Quantitative analysis of phosphate accumulation in PHO regulatory system-mutant strains of Saccharomyces cerevisia

Quantitative analysis of phosphate accumulation in PHO regulatory system-mutant strains of *Saccharomyces cerevisiae*

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Running Title: Phosphorus accumulation in yeast

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Keywords: *Saccharomyces cerevisiae*; *PHO4*; *PHO81*; mutation; phosphorus; polyphosphate

ABSTRACT

PHO-mutant strains of *Saccharomyces cerevisiae*, NOF-1 and NBD82-1, which constitutively express *PHO81* and *PHO4* respectively, have been reported to accumulate phosphate even in high-phosphate conditions. However, detailed analysis, including a quantitative evaluation of the accumulated phosphate, has not been performed for these mutants. In this study, NOF-1 and NBD82-1 mutant and double mutant strains were cultured in a high-phosphate medium to quantitatively analyze the amount, accumulation form, and physiological use of the accumulated phosphate in the cells. In control strains (BY4741 and NBW7), the percentage of phosphorus in total dry weight of cell was approximately 2%TDW; for the NBD82-1 mutant and double mutant strains, it was approximately 6%TDW; and for strain NOF-1 it was 8.5%TDW. When cells of the mutant strains were stained with 4′,6-diamidino-2-phenylindole (DAPI) they showed a fluorescence peak at 540 nm, suggesting that phosphate accumulated as polyphosphoric acid (polyP). Quantitative evaluation revealed that for strain NOF-1, the percentage of phosphorus exiting as polyP in total dry weight of cell was approximately 5.0%TDW, equivalent to 60% of the total phosphorus in the cells. We also demonstrated that the mutant strains could grow well in phosphate-free medium, suggesting that phosphate accumulated in the cells was used as a phosphorus source. This is the first report concerning the quantitative analysis of phosphate accumulation and the properties of PHO regulatory system-mutant strains of *Saccharomyces cerevisiae*.

INTRODUCTION

Phosphorus is an essential nutrient for all organisms and is used as phosphate in the biosynthesis of diverse cellular components, including nucleic acids, proteins, lipids, and sugars. In *Saccharomyces cerevisiae*, phosphorus accumulates as phosphate via the action of the *PHO* genes, which are regulated by several phosphate-responsive genes (Persson et al. 2003). The PHO regulatory system consists of five PHO-specific regulatory proteins, the transcriptional activators Pho2 and Pho4, the cyclin–dependent protein kinase (CDK) complex Pho80–Pho85, and the CDK inhibitor Pho81. Pho84 is a high-affinity phosphate transporter localized to the plasma membrane (Bun-Ya et al. 1991; Sasano et al. 2018) whose expression is activated by a phosphate-starvation signal mediated by the PHO regulatory system. *PHO5* encodes a repressible acid phosphatase localized to the periplasmic space (Andreeva et al. 2019). Active Pho80–Pho85 catalyzes hyperphosphorylation of Pho4 (Schneider et al. 1994; Ogawa et al. 1995). Meanwhile, hypophosphorylated Pho4 is preferentially localized to the nucleus, where it activates target gene transcription together with Pho2 (Kaffman et al. 1998; Komeili and O'Shea 1999). When the phosphate concentration in the culture medium is low (~0.2 mM), Pho81 inhibits the kinase activity of Pho80–Pho85; in contrast, when the phosphate concentration is high (~10 mM), the Pho80–Pho85 kinase phosphorylates Pho4, and thus the PHO regulatory pathway is downregulated (Oshima 1997).

Previous studies have reported that mutations in *PHO81* and *PHO4* activate the expression of Pho84 and Pho5 even in high-phosphate conditions (Ogawa and Oshima 1990; Ogawa et al. 1995; Ogawa et al. 2000). In the case of the *PHO81* mutant (NOF-1), the point mutation GGA (Gly) to AGA (Arg) at bp 441–443 results in a constitutive expression phenotype, regardless of the phosphate concentration (*i.e.*, *PHO81*^{c}).

Similarly, in the *PHO4* mutant (NBD82-1), the point mutation CCC (Pro) to CTC (Leu) at bp 522–524 results in a constitutive phenotype (*PHO4*^{c}). These mutant strains both constantly express Pho5 and Pho84 (Ogawa et al. 2000). Another study demonstrated that NOF-1 and NBD82-1 strains showed increased phosphate uptake from culture medium compared with the parental strain (Watanabe et al. 2008). However, the amounts of phosphate in these mutant cells have not been evaluated quantitatively. Furthermore, *S. cerevisiae* accumulates phosphate in the form of polyphosphoric acid (polyP), a linear polymer of up to hundreds of phosphate residues linked by phosphoanhydride bonds (Persson et al. 2003; Bru et al. 2016; Gerasimaitė and Mayer 2016). PolyP comprises 37% of the total cellular phosphate in *S. cerevisiae*, and >90% of polyP is localized to vacuoles (Urech et al. 1978; Nguyen et al. 2019). Therefore, it is important to clarify the amount and accumulation form of phosphate for the application of these mutant strains.

This study included a quantitative comparison of the phosphate content in cells of *PHO4* and *PHO81* mutant and double mutant strains. We confirmed the existence of polyP in these phosphate-accumulating strains. The amount of polyP was determined by an enzymatic method using yeast pyrophosphatase (Ppn1) and exopolyphosphatase (Ppx1). Finally, cell growth ability of the mutant strains in phosphate-free conditions was assessed.

