

Published in final edited form as:

*J Microbiol Methods*. 2011 August ; 86(2): 156–159. doi:10.1016/j.mimet.2011.04.014.

## Efficient implementation of RNA interference in the pathogenic yeast *Cryptococcus neoformans*

Indrani Bose and Tamara L. Doering\*

### Abstract

An improved method has been developed for RNA interference in *Cryptococcus neoformans*, using opposing promoters to facilitate cloning and RNA interference targeting *URA5* to allow selection of cells in which silencing is most effective. These advances significantly reduce the variability of silencing and the effort required for interference plasmid construction.

### Keywords

*Cryptococcus neoformans*; RNA interference; pathogenic fungus

*Cryptococcus neoformans* is a basidiomycetous yeast that causes serious opportunistic infections, leading to over 600,000 deaths annually (Park et al., 2009). The fascinating biology of this organism and its major impact on world health have motivated intensive study of cryptococcal virulence factors and pathogenicity. One valuable molecular tool in this work is targeted gene silencing mediated by RNA interference (RNAi) (Liu et al., 2002; Panepinto et al., 2009). Unfortunately, the utility of this method has been limited by variability in RNAi penetrance and the labor required to generate silencing plasmids. We have now developed a double promoter construct for RNAi in *C. neoformans* to simplify cloning. We have also incorporated *URA5* gene silencing to allow selection for cells that robustly perform RNAi.

The plasmids we developed contain two cryptococcal promoters in opposing orientation, between which the sequence to be silenced can be inserted. Placing the target sequence between opposing promoters allows it to be transcribed from both directions simultaneously, producing complementary transcripts. These transcripts can hybridize to form the double-stranded RNA that activates the RNAi system. The opposing promoters of the plasmids are separated by restriction sites, allowing simple insertion of the gene segment(s) to be bidirectionally transcribed, and are flanked by terminators (Fig. 1A). This plasmid design eliminates the need to clone the target sequence twice in opposite orientations to yield a hairpin RNA, which is another way to produce double-stranded RNA and activate the RNAi pathway in fungi (Liu, 2002). The vectors also contain a selectable marker (*ADE2*, *URA5*, or *neo<sup>R</sup>* which confers geneticin-resistance) and telomeric (*TEL*) sequences (Edman, 1992); the latter become terminal when the vector is digested with *I-SceI*, thus stabilizing the plasmid (Fig. 1A).

© 2010 Elsevier B.V. All rights reserved.

\*Corresponding author: Department of Molecular Microbiology, Washington University School of Medicine, Campus Box 8230, 660 South Euclid Avenue, St. Louis, MO 63110, 314-747-5597, fax 314-362-1232, doering@wustl.edu.

**Publisher's Disclaimer:** This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

We first tested *URA5*-marked plasmids with opposing copies of the constitutively active *ACT1* promoter (Cox et al., 1995) (pIBB24), opposing copies of the regulatable *GAL7* promoter (Wickes and Edman, 1995) (pIBB1), or one of each promoter (pIBB5). (Table 1 summarizes these plasmids and others discussed below.) As a target sequence each plasmid included a ~500 bp *BglII* fragment of *ADE2* (Sudarshan et al., 1999), from the plasmid pADE2i (Liu et al., 2002). Successfully silencing *ADE2* (which encodes phosphoribosylaminoimidazole carboxylase) yields pink colonies, due to accumulation of a purine biosynthetic intermediate. Each plasmid was electroporated into a *ura5* serotype D strain (JEC43, Wickes et al., 1997) and transformants were selected on medium without uracil. The selection medium contained 2% galactose as the carbon source for plasmids with one or two copies of the *GAL7* promoter and 2% glucose for other transformations. Transformation with pIBB24 and pIBB5 yielded uniformly white colonies (not shown), suggesting that *ADE2* silencing was not effectively mediated by these constructs. In contrast, >80% of transformants carrying the pIBB1 plasmid were pink to red in color after 5 days of incubation at 30°C (Fig. 1B, left) demonstrating that the *ADE2* gene had been effectively silenced by the plasmid with opposing *GAL7* promoters.

We observed wide variation in the colony phenotypes of cells transformed with pIBB1, with colors ranging from cream to dark red (Fig. 1B, left). Results were similar when we modified this plasmid to target *LAC1* (pIBB29); this gene encodes a laccase responsible for melanin production that causes colonies to appear brown on appropriate media. While many transformants in this case appeared white, indicating successful silencing, a substantial minority displayed various levels of melanization (Fig. 1B, right). The apparent variability in RNAi efficacy might confound interpretation of studies employing RNAi. We therefore developed a system to eliminate those transformants in which silencing is inefficient.

