RESEARCH ARTICLE

De novo generation of a phosphate starvation-regulated promoter in *Candida glabrata*

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Abstract

What steps are required for a promoter to acquire regulation by an environmental condition? We address this question by examining a promoter in *Candida glabrata* that is regulated by phosphate starvation and the transcription factor Pho4. The gene *PMU2* encodes a secreted acid phosphatase that resulted from gene duplication events not present in other *Ascomycetes*, and only this gene of the three paralogs has acquired Pho4 regulation. We observe that the *PMU2* promoter from *C. glabrata* is not functional in *Saccharomyces cerevisiae*, which is surprising because it is regulated by Pho4, and Pho4 is regulated in a similar manner in both species – through phosphorylation and localization. Additionally, we determine that phosphate starvation-regulated promoters in *C. glabrata* do not require the coactivator Pho2, which is essential to the phosphate starvation response in *S. cerevisiae*. We define a region of the *PMU2* promoter that is important for Pho4 regulation, and this promoter region does not contain the canonical CACGTX sequence that *Sc*Pho4 utilizes for phosphate starvation-dependent transcription. However, *Cg*Pho4 utilizes CACGTX in the *CgPHO84* promoter, as mutation of this sequence decreases transcription. We conclude that the acquisition of *PMU2* has expanded the binding specificity of *Cg*Pho4 relative to *Sc*Pho4.

Introduction

Promoter structure in closely related eukaryotes has been addressed in many bioinformatics and genomic approaches (Gasch *et al.*, 2004; Ihmels *et al.*, 2005; Wohlbach *et al.*, 2009). For example, the transition in *Ascomycetes* from using Rap1 as the main transcription factor required for ribosomal protein gene expression in *Saccharomyces cerevisiae* demonstrates that over evolutionary time, there can be numerous *cis* and *trans* changes (Tanay *et al.*, 2005). Transitions are important as they allow for redundancy and exploration of different transcription factor combinations (Kellis *et al.*, 2004). Promoter structure requires specific nucleotide sequences (*cis*) that are recognized by protein-binding (*trans*) factors. Additionally, the interaction of *cis* and *trans* factors with nucleosome positioning is an important determinant of promoter activity (Segal *et al.*, 2006). We addressed the question of how a regulated promoter acquires sequences that bind appropriate transcription factors, and how this behavior evolved from an ancestral promoter that was not regulated. The promoter of the *PMU2* gene in *C. glabrata* is an example of a gene that initially was basally expressed, but acquired regulation by phosphate starvation (Orkwis *et al.*, 2010).

PMU2 was identified as a *C. glabrata* gene that rescued the phosphatase deficiency of a *pho5*Δ strain in *S. cerevisiae* (Orkwis *et al.*, 2010). *PHO5* encodes a well-studied acid phosphatase that is highly induced during phosphate starvation and is regulated by the transcription factor *Sc*Pho4 (Lau *et al.*, 1998; Haswell & O'Shea, 1999; Zhou & O'Shea, 2011). *PMU2* also encodes an acid phosphatase and is regulated by Pho4, but Pmu2 shares no sequence similarity with Pho5; thus, the two are analogs of one another. Further studies of *PMU2* indicated that it was likely a consequence of small-scale gene duplication in *C. glabrata* and in no other *Ascomycetes*, resulting in three copies (*PMU1*, *PMU2*, and *PMU3*). *PMU2* neofunctionalized into a broad-specificity phosphate starvation-regulated acid phosphatase, while the other copies have narrower specificity and are not regulated by phosphate starvation (Orkwis *et al.*, 2010). The rate of nucleotide substitution in the

promoter is much higher than in the ORF, suggesting selective pressure to maintain all three ORFs functional. The three ORFs are approximately 70% identical to one another and encode phosphatase activity; however, Pmu2 has 20 × the V_{max} of the other two proteins against p-nitrophenylphosphate (Orkwis *et al.*, 2010). Additionally, only the promoter of *PMU2* is regulated by extracellular phosphate concentrations and by *Cg*Pho4.

*Cg*Pho4 is a transcription factor that is 53% identical to the well-characterized *Sc*Pho4 (Kerwin & Wykoff, 2009; Zhou & O'Shea, 2011). However, almost all of this identity is in the basic-helix-loop-helix domain, which is required for dimerization and DNA binding (Shimizu *et al.*, 1997; Komeili & O'Shea, 1999). Recent studies have elucidated the requirements for *Sc*Pho4 binding in the genome of *S. cerevisiae* (Zhou & O'Shea, 2011). There are multiple requirements for binding, including the presence of a CACGTX binding site, a Pho2 binding site nearby, and at least one accessible CACGTX motif that is not occluded by a nucleosome (Zhou & O'Shea, 2011). Whereas *Cg*Pho4 is required for the induction of a number of phosphate starvation-regulated genes, there are significant differences, including the lack of CACGTX motifs in many Pho4-regulated promoters and the apparent lack of a requirement of Pho2. *Cg*Pho4 is almost twice the size of *Sc*Pho4, and this increased size correlates with Pho2 independence in a number of Ascomycete species (Kerwin & Wykoff, 2009). Previous work has demonstrated that both species have a similar genetic cascade that regulates Pho4. The cyclin/cyclin-dependent kinase Pho80/Pho85 negatively regulates Pho4 through demonstrated phosphorylation of *Sc*Pho4 and hypothesized phosphorylation of *Cg*Pho4 (O'Neill *et al.*, 1996; Komeili & O'Shea, 1999; Kerwin & Wykoff, 2009). In *S. cerevisiae*, Pho81 binds to the cyclin/CDK complex regardless of external phosphate concentration and inhibits the kinase activity during phosphate starvation through the binding of a small metabolite IP_7. (Lee *et al.*, 2007, 2008).

