The glycosylphosphatidylinositol-linked aspartyl protease Yps1 is transcriptionally regulated by the calcineurin-Crz1 and Slt2 MAPK pathways in *Candida glabrata*

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Abstract

In the pathogenic fungus *Candida glabrata*, the *YPS1* gene, which encodes a glycosylphosphatidylinositol-linked aspartyl protease, is required for cell wall integrity and virulence. Although the expression of *YPS1* has been studied in *Saccharomyces cerevisiae*, the transcriptional regulation of this gene in *C. glabrata* is not well understood. Here, we report that *C. glabrata* Yps1 is required for cell growth at elevated temperatures, and that the heat-induced expression of *YPS1* is regulated predominantly by the calcineurin-Crz1 pathway and partially by the Slt2 MAPK pathway. Although a total of eleven *YPS* genes are present in the *C. glabrata* genome, loss of transcriptional induction in a calcineurin mutant was observed only for *YPS1*. The results of a *YPS1* promoter-*lacZ* reporter assay using a series of constructs with mutated promoter elements indicated that the transcription factor Crz1 binds to multiple sites in the promoter region of *YPS1*. To date, as none of the putative Crz1 targets in *C. glabrata* have been characterized using a Δ*crz1* mutant, monitoring the expression of *YPS1* represents an effective method for measuring the activity of the calcineurin-Crz1 signaling pathway in this fungus.

Introduction

In *Saccharomyces cerevisiae*, a family of five glycosylphosphatidylinositol-linked aspartyl proteases (Yps1-3, Yps6, and Yps7), known as yapsins, are required for maintaining cell wall integrity (Krysan, *et al.*, 2005, Gagnon-Arsenault, *et al.*, 2006). Yapsins have also been identified in the opportunistic fungal pathogen *Candida glabrata*, which contains eleven *YPS* genes (*YPS1*, *YPS2*, *YPS7*, and a cluster of eight *YPS* genes), encoding Yps proteases that play an important role in cell wall remodeling by removing glycosylphosphatidylinositol-anchored cell wall proteins, such as the adhesin Epa1, from the cell wall (Kaur, *et al.*, 2007). Kaur et al. (2007) have also demonstrated that among the eleven *YPS* genes, *YPS1* serves a primary role for survival of *C. glabrata* within macrophages and virulence in a mouse model of disseminated candidiasis. Although the expression of *S. cerevisiae YPS1* is induced in association with active cell wall synthesis or remodeling under cell wall-damaging conditions, and is co-regulated by the Pkc1-Mpk1/Slt2 and calcineurin-signaling pathways (Krysan, *et al.*, 2005, Gagnon-Arsenault, *et al.*, 2006), it is not known if these regulation mechanisms are active in *C. glabrata*. In this study, we therefore attempted to determine if the Slt2 pathway and calcineurin are involved in the transcriptional regulation of *YPS1* in *C. glabrata*.

Materials and methods

Strains and culture conditions

The *C. glabrata* strains used in this study are listed in Table 1. *C. glabrata* cells were routinely propagated at 30°C in synthetic complete medium (SC), or SC lacking uracil (SC-ura) or tryptophan (Sc-trp) to maintain plasmid selection (Kaiser, *et al.*, 1994).

Deletion and complementation of *YPS1* in *C. glabrata*

Sequence information of *C. glabrata* genes was obtained from the *C. glabrata* genome database Genolevures (http://www.genolevures.org/). The primers and plasmids used in this study are listed in Tables 2 and 3, respectively. The plasmids constructed using PCR products were verified by sequencing before use. Transformation of *C. glabrata* was performed using a lithium acetate protocol, as described previously (Cormack & Falkow, 1999).

A *C. glabrata YPS1* deletion strain was generated using a one-step PCR–based technique, as described previously (Miyazaki, *et al.*, 2010). Briefly, a deletion construct was amplified from pBSK-HIS (Miyazaki, *et al.*, 2010) with the primer pair CgYPS1 100-F and CgYPS1 100-R. *C. glabrata* strain 2001HT (Δ*his3*, Δ*trp1*) (Kitada, *et al.*, 1995) was then transformed with the deletion construct, and the resulting transformants were selected by histidine prototrophy. Both PCR and Southern blotting were performed to verify that the desired homologous recombination occurred at the target locus without ectopic integration of the deletion construct. One successful deletion strain (Δ*yps1*) was selected and designated as TG191.

To generate a complementation plasmid, a 1836-bp DNA fragment containing the *C. glabrata YPS1* gene was amplified from the genomic DNA of the *C. glabrata* wild-type strain CBS138 (Dujon, *et al.*, 2004) with the primer pair CgYPS1-Xba-F and CgYPS1-Xba-R. The PCR product was digested with XbaI and inserted into the XbaI site of pCgACT-P to generate pCgACT-PY1. Strain TG191 was then transformed with pCgACT-P and pCgACT-PY1 to generate strains TG192 and TG193, respectively.

Spot dilution test

To examine sensitivity to high temperature, a spot dilution test was performed as described previously (Miyazaki, *et al.*, 2010). Briefly, logarithmic-phase cells grown in SC-trp broth were harvested and adjusted to 2×10^7 cells/ml. Serial 10-fold dilutions were then prepared, and 5 µl of each dilution was spotted onto SC-trp agar plates in the presence and absence of 1 M sorbitol. Plates were incubated for 48 h at 30 or 41°C. The spot dilution test was repeated twice on independent occasions.

Quantitative real-time RT-PCR

To examine expression levels of the *YPS* genes, logarithmic-phase cells grown in SC-trp broth at 30°C were adjusted to 1×10^7 cells/ml and then further incubated at 41°C with agitation. Total RNA was extracted using a FastRNA Red Kit (Qbiogene, Carlsbad, CA). Quantitative real-time RT-PCR was performed as described previously (Saijo, *et al.*, 2010). Briefly, first strand cDNA was synthesized with a QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA) from 1 µg of total RNA in a final volume of 20 µl, and 3 µl of resulting cDNA was then used as the template for individual PCR with gene-specific primers

(Table 2), using a QuantiTect SYBR Green PCR kit (Qiagen). Quantitative real-time RT-PCR was performed in triplicate in a 96-well plate format, using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The mRNA abundance of the *YPS* genes was normalized to *ACT1* transcript levels. The real-time RT-PCR assays were repeated twice on independent occasions.

