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Specialized Sugar Sensing in Diverse Fungi

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Summary

S. cerevisiae senses glucose and galactose differently. Glucose is detected through sensors that reside in the cellular plasma membrane. When activated, the sensors initiate a signal transduction cascade that ultimately inactivates the Rgt1 transcriptional repressor by causing degradation of its co-repressors Mth1 and Std1 [1,2]. This results in expression of many *HXT* genes encoding glucose transporters [3]. The ensuing flood of glucose into the cell activates Mig1, a transcriptional repressor that mediates 'glucose repression' of many genes, including the *GAL* genes; hence, glucose sensing hinders galactose utilization [4-6]. Galactose is sensed in the cytoplasm *via* Gal3. Upon binding galactose (and ATP), Gal3 sequesters the Gal80 protein, thereby emancipating the Gal4 transcriptional activator of the *GAL* genes [7]. Gal4 also activates expression of *MTH1* encoding a co-repressor critical for Rgt1 function [8]. Thus, galactose inhibits glucose assimilation by encouraging repression of *HXT* genes. *C. albicans* senses glucose similarly to *S. cerevisiae*, but does not sense galactose through Gal3/Gal80/Gal4 [9]. Its genome harbors no *GAL80* orthologue, and the severely truncated CaGal4 does not regulate *CaGAL* genes [9,10]. We present evidence that *C. albicans* senses galactose with its Hgt4 glucose sensor, a capability that is enabled by transcriptional 'rewiring' of its sugar-sensing signal transduction pathways (Fig. 1). We suggest that galactose sensing through Hgt4 is ancestral in fungi.

Results and Discussion

Hgt4 affects cell growth and filamentation on galactose

C. albicans Δhgt4 mutants cannot grow on glucose in the presence of the respiration inhibitor antimycin A [11], which forces cells to ferment glucose and demands a high rate of glucose influx. Because galactose and glucose are structurally similar, it seemed plausible that the Hgt4 glucose sensor might sense galactose. Indeed, *Δhgt4* cells have a marked growth defect on galactose with antimycin A (Fig. S1A), suggesting that Hgt4 is required for galactose utilization (See Table S3 for strains used in this study). Galactose induces robust filamentation (yeast-to-hyphal morphogenesis) of *C. albicans* cells, and the *Δhgt4* cells are also defective in this response (Fig. S1B). Thus, in the absence of Hgt4, *C. albicans* cells display growth and morphological defects in galactose.

Galactose and glucose induce expression of the same genes

Expression of 49 genes increased by 2-fold or greater (Table 1, Groups I-III) in response to 2% galactose (compared to glycerol). Most of these galactose-induced genes (40, or 82%) are

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also significantly induced by 2% glucose (Table 1, Group I). Six of the nine genes that were not induced by 2 % glucose are in fact induced by low glucose levels (<0.2%), but have been shown to be repressed in cells exposed to the high level of glucose used here (Table 1, Group II) [11-15]. Only three genes are modestly induced by galactose but not induced by glucose (Table 1, Group III). Therefore, 94% (46/49) of the characterized genes that are induced in response to galactose are also induced in response to low or high levels of glucose.

Hgt4 affects the transcriptional response to galactose

Expression of five of the top genes listed in Table 1 (Group I) was re-examined by RT-PCR analysis. In cells grown on glycerol, these genes are either not expressed (*HGT7, QDR1, AOX2*) or expressed at low levels (*CMK1, HXK2*), and all five are induced in response to galactose in an Hgt4-dependent manner (Fig. 2). *HGT12*, encoding a glucose transporter related to Hgt4 [11,16], does not affect the expression of these genes. Induction of *GAL1* expression by galactose is significantly diminished in the *Δhgt4* mutant (Fig. S2), consistent with the previous observation that the Hgt4 signal increases *GAL1* and *GAL7* expression two-fold [11,12]. Galactose still induces *GAL1* expression in *Δhgt4* cells, indicating another signaling pathway contributes to *GAL1* expression, possibly by acting upon Cph1 (a *C. albicans* homologue of *S. cerevisiae* Ste12) [9].

The *CaHGT7* gene, encoding a hexose transporter, is highly induced – over 30-fold – by both galactose and glucose (Table 1). *HGT7* expression is activated by low levels (0.04%) of glucose, fructose, or mannose (Fig. 3A, top), and by a high level (1.6%) of galactose (Fig. 3A, bottom). *HGT7* expression in response to sugars is entirely dependent on *HGT4* (Fig. 3B), and Hgt4 mediates the dose-dependent galactose induction *of HGT7* expression at concentrations as low as 0.6% (Fig. S3).

Galactose-induced genes have Rgt1-binding sites

Of the 50 genes most highly induced by galactose, 34 of them (68%) contain at least one consensus Rgt1 consensus DNA-binding motif (5′-CGGANNA-3′) within 1 kilobase upstream of the translational start codon (Table S1). This is a significant enrichment ($p<10^{-3}$) — only 46% of promoters genome-wide harbor an Rgt1 motif — that is similar to the enrichment of consensus Rgt1-binding sites upstream of genes regulated by glucose *via* Hgt4 and CaRgt1 (66%, $p<10^{-5}$) (See Experimental Procedures in Supplementary Material for statistical methods). The CaCph1 transcription factor has also been implicated in the expression of the *CaGAL* genes in response to galactose [9], but its binding-site is not enriched in other galactose-induced genes (Table S1). The promoters of the *GAL1-10* and *GAL7* genes (encoding the enzymes for galactose metabolism) each contain both a perfect Cph1 response element (5′-TGTAACGTTACA-3′) [9] and two Rgt1 recognition sequence motifs, consistent with the idea that Rgt1 and Cph1 coordinately regulate these genes in response to galactose.