Given the similarities and differences between the requirements for a transcriptional induction of genes during phosphate starvation in the two relatively closely related yeasts, we investigated the *cis* and *trans* requirements for the regulation of a newly formed Pho4-regulated promoter, *PMU2*, with the goal of understanding the changes that converted a basal promoter into a phosphate starvation-regulated promoter.

Materials and methods

Yeast strains and growth conditions

Yeast strains used in this study are listed in Supporting Information, Table S1. Mutants were generated using antibiotic resistance genes *KANMX6* and *NATMX6* or the gene *URA3* and homologous recombination to precisely delete ORFs (Kerwin & Wykoff, 2009). Deletion was confirmed by PCR and a phosphatase plate assay of multiple isolates. Table S2 contains all of the primers used to generate strains.

Yeast strains were grown in YEPD medium or synthetic dextrose (SD) medium with complete supplement mixture (CSM), either with or without histidine (Kerwin & Wykoff, 2009; Sunrise Science Products; San Diego). For induction of phosphate starvation genes and promoters, strains were grown at 30° to logarithmic growth phase (OD_{600} *c.* 0.2–0.5) in SD medium with 10 mM phosphate (KH_2PO_4). Cells were harvested by centrifugation, washed three times with medium lacking phosphate, and transferred to SD medium without phosphate (no phosphate conditions) or to SD medium with 10 mM KH_2PO_4 added (high phosphate conditions). Strains were grown in these conditions for 3 h (for quantitative reverse-transcription PCR analysis) or 4 h [for fluorescence-activated cell sorting (FACS) analysis and microscopy].

Plasmid construction and integration into the genome

To construct plasmids containing genes, the promoter and the ORF of the gene were amplified (primers in Table S2) and cloned by homologous recombination into a pRS313 (*HIS3*) vector (Sikorski & Hieter, 1989). The genomic clone of *PMU2* from the *C. glabrata* genomic library contained 3.6 kb of the promoter with the ORF. The *Cg*Pho4^{nophos} plasmid was purchased from GENEWIZ (in pUC57). All of the serine–proline (SP) and threonine–proline (TP) sites in *Cg*Pho4 were mutated to alanine–proline (AP) sites, creating a protein that cannot be phosphorylated by the Pho80–Pho85 complex. The *Cg*Pho4^{nophos} ORF in pUC57 was then amplified and cloned by homologous recombination into a pRS313-myc vector (Wykoff & O'Shea, 2005). To examine localization of *Cg*Pho4, a *Cg*Pho4-yellow fluorescent protein (YFP) C-terminal fusion protein was made by amplifying *YFP::KANMX6* from a plasmid and integrating the product into the genome, selecting for G-418 resistance (Longtine *et al.*, 1998).

To replace *ScPHO4* with *CgPHO4*, the *ScPHO4* ORF was deleted with *ScURA3*, and inactivation was confirmed by PCR and a semi-quantitative phosphatase plate assay. *CgPHO4* was then amplified and integrated into this *URA3* locus using 5-FOA selection, generating a strain in which *S. cerevisiae* contains *Cg*Pho4. Only the ORF was precisely replaced, and there were no other changes to the genome.

To construct truncated *CgPMU2* and *CgPHO84* promoters driving expression of YFP in a plasmid (*CgPMU2*pr-YFP and *CgPHO84*pr-YFP plasmids), varying lengths of the promoter upstream of the ATG start codon (100- and 20-bp increments) were amplified and cloned by homologous recombination into pRS313 with vYFP in the polylinker (Sikorski & Hieter, 1989; Orkwis *et al.*, 2010). For the mutated Pho4-binding site in the *CgPHO84* promoter, fusion PCR was used to mutate the CACGTG to GAGCTC in a 400-bp promoter construct and confirmed by sequencing. For the mutated CCCGTG site in the *CgPMU2* promoter, fusion PCR was used to delete GTG in a 1-kb promoter construct and confirmed by sequencing. These plasmids were then used as a template to amplify the promoter-YFP to integrate into the *CgURA3* locus of a *C. glabrata* wild-type strain using 5-FOA selection. This genome integration was performed to prevent plasmid copy number effects.

Assay for phosphatase activity

For a semi-quantitative assay of phosphatase activity, agar plates with colonies were overlaid with Fast Blue Salt B stain, 1-naphthyl phosphate (1-NP), and 0.1 M sodium acetate (pH 4.2; Wykoff *et al.*, 2007). This assay causes a colony to turn red (dark color on plate) when there is phosphatase activity and to remain white when there is no activity.

Quantitative reverse-transcription PCR

RNA was extracted using a standard phenol–chloroform protocol (Huang & O'Shea, 2005) and converted to cDNA by a reverse-transcription reaction (Bio-Rad iScript cDNA synthesis kit). Quantitative PCR was performed with a Chromo-4 PCR machine (Bio-Rad) using SyberGreen I dye in a 50-μL reaction. Primers were designed for phosphate starvation genes *CgPHO84*, *CgPMU2*, *ScPHO84*, *ScPHO5*, as well as for *CgACT1* and *ScACT1* (Table S2). The amount of transcript for each gene was normalized to *ACT1* because the expression of *ACT1* does not change in response to phosphate starvation (Kerwin & Wykoff, 2009). Each gene was equally amplified using 10-fold genomic DNA dilutions as an amplification control.

