

A lncRNA-regulated gene expression system with rapid induction kinetics in the fission yeast *Schizosaccharomyces pombe*

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ABSTRACT

The fission yeast *Schizosaccharomyces pombe* is an excellent model organism for the study of eukaryotic cellular physiology. The organism is genetically tractable and several tools to study the functions of individual genes are available. One such tool is regulatable gene expression and overproduction of proteins. Limitations of currently available overexpression systems include delay in expression after induction, narrow dynamic range, and system-wide changes due to induction conditions. Here I describe a new long noncoding RNA (lncRNA)-regulated, thiamine-inducible expression system that integrates lncRNA-based transcriptional interference at the fission yeast *tgp1* promoter with the fast repression kinetics of the thiamine-repressible *nmt1* promoter. This hybrid system has rapid induction kinetics, broad dynamic range, and tunable expression via thiamine concentration. The lncRNA-regulated thiamine-inducible system will be advantageous for the study of individual genes and for potential applications in the production of heterologous proteins in fission yeast.

Keywords: regulated expression; thiamine-induced; lncRNA-regulation; rapid induction; tunable expression

INTRODUCTION

Fission yeast *Schizosaccharomyces pombe* is an excellent model system for the study of eukaryotic cell biology (Hoffman et al. 2015; Hayles and Nurse 2018). Fission yeast is also an appealing host for the production of heterologous eukaryotic proteins (Celik and Calik 2012). Regulatable gene expression systems are a major tool in understanding the function of individual genes and their products. Rapid induction kinetics, reproducible and titratable induction levels, and low reagent costs are advantageous features of expression systems for use in basic research and for industrial-scale protein production. Currently, the thiamine-repressible *nmt1* (no message in thiamine) gene promoter is the most widely used regulatable gene expression system in fission yeast (Maundrell 1990; Basi et al. 1993; Forsburg 1993). Upon addition of thiamine, the *nmt1* transcript level declines after 1 h and is undetectable by 3 h. When cells are transferred from thiamine-replete to thiamine-free medium, the expression of *nmt1* mRNA is de-repressed after 14–16 h (Maundrell 1990). The *nmt1* promoter system has been adapted to overexpress genes placed under its control on an extrachromosomal circular DNA (Basi et al. 1993; Forsburg

1993). Despite its popularity, the *nmt1* system has the following limitations: (i) It takes 14–20 h to induce gene expression; (ii) it requires harvesting cells grown in medium with thiamine, conducting extensive washes and resuspending in thiamine-free medium, making it cumbersome for large cultures; and (iii) it has high basal expression in the repressed state when placed on a high-copy plasmid.

Several other regulatable fission yeast expression systems have been developed, variously entailing glucose depletion (*fbp1* or *inv1* promoter) (Hoffman and Winston 1989; Iacovoni et al. 1999), ethanol induction (*gld1* promoter) (Matsuzawa et al. 2013), heat-shock induction (*hsp16* promoter) (Fujita et al. 2006), copper-based regulation (*ctr4* promoter) (Bellemare et al. 2001), tetracycline-induction (tetracycline-regulatable cauliflower mosaic virus 35S promoter) (Faryar and Gatz 1992; Forsburg 1993), uracil-based regulation (*urg1* promoter) (Watt et al. 2008; Watson et al. 2011), estradiol-regulated induction (Ohira et al. 2017), and pheromone induction (*sxa2/rep1* promoters) (Hennig et al. 2018). Many of these methods have limitations such as narrow dynamic range (e.g., tetracycline-

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induction); strain construction (uracil-based regulation); self-limiting gene expression (*inv1* promoter); induction-based growth arrest (pheromone induction); or induction of stress and/or system-wide transcriptional changes (e.g., heat-shock induction, copper chelation, glucose depletion, and ethanol induction) (Chen et al. 2003; Rustici et al. 2007; Vassiliadis et al. 2019). To circumvent the limitations posed by current expression systems, I combined the properties of the thiamine-based *nmt1* promoter repression and regulation of the phosphate homeostasis gene *tgp1* via upstream long noncoding RNA (lncRNA) transcription to build a vector with rapid and reliable kinetics of gene induction.

Fission yeast phosphate homeostasis is achieved by transcriptional control of the *PHO* regulon comprising three genes—*pho1*, *pho84*, and *tgp1*—encoding proteins involved in extracellular phosphate acquisition. Pho1 is a cell surface acid phosphatase, Pho84 is an inorganic phosphate transporter, and Tgp1 is a glycerophosphate transporter (Carter-O’Connell et al. 2012). The expression of these genes is dependent on Pho7, a member of the Zn₂Cys₆ family of transcription factors (Carter-O’Connell et al. 2012; Schwer et al. 2017; Garg et al. 2018a, 2019). In phosphate-rich conditions, the expression of each of these genes is repressed by the transcription of a 5’ adjacent *cis*-acting lncRNA: *prt* for *pho1*, *prt2* for *pho84*, and *nc-tgp1* for *tgp1* (Lee et al. 2013; Ard et al. 2014; Shah et al. 2014; Schwer et al. 2015; Chatterjee et al. 2016; Garg et al. 2018b; Sanchez et al. 2018). The model of repression is that RNA polymerase II transcribing the lncRNA traverses the promoter of the downstream *PHO* gene, thereby ejecting Pho7 from the mRNA promoter and interfering with *PHO* gene expression (Chatterjee et al. 2016; Schwer et al. 2017; Sanchez et al. 2018). The current view of induction in phosphate-starved conditions hypothesizes that transcriptional shut-off of the lncRNA relieves transcriptional interference and thus de-represses the downstream phosphate homeostasis gene.

In this study, I describe a regulatable expression vector wherein the *nmt1* promoter controls the synthesis of the lncRNA *nc-tgp1* upstream of the *tgp1* promoter, which in turn directs the transcription of a gene of interest. The addition of thiamine quickly halts synthesis of the *nc-tgp1* lncRNA, thereby de-repressing the gene of interest. I further describe the tunability of this vector by titration of thiamine concentration as well as an alternative vector that affords a lower target gene expression level.