Hgt4 senses galactose in *S. cerevisiae*

In *S. cerevisiae*, *HXT* genes are induced by glucose, fructose, and mannose, but not by galactose, ostensibly because Snf3 and Rgt2 do not bind galactose [17]. If Hgt4 binds galactose, then expressing it in *S. cerevisiae* should cause galactose-induction of *HXT* genes. The *HGT4* sugar-binding domain (codon optimized) was expressed in *S. cerevisiae* from the *RGT2* promoter (see Experimental Procedures in Supplementary Material). Because the C-terminal cytoplasmic tails of the glucose sensors have diverged almost completely in the ~200 million years since the *C. albicans* and *S. cerevisiae* lineages diverged, the Hgt4 sugar-binding domain was fused to the Rgt2 tail to enable coupling of the sensor to the *S. cerevisiae* signal transduction pathway (Fig. S4) [11]. Exchanging the intracellular signaling tails of glucose sensors does not affect their response to glucose (V. Brown unpublished data; V. Brachet, unpublished data), so we are confident that the Hgt4-Rgt2 chimera retains the sugar-sensing

specificity of Hgt4. In *S. cerevisiae* cells expressing the Hgt4 chimera, *HXT1* is not induced by galactose (Fig. 4A, black bars;Fig. S5, third row). However, galactose induces *MTH1* expression in *S. cerevisiae via* Gal4 [8], and the resulting increase in Mth1 levels would be expected to reinforce Rgt1-mediated repression of *HXT1*, effectively masking any galactose signal generated by Hgt4 in *S. cerevisiae*. Deleting *ScGAL4* eliminates this control element, and reveals robust activation of the *HXT1-lacZ* reporter in response to galactose in cells expressing the Hgt4 chimera (Fig. 4A, blue bars;Fig. S5, bottom row) [18]. In contrast, neither Rgt2 nor Snf3 (which are present in these strains) respond to galactose (indicated by cells with the vector control, Fig. 4A, grey bars; Fig. S5, first column). Thus, expression of the Hgt4 sugar-binding domain in *S. cerevisiae* confers a novel galactose response upon baker's yeast.

Galactose induces *MTH1* expression in diverse fungi

C. albicans did not undergo a whole genome duplication, so it has one homologue of the *S. cerevisiae MTH1* and *STD1* paralogues (*CaSTD1*). CaStd1 (orf19.6173), is 27% identical to both the *S. cerevisiae* Std1 (43% similar) and Mth1 (41% similar), and harbors a conserved motif (SxSxxSSIFS, residues 62-71) that is critical for glucose-induced phosphorylation of ScStd1 and ScMth1 (which leads to their degradation) [19]. We surmised that since Hgt4 functions as a galactose sensor in *C. albicans*, *CaSTD1* expression must not be induced by galactose. Indeed, *CaSTD1* expression is unaffected by galactose (Table 1 and data not shown), a result confirmed by RT-PCR and RT-qPCR analyses (Figs. S6 and 4C respectively). To assess the evolutionary conservation of this galactose response, we measured expression of *MTH1* orthologues in a diverse sampling of fungi spanning ~200 million years of evolution (Fig. 4B). In all species tested except *C. albicans*, expression of *MTH1* is induced in response to galactose (Fig. 4C and S6). Induction occurs even in *C. glabrata*, which has lost the *GAL4* gene, as well as in *K. lactis*, which lacks canonical Gal4 binding sites in the promoter of its *MTH1* orthologue (Table S2). Galactose-induced activation of *ScMTH1* expression by ScGal4 in *S. cerevisiae* [8] appears to antagonize the galactose signal generated by Hgt4, and such antagonism is likely in the four fungi in the *S. cerevisiae* to *K. lactis* clade that we analyzed.

These data illuminate the evolution of galactose sensing in fungi. Sensing galactose through both the Gal4 and the Hgt4/Snf3/Rgt2 pathways seems imprudent because it would lead to cross-repression of genes in both pathways (see Summary and Fig. 1). Within the Ascomycetes, *Candida glabrata*, *Kluyveromyces waltii*, and *Ashbya gossypii* have no canonical galactose sensor because *GAL4*, or *GAL80*, or both are absent, but they have also lost all galactose utilization pathway enzymes (*GAL1*, *GAL7*, and *GAL10*), and thus cannot utilize galactose in any case [10,20,21]. The Gal4-mediated galactose-sensing pathway is intact in a few yeasts that diverged before the duplication, such as *K. lactis* and *S. kluyveri* [22-24]. *Debaromyces hansenii* and *Pichia stipitus* have *GAL4* homologues, but no obvious *GAL80* homologues. In contrast, all the *Candida* species we surveyed (except *C. glabrata*), as well as *Yarrowia lipolytica*, and *Lodderomyces elongisporus*, harbor genes encoding the enzymes for galactose metabolism, but their *GAL4* genes are more similar to *CaGAL4* (than *ScGAL4*), and they all lack a *GAL80* functional homologue. The implication is that the Ascomycetes that can metabolize galactose, but have no Gal4 or Gal80 regulators, utilize an Hgt4-like sensing pathway to control galactose-response genes. This supports the notion that the Gal4-Gal80 control circuit arose prior to the origin of the *S. cerevisiae – K. lactis* clade, but after this clade and *Candida* species diverged from their common ancestor (Fig. 4B, white dot), and suggests that Hgt4 represents an ancestral sensor of galactose. In *C. albicans*, the altered specificity of the Hgt4 glucose sensor in combination with the absence a canonical Gal4 pathway has enabled this fungus to sense galactose through Hgt4.