For Fig. 1, quantitative PCR was performed on native genes, such as *PHO84*, in each strain to confirm induction during growth conditions (data not shown). We also confirmed that the differences in behavior of the *PMU2* and *PHO5* promoters were a consequence of the promoter and not the individual ORFs by placing the promoters under the control of YFP and noting identical expression patterns to the native ORF (data not shown).

Microscopy

Strains were phosphate-starved as previously described, and cells were concentrated by centrifugation. YFP was visualized using a Leica laser microdissection microscope with a YFP-specific filter and exposure of 2 s. Contrast was adjusted equally for each strain in both growth conditions using Adobe Photoshop CS5. Nuclear staining was performed by incubating phosphate-starved cells fixed in 1% fresh formaldehyde overnight with 0.5 mg mL^{-1} zymolyase and 0.5 ng mL^{-1} DAPI in 1.2 M sorbitol to minimize background fluorescence. Cells were pelleted the next day and resuspended in sorbitol and imaged after 15 min. Nuclei were imaged with a 50-ms exposure using a DAPI-specific filter.

Fluorescence-activated cell sorting

Strains were phosphate-starved as previously described, but at a low cell density ($< 10^5$ cells mL^{-1}). Cells were fixed in 1% formaldehyde. Ten thousand cells were counted, and the mean fluorescence was determined. Average auto-fluorescence (*C. glabrata* wild-type) was subtracted from the mean for each strain. For *CgPMU2*pr-YFP constructs, each replicate set of data was normalized to 3-kb *CgPMU2*pr-YFP such that this strain was 100% and the average of the normalized replicates was graphed. For *CgPHO84*pr-YFP constructs, the average of the replicates was normalized to 400-bp *CgPHO84*pr-YFP such that this strain was 100% and graphed. Error is standard error of the mean with $n = 3$, unless otherwise noted.

Chromatin immunoprecipitation of *Cg*Pho4-myc and *Sc*Pho4-myc

*Cgpho4*Δ strains containing either *Cg*Pho4-myc or *Sc*Pho4-myc were grown for 3 h in no phosphate medium and fixed with 1% fresh formaldehyde and processed as previously described (Lam *et al.*, 2008). For each experiment, approximately 0.75 mg of protein extract was immunoprecipitated with 8 μL of α-myc antibody (9E10.3; Invitrogen) and protein G dynabeads (Life technologies). Immunoprecipitated DNA was quantified relative to input DNA by qPCR and presented as the % immunoprecipitated relative to input.

Results

The *PMU2* promoter does not function appropriately in *S. cerevisiae*

To characterize phosphate starvation-regulated promoters, we cloned 1 kb of the *PHO5* promoter and the *PMU2*

