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.

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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 (Tsong *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

Abbreviations: 5-FOA, 5-fluororotic acid; NCR, non-coding region; UAS_{INO}, upstream activator sequence for inositol regulation.

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-vav 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 (UAS_{INO}) in the promoters of target genes such as *ScINO1* (Ambroziak & Henry, 1994; Bachhawat *et al.*, 1995; Loewen *et al.*, 2003; Lopes & Henry, 1991; Nikoloff & 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 UAS_{INO} 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

Fig. 1. The inositol regulon in *S. cerevisiae* controls transcription of phospholipid biosynthetic genes like *ScINO1* in response to the level of extracellular inositol. (a) The ScIno2p-ScIno4p heterodimer activates transcription of *ScINO1* and other target genes in the absence of extracellular inositol. ScIno2p-ScIno4p binds UAS_{INO} present in the promoters of genes like *ScINO1*. (b) In the presence of extracellular inositol, ScOpi1p binds to ScIno2p and prevents it from activating transcription of *ScINO1* and other targets.

Table 1. Strains used in this study

Strain	Genotype	Reference
BG14	<i>Cgura3::Tn903neoR</i>	Cormack & Falkow (1999)
BG2	<i>CgURA3</i> , parent of BG14	Cormack & Falkow (1999)
Cg11	<i>Cgura3::Tn903neoR</i> pCgOPI1	This study
Cg12	<i>Cgura3::Tn903neoR</i> pGRB2.1	This study
Cg14	<i>Cgura3::Tn903neoR Cgopi1Δ</i> pCgOPI1	This study
Cg5	<i>Cgura3::Tn903neoR Cgino2Δ</i>	This study
EBCg019	<i>Cgura3::Tn903neoR Cgino2Δ::CgINO2</i>	This study
Cg23	<i>Cgura3::Tn903neoR Cgino2Δ</i> pCgOPI1	This study
Cg18	<i>Cgura3::Tn903neoR Cgino2Δ Cgopi1Δ</i> pCgOPI1	This study
EBCg005	<i>Cgura3::Tn903neoR Cgino4Δ</i>	This study
EBCg008	<i>Cgura3::Tn903neoR Cgino4Δ::CgINO4</i>	This study
EBCg014	<i>Cgura3::Tn903neoR Cgino1Δ</i>	This study
EBCg017	<i>Cgura3::Tn903neoR Cgino1Δ::CgINO1</i>	This study
Cg33	<i>Cgura3::Tn903neoR Cghis3Δ</i>	This study
EBCg046	<i>Cgura3::Tn903neoR Cghis3Δ Cgtrp1Δ</i>	This study
EBCg048	<i>Cgura3::Tn903neoR Cghis3Δ Cgtrp1Δ</i> pINTG4 pEB48	This study
EBCg049	<i>Cgura3::Tn903neoR Cghis3Δ Cgtrp1Δ</i> pINTG4 pEB48	This study

Southern blotting with the *tetO::HOP1::CgOPI1* strains. Strain BG14, which is a uracil auxotroph (Table 1), was a gift from Brendan Cormack and was used to generate all of the strains reported in this study. Disruption of *CgINO2*, *CgINO4* and *CgINO1* was performed in BG14 (Table 1) utilizing a two-step gene disruption 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 *ScURA3* marker that can be selected for on media lacking uracil and counterselected against using media containing 5-fluororotic acid (5-FOA). Reconstitution of gene disruption mutants was done by transforming the strains with *ScURA3*-marked integrating plasmids carrying the wild-type gene (Table 3).

The *Cgopi1Δ* mutant (Cg14, Table 1) was generated in BG14 by transforming it with the pCgOPI1 episomal plasmid (*ScURA3* marker, Table 3), and then disrupting the *CgOPI1* gene on the chromosome with the *Cgopi1::NAT1* construct amplified from plasmid pCgopi1Δ (Table 3). The *Cgopi1Δ Cgino2Δ* double mutant was made in the same manner as the *Cgopi1Δ* mutant except it was done in strain Cg5 (*Cgino2Δ*).

The *tetO::HOP1::CgOPI1* strains EBCg048 and EBCg049 (Table 1) were generated by first making BG14 a histidine and tryptophan auxotroph (*Cghis3Δ Cgtrp1Δ*). This was done by disrupting *CgHIS3* in BG14 as described above for the *Cgino2Δ* gene to create strain Cg33. The *CgTRP1* gene was disrupted in Cg33 using a plasmid from Karl Kuchler's lab to create strain EBCg046. EBCg046 was used to create the *tetO::HOP1::CgOPI1* strains EBCg048 and EBCg049 by first integrating plasmid pINTG4 carrying the *tetR::GAL4AD* repressor-activator into its genome as described by Nakayama *et al.* (1998). Then the *tetO::HOP1::CgOPI1* construct was PCR-amplified from plasmid pEB48 (Table 3) and was used to replace the *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.

