The inositol regulon controls viability in *Candida glabrata*

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Inositol is essential in eukaryotes, and must be imported or synthesized. Inositol biosynthesis in *Saccharomyces cerevisiae* is controlled by three non-essential genes that make up the inositol regulon: *ScINO2* and *ScINO4*, which together encode a heterodimeric transcriptional activator, and *ScOPI1*, which encodes a transcriptional repressor. ScOpi1p inhibits the Scino2-Scino4p activator in response to extracellular inositol levels. An important gene controlled by the inositol regulon is *ScINO1*, which encodes inositol-3-phosphate synthase, a key enzyme in inositol biosynthesis. In the pathogenic yeast *Candida albicans*, homologues of the *S. cerevisiae* inositol regulon genes are 'transcriptionally rewired'. Instead of regulating the *CaINO1* gene, *CaINO2* and *CaINO4* regulate ribosomal genes. Another *Candida* species that is a prevalent cause of infections is *Candida glabrata*; however, *C. glabrata* is phylogenetically more closely related to *S. cerevisiae* than *C. albicans*. Experiments were designed to determine if *C. glabrata* homologues of the inositol regulon genes function similarly to *S. cerevisiae* or are transcriptionally rewired.

*CgINO2*, *CgINO4* and *CgOPI1* regulate *CgINO1* in a manner similar to that observed in *S. cerevisiae*. However, unlike in *S. cerevisiae*, *CgOPI1* is essential. Genetic data indicate that *CgOPI1* is a repressor that affects viability by regulating activation of a target of the inositol regulon.

INTRODUCTION

Fungi of the genus *Candida* are the most common cause of human fungal infections and can lead to both mucosal and systemic infections (Calderone, 2002). *Candida albicans* is the most common cause of these infections, but non- *albicans* *Candida* species are increasingly associated with disease (Coleman et al., 1998). One of these species, *Candida glabrata*, is now the second most common cause of both mucosal and systemic *Candida* infections (Kaur et al., 2005).

Phylogenetically, *C. glabrata* is more closely related to the non-pathogenic yeast *Saccharomyces cerevisiae* than most of the other common species of *Candida* associated with human disease (Kaur et al., 2005). *C. glabrata* lacks a number of the virulence factors associated with *Candida* pathogens, such as secreted hydrolases and hyphal growth (Kaur et al., 2005). Despite this, *C. glabrata* is a growing challenge in clinical settings where it causes mucosal infections and is associated with approximately 15% of all *Candida*-related systemic bloodstream infections (Pfaller & Diekema, 2004). These observations are interesting in light of the fact that *C. glabrata* is more closely related to *S. cerevisiae*, but is still a pathogen, whereas *S. cerevisiae* is only very rarely associated with infection (Piarroux et al., 1999). A clearer understanding of how *S. cerevisiae* and *C. glabrata* differ from one another may help shed light on why one is a significant human pathogen and the other is not.

A number of recent studies have shown that *C. albicans* is transcriptionally rewired compared to *S. cerevisiae*. For example, the genes encoding enzymes of the Leloir pathway for galactose catabolism in *S. cerevisiae* are regulated by the ScGal4p transcription factor. However, in *C. albicans* these genes are regulated by the transcription factor Cph1p, while the *C. albicans* CaGal4p homologue regulates TCA cycle genes such as *CaLAT1* (Martchenko et al., 2007). Other examples of transcriptional rewiring between *C. albicans* and *S. cerevisiae* include regulatory systems controlling mating type (Tseng et al., 2003), mitochondrial ribosomal genes (Ihmels et al., 2005) and *de novo* myo-inositol biosynthesis genes (Hoppen et al., 2007; Y. L. Chen & T. B. Reynolds, unpublished). Myo-inositol will be referred to as inositol throughout the rest of this article.

Since there are several examples of transcriptional rewiring between these two more distantly related yeasts (*S. cerevisiae* and *C. albicans*), it was of interest to determine if similar rewiring is present between the more closely
related yeasts *C. glabrata* and *S. cerevisiae*. The Leloir enzymes for galactose metabolism are not present in *C. glabrata* (Kaur et al., 2005), and this yeast is a galactose auxotroph (Kreger-van Rij, 1984), so this pathway is unavailable for comparison. Mating has never been described for *C. glabrata*, thus this pathway is not useful for study either. In contrast to these pathways, the inositol regulon appears to be an excellent pathway to compare between these two yeasts. The inositol regulon is a very well-studied transcriptional regulon in *S. cerevisiae* (reviewed by Chen et al., 2007; Greenberg & Lopes, 1996), and there are *C. glabrata* orthologues for both the transcription factors and targets of the *S. cerevisiae* inositol regulon (see Results and Discussion).