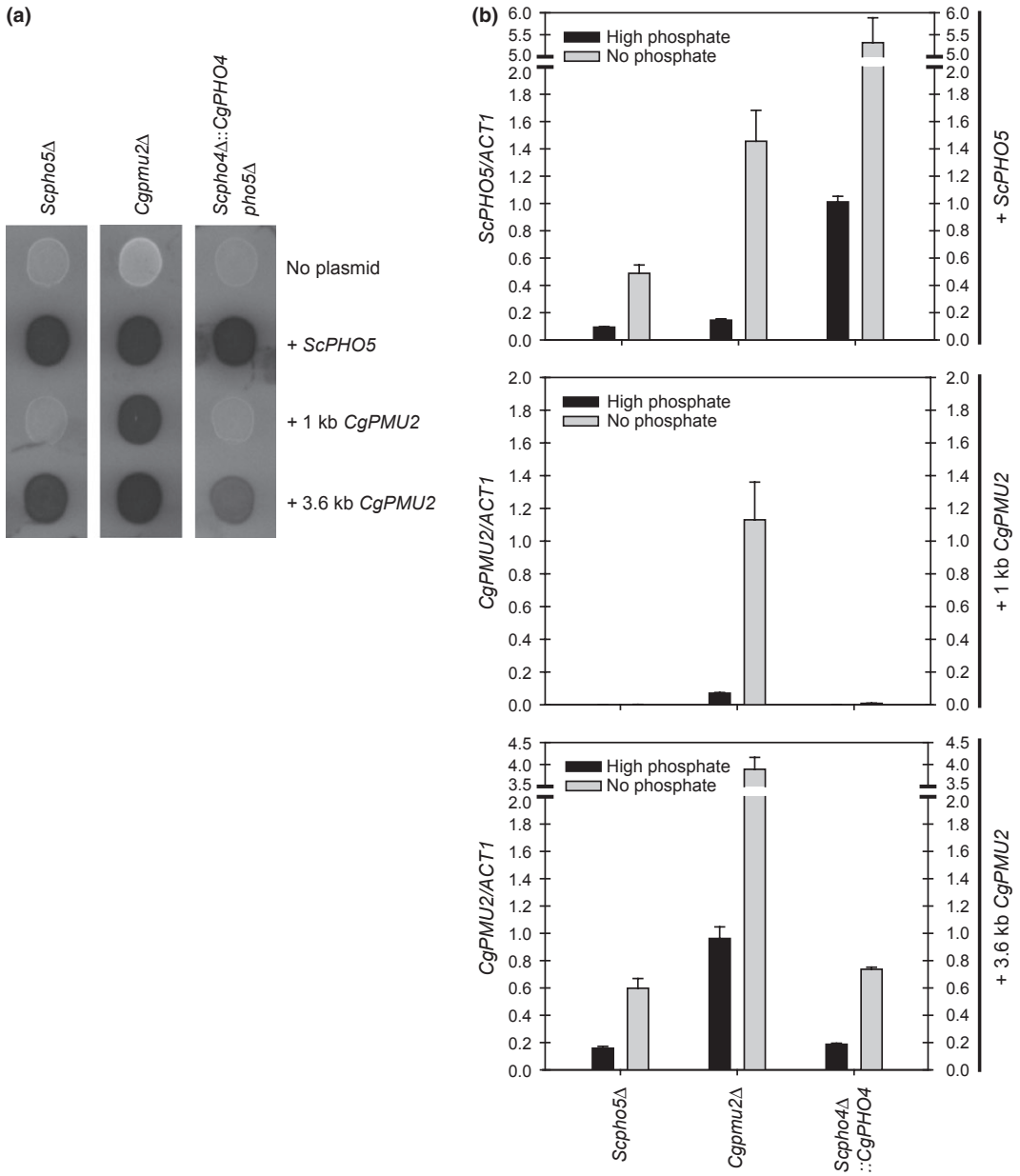

Fig. 1. *CgPMU2* promoter does not function in *Saccharomyces cerevisiae*. (a) Phosphatase plate assay in phosphate starvation conditions of strains containing *ScPHO5* and *CgPMU2*, with either 1 or 3.6 kb (genomic clone) upstream of the *PMU2* ORF. Dark color indicates phosphatase activity. (b) Transcript analysis of strains in (a). Strains are the same for each graph with the exception of *Scpho4Δ::CgPHO4+ ScPHO5*; *ScPHO5* was also deleted in this strain to quantify transcription from the plasmid, and not the native *PHO5*. We confirmed that the observed differences were not a consequence of lack of induction of native genes and that the differences were not dependent on the ORF (see Materials and methods). Data in this figure and all following figures are expressed as mean ± SEM, $n = 3$ for each strain unless noted.

promoter in front of their native ORFs into plasmids and transformed them into both species. We expected that both promoters would be functional and appropriately regulated by external phosphate concentrations in either species. Surprisingly, moving a 1-kb promoter fragment of *PMU2* that was regulated in *C. glabrata* did not function in *S. cerevisiae* (Fig. 1a). This was surprising because *PMU2* was identified as a gene that rescued the *Scpho5*Δ strain, meaning that *PMU2* should be expressed in *S. cerevisiae*. Only clones that contained 3.6 kb of the promoter (genomic clones) were functional in *S. cerevisiae* (Fig. 1a and b); this large promoter contained a CACGTG sequence, and potentially, this is the reason for its ability to be regulated (Lam *et al.*, 2008).

The 1-kb *PMU2* promoter, which was functional in *C. glabrata* and dependent on *Cg*Pho4 (data not shown), does not contain a CACGTX sequence, suggesting that *Cg*Pho4 might have a different binding requirement relative to *Sc*Pho4.

To determine whether the inability of the 1-kb *PMU2* promoter to express during phosphate starvation in *S. cerevisiae* is a consequence of *Sc*Pho4 being unable to bind the 1-kb *PMU2* promoter, we replaced the *ScPHO4* ORF precisely with the *CgPHO4* ORF (Fig. 1a and b). We observed that moving *CgPHO4* was not sufficient to restore expression of the 1-kb *PMU2* promoter, suggesting that *C. glabrata* contains other proteins required for induction of the *PMU2* promoter. Given the lack of a canonical CACGTX site in this 1 kb of promoter, we hypothesized that *Cg*Pho4 is capable of binding non-CACGTX sequences in the *PMU2* promoter possibly with the help of other protein factors in *C. glabrata*. Because a larger form of the *PMU2* promoter (3.6 kb) is functional in *S. cerevisiae*, we conclude that this additional factor is not required when there is a CACGTG present in the promoter.

Similarities between the two species – *Cg*Pho4 is regulated by phosphorylation and localization

Previously, we determined that *Cg*Pho4 appeared to be regulated by the cyclin-dependent kinase complex *Cg*Pho80-*Cg*Pho85-*Cg*Pho81; however, we did not directly demonstrate that this complex regulated *Cg*Pho4 in a manner similar to *Sc*Pho4, that is, phosphorylation (Komeili & O'Shea, 1999; Kerwin & Wykoff, 2009). Pho85 recognizes SP and TP dipeptides in proteins (O'Neill *et al.*, 1996), and *Cg*Pho4 contains 13 such sites. To confirm that *Cg*Pho4 is regulated by phosphorylation, we mutated all SP or TP dipeptides to AP sites (called *Cg*Pho4^{nophos}) and determined the effect of this mutant form of *Cg*Pho4 on the regulation of phosphate starvation genes (Fig. 2). As expected, *Cg*Pho4^{nophos} was de-repressed for expression of these genes in high phosphate conditions. Additionally, placing this mutant in a *Cgpho80*Δ strain did not increase the expression of phosphate starvation genes, indicating that gene activation because of a loss of CDK activity must act through phosphorylation of *Cg*Pho4 (Fig. S1). It is worth noting that *Scpho80*Δ mutants appear hyperinducible in *S. cerevisiae* (Lemire *et al.*, 1985; Huang & O'Shea, 2005) and that mutants that are considered constitutive (i.e. *msn5*Δ and *pho80*Δ) are not as de-repressed in high external phosphate conditions as low phosphate conditions (Kerwin & Wykoff, 2009). While the mechanism for this hyperinducibility is not known, our data with the *pho80*Δ strain are consistent with previous results.

