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ORIGINAL PAPER

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Studies on inositol-mediated expression of *MAL* gene encoding maltase and phospholipid biosynthesis in *Schizosaccharomyces pombe*

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Abstract In this study, the effects of inositol addition on expression of the *MAL* gene encoding maltase and phosphatidylinositol (PI) biosynthesis in *Schizosaccharomyces pombe* (a naturally inositol-requiring strain) were examined. We found that specific maltase activity was at its maximum when the concentration of added inositol reached 6 μg ml^{-1} in a synthetic medium containing 2.0% (w/v) glucose. When the concentration of added inositol was 1 μg ml^{-1} in the medium, repression of *MAL* gene expression occurred at glucose concentration higher than 0.2% (w/v). However, when *S. pombe* was cultured in the synthetic medium containing 6 μg ml^{-1}, repression of maltase gene expression occurred only at initial glucose concentration above 1.0% (w/v). More mRNA encoding maltase was detected in the cells grown in the medium with 6 μg ml^{-1} inositol than in those grown in the same medium with 1 μg ml^{-1} inositol. These results demonstrate that higher inositol concentrations in the synthetic medium could derepress *MAL* gene expression in *S. pombe*. PI content of the yeast cells grown in the synthetic medium with 6 μg ml^{-1} of inositol was higher than that of the yeast cells grown in the same medium with 1 μg ml^{-1} of inositol. This means that PI may be involved in the derepression of *MAL* gene expression in *S. pombe*.

Keywords Expression of *MAL* gene · Phosphatidylinositol · Derepression · *Schizosaccharomyces pombe*

Abbreviations PC: Phosphatidylcholine · PE: Phosphatidylethanolamine · PA: Phosphatidyl acid · PI: Phosphatidylinositol · PS: Phosphatidylserine · CL: Cardiolipin

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Introduction

It has been shown that phosphatidylinositol (PI) has many functions in yeast cells; it can play important roles in cell viability, cell growth, cell morphology, metabolism and signal transduction [1–4]. PI biosynthesis in yeast cells is significantly regulated by inositol available in the medium [2, 5]. In recent years, many advances in research on glucose repression and enzyme secretion in yeasts have been made [6, 7]. However, there is still little known about the relationship between lipid composition and gene expression in yeasts. In previous studies [8], we demonstrated that inositol supplementation could derepress invertase secretion to some extent in *Saccharomyces* sp. W4. Much more mRNA encoding secreted invertase was detected in the glucose-derepressed cells with higher PI content than in the glucose-repressed cells with less PI content. We thought that a PI-type signaling pathway may be involved in the activation of transcription of the gene encoding invertase in the yeast strain [9]. At the same time, we found [10, 11] that when the *INO1* gene encoding inositol-phosphate synthase was transformed into wild type *S. pombe*, a natural inositol auxotroph, the inositol prototrophic transformants obtained not only can grow in the synthetic medium lacking inositol, but also can secrete a large amount of inositol into the medium. Higher invertase secretion occurs when PI synthesis is enhanced in the inositol over-producing cells. It was also found that more mRNA encoding secreted invertase and more PI and less phosphatidylserine (PS) exist in the transformant *Sch.p*944 with inositol over-production than those in the transformant *Sch.p*1025 which produces less inositol [10]. The results show that the increase in produced inositol in the cells could lead to an increase of PI and a decrease of PS. These results also mean that PI may be involved in derepression of invertase secretion at the transcriptional level.

In a recent study [12], we obtained evidence that invertase secretion was enhanced in the cells of

Schizosaccharomyces pombe cultivated at 8 μg ml^{-1} of inositol compared with that in the cells of the same yeast strain grown at 1 μg ml^{-1} of inositol. More mRNA encoding secreted invertase was detected in the cells grown in the 8 μg ml^{-1} inositol-containing medium than in those grown in 1 μg ml^{-1} inositol-containing medium. These results demonstrate that higher concentration of added inositol in the synthetic medium could derepress INV^+ gene expression in *S. pombe*. PI content of the yeast cells grown in the 8 μg ml^{-1} inositol-containing medium was higher than that of the yeast cells grown in 1 μg ml^{-1} inositol-containing medium. This means that PI may be involved in derepression of INV^+ gene expression in *S. pombe*.

