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Candida glabrata PHO4 Is Necessary and Sufficient for Pho2-Independent Transcription of Phosphate Starvation Genes

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ABSTRACT

Comparative genomic analyses of *Candida glabrata* and *Saccharomyces cerevisiae* suggest many signal transduction pathways are highly conserved. Focusing on the phosphate signal transduction (PHO) pathway of *C. glabrata*, we demonstrate that components of the pathway are conserved and confirm the role of *CgPHO81*, *CgPHO80*, *CgPHO4*, and *CgMSN5* in the PHO pathway through deletion analysis. Unlike *S. cerevisiae*, *C. glabrata* shows little dependence on the transcription factor, Pho2, for induction of phosphate-regulated genes during phosphate limitation. We show that the *Cg*Pho4 protein is necessary and sufficient for Pho2-independent gene expression; *Cg*Pho4 is capable of driving expression of PHO promoters in *S. cerevisiae* in the absence of *ScPHO2*. On the basis of the sequences of *PHO4* in the hemiascomycetes and complementation analysis, we suggest that Pho2 dependence is a trait only observed in species closely related to *S. cerevisiae*. Our data are consistent with *trans*-regulatory changes in the PHO pathway via the transcription factor Pho4 as opposed to *cis*-regulatory changes (the promoter).

DIFFERENT species exploit specific niches. Gene expression programs have evolved to allow for optimal growth in these niches. Many gene expression programs are common to species (*e.g.*, DNA damage response, or nutrient starvation response); however, it is unclear how signal transduction pathways have evolved to tailor gene expression programs to their niche (Fry *et al.* 2006; Gasch 2007). Traditional signal transduction studies utilize conservation of signaling components in one species to infer conservation of functional output and this has been successful at predicting behaviors of important signal transduction pathways in organisms ranging from humans to bacteria (Simon 2001; Pinter *et al.* 2005). However, recent work has demonstrated that even when components are highly conserved between organisms, the network architecture, or how the components may interact, is different. For example, chemotaxis in *Escherichia coli* and *Bacillus subtilis* relies on the same components but the interactions are different, resulting in divergent behavior (Alon *et al.* 1999; Rao *et al.* 2004). This is underscored by examples of signal transduction pathways that behave differently between humans and mice (Migeon *et al.* 2005; Garofalo 2006). Thus, comparative analyses of signaling pathways are the first step in establishing the evolutionary pressures regulating speciation. Multicellular systems (notably the drosophilids) present a diversity of sequenced organisms and molecular genetic tools; however, the complexity of pathways, the number of components, and the exponential number of possible network interactions makes a thorough comparative analysis difficult (Yeger-Lotem *et al.* 2004; Moses *et al.* 2006).

The complete genomic sequence of many ascomycetes has begun to allow for a comparative genomic approach to understand the evolutionary steps required for speciation (Cliften *et al.* 2003; Dujon *et al.* 2004). For example, if a species does not experience an environmental condition during evolutionary time, the pathway required to respond to that condition can decay (Hittinger *et al.* 2004). Furthermore, promoters responsive to important signaling pathways, such as ribosomal protein biogenesis, can drift and acquire the ability to bind different transcription factors (Tsong *et al.* 2003; Butler *et al.* 2004; Ihmels *et al.* 2005). In this study, we have utilized a comparative genomic approach to study the PHO pathway in *Candida glabrata* and contrast its signaling pathway with the well studied PHO pathway of *Saccharomyces cerevisiae*.

The PHO pathway in *S. cerevisiae* activates the transcription of at least 20 genes during phosphate starvation (Lenburg and O'Shea 1996; Oshima 1997; Carroll *et al.* 2001). The PHO pathway consists of upstream signaling components (Pho81, Pho80, Pho85, Pho4, and Pho2) and a downstream transcriptional output. For the purposes of this study, we examine the transcription of *PHO5* and *PHO84* in *S. cerevisiae* and *PHO84* and *GIT1* in *C. glabrata*. Core to the signaling pathway is Pho4, a transcription factor regulated by a

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cyclin/cyclin-dependent kinase (CDK)/CDK inhibitor complex composed of Pho80, Pho85, and Pho81. In high phosphate conditions, the Pho80/Pho85 complex phosphorylates Pho4 on four key serine residues, causing export of Pho4 by Msn5 from the nucleus and cytoplasmic localization (O'Neill *et al.* 1996; Komeili and O'Shea 1999). In low phosphate conditions, Pho81 inhibits the kinase complex causing dephosphorylated Pho4 to accumulate in the nucleus via the import receptor Pse1, allowing Pho4 to cooperatively bind phosphate starvation promoters with the transcriptional coactivator Pho2 (Vogel *et al.* 1989; Yoshida *et al.* 1989a; Schneider *et al.* 1994).

We examined the PHO pathway in *C. glabrata*, because it is closely related to *S. cerevisiae* in the ascomycete lineage; however, both species experienced an ancestral whole genome duplication supplying the raw material for neofunctionalization (Wolfe 2001; Dujon *et al.* 2004). Because these species diverged from a common ancestor ~10 MYA, share ~75% protein sequence identity, and share many signal transduction pathways, we hypothesized *C. glabrata* would have many similarities to *S. cerevisiae*, with the notable difference of environment; *C. glabrata* is a commensal pathogen with mammals (Redondo-Lopez *et al.* 1990; Cormack and Falkow 1999; Cormack *et al.* 1999; Domergue *et al.* 2005).

We demonstrate that whereas most components of the PHO pathway are conserved between the two species, an important difference exists that may affect speciation. Pho2 is not as important for the transcriptional response to phosphate starvation in *C. glabrata* as it is in *S. cerevisiae*. We propose this Pho2 requirement (or lack of) has evolved in *trans* and that mutations in promoters have had a minimal impact on the Pho2 requirement as is evidenced by the sufficiency of *Cg*Pho4 to circumvent the Pho2 requirement in *S. cerevisiae*. (We abbreviate the derivation of genes from the two organisms as *Sc*, *S. cerevisiae* and *Cg*, *C. glabrata*.) Comparative genomic analysis of the hemiascomycetes suggests that the requirement for Pho2 in the transcriptional induction of PHO genes in *S. cerevisiae* is a derived trait, and the inclusion of Pho2 into the PHO pathway could have allowed for the Saccharomyces genus to take advantage of low inorganic phosphate conditions in nature.

MATERIALS AND METHODS

Strain construction: *C. glabrata* mutants were generated using antibiotic resistance genes *KANMX6* or *NATMX6* and homologous recombination to inactivate various phosphate signaling genes in a *C. glabrata his3*$^-$ background (Longtine *et al.* 1998; Cormack and Falkow 1999; Hentges *et al.* 2005). See Table 1 for a summary of strains and supporting information, Table S1, for a description of primers utilized to generate strains and plasmids. Deletion of genes was confirmed by PCR. The mutant strains were further confirmed by a semiquantitative phosphatase assay to confirm that multiple isolates behaved similarly.

TABLE 1

Strains used in this study

Strain	Genotype	Reference
	S. cerevisiae	
EY57	K699 with *MAT***a**	Wykoff and O'Shea (2001)
EY131	EY57 with *pho4*Δ*TRP1*	Wykoff *et al.* (2007)
EY337	EY57 with *pho2*Δ*LEU2*	Wykoff *et al.* (2007)
EY338	EY57 with *pho4*Δ*TRP1 pho2*Δ*LEU2*	Wykoff *et al.* (2007)
	C. glabrata	
BG99	*his3*Δ(1 + 631)	Cormack and Falkow (1999)
DG2	BG99 with *Cgpho4*Δ*KANMX6*	This study
DG3	BG99 with *Cgpho2*Δ*KANMX6*	This study
DG6	BG99 with *Cgpho81*Δ*KANMX6*	This study
DG8	BG99 with *Cgpho80*Δ*NATMX6*	This study
DG9	BG99 with *Cgmsn5*Δ*NATMX6*	This study
DG11	BG99 with *Cgpho4*Δ*KANMX6 Cgpho2*Δ*NATMX6*	This study

The genotype of K699 is *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3*.