To determine whether *Cg*Pho4 was regulated by localization, we C-terminally tagged *Cg*Pho4 with YFP in the genome and observed localization of *Cg*Pho4-YFP during high and no external phosphate conditions (Fig. 3). As expected, *Cg*Pho4 is regulated by localization in a similar manner as *Sc*Pho4 – that is, Pho4 is nuclear-localized during phosphate starvation and cytoplasmic under

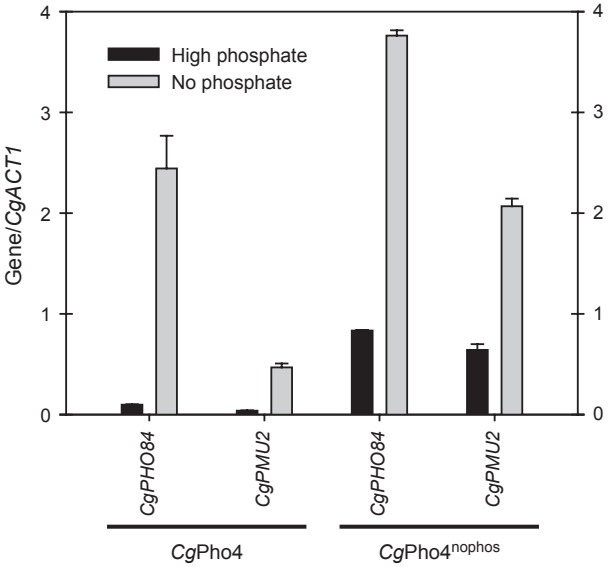

Fig. 2. *Cg*Pho4^{nophos} is de-repressed for *PHO84* and *PMU2* expression in high phosphate conditions, indicating that *Cg*Pho4 is regulated by phosphorylation. All SP and TP sites in *Cg*Pho4 were mutated to AP sites, generating a protein that cannot be phosphorylated by the Pho80-Pho85-Pho81 complex (CgPho4^{nophos}). Wild-type *CgPHO4*- and *CgPHO4*^{nophos}- containing plasmids were transformed into a *Cgpho4Δ*, and quantitative PCR was performed.

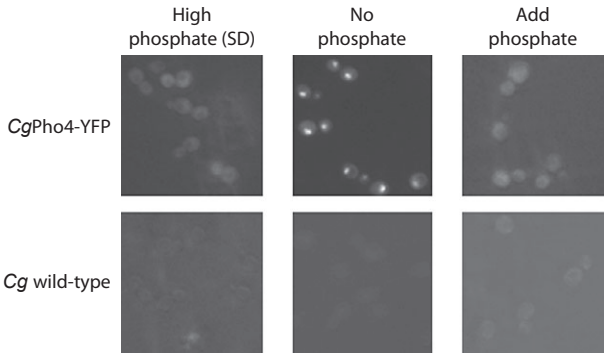

Fig. 3. *Cg*Pho4 is regulated by localization. Cells were grown in SD medium containing phosphate or SD medium lacking phosphate, and then phosphate was added back to the phosphate starvation condition. Auto-fluorescence in a *C. glabrata* wild-type strain serves as a negative control. As phosphate conditions fluctuate, *Cg*Pho4 shuttles into and out of the nucleus to regulate gene expression.

conditions of high external inorganic phosphate (O'Neill *et al.*, 1996; Komeili & O'Shea, 1999). We confirmed nuclear localization by fixing cells and staining cells with DAPI (Fig. S2). Additionally, because deletion of *CgMSN5*, a putative exportin, also results in de-repression of phosphate starvation genes to a similar extent as the *Cg*Pho4^{nophos} mutant, it is likely that localization by differential phosphorylation is regulated similarly for both *Cg*Pho4 and *Sc*Pho4 (Kaffman *et al.*, 1998; Kerwin & Wykoff, 2009).

Differences between the species – *Cg*Pho2 is not necessary for the induction of *PMU2*

To determine whether the induction of *PMU2* during phosphate starvation is regulated by *Cg*Pho2, we examined transcript abundance in the *Cgpho2*Δ strain in high and no phosphate conditions. As expected from previous work where only phosphatase activity was measured in the *Cgpho2*Δ strain (Kerwin & Wykoff, 2009), *CgPHO2* does not appear to be required for *PMU2* induction (Fig. 4).

Identification of region of *PMU2* promoter required for Pho4-dependent expression

To determine which region of the *PMU2* promoter was required for Pho4-dependent regulation, we generated truncated promoter-YFP constructs and measured expression from the constructs under high and no phosphate conditions. These constructs were integrated into the *CgURA3* locus using 5-FOA selection to prevent plasmid copy number effects. Analysis by microscopy (data not shown) and FACS (Fig. 5a) indicate a loss of expression in the range of − 800 to − 700 bp upstream from the ATG. Whereas other regions also appear important for expression (e.g. − 500 to − 400 bp), this was the only region with statistical support. We further subdivided this region into 20-bp increments and observed a decline in promoter activity upon deleting the region around − 740 to − 720 bp (Fig. 5b). There is a CCCGTG sequence in this region that we hypothesized could be the binding site for *Cg*Pho4, as it is only one nucleotide different from the CACGTG consensus recognized by *Sc*Pho4. We generated a − 730 bp *CgPMU2*pr-YFP construct that truncated the CCC from the site, and we deleted three nucleotides (GTG) from the CCCGTG site in the context of the 1-kb promoter (mutated 1-kb *CgPMU2*pr-YFP), and both constructs seemed to only have mild defects relative to their proper control (Fig. 5c). These analyses begin to identify important sequence elements; however, there is likely more complexity to be uncovered in the future.