Mehta et al. [13] reported that it seems that maltase in *std* mutant of *S. pombe* is an external enzyme as the *std* mutation of *S. pombe* revealed the extracellular hydrolysis of maltose to glucose. However, after a search for the secretion leader in the deduced protein sequence of cloned *MAL* gene (Accession number: DQ019991) in *S. pombe* used in this study by using the methods as described in SignalP3.0 Server at http://www.cbs.dtu.dk/services/SignalP, it was found that there is no secretion leader in the deduced protein sequence. Therefore, the maltase in this yeast strain is not a secretable enzyme. In order to determine if expression of the *MAL* gene encoding intracellular maltase in *S. pombe* is mediated by inositol and PI, the effect of the added inositol on maltase activity and *MAL* gene expression and phospholipid biosynthesis in *S. pombe* were examined.

Materials and methods

Yeast strain

Schizosaccharomyces pombe, a naturally inositol-requiring strain was kindly supplied by Dr. Olaf Nielsen at University of Copenhagen, Denmark [10, 12]. The yeast strain was maintained at 4°C on YPD agar which contained 2.0% glucose, 2.0% peptone, 1.0% yeast extract and 2.0% agar.

Medium

A completely synthetic medium without inositol addition at pH 5.5 was used in this study [14]. Before autoclaving, different concentrations of inositol were added to the synthetic medium.

Growth conditions for effects of inositol addition on maltase activity

In order to determine the effects of inositol addition on maltase gene expression, mRNA biosynthesis and phospholipids biosynthesis, different concentrations of glucose were added to the synthetic medium with different concentrations of inositol. After that, the cells were shaken vigorously on a rotary shaker (170 rpm) at 30°C for 44 h. In order to determine the time course of cell growth and maltase activity, the cells in the culture were collected by centrifugation for determination of cell dry weight and maltase activity and the supernatant was used for the measurement of residual reducing sugar at the time interval of 8 h.

Lipid extraction

Yeast cells were harvested by centrifugation and washed three times with deionized water. The washed cells were resuspended in 10 ml of deionized water. The cell disruption and lipid extraction were carried out according to the methods [1].The lipid sample was stored at −20°C.

Phospholipid analysis

Individual phospholipids in the lipid extract were separated by two-dimensional thin-layer chromatography, in the first direction with chloroform–methanol–25% ammonia (65:35:5 v/v) and in the second direction with chloroform–acetone–methanol–acetic acid–water (50:20:10:10:5 v/v). Spots of the different phospholipids were located by exposing the plates to iodine vapor, marking the spots with pins and scraping them off the plates. Phospholipids were extracted from the spots with chloroform–methanol (1:4) and dried under a stream of nitrogen. Phospholipid content was determined by assaying the amount of phosphorus in the phospholipid extract [1, 15].

Measurement of cell dry weight

Yeast cell culture (5 ml) was harvested by centrifugation and washed two times with deionized water, then dried at 100°C until constant weight was established.

Determination of intracellular maltase activity

Intracellular maltase activity was determined in situ by following the conversion of *para*-nitrophenyl glucoside (PNPG) to paranitrophenol (PNP) at 410 nm. Briefly, pelleted cells were permeabilized by freezing in liquid nitrogen for 30 s and thawing at 37°C. The reaction mixture containing 50 μl of the permeabilized cells suspended in 50 mM phosphate buffer pH 6.8 and 1.0 ml of 5.0 mM PNPG in the same buffer was incubated at 30°C. The reaction was stopped by addition of 1.0 ml of 0.1 M Na_2CO_3. After a rapid low-speed centrifugation, the release of *para*-nitrophenol was followed by measuring the absorbance at 410 nm. One unit of maltase

activity was defined as the amount of enzyme that hydrolyzes PNPG to liberate one microgram of PNP per minute at 30°C and pH 6.8. The specific maltase activity was defined as one unit of per gram of cell dry weight [16].

Assay of reducing sugar in the medium

The amount of reducing sugar (glucose) in the medium was determined by the Nelson–Somogyi method [17].

Primers design for cloning of the gene encoding maltase in *S. pombe*

The primers design for cloning of the gene encoding maltase in *S. pombe* was carried out according to the conserved sequence of maltase genes in *Saccharomyces carlsbergensis*, *Hansenula polymorpha*, *Candida albicans* and the complete sequence of genomic DNA in *S. pombe* [18–21].