Construction of lacZ-reporter plasmids

A 0.4-kb fragment containing the 355-bp upstream region and first 12 codons of *C. glabrata YPS1* was amplified from the genomic DNA of *C. glabrata* CBS138 with primers CgYPS1-355Bam-F and CgYPS1-38Kpn-R. The resulting PCR product was digested with BamHI and KpnI and inserted into the corresponding sites of pEM14 (El Barkani, *et al.*, 2000) to yield pPYPS1. Various deletions were introduced into the *YPS1* promoter sequence in pPYPS1 using the KOD-Plus-Mutagenesis Kit (Toyobo, Osaka, Japan) and mutagenic primers (Table 2 and Fig. 3b). Plasmid pPYPS1-D2 was used as the template to generate plasmids pPYPS1-D42 and pPYPS1-D52. All plasmids generated by site-directed deletion mutagenesis were verified by DNA sequencing.

β-galactosidase assay

C. glabrata strain 2001TU (Δ*trp1*, Δ*ura3*) (Kitada, *et al.*, 1995) was transformed with the series of constructed *YPS1* promoter-*lacZ* reporter plasmids (Fig. 3b). Strain TG174 (Δ*crz1*, Δ*trp1*, Δ*ura3*), which was obtained by plating strain TG171 (Δ*crz1*, Δ*trp1*) (Miyazaki, *et al.*, 2010) on 5-fluoroorotic acid-containing media as previously described (Boeke, *et al.*, 1984), was transformed with pPYPS1. Transformants were selected by uracil prototrophy and

verified by PCR using plasmid-specific primers. Logarithmic-phase cells grown in SC-ura broth were adjusted to 1×10^7 cells/ml and subjected to a temperature shift from 30 to 41°C. After 3 h of incubation at 41°C, the cell cultures were harvested and washed twice with ice-cold phosphate-buffered saline. Cells (100 µl) were re-suspended in 300 µl Reporter Lysis Buffer (Promega, Madison, WI) containing 5 µl Protease Inhibitor Cocktail (Sigma, St. Louis, MO). Cell extracts were prepared using acid-washed glass beads (Sigma) and cleared by centrifugation at 14,000 x *g* for 30 min at 4°C. Protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA) using bovine serum albumin as a standard. β-galactosidase activity was measured using the β-Galactosidase Enzyme Assay System (Promega) according to the manufacturer's instructions. All assays were performed in triplicate on separate days. β-galactosidase activities of each reporter construct were calculated in Miller units (nmoles/min/mg of protein) at 37°C (Miller, 1972). The activity of each mutated promoter is expressed relative to the value obtained with the intact promoter.

Results and discussion

C. glabrata requires Yps1 for cell growth at elevated temperatures

Several phenotypic differences in the Δ*yps1* strains of *S. cerevisiae* and *C. glabrata* have been reported. For example, while the loss of Yps1 results in decreased tolerance to Congo red and caspofungin in *S. cerevisiae* (Krysan*, et al.*, 2005), a similar sensitivity is not displayed by *C. glabrata* (Kaur*, et al.*, 2007). As the effect of *YPS1* deletion on temperature sensitivity has not been reported in *C. glabrata*, we examined the growth of the *C. glabrata* Δ*yps1* strain at 30 and 41°C using a spot dilution assay (Fig. 1). The growth of the mutant was similar to that of the wild-type strain at 30°C, but it was drastically impaired at 41°C. However, either reintroduction of the wild-type *YPS1* gene or the addition of 1 M sorbitol, an osmotic stabilizer, into the medium could effectively restore growth of the mutant. These results suggest that *C. glabrata* Yps1 plays a role in maintaining cell wall integrity to prevent cell lysis at elevated temperatures.

Heat-induced expression of *YPS1* is predominantly regulated by the calcineurin-Crz1 pathway in *C. glabrata*

To examine whether transcription of *C. glabrata YPS1* is upregulated in response to heat stress, we monitored the expression levels of *YPS1* mRNA using real-time RT-PCR. Since the signaling pathways controlling *YPS1* expression in *C. glabrata* are unclear, we also examined *YPS1* expression levels in mutants lacking a key component of two cell wall-associated signaling pathways, including the last member of the PKC1-MAPK cascade (Slt2), the regulatory B subunit of the serine-threonine-specific protein phosphatase calcineurin (Cnb1), and the calcineurin-regulated transcription factor Crz1. The expression

level of *C. glabrata YPS1* increased in the wild-type strain 1 h after a temperature shift from 30 to 41°C, and this upregulation was sustained for the duration of the 4-h experiment (Fig. 2a), which is consistent with the kinetics of most genes involved in cell wall remodeling (Garcia*, et al.*, 2004). Notably, the transcriptional upregulation of *YPS1* was not observed in the Δ*cnb1* or Δ*crz1* mutants (Fig. 2a). In *S. cerevisiae*, treatment of wild-type cells with sodium dodecyl sulfate or calcofluor white leads to increased expression of *YPS1*, but has no effect in a strain lacking *MPK1*/*SLT2* (Krysan*, et al.*, 2005, Gagnon-Arsenault*, et al.*, 2006). Here, we observed that the deletion of *SLT2* in *C. glabrata* impaired the heat-induced expression of *YPS1* compared to the wild-type strain, however expression was not completely abolished (Fig. 2a). Rlm1 is a transcription factor downstream of Slt2 and responsible for most of the transcriptional activation of genes required for cell wall integrity in *Saccharomyces cerevisiae* (Garcia*, et al.*, 2004). However, the *YPS1* expression levels in a *C. glabrata* Δ*rlm1* mutant (Miyazaki*, et al.*, 2010) were similar to those in the wild-type strain after 3 h of incubation at 41°C (data not shown), indicating that Slt2 regulated *YPS1* expression independently of Rlm1 in *C. glabrata*. The results were consistent with previous findings in *S. cerevisiae* that induction of *YPS1* expression after exposure to calcofluor white requires Slt2 but not Rlm1 (Krysan*, et al.*, 2005). Further studies investigating how Slt2 regulates *YPS1* expression in these fungi are warranted.