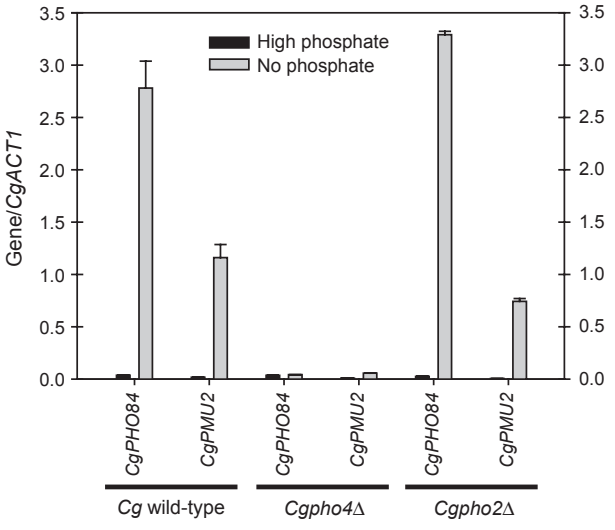

Fig. 4. *Cg*Pho2 is not required for the induction of *PMU2*. *CgPMU2* is regulated by phosphate starvation, and *Cg*Pho4, as *CgPMU2*, is induced during phosphate starvation compared to high phosphate; there is no induction when *CgPHO4* is deleted. There are near wild-type levels of *CgPHO84* and *CgPMU2* transcript in the *pho2Δ*, demonstrating that transcription is less dependent on *Cg*Pho2. Transcript abundance was measured by quantitative PCR.

As a control, we made *CgPHO84*-promoted YFP constructs, integrated them into the genome, and observed that YFP expression was regulated by phosphate starvation until the CACGTG site (at − 350 bp upstream) was removed from the promoter as expected (Fig. 6). Additionally, we mutated this site from CACGTG to GAGCTC to prevent *Cg*Pho4 binding and observed a 65% loss in expression of the *CgPHO84* promoter (Fig. 6). The results with the *CgPHO84* promoter indicate that CACGTG is utilized by *Cg*Pho4, but that there may be other sequences required for Pho4 dependence. Thus, *Cg*Pho4 utilizes the CACGTG to regulate expression of *PHO84*, but *Cg*Pho4 must be binding to a different DNA element in the *PMU2* promoter and possibly in the *PHO84* promoter, to which *Sc*Pho4 is unable to bind. These results were unexpected, but demonstrate that further work is needed to understand the *PMU2* promoter in detail, including the identification of transcriptional cofactors that may interact with CgPho4.

Chromatin immunoprecipitation demonstrates Pho4 binding to the *CgPMU2* and *CgPHO84* promoters

To confirm that *Cg*Pho4 and *Sc*Pho4 bind to the *CgPMU2* and *CgPHO84* promoters directly, we quantified the DNA associated with *Cg*Pho4-myc and *Sc*Pho4-myc during phosphate starvation in *C. glabrata* using chromatin immunoprecipitation. *Cg*Pho4-myc is enriched at the