The inositol regulon in *S. cerevisiae* has been well-described (Chen et al., 2007; Greenberg & Lopes, 1996), and consists of three main transcription factors that regulate target gene expression in response to extracellular inositol levels (Fig. 1). The roles of each of these transcription factors in controlling this regulon are described in more detail below. Transcriptional targets of the inositol regulon include a number of phospholipid biosynthetic genes, but the most highly expressed and well-characterized of these targets is the *S. cerevisiae* INO1 (*ScINO1*) gene. *ScINO1* encodes an enzyme that occupies the rate-limiting step in *de novo* inositol biosynthesis. Inositol is essential and is required for the synthesis of phosphatidylinositol (PI) which is a precursor for several essential lipids, including inositol-phosphate signalling lipids, glycosylphosphatidylinositol (GPI) anchors and sphingolipids (Dickson & Lester, 1999; Michell, 2008; Strahl & Thorner, 2007).

*ScIno1p* is an inositol-3-phosphate synthase which converts glucose 6-phosphate to inositol 3-phosphate (Majumder et al., 1997). Inositol 3-phosphate is dephosphorylated by the inositol monophosphatases ScInm1p or ScInm2p to create inositol (Lopez et al., 1999). An *ScIno1Δ* mutant cannot make inositol *de novo* and is an inositol auxotroph. In the absence of extracellular inositol (or at low concentrations like 10 μM) *ScINO1* is expressed, and in the presence of higher concentrations of extracellular inositol *ScINO1* is repressed (Graves & Henry, 2000) (Fig. 1).

The inositol regulon transcription factors *ScIno2p* and *ScIno4p* form a heterodimeric transcriptional activator that binds to the upstream activator sequence (*UASINO*) in the promoters of target genes such as *ScINO1* (Ambroziak & Henry, 1994; Bachhawat et al., 1995; Loewen et al., 2003; Lopes & Henry, 1991; Nikolo夫 & Henry, 1994; Schwank et al., 1995). Both *ScIno2p* and *ScIno4p* are absolutely required for transcription of *ScINO1*, so *Scino2Δ* and *Scino4Δ* mutants are inositol auxotrophs.

The regulation of *ScINO1* in response to extracellular inositol is dependent on the repressor protein *ScOpi1p* (Heyken et al., 2005; Jiranek et al., 1998; Loewen et al., 2003, 2004; Loewen & Levine, 2005). *ScOpi1p* senses the level of extracellular inositol indirectly based on the level of the PI precursor lipid phosphatidic acid (PA). Inositol is the rate-limiting metabolite in PI synthesis, and when there is abundant extracellular inositol, PI synthesis is maximal, and PA levels are lower because PA is consumed during synthesis of PI. In these conditions *ScOpi1p* binds to *ScIno2p* and represses transcription of *ScINO1*.

When extracellular inositol is not available (or greatly decreased), PI synthesis slows, and PA levels increase in the endoplasmic reticulum (ER). *ScOpi1p*, which binds to PA, is recruited to the ER where it also binds Scs2p. Thus, the *ScIno2p-ScIno4p* heterodimer is freed to transcribe *ScINO1*. A mutation that deletes *ScOPI1* results in constitutive overexpression of *ScINO1* and other genes carrying the *UASINO* sequence in their promoters.

The purpose of this study was to determine if *C. glabrata* carried an inositol regulon that is similar to that in *S. cerevisiae*, or if these yeasts are transcriptionally rewired for inositol regulation. In order to do this, *C. glabrata* homologues of the *S. cerevisiae* inositol regulon proteins were identified and disrupted. Analysis of these mutants revealed the surprising finding that *CgOPI1* is essential. *CgIno2p* and *CgIno4p* (which are not essential) are activators of *CgINO1*, and *CgOpi1p* is a transcriptional repressor of *CgINO1*. This is similar to the inositol regulon in *S. cerevisiae*. However, unlike in *S. cerevisiae*, *CgOPI1* is required for viability. This difference from *S. cerevisiae* may indicate that the inositol regulon in *C. glabrata* has some additional or different targets than in bakers’ yeast, or *C. glabrata* may be metabolically rewired compared to *S. cerevisiae*.