To generate *PHO4* and *PHO2* containing plasmids, the ORF for the gene with at least 500 bp upstream was PCR amplified with primers that created a *Not*I restriction site at the 5′ end and a *Pac*I restriction site at the 3′ end of the gene. Primers used in this study are described in Table S1. The PCR fragment was digested with *Not*I and *Pac*I and ligated in frame into pRS313-13myc, so that the myc epitope was C terminal (Sikorski and Hieter 1989; Wykoff and O'Shea 2005). For Figure 6, all *PHO4* genes were amplified with a stop codon and were not in frame with the myc epitope. The *PHO4* and *PHO2* plasmids, for both *S. cerevisiae* and *C. glabrata*, were transformed into yeast strains using a standard lithium acetate yeast transformation protocol with selection on medium lacking histidine (Wykoff and O'Shea 2001; Guthrie and Fink 2002).

Media and growth conditions: Yeast strains defective in PHO pathway genes, but without plasmids, were grown in synthetic dextrose (SD) media with complete supplement mixture (CSM) amino acids (Sunrise Science Products, San Diego, CA) at 30° until logarithmic growth phase (see Guthrie and Fink 2002 for media components). Logarithmic growth is an OD_{600} ~0.5. Yeast strains defective in pathway genes and containing *HIS3*$^+$ plasmids were grown in SD +CSM −histidine. For all described experiments, cells were grown to logrithmic phase, pelleted by centrifugation, washed three times in medium lacking phosphate, then transferred to media lacking phosphate or media with 10 mm KH_2PO_4 (for phosphate replete conditions) and grown at 30° for 3 hr.

Comparative genomic analysis: Orthologs of each *S. cerevisiae* PHO pathway component were identified in *C. glabrata* on the basis of sequence similarity using the "Blastp *vs.* Fungi" feature in the Saccharomyces Genome Database (http://www.yeastgenome.org/). The result with the lowest expect (*E*) value (specifically a cutoff of $E = 10^{-2}$) was determined to be the ortholog in *C. glabrata*. To obtain values useful in comparing the two species, the sequences for the orthologs in the two species were aligned using the National Center for Biotechnology Information (NCBI) Blastp and the BLOSUM62 matrix (http://www.ncbi.nlm.nih.gov/). The relevant values were recorded; specifically, percentage of amino acid iden-

TABLE 2

Genomic sequence predicts a *C. glabrata* PHO pathway very similar to *S. cerevisiae*

Gene	*Cg* systematic name	*Sc* to *Cg*		Length of protein	
		Identity (%)	Expect	*Sc*	*Cg*
MSN5	CAGL0M01144g	935/1219 (76)	0	1225	1221
PHO84	CAGL0B02475g	448/586 (76)	0	588	580
PHO87	CAGL0F02387g	649/966 (67)	0	924	952
PHO90	CAGL0F02387g	624/957 (65)	0	882	952
PSE1	CAGL0M13871g	754/1091 (69)	0	1090	1091
VIP1	CAGL0M09823g	832/1113 (74)	0	1147	1128
PHO85	CAGL0L12474g	273/299 (91)	3*E*-159	306	302
PHO81	CAGL0L06622g	344/900 (38)	5*E*-144	1179	1137
ADK1	CAGL0K11418g	201/222 (90)	6*E*-113	223	222
PHO23	CAGL0G06556g	197/336 (58)	5*E*-88	331	316
PHO86	CAGL0L05456g	163/300 (54)	5*E*-86	312	306
PHO2	CAGL0L07436g	180/384 (46)	8*E*-85	560	515
PHO80	CAGL0E02541g	138/261 (52)	7*E*-73	294	342
PHO88	CAGL0K12276g	134/196 (68)	8*E*-68	189	194
PHO4	CAGL0D05170g	32/60 (53)	4*E*-08	313	533
SPL2		No ortholog			
PHO89		No ortholog			
NCP1	CAGL0D04114g	457/693 (65)	0	692	687
PHM1	CAGL0F02145g	499/831 (60)	0	829	816
PHM2	CAGL0F02145g	489/854 (57)	0	836	816
PHM3	CAGL0G06952g	582/719 (80)	0	722	717
PHO8	CAGL0H07359g	322/488 (65)	4*E*-176	567	545
AUT4	CAGL0B00770g	311/538 (57)	1*E*-162	529	541
PHO91	CAGL0I05632g	289/690 (41)	8*E*-133	895	886
SDT1	CAGL0H09218g	158/265 (59)	6*E*-90	281	279
IRC15	CAGL0F01947g	183/471 (38)	5*E*-79	500	493
GIT1	CAGL0A01243g	146/483 (30)	3*E*-60	519	531
PHM8	CAGL0C02321g	136/292 (46)	2*E*-58	322	314
PHM4	CAGL0M12705g	110/124 (88)	1*E*-53	130	127
CTF19	CAGL0F02035g	105/334 (31)	6*E*-32	370	373
PHM6		No ortholog			
PHO5		No ortholog			
PHO11		No ortholog			
PHO12		No ortholog			

Orthologs of the PHO pathway components, both regulators and transcriptional output, in *S. cerevisiae* (*Sc*) were determined for *C. glabrata* (*Cg*) on the basis of sequence similarity. Components are listed from lowest expect value to highest expect value and components with no ortholog are listed last. *MSN5* through *PHO89* are signaling components and *NCP1* through *PHO12* are genes upregulated in response to phosphate starvation.

tity, the number of amino acids over which the percentage of amino acid identity was measured, expect value, as well as the sizes of the proteins in *S. cerevisiae* and *C. glabrata* (Table 2).

Detection of phosphatase activity: For a semiquantitative phosphatase assay, the agar plates with colonies were overlaid with α-naphthyl phosphate, Fast Blue Salt B stain, and 0.1 M sodium acetate (pH 4.2) (Wykoff *et al.* 2007). This assay causes a colony to turn red when phosphatase is secreted and remain white in the absence of phosphatase. For quantification of *p*-nitrophenyl phosphatase activity, strains were grown in high or no phosphate conditions in liquid media for 16 hr, with high phosphate samples diluted to stay in logarithmic phase. One mL of cells (OD_{600} ~0.5) was pelleted and resuspended in sterile water. Measured units of phosphatase activity were expressed as OD_{400}/OD_{600} (Huang and O'Shea 2005). Data were normalized to either wild type grown in phosphate starvation or *pho4*Δ + *PHO4* such that induction for these strains was 100% (maximal induction expected).

Quantitative reverse-transcription PCR: RNA was extracted by a standard phenol–chloroform protocol (Huang and O'Shea 2005). RNA was converted to cDNA with a reverse-transcription reaction (BIO-RAD iScript cDNA synthesis kit). Quantitative PCR using a 50-μl PCR reaction with Sybr Green I (Sigma-Aldrich, St. Louis) was performed. Primers were designed for *ACT1*, *PHO84*, and *PHO5* for *S. cerevisiae* and *ACT1*, *PHO84*, and *GIT1* for *C. glabrata* (see Table S1). Data were normalized to expression of ACT1 to control for loading differences, and we confirmed that *ACT1* transcript abundance does not change dramatically during phosphate starvation (data not shown). These values were then normalized as described above to 100% induction.