Determination of *MAL* gene expression by RT-PCR

Five milliliters of the cell culture at 44 h of incubation were collected and washed by centrifugation at 5,000 *g* for 10 min. Total RNA in the yeast cells was extracted by using a Rneasy Mini Kit (purchased from QIAGEN company) according to the procedures offered by the manufacturer. RNA content in the sample was measured at 260 nm. *MAL* gene expression was determined by using the SuperScript™ One-Step System with Platinum®Taq DNA Polymerase kit (Invitrogen). The primers for RT-PCR are: Primer K1 : 5′-ATGAAAGTTGTGCCAAGCGAAAAAA-3′ and Primer K2 : 5′-TTAGAGCTTTAACAAGACTGCTTGA-3′. The conditions for RT-PCR amplification were as follows: initial denaturation at 50°C for 30 min and 94°C for 2 min, denaturation at 94°C for 15 s, annealing temperature at 50°C for 30 s, Extension at 72°C for 1 min, final extension at 72°C for 10 min. The RT-PCR was run for 30 cycles. RT-PCR cycler was GeneAmp® PCR System 2400 made by Perkin-Elmer. The amount of RT-PCR products was estimated by using the Automated Gel Documentation & Analysis System (GeneGenius, USA) according to the methods offered by the manufacturer.

Results and discussion

The effects of varying inositol concentrations on maltase gene expression and cell growth

Mehta et al. [13] reported that maltase in an *std* mutant of *S. pombe* is an external enzyme as the *std* mutation of *S. pombe* resulted in the extracellular hydrolysis of maltose to glucose. In order to determine the *MAL* gene sequence in *S. pombe* used in this study, the gene was cloned using the methods described in Materials and methods, and the sequence (data not shown) was deposited at the NCBI server (Accession number is DQ019991). A search for the secretion leader in the deduced protein sequence of the cloned *MAL* gene in *S. pombe* used in this study, using the methods described in the SignalP3.0 Server (http://www.cbs.dtu.dk/services/SignalP) revealed that there is no secretion leader in the deduced protein sequence. Therefore, the maltase in this yeast strain is not a secretable enzyme. Many results from our previous studies [10, 12] have demonstrated that the secreted invertase in this yeast strain is repressed by glucose. However, it is still uncertain if intracellular maltase in *S. pombe* is repressed by glucose.

Since *S. pombe* used in this study has an absolute requirement for inositol, we need to know the effects of different concentrations of inositol on maltase gene expression and cell growth in the synthetic medium. The results in Fig. 1 show that different concentrations of inositol had great influence on cell growth and maltase activity. As inositol concentration in the synthetic medium containing initial 2.0% (w/v) glucose was increased from 0.5 to 12 μg ml^{-1}, cell dry weight increased continuously from 0.52 to 2.60 mg ml^{-1}. However, specific maltase activity increased from 15.4 to 243.3 U g^{-1} as initial inositol concentration was increased from 0.50 to 6 μg ml^{-1}, while specific maltase activity was reduced as initial inositol concentration was increased from 6 to 12 μg ml^{-1}. This means that maltase activity reached the highest level when the synthetic medium contained 6 μg ml^{-1} of inositol. However, secreted invertase in the same strain reaches the highest level when the synthetic medium contains 8 μg ml^{-1} of inositol [11]. From Fig. 1 it also can be seen that the cells could grow in the synthetic medium with 1 μg ml^{-1} of inositol. However, the cells synthesized the lower level of maltase under this condition. Thus, the two concentrations of inositol (6 and 1 μg ml^{-1}) were used for all subsequent studies.