C. albicans Std1 functions in the sugar sensing pathway

Since the Gal4 signaling pathway is structured differently in *C. albicans*, it was possible that the Hgt4 pathway had also changed. If sugar sensing by *C. albicans* is analogous to *S. cerevisiae* glucose-sensing, the CaStd1 co-repressor should be a key protein in the pathway (Fig. 1). Indeed it is, because homozygous *Δstd1* null mutant cells have the same hyper-filamented morphology as *Δrgt1* mutant cells (Fig. S7A), and as cells carrying the constitutively signaling *HGT4-1* mutation (Fig. S7B), and this phenotype is reversed by reintroducing one wild-type allele into these cells (Fig. S7B). This result supports previous observations that implicated the Hgt4 pathway in *C. albicans* filamentation [11,12]. Further, *HGT7* expression is constitutive in the *Δstd1* mutant (just like in the *HGT4-1* mutant), and reintroducing one copy of *CaSTD1* into this mutant reverses this phenotype (Fig. 5A). Thus, although the galactose-sensing pathways are completely different between *C. albicans* and *S. cerevisiae*, the glucose-sensing pathway remains the same (Hgt4-CaStd1-CaRgt1).

Examining CaStd1 function in *C. albicans* sheds light on the separate functions of the *S. cerevisiae* paralogues. *CaSTD1* expression resembles that of *ScSTD1*, not *ScMTH1*: it is induced by glucose but not by galactose (Figs. 5B and 4C respectively) [25]. This implies that ScStd1 has a more ancestral role, and ScMth1 a more derived role, in this signal transduction pathway. The Hgt4/Snf3/Rgt2 sugar sensing pathway may be universally involved in fungal morphology: disrupting *ScMTH1* represses filamentous growth in baker's yeast in the Σ1278b pseudohyphal strain [26]. Further studies on pre- and post-duplication yeast species will be necessary to determine whether Mth1 and Std1 function redundantly, cooperatively, or in opposition to each other, and whether they affect fungal morphogenesis throughout this kingdom.

It seems clear that the glucose and galactose sensing systems in fungi work *together* as a network to regulate transcription of genes such as *GAL1* in *C. albicans* and *HXT1* in *S. cerevisiae*. In fact, transcriptional regulation of the *HXT* genes in *S. cerevisiae* is the result of at least seven interconnected signal transduction cascades: (I.) glucose-sensing through Snf3/Rgt2 [27], (II.) sugar sensing through the Gpr1 G-protein coupled receptor [28], (III.) osmo-sensing through the Hog1 MAP kinase pathway [29], (IV.) glucose repression mediated by Mig1 and Mig2 [6], (V.) the TOR1 protein kinase pathway [30], (VI.) oxygen availability [31], and finally (VII.) galactose-sensing through Gal4. These signal transduction pathways provide a malleable framework for responding to extracellular nutrients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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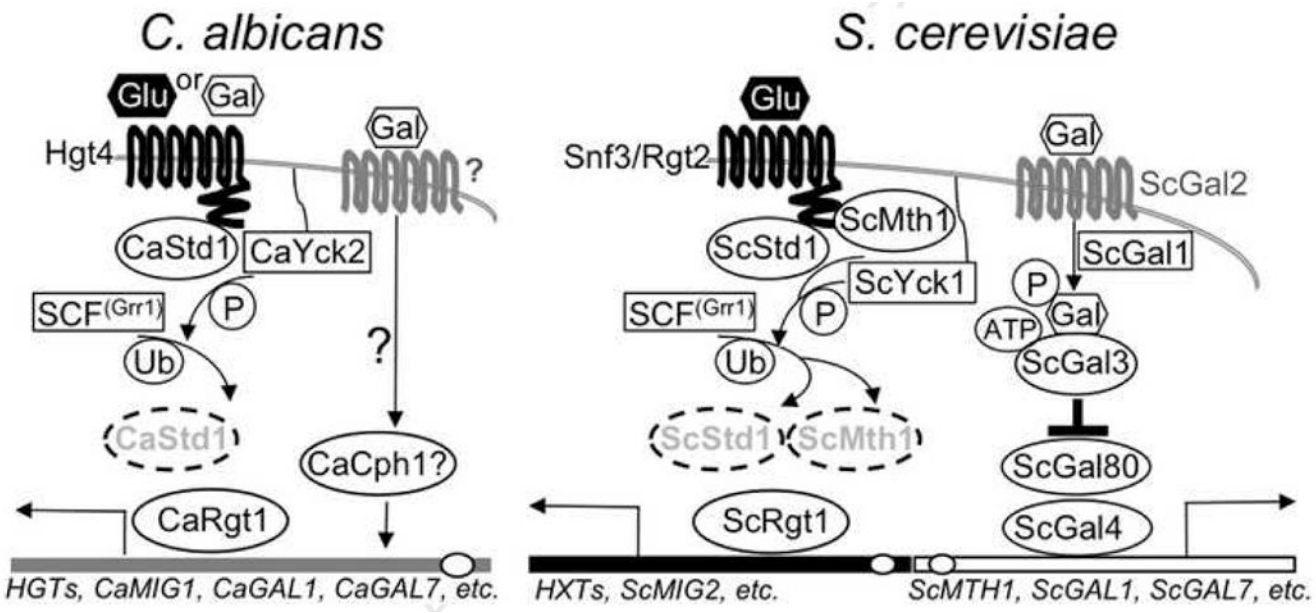