In our earlier studies of RNAi in *C. neoformans*, we proposed simultaneous silencing of *ADE2* and a gene of interest, in order to use the red pigment that accumulates upon *ADE2* silencing as an indicator of RNAi efficacy (Liu et al., 2002). GFP has also been used as an RNAi “sentinel” in *Histoplasma capsulatum* to identify individual transformants in which RNAi is most robust (Rappleve et al., 2004). Both of these indicators are useful, but we wished to develop a way to select transformants in which RNAi is most effective. We chose to target *URA5* (Edman and Kwon-Chung, 1990) because silencing this gene would allow transformants to survive treatment with 5-fluoroorotic acid (5-FOA). (5-FOA is converted to 5-fluorouracil in strains bearing a functional *URA5* gene; this product is toxic to yeast (Boeke et al., 1987).) To target *URA5*, we cloned a 610-bp *Sall* fragment of the gene between the *GAL7* promoters of pIBB1 and replaced the existing *URA5* marker with an *ADE2* marker. We electroporated the resulting plasmid (pIBB32) into a serotype D *ade2* mutant strain (JEC50) and grew the transformed cells for 3 h in medium lacking adenine (to select against untransformed cells) and containing galactose (to induce synthesis of double-stranded RNA corresponding to the *URA5* sequence). We next treated the cells for 1 h with 1 mg/ml 5-FOA to select for cells in which interference was active and plated them on galactose medium lacking adenine. We restreaked the colonies onto the same medium, and then replica-plated onto galactose media either lacking uracil (so that cells with functioning RNAi cannot grow) or containing 1 mg/ml 5-FOA (so that wild-type cells cannot grow). Virtually all of the recovered colonies (100 of 101 tested) grew on 5-FOA (Fig. 2A and data not shown), confirming effective silencing of the *URA5* gene. The telomeric vector remained episomal in these strains, as shown by plasmid loss upon plating on non-selective medium (data not shown). These results suggested that *URA5* silencing with 5-FOA selection is an efficient way to isolate cells that are effectively performing RNAi. We confirmed this finding by using this strategy to target the *LAC1* gene in tandem with *URA5* (pIBB69). The colony color of transformants in this experiment matched that of a *lac1* mutant (Fig. 2B) and was significantly ( $p < 0.0002$ ) more consistent (0% melanin-producing

colonies, n=3) than in similar studies without the selection step (12 – 15% melanin-producing colonies, n=3) (Fig. 1B, right panel).

In our initial studies we used 500- to 700-bp segments of chromosomal DNA to direct silencing of designated target genes (*ADE2*, *URA5*, and others not shown). Although we generally chose regions that were primarily exonic, the multiple short exons typical of *C. neoformans* genes (Tenney et al., 2004; Loftus et al., 2005) meant that the target regions often contained some intronic sequence as well. This raised the question of the optimum length of sequence for efficient silencing. To test this we amplified a series of fragments from genomic DNA using primer IB077 [5'-CCATTAATCAAGCTGGACAATTCCGAC-3'] as the reverse primer and forward primers designed to amplify a total of 100, 200, 300, 400, or 500 bp of the *URA5* gene. We replaced the longer *URA5* segment in pIBB32 with each fragment, transformed JEC50 with the resulting plasmids, and tested the transformants for resistance to 1 mg/ml 5-FOA as an assay of RNAi efficacy. In one representative experiment the fraction of colonies demonstrating robust RNAi was 0, 91, 95, 67, and 62% for plasmids containing target fragments of 100, 200, 300, 400, and 500 bp, respectively. Based on these and other similar results (not shown), we performed subsequent studies using the pIBB32-derived vector pIBB75 (Table 1), which bears a 250-bp *URA5* target fragment amplified using primers IBO77 and IBO78 [5'-CCATTAATCAAGCTGGACAATTCCGAC-3'].

In all organisms studied so far, RNAi employs conserved proteins of the Dicer and Argonaute families; in many cases RNA-dependent RNA polymerases also play a major role in this phenomenon. We identified two orthologs of each of the Argonaute and Dicer genes and one RNA-dependent RNA polymerase (RdRP) gene in the JEC21 serotype D strain of *C. neoformans*, consistent with observations by Nakayashiki et al. (2006) and Janbon et al. (2010). To confirm the requirement for an RdRP in cryptococcal RNAi, we replaced the *RDPI* gene (CNG01230) in serotype D strain JEC50 with a nourseothricin resistance cassette (McDade and Cox, 2001). We then transformed these and wild-type cells with pIBB103 (Genbank accession number HQ455038), a plasmid identical to pIBB75 but with a neomycin resistance marker that enables growth on medium containing G418 (Fig. 3, top). Wild-type transformants effectively silenced the *URA5* gene and grew on media containing 5-FOA, while the *rdp1*Δ strain was not viable on 5-FOA-containing medium (Fig. 3, bottom). Complementing the *rdp1*Δ with wild-type *RDPI* at the endogenous locus restored wild-type growth on 5-FOA (Fig. 3, bottom). RT-PCR of these strains confirmed that *URA5* mRNA is reduced in the wild type and *RDPI* complemented strains compared to the *rdp1*Δ strain (not shown). Together, our studies show that (1) the *RDPI* gene is required for RNAi in *C. neoformans*, (2) opposing promoter constructs mediate RNAi, (3) 200–300 bp is the optimum length for target inserts, and (4) *URA5* targeting is an effective strategy to select transformants that efficiently perform RNAi. We have now used the opposing promoter constructs described here to effectively silence multiple genes in serotype D *C. neoformans* (this report, Reilly (2010), and unpublished data from our laboratory), suggesting that the approach is target-independent. These findings should advance use of RNAi as a powerful tool in studies of an important pathogenic fungus.