Immunoblot analysis: Abundance of *S. cerevisiae* and *C. glabrata* Pho4 protein was analyzed by immunoblot. The *pho4*Δ + *PHO4* strains for both species were grown in high- and no-phosphate conditions as described. Protein was extracted and quantified and 30 μg of protein were subjected to SDS–PAGE and analyzed as described previously (Wykoff and O'Shea 2005).

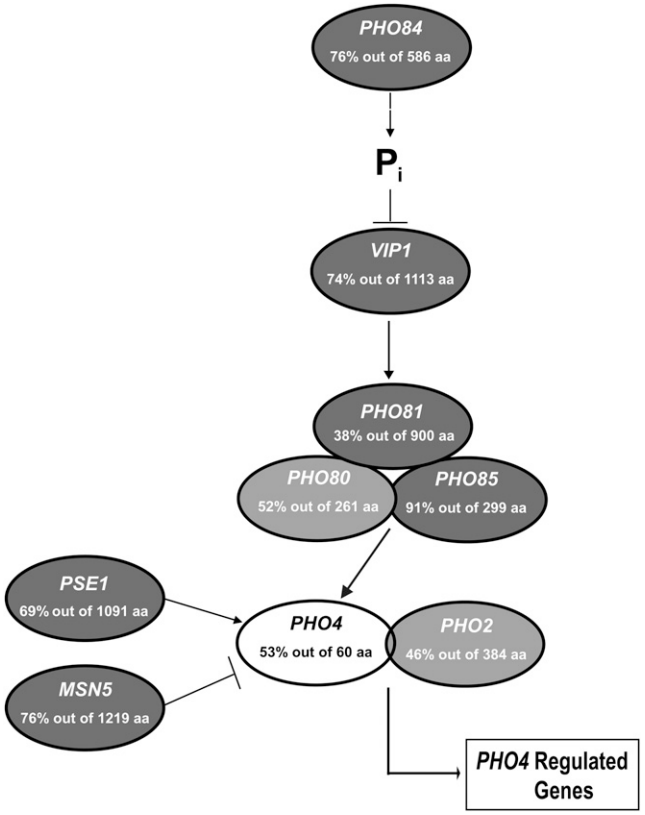

Figure 1.—Genomic sequence predicts a *C. glabrata* PHO pathway similar to *S. cerevisiae*. The values indicate the percentage of amino acid identity and the number of amino acids over which the identity was measured by BLASTP alignment. Shading of each component differs on the basis of the expect (*E*) value for each *C. glabrata* ortholog. Components with an *E* value $<10^{-100}$ are darkly shaded; *E* values $>10^{-100}$ are lightly shaded; and *E* values $>10^{-10}$ are open. Systematic *C. glabrata* names are given in Table 2.

RESULTS

Comparative genomic analysis of PHO pathway in *S. cerevisiae* and *C. glabrata*: To determine PHO pathway conservation in *C. glabrata*, we identified orthologous sequences for each component of the pathway using BLASTP and the yeast genome order browser (Byrne and Wolfe 2005). Utilizing a predetermined cutoff value of $E = 10^{-2}$, most of the components of the PHO pathway in *S. cerevisiae* have clear orthologs in *C. glabrata* (Figure 1 and Table 2), suggesting that a PHO pathway of similar architecture is present. However, *C. glabrata* does not contain orthologs of the acid phosphatases present in *S. cerevisiae* (*PHO5*, *PHO11*, and *PHO12*) or the Pho89 high-affinity phosphate transporter, nor is there obvious positive feedback through *SPL2* (Persson *et al.* 2003; Wykoff *et al.* 2007). It is unclear what genes encode the inducible acid phosphatase activity because there are no homologs of acid phosphatases in the *C. glabrata* genome, but there is clear inducible acid phosphatase activity (Figure 2A). The transcription factor Pho4 is the least conserved component of the pathway and is identifiable by amino acid identity in the C-terminal DNA binding domain (Figure S1).

Confirmation of PHO pathway functions in *C. glabrata*: To confirm that the identified orthologs regulate a phosphate-starvation-inducible acid phosphatase, we inactivated candidate genes with antibiotic resistance genes *KANMX6* or *NATMX6* (Longtine *et al.* 1998). Assaying these mutants by a semiquantitative assay for acid phosphatase activity, we confirmed that *Cg*Pho4 is required for induction of phosphatase activity during phosphate starvation (Figure 2A). We further determined that *Cg*Pho4 is regulated by the orthologous cyclin/CDK/CDK inhibitor complex and that *Cg*Pho4 localization is likely one mode of regulation as deletion of *CgMSN5*, a putative exporter of *Cg*Pho4, results in partial activation of the pathway. Notably, deletion of *CgPHO2* does not abolish phosphatase induction, as it does in *S. cerevisiae* (Yoshida *et al.* 1989a; Springer *et al.* 2003).

Using quantitative PCR, we quantified the amount of transcript of two genes we hypothesized were regulated by extracellular phosphate status, *CgPHO84* and *CgGIT1*, and confirmed that they are transcriptionally induced during phosphate starvation (Figure 2B). Quantitative analysis of the mutant strains supported the semiquantitative conclusions, although the two promoters behaved slightly differently from one another. Because the acid phosphatase gene has not been identified, we cannot directly compare transcriptional induction of these two genes with induction of phosphatase activity. Deletion of *PHO4* in *C. glabrata* results in no increase in gene expression during phosphate starvation, which is true in *S. cerevisiae*. Unlike *S. cerevisiae*, *C. glabrata* exhibits a reduced dependence on the Pho2 transcription factor. Although not at wild-type levels, gene expression of the starvation-regulated promoters still occurs when *PHO2* is deleted in *C. glabrata*.

***C. glabrata* Pho4 is sufficient for Pho2 independence:** To determine whether the Pho2 independence in *C. glabrata* is due to alterations in the *C. glabrata* Pho4 transcription factor (in *trans*) or alterations to *C. glabrata* promoters (in *cis*), we characterized the ability of Pho4, from both *S. cerevisiae* and *C. glabrata*, to induce gene expression independent of Pho2 in either species. Plasmids containing *PHO4* or *PHO2* genes from either *S. cerevisiae* or *C. glabrata* were generated and transformed into *S. cerevisiae* and *C. glabrata* mutants defective in one or both transcription factors (*pho4*Δ, *pho2*Δ, or *pho4*Δ*pho2*Δ). *PHO4* from either species complemented or cross-complemented *pho4*Δ strains when subjected to the semiquantitative phosphatase assay, suggesting the plasmids contain functional genes (Figure 3). We also confirmed cross-complementation with *PHO2*, although *CgPHO2* complemented *S. cerevisiae* to a lower extent than the converse condition (Figure 4, A and B and Figure S2). We confirmed our semiquantitative data by quantifying the amount of *ScPHO84* and *ScPHO5* transcript in *S. cerevisiae*