Figure 1. Sugar sensing pathways in *C. albicans* and *S. cerevisiae*

Glucose signaling begins at the cell surface with the sensors (CaHgt4, or ScSnf3 and ScRgt2), and ends in the nucleus with deactivation of the Rgt1 transcriptional repressor [1,11]. The keystone proteins are the transcriptional co-repressors (CaStd1, or ScStd1 and ScMth1), which associate with both the sensor and the transcriptional repressor, and it is the levels of these proteins that translate the environmental signal into gene expression changes. Sugar binding to a sensor activates yeast casein kinase (Yck), which then phosphorylates Std1 and Mth1, thereby marking them for ubiquitylation by the SCF^{Grr1} complex, and dooming them to destruction by the proteasome. Depletion of the co-repressors renders Rgt1 impotent, which results in transcriptional derepression of downstream genes. In *S. cerevisiae*, galactose enters the cell, is phosphorylated and binds (with ATP) to the Gal3 protein. This complex binds and sequesters Gal80, and relieves the inhibition of the Gal4 transcriptional activator. In *C. albicans*, CaGal4 does not regulate the *GAL* genes. Instead, galactose is sensed by the Hgt4 glucose sensor, and likely also through Cph1 (a homologue of the *S. cerevisiae* Ste12).

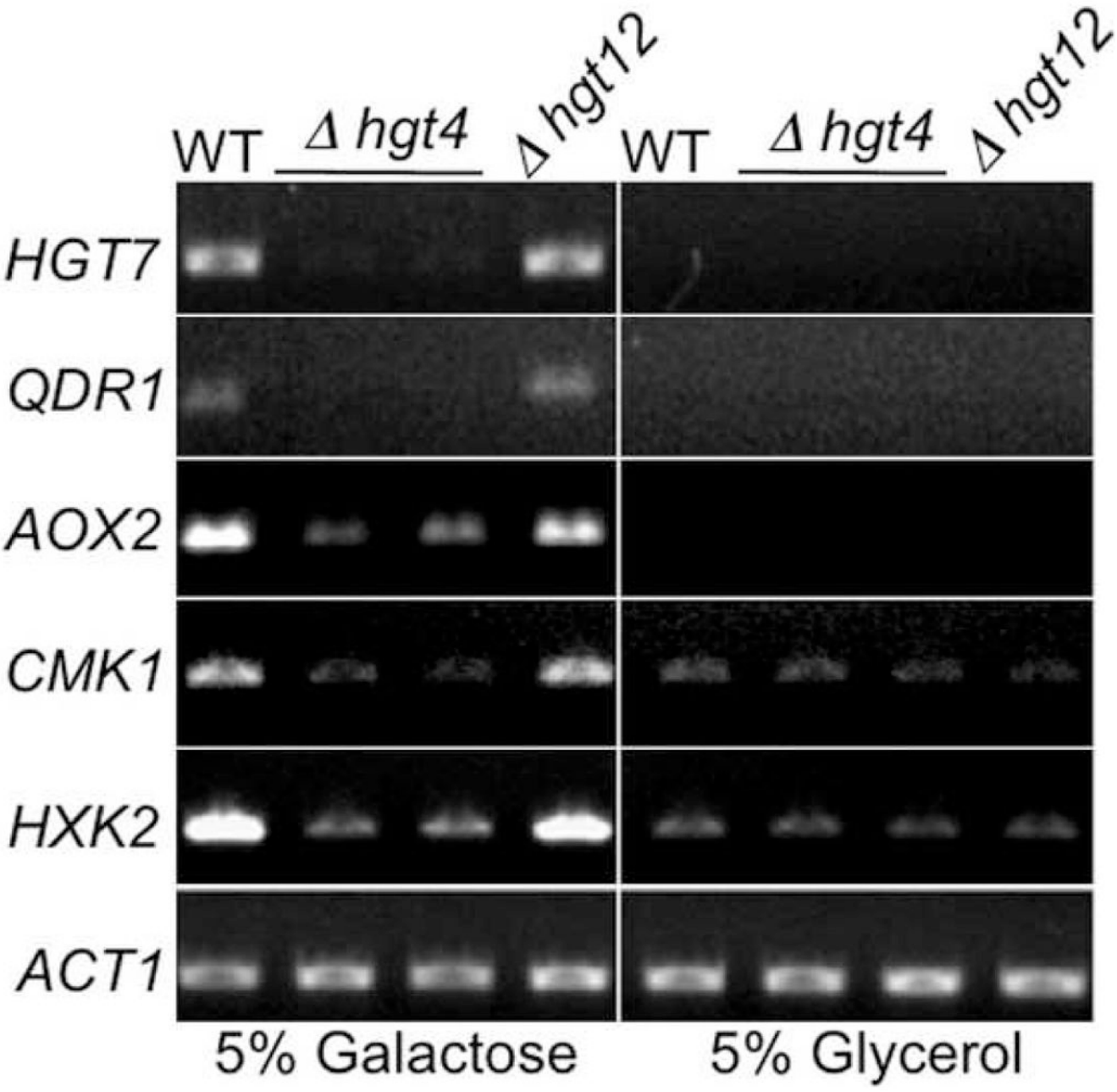

Figure 2. Hgt4 regulates galactose-induced genes

