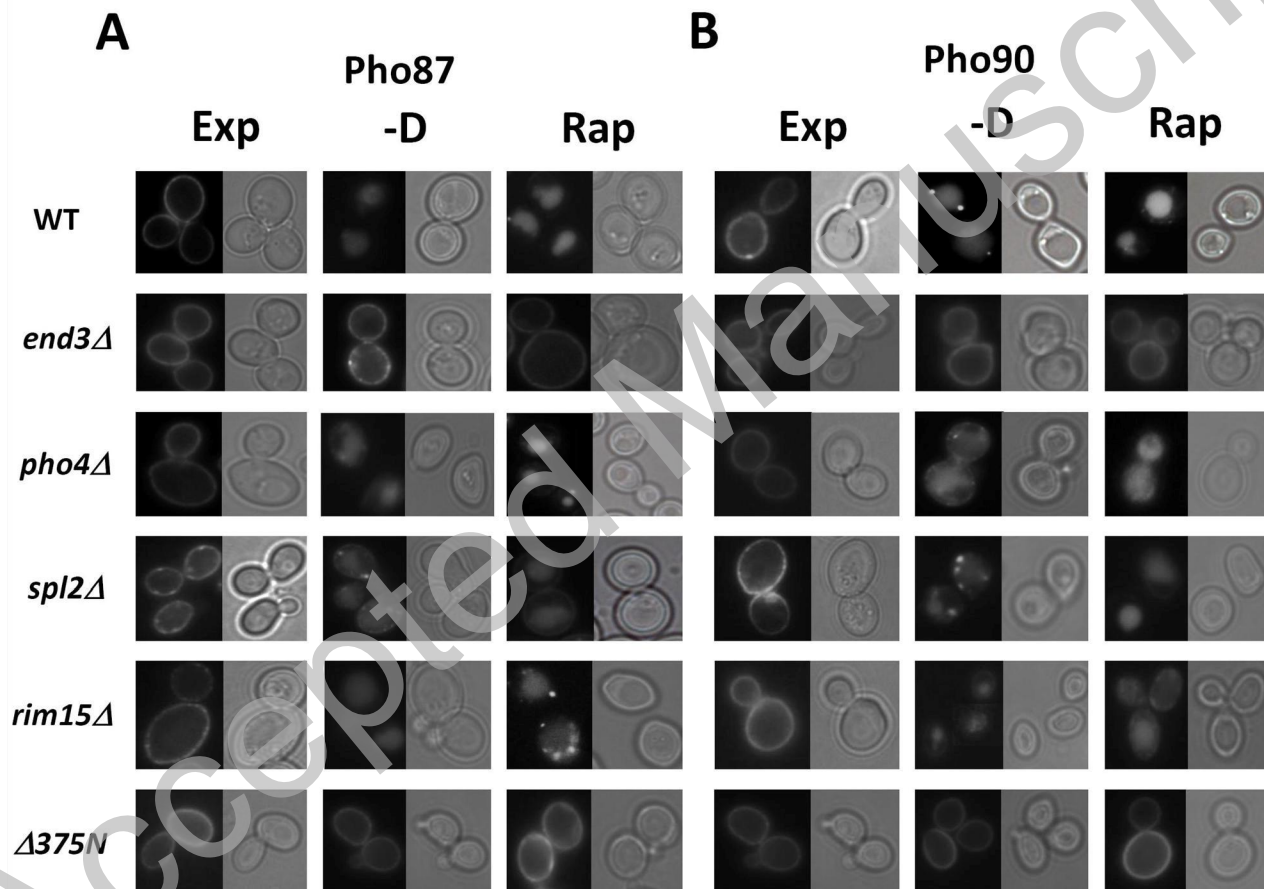


Figure 4

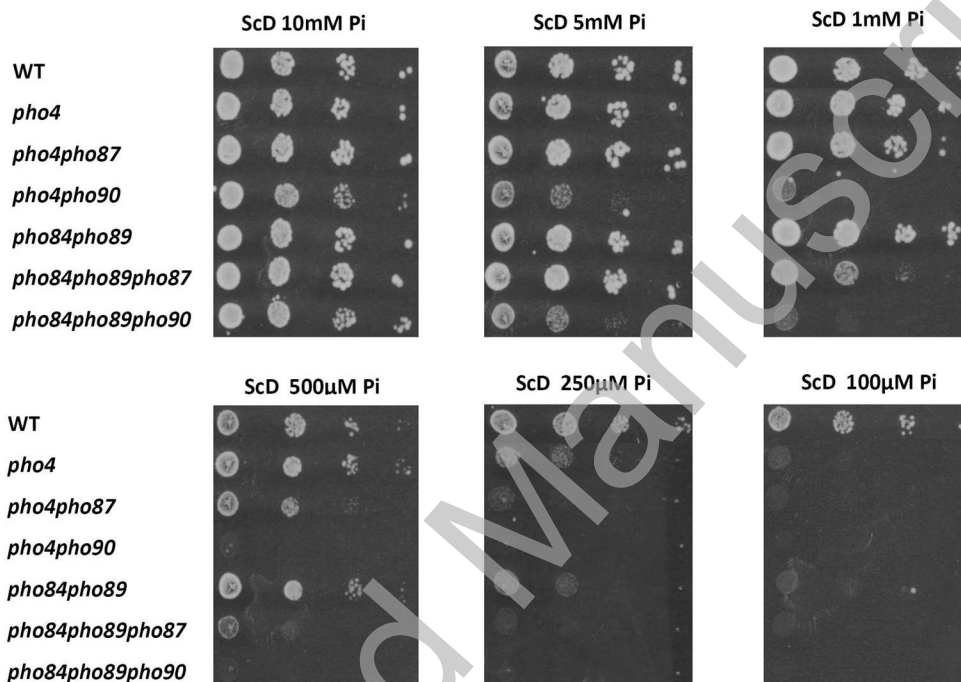
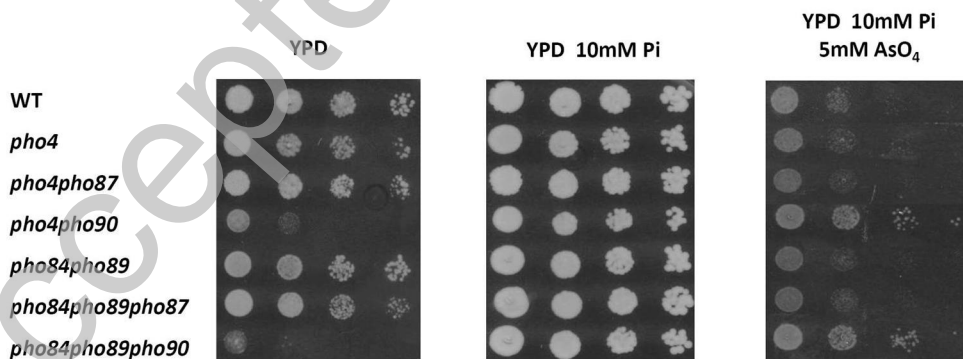
A**B**

Figure 5