RESULTS

lncRNA-regulated thiamine-inducible expression system

Of the three phosphate homeostasis genes, *tgp1* is the most tightly repressed in phosphate-rich conditions

(Marguerat et al. 2012). Repression occurs via transcriptional interference with the *tgp1* promoter by synthesis in *cis* of an upstream adjacent *nc-tgp1* lncRNA (Ard et al. 2014; Sanchez et al. 2018). I used this property of the *nc-tgp1-tgp1* gene cassette to engineer a regulatable promoter system on a plasmid.

The design involves placement of the *nmt1* promoter upstream of the *nc-tgp1* lncRNA transcription start site (TSS), which confers thiamine-based control of *nc-tgp1* lncRNA expression (Figs. 1, 2A). Thus, in the absence of thiamine, *nc-tgp1* is at peak expression, thereby repressing the downstream *tgp1* promoter. The addition of thiamine represses lncRNA expression and that in turn de-represses the downstream *tgp1* promoter (Fig. 2A). To increase the repressive effect of *nc-tgp1* transcription, I introduced mutations in a cluster of DSR elements in the *nc-tgp1* lncRNA that are binding sites for Mmi1 (Figs. 1, 2A; Harigaya et al. 2006; Chen et al. 2011; Yamashita et al. 2012; Kilchert et al. 2015; Sanchez et al. 2018). The DSR mutations enhance lncRNA repression of the *tgp1* promoter (Sanchez et al. 2018). The lncRNA-regulated thiamine-inducible expression system will henceforth be called the pTIN system (thiamine inducible) and the base vector will be referred to as pTIN.

To assay the activity of this promoter system, I utilized *pho1* as the reporter gene driven by the *tgp1* promoter (Fig. 2A). Cell surface-associated Pho1 activity is readily assayable colorimetrically via the conversion of *p*-nitrophenylphosphate to *p*-nitrophenol. The *pho1* gene was cloned downstream from the *tgp1* 5’ UTR in the pTIN vector with its native 3’ flanking DNA to yield the pTIN-*pho1* plasmid (Fig. 2A). To test background acid phosphatase levels, a promoter-less pTIN-*pho1* vector was constructed that lacked the DNA segment spanning the regulatory *nmt1*-promoter-*nc-tgp1-tgp1* promoter region as well as the *tgp1* 5’ UTR and only contained the *pho1* ORF. These plasmids were introduced into a strain in which the chromosomal *pho1* and *pho4* genes encoding the cell-surface acid phosphatases Pho1 and Pho4 were deleted. Plasmid-bearing *pho1Δ pho4Δ* cells were grown to mid-log phase in Leu⁻ PMG medium without thiamine and subsequently diluted and grown in Leu⁻ PMG medium with or without 15 μM thiamine. Pho1 acid phosphatase activity was measured by incubating dilutions of cells with *p*-nitrophenylphosphate and measuring its conversion to *p*-nitrophenol for 5 min at 30°C. The amount of product formed (within the linear range of detection) was normalized to cell density and is plotted on the y-axis in Figure 2B. In Leu⁻ PMG medium with or without thiamine, the cells containing promoter-less pTIN-*pho1* had very low acid phosphatase activity (Fig. 2B). Cells bearing the pTIN-*pho1* plasmid had 3.5-fold higher acid phosphatase activity in thiamine-free medium compared to the promoter-less pTIN-*pho1* control. Inclusion of thiamine elicited a 58-fold increase in acid phosphatase activity (Fig. 2B).

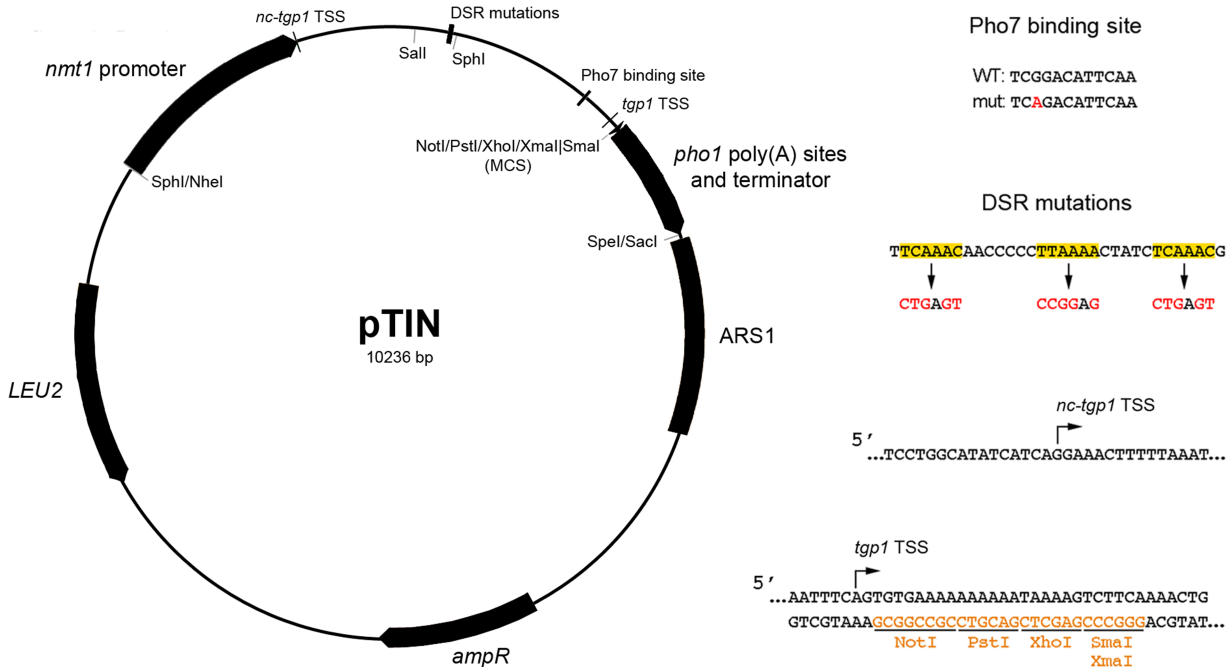


FIGURE 1. pTIN/pTIN7m plasmid map. Features of the pTIN/pTIN7m vector are depicted. The positions of the indicated restriction sites are shown. The DNA sequences of the WT or mutant (mut) Pho7 DNA binding site present in the pTIN or pTIN7m vector, respectively, and the base-specific changes for mutations in the DSR element cluster are indicated on the *top right*. The sequences of the following regions are shown at the *bottom right*—the *nmt1* promoter fused to the *nc-tgp1* lncRNA transcription start site (TSS), and the *tgp1* 5' UTR with the multiple cloning site polylinker (MCS), where the black bent arrow marks the initiating nucleotide of the lncRNA or the mRNA as indicated.