**METHODS**

**Strains and media.** Strains are listed in Table 1. Integrations and manipulations were confirmed by PCR (Table 2) in most cases and by
The \textit{Cgopi1} gene was disrupted in \textit{C. glabrata} strain BG14 (Cormack & Falkow, 1999; Cormack et al., 1999) utilizing a two-step gene disruption strategy (disruption cassette plasmids were used to replace the respective wild-type alleles on the chromosome of BG14 by the two-step deletion strategy (Cormack & Falkow, 1999; Cormack et al., 1999; Rothstein, 1991)) with disruption constructs built in the pRS306 vector (Sikorski & Hieter, 1989; Table 3) carrying the \textit{ScURA3} marker that can be selected for on media lacking uracil and counterselected against using media containing 5-fluororacil acid (5-FOA). Reintegration of gene disruption mutations was done by transforming the strains with \textit{ScURA3}-marked integrating plasmids carrying the wild-type gene (Table 3).

The \textit{Cgopi1\Delta} mutant (Cg14, Table 1) was generated in BG14 by transforming it with the \textit{pCgOPI1} episomal plasmid (\textit{ScURA3} marker, Table 3), and then disrupting the \textit{CgOPI1} gene on the chromosome with the \textit{Cgopi1::NAT1} construct amplified from plasmid pCgOPI1 (Table 3). The \textit{Cgopi1\Delta Cgino2\Delta} double mutant was made in the same way as the \textit{Cgopi1\Delta} mutant except it was done in strain Cg5 (\textit{Cgino2\Delta}).

The \textit{tetO::HOP1::CgOPI1} strains EBCg048 and EBCg049 (Table 1) were generated by first making BG14 a histidine and tryptophan auxotroph (\textit{CgHis3\Delta Cgtrp1\Delta}). This was done by disrupting \textit{CgHis3} in BG14 as described above for the \textit{Cgino2\Delta} gene to create strain Cg33. The \textit{CgTRP1} gene was disrupted in Cg33 using a plasmid from Karl Kuchler’s lab to create strain EBCg046. EBCg046 was used to create the \textit{tetO::HOP1::CgOPI1} strains EBCg048 and EBCg049 by first integrating plasmid \textit{pINTG4} carrying the \textit{tetR::GAL4AD} repressor-activator into its genome as described by Nakayama et al. (1998). Then the \textit{tetO::HOP1::CgOPI1} construct was PCR-amplified from plasmid pEB48 (Table 3) and used to replace the \textit{CgOPI1} locus on the chromosome.

Media used in this study included 2% agar plates or liquid media made with YPD, YNB, or inositol-free media (Styles, 2002) that included supplements of amino acids, nucleotides, inositol, doxycycline, 5-FOA, etc., as described in the text. For inositol-free agar plates, Bactoagar was used because it does not contain trace amounts of inositol.

\textbf{Plasmids and constructs.} The gene disruption plasmids (pRS306-ino2\Delta, pRS306-ino4\Delta and pRS306-ino1\Delta) were made by PCR-amplifying DNA corresponding to approximately 500 bp of non-coding DNA that flank the 5' and 3' edges of each ORF (5' or 3' NCRs), and then cloning them into pRS306 adjacent to one another to create a disrupted allele (primers and restriction sites used for cloning are listed in Table 2). The pRS306-his3\Delta disruption plasmid was created in a similar manner by subcloning the \textit{CgHis3} 5' NCR into plasmid pGRB2.1 (Friedman et al., 2002) upstream of the 3' NCR of \textit{CgHis3} contained in this plasmid. The whole 5' and 3' NCR disruption cassette was then subcloned into the pRS306 integrating vector (Sikorski & Hieter, 1989) as an Xbal-KpnI fragment to create pRS306-his3\Delta. The disrupted alleles from all of the pRS306 disruption cassette plasmids were used to replace the respective wild-type alleles on the chromosome of BG14 by the two-step deletion strategy (Cormack & Falkow, 1999; Cormack et al., 1999; Rothstein, 1991). For example, the pRS306-ino1\Delta cassette was cut with \textit{NruI} in the 5' NCR to linearize it and target it to recombine upstream of \textit{CgINO1}. PCR was used to confirm correct integrations and disruptions. In a similar manner, plasmids pRS306-ino4\Delta, pRS306-ino1\Delta and pRS306-his3\Delta were cut with \textit{PmlI}, \textit{SnaBI} and \textit{BglII}, respectively. Following replacement of the wild-type genes with the disrupted alleles, the mutant alleles were confirmed based on phenotype and PCR. Reintegration constructs were generated for each of the above disruptants in the vector pRS306 by PCR-amplifying the corresponding ORFs plus ~500 bp 5' and 3' NCRs with the primers \textit{BcoIII} and \textit{BcoII} for \textit{CgINO2}, USO1 and USO4 for \textit{CgINO4}, and \textit{Mho3} and \textit{Mho2} for \textit{CgINO1}. Cut sites for subcloning are listed in Table 2. Plasmids were linearized with the enzymes mentioned above, and transformed into their respective disruptant strains. Correct integrations were confirmed by PCR with primers listed in Table 2.