Log phase cultures of WT (BWP17), *Δhgt4* (CM9 and CM10), or *Δhgt12* (CM64) cells were split and incubated in fresh media containing 5% galactose or 5% glycerol at 30°C for 2 hours. Total RNA was reverse transcribed and PCR amplified with primers for *HGT7* (orf19.2023), *QDR1* (orf19.508), *AOX2* (orf19.4773), *CMK1* (orf19.5911), *HXK2* (orf19.542), and *ACT1* (orf19.5007). Control reactions lacking reverse transcriptase yielded no products (not shown).

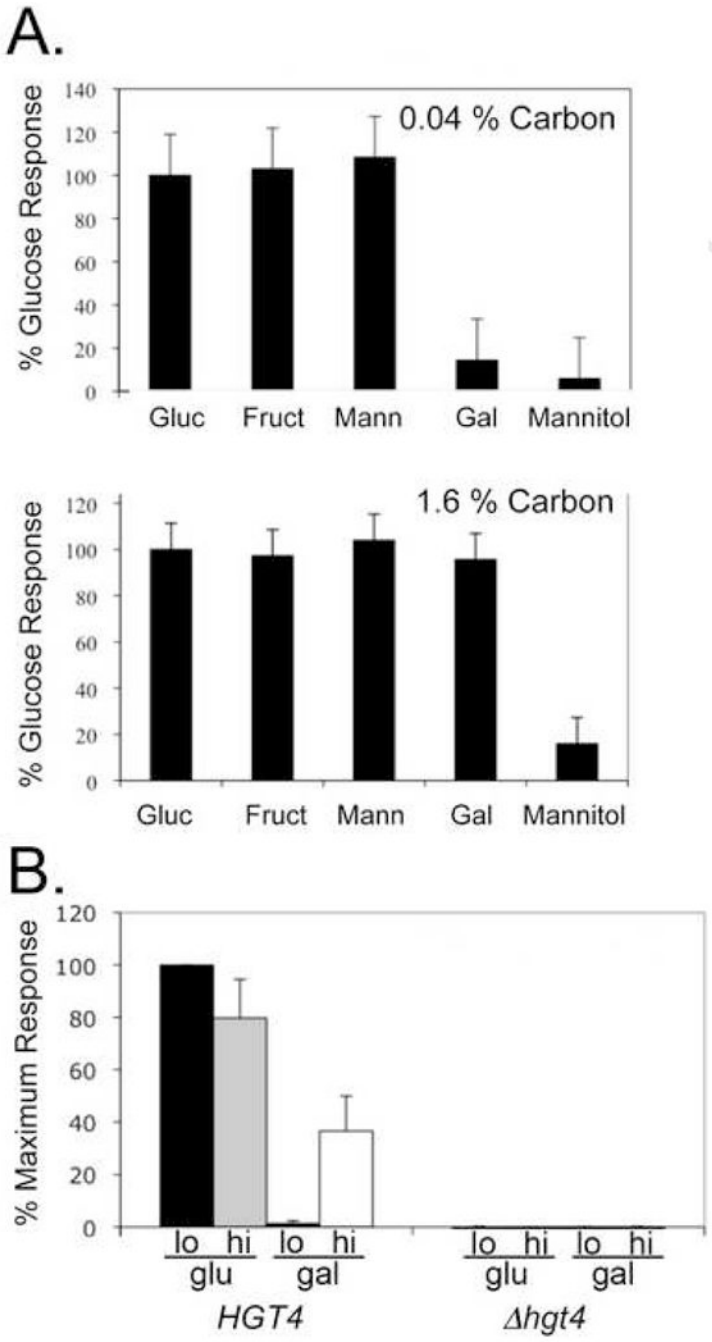

Figure 3. *HGT7* is induced in response to galactose

(**A**) The *HGT7* promoter was fused to *Streptococcus thermophilus lacZ* gene, and this construct was integrated into the *C. albicans* genome at the native *HGT7* locus. Cells with *HGT7::HGT7-lacZ* (CM79 and CM80) were grown in glycerol media, split, and incubated at 30°C for 2 hours in fresh media containing glycerol or 0.04% (top panel) or 1.6% (bottom panel) of the sugars indicated. Cells were lysed, assayed for *β*-galactosidase activity (in quadruplicate (top) or in triplicate (bottom)), and the results were normalized to the *lacZ* activity in the glycerol media. Data are presented as the mean +/- one standard deviation. (**B**) Cells with *HIS1::HGT7-lacZ* (*HGT4;* CM230 and CM 231), (*Δhgt4;* CM232 and CM233) were grown in media with glycerol as carbon source, split, and incubated at 30°C for 2 hours in fresh media lacking histidine but