Cultures of *pho1Δ pho4Δ* cells bearing either the pTIN empty vector or pTIN-*pho1* were serially diluted and spotted on Leu⁻ PMG agar medium with or without 15 μM thiamine. After 3 d of incubation at 30°C, there was no difference in growth between cells harboring either the empty pTIN or the pTIN-*pho1* plasmid, as gauged by colony size. Colony sizes were larger on thiamine-containing medium compared to medium lacking thiamine (Fig. 2C), consistent with the observation that thiamine is limiting for *S. pombe* growth (Maundrell 1990). The plates were subsequently processed for agarose overlay acid phosphatase assay. This assay follows the acid phosphatase-catalyzed conversion of α-naphthyl phosphate to 1-naphthol, which in the presence of Fast Blue B salt forms a colored azo-dye, presenting as red coloration around the cells. *pho1Δ pho4Δ* cells bearing the empty pTIN plasmid remained white in the presence or absence of thiamine, indicating low background signal (Fig. 2C). *pho1Δ pho4Δ* cells bearing pTIN-*pho1* were similarly white when grown on medium lacking thiamine but were intensely red when grown on medium with thiamine (Fig. 2C).

Kinetics of thiamine-induced gene expression

I determined Pho1 acid phosphatase activity in *pho1Δ pho4Δ* cells bearing the pTIN-*pho1* plasmid as a function of time after addition of thiamine (Fig. 3A). Pho1 activity in-

creased linearly between 1 and 7 h and continued to increase between 7 and 11 h at 58% of the initial rate. Extending the time course of thiamine induction to 23 h resulted in a 53-fold increase in Pho1 level over the uninduced state (Fig. 3A). To gauge the level of *pho1* mRNA, I performed primer extension analysis using a *pho1*-specific radiolabeled primer for reverse-transcription, in parallel with an actin gene-specific primer as a control. The *pho1* mRNA was induced 25-fold at 30 min after the addition of thiamine and attained a maximum level of 61-fold by 1 h (Fig. 3B). The levels of *pho1* mRNA remained at ~60-fold higher than the uninduced state at all times assayed after 1 h (Fig. 3B; time points from 4 to 9 h not shown). The assessment of the kinetics of gene expression from the pTIN system show that the thiamine-dependent de-repression of the *tgp1* promoter allowed rapid transcriptional up-regulation, where peak levels of the *pho1* mRNA were achieved by 1 h. Continuous translation of the available *pho1* mRNA resulted in the linear accumulation of Pho1 at the cell surface.

Comparison to the thiamine-repressible *nmt1* expression system

To test the differences in the kinetics and absolute levels of induction of the pTIN system with the thiamine-repressed *nmt1* expression system (Forsburg 1993), I measured acid