The episomal plasmid \textit{pCgOPI1} carrying \textit{CgOPI1} plus 215 bp of upstream DNA and 439 bp of downstream DNA was generated by amplifying the \textit{CgOPI1} ORF and flanking sequences from purified BG14 DNA using primers TRO605 and TRO608, and cutting the PCR product with \textit{BamHI} and \textit{SpeI} enzymes. The \textit{SpeI} site was introduced by primer TRO608, and the \textit{BamHI} site was internal to the amplified DNA fragment. This PCR product was cloned into the \textit{ScURA3}-bearing \textit{C. glabrata CEN/ARS} plasmid pGRB2.1 (Friedman et al., 2002) using \textit{SpeI} and \textit{BamHI}. Plasmid pCgOPI1 was generated by subcloning the \textit{NAT1} cassette from pAG25 (Goldstein & McCusker, 1999) with primers TRO652 and TRO653 into an \textit{EcoRI} site between a 469 bp fragment containing the 5' NCR of \textit{CgOPI1} (cloned by

\begin{table}
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Strain} & \textbf{Genotype} & \textbf{Reference} \\
\hline
BG14 & \textit{Cgura3::Tn903neoR} & Cormack & Falkow (1999) \\
BG2 & \textit{CgURA3, parent of BG14} & Cormack & Falkow (1999) \\
Cg11 & \textit{Cgura3::Tn903neoR \textit{pCgOPI1}} & This study \\
Cg12 & \textit{Cgura3::Tn903neoR \textit{pGRB2.1}} & This study \\
Cg14 & \textit{Cgura3::Tn903neoR \textit{Cgopi1\Delta \textit{pCgOPI1}}} & This study \\
Cg5 & \textit{Cgura3::Tn903neoR \textit{Cgino2\Delta}} & This study \\
EBCg019 & \textit{Cgura3::Tn903neoR \textit{Cgino2\Delta::CgINO2}} & This study \\
Cg23 & \textit{Cgura3::Tn903neoR \textit{Cgino2\Delta \textit{pCgOPI1}}} & This study \\
Cg18 & \textit{Cgura3::Tn903neoR \textit{Cgino2\Delta \textit{pCgOPI1}}} & This study \\
EBCg005 & \textit{Cgura3::Tn903neoR \textit{Cgino4\Delta}} & This study \\
EBCg008 & \textit{Curer3::Tn903neoR \textit{Cgino4\Delta::CgINO4}} & This study \\
EBCg014 & \textit{Cgura3::Tn903neoR \textit{Cgino1\Delta}} & This study \\
EBCg017 & \textit{Cgura3::Tn903neoR \textit{Cgino1\Delta::CgINO1}} & This study \\
Cg33 & \textit{Cgura3::Tn903neoR \textit{Cgino3\Delta}} & This study \\
EBCg046 & \textit{Cgura3::Tn903neoR \textit{Cgino3\Delta \textit{Cgtrp1\Delta}}} & This study \\
EBCg048 & \textit{Cgura3::Tn903neoR \textit{Cgino3\Delta \textit{Cgtrp1\Delta \textit{pINTG4 \textit{pEB48}}}}} & This study \\
EBCg049 & \textit{Cgura3::Tn903neoR \textit{Cgino3\Delta \textit{Cgtrp1\Delta \textit{pINTG4 \textit{pEB48}}}}} & This study \\
\hline
\end{tabular}
\caption{Strains used in this study}
\end{table}
Table 2. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)*</th>
<th>Restriction site</th>
<th>Purpose†</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRO605</td>
<td>AAAAACGTTGCCCTCTTATACGTTAACAA</td>
<td>HinIII</td>
<td>Forward for 5’ NCR of CgOPI1</td>
</tr>
<tr>
<td>TRO606</td>
<td>AAAAAGTCTCCAGCACGACTGTTATTC</td>
<td>EcoRI</td>
<td>Reverse for 5’ NCR of CgOPI1</td>
</tr>
<tr>
<td>TRO607</td>
<td>AAAAGGAATTCTCCTCCTGATATAATATATTACGAC</td>
<td>EcoRI</td>
<td>Forward for 3’ NCR of CgOPI1</td>
</tr>
<tr>
<td>TRO608</td>
<td>AAAAACGTTGCCCTCTTATACGTTAACAA</td>
<td>SphI</td>
<td>Reverse for 3’ NCR of CgOPI1</td>
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<tr>
<td>TRO652</td>
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<td>EcoRI</td>
<td>Forward for NAT1 gene</td>
</tr>
<tr>
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<td>Reverse for NAT1 gene</td>
</tr>
<tr>
<td>VSO1</td>
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<td>XhoI</td>
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<tr>
<td>VSO2</td>
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</tr>
<tr>
<td>VSO3</td>
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</tr>
<tr>
<td>VSO4</td>
<td>AAAAACGTTGCCCTCTTATACGTTAACAA</td>
<td>BamHI</td>
<td>Reverse for 3’ NCR CgINO4</td>
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<td>MHO3</td>
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<td>MHO4</td>
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<td>SmaI</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<tr>
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</tr>
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</tr>
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*Underline shows the restriction site.
†NCR, Non-coding region that is either 5’ or 3’ to the designated ORF.