MATERIALS AND METHODS

Microbial strains, media, and culture conditions

Table 1 summarizes the microbial strains and plasmids used in this study. *S. cerevisiae* BY4741 and NBW7 were used as control strains (Ogawa and Oshima 1990;

Brachmann et al. 1998). BY4741 is a typical model strain and NBW7 is a parental strain of NOF-1 and NBD82-1. NOF-1 and NBD82-1 are mutant strains with a point mutation giving rise to the phenotypes *PHO81*^{c} and *PHO4*^{c}, respectively (Ogawa and Oshima 1990; Ogawa et al. 2000). Strains NOF-1 and BY22297 were used to construct a double mutant. Mating, sporulation, and tetrad analysis were performed as previously described (Sheman et al. 1982). *S. cerevisiae* strains SH682 and SH683 were used to determine the mating type (Nakazawa et al. 1991). The genomic DNA of double mutant was extracted and sequence analysis was performed to confirm mutations.

Yeast extract–peptone–dextrose (YPD) medium (1% [w/v] yeast extract, 2% [w/v] peptone, and 2% [w/v] glucose) was used to culture yeast strains. KH_2PO_4 (0.4% [w/v]) was added to YPD medium to generate the phosphate-rich condition (YPDP medium). Cells were precultured at 30°C overnight in test tubes containing 4 mL YPDP medium and then inoculated into Sakaguchi flasks containing 100 mL YPDP medium to optical density at 600 nm = 0.2 (OD_{600} = 0.2) and cultured at 30°C with shaking at 140 rpm.

Plasmid construction and Ppx1 purification

An *Escherichia coli* expression system was constructed to prepare Ppx1 for quantitative measurement of polyP. *PPX1* was PCR-amplified from *S. cerevisiae* genomic DNA using primers 5′-AAAGAATTCATGTCGCCTTTGAGAAAGAC-3′ and 5′-AAACTCGAGCTCTTCCAGGTTTGAGTACG-3′. The *PPX1* amplicon was treated with *Eco*RI and *Xho*I and cloned into pET26b. DNA sequencing confirmed the insertion, and the constructed plasmid was named pET26b-PPX1, which was introduced into *E. coli* BL21 (DE3) to give *E. coli* strain BL21 (DE3)/PPX1.

To purify recombinant Ppx1, precultured *E. coli* BL21 (DE3)/PPX1 cells were

inoculated into baffled flasks containing 100 mL lysogeny broth (10 g/L Bacto™ Tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 75 mg/L kanamycin) at 37°C with shaking at 140 rpm. When OD_{660} reached ~0.5, recombinant protein expression was induced by 100 mM isopropyl β-D-1-thiogalactopyranoside and incubating for 4 h at 30°C. Cells were harvested by centrifugation at 10,000 × g for 5 min at 4°C. The resultant cells were resuspended in pure water and ultrasonicated in an ice bath. The supernatant was collected by centrifugation at 10,000 × g for 5 min at 4°C and then applied to a Ni-NTA affinity column, HisTrapHP-1 mL (GE Healthcare Life Science, Buckinghamshire, UK). After washing with 10 mM phosphate buffer (pH 7.4) containing 30 mM imidazole, the target protein was eluted with 10 mM phosphate buffer (pH 7.4) containing 500 mM imidazole.

Purification of Ppx1 was confirmed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% resolving gel) with Coomassie Brilliant Blue staining and western blotting. Western blotting was performed by a standard method using a polyvinylidene fluoride membrane (Immobilon-P, Merck Millipore, Billerica, MA, USA) with mouse IgG anti-His-tag monoclonal antibody (Medical & Biological Laboratories, Nagoya, Japan) as the primary antibody and goat anti-mouse IgG-alkaline phosphatase (Sigma-Aldrich, St. Louis, MO, USA) as the secondary antibody, along with Can Get Signal® (Toyobo, Osaka, Japan) as an immunoreaction enhancer solution and a BCIP-NBT Solution Kit (Nacalai Tesque, Kyoto, Japan).

Blue–white screening for acid phosphatase hydrolysis

Screening of mutants for high acid phosphatase hydrolysis activity used 5-bromo-4-chloro-3-indolyl disodium phosphate 1.5 hydrate (X-phosphate) (Morohoshi

et al. 2002; Watanabe et al. 2008). Each yeast strain was cultured overnight at 30°C in test tubes containing 4 mL of YPDP medium with shaking. The culture was diluted with 10 mM phosphate-buffered saline (PBS) and inoculated on YNB-agar medium (0.67% [w/v] yeast nitrogen base without amino acids and ammonium sulfate, 0.5% [w/v] ammonium sulfate, 2% [w/v] glucose, 1% agarose) containing 35 μg/mL X-phosphate and supplemented with appropriate amino acids (Table S1). Cells were incubated for 3 days at 30°C, and the color of any resultant colonies was observed.