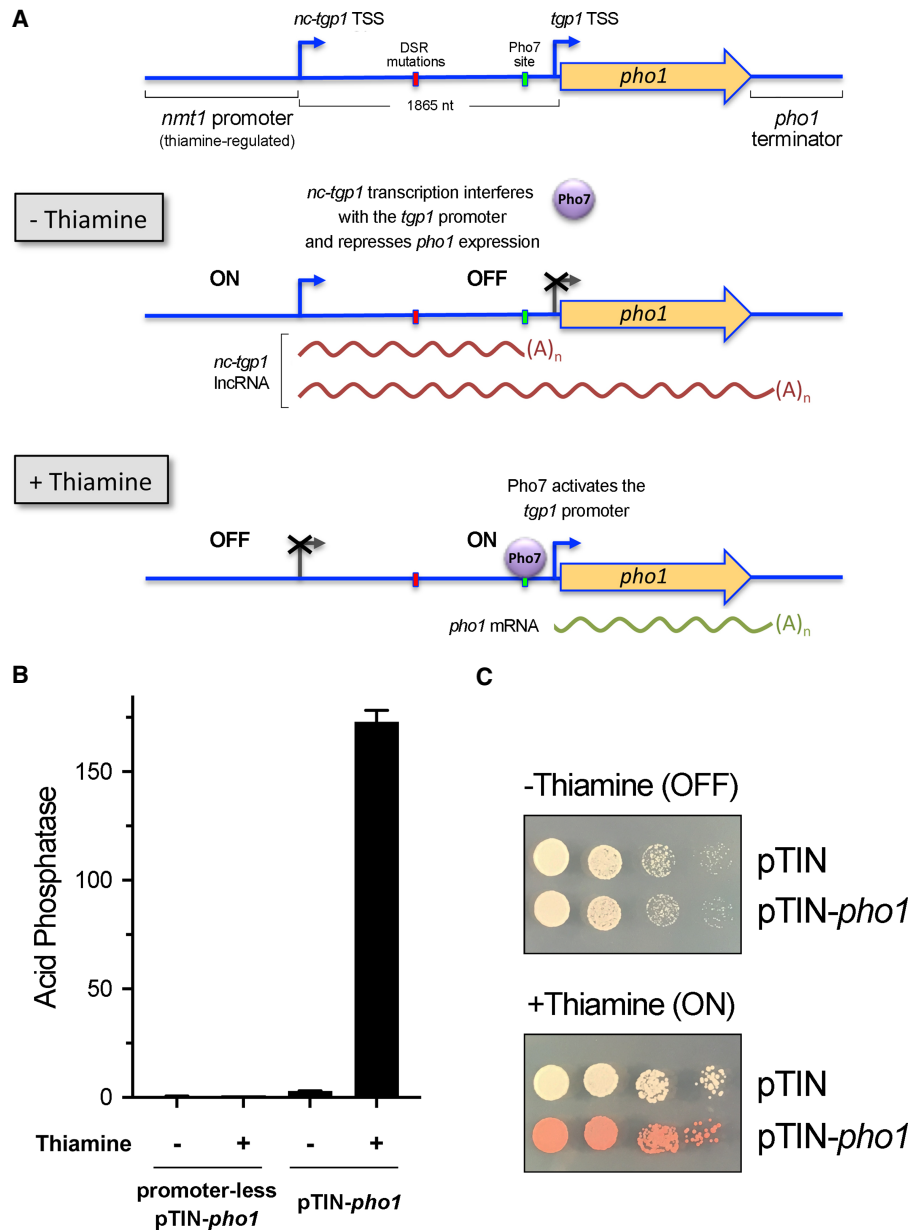


FIGURE 2. The pTIN system. (A) A schematic illustration of the lncRNA-regulated thiamine-inducible system using Pho1 acid phosphatase as a reporter (pTIN-*pho1*) is shown at the top. Transcription start sites are shown as bent arrows. The positions of the *nc-tgp1* TSS, *nc-tgp1* lncRNA DSR mutations (red box), *tgp1* promoter Pho7 DNA binding site (green box), and *tgp1* TSS are indicated. The distance between the *nc-tgp1* TSS and the -1 nt relative to the *tgp1* translation start site is indicated by the bracket. The *pho1* ORF is denoted by a gold horizontal arrow in the direction of mRNA synthesis. The mechanism of the lncRNA-regulated thiamine-inducible system is depicted below, where the thiamine-starved condition ($-$ Thiamine) is the repressed state and thiamine-replete condition ($+$ Thiamine) is the induced state for *pho1* expression. The two predominant *nc-tgp1* lncRNA isoforms are indicated. (B) Single colonies of *pho4Δ pho1Δ* cells transformed with promoter-less pTIN-*pho1* or pTIN-*pho1* were pooled (≥ 20) and grown in Leu⁻ PMG medium. Cells were diluted in Leu⁻ PMG medium without or with 15 μ M thiamine, and acid phosphatase activity was measured after incubation at 30°C for 21 h. The acid phosphatase activity is the average \pm SEM from three independent cultures. (C) A representative culture of *pho4Δ pho1Δ* cells bearing the pTIN empty vector or pTIN-*pho1* were grown in Leu⁻ PMG medium without thiamine at 30°C. The cultures were adjusted to A₆₀₀ of 0.1, and fivefold serial dilutions were spotted on Leu⁻ PMG agar medium without or with 15 μ M thiamine and incubated at 30°C for 3 d. The cells were overlaid with 1% agarose containing 0.015% α -naphthyl phosphate and 0.15% Fast Blue B Salt in 0.1 M sodium acetate (pH 4.2). The plates were photographed after incubation for 2 min at room temperature.

phosphatase activity of *pho1Δ pho4Δ* cells bearing the pTIN-*pho1*, pREP3X-*pho1*, pREP41X-*pho1*, or the pREP81X-*pho1* plasmid as a function of time after induc-

tion (pTIN system: addition of thiamine; pREP-series: withdrawal of thiamine). In repressed conditions, the acid phosphatase activity of cells bearing pTIN-*pho1* was