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RESULTS

CgINO2 and CgINO4 encode transcriptional activators of the CgINO1 gene and control de novo inositol biosynthesis

A homologue of ScINO1 was identified in C. glabrata by BLAST analysis of the ScINO1 translated protein sequence...
against the *C. glabrata* genome at the Genolevures website (www.genolevures.org). The BLAST search revealed that the gene *CAGL0106050g* encoded a protein that was 73.9% identical to ScIno1p. We refer to *CAGL0106050g* as *CgIno1* based on the experiments described below.

*CgIno1* expression was examined in media containing or lacking inositol by Northern blotting which revealed that *CgIno1* was highly expressed in medium lacking inositol, but it was poorly expressed in medium containing 75 μM inositol (Fig. 2). This is similar to what has been observed for *ScINO1* in *S. cerevisiae* (Graves & Henry, 2000). In order to determine if *CgIno1* was required for *de novo* inositol biosynthesis, the *CgIno1* ORF was disrupted by homologous recombination using a two-step gene deletion strategy (Cormack & Falkow, 1999; Cormack et al., 1999). The resulting *Cgino1Δ* mutant was unable to grow on inositol-free medium (Fig. 3). However, when the *CgIno1* gene was reintegrated into the genome at the *CgIno1* locus, the reconstituted strain (*Cgino1Δ::CgIno1*) could grow in medium lacking inositol (Fig. 3).

The *CgIno1* gene is regulated in a similar manner as the *ScINO1* gene in synthetic medium containing or lacking inositol, which suggests that the inositol regulon that controls *ScINO1* in *S. cerevisiae* might be conserved in *C. glabrata*. In order to test this, homologues of the *ScINO2* and *ScINO4* transcriptional activator genes were identified by BLAST searches, querying the protein sequences of ScIno2p and ScIno4p against the *C. glabrata* genome at the Genolevures website. The BLAST searches revealed only one strong homologue for each protein, and these were encoded by the genes *CAGL001947g* for ScIno2p (35.6% identity) and *CAGL007355g* for ScIno4p (44.5% identity). These genes, *CAGL001947g* and *CAGL007355g*, were named *CgIno2* and *CgIno4*, respectively. *CgIno2* and *CgIno4* were disrupted using the two-step gene disruption method which completely removed the ORF of each gene. The resulting *CgIno2Δ* and *CgIno4Δ* mutants were tested to determine if they could grow in the absence of inositol in the medium. As seen for orthologous *Saccharomyces* mutants, the *CgIno2Δ* and *CgIno4Δ* mutants were unable to grow on inositol-free medium (Fig. 3). These data suggest that *CgIno2* and *CgIno4* control the expression of the *CgIno1* gene. Northern blotting revealed that *CgIno2Δ* and *CgIno4Δ* mutants showed a complete lack of *CgIno1* expression even in inositol-free medium (Fig. 2). Reconstituted *CgIno2Δ::CgIno2* and *CgIno4Δ::CgIno4* strains, conversely, grew well on medium lacking inositol (Fig. 3) and regulated *CgIno1* much like the wild-type strain (Fig. 2).

**CgOPI1 is an essential gene in *C. glabrata***

In *S. cerevisiae*, the *SCOPI1* gene encodes the main regulator of *de novo* inositol biosynthesis, and its homologue was identified in a BLAST search against the *C. glabrata* genome at Genolevures as described above. The protein encoded by *CAGL003267g* was found to be 52.3% identical to ScOp11p. An attempt was made to disrupt the *CgOPI1* gene by the two-step gene disruption strategy; however, this method continuously yielded strains carrying a wild-type ORF of *CgOPI1*.