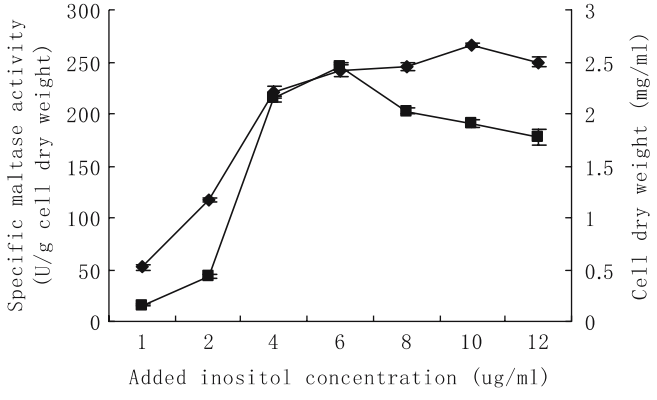

Fig.1 Effects of varying inositol concentrations on maltase activity (*filled square*) and cell growth (*filled triangle*) in the presence of 2.0% glucose

Effects of the two concentrations of inositol on maltase activity and glucose consumption at different concentrations of glucose

In previous studies [8], we found that glucose repression of invertase secretion in *Saccharomyce* sp. W4 occurred at initial glucose concentrations above 2.0% (w/v) when the yeast cells were grown in the inositol-supplemented medium and in *S. pombe*, glucose repression on invertase secretion occurred at initial glucose concentrations above 1.0% when the cells were grown in the synthetic medium plus 8 μg ml^{-1} of inositol [12]. The results in Fig. 2 indicate that glucose repression on maltase activity in *S. pombe* also occurred at initial glucose concentrations above 1.0% (w/v), when the fission yeast cells were grown in the synthetic medium plus 6 μg ml^{-1} of inositol. When the fission yeast cells were grown in the synthetic medium plus 1 μg $ml^{-1}$of inositol, maltase activity decreased continuously as initial glucose concentrations were increased from 0.2 to 5.0% (w/v) (Fig. 2). Furthermore, it can be observed from the results in Fig. 3 that residual reducing sugar in the culture with 1 μg ml^{-1} of inositol was much higher than that in the culture with 6 μg ml^{-1} of inositol, when initial glucose concentration in the medium was higher than 2.0%. However, when the initial glucose concentration in the medium was 1.0%, the residual glucose concentration in the culture with 1 μg ml^{-1} of inositol was almost the same as that in the culture 6 μg ml^{-1} of inositol after cultivation for 44 h under the conditions used in this study. Therefore, these results demonstrate that higher concentrations of inositol added in the medium could also derepress the intracellular maltase in *S. pombe*.

Time course of cultivation of *S. pombe* cultivated on the media containing 6 μg ml^{-1} of inositol and 1 μg $ml^{-1}$of inositol

As shown in Fig. 2, in cells grown in the synthetic medium with both 1.0% glucose and 6 μg ml^{-1} inositol, maltase activity reached the highest level. In contrast, in cells grown in the synthetic medium with 1 μg ml^{-1} of inositol, maltase activity declined continuously as glucose concentrations were increased from 0.2 to 5.0% (w/v). In order to know when maltase activity in *S. pombe* would be derepressed in the medium containing 1.0% (w/v) glucose during the batch growth, cell growth and specific maltase activity were monitored.

Fig. 2 Effects of the two concentrations of inositol on specific maltase activity at different concentrations of glucose: 1 μg ml^{-1} of inositol (*filled triangle*), 6 μg ml^{-1} of inositol (*filled square*)

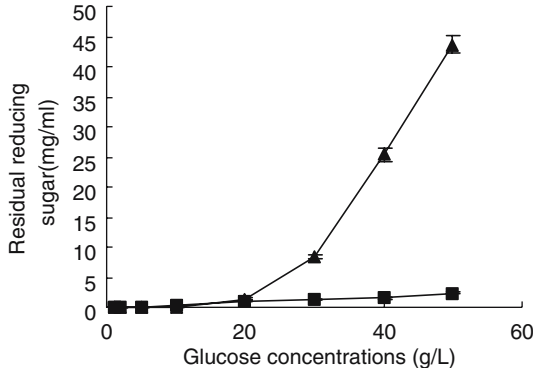

Fig.3 The residual reducing sugar left in cultures at two concentrations of inositol and different glucose concentrations:1 μg ml^{-1} of inositol (*filled triangle*); 6 μg ml^{-1} of inositol(*filled square*)

The results in Figs. 4 and 5 show that specific maltase activity dropped when the washed cells were transferred to the fresh medium with 1.0% (w/v) glucose and 6 μg ml^{-1} of inositol. When the cell growth reached the log phase, maltase activity increased sharply (Fig. 5). After 44 h of cultivation, when the cell growth reached the early stationary phase, the amount of maltase was maximal. However, when the cells were grown in the same medium with 1.0% (w/v) glucose and 1 μg ml^{-1} of inositol, maltase activity decreased steadily even though cell growth increased (Fig. 4 and 5).