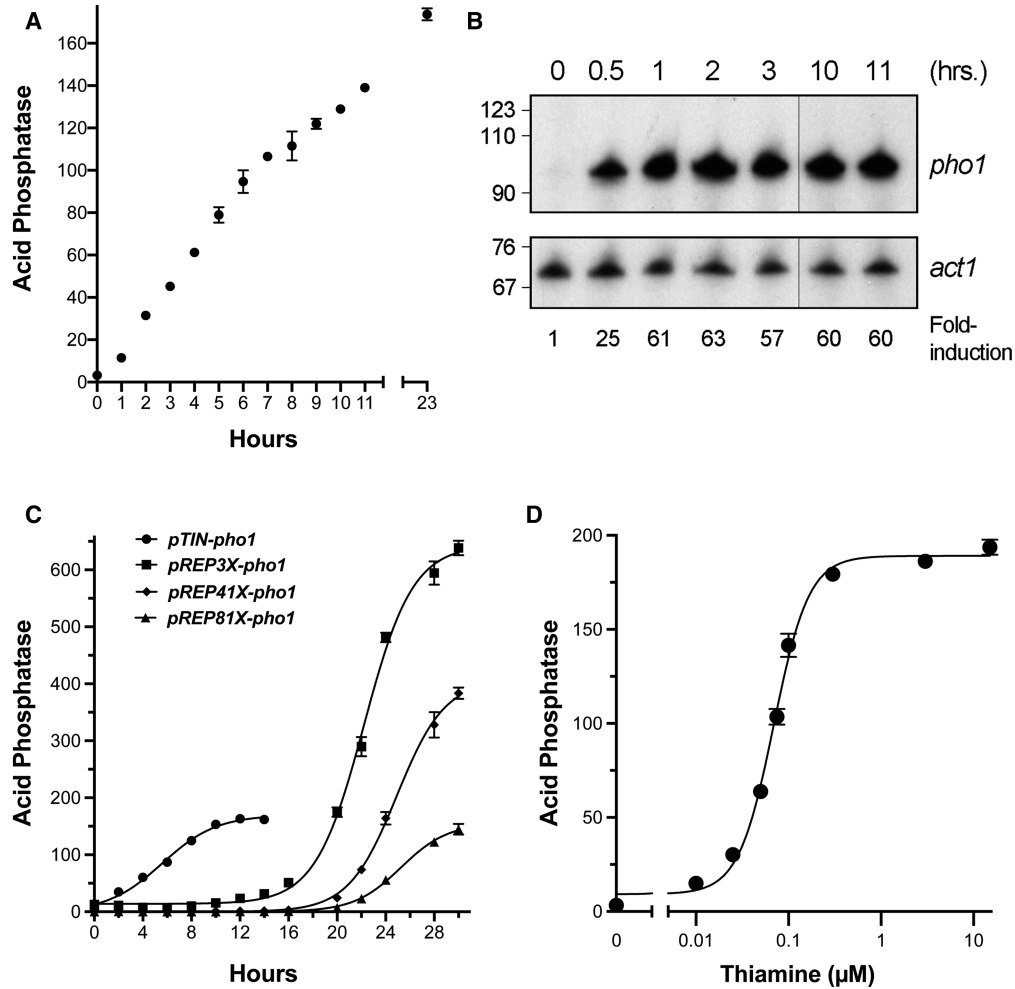


FIGURE 3. Kinetics of induction and titration of expression. (A) Single colonies of *pho4Δ pho1Δ* cells transformed with the pTIN-*pho1* plasmid were pooled (≥ 20) and grown at 30°C in Leu⁻ PMG without thiamine. Acid phosphatase activity was measured from an aliquot of the culture (time-0) and the remaining culture was adjusted to 15 μM thiamine. Acid phosphatase activity was measured at the indicated times after the addition of thiamine. Each data point is the average \pm SEM from three independent cultures. (B) A representative culture of *pho4Δ pho1Δ* cells bearing the pTIN-*pho1* plasmid was grown as described in Figure 3A, and aliquots were harvested before (time-0) or at the indicated times after the addition of thiamine. Total RNA prepared from harvested samples was analyzed by reverse transcription primer extension using a mixture of radiolabeled primers complementary to the *pho1* (top panel) or the *act1* (bottom panel) mRNAs. The reaction products were resolved by denaturing urea-PAGE and visualized by autoradiography. The images shown in the top and bottom panels are from a single exposure of one gel. For conciseness of the figure, the intervening lanes of the gel were cropped (represented by the thin line separating the 3- and 10-h samples). The positions and sizes (in nucleotides) of DNA markers are indicated on the left. Fold-induction reflects the ratio of the *act1*-normalized *pho1* signal at the indicated time to time-0. (C) Single colonies of *pho4Δ pho1Δ* cells transformed with the indicated plasmids were pooled (≥ 20), where the pTIN-*pho1* or the pREP(3 \times ; 41 \times ; 81 \times)-*pho1* bearing cells were grown in Leu⁻ PMG medium lacking thiamine or containing 15 μM thiamine, respectively. Pho1 expression was induced by adjusting thiamine concentration to 15 μM for the pTIN-*pho1* bearing cells or by pelleting cells, washing twice with water and resuspending in Leu⁻ PMG medium lacking thiamine for the pREP-series-*pho1* bearing cells. Acid phosphatase activity was measured in repressed conditions (time-0) and at the indicated times after induction. Each data point is the average \pm SEM from three independent cultures. The data were fit to a sigmoidal model (Boltzmann) in Graphpad Prism with a goodness of fit correlation coefficient of 0.99 for each induction curve. (D) *pho4Δ pho1Δ* cells bearing the pTIN-*pho1* plasmid were propagated at 30°C in Leu⁻ PMG medium lacking thiamine. The cells were then diluted and grown for 20–23 h in Leu⁻ PMG medium with the indicated thiamine concentration. The acid phosphatase activity is the average \pm SEM from three independent cultures. The data were fit to a dose-response model (EC₅₀ shift) in Graphpad Prism with a goodness of fit correlation coefficient of 0.99, Hill slope of 2.29, and dose of half maximal response (EC₅₀) of 0.069 \pm 0.005 μM thiamine.

fourfold lower than pREP3X-*pho1* bearing cells, and fourfold or 15-fold higher than that of the pREP41X-*pho1* or pREP81X-*pho1* bearing cells, respectively (Fig. 3C). The pTIN-*pho1* bearing cells induced Pho1 activity without a detectable lag period compared to the pREP3X-*pho1*,

pREP41X-*pho1*, or pREP81X-*pho1* bearing cells, which induced Pho1 activity after a lag of 14, 16, or 20 h, respectively (Fig. 3C). The data were fit to the Boltzmann sigmoidal model which specified the following times for half maximal induced Pho1 activity—pTIN-*pho1*: 5.6 \pm

0.4 h, pREP3X-*pho1*: 22.2 ± 0.2 h, pREP41X-*pho1*: 24.9 ± 0.3 h, pREP81X-*pho1*: 25.3 ± 0.4 h. The model-derived maximum induced acid phosphatase activity of the pTIN-*pho1* bearing cells was fourfold lower, 2.5-fold lower or equivalent to the pREP3X-*pho1*, pREP41X-*pho1*, or pREP81X-*pho1* bearing cells, respectively.

Effects of thiamine concentration

The levels of expression from the *nmt1* promoter can be titrated by varying the thiamine concentration in the growth medium (Javerzat et al. 1996). To test the effects of thiamine concentration on the pTIN system, I propagated *pho1Δ pho4Δ* cells bearing the pTIN-*pho1* plasmid in Leu⁻ PMG medium either lacking thiamine or containing different concentrations of thiamine. Pho1 activity was induced by 59-fold for cells grown in Leu⁻ PMG medium with 15 μM thiamine compared to medium lacking thiamine. Cells grown in Leu⁻ PMG medium with 0.01, 0.025, 0.05, 0.075, 0.1, 0.3, and 3 μM thiamine had Pho1 activities that were 8%, 16%, 33%, 54%, 73%, 93%, and 96%, respectively, of the level attained with 15 μM thiamine (Fig. 3D). Fitting the data to a dose-response curve indicated that half-maximal induction was attained at 0.069 ± 0.005 μM thiamine and was saturated at ≥ 0.3 μM thiamine. Thus, expression of the target protein can be titrated across a wide range by varying the thiamine concentration in the growth medium.

Effects of Pho7 site mutation on the pTIN system

The *tgp1* promoter has one binding site for the transcription factor Pho7 and retains 15% of its activity in the absence of Pho7 (Schwer et al. 2017). A single nucleotide change of the Pho7 site from 5'-TCGGACATTCAA to 5'-TCAGACATTCAA severely weakens its interaction with the Pho7 DNA binding domain in vitro and reduces in vivo *tgp1* promoter activity to 20% of the wild-type (Garg et al. 2018a).