The above results suggested that *CgOPI1* might be essential, and this was shown to be the case using a counterselectable episomal plasmid expressing *CgOPI1*. *CgOPI1* was cloned, along with non-coding DNA flanking both 5′ and 3′ of the ORF (including the transcriptional promoter and terminator, respectively), into the *CEN/ARS* vector pGRB2.1 (Frieman et al., 2002) to create plasmid pCgOPI1. Plasmid pCgOPI1, which carries the *S. cerevisiae* *URA3* gene, was transformed into the wild-type strain, and the chromosomal *CgOPI1* ORF was disrupted by homologous recombination using a construct in which the *CgOPI1* ORF is replaced with the nourseothricin resistance marker cassette NAT1 (Goldstein & McCusker, 1999). The resulting pCgOPI1 *Cgopi1Δ* strain was then streaked onto medium containing 5-FOA. Processing of 5-FOA by the

### Table 3. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Type</th>
<th>Restriction site</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS306-ino2Δ</td>
<td><em>CgIno2</em> knockout</td>
<td>Integrating</td>
<td><em>SnBI</em></td>
<td>This study</td>
</tr>
<tr>
<td>pRS306-CgIno2</td>
<td><em>CgIno2</em> reinteg.</td>
<td>Integrating</td>
<td><em>SnBI</em></td>
<td>This study</td>
</tr>
<tr>
<td>pEB19</td>
<td><em>CgIno1</em> knockout</td>
<td>Integrating</td>
<td><em>Nrl</em></td>
<td>This study</td>
</tr>
<tr>
<td>pEB21</td>
<td><em>CgIno1</em> reinteg.</td>
<td>Integrating</td>
<td><em>Nrl</em></td>
<td>This study</td>
</tr>
<tr>
<td>pEB13</td>
<td><em>CgIno4</em> knockout</td>
<td>Integrating</td>
<td><em>PmlII</em></td>
<td>This study</td>
</tr>
<tr>
<td>pEB17</td>
<td><em>CgIno4</em> reinteg.</td>
<td>Integrating</td>
<td><em>PmlII</em></td>
<td>This study</td>
</tr>
<tr>
<td>pCgOPI1</td>
<td>Contains <em>CgOPI1</em></td>
<td>Episomal</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>pCgopi1Δ</td>
<td><em>cgopi1::NAT1</em></td>
<td>PCR template</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>pRS306-his3Δ</td>
<td><em>CgHis3</em> knockout</td>
<td>Integrating</td>
<td><em>BglII</em></td>
<td>This study</td>
</tr>
<tr>
<td>pGRB2.1</td>
<td>ScURA3 vector</td>
<td>Episomal</td>
<td></td>
<td>Frieman et al. (2002)</td>
</tr>
<tr>
<td>pAG25</td>
<td>NAT1 cassette</td>
<td>PCR template</td>
<td></td>
<td>Goldstein &amp; McCusker (1999)</td>
</tr>
<tr>
<td>pEB48</td>
<td>tetR::HOP1::CgOPI1</td>
<td>Integrating</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>pINTG4</td>
<td>tetR::GAL1AD</td>
<td>Integrating</td>
<td></td>
<td>Nakayama et al. (1998)</td>
</tr>
<tr>
<td>pRS306</td>
<td>Shuttle vector</td>
<td>Integrating</td>
<td></td>
<td>Sikorski &amp; Hieter (1989)</td>
</tr>
</tbody>
</table>
URA3 gene product from *S. cerevisiae* or *C. glabrata* leads to production of 5-fluorouracil which is toxic to *C. glabrata* (Boeke *et al.*, 1987; Cormack & Falkow, 1999). If the Cgopi1Δ mutation is lethal, then no Cgopi1Δ pCgOPI1 colonies should grow on 5-FOA medium because the 5-FOA would select against the cells carrying pCgOPI1. In Fig. 4 it is clear that the Cgopi1Δ pCgOPI1 strain cannot grow on 5-FOA. In contrast, colonies from the parental strain carrying pCgOPI1 or the empty vector grew well on this medium, indicating that they had lost the plasmid but were still viable.

These experiments were performed in the BG2 strain background (Cormack & Falkow, 1999). To be sure that this phenotype was not strain-specific, the above strategy was used to test the essentiality of CgOPI1 in the ATCC 2001 strain background (a gift from Karl Kuchler). It was found that CgOPI1 was essential in strain ATCC 2001 as well, which suggested that the essentiality of CgOPI1 is not just a BG2 strain-specific phenomenon (data not shown).