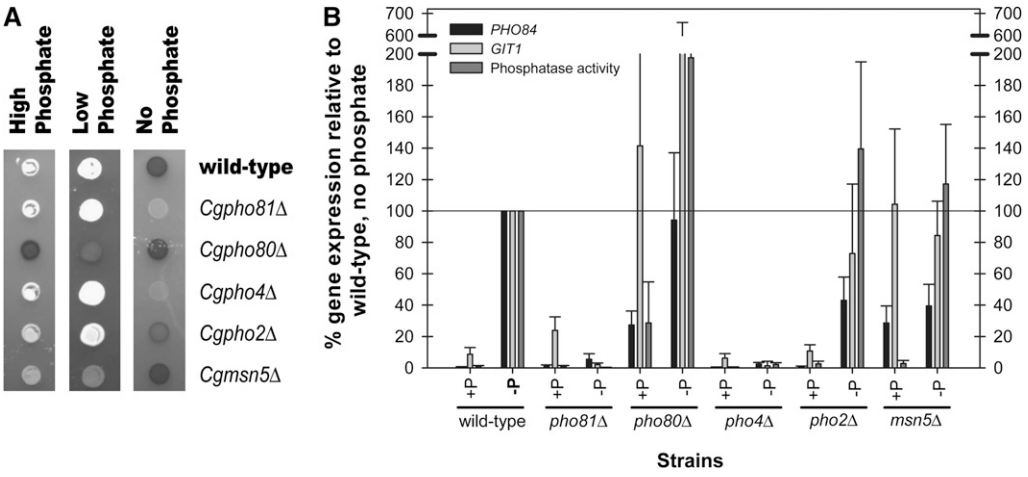

FIGURE 2.—*C. glabrata* mutants defective in the PHO pathway components have similar phenotypes as *S. cerevisiae* mutants, except for *Cgpho2*Δ. (A) Acid phosphatase activity of *C. glabrata* mutants defective in major PHO pathway components. Strains were grown in high (SD), low (YEPD), and phosphate starvation (SD no phosphate) conditions and solid media was overlaid with substrate to detect acid phosphatase activity. Dark shaded colonies have acid phosphatase activity and white colonies have reduced phosphatase activity. (B) Induction of *PHO84* and *GIT1* and phosphatase activity of *C. glabrata* mutants. Transcription of *PHO84* and *GIT1* was measured with reverse-transcription quantitative PCR. Data were normalized to wild-type cells grown in phosphate starvation such that expression for this strain was 100% (maximal induction expected during starvation). *p*-nitrophenyl phosphatase activity was quantified and normalized to wild type. Data for *PHO84* and *GIT1* are representative of four independent experiments and data for phosphatase activity are representative of three independent experiments. Standard error was calculated for *PHO84*, *GIT1*, and phosphatase activity. The value for *GIT1* in *pho80*Δ without phosphate is 380 ± 279%. The errors not shown are 63% for *GIT1* in *pho80*Δ with phosphate, and 135% for phosphatase activity in *pho80*Δ without phosphate.

strains (Figure 4A) and the amount of *CgPHO84* and *CgGIT1* transcript in *C. glabrata* strains (Figure 4B).

We hypothesized that if Pho2 dependence is a consequence of alterations in the Pho4 protein, then only *Cg*Pho4 should induce phosphate starvation genes in the *pho4*Δ*pho2*Δ mutant in both *S. cerevisiae* and *C. glabrata*. However, if the Pho2 dependence is due to changes in the promoter regions (*cis*-regulatory regions), then both *Sc*Pho4 and *Cg*Pho4 should induce starvation genes in the *C. glabrata pho4*Δ*pho2*Δ, but neither *Sc*Pho4 nor *Cg*Pho4 would induce transcription in the *S. cerevisiae pho4*Δ*pho2*Δ. Finally, if the Pho2 dependence is due to changes in both the Pho4 protein and the promoter regions, then both *Sc*Pho4 and *Cg*Pho4 should induce starvation genes in *C. glabrata pho4*Δ*pho2*Δ and only *Cg*Pho4 should induce starvation genes in *S. cerevisiae pho4*Δ*pho2*Δ.

Analysis of gene expression during phosphate starvation demonstrates that only *Cg*Pho4, and not *Sc*Pho4, can dramatically induce starvation genes in the absence of the Pho2 in either species (Figure 4, A and B). Specifically, *Cg*Pho4 suppresses the Pho2 dependence in *S. cerevisiae* and *Sc*Pho4 is not sufficient in *Cgpho4*Δ*pho2*Δ. These results suggest that the Pho2 dependence is a consequence of alterations in the Pho4 protein and that *Cg*Pho4 obviates the need for Pho2. The *Cg*Pho4 generation of Pho2 independence is most clear in *S. cerevisiae* strains; with *Cg*Pho4, the *S. cerevisiae* promoters do not require Pho2. While not as dramatic, *C. glabrata* promoters exhibit similar behavior with *Sc*Pho4 being dependent on Pho2 and *Cg*Pho4 not requiring Pho2, but we cannot eliminate the possibility that there are subtle *cis* changes to phosphate-regulated promoters.

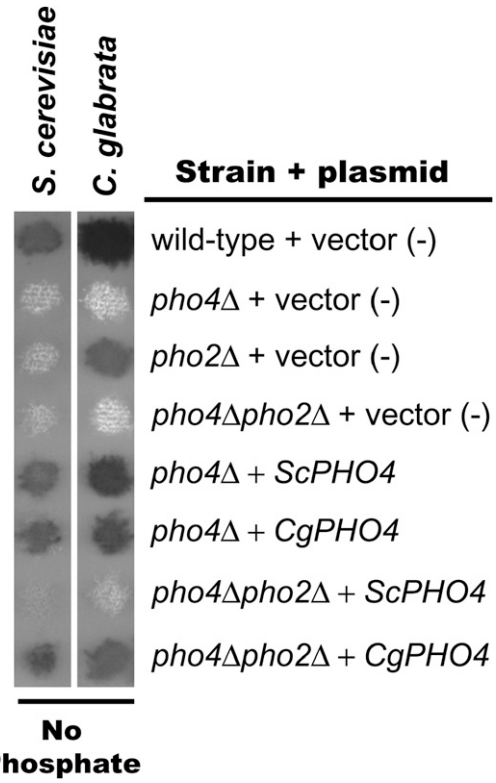

FIGURE 3.—Semiquantitative phosphatase assay demonstrating that *PHO4* plasmids are functional and *CgPHO4* is sufficient for Pho2 independence. *S. cerevisiae* and *C. glabrata* mutants lacking one or both transcription factors (*pho4*Δ, *pho2*Δ, or *pho4*Δ*pho2*Δ) contain either empty vector (pRS313), *ScPHO4*, or *CgPHO4* plasmids. These strains were grown on solid media lacking phosphate and overlaid with phosphatase substrate.