We also compared the heat-induced expression levels of *YPS1* in the *CNB1*-, *CRZ1*-, and *SLT2*-overexpressing strains to that of the wild-type *C. glabrata* strain (Fig. 2b). Although the expression levels of *YPS1* in the *CNB1*- and *SLT2*-overexpressing strains were comparable to that of wild-type, expression of *YPS1* in the *CRZ1*-overexpressing strain increased approximately 5-fold even in the absence of heat stress, and increased more than 20-fold at

41°C (Fig. 2b). Taken together, these results suggest that stimulated expression of *YPS1* in *C. glabrata* in response to elevated growth temperature is regulated by both the Slt2 MAPK pathway and calcineurin signaling via the Crz1 transcription factor, with the latter representing the dominant mechanism.

Dissection of the *C. glabrata YPS1* promoter

To further validate the speculation that Crz1 regulates transcription of *YPS1* in *C. glabrata*, the *YPS1* promoter elements were dissected using a *lacZ* reporter system. The 355-bp sequence immediately upstream of the *YPS1* open reading frame and the putative transcription factor binding sites in this region are shown in Fig. 3a. The intact 355-bp promoter region and first 12 codons of *YPS1* were fused to *lacZ* in pEM14 to generate the pPYPS1 reporter construct. Site-directed deletion mutagenesis was then used to sequentially introduce mutations into the promoter region to construct the reporter plasmids illustrated in Fig. 3b. The β-galactosidase activities of these reporter constructs in strains 2001TU and TG174 were then compared after 3 h of incubation at 41°C (Fig. 3c). Interestingly, we identified a sequence, 5'-ATGACGTAAT-3', in the upstream region (-306 to -297) of *C. glabrata YPS1* matching the consensus sequence of the *S. cerevisiae* Sko1-binding site, 5'-ATKACGTMAT-3' (Proft, *et al.*, 2005) (Fig. 3a). The transcription factor Sko1 has been characterized as a downstream target of the Hog1 signaling pathway in *S. cerevisiae* (Proft, *et al.*, 2001, Proft & Struhl, 2002). However, Sko1-mediated transcriptional regulation of *YPS1* was not evident, since deletion of this sequence did not affect promoter activity, as evidenced by the high level induction of β-galactosidase activity in the strain containing pPYPS1-D1 (Fig. 3c).

We also identified the sequence 5'-GGGGCTCC-3', which was similar to the consensus Crz1-binding site 5'-GNGGC(G/T)CA-3' (Yoshimoto, *et al.*, 2002), in the upstream region (-166 to -159) of *C. glabrata YPS1* (Fig. 3a). Deletion of this sequence resulted in only a slight decrease of promoter activity, displaying 90% activity of the intact promoter at 41°C (pPYPS1-D2 in Fig. 3c). A genome-wide analysis in *S. cerevisiae* previously revealed that the promoters of most calcineurin-dependent genes contain 1-6 copies of the Crz1-binding sequence 5'-GNGGC(G/T)-3', with any base substitution in the core "GGCT" sequence resulting in the elimination of Crz1p binding, except the T to G change (Yoshimoto, *et al.*, 2002). Similarly, 5 copies of the core Crz1-binding sequence "GGCT" were present within the 355-bp upstream region of *C. glabrata YPS1* ("GGCG" was not found), as shown in Fig. 3a. Therefore, we extended the originally deleted regions in the *YPS1* promoter to sequentially remove the core Crz1-binding sequences, as summarized in Fig. 3b. The pPYPS1-D4 construct, in which the first and second "GGCT" sites were deleted, showed 72% activity of the intact promoter at 41°C (Fig. 3c). The induction of promoter activity of pPYPS1-D42, which contained the additional deletion of the putative Crz1 binding sequence, 5'-GGGGCTCC-3', was significantly impaired compared to the results with pPYPS1-D4 and pPYPS1-D2, suggesting that both regions contribute to the full activity of *YPS1* promoter. Compared with the activity levels of pPYPS1, pPYPS1-D42 displayed 54% activity at 30°C but only 24% activity at 41°C. The induction ratios of pPYPS1 and pPYPS1-D42 were 4.10 and 1.86, respectively. The results suggested that the deletions in pPYPS1-D42 did not drastically affect the basal activity of the *YPS1* promoter but impaired induction of promoter activity at elevated temperature. On the other hand, the absence of the putative TATA box, 5'-TATATAT-3', or the first three "GGCT" sites impaired not only

induction but also the basal activity of the *YPS1* promoter (pPYPS1-D3 and pPYPS1-D5, respectively). To examine whether the drastically decreased promoter activity of pPYPS1-D5 was solely due to the loss of the -221 to -200 region containing the third "GGCT", we constructed pPYPS1-D5-4, in which only the -221 to -200 region was deleted. The pPYPS1-D5-4 construct displayed 83% activity of the intact promoter at 41°C, suggesting that multiple sites were involved in the induction of the *YPS1* promoter activity. As expected, the promoter activity was completely lost by the simultaneous deletion of the putative Crz1-binding sequence and the first three or four "GGCT" sites (pPYPS1-D52 and pPYPS1-D6, respectively). The results from these promoter assays suggest that *C. glabrata* Crz1 regulates *YPS1* transcription via multiple binding sites in the promoter region of this gene. Consistent with the results of the real-time RT-PCR assay, no β-galactosidase activity was detected in the Δ*crz1* mutant containing pPYPS1.