Total phosphorus determination in yeast cells

Total phosphorus in yeast cells was determined as previously described with some modifications (Tong et al. 2019). Briefly, cells were cultured in YPDP medium at 30°C with shaking at 140 rpm for 24 h and then harvested by centrifugation (3,000 × *g*, 5 min). The cells were washed three times with pure water and then freeze-dried. Dry cells (20 mg) were suspended in 25 mL of deionized water. After adding 2 mL HNO_3, the suspension was concentrated to 10 mL by heating. Then, 3 mL of perchloric acid was added to the cooled suspension, which was further concentrated to 3 mL by heating. The pH of the resultant solution was adjusted to 1–2 using NaOH. The phosphorus concentration was measured using a Varian Vista MPX simultaneous ICP optical emission spectrometer (Agilent Technologies, Santa Clara, CA, USA). The percentage of phosphorus in total dry weight of cell was calculated as the % total dry weight (%TDW), the value takes 100%.

Detection and observation of polyP in yeast cells

Cells were cultured in YPDP medium at 30°C with shaking at 140 rpm for 24 h and then harvested by centrifugation (3,000 × *g*, 5 min) and washed twice with PBS. Collected cells were resuspended to OD_{600} = 5 and stained with 50 µg/mL 4',6-diamidino-2-phenylindole (DAPI) at 30°C for 30 min. After washing twice with PBS, the fluorescence spectrum from 460 to 640 nm was measured using an Infinite® 200 PRO spectrofluorophotometer (TECAN).

Quantitative evaluation of polyP

Extraction and analysis of polyP were conducted as previously described, with some modifications (Christ and Blank 2018a; Christ and Blank 2018b). Briefly, cells were harvested after culturing in YPDP, washed three times with pure water, and then lyophilized. Dried cells (25 mg) were resuspended in 400 µL of ME buffer (25 mM MOPS, 2.5 mM Na_2EDTA, pH 7.0 with HCl/NaOH, filtered [0.2-µm pores]) and vortexed for 20 s. TE-saturated phenol (300 µL) was added, and the mixture was vortexed for 20 s. After incubation at 45°C for 10 min, the mixture was cooled in an ice bath for 2 min, and then 800 µL chloroform was added, followed by vortexing for 20 s. The aqueous (upper) layer was transferred to a Phase Lock Gel Light (Funakoshi) and centrifuged (12,000 × *g*, 5 min). The entire upper layer was then collected and stored at −20°C. To estimate the polymeric degree phosphate (chain length) of polyP in yeast cells, 20 µL of the sample was mixed with 4 µL of 6× Orange G loading buffer. Samples (10 µL) were loaded onto 20% acrylamide gels in 1× TBE, and commercial polyP with an average chain length of 60, 140 (10 µL; FUJIFILM Wako) was used as a standard. Electrophoresis was performed at 30 mA and 150 V for 1 h. The gel was soaked in

0.05% toluidine blue (pH 4.1), stained for 1 h, and then shaken overnight in pure water to decolorize.

To quantify polyP, a mixture of 80 µL polyP extract, 50 µL enzyme reaction buffer [22.5 mM $(CH_3COO)_2Mg$, 60 mM Tris, 150 mM CH_3COONH_4, pH 7.5], 5 µL Ppn1 (Biolabs), and 50 µL purified Ppx1 was stirred and incubated at 37°C for 24 h. The free phosphoric acid concentration was measured using a Malachite Green Phosphate Assay Kit (Funakoshi). The amount of total phosphorus exiting as polyP was calculated based on a negative control prepared without Ppn1 and Ppx1. The percentage of phosphorus exiting as polyP in total dry weight of cell was calculated as %TDW, the value takes 100%.

Culture of phosphate-accumulating strain under phosphate-free condition

To accumulate the phosphate in the cells, each strain was precultured in YPDP medium for 24 h. The cells of each strain were collected by centrifugation (3,000 × *g*, 5 min) and washed three times with pure water. Cells were inoculated into a test tube containing 4 mL phosphate-free MOPS medium (Supplementary Table S1) to an OD_{600} = 0.2 and then shaken at 30°C for 144 h. The OD_{600} was measured every 24 h. After 48 h inoculation, the appropriate amino acid mixture (Table S1) was added to initiate cell growth under phosphate-free conditions.

RESULTS AND DISCUSSION

Estimation of phosphate accumulation in each mutant strain

We first estimated the phosphate accumulation in each mutant strain after growth on YNB-agar medium containing X-phosphate. Both acid phosphatase Pho5 and phosphate

transporter Pho84 are regulated by the same transcriptional activator, Pho4. A bluer colony, caused by high acid phosphatase activity which degrades the X-phosphate, suggests increased phosphate transporter activity, followed by the accumulation of more phosphate in the cells. In this experiment, the cells were diluted by 10 mM PBS and inoculated on YNB-agar. The presence of phosphate during dilution might affect the phosphate accumulation in the cells. However, all strains were diluted under the same conditions, so it is considered reliable as a relative evaluation. Figure 1 shows that colony of the control strains (BY4741 and NBW7) was cream-colored. Colony of the mutant strain NBD82-1 (a *PHO4*^{c} mutant) and the double mutant was bluer, while colony of NOF-1 (a *PHO81*^{c} mutant) was the bluest. These results indicate that strain NOF-1 accumulated the most phosphate and the double mutation endowed no additive/synergistic effect.

Phosphate accumulation was then quantitatively evaluated after cell culture in a high-phosphate medium. The total phosphorus in cells of each strain was determined using ICP-OES analysis after extracting phosphorus from the cells by nitric acid treatment (Fig. 2B). In the case of strains BY4741 and NBW7, the percentage of phosphorus in total dry weight of cell was approximately 2%TDW. This value is slightly higher than that (approximately 1%TDW) reported previously (Groombridge et al. 2013). The higher phosphorus accumulation by the control strains in this study was because the cells were cultured in a high-phosphate medium.