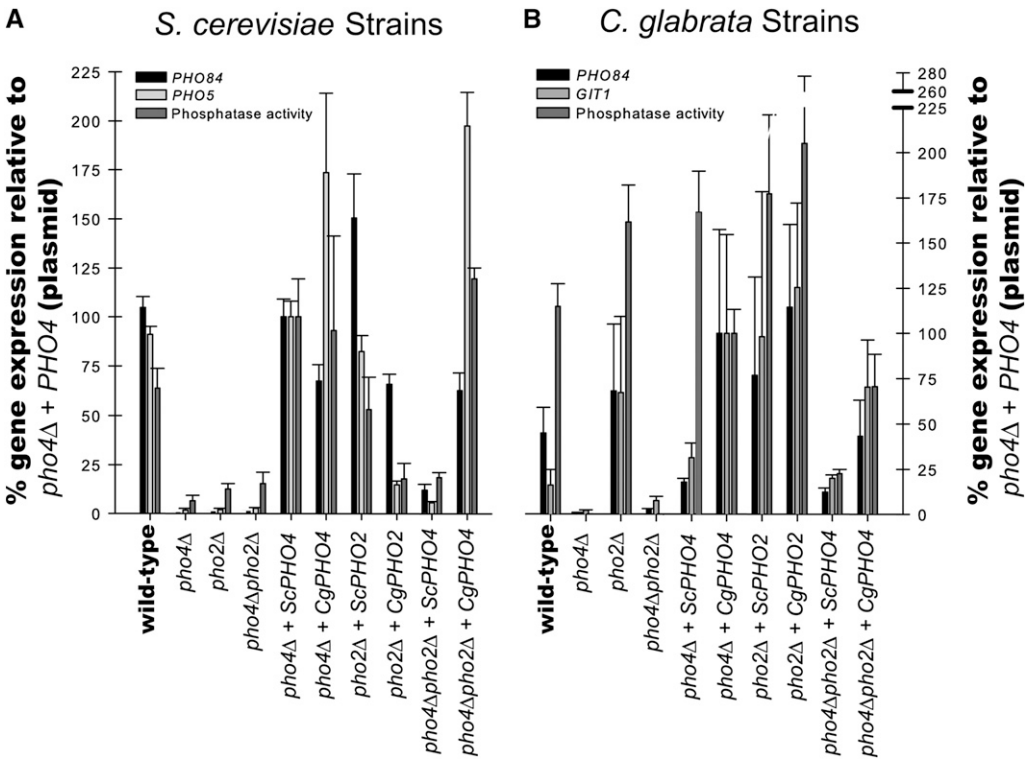

FIGURE 4.—Quantification of phosphate responsive genes in *S. cerevisiae* strains and *C. glabrata* strains. (A) *S. cerevisiae* mutants lacking one or both transcription factors (*pho4*Δ, *pho2*Δ, or *pho4*Δ*pho2*Δ) were generated to contain either empty vector (pRS313), *ScPHO4*, *CgPHO4*, *ScPHO2*, or *CgPHO2* plasmids. These strains were grown in media lacking phosphate and quantitative reverse-transcription PCR was used to measure amount of *PHO84* and *PHO5* transcript. Data were normalized to *Scpho4*Δ + *ScPHO4* (such that expression for this strain was 100%) rather than to wild type because the plasmid alters the copy number of the *PHO4*. *p*-nitrophenyl phosphatase activity was also normalized to *Scpho4*Δ + *ScPHO4*. (B) *C. glabrata* mutants lacking one or both transcription factors with the same plasmids as in A. These strains were treated as described for *S. cerevisiae* strains except that induction of *GIT1* was measured for *C. glabrata* rather than *PHO5*. Data were normalized to *Cgpho4*Δ + *CgPHO4* for both the quantitative reverse-transcription PCR and the phosphatase assay. The standard error is of at least three independent replicates.

While Pho2 may serve an ancillary role in *C. glabrata* and the promoters in *C. glabrata* have a low affinity for *Cg*Pho2, *Sc*Pho4 must recruit *Cg*Pho2 to some *C. glabrata* promoters because *Sc*Pho4 is functional in a *Cgpho4*Δ background. Because the consensus binding site for Pho2 is an A/T-rich region (BARBARIC *et al.* 1996), we are not able to determine whether there are Pho2 binding sites within the phosphate-regulated promoters.

***C. glabrata* likely confers Pho2 independence as a consequence of its increased size and not because of overexpression:** Previous studies have demonstrated that when Pho4 is overexpressed in *S. cerevisiae* (~20-fold higher than wild-type expression), transcription of *PHO5* can be induced in the absence of Pho2, although expression is only ~25% of wild-type levels (YOSHIDA *et al.* 1989a,b; BARBARIC *et al.* 1996). This situation appears to resemble that of a *Cgpho2*Δ strain, where phosphate-regulated promoters are induced, but less than wild-type phosphate starvation levels. To determine whether the Pho2 independence in *C. glabrata* is a consequence of overexpression of the *PHO4* during phosphate starvation conditions, we examined the abundance of the *S. cerevisiae* and *C. glabrata* Pho4 proteins in high- and no-phosphate conditions by immunoblot analysis (Figure 5).

We demonstrate that neither *Sc*Pho4 nor *Cg*Pho4 change abundance dramatically in high- and no-phosphate conditions. Although *Cg*Pho4 is much fainter for *Scpho4*Δ + *CgPHO4*, the amount of protein is still the same for both phosphate conditions. The lack of abundance changes is unlikely to be an artifact of C-terminally tagging the proteins with a myc epitope because the myc-tagged genes complement many deletion strains (Figures 3 and 4). These results suggest that the Pho2 independence is not a consequence of overexpression of *Cg*Pho4.

Our results demonstrate that only *Cg*Pho4, and not *Sc*Pho4, can suppress the Pho2 dependence in either species. This difference prompted us to look at the difference between the Pho4 transcription factors from the two species. One major difference is the size of the Pho4 proteins: *C. glabrata* Pho4, which is 533 amino acids, is much larger than *S. cerevisiae* Pho4, which is only 313 amino acids. Of all of the orthologs that we identified between the two species, this is the greatest difference in size (Table 2). An examination of the ascomycete lineage reveals that the Pho4 protein in the Saccharomyces genus is small relative to all of the other sequenced ascomycetes. Furthermore, an extensive analysis of *Neurospora crassa* mutants identified many shared components of the PHO pathway, except that *NcPHO2* was never demonstrated to be important for phosphatase induction during phosphate starvation (PELEG *et al.* 1996a,b; YANG *et al.* 1991). We hypothesized that the Pho2 dependence is a trait that has only appeared in species closely related to *S. cerevisiae* with relatively small Pho4 proteins.

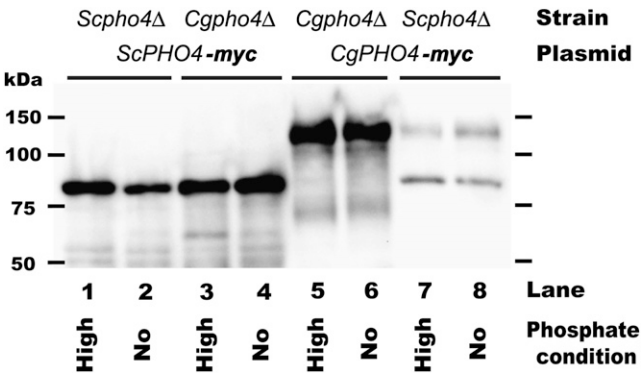

FIGURE 5.—Immunoblot analysis demonstrating little change in Pho4 abundance between high- and no-phosphate conditions. *S. cerevisiae* and *C. glabrata pho4*Δ strains with a *PHO4* plasmid with 13-myc epitope were grown to log phase, washed, and then transferred to high- and no-phosphate conditions for 3 hr. Pho4 was detected with 9E10 as described previously (WYKOFF and O'SHEA 2005). The expected sizes of Pho4 are 54 kDa in *S. cerevisiae* and 79 kDa in *C. glabrata* (size of Pho4 + 13-myc tag); however, previous studies demonstrate *Sc*Pho4 migrates ~25 kDa heavier than expected and has a degradation product (KAFFMAN *et al.* 1998; O'NEILL *et al.* 1996), and *Cg*PHO4 also appears to migrate aberrantly. This immunoblot is representative of three independent experiments.

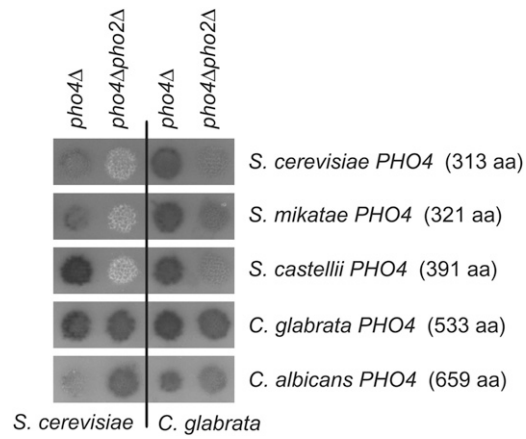

FIGURE 6.—Phosphatase activity in *S. cerevisiae* and *C. glabrata* mutants containing the Pho4 transcription factor from various hemiascomycetes. Plasmids containing the *PHO4* gene from various hemiascomycetes (labeled on the right) were transformed into *S. cerevisiae* (left two columns) and *C. glabrata* mutants (right two columns) defective in one or both transcription factors (*pho4*Δ and *pho4*Δ*pho2*Δ). Phosphatase activity was determined as described in Figure 2 and is representative of multiple transformants.