Effects of the two concentrations of inositol on phospholipid biosynthesis

Phospholipid biosynthesis in *Saccharomyces* cells can be greatly influenced by the inositol available in the

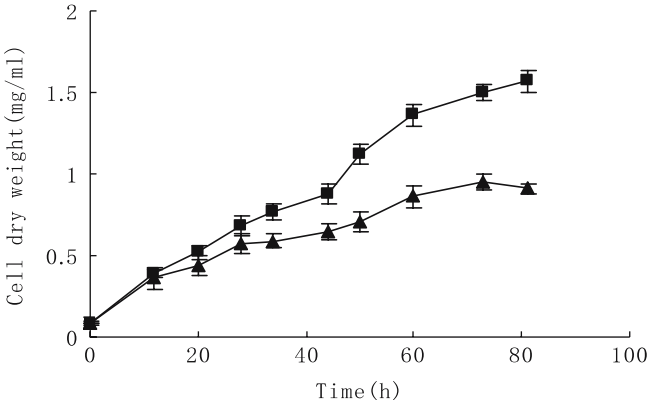

Fig.4 Time course of cell growth of *S. pombe* in the media containing 1 μg ml^{-1} (*filled triangle*) and 6 μg ml^{-1} (*filled square*) of inositol and 1% glucose

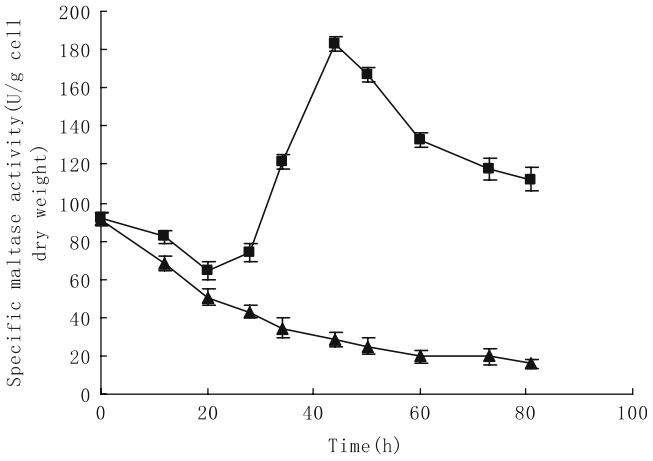

Fig.5 Time course of specific maltase activity of *S. pombe* in the media containing 1 μg ml^{-1} (*filled triangle*) and 6 μg ml^{-1} (*filled square*) of inositol and 1% glucose

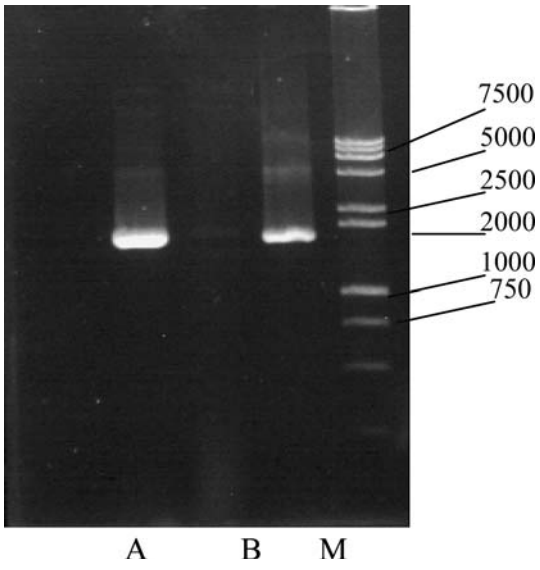

Fig.6 The amount of mRNA encoding maltase at the two concentrations of inositol. *A* 6 μg ml^{-1} of inositol, *B* 1 μg ml^{-1} of inositol, *M* DNA marker