Plasmids and constructs. The gene disruption plasmids (pRS306-ino2Δ, pRS306-ino4Δ and pRS306-ino1Δ) were made by PCR-

amplifying DNA corresponding to approximately 500 bp of non-coding DNA that flanked 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-his3Δ disruption plasmid was created in a similar manner by subcloning the *CgHIS3* 5' NCR into plasmid pGRB2.1 (Frieman *et al.*, 2002) upstream of the 3' NCR of *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 *XbaI-KpnI* fragment to create pRS306-his3Δ. 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-ino1Δ cassette was cut with *NruI* in the 5' NCR to linearize it and target it to recombine upstream of *CgINO1*. PCR was used to confirm correct integrations and disruptions. In a similar manner, plasmids pRS306-ino4Δ, pRS306-ino1Δ and pRS306-his3Δ were cut with *PmlI*, *SnaBI* and *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 BCO3 and BCO2 for *CgINO2*, USO1 and USO4 for *CgINO4*, and MHO3 and MHO2 for *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 pCgOPI1 carrying *CgOPI1* plus 215 bp of upstream DNA and 439 bp of downstream DNA was generated by amplifying the *CgOPI1* ORF and flanking sequences from purified BG14 DNA using primers TRO605 and TRO608, and cutting the PCR product with *BamHI* and *SpeI* enzymes. The *SpeI* site was introduced by primer TRO608, and the *BamHI* site was internal to the amplified DNA fragment. This PCR product was cloned into the *ScURA3*-bearing *C. glabrata* CEN/ARS plasmid pGRB2.1 (Frieman *et al.*, 2002) using *SpeI* and *BamHI*. Plasmid pCgopi1Δ was generated by subcloning the *NAT1* cassette from pAG25 (Goldstein & McCusker, 1999) with primers TRO652 and TRO653 into an *EcoRI* site between a 469 bp fragment containing the 5' NCR of *CgOPI1* (cloned by