The mutant strains showed a higher phosphate accumulation than the control strains; the phosphate accumulation in strain NOF-1 was 8.5%TDW, which was the highest among the tested strains. Phosphate accumulation by strain NBD82-1 was approximately 6%TDW, suggesting that the mutation in *PHO81* had a greater impact on

phosphate accumulation than the mutation in *PHO4*. The possible reason for this difference is that *PHO81* is more upstream regulator than *PHO4* in the PHO regulatory system (Ogawa et al. 2000). Therefore, there is a possibility that *PHO81* controls the phosphate regulation other than through *PHO4*. From this point, the double mutant only accumulated phosphate to 6%TDW. Here, since the genotype at mating type of the constructed double mutant was MATα, it was desirable to align the mating type to parent strains. However, given that it had no effect on cell growth, the differences in mating type are unlikely to have a large effect on the phenotype of phosphate accumulation in *S. cerevisiae*. Therefore, it was concluded that the cumulative gene mutation did not enhance the phosphate accumulation. We expected the additive/synergistic effect of double mutation by considering the possibility that *PHO81* controls the phosphate regulation other than through *PHO4* or *PHO4* is partially controlled by *PHO81*. However, our results suggest that mutation in *PHO4* has a more dominant effect on phosphate accumulation.

Quantitative evaluation of polyP

Quantitative evaluation of phosphate in cells revealed that the mutant strains accumulated up to four times as much as that found in the control strains. Meanwhile, another study reported that a portion of the accumulated phosphate is converted to polyP in vacuoles of *S. cerevisiae* (Urech et al. 1978; Persson et al. 2003). DAPI staining was used to confirm the presence of polyP in cells of the mutants. DNA was also strained with DAPI, however, these can be distinguished because emission wavelength are 400 and 540 nm for DNA and polyP, respectively. Figure 3 shows the fluorescence spectra of cells of each strain stained with DAPI at > 460 nm. There was no fluorescence peak

for either control strain (BY4741 and NBW7), suggesting that polyP was absent from these cells. In contrast, the three mutant strains showed a clear fluorescence peak at approximately 540 nm, indicating that some of their accumulated phosphate occurred as polyP. The peak value was highest for NOF-1, indicating that NOF-1 accumulated the most polyP, consistent with the results from the quantitative evaluation of total phosphorus in the cells (Fig. 2B).

Next, the amount of polyP was determined according to a previous report (Christ and Blank 2018a; Christ and Blank 2018b); polyP was extracted from cells, and the amount of phosphate was determined using an enzymatic method. For the reason to use BY4741 as control strain, there was almost no difference in phosphate accumulation between BY4741 and NBW7 (Fig. 2). To further conduct the quantitative evaluation of polyP amount, we decided that BY4741, a general model strain of *S. cerevisiae*, would be suitable to compare and discuss the results in previous reports.

Confirmation of polyP extraction and estimation of the polyP chain length were conducted by PAGE with toluidine blue staining (Fig. 4A). For strain BY4741, three clear bands appeared at positions longer than that of $polyP_{140}$ (i.e., polyP with chain length 140) and were considered to be 26S rRNA, 28S rRNA, and tRNA, but polyP was not clearly observed in this strain. For the sample from strain NOF-1, a heavier band than that from $polyP_{140}$ was observed (Fig. 4A, lane 1), suggesting that the chain length of polyP in NOF-1 was similar to that of $polyP_{140}$ or longer. The band position did not change after incubation in buffer without enzymes Ppn1 or Ppx1 (lane 2). However, the band was wholly degraded by Ppn1 and Ppx1 treatment (lane 3). Though the bands from ribosomal RNAs also disappeared from both samples on enzymatic treatment, it seems that the amount of RNA was negligible compared with that of polyP based on the

relative band intensities in PAGE. Therefore, the amount of polyP was quantified by determining the difference in the amount of free phosphate released with and without enzyme treatment. The weight percentage of phosphorus present as polyP is shown in Fig. 4B. For strain BY4741, even though the ployP was not clearly detected by the fluorescence analysis with DAPI or PAGE analysis, the percentage of phosphorus exiting as polyP in total dry weight of cell was determined as 0.6%TDW by the quantitative analysis. This means that quantitative evaluation has higher analysis accuracy. Because our result suggests that approximately 26% of total phosphorus in these control cells was converted to polyP, this value is consistent with a previous report in which *S. cerevisiae* accumulated polyP in vacuoles to comprise 37% of the total cellular phosphate (Urech et al. 1978). In contrast, the percentage of phosphorus exiting as polyP in total dry weight of cell in strain NOF-1 was 5.0%TDW, significantly higher than that in the control strain and accounting for approximately 60% of total phosphorus in strain NOF-1.