Transcription of yapsin genes other than *YPS1* is not regulated by the calcineurin-Crz1 or Slt2 pathway in *C. glabrata*

In addition to *YPS1*, the expression levels of the other ten *YPS* genes in *C. glabrata* were also examined by quantitative real-time RT-PCR in the wild-type, Δ*cnb1*, Δ*crz1*, and Δ*slt2* strains after a 2-h incubation at 41°C (Fig. 4). Although *YPS3* and *YPS5* were not transcriptionally induced in response to elevated growth temperature, the expression levels of the remaining eight *YPS* genes (*YPS2, YPS4* and *YPS6-11*) were increased at 41°C in all the strains tested in this study compared to the respective control data obtained at 30°C. The results suggested that the upregulated *YPS* genes might be involved in cellular adaptation to high temperature independently of the calcineurin-Crz1 and Slt2 pathways. In agreement with

the results of the gene expression assay, a putative Crz1p-binding motif could not be found in the 1-kb upstream regions of the *YPS* genes other than *YPS1*.

Although the induced expression levels of *YPS4* and *YPS10* in the Δ*cnb1* mutant were higher than those in the wild-type strain, this phenomenon was not observed in the Δ*crz1* mutant. In addition, none of the *YPS* genes were compensatory upregulated in the Δ*yps1* mutant and overexpression of *YPS1* in the Δ*cnb1* mutant did not affect the expression levels of *YPS4* and *YPS10* (data not shown). These results suggested that *YPS4* and *YPS10* were upregulated as a general response to loss of calcineurin independently of *YPS1* expression levels.

Concluding remarks

Although a previous genome-wide analysis in *S. cerevisiae* revealed that the expression of more than 160 genes is regulated in a calcineurin-Crz1-dependent manner (Yoshimoto, *et al.*, 2002), none of the orthologous genes have been studied in a *C. glabrata* Δ*crz1* mutant. Our present study has provided evidence that Yps1 is required for the growth of *C. glabrata* at high temperatures and that the heat-induced expression of the *YPS1* gene is dependent on the calcineurin-Crz1 pathway. The results of the β-galactosidase assays performed using mutated *YPS1* promoters suggest that *C. glabrata* Crz1 may bind to multiple sites in the *YPS1* promoter region to induce transcription of this gene. The *YPS1* promoter-*lacZ* reporter system established in this study will be a useful tool for monitoring activation of the calcineurin-Crz1 signaling pathway in *C. glabrata*.

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Table 1. *Candida glabrata* strains used in this study

Strain	Genotype or description	Reference or source
CBS138	Wild-type	(Dujon, *et al.*, 2004)
2001T	Δ*trp1*	(Kitada, *et al.*, 1995)
2001HT	Δ*his3*, Δ*trp1*	(Kitada, *et al.*, 1995)
2001TU	Δ*trp1*, Δ*ura3*	(Kitada, *et al.*, 1995)
TG11	2001T containing pCgACT-P	(Miyazaki, *et al.*, 2010)
TG151	Δ*slt2*::*HIS3*, Δ*trp1* (made from 2001HT)	(Miyazaki, *et al.*, 2010)
TG152	TG151 containing pCgACT-P	(Miyazaki, *et al.*, 2010)
TG153	TG151 containing pCgACT-PS2	(Miyazaki, *et al.*, 2010)
TG161	Δ*cnb1*::*HIS3*, Δ*trp1* (made from 2001HT)	(Miyazaki, *et al.*, 2010)
TG162	TG161 containing pCgACT-P	(Miyazaki, *et al.*, 2010)
TG163	TG161 containing pCgACT-PNB	(Miyazaki, *et al.*, 2010)
TG164	TG161 containing pCgACT-PY1	This study
TG171	Δ*crz1*::*HIS3*, Δ*trp1* (made from 2001HT)	(Miyazaki, *et al.*, 2010)
TG172	TG171 containing pCgACT-P	(Miyazaki, *et al.*, 2010)
TG173	TG171 containing pCgACT-PRZ	(Miyazaki, *et al.*, 2010)
TG174	Δ*crz1*::*HIS3*, Δ*trp1*, Δ*ura3*	This study
TG181	Δ*rlm1*::*HIS3*, Δ*trp1* (made from 2001HT)	(Miyazaki, *et al.*, 2010)
TG182	TG181 containing pCgACT-P	(Miyazaki, *et al.*, 2010)
TG191	Δ*yps1*::*HIS3*, Δ*trp1* (made from 2001HT)	This study
TG192	TG191 containing pCgACT-P	This study
TG193	TG191 containing pCgACT-PY1	This study
TU-EM14	2001TU containing pEM14	This study
TU-Y1	2001TU containing pPYPS1	This study
TU-YD1	2001TU containing pPYPS1-D1	This study
TU-YD2	2001TU containing pPYPS1-D2	This study
TU-YD3	2001TU containing pPYPS1-D3	This study
TU-YD4	2001TU containing pPYPS1-D4	This study
TU-YD42	2001TU containing pPYPS1-D42	This study
TU-YD5	2001TU containing pPYPS1-D5	This study
TU-YD5-4	2001TU containing pPYPS1-D5-4	This study
TU-YD52	2001TU containing pPYPS1-D52	This study
TU-YD6	2001TU containing pPYPS1-D6	This study
TG174-Y1	TG174 containing pPYPS1	This study