Here, I introduced this single-nucleotide mutation at the Pho7 site in the *tgp1* promoter of the pTIN plasmid, thereby generating a second-generation vector pTIN7m, into which I cloned the *pho1* reporter gene (Fig. 4A). *pho1Δ pho4Δ* cells bearing the pTIN7m empty vector or pTIN7m-*pho1* plasmids were grown in Leu⁻ PMG medium without or with thiamine and assayed for acid phosphatase activity. As expected, there was negligible Pho1 acid phosphatase generated in cells bearing the pTIN7m vector (Fig. 4B). The key findings were as follows: (i) The uninduced level of Pho1 activity from pTIN7m-*pho1* was 12-fold lower than that derived from the pTIN-*pho1* with an intact Pho7 site; (ii) addition of thiamine resulted in a 172-fold increase in Pho1 activity; and (iii) the level of thiamine induced Pho1 expression from pTIN7m-*pho1* was 24% of that derived from the pTIN-*pho1* (Fig. 4B, com-

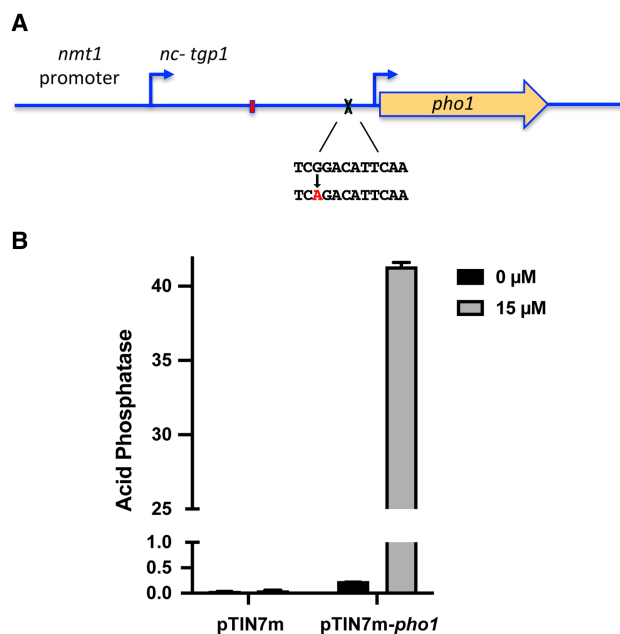


FIGURE 4. Effects of Pho7 site mutation on the pTIN system. (A) In the pTIN7m-*pho1* plasmid, the lncRNA-regulated thiamine-inducible regulatory region with a mutated Pho7 binding site fused to the *pho1* ORF is shown as a schematic. The regulatory region is described in Figure 2A with the following exception: the green box with a black cross represents a mutated Pho7 DNA binding site and the base-specific change is indicated. (B) Single colonies of *pho4Δ pho1Δ* cells transformed with pTIN7m or pTIN7m-*pho1* were pooled (≥ 20) and grown at 30°C in Leu⁻ PMG medium. The cultures were diluted and grown for 22 h in Leu⁻ PMG medium with the indicated thiamine concentration and acid phosphatase activity was measured. Each data point is the average \pm SEM of three independent cultures.

pared to Fig. 2B). These results show that the amplitude of the pTIN system can be dampened without adversely affecting the fold induction in response to thiamine.

pTIN-based overexpression of the mitotic transcription factors Sak1 and Fkh2

I exploited the pTIN system to overexpress the fission yeast transcription factors Sak1 and Fkh2 that regulate the mitotic (M) phase of the fission yeast cell cycle (Garg et al. 2015). Overexpression of Sak1 or Fkh2 by other methods leads to abnormal cell cycle phenotypes and growth inhibition (Buck et al. 2004; Vachon et al. 2013; Garg et al. 2015). Here I find that Sak1 or Fkh2 expression via the pTIN system prevented growth and that this was attributable to their overexpression, insofar as (i) the pTIN empty vector had no effect on growth; and (ii) growth inhibition was seen in the presence of thiamine when the lncRNA-regulated *tgp1* promoter was on, but not in the absence of thiamine when the lncRNA-regulated *tgp1* promoter was off (Fig. 5A). Note that cells bearing pTIN-*sak1* grew more slowly than cells with the pTIN empty vector as gauged by colony size (discussed below).