Growth of phosphate-accumulating strains in phosphate-free conditions

To confirm the physiological use of accumulated phosphate in cells, the growth of the phosphate-accumulating strains was examined in phosphate-free culture conditions. To accumulate the phosphate in the cells, each strain was precultured in YPDP medium for 24 h. The cells of each strain were moved to a phosphate-free MOPS medium to an OD_{600} = 0.2. After confirming minimal cell growth for 48 h, cell growth was induced by adding an amino acid mixture (Table S1). The control strains started growing following the amino acid addition and stopped growth at OD_{600} <1.0 after 100 h in culture. As noted above, approximately 2%TDW phosphate accumulation was confirmed for these

strains after culture in the high-phosphate condition (Fig. 2); therefore, the control strains were limited to one or two cell division using 2%TDW phosphate accumulated during the pre-culture. In contrast, however, the mutants NOF-1 and NBD82-1 continued to grow in the phosphate-free MOPS medium with added amino acids, and reached OD_{600} values of 2.2 and 1.7, respectively. These values were significantly higher than those for the control strains. Considering that the initial OD_{600} = 0.2, these mutant strains were capable of three or four cell divisions using the excess phosphate. The double mutant showed almost the same growth as the control strains. Therefore, the cell proliferation amount was consistent with the amount of total phosphorus in the cells (Fig. 2). Thus, the accumulated phosphate was found to be used as a source of phosphate and had a physiological function in phosphate metabolism in *S. cerevisiae* cells.

CONCLUSIONS

This study quantitatively analyzed the amount and form of stored phosphorus in phosphate-accumulating *S. cerevisiae* strains and the physiological use of accumulated phosphate. the percentage of phosphorus in total dry weight of cell was approximately 2%TDW in the control strains under high-phosphate conditions. This increased to approximately 6%TDW in strain NBD82-1 and the double mutant, and was highest in strain NOF-1 (8.5%TDW). The accumulation of polyP in mutant cells was confirmed by a fluorescence peak at 540 nm, following DAPI staining. Quantitative evaluation revealed that the percentage of phosphorus exiting as polyP in total dry weight of cell in strain NOF-1 was 5.0%TDW, which was equivalent to 60% of the total phosphorus in the cells. Finally, the accumulated phosphate could be used as a phosphorus source by

growing cells in phosphate-free medium. This is the first report concerning the quantitative analysis of phosphate accumulation and the properties of PHO regulatory system-mutant strains of *Saccharomyces cerevisiae*.

ACKNOWLEDGMENTS

Thanks to Dr. Y. Kaneko for helpful discussions. We thank Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

AUTHOR CONTRIBUTIONS

YO and MA proposed the research concept and provided necessary tools for experiments and experimental instructions. YO wrote the manuscript. KN and RA designed and conducted the experiments and analyzed data. All authors read and approved the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

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FIGURE LEGENDS

Fig. 1 Photographs of colonies of *Saccharomyces cerevisiae* strains grown on YNB-agar medium containing X-phosphate. Blue indicates the degradation of X-phosphate and thus high acid phosphatase activity.

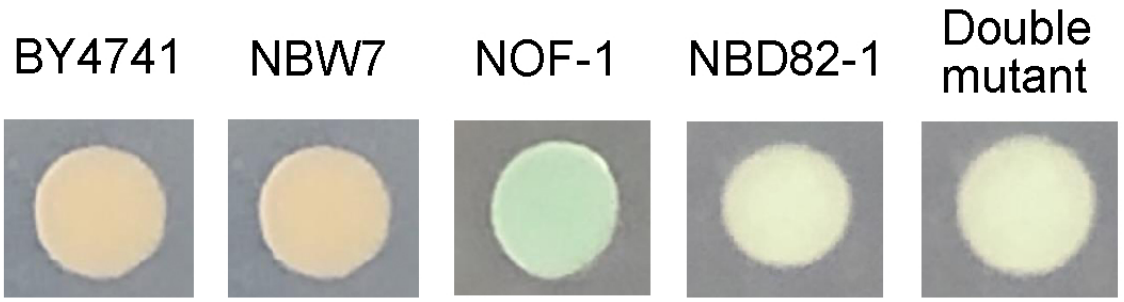
BY4741
NBW7
NOF-1
NBD82-1
Double mutant

Fig. 2 The percentage of phosphorus in total dry weight of cell (%TDW) after culture for 24 h in a high-phosphate medium. Each datum represents the average of three independent assays ± SD. Significant differences ($p < 0.05$) are marked with asterisks.