To begin testing this hypothesis, *S. cerevisiae* and *C. glabrata* mutants defective in one or both transcription factors (*pho4*Δ and *pho4*Δ*pho2*Δ) were transformed with plasmids containing *PHO4* from various ascomycetes (Figure 6). These strains were tested for phosphatase activity. The *pho4*Δ mutants into which *PHO4* plasmids were transformed demonstrate that the plasmids contain functional genes; the defect caused by the deletion of *ScPHO4* or *CgPHO4* is removed and phosphatase activity is detected. The results for the *pho4*Δ *pho2*Δ mutants with *PHO4* plasmids resemble the data obtained for *Sc*Pho4 and *Cg*Pho4 in the *pho4*Δ *pho2*Δ mutants. Only the larger Pho4 transcription factors [*i.e.*, *C. glabrata* (533 aa) and *C. albicans* (659 aa)] induce phosphatase activity independent of Pho2 in both species. The smaller Pho4 transcription factors [*i.e.*, *S. cerevisiae* (313 aa), *S. mikatae* (321 aa), *S. castellii* (391 aa)], however, do not induce the phosphatase in the absence of Pho2. These results suggest that Pho2 independence is likely a consequence of the increased size of the Pho4 transcription factor.

DISCUSSION

We demonstrate that genomic sequence is highly predictive of the PHO signal transduction pathway in *C. glabrata*. Through mutant analysis, *Cg*Pho4 is likely regulated by the cyclin/CDK/CDK inhibitor complex Pho80/Pho85/Pho81 and likely regulated by localization in the same fashion as *S. cerevisiae*. This is supported by deletion of *PHO81*, which is unable to induce phosphatase expression during phosphate starvation in both *S. cerevisiae* and *C. glabrata*, and deletion of *PHO80* resulting in a constitutive phenotype. It is unlikely that these phenotypes are independent of the kinase activity of this complex. Furthermore, the known phosphorylation sites of *Sc*Pho4 are moderately conserved in *Cg*Pho4 also suggesting that these sites are used in *C. glabrata* (Figure S1). Finally, the expression of the phosphatase is increased in high phosphate conditions in a *Cgmsn5*Δ strain, identical to *Scmsn5*Δ strains. Whereas we have not directly demonstrated that *Cg*Pho4 is a substrate for the kinase complex, the simplest explanation is that *Cg*Pho4 is regulated in a similar fashion to *S. cerevisiae*.

Switching Pho4 between species demonstrates that *Cg*Pho4 is necessary and sufficient for Pho2-independent induction of phosphate starvation-regulated promoters in both species. The Pho2 independence of PHO promoters in *C. glabrata* was an unexpected result and highlights the need for studies in nonmodel species. Interestingly, there are additional differences between the PHO pathway of *S. cerevisiae* and *C. glabrata*, including the lack of a known acid phosphatase gene and the relative dearth of canonical Pho4 binding sites (CAC GTG) in many putative phosphate-regulated genes in *C. glabrata* (data not shown), suggesting that there are many subtle differences in how the two organisms regulate their phosphate starvation response.

The view of speciation being influenced by subtle changes to promoters has been disputed by many recent studies and our study supports the idea that species have exploited changes in *trans* in the PHO pathway of ascomycetes (KING and WILSON 1975; PIANO *et al.* 1999; WITTKOPP *et al.* 2004, 2008; HOEKSTRA *et al.* 2006; CHANG *et al.* 2008). On the basis of size and

conservation of Pho4 in ascomycetes that did not experience a whole genome duplication event, we hypothesize that Pho2 independence is an ancestral trait, which is supported by small Pho4 proteins appearing to be Pho2 dependent. Because of the relative promiscuousness of Pho2 binding (it binds to A/T-rich sequences of DNA) (Barbaric *et al.* 1996; Zhang *et al.* 1997), we cannot exclude the possibility that promoter changes have occurred, but clearly the Pho2 independence depends on domains within *Cg*Pho4. We expect that the Saccharomyces species that appear Pho2 dependent likely experienced a selective advantage because of this increased pathway complexity.

C. glabrata, as a commensal pathogen of mammals (Redondo-Lopez *et al.* 1990; Cormack and Falkow 1999), likely experiences two inorganic phosphate conditions: high phosphate conditions during growth in mammalian tissues and extremely low phosphate conditions on external epithelium. *S. cerevisiae*, however, likely experiences a different condition: growth on decaying organic matter that might provide organic phosphates and moderate levels of inorganic phosphate. Contrasting these niches provides for a possible selective advantage for Saccharomyces species. During conditions of low inorganic phosphate, *S. cerevisiae* through Pho4 is able to induce a subset of phosphate starvation responsive genes, including the high-affinity phosphate transporter *PHO84*, but maintain relative repression of the acid phosphatase gene *PHO5* (Springer *et al.* 2003). This alternate gene regulation program results from an isoform of Pho4 that is unphosphorylated except on one site (Springer *et al.* 2003). Phosphorylation of serine 223 decreases the interaction between Pho4 and Pho2 (O'Neill *et al.* 1996; Komeili and O'Shea 1999). Because the acid phosphatase gene in *C. glabrata* is unidentified, we are unable to determine whether this same intermediate phosphate starvation response occurs in *C. glabrata*, but future studies will explore this question.

We observe a different signaling architecture in the PHO pathway of *C. glabrata* relative to budding yeast and others have observed differences between pathways such as galactose metabolism and mating pathways between the two species (Tsong *et al.* 2003; Butler *et al.* 2004; Hittinger *et al.* 2004). The two species inhabit different environments and require divergent responses for optimal growth. It is appealing to hypothesize that *trans*-regulatory changes to signaling pathways have allowed the two different species to exploit their environmental niches through changes in the transcription factors that regulate entire gene expression programs.

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GENETICS

Supporting Information
http://www.genetics.org/cgi/content/full/genetics.109.101063/DC1

Candida glabrata PHO4 Is Necessary and Sufficient for Pho2-Independent Transcription of Phosphate Starvation Genes