containing glycerol or the sugar indicated, (*n*=10 for *HGT4; n*=10 for *Δhgt4*). Black bar: 0.04% glucose; grey bar: 1.6% glucose; striped bar: 0.04% galactose; white bar: 1.6% galactose. All values were normalized to activity in glycerol, and expressed as the percent of the maximum response in 0.04% glucose. Data are the mean +/- one standard deviation.

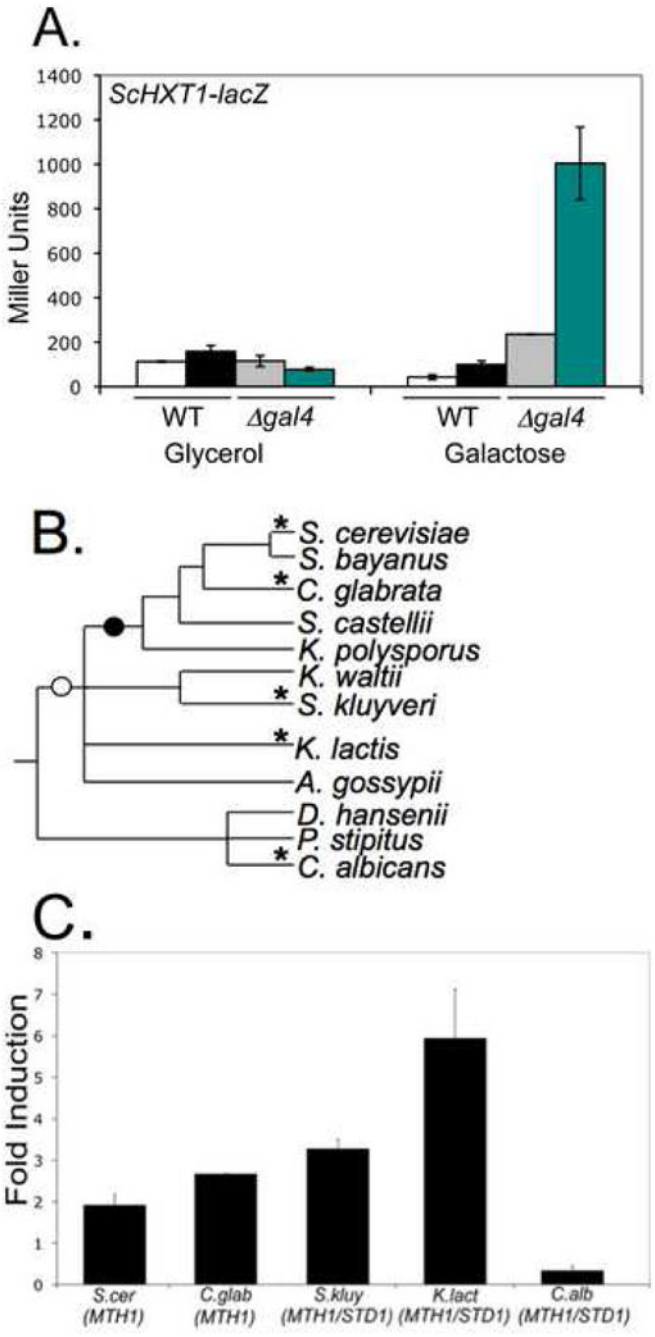

Figure 4. *C. albicans HGT4* confers a novel galactose-response upon *S. cerevisiae*

(**A**) *S. cerevisiae* strains were grown in media containing glycerol, cell densities were normalized, and the culture was split and incubated overnight at 30°C in fresh media containing 5% glycerol or 5% galactose, then lysed and *β*-galactosidase activity was assayed (Experimental Procedures in Supplementary Material). Data are the average of biological duplicates. White bars: wild-type cells + pRS316 vector (YM7642); black bars: wild-type cells +Hgt4-Chimera (YM7643); grey bars: *Δgal4* cells + pRS316 vector (YM7644); blue bars: *Δgal4* cells + Hgt4-Chimera (YM7645). (**B**) ***MTH1* orthologues are galactose-induced in diverse fungi.** A phylogenetic tree showing the relationship of yeasts spanning ~200 million

years of evolution is shown [42-45]. Characteristics of the galactose-sensing pathways in these species are described in Table S2. The black circle represents a whole genome duplication event, the white circle represents the proposed appearance of the Gal4-Gal80 gene regulatory mechanism; asterisks indicate the species analyzed in (C). (**C**) Each species was grown overnight in glycerol media, and incubated in fresh media containing 5% glycerol or 5% galactose at 30°C for 3 hours. RT-PCR was performed on total RNA using species-specific primers for either *ACT1* or the *MTH1/STD1* orthologue (Fungal strains are described in Experimental Procedures Supplementary Material). First strand cDNAs served as templates for quantitative PCR. Each *MTH1/STD1* signal was normalized to the *ACT1* signal in that sample, and the $\Delta\Delta$Ct values are expressed as 'Fold Induction' of expression in galactose relative to expression in glycerol ($2^{\Delta\Delta Ct}$). Data are the average of duplicates. Separate experiments were performed using semi-quantitative PCR to confirm the results (see Fig. S6).