Table 2. Primers used in this study

Primer	Sequence (5'–3')*	Restriction site	Purpose†
TRO605	AAAAAAGCTTTGCCTCCTTATCGGTAACAA	<i>Hind</i> III	Forward for 5' NCR of <i>CgOPII</i>
TRO606	AAAAGAATTCTCCAGCAGCACAGTTTATTCA	<i>Eco</i> RI	Reverse for 5' NCR of <i>CgOPII</i>
TRO607	AAAAGAATTCTCTTCAAATTGAAAACGTTACGAC	<i>Eco</i> RI	Forward for 3' NCR of <i>CgOPII</i>
TRO608	AAAACTAGTGATTTCTGTTTGACTATTGGTTCTC	<i>Spe</i> I	Reverse for 3' NCR of <i>CgOPII</i>
TRO652	AAAAGAATTCAGCTGAAGCTTCGTACGC	<i>Eco</i> RI	Forward for <i>NAT1</i> gene
TRO653	AAAAGAATTCGCATAGGCCACTAGTGGATCTG	<i>Eco</i> RI	Reverse for <i>NAT1</i> gene
VSO1	AAAAGCTCGAGCCTTTACACCTGTGAACACAGTCA	<i>Xho</i> I	Forward for 5' NCR <i>CgINO4</i>
VSO2	AAAAGAATTCTCCGACTTTTGAAATGGGGT	<i>Eco</i> RI	Reverse for 5' NCR <i>CgINO4</i>
VSO3	AAAAGAATTCTGCCTGTAATTGAGAAATCCTATTG	<i>Eco</i> RI	Forward for 3' NCR <i>CgINO4</i>
VSO4	AAAAGGATCCTTGAAAAGAGAAGTTAAAACAGAGG	<i>Bam</i> HI	Reverse for 3' NCR <i>CgINO4</i>
MHO3	AAAAGCTCGAGTGTCCCTTTTGTGGTCC	<i>Xho</i> I	Forward for 5' NCR <i>CgINO1</i>
MHO4	AAAACCCGGGTCGTGTGGTAAGTGTAGTTGGTCA	<i>Sma</i> I	Reverse for 5' NCR <i>CgINO1</i>
MHO1	AAAACCCGGGCAGACTTCCCAATGAGGGAAA	<i>Sma</i> I	Forward for 3' NCR <i>CgINO1</i>
MHO2	AAAACCCGGGCGTTCGTGGCGAAACTTTT	<i>Sac</i> I	Reverse for 3' NCR <i>CgINO1</i>
BCO3	AAAAAAGCTTTGCGCCGTCTGAAAAAAA	<i>Hind</i> III	Forward for 5' NCR of <i>CgINO2</i>
BCO4	AAAAGAATTCGGTTCGTGTATTAAATTAAGCACTC	<i>Eco</i> RI	Reverse for 5' NCR of <i>CgINO2</i>
BCO1	AAAAGAATTCCTACTGACTGTATGTTAGGCTGCAA	<i>Eco</i> RI	Forward for 3' NCR of <i>CgINO2</i>
BCO2	AAAAGGATCCCAAACTCTTCTTTGAATGACTTTG	<i>Bam</i> HI	Reverse for 3' NCR of <i>CgINO2</i>
TRO665	AAAATCTAGAATTCCTCCCATGTACCACAGTC	<i>Xba</i> I	Forward for 5' NCR of <i>CgHIS3</i>
TRO667	AAAAGGATCCTTGCTCGATGCTTCTCTTTG	<i>Bam</i> HI	Reverse for 5' NCR of <i>CgHIS3</i>
EBO24	GTTGCTGTAAAGTATGTTGA		Check insertion of pEB13 (ino4Δ)
EBO27	TGGCAACTAGAATTTTCACATGC		Check insertion of pEB19 (ino1Δ)
TRO668	TACGTTGTTACCCACACGATT		Check insertion of pRS306-his3Δ
TRO623	ACAGTCATCCAAAGGTGACTCTCAT		Check insertion of pRS306-ino2Δ
TRO536	CCAGGGTTTCCCAAGTCA		Reverse for primers EBO24, EBO27 and TRO623
TRO614	GAAGGAATCTCAAAAGTGCGGA		Check insertion of pCgopi1Δ
TRO461	GTGCGCAGAAAGTAATATC		Reverse primer for TRO614
EBO64	AAAAGAATTCACCATGGACACAAGGCGTG	<i>Eco</i> RI	Forward for <i>CgOPII tetO</i> promoter
EBO65	AAAAGGGCCCGTAGATGTAGGTTCTCCTTTTCATTA	<i>Ap</i> I	Reverse for <i>CgOPII tetO</i> promoter
EBO40	ATGACTGTGAATAAAGGTATTAGCATTC		Forward for <i>CgINO1</i> probe
EBO41	CTATTTCAATCTTTCTTCGAATCTCAG		Reverse for <i>CgINO1</i> probe
TRO656	GCCGGTTTCGCCGGTGACG		Forward for <i>CgACT1</i> probe
TRO636	CCAAAGCGACGTAACATAGCTTT		Reverse for <i>CgACT1</i> probe
EBO12	GACACAAGGCGTGCGGTG		Forward for <i>CgOPII</i> probe
EBO11	TCATACCTTTTGTAATGCATA		Reverse for <i>CgOPII</i> probe

*Underline shows the restriction site.

†NCR, Non-coding region that is either 5' or 3' to the designated ORF.

primers TRO605 and TRO606) and a 439 bp fragment containing the 3' NCR of *CgOPII* (cloned by primers TRO607 and TRO608), both carried on the pRS306 vector. The pINT4 vector was a gift from Hironobu Nakayama (Nakayama *et al.*, 1998) and was integrated into the EBCg046 genome after cutting it with *Eco*RV (Nakayama *et al.*, 1998). Plasmid pEB48 was generated by subcloning an ~500 bp 5' NCR of the *CgOPII* ORF into p97CGH upstream (5') of the *HIS3 tetO-HOP1* cassette using primers EBO76 and EBO77. The *CgOPII* ORF was then subcloned 3' to the *HIS3 tetO-HOP1* cassette using a PCR product made by primers EBO64 and EBO65. This whole segment from pEB48, containing 5'-*CgOPII*, NCR-*HIS3* and the *tetO-HOP1-CgOPII* ORF was then transformed into strain EBCg046 carrying pINTG4 to replace the *CgOPII* promoter with the *tetO-HOP1* cassette.

Northern blotting. Northern blotting was performed essentially as described by Reynolds (2006) using probes generated from primers EBO40 and EBO41 for *CgINO1*, EBO11 and EBO12 for *CgOPII*,

TRO656 and TRO636 for *CgACT1*, TRO480 and TRO648 for *ScACT1*, EBO56 and EBO57 for *CgCHO1*, EBO54 and EBO55 for *CgOPI3*, and EBO58 and EBO59 for *CgCHO2*. Probes specific for *ScACT1* or *CgACT1* were used to reprobe Northern blots for *CgACT1* expression in *C. glabrata* for normalization, and both probes appeared to work well.