**The regulation of viability by CgOPI1 is dependent on the CgINO2 transcription factor**

Based on the model from *S. cerevisiae* (Fig. 1), it was hypothesized that disruption of CgOPI1 causes overexpression of a downstream target of the inositol regulon that then results in a loss of viability. In *S. cerevisiae*, overexpression of ScINO1 in the Scopi1Δ mutant is due to unpressed transcriptional activation by ScIno2p (Fig. 1). The ScINO1 overexpression phenotype of the Scopi1Δ mutant can be blocked by a Scino2Δ mutation. The Scopi1Δ Scino2Δ double mutant acts like the Scino2Δ single mutant and fails to express ScINO1 because the Scino2Δ mutation is epistatic to the Scopi1Δ mutation (Graves & Henry, 2000).

If the Cgopi1Δ mutant compromises viability due to overexpression of a downstream target of CgIno2p, then
a Cgino2Δ mutation should restore the viability of a Cgopi1Δ mutant by blocking expression of this putative target. In order to test this hypothesis, the Cgino2Δ mutant was transformed with plasmid pCgOPI1, and the chromosomal ORF of CgOPI1 was disrupted as described above. The resulting Cgopi1Δ Cgino2Δ pCgOPI1 strain was streaked onto 5-FOA medium, and it was found to grow like the wild-type strain carrying pCgOPI1 or empty vector (Fig. 5). The Cgino2Δ strain also grew like the wild-type strain (Fig. 5).

The CgOPI1 gene product represses expression of CgINO1

The results above suggest that CgOpi1p can act as a repressor of CgIno2p targets such as CgINO1. To determine if CgOpi1p could repress CgINO1, CgOPI1 was placed under the control of a doxycycline-repressible promoter (Nakayama et al., 1998). C. glabrata strain BG14 (Cgura3Δ) was modified by disrupting both the CgHIS3 gene (making it a histidine auxotroph) and the CgTRP1 gene (making it a tryptophan auxotroph). Using the resulting triple auxotroph (Cgura3Δ Cghis3Δ Cgtrp1Δ), the promoter of the CgOPI1 gene was replaced on the chromosome by homologous recombination with the tetO-HOP1 promoter construct derived from plasmid p97CGH (CgHIS3) (Nakayama et al., 1998). This strain was further modified by integration of plasmid pINTG4 (CgTRP1) carrying the tetR::GAL4AD-encoded repressor-activator that activates the tetO-HOP1 chimeric promoter in the absence of doxycycline, but represses it in the presence of doxycycline.

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**Fig. 3.** The CgINO1, CgINO2 and CgINO4 genes are all required for de novo inositol biosynthesis, like their S. cerevisiae homologues. The Cgino1Δ, Cgino2Δ and Cgino4Δ mutants and their respective reconstituted strains (along with the wild-type control) were streaked onto inositol-free media supplemented with 0 (−) or 75 μM inositol (+), and grown at 30 °C for 3 days.

**Fig. 4.** The CgOPI1 gene is essential. Wild-type (WT) and Cgopi1Δ strains carrying CgOPI1 on a URA3 plasmid (pCgOPI1) were grown for 3 days on medium with (+) or without (−) 5-FOA at 30 °C.
The resulting strain was then tested for growth in the presence and absence of doxycycline, and it was found that this strain showed very poor growth in 10 μg doxycycline ml⁻¹, but grew quite well in the absence of doxycycline (Fig. 6b). These experiments were performed on synthetic minimal medium, but similar results were seen in YPD (rich) medium (E. K. Bethea & T. B. Reynolds, unpublished). When CgOPII levels were assessed in this strain by Northern blotting, it was found that CgOPII was expressed in the absence of doxycycline, but was not expressed in the presence of the drug (Fig. 6a).

CgOpi1p is a repressor of CgINO1. When CgOPII is repressed by doxycycline, CgINO1 expression increases substantially, but when CgOPII is expressed in the absence of doxycycline, then CgINO1 mRNA is very low or undetectable (Fig. 6a). This indicates that CgOpi1p acts as a repressor of CgINO1. We were also able to demonstrate the repression of CgINO1 expression by CgOpi1p using the C. glabrata copper-inducible MTII promoter (El Barkani et al., 2000) as well (E. K. Bethea & T. B. Reynolds, unpublished).

**Overexpression of CgINO1 is not responsible for the loss of viability in the Cgopi1Δ mutant**

Since CgINO1 is overexpressed in the absence of CgOPII, it seemed possible that CgINO1 overexpression is responsible for the loss of viability. This was tested by creating a Cgopi1Δ Cgino1Δ pCgOPI1 double mutant. However, when this double mutant is streaked onto 5-FOA it fails...
to grow (E. K. Bethea & T. B. Reynolds, unpublished) indicating that the disruption of CgINO1 is not sufficient to restore viability as seen with CgINO2 (Fig. 5).