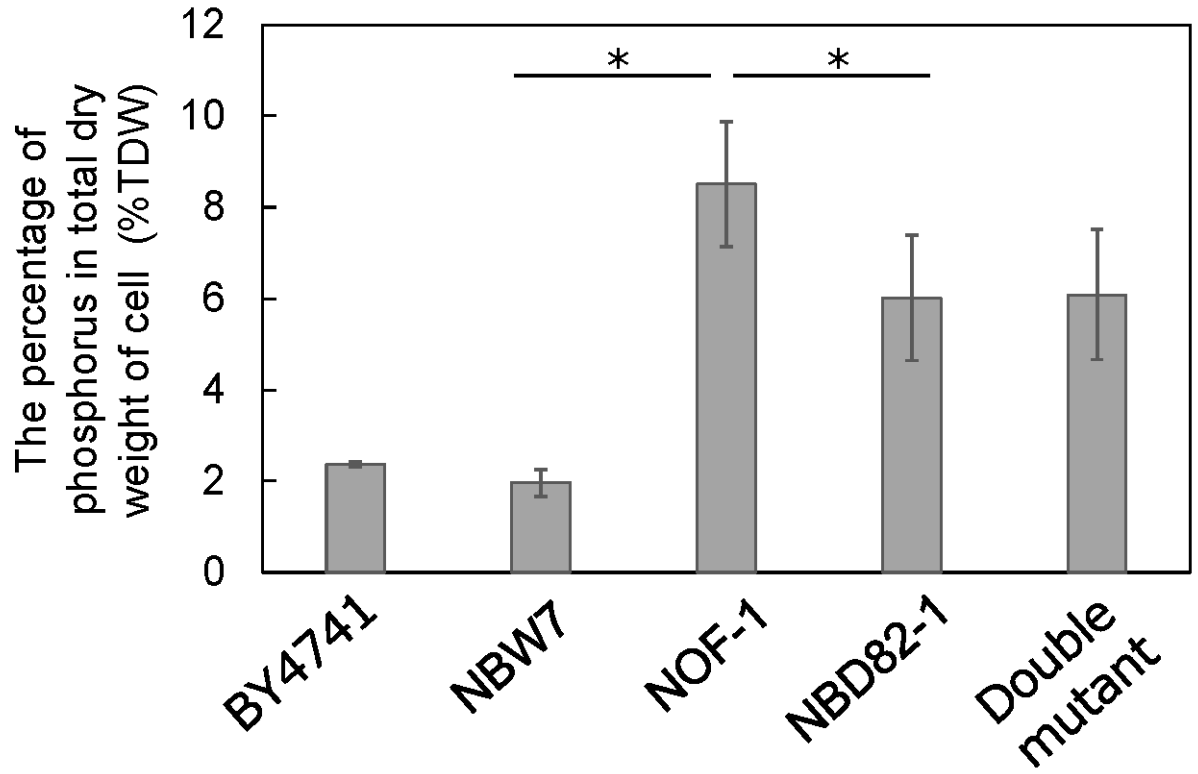

Fig. 3 Fluorescence spectrum of yeast cells stained with 4′,6-diamidino-2-phenylindole (DAPI). Cells of each strain were collected after culture for 24 h in a high-phosphate medium. PolyP stained with DAPI has a fluorescence maximum at 540 nm.