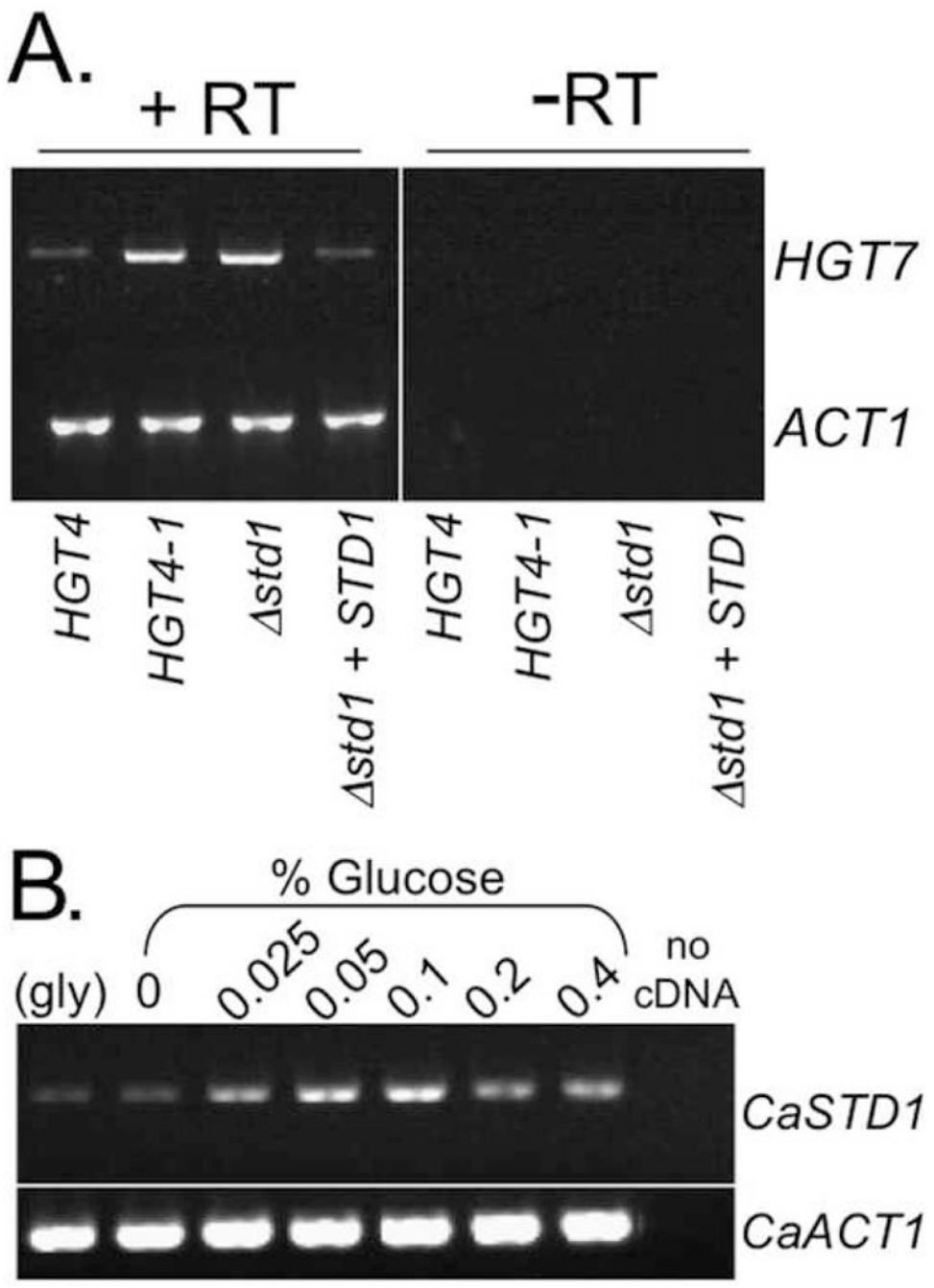

Figure 5. *CaSTD1* and *ScSTD1* function similarly

(**A**) *CaSTD1* plays a role in the *HGT4* pathway. Isogenic strains [*HGT4* (CM87) vs. *HGT4-1* (CM36) and *Δstd1* (CM222) vs. *STD1* (CM224)] were grown at 30°C to log phase in media containing glycerol. Cells were harvested, snap frozen, and total RNA was purified for RT-PCR analysis of *HGT7* (orf19.2023) *or ACT1* (orf19.5007). (**B**) *CaSTD1* is glucose-induced. C. *albicans* cells (SC5314) were grown to log-phase in media containing glycerol, then incubated at 30°C for 2 hours in fresh media with glycerol (gly), or with the indicated concentrations of glucose (0 indicates no carbon source). Cells were harvested, snap frozen, and total RNA was purified for RT-PCR analysis using primers for *CaSTD1* (orf19.6173) or *ACT1* (orf19.5007).