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

Table 3. Plasmids used in this study

Plasmid	Description	Type	Restriction site	Reference
pRS306-ino2Δ	<i>CgINO2</i> knockout	Integrating	<i>SnaBI</i>	This study
pRS306-CgINO2	<i>CgINO2</i> reintegration	Integrating	<i>SnaBI</i>	This study
pEB19	<i>CgINO1</i> knockout	Integrating	<i>NruI</i>	This study
pEB21	<i>CgINO1</i> reintegration	Integrating	<i>NruI</i>	This study
pEB13	<i>CgINO4</i> knockout	Integrating	<i>PmlI</i>	This study
pEB17	<i>CgINO4</i> reintegration	Integrating	<i>PmlI</i>	This study
pCgOPI1	Contains <i>CgOPI1</i>	Episomal		This study
pCgopi1Δ	<i>Cgopi1::NAT1</i>	PCR template		This study
pRS306-his3Δ	<i>CgHIS3</i> knockout	Integrating	<i>BglII</i>	This study
pGRB2.1	<i>ScURA3</i> vector	Episomal		Frieman <i>et al.</i> (2002)
pAG25	<i>NAT1</i> cassette	PCR template		Goldstein & McCusker (1999)
pEB48	<i>tetO::HOP1::CgOPI1</i>	Integrating		This study
pINTG4	<i>tetR::GAL4AD</i>	Integrating		Nakayama <i>et al.</i> (1998)
pRS306	Shuttle vector	Integrating		Sikorski & Hieter (1989)

against the *C. glabrata* genome at the Genolevures website (www.genolevures.org). The BLAST search revealed that the gene *CAGL0I06050g* encoded a protein that was 73.9 % identical to *ScIno1p*. We refer to *CAGL0I06050g* 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 *CAGL0B01947g* for *ScIno2p* (35.6 % identity) and *CAGL0I07359g* for *ScIno4p* (44.5 % identity). These genes, *CAGL0B01947g* and *CAGL0I07359g*, 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 *CAGL0K03267g* was found to be 52.3 % identical to *ScOpi1p*. 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

Fig. 2. *CgINO2* and *CgINO4* are required to express *CgINO1* in the absence of exogenous inositol. (a) Northern blotting was used to assess the expression of *CgINO1* in wild-type (WT), mutant and reconstituted strains in the presence (+) or absence (-) of exogenous inositol. *CgACT1* was used as a loading control. Strains were grown overnight at 30 °C in inositol-free medium supplemented with 75 μM inositol, washed with water, and resuspended in inositol-free media containing either 0 or 75 μM inositol, after which the cultures were incubated with shaking for 6 h before collecting total RNA for Northern blotting. (b) A Storm Phosphorimager was used to quantify expression of *CgINO1* in each strain relative to the wild-type strain grown in inositol-free medium, which was arbitrarily set at 100 %. Expression was normalized to *CgACT1* expression. These results are representative of four different experiments.

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 unrepressed 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

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.

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.

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.

Fig. 5. The viability defect of the *Cgopi1Δ* mutant is dependent on the *CgINO2* transcriptional activator. The wild-type (WT), *Cgopi1Δ*, *Cgino2Δ* and *Cgopi1Δ Cgino2Δ* strains containing plasmid pCgOPI1 (URA3-based) were streaked onto synthetic medium plates with (+) or without (–) 5-FOA. The WT strain containing the empty vector was included as a control.

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 *CgOPI1* levels were assessed in this strain by Northern blotting, it was found that *CgOPI1* 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 *CgOPI1* is repressed by doxycycline, *CgINO1* expression increases substantially, but when *CgOPI1* 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 *CgOPI1*, 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

Fig. 6. CgOpi1p is a transcriptional repressor of *CgINO1*. The promoter of the chromosomal copy of *CgOPI1* was replaced with the doxycycline-repressible *tetO::HOP1* promoter. (a) Expression of *CgOPI1*, *CgINO1* and *CgACT1* (loading control) were tested by Northern blotting in the presence (+) and absence (–) of doxycycline. Cells were grown to saturation overnight in synthetic medium (containing $\sim 11 \mu\text{M}$ inositol) lacking doxycycline, washed with water, and were then resuspended in fresh synthetic medium containing 0 or 10 μg doxycycline ml^{-1} and grown for ~ 6 h at 30 $^{\circ}\text{C}$ with shaking at which time samples were taken for Northern blotting. (b) Cells were grown on plates containing 0 (–) or 10 μg doxycycline ml^{-1} (+) to confirm that loss of *CgOPI1* expression decreased viability. Two different transformants were tested for the doxycycline repressible promoter.

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 *CgIno2p*–*CgIno4p* 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 UAS_{INO} consensus sequence CATGTGAAAT 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 CATGTG (the most important part of the UAS_{INO} 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 *Opi1p* 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, Ca*Opi1p* (*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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