Table 2. Primers used in this study

For strain and plasmid construction	
Primer [a]	Sequence (5' – 3') [b]
CgYPS1 100-F	*CACGCTAATGGCTTGGGGGGGAAGGGCAAGGGCATTAACT GCTGATGGGGCTCCCCTGCAAGGCTTGGATCTTGATAATTG CCATATATATAGCTTGTA***TAATACGACTCACTATAGGGC**
CgYPS1 100-R	*GCATTTAAAGAACTCCAGCTTTGCTTAATATCAAATGAACAAA GAAATCCTCACCAGGAGCACCGGTTGATGCTATTCCAGGGT GAGAGCCATCTTCAG***CGCTCTAGAACTAGTGGATCC**
CgYPS1-Xba-F	GCTCTAGAATGAAGTTTAGTTCGCTATGTATGC
CgYPS1-Xba-R	GCTCTAGAGGGTGAGAGCCATCTTCAGAATG
CgYPS1-355Bam-F	CGGGATCCTGAAGCCCGAGAGAAATCCC
CgYPS1-38Kpn-R	AAGGTACCGCAACAGACGCCAGCATACATAG
YPS1prm-F1	TTGCGCATCTTGAAAAAACAA
YPS1prm-F2	CCCTGCAAGGCTTGGATCT
YPS1prm-F3	CTTGTAAAATGTGATTCCTGAATACC
YPS1prm-F4	TTTCTTTTTCACGCTAATGGC
YPS1prm-F5	TGGGGGGGAAGGGCAAGG
YPS1prm-F6	TGGATCTTGATAATTGCCA
YPS1prm-R1	GTGCTTTGCTTCTTTGCTTC
YPS1prm-R2	ATCAGCAGTTAATGCCCTTGC
YPS1prm-R3	GGCAATTATCAAGATCCAAGC
YPS1prm-R4	GCTTTAATTTGAATTTTCTTC
YPS1prm-R5	TTTTCAAGCCCTTTTCAAGCCCTTT
For real-time PCR	
Primer [a]	Sequence (5' – 3')
CgYPS1-F1281	CGGATTCCACATCAACGCTC
CgYPS1-R1436	TCGTAGTTTTCCAGGTCGTAGACG
CgYPS2-F1518	CACTGAGTTCCCATCTTCTTATGCC
CgYPS2-R1647	CGTGTTTGTGTTTCTGCTTGTCC

CgYPS3-F944	ACGCACCAAAGCAAGTCGTC
CgYPS3-R1079	GCACCAGCAAAGTTGAAGATAAGC
CgYPS4-F490	AATGGCGTGAAGGTTGATAACG
CgYPS4-R603	GGCAGCAAGTTGATTTGTTGTCTC
CgYPS5-F312	TGATGGATTCCCCACTTCGC
CgYPS5-R461	GCAAATGTCTCGTCACCGTAGC
CgYPS6-F305	CTGGCTTTCCAACAAACCCTG
CgYPS6-R495	CAATCCACCCAAACTAACCACATC
CgYPS7-F438	CAGCGGTGTGAGTTTCAGGAAC
CgYPS7-R602	AGGAAGAAGAACGAGTGGTTGATG
CgYPS8-F510	TGTGAACTTTGCCTTGGGGAG
CgYPS8-R632	GAATCGCTGATGTTTTTCCTGC
CgYPS9-F70	AAGACCCCAGGCAGTGATTTG
CgYPS9-R198	TTGTAGCAAGACCTTCTGAGGAGG
CgYPS10-F310	GCACAGGTTCCCAAATCGTTG
CgYPS10-R439	CAGCGTAGGATGAGTCCAAGTAGC
CgYPS11-F1269	TGACACTTTGCCAGAAGACATCG
CgYPS11-R1414	TTGTGCTGCCGTTTGAGGAG
CgACT1-F163	GGTATGGGTCAAAAGGACTCTTACG
CgACT1-R305	TCGTTGTAGAAAGTGTGATGCCAG

[a] "F" and "R" indicate forward and reverse primers, respectively.

[b] Sequences homologous to flanking regions of the *YPS1* open reading frame are shown in italics. Sequences shown in boldface are present in pBSK-HIS. Restriction sites are underlined.

Table 3. Plasmids used in this study [a]

Plasmid	Description	Reference or source
pBSK-HIS	A 1-kb XhoI fragment containing *C. glabrata HIS3* was inserted into the XhoI site of pBluescript II SK+ (Stratagene).	(Miyazaki, *et al.*, 2010)
pCgACT-P	A 1-kb SacI-KpnI fragment containing the *S. cerevisiae PGK1* promoter, polylinker and *C. glabrata HIS3* 3'UTR was excised from pGRB2.2 (Frieman, *et al.*, 2002) and inserted into the SacI-KpnI site of pCgACT (Kitada, *et al.*, 1996).	(Miyazaki, *et al.*, 2010)
pCgACT-PS2	A 1625-bp fragment containing *C. glabrata SLT2* was inserted into the SalI site of pCgACT-P	(Miyazaki, *et al.*, 2010)
pCgACT-PNB	A 580-bp fragment containing *C. glabrata CNB1* was inserted into the SmaI site of pCgACT-P	(Miyazaki, *et al.*, 2010)
pCgACT-PRZ	A 1938-bp fragment containing *C. glabrata CRZ1* was inserted into the EcoRI site of pCgACT-P	(Miyazaki, *et al.*, 2010)
pCgACT-PY1	A 1836-bp fragment containing *C. glabrata YPS1* was inserted into the XbaI site of pCgACT-P	This study
pEM14	*C. glabrata* centromere and autonomously replicating sequence-based plasmid containing *S. cerevisiae URA3* and *E. coli lacZ*	(El Barkani, *et al.*, 2000)
pPYPS1	A 0.4-kb fragment containing the 355-bp promoter region and the first 12 codons of *C. glabrata YPS1* was inserted into the BamHI-KpnI site of pEM14.	This study

[a] The series of lacZ-reporter plasmids constructed from pPYPS1 are summarized in Fig. 3b.

Figure legends

Fig. 1. Yps1 is required for cell growth at elevated temperature in *C. glabrata*. After logarithmic-phase cells were adjusted to 2×10^7 cells/ml, 5 μl of serial 10-fold dilutions was spotted onto an SC-trp agar plate in the presence and absence of 1 M sorbitol. Plates were photographed after 48 h of incubation at the indicated temperatures. Representative results of two independent experiments are shown. *C. glabrata* strains: wild-type, 2001T containing an empty vector (strain TG11); Δ*yps1*, a Δ*yps1* strain containing an empty vector (strain TG192); and Δ*yps1* + *YPS1*, a Δ*yps1* strain containing pCgACT-PY1 (strain TG193).