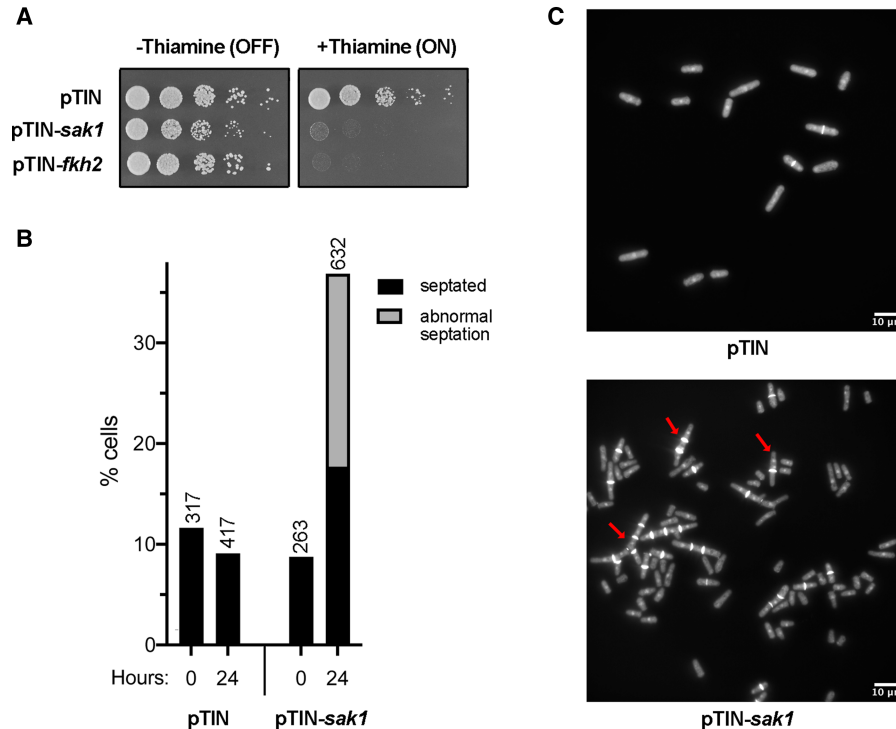


FIGURE 5. Overexpression of the mitotic transcription factors Sak1 and Fkh2. (A) Single transformants of WT cells bearing the pTIN, pTIN-*sak1*, or pTIN-*fkh2* plasmids were propagated in Leu⁻ PMG medium without thiamine. Serial dilutions of cells were spotted on Leu⁻ PMG medium with or without 15 μ M thiamine and were incubated at 30°C for 3 or 4 d, respectively. (B) A representative culture of WT cells bearing either the pTIN empty vector or pTIN-*sak1* plasmid were grown at 30°C in Leu⁻ PMG medium lacking thiamine. An aliquot of the cultures was fixed (time 0) and the remaining volume of cultures was adjusted to 15 μ M thiamine. The cultures were incubated at 30°C and an aliquot of each culture was fixed after 24 h. Fixed samples were processed for DAPI and Calcofluor White staining and the proportion of septated cells was quantified by fluorescence microscopy. The total number of cells counted for each sample is indicated above the bar. The Z-test two tailed *P*-values comparing the proportion of total septated cells of pTIN and pTIN-*sak1* samples are 0 h: 0.30; 24 h: 9.18×10^{-24} . (C) Fluorescence images (DAPI and Calcofluor) of cells bearing the indicated plasmids (described in Fig. 5B) under de-repressed (+thiamine) conditions at 24 h. Red arrows indicate examples of cells with aberrant mitosis.

It has been shown that Sak1 overexpressed from the *nmt1* promoter system advances mitosis leading to a higher septation index (proportion of cells with a single medial septum) and shorter cell lengths at septation (Garg et al. 2015). To observe phenotypes of overexpressing Sak1 from the pTIN system, cells bearing either the pTIN or pTIN-*sak1* plasmid were visualized by fluorescent microscopy staining the nucleus with DAPI and the septum with Calcofluor in the uninduced state and at 24 h after thiamine induction (Fig. 5B,C). In noninducing conditions, pTIN-*sak1* bearing cells had a septation index similar to the pTIN vector control (Fig. 5B), but had an average cell length at septation that was shorter by $1.28 \pm 0.40 \mu$ m (two-tailed t-test *P*-value: 0.002). Thus the slower growth rate of the pTIN-*sak1* bearing cells in the absence of thiamine may be attributable to some degree of mitotic advancement by basal Sak1 expression. Inducing Sak1 expression led to an increase in the septation index and abnormal septation (percentage of septated cells that had two or more septa, missegregated nuclei and/or were misshaped) (Fig. 5B,C). The septation index of con-

trol cells bearing the pTIN empty vector was unaffected by thiamine (Fig. 5B,C). Thus, in accordance with prior observations, Sak1 overexpression-based increase in the septation index and abnormal septation indicated cell cycle defects.

DISCUSSION

The present study describes the design and implementation of a lncRNA-regulated thiamine-inducible expression system in the fission yeast *Schizosaccharomyces pombe*. This system has several advantages. Rapid kinetics allow peak target mRNA levels to accumulate by 1 h after induction (Fig. 3B) and makes it possible to follow the effects of overproducing a gene product within one generation time of the fission yeast life cycle. The target protein is translated continuously and accumulates progressively after the induced steady-state mRNA level is achieved (Fig. 3A,C). The pTIN system has a broad dynamic range, seen as a 60-fold transcriptional induction when fully de-repressed by the addition of thiamine (Figs. 2B, 3B). The level of

protein expression can be tuned by varying the thiamine concentration (Fig. 3D). The pTIN system has a clear advantage of induction kinetics over the *nmt1*-based expression system as evidenced by the induction of half-maximal target protein levels 16.6 h, 19.3 h, or 19.7 h earlier than the pREP3X, pREP41X, or pREP81X expression vectors, respectively (Fig. 3C). Induction by simple addition of thiamine to the medium is more convenient than the standard *nmt1* promoter-driven overexpression system, which necessitates harvesting, washing, and resuspending cells in thiamine-free medium.

The pTIN system is modular, that is, the *nmt1* promoter guides the expression of a repressive lncRNA that in turn controls the expression of the target gene. In principle, any one component may be changed to influence the expression level of the system; for example, the pTIN7m vector has a Pho7 binding site mutation in the *tgp1* promoter, the effect of which is to reduce basal expression in the absence of thiamine and dampen the extent of protein overproduction in the presence of thiamine. This variant has potential applications in cases where tighter repression is called for, for example, when working with endogenous or heterologous proteins that are toxic at high gene dosage (i.e., when expressed from multicopy fission yeast plasmids). Importing the pTIN system as a single integrated copy on the chromosome may also control gene dosage.

The pTIN system also has potential industrial applications. *S. pombe* has considerable advantages as a host for the industrial-scale production of eukaryotic proteins related to transactions conserved in higher eukarya (RNA splicing, RNA interference pathway, etc.) and advantages in glycosylation of proteins (Celik and Calik 2012; Gunduz Ergun et al. 2019). The pTIN system will allow convenient usage of a regulatable expression vector, where fermentation scale cultures may be grown in noninducing conditions and gene expression turned on by the addition of thiamine. The use of thiamine as an inducer has clear advantages due to its availability and low cost whereby 100 g of thiamine hydrochloride suffices for induction of 20,000 liters of fission yeast culture.