Table 1

Genes Induced in Response to Sugars

GROUP I (Genes induced in 2% galactose and in 2% glucose)*		Galactose	Glucose	
Name	**ORF ID**	**Fold Up**	**Fold Up**	**Annotation**
HGT7	orf19.2023	32.2	30.8	Putative glucose transporter
QDR1	orf19.508	18.6	56.1	Antibiotic resistance transporter
AOX2	orf19.4773	13.7	8	Alternative oxidase
CRZ2	orf19.2356	10.1	12.8	Putative transcription factor
FET99	orf19.4212	10	19.1	Multicopper oxidase family
RHR2	orf19.5437	9.2	25.1	Putative glycerol 3-phosphatase
RNR22	orf19.1868	8.1	25.4	Ribonucleoside di-Phosphate reductase
TPO3	orf19.4737	7.4	16.4	Possible polyamine transporter
PDC11	orf19.2877	6.2	9.1	Similar to pyruvate decarboxylase
HGT6	orf19.2020	5.3	3.2	Putative glucose transporter
MNN22	orf19.3803	5.3	9.1	Golgi alpha-1, 2-mannosyltxferase
FMA1	orf19.6837	5.2	9.9	Membrane-assoc. protein
HAK1	orf19.6249	5.1	3.4	Putative potassium transporter
HXK2	orf19.542	5	8	Hexokinase II
TYE7	orf19.4941	4.4	5.7	Putative bHLH transcription factor
GDH3	orf19.4716	4.3	20.2	NADP-glutamate dehydrogenase
CMK1	orf19.5911	4.1	5	Ca2+/Calmodulin-dependent kinase
FET34	orf19.4215	3.8	6.8	Similar to multicopper ferroxidase
STP4	orf19.909	3.8	5.7	Putative transcription factor
AOX1	orf19.4774	3.7	3.3	Alternative oxidase
EHT1	orf19.3040	3.4	8.4	Similar to Eht1p
PFK1	orf19.3967	3.2	7	α-subunit of phosphofructokinase
CRP1	orf19.4784	3.2	6.9	Copper transporter
PFK2	orf19.6540	3.1	5.7	β-subunit of phosphofructokinase
PHO15	orf19.4444	3	5.8	4-nitrophenyl phosphatase
UBC15	orf19.5337	2.9	3	Ub-conjugation, DNA repair
MIG1	orf19.4318	2.9	3.2	Transcriptional repressor

GROUP I

(Genes induced in 2% galactose and in 2% glucose)*

Name	ORF ID	Galactose Fold Up	Glucose Fold Up	Annotation
AHP1	orf19.2762	2.9	6.3	Putative alkyl hydroperoxide reductase
ARG1	orf19.7469	2.7	4	Similar to argininosuccinate synthase
PHO113	orf19.2619	2.6	3.6	Constitutive acid phosphatase
NDE1	orf19.339	2.5	2.7	Putative NADH dehydrogenase
GPX2	orf19.85	2.5	2.25	Similar to glutathione peroxidase
ROD1	orf19.1509	2.4	3.8	Drug tolerance; Rgt1-repressed
FCR1	orf19.6817	2.3	2.8	Put. Zn-cluster transcription factor
OPT9	orf19.2584	2.1	3.1	Probable pseudogene
EBP7	orf19.5816	2	1.9	Stress-induced via Cap1p
ARG5	orf19.4788	2	5	Arginine biosynthetic enzyme
ARG4	orf19.6689	2	3.9	Argininosuccinate lyase
DOG1	orf19.3392	1.9	2.6	Put. 2-deoxygluc-6-phosphatase
YIM1	orf19.847	1.9	1.8	Similar to mitochondrial protease

GROUP II

(Genes induced in 2% galactose, but repressed in 2% glucose)

Name	ORF ID	Galactose Fold Up	Glucose Fold Up	Annotation
HGT12	orf19.7094	6.74	0.07	Glucose, fructose, mannose transporter
HXT10	orf19.4384	2.6	0.21	Sugar transporter
HGT2	orf19.3668	2	0.02	Putative glucose transporter
GAL1	orf19.3670	6.1	0.8	Galactokinase
GAL10	orf19.3672	6	0.7	UDP-glucose 4-epimerase
GAL7	orf19.3675	5.9	0.9	UDP-hexose-1-P uridylyltransferase

GROUP III

(Genes induced in 2% galactose, *NOT* induced in 2% glucose)

Name	ORF ID	Galactose Fold Up	Glucose Fold Up	Annotation

GROUP I

(Genes induced in 2% galactose and in 2% glucose)*

		Galactose	Glucose	
Name	**ORF ID**	**Fold Up**	**Fold Up**	**Annotation**
PGA37	orf19.3923	3.2	1.1	Putative GPI-anchored protein
HSP30	orf19.4526	2.9	0.48	Similar To heat shock protein
STB3	orf19.203	2.3	1.4	Predicted Sin3 Binding protein

* Fold induction indicates gene expression in each sugar relative to its expression in glycerol cultures. For all genes listed, $p<0.05$ (student's t-test). Uncharacterized open reading frames induced in galactose and glucose are shown in Table S4.