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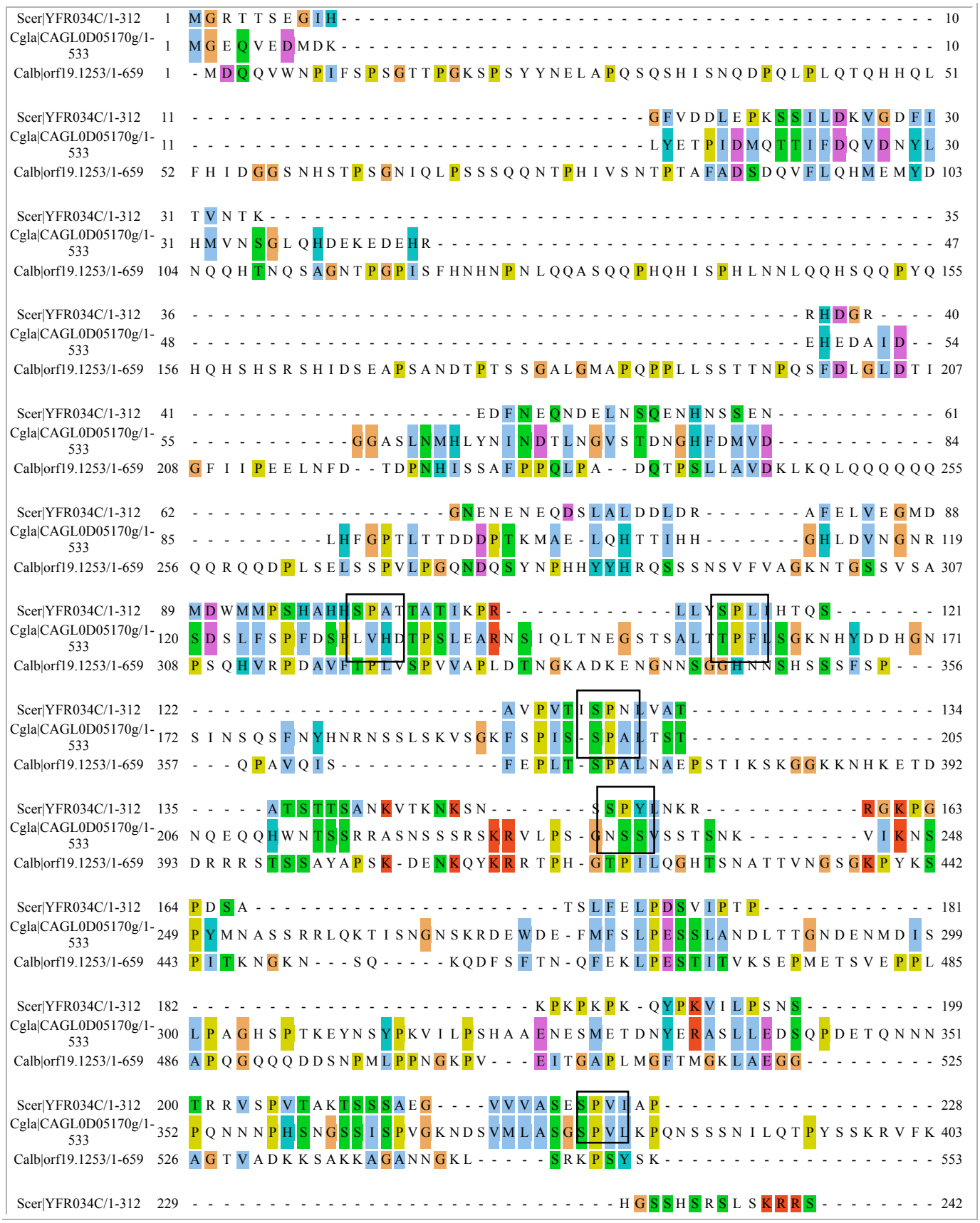
DOI: 10.1534/genetics.109.101063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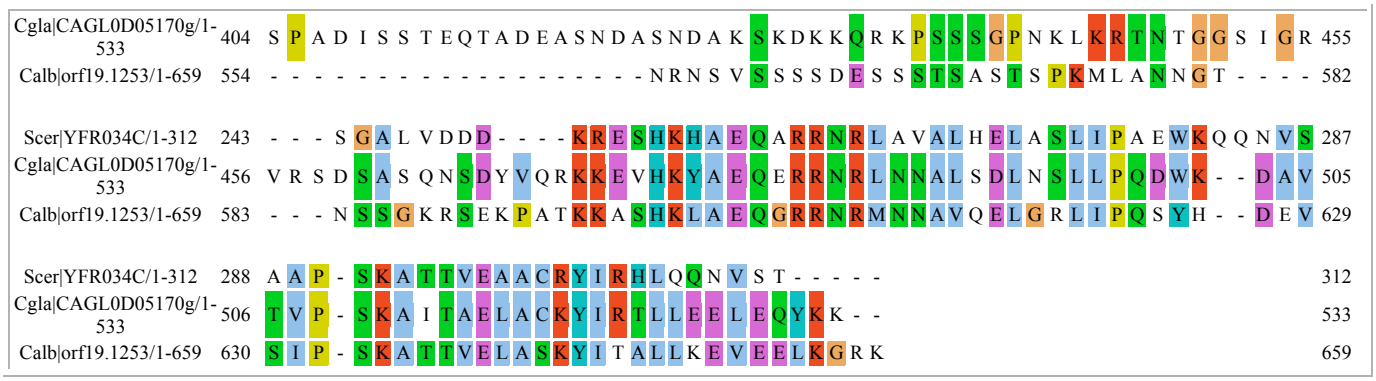

FIGURE S1.—Alignment of *Sc*Pho4 with *Cg*Pho4 and *Ca*Pho4. Utilizing a tree-assisted alignment of OG9983 which is the orthogroup of Pho4, we removed the other 16 species after CLUSTAL alignment, removed gaps common to all three proteins and colored residues according to the clustal option in Jalview. The most conserved portion of the proteins is in the C-terminal DNA binding domain. The known phosphorylation sites of *Sc*Pho4 are surrounded by boxes.

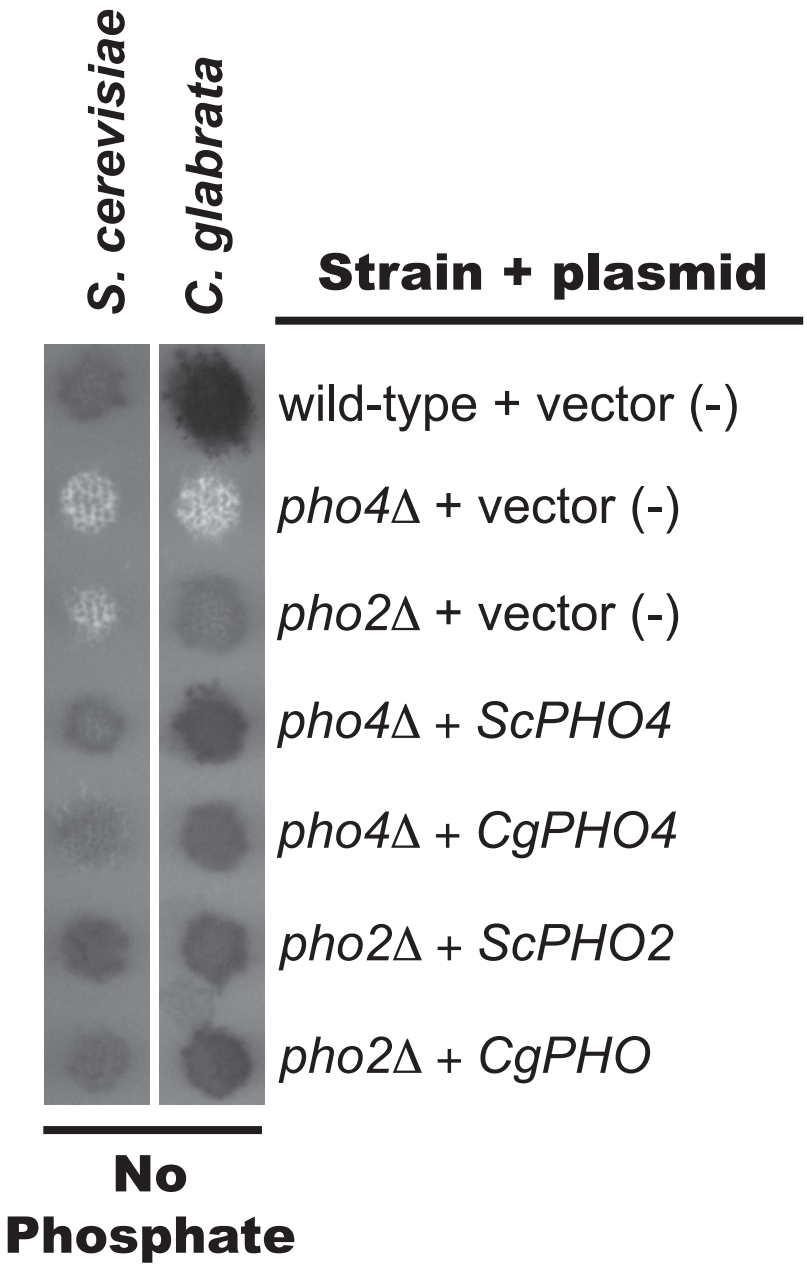

FIGURE S2.—Semi-quantitative phosphatase assay demonstrating that *PHO4* and *PHO2* plasmids are functional. *S. cerevisiae* and *C. glabrata* mutants lacking the Pho4 transcription factor (*pho4*Δ) contain either empty vector (pRS313), *ScPHO4*, or *CgPHO4* plasmids. *S. cerevisiae* and *C. glabrata* mutants lacking the Pho2 transcription factor (*pho2*Δ) contain either empty vector (pRS313), *ScPHO2*, or *CgPHO2* plasmids. These strains were grown on solid media lacking phosphate and overlaid with phosphatase substrate.