MATERIALS AND METHODS

Generation of pTIN system vectors

The thiamine-inducible vector (pTIN) consists of four elements in series from 5' to 3' (Fig. 1): (i) the *nmt1* promoter, comprising a 1108-bp segment of genomic DNA consisting of nucleotides -1177 to -70 relative to the +1 *nmt1*⁺ translation start site; (ii) *nc-tgp1* and the *tgp1* promoter, a 1865-bp genomic DNA segment from the transcription start site of *nc-tgp1* lncRNA to the -1 nt relative to the *tgp1*⁺ translation start site; mutations of a cluster of DSR (determinant of selective removal) elements were included as shown in Figure 1; (iii) a poly-linker consisting of the

restriction sites 5'-NotI, PstI, XhoI, and SmaI/XmaI; and (iv) a 647-bp segment of genomic DNA 3' of the *pho1*⁺ stop codon consisting of poly(A) site(s) and a transcription terminator followed by an SmaI restriction site. The SacI-SphI *LEU2*-containing DNA fragment of pREP3X served as the plasmid backbone (Forsburg 1993). Two-stage overlap-extension PCR was used to fuse the *nmt1*⁺ promoter to the *nc-tgp1* transcription start site. To generate pTIN7m, the alternative expression vector with lower target gene expression, the Pho7 binding site of the pTIN vector was mutated from 5'-TCGGACATTCAA to 5'-TCAGACATTCAA (Fig. 1). A segment of genomic DNA starting at the +1 position of the *pho1* open reading frame (ORF) to 647-bp 3' of the *pho1*⁺ stop codon that had the NotI and SmaI sites upstream and downstream from this region, respectively, was cloned between the aforementioned sites in the pTIN or pTIN7m vectors. The *fkh2* and *sak1* ORFs were PCR amplified from *S. pombe* cDNA so as to introduce NotI and XhoI sites immediately upstream and downstream from the translation start and stop codon, respectively, and subsequently cloned in the pTIN vector using the aforementioned sites. The promoter-less pTIN-*pho1* was generated by digesting the pTIN-*pho1* vector with NheI (restriction site 5' of the *nmt1* promoter; Fig. 1) and NotI, followed by fill-in of overhangs with DNA Polymerase I Klenow fragment (NEB) and blunt-end ligation of the vector backbone. All plasmid constructs were sequenced to exclude unwanted mutations.

Generation of the pREP series *pho1* reporter constructs

The *pho1* ORF was PCR amplified from *S. pombe* genomic DNA so as to introduce XhoI and XmaI sites immediately upstream and downstream from the translation start and stop codon, respectively, and subsequently cloned in pREP3X, pREP41X, or pREP81X vectors (Forsburg 1993) using the aforementioned sites. All plasmid constructs were sequenced to exclude unwanted mutations.

Deletion of *pho4* in [*prt2-pho84-prt-pho1*] Δ cells

pho4 DNA segments [-452 to -1 and +1845 to +2315 relative to the *pho4*⁺ translational start codon (+1)], were PCR amplified and cloned upstream and downstream, respectively, of the *natMX* antibiotic resistance cassette in a bacterial plasmid. The linear *pho4::natMX* gene disruption cassette was excised from the plasmid and transfected into haploid *S. pombe* [*prt2-pho84-prt-pho1*] Δ cells (Garg et al. 2018b). Nourseothricin-resistant transformants were selected and analyzed by Southern blotting to confirm correct integration at the *pho4* locus.

Plasmid transformation

Plasmids were transfected as specified by the lithium acetate method (Forsburg and Rhind 2006) into WT (*leu1-32 ura4-D18 ade6-M216 his3-D1*) or *pho4* Δ *pho1* Δ (*pho4::natMX* [*prt2-pho84-prt-pho1*]:*hygMX* *leu1-32 ura4-D18 ade6[M210 or M216] his3-D1*) cells cultured in thiamine-free PMG (pombe minimal glutamate) medium, pH 5.6, supplemented with 250 mg/L each of adenine, uracil, leucine, histidine, and lysine. Note that the plasmids must be transfected in cells cultivated in thiamine-

free medium to prevent any de-repression of the target gene post-transformation due to residual intracellular thiamine. Transformants were selected on Leu⁻ PMG agar medium at 30°C.

Acid phosphatase activity assay

Aliquots of exponentially growing cultures were harvested, washed with water, and resuspended in water. To quantify acid phosphatase activity, reaction mixtures (200 µL) containing 100 mM sodium acetate (pH 4.2), 10 mM *p*-nitrophenyl phosphate (NEB), and cells (ranging from 0.001 to 0.1 A₆₀₀ units) were incubated for 5 min at 30°C. The reactions were quenched by addition of 1 mL of 1 M sodium carbonate; the cells were removed by centrifugation and the absorbance of the supernatant was measured at 410 nm. Acid phosphatase activity is expressed as the ratio of A₄₁₀ (*p*-nitrophenol production) to A₆₀₀ (cells). The data are averages (± SEM) of at least three assays using cells from three independent cultures.

RNA analysis

Total RNA was extracted via the hot phenol method (Köhler and Domdey 1991) from three A₆₀₀ units of harvested yeast cells. For analysis of specific transcripts by primer extension, aliquots (5 µg) of total RNA were templated for M-MuLV reverse transcriptase-catalyzed extensions of 5' ³²P-labeled oligodeoxynucleotide primers complementary to the *pho1* and *act1* mRNAs in one reaction mix. The primer sequence for *act1* was 5'-GATTTCTTCTCC ATGGTCTTGTC, and for *pho1*, 5'-GTTGGCACAAACGACG GCC. The primer extension reactions were performed as described previously (Schwer et al. 1998), and the products were analyzed by electrophoresis of the reaction mixtures through a 22-cm 8% polyacrylamide gel containing 7 M urea in 80 mM Tris-borate, 1.2 mM EDTA. The ³²P-labeled primer extension products were visualized by autoradiography of the dried gel. Quantification of *pho1* and *act1* mRNA levels was done by scanning the dried gel with a phosphorimager and analyzing the data in ImageQuant software.

Fluorescence microscopy

Aliquots of cultured cells were fixed in 70% ethanol as described in the text. Fixed cells were harvested, washed twice with water, and resuspended in water. Equal volumes of the cell suspension and mounting solution [75 µg/mL DAPI (4',6-diamidino-2-phenylindole dihydrochloride; Sigma 32670); 4.6 mg/mL Calcofluor White (Sigma F3543) in mounting medium (Vectashield H-1000)] were mixed and incubated for 5 min followed by slide preparation. Cells were observed by fluorescent microscopy and 30–60 independent successive frames were imaged and subsequently counted in ImageJ.

DATA DEPOSITION

The DNA sequence of the pTIN plasmid utilized in this publication has been deposited in NCBI's GenBank database and is accessible through GenBank accession number MT740318 (<https://www.ncbi.nlm.nih.gov/nuccore/MT740318>).

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