Fig. 2. Time-course analysis of *YPS1* expression in mutant (a) and overexpressing (b) strains of *CNB1*, *CRZ1*, and *SLT2* at elevated growth temperature. Logarithmic-phase cells grown at 30°C were adjusted to 1×10^7 cells/ml and then further incubated at 41°C. Total RNA was extracted at the indicated time points, and *YPS1* mRNA was measured in triplicate by quantitative real-time RT-PCR. Results represent the average of two independent experiments. Error bars are standard deviations. *C. glabrata* strains: wild-type, strain TG11; Δ*cnb1*, strain TG162; Δ*crz1*, strain TG172; Δ*slt2*, strain TG152; *CNB1*-OE, a *CNB1*-overexpressing strain (TG163); *CRZ1*-OE, a *CRZ1*-overexpressing strain (TG173); and *SLT2*-OE, an *SLT2*-overexpressing strain (TG153).

Fig. 3. Dissection of putative promoter elements in the upstream region of *C. glabrata YPS1*.

(a) The 355-bp sequence upstream of *C. glabrata YPS1*. Nucleotide sequence numbers relative to the transcription start site (A of ATG) are indicated. A putative Sko1-binding site, 5'-ATGACGTAAT-3', is underlined (-306 to -297), while a putative Crz1-binding site, 5'-GGGGCTCC-3', is shown in a grey box (-166 to -159). Five copies of the core Crz1-binding sequence "GGCT" are italicized and underlined. A putative TATA box,

5'-TATATAT-3', is shown in bold and underlined (-128 to -122). (b) A summary of the deletions introduced into the *YPS1* promoter sequences. Symbols: open box, putative Sko1-binding site; shaded circle, core Crz1-binding sequence; shaded box, putative TATA box. (c) β-Galactosidase assay. *C. glabrata* wild-type strain (2001TU) containing a single copy of the reporter constructs was incubated at 41°C for 3 h. The Δ*crz1* mutant containing pPYPS1 was also included as a control. β-galactosidase activities were calculated in Miller units (nmoles/min/mg of protein) at 37°C. The activity of each reporter construct is expressed relative to that of pPYPS1 in the wild-type strain at 30°C. Three independent transformants for each reporter construct were assayed in triplicate. Error bars represent standard deviations from the means of three independent experiments.

Fig. 4. Relative mRNA abundance of *YPS* genes in response to elevated growth temperature. Logarithmic-phase cells grown in SC-trp broth at 30°C were further incubated at 41°C for 2 h, and total RNA was then extracted. Relative mRNA abundance was measured by quantitative real-time RT-PCR. Results are expressed as the mean $\pm$ standard deviation. The quantitative real-time RT-PCR was performed in triplicate and representative data of two independent experiments are shown. *C. glabrata* strains: wild-type, strain TG11; Δ*cnb1*, strain TG162; Δ*crz1*, strain TG172; and Δ*slt2*, strain TG152.

Fig. 1.

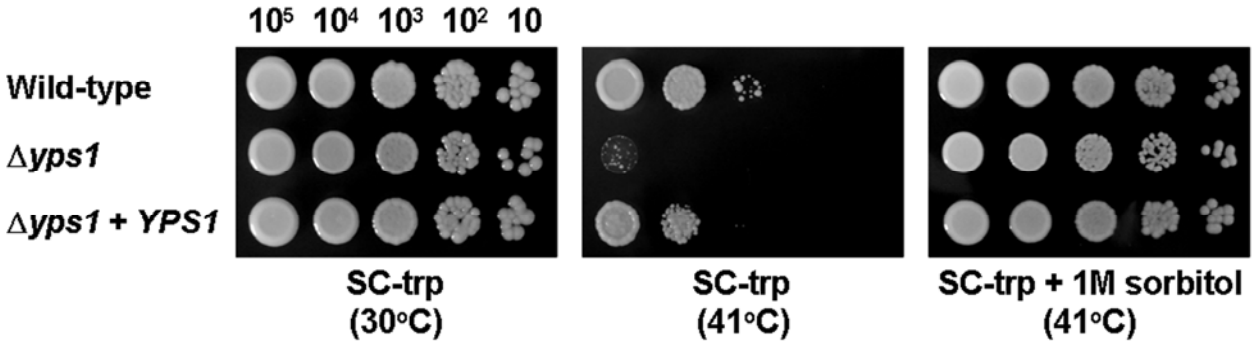

10⁵ 10⁴ 10³ 10² 10
Wild-type
Δyps1
Δyps1 + YPS1
SC-trp (30°C)
SC-trp (41°C)
SC-trp + 1M sorbitol (41°C)

Fig. 2.