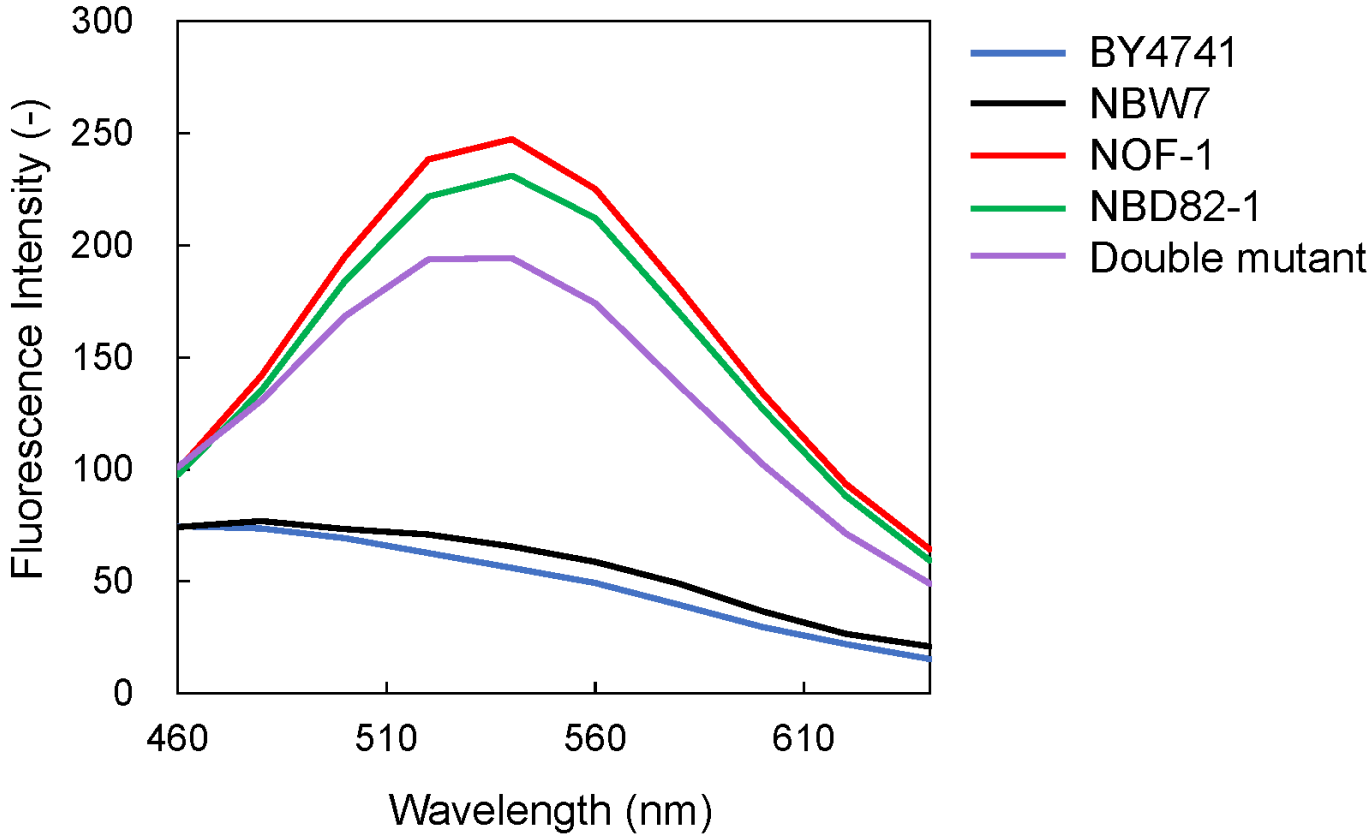

Fig. 4 Analysis of polyP in *S. cerevisiae* strains BY4741 and NOF-1. (A) PAGE analysis of polyP isolated from cells of each yeast strain. Lane 1: the extracted sample of polyP; lane 2: the sample incubated in buffer only; lane 3: the sample incubated in buffer with Ppx1 and Ppn1; $PolyP_{60}$ and $PolyP_{140}$ are commercial polyP preparations with average chain lengths of 60 and 140, respectively, that were used as standards. (B) The percentage of phosphorus exiting as polyP in total dry weight of cell (%TDW). Each datum represents the average of three independent assays ± SD. Significant difference ($p < 0.05$) is marked with asterisks.

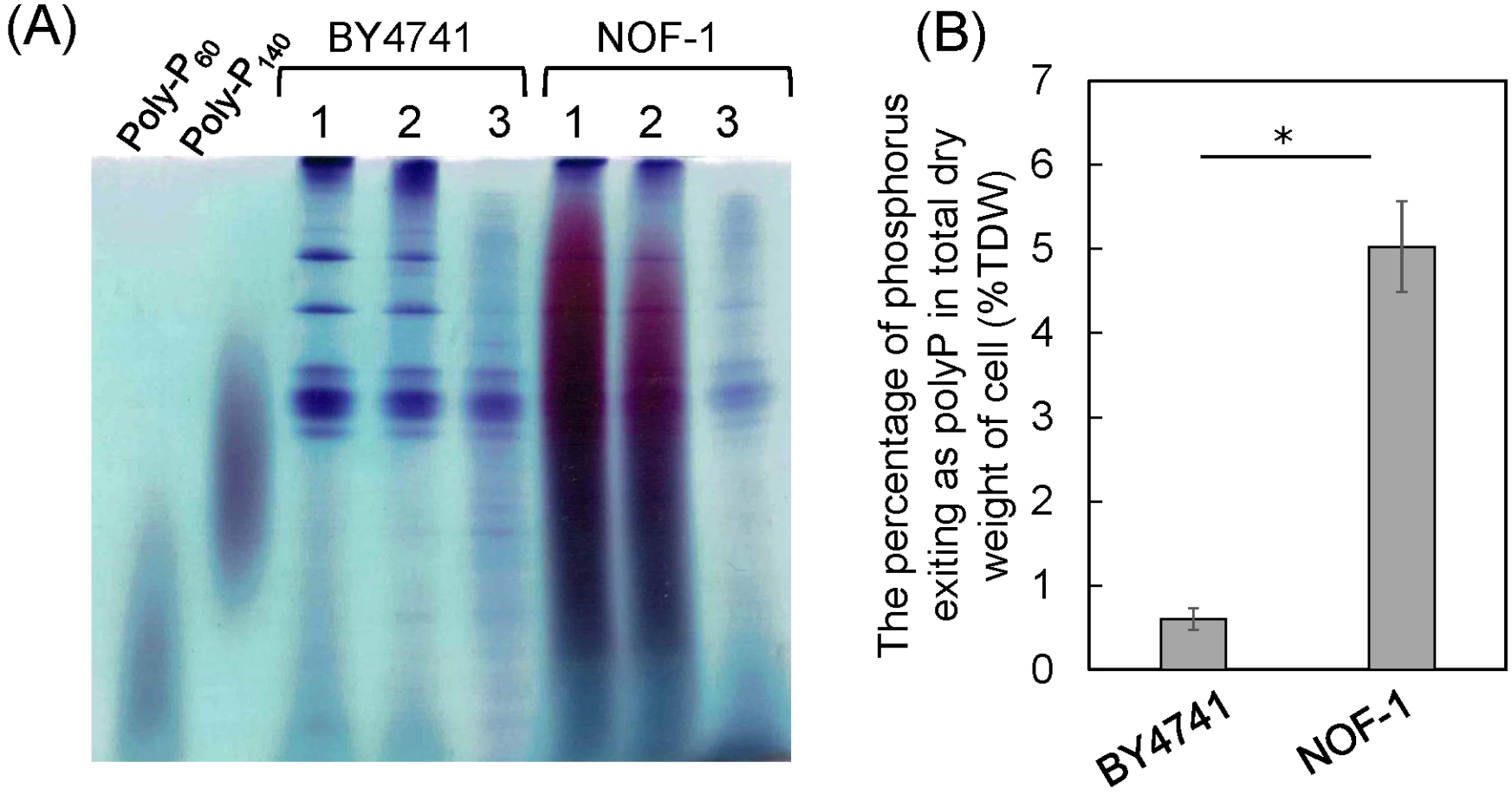
(A)
Poly-P 60
Poly-P 140
BY4741
NOF-1
1 2 3 1 2 3
(B)
The percentage of phosphorus exiting as polyP in total dry weight of cell (%TDW)
0 1 2 3 4 5 6 7
*
BY4741
NOF-1