medium. In general, the presence of inositol in the growth medium could cause an increase in PI content and a decrease in PS in this yeast [4, 5]. In order to know if a similar phenomenon happened in the cells of *S. pombe*, the phospholipid composition of *S. pombe* cells was examined in the cultures with inositol concentrations of 1 and 6 μg ml^{-1}, respectively, when the cells were grown in the medium with an initial glucose concentration of 1.0% for 44 h. The results in Table 1 indicate that in cells grown with 6 μg ml^{-1} of inositol and 1.0% (w/v) of glucose for 44 h, PI content was 15.50% of total phospholipids and the PI/PS ratio was 1.98, but PI content was 6.64% of the total phospholipids and the PI/PS ratio was 0.47 in cells grown at 1 μg ml^{-1} of inositol and 1.0% (w/v) of glucose for 44 h, respectively. However, our previous studies [9] showed that in cells of the same strain grown at 8 μg $ml^{-1}$of inositol and 1.0% (w/v) of glucose, PI content is 13.80% of total phospholipids and the PI/PS ratio is 1.27, but PI content is 6.2% of the total phospholipids and the PI/PS ratio is 0.44 in the cells grown at 1 μg ml^{-1} of inositol and 1.0% (w/v) of glucose. Inositol concentration had little effect on the relative amounts of other phospholipids (Table 1). Thus, the presence of inositol in the growth medium could also cause an increase in PI content and a decrease in PS in this yeast. The results in Fig. 2 indicated that intracellular maltase activity in the cells grown at 6 μg ml^{-1} of inositol and 1.0% (w/v) of glucose was much higher than that in the cells grown at 1 μg ml^{-1} of inositol and 1.0% (w/v) of glucose under the same conditions. This means that the maltase activity in the yeast may be related to PI content.

Effects of the two concentrations of inositol on *MAL* gene expression

Experiments were carried out to determine whether higher PI content in the derepressed cells was involved in higher maltase at transcriptional level. Total RNA from the cells grown in the synthetic medium with 1 μg ml^{-1} inositol, and those grown in the same medium with 6 μg ml^{-1} inositol was isolated, and the isolated mRNA was used as templates for RT-PCR amplification of the *MAL* gene by using the primers described in Materials and methods. In Fig. 6, it can be seen that the derepressed cells with higher PI content contained more mRNA encoding maltase than the repressed cells with lower PI content. These results demonstrate that PI is involved in derepression of *MAL* gene expression in this yeast.

To date, we have additional evidence to show that glucose derepression and intracellular maltase gene expression in the fission yeast were also mediated by PI. According to review articles on yeast carbon catabolite repression [6, 7], we believe that an increase in PI content

Table 1 Phospholipid compositions of *Schizosaccharomyces pombe* in the presence of various concentrations of inositol

Inositol (μg ml^{-1})	Percentage of the total phospholipids(%)					
	PI	PS	PC	PE	PA	CL
1	6.64 ± 0.19	14.19 ± 0.43	47.79 ± 1.01	17.19 ± 0.57	2.21 ± 0.16	7.68 ± 0.18
6	15.50 ± 0.3	7.83 ± 0.18	48.10 ± 0.61	1 9.80 ± 1.04	3.10 ± 0.31	5.82 ± 0.38

may finally cause phosphorylation of Mig1 by a PI-type signaling pathway and its translocation to the cytoplasm in *S. pombe* in which the similar glucose catabolite repression occurs. Then, the activators activate transcription of the *MAL* gene and cause an increase in mRNA encoding intracellular maltase even in the presence of a high concentration of glucose (Fig. 6 A). Therefore, glucose derepression in *S. pombe* occurred at glucose concentrations below 1.0% (w/v) when the fission yeast cells were grown in the synthetic medium plus 6 μg ml^{-1} of inositol (Fig. 2), due to the high content of PI in the cells (Table 1). When the yeast cells were grown in the synthetic medium plus 1 μg ml^{-1} of inositol, the decrease in PI content may cause dephosphorylation of Mig 1, and the Mig 1 in the nucleus repressed the transcription of *MAL* gene, so that the decrease in mRNA encoding maltase occurred in the presence of high concentrations of glucose (Fig. 6B). Therefore, specific maltase activity decreased continuously as glucose concentration was increased from 0.2 to 5.0% because of the lower content of PI in the cells (Fig. 2 and Table 1) [7, 11, 22]. But it is still unknown how glucose derepression and gene expression in yeasts are mediated by PI. Therefore, the regulatory mechanisms of gene expression and glucose repression mediated by PI are under investigation in this laboratory.

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