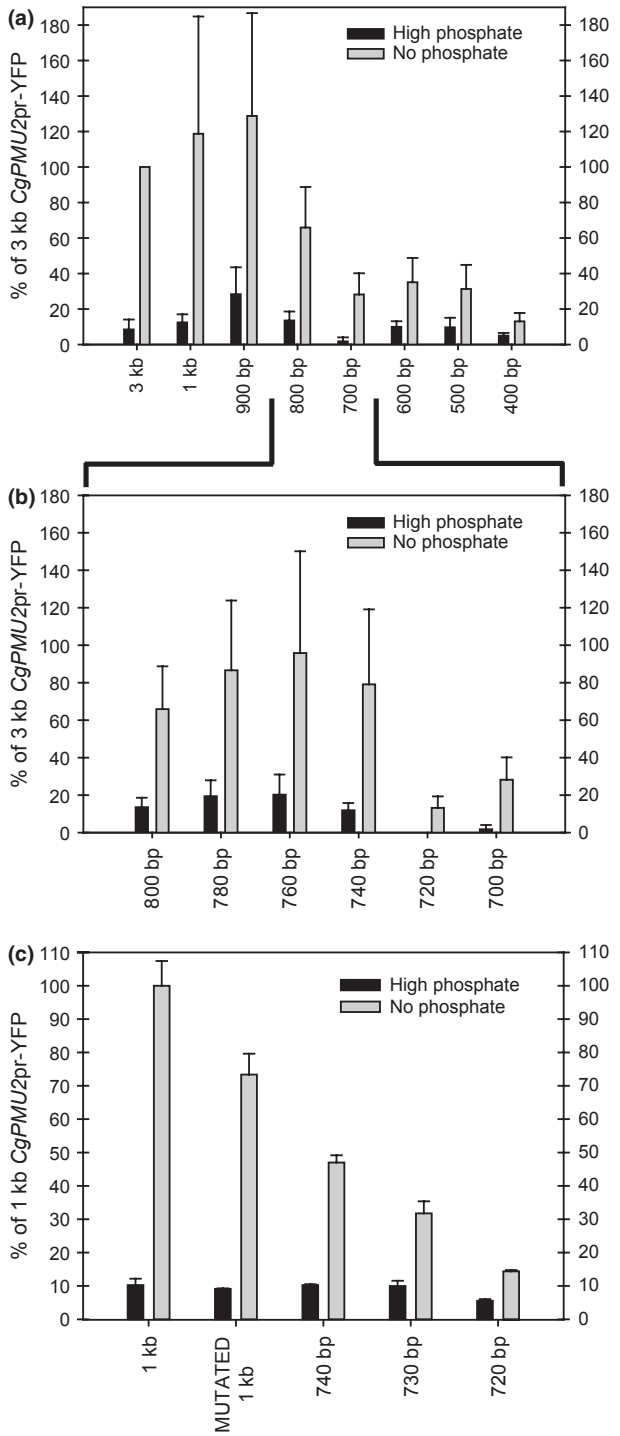

Fig. 5. Region of *CgPMU2* promoter required for Pho4-dependent expression is between − 740 and − 720 bp upstream of start codon. Truncated *CgPMU2*pr-YFP constructs (100- and 20-bp increments) driving YFP expression were generated, and expression was measured using FACS. Average arbitrary fluorescence units were normalized to 3-kb *CgPMU2*pr-YFP. For (a), $n = 4$. (a) There is a loss of *CgPMU2*pr-YFP expression in the range of − 800 and − 700 bp upstream from the start codon. (b) The region between − 800 and − 700 bp was divided into 20-bp increments, and a decline in expression was observed between − 740 and − 720 bp upstream (Student's *t*-test, $P = 0.0001$). (c) *Cg*Pho4 utilizes the sequence CCCGTG in the *PMU2* promoter. Average arbitrary fluorescence units for each construct were normalized to 1-kb *CgPMU2*pr-YFP. For the mutated promoter, three nucleotides (GTG) were deleted from the CCCGTG site in a 1-kb promoter construct. The 730-bp *PMU2*pr-YFP construct truncated the CCC from the site. *CgPMU2*pr-YFP expression decreases when the CCCGTG site is mutated to prevent *Cg*Pho4 binding (Student's *t*-test, $P = 0.02$).

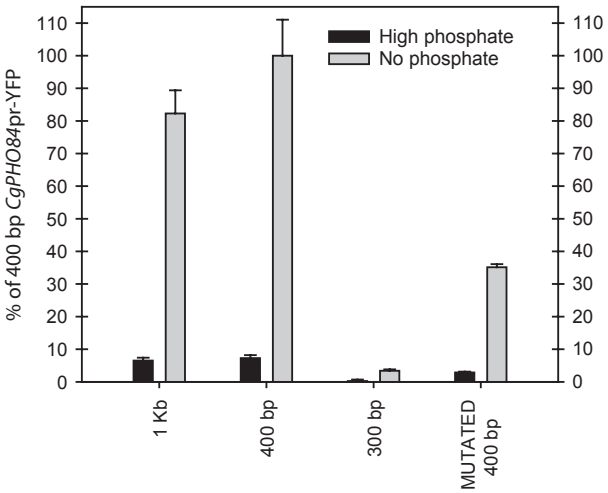

Fig. 6. *CgPHO84* promoter is regulated by starvation until the CACGTG site at − 350 bp upstream is removed from the promoter. Truncated *CgPHO84*pr-YFP constructs were generated, and expression was measured using FACS as in Fig. 5. The average of arbitrary fluorescence units for each construct was normalized to 400 bp *CgPHO84*pr-YFP. For the mutated promoter, the CACGTG site at − 350 bp was mutated to GAGCTC in a 400-bp promoter construct. When the CACGTG site is mutated to GAGCTC to prevent *Cg*Pho4 binding, we observed a loss in expression of the *CgPHO84* promoter (Student's *t*-test, $P = 0.004$).

CgPHO84 promoter relative to control sequences, as is *Sc*Pho4-myc to a lesser extent (Fig. 7a). There is significantly less enrichment for both proteins at sites far from the CACGTG site and at non-Pho4-regulated DNA (i.e. +1500 bp and *CgACT1*). We conclude that both proteins bind the *CgPHO84* promoter and that *Cg*Pho4-myc appears to have a higher affinity for the *CgPHO84* promoter relative to *Sc*Pho4.

The same samples were quantified for binding to the *PMU2* promoter, and in all cases, the binding of both proteins was significantly reduced. The fold enrichment relative to negative controls was two to fivefold. We had expected similar enrichment for both the *CgPHO84* and the *PMU2* promoters with *Cg*Pho4-myc, and *Sc*Pho4-myc