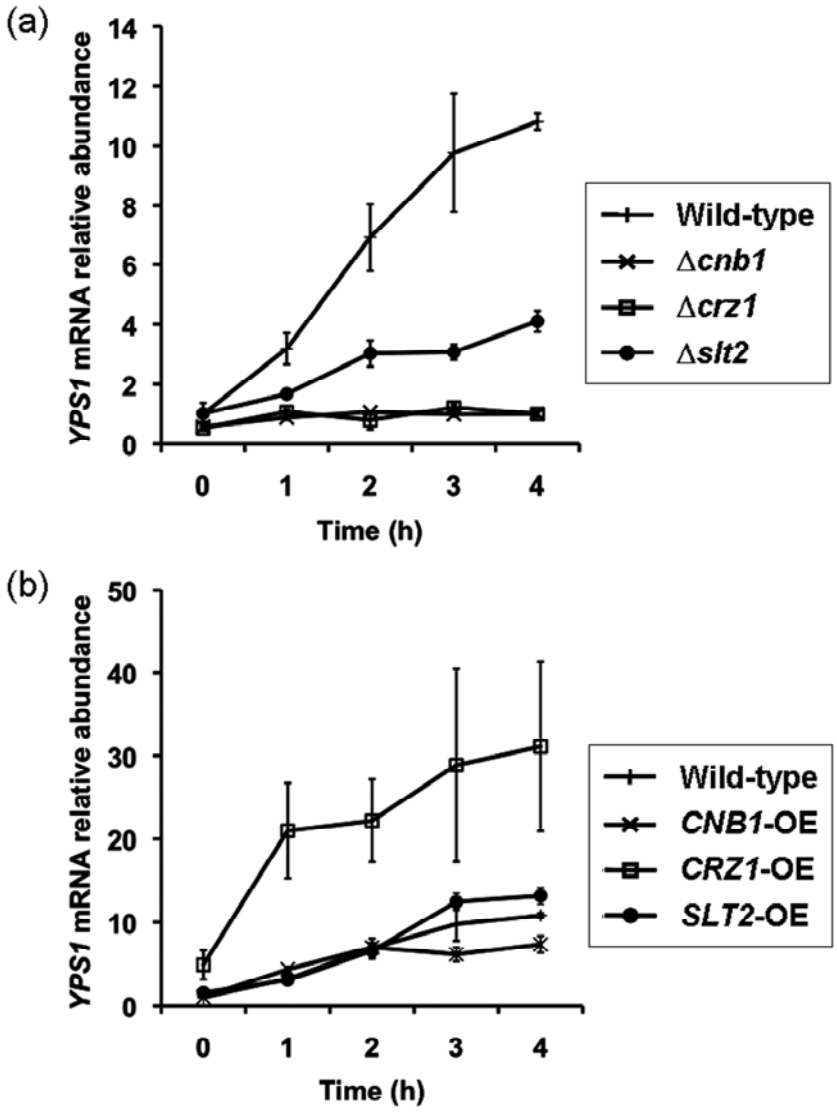

(a)
YPS1 mRNA relative abundance
Time (h)
Wild-type
Δcnb1
Δcrz1
Δslt2
(b)
YPS1 mRNA relative abundance
Time (h)
Wild-type
CNB1-OE
CRZ1-OE
SLT2-OE

Fig. 3.

(a)

(-350) (-301)
CCCGAGAGAAATCCCGCACAAGAGAAGCAAAGAAGCAAAGCACAATGACG

(-300) (-251)
TAATTTGCGCATCTTGAAAAAACAAATTTGAAGAAAATTCAAATTAAAGC

(-250) (-201)
TGAAAAGGGCTTGAAAAGGGCTTGAAAAGTTTCTTTTTCACGCTAATGGC

(-200) (-151)
TTGGGGGGGAAGGGCAAGGGCATTAACTGCTGATGGGGCTCCCCTGCAAG

(-150) (-101)
GCTTGGATCTTGATAATTGCCATATATATAGCTTGTAAAATGTGATTCCT

(-100) (-51)
GAATACCTTTACTCTAGTACCAGAACTAGAACTAGAACTAGAACCAGAAC

(-50) (-1)
TACAAGTAAAACTAGAACTAGAACTACAAGTACAAACACACATACAGAAT

(b)

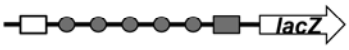
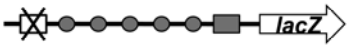
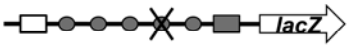
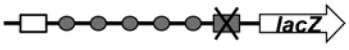
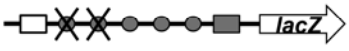
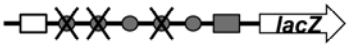
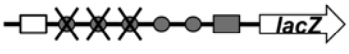
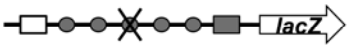
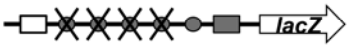
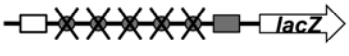
	Plasmid name	Mutagenic Primer	Deleted region
	pPYPS1	N/A	N/A
	pPYPS1-D1	YPS1prm-F1 & YPS1prm-R1	-307 to -297
	pPYPS1-D2	YPS1prm-F2 & YPS1prm-R2	-166 to -160
	pPYPS1-D3	YPS1prm-F3 & YPS1prm-R3	-129 to -120
	pPYPS1-D4	YPS1prm-F4 & YPS1prm-R4	-250 to -222
	pPYPS1-D42	YPS1prm-F4 & YPS1prm-R4	-250 to -222, -166 to -160
	pPYPS1-D5	YPS1prm-F5 & YPS1prm-R4	-250 to -200
	pPYPS1-D5-4	YPS1prm-F5 & YPS1prm-R5	-222 to -200
	pPYPS1-D52	YPS1prm-F5 & YPS1prm-R4	-250 to -200, -166 to -160
	pPYPS1-D6	YPS1prm-F6 & YPS1prm-R4	-250 to -148

Fig. 3.