Fig. 5 Cell growth of each yeast strain in phosphate-free culture conditions. Cells were precultured in high-phosphate medium (YPDP) for 24 h to accumulate phosphate in the cells. The arrow shows the timing of the addition of amino acid mixture to the culture. Each datum represents the average of three independent assays ± SD

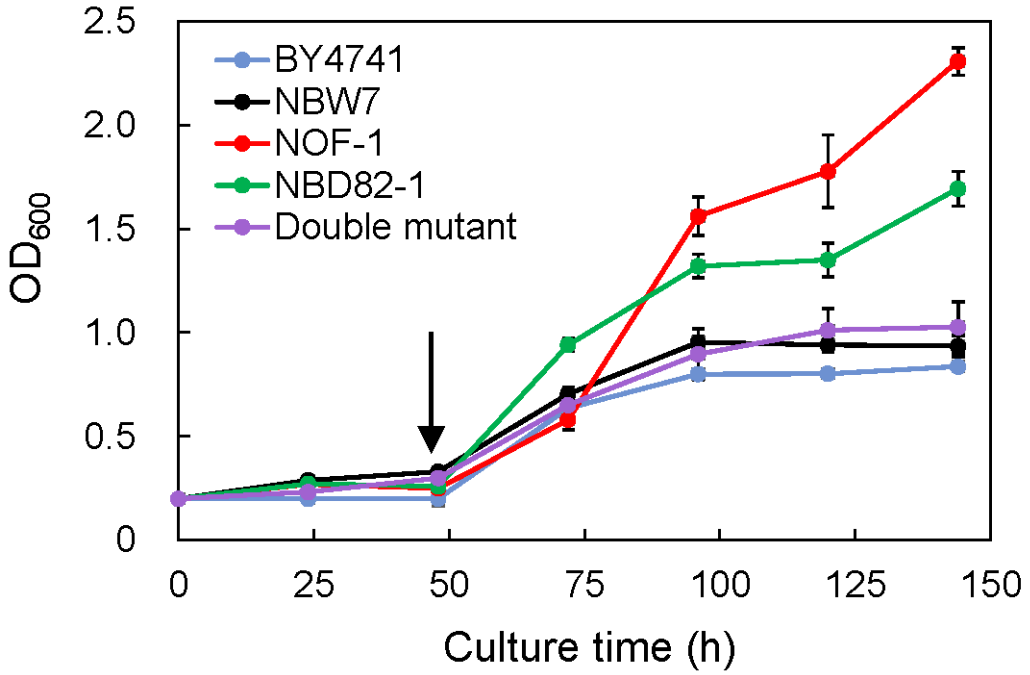

Table 1 Strains and plasmids used in this study

Strains or plasmids	Genotype	Reference
Strains		
S. cerevisiae BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	Brachmann et al. 1998
S. cerevisiae NBW7	MATa ade2 his3 leu2 trp1 ura3 pho3-1	Ogawa and Oshima. 1990
S. cerevisiae NOF-1	MATa ade2 his3 leu2 trp1 ura3 pho3-1 $PHO81^{c-}1$	Ogawa et al. 2000
S. cerevisiae NBD82-1	MATa ade2 his3 leu2 trp1 ura3 pho3-1 $PHO4^{c-}1$	Ogawa and Oshima. 1990
S. cerevisiae BY22297	MATα $PHO4^{c-}1$ pho3-1	NBRP
Double mutant	MATα ura3 pho3-1 $PHO81^{c}$-1, $PHO4^{c}$-1	This study
S. cerevisiae SH682	MATa ura1 ura2 trp3 lys1 pho3 pho5	Nakazawa et al. 1991
S. cerevisiae SH683	MATα ura1 ura21 trp3 lys1 pho3 pho5	Nakazawa et al. 1991
E. coli BL21(DE3)	F－, ompT, hsdSB(rB－ mB－), gal(λcI 857, ind1, Sam7, nin5, lacUV5-T7gene1), dcm(DE3)	Novagen
Plasmids		
pET26b	*E. coli* expression plasmid with T7 promoter	Novagen
pET26b-PPX1	pET26b carrying PPX1	This study

Supplementary Table S1

MOPS medium

Components	Concentration
MOPS	4.0×10^{-1} M
Tricine	4.0×10^{-2} M
Thiamin·HCl	1.8×10^{-3} M
$FeSO_4·7H_2O$	1.0×10^{-6} M
NH_4Cl	9.5×10^{-2} M
K_2SO_4	2.8×10^{-3} M
$CaCl_2·2H_2O$	5.0×10^{-6} M
$MgCl_2$	5.3×10^{-3} M
NaCl	5.0×10^{-1} M
$(NH_4)_6Mo_7O_{24}·4H_2O$	2.9×10^{-8} M
H_3BO_4	4.0×10^{-6} M
$CoCl_2$	3.0×10^{-7} M
$CuSO_4·5H_2O$	9.6×10^{-8} M
$MnCl_2$	8.1×10^{-7} M
$ZnSO_4$	9.7×10^{-8} M

Amino acid mixture for BY4741

Components	Concentration
Leu	60 mg/L
His	20 mg/L
Met	10 mg/L
Lys	30 mg/L
Ura	20 mg/L

Amino acid mixture for other strains

Components	Concentration
Leu	60 mg/L
His	20 mg/L
Trp	20 mg/L
Ade	10 mg/L
Ura	20 mg/L