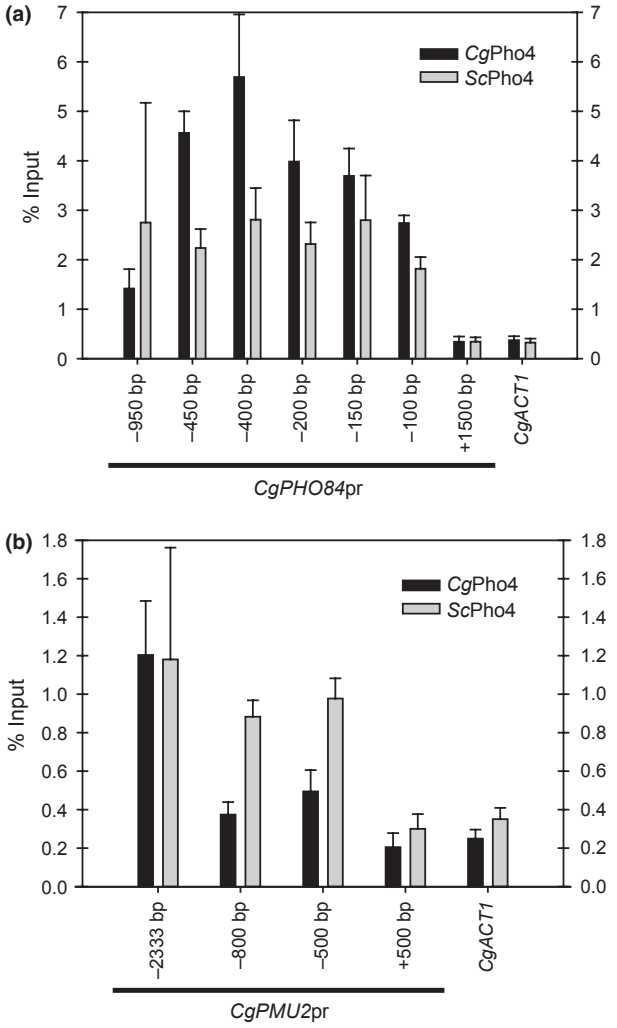

Fig. 7. Chromatin immunoprecipitation of *Cg*Pho4-myc and *Sc*Pho4-myc. Immunoprecipitated DNA was quantified relative to input DNA by qPCR and presented as the % immunoprecipitated relative to input. The locations presented are the middle base pairs of approximately 100-bp PCR products. The DNA was sheared to an average size of 1 kb. Three independent chromatin immunoprecipitation experiments were performed for each myc-tagged protein and + 1500 or + 500 bp from the start codon as well as *CgACT1* primer sets serve as controls for regions that were not expected to be immunoprecipitated. Primer sets presented correspond to (a) *PHO84* and (b) *CgPMU2* promoter sequences.

to only bind CACGTG sites; however, *Sc*Pho4 appears to bind to the *PMU2* promoter in regions that are required for expression (i.e. − 500 and − 800 bp), but we know that this binding does not result in productive transcription. The proteins bind best to the CACGTG sequence-2333 bp upstream from the start codon of *PMU2*. These results suggest that both Pho4 proteins bind the *CgPHO84* promoter effectively and probably directly. We hypothesize that binding to the *PMU2* promoter by both Pho4 proteins may be indirect or that other transcription factors interfere with efficient crosslinking. *Sc*Pho4 may bind to the − 500- and − 800-bp regions as a consequence of other proteins binding to the promoter (such as Pho2), but does not drive transcription because of missing transcriptional machinery.

Discussion

We began this work to understand how a relatively new phosphate starvation-regulated promoter acquired regulation by Pho4. Because *PMU2* is unique to *C. glabrata* in the *Ascomycetes*, and it is regulated by Pho4, there must have been a transition from the ancestral nonregulated promoter to a promoter being regulated by Pho4. Our data demonstrate that *PMU2*, and other phosphate-regulated promoters in *C. glabrata*, does not require Pho2. We also demonstrate that while phosphate starvation-regulated promoters in *S. cerevisiae* are able to function in *C. glabrata*, the *PMU2* promoter does not function well in *S. cerevisiae*. Moving 1 kb of the *PMU2* promoter into *S. cerevisiae* does not allow for transcription even though the same plasmid construct is regulated appropriately in *C. glabrata*.

To determine the site of *Cg*Pho4 binding in the *PMU2* promoter, we truncated the promoter and identified a approximately 20-bp region that influences expression; however, our data indicate that the *PMU2* promoter does not have a single definable site for transcriptional activation. Interestingly, the *PHO84* promoter in *C. glabrata* appears to be regulated by a known *Sc*Pho4-binding site, CACGTG, as when we mutate this site to a GAGCTC, expression declines over 60%. On the basis of the whole of the results, we hypothesize that the binding requirements of Pho4 are different in *C. glabrata* and that these requirements are influenced by an unknown factor. The major support for this argument is that moving *CgPHO4* into *S. cerevisiae* does not allow for expression of the *PMU2* promoter, indicating that another factor is present in *C. glabrata*.

On the basis of *S. cerevisiae* and *Neurospora crassa*, it appears that the ancestral state of Pho4 is the ability to bind to CACGTG sequences in yeast genomes (Peleg & Metzenberg, 1994; Barbaric *et al.*, 1998; Zhou & O'Shea, 2011). The acquisition of Pho2 to allow for cooperative binding at PHO promoters appears to be a derived trait only present in the *sensu stricto* species of *Saccharomyces* because Pho2 appears unimportant for the phosphate starvation response in *C. glabrata* and *Candida albicans* (Kerwin & Wykoff, 2009; Romanowski *et al.*, 2012). It is unclear whether there is always a protein partner of Pho4 that influences its binding affinity or whether *C. glabrata* alone acquired a protein cofactor independent of Pho2.

We speculate that there were a number of selective pressures that *C. glabrata* experienced with the loss of *PHO5* and the gaining of *PMU2*. The wholesale altering of the *trans* factor *Cg*Pho4 is unlikely because cells could not cope with a complete loss of expression of the other phosphate starvation genes. Instead, the promoter of *PMU2* might have acquired sequences *de novo* that were related to the *Cg*Pho4-binding site, and at the same time, *Cg*Pho4 may have acquired the ability to bind with a protein coactivator. These steps would allow for subtle changes of the binding specificity of Pho4, allowing for expression of the other phosphate starvation genes and the new phosphate starvation-regulated *PMU2*. Over time, the majority of the phosphate-regulated promoters would alter their binding specificity for optimal expression and potentially lose CACGTX sites.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Activation of phosphate starvation genes by loss of CDK activity acts through phosphorylation of *Cg*Pho4.

Fig. S2. Localization of *Cg*Pho4-YFP is consistent with nuclear localization during growth in phosphate starvation conditions.

Table S1. Strains used in this study.

Table S2. Primers used in this study.

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