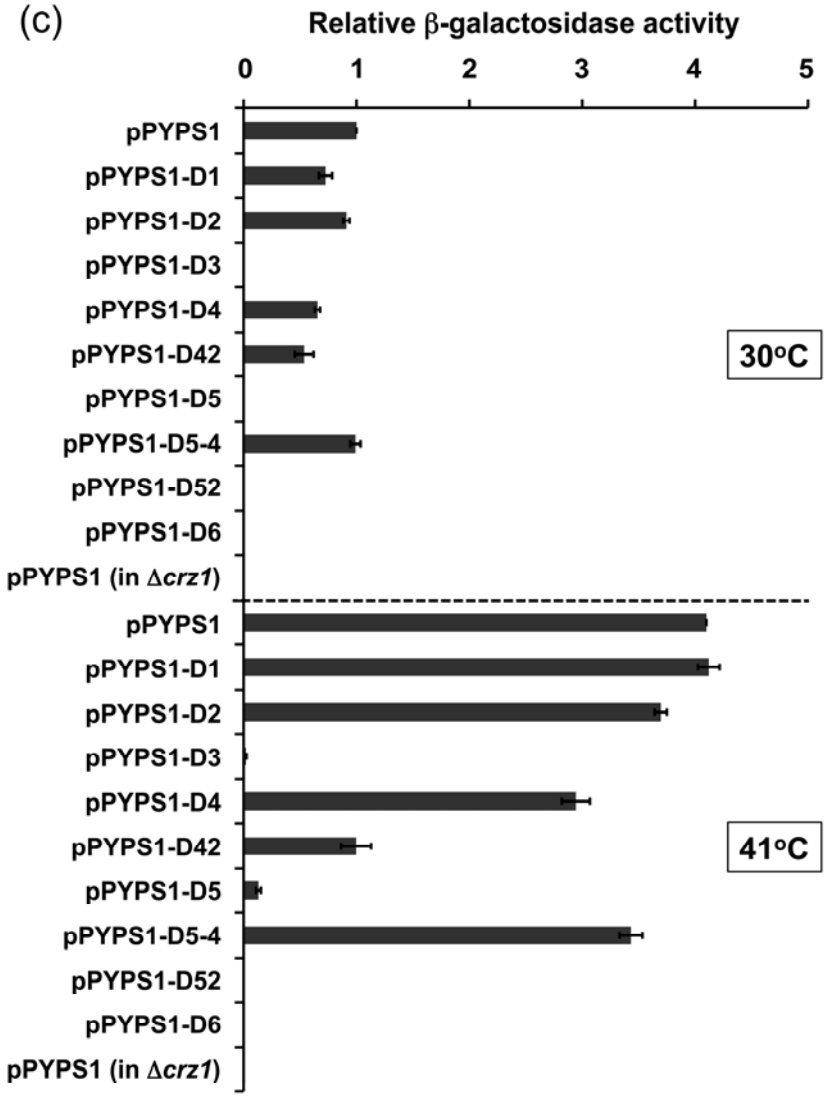
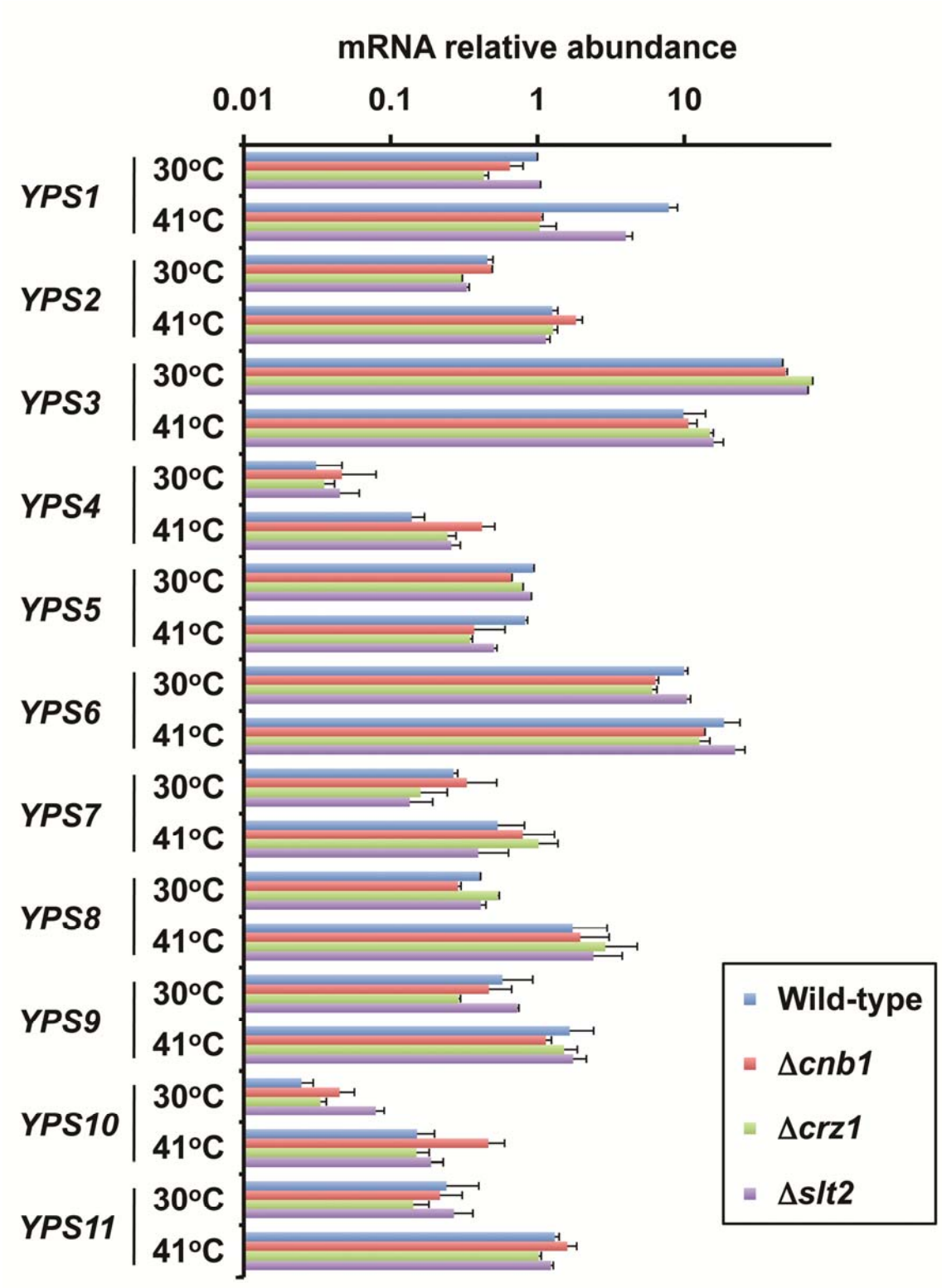

(C)
Relative β-galactosidase activity
0
1
2
3
4
5
pPYPS1
pPYPS1-D1
pPYPS1-D2
pPYPS1-D3
pPYPS1-D4
pPYPS1-D42
pPYPS1-D5
pPYPS1-D5-4
pPYPS1-D52
pPYPS1-D6
pPYPS1 (in Δcrz1)
30°C
pPYPS1
pPYPS1-D1
pPYPS1-D2
pPYPS1-D3
pPYPS1-D4
pPYPS1-D42
pPYPS1-D5
pPYPS1-D5-4
pPYPS1-D52
pPYPS1-D6
pPYPS1 (in Δcrz1)
41°C

Fig. 4.


mRNA relative abundance
0.01
0.1
1
10
YPS1
YPS2
YPS3
YPS4
YPS5
YPS6
YPS7
YPS8
YPS9
YPS10
YPS11
30°C
41°C
Wild-type
Δcnb1
Δcrz1
Δslt2