TABLE S1

Primers used in this study

Primer	Sequence	Purpose
OD16	tgactgggattccgtgagacggac	Delete *C. glabrata PHO4* with *KANMX6*
OD17	ttaattaacccggggatccgacctgttcacccatcactaacag	
OD18	ctgttagtgatgggtgaacaggtcggatccccgggttaattaa	
OD19	gtttaaacgagctcgaattctacagatactccaagacaaaac	
OD20	gttttgtcttggagtatctgtagaattcgagctcgtttaaac	
OD21	atcttgtgccctccaaacagaactg	
OD25	gctcgctctccattgcctattgtag	Delete *C. glabrata PHO2* with *KANMX6*
OD26	ttaattaacccggggatccgccatccatttaactacaaagttggc	
OD27	gccaactttgtagttaaatggatggcggatccccgggttaattaa	
OD28	gtttaaacgagctcgaattcgctacgaatattcatgtacat	
OD29	atgtacatgaatattcgtagcgaattcgagctcgtttaaac	
OD30	tcccatccaatcttatccggtgc	
O1	gacagtcaaagtggtaaaggg	Delete *C. glabrata PHO81* with *KANMX6*
O2	ttaattaacccggggatccgcagatatttaccaaacttcat	
O3	atgaagtttggtaaatatctgcggatccccgggttaattaa	
O4	GTTTAAACGAGCTCGAATTCgacgataaaaacctgatgctt	
O5	aagcatcaggtttttatcgtcGAATTCGAGCTCGTTTAAAC	
O6	taatctagatcttcaaacgtc	
O7	ctgtcctggtcttggtgtggc	
O15	caccagacatccttagtggat	Delete *C. glabrata PHO80* with *NATMX6*
O16	ttaattaacccggggatccgctgagcatcttggggagtcat	

O17	atgactccccaagatgctcagcggatccccgggttaattaa	
O18	GTTTAAACGAGCTCGAATTCagcatatatccttcatctata	
O19	tatagatgaaggatatatgctGAATTCGAGCTCGTTTAAAC	
O20	cgtgtgaaatacacacctctg	
O21	cgtgtcaaaacttgattccag	
O29	ggcacttaatgtaatatattc	Delete *C. glabrata MSN5* with *NATMX6*
O30	ttaattaacccggggatccgactggccaacttctcccttta	
O31	taaaggagaagttggccagtcggatccccgggttaattaa	
O32	GTTTAAACGAGCTCGAATTCctgatagcgatggctggttc	
O33	gaaccagccatcgctatcagGAATTCGAGCTCGTTTAAAC	
O34	acacgagcaccataaatcca	
O35	atgaatgtcggggctaatcaag	
OD18	ctgttagtgatgggtgaacaggtcggatccccgggttaattaa	Delete *C. glabrata PHO4* with *NATMX6* in DG3
OD20	gttttgtcttggagtatctgtagaattcgagctcgtttaaac	
DWO1	CGATCGGCGGCCGCTGCATTATTTAGATCGGAAAAAGTC	Amplify *S. cerevisiae PHO4* with 5' *Not*I and 3' *Pac*I restriction sites
DWO2	CGACACTTAATTAACGTGCTCACGTTCTGCTGTAGGTG	for cloning into pRS313-13myc
DWO7	CGATCGGCGGCCGCGATGAGCTCTTTTCATCACTTCTCG	Amplify *S. cerevisiae PHO2* with 5' *Not*I and 3' *Pac*I restriction sites
DWO8	CGACACTTAATTAATATCCATCTATGCTCGTCAGTTAG	for cloning into pRS313-13myc
DWO5	CGATCGGCGGCCGCGTATGCATTACCTTCTCAATTAGG	Amplify *C. glabrata PHO4* with 5' *Not*I and 3' *Pac*I restriction sites
DWO6	CGACACTTAATTAATTTCTTGTACTGCTCAAGCTCTTCG	for cloning into pRS313-13myc
DWO11	CGATCGGCGGCCGCAGCAACCACCCTGGCGTCCTGCGC	Amplify *C. glabrata PHO2* with 5' *Not*I and 3' *Pac*I restriction sites
DWO12	CGACACTTAATTAATATCCAACGGTTGTCTTCGTTTAC	for cloning into pRS313-13myc

O163	CGATCGGCGGCCGCtgcattatttagatcggaaaaagtc	Amplify *S. cerevisiae PHO4* with 5' *Not*I and 3' *Pac*I restriction sites for cloning into
O164	CGACACTTAATTAAtcacgtgctcacgttctgctgtaggtg	pRS313-13myc, with stop codon
O128	CGATCGGCGGCCGCtagtgaagccagtccttcacgccca	Amplify *S. mikatae PHO4* with 5' *Not*I and 3' *Pac*I restriction sites for cloning into
O161	CGACACTTAATTAAtcacgtgctcccattccgttgtagatga	pRS313-13myc, with stop codon
O174	CGATCGGCGGCCGCaggtagaggaggaggcagag	Amplify *S. castellii PHO4* with 5' *Not*I and 3' *Pac*I restriction sites for cloning into
O175	CGACACTTAATTAAggaatttgcttagattcctaatcg	pRS313-13myc, with stop codon
O167	CGATCGGCGGCCGCgtatgcattaccttctcaattagg	Amplify *C. glabrata PHO4* with 5' *Not*I and 3' *Pac*I restriction sites for cloning into
O168	CGACACTTAATTAAtcatttcttgtactgctcaagctcttcg	pRS313-13myc, with stop codon
O157	CGATCGGCGGCCGCcagccatgtactcatgatactg	Amplify *C. albicans PHO4* with 5' *Not*I and 3' *Pac*I restriction sites for cloning into
O158	CGACACTTAATTAAttacttccttcctttcaactcctcaac	pRS313-13myc, with stop codon
O155	TCCTACGAACTTCCAGATGGT	Quantify *S. cerevisiae ACT1* with quantitative reverse-transcription PCR
O156	GGCAGATTCCAAACCCAAAA	
O153	GCCGGTTCCAAAAACGTTTA	Quantify *S. cerevisiae PHO84* with quantitative reverse-transcription PCR
O154	GACAGTGAAGACGGATACCCA	
O151	TGCTTGTAACTCATGTCCTGC	Quantify *S. cerevisiae PHO5* with quantitative reverse-transcription PCR
O152	TTGAGGTCAAGTTCAAACCCT	
O75	gaccaaactacttacaactcc	Quantify *C. glabrata ACT1* with quantitative reverse-transcription PCR
O76	ccactttcgtcgtattcttgcttg	
O78	gccttgggtactctgatcgacc	Quantify *C. glabrata PHO84* with quantitative reverse-transcription PCR
O79a	gtgctgtcggcgacagtaac	
O81	ggttaacgccacaccagtaagagc	Quantify *C. glabrata GIT1* with quantitative reverse-transcription PCR
O82	gatagtggacaattcactctcgtc	