DISCUSSION

Transcriptional rewiring appears to exist between the transcription factor homologues of the inositol regulons of S. cerevisiae and C. albicans (Hoppen et al., 2007; Y. L. Chen & T. B. Reynolds, unpublished). C. glabrata is much more closely related to S. cerevisiae than C. albicans based on phylogenetic trees comparing 18S ribosomal sequences (Kaur et al., 2005). Our analysis suggests that the C. glabrata inositol regulon is not transcriptionally rewired compared to S. cerevisiae, at least for CgINO1 regulation. However, a major difference between the two species is that the OPI1 homologue in C. glabrata appears to be essential (Figs 4–6), whereas it is not in S. cerevisiae.

It is not clear why the Cgopi1Δ mutation is lethal, but our data suggest that the Cgopi1Δ mutation causes overexpression of Cglno2p-Cglno4p target genes and one of these targets causes a loss in viability when overexpressed. In support of this hypothesis, disruption of CgINO2 in the Cgopi1Δ strain rescues growth of the Cgopi1Δ mutant, presumably because the downstream target is no longer overexpressed (Fig. 5).

One possible target for compromising viability appeared to be CgINO1. However, disruption of CgINO1 in the Cgopi1Δ strain did not rescue viability on 5-FOA plates (E. K. Bethea & T. B. Reynolds, unpublished). This indicates that CgINO1 overexpression is not toxic.

There are two main models to explain the loss of viability of the Cgopi1Δ mutant. (1) A direct downstream target gene involved in phospholipid biosynthesis is overexpressed, and C. glabrata is particularly sensitive to this imbalance in lipid biosynthesis and loses viability. (2) Expression of a direct target gene not involved in lipid biosynthesis is affected by Cgopi1Δ and results in a loss of viability.

In the first model, there are several phospholipid biosynthetic genes that may be targets of the C. glabrata inositol regulon based on sequence similarity to homologues in S. cerevisiae. Direct downstream targets of the S. cerevisiae inositol regulon have been identified by the presence of the UASINO consensus sequence CATGGAAAT in their promoters and their misregulation in Scino2Δ, Scino4Δ and Scopi1Δ mutants (Bailis et al., 1992; Graves & Henry, 2000; Jackson & Lopes, 1996; Lai & McGraw, 1994; Li & Brendel, 1993). Using these genes as a guide, there are five genes in C. glabrata that contain the sequence CATGG (the most important part of the UASINO consensus sequence; Greenberg & Lopes, 1996) in their promoters. These genes include CgINO1, CgOPI3, CgCHO1, CgCHO2 and CgITR2. In addition to these five genes, two other genes, CgERG20 and CgCKI1, contain the sequence CATGTT, which differs by only one base and could possibly also respond to the inositol regulon. In the second model, the target may be unrelated to phospholipid biosynthesis and/or may not have a homologue that is regulated by the inositol regulon in S. cerevisiae. Such a gene might also not be the direct cause of the loss of viability, but might itself regulate a downstream effector and cause the loss of viability. For example, if CgOPI1 were to repress a transcriptional repressor of an essential gene, then loss of CgOPI1 could result in loss of expression of the essential gene and compromise viability.

The essential nature of Opi1p in C. glabrata appears to be unique among the few characterized members of the growing Op1 family of proteins, a family that is specific to fungi (Hirakawa et al., 2009). Opi1p homologues in S. cerevisiae, C. albicans, and Yarrowia lipolytica (Yas3p) are not essential (Hirakawa et al., 2009; Jiranek et al., 1998) (Y. L. Chen & T. B. Reynolds, unpublished). It is of interest to note that in S. cerevisiae and C. glabrata, two closely related yeasts, the Opi1p proteins both regulate inositol biosynthesis, whereas in the more distantly related yeasts C. albicans and Y. lipolytica, their Opi1p family members, CaOpi1p (C. albicans) and Yas3p (Y. lipolytica) do not regulate inositol biosynthesis (Endoh-Yamagami et al., 2007; Hirakawa et al., 2009; Yamagami et al., 2004) (Y. L. Chen & T. B. Reynolds, unpublished). Thus, the Opi1p family of proteins may have a number of diverse functions.

We are currently investigating the mechanism by which CgOPI1 controls viability in C. glabrata. Considering that the Opi1p family of proteins is unique to fungi, CgOpi1p may be a possible future drug target for treating C. glabrata infections.

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