#### Research Highlights

- An improved method has been developed for RNA interference in *Cryptococcus neoformans*
- The method simplifies construction of interference plasmids
- The method allows selection of cells in which RNAi is effective

-- These improvements reduce effort and improve results for RNAi in *C. neoformans*

## Abbreviations

<b>RNAi</b>	RNA interference
<b>5-FOA</b>	5-fluoroorotic acid
<b>Ade</b>	adenine
<b>Gal</b>	galactose
<b>Ura</b>	uracil

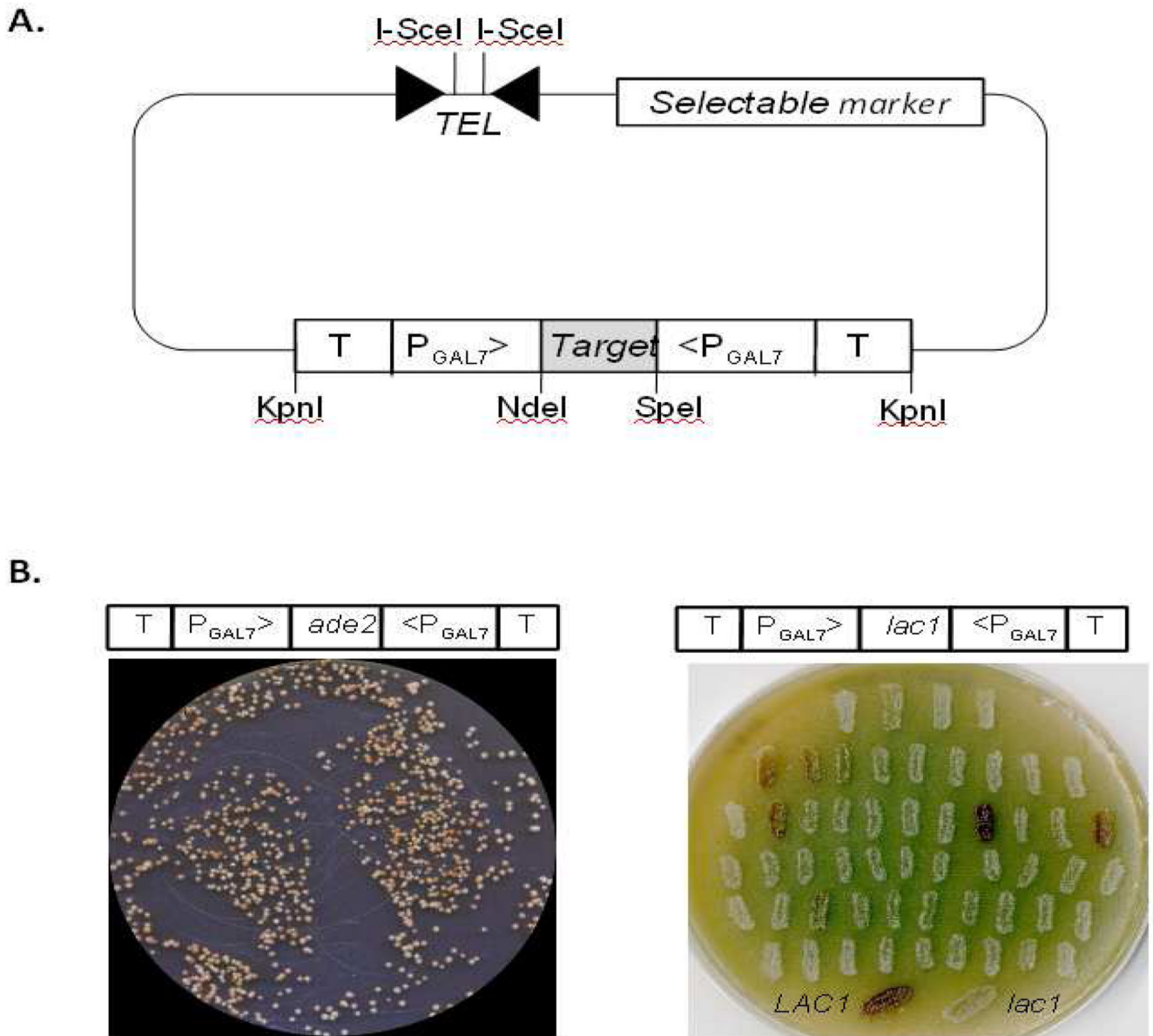
## Acknowledgments

This work was supported by National Institutes of Health grant GM071007 and an Award from the Edward J. Mallinckrodt, Jr. Foundation. The authors are grateful to Morgann C. Reilly for plasmid sequencing, Michael L. Skowyrza for comments on the manuscript and sequence submission, and Doering lab members for helpful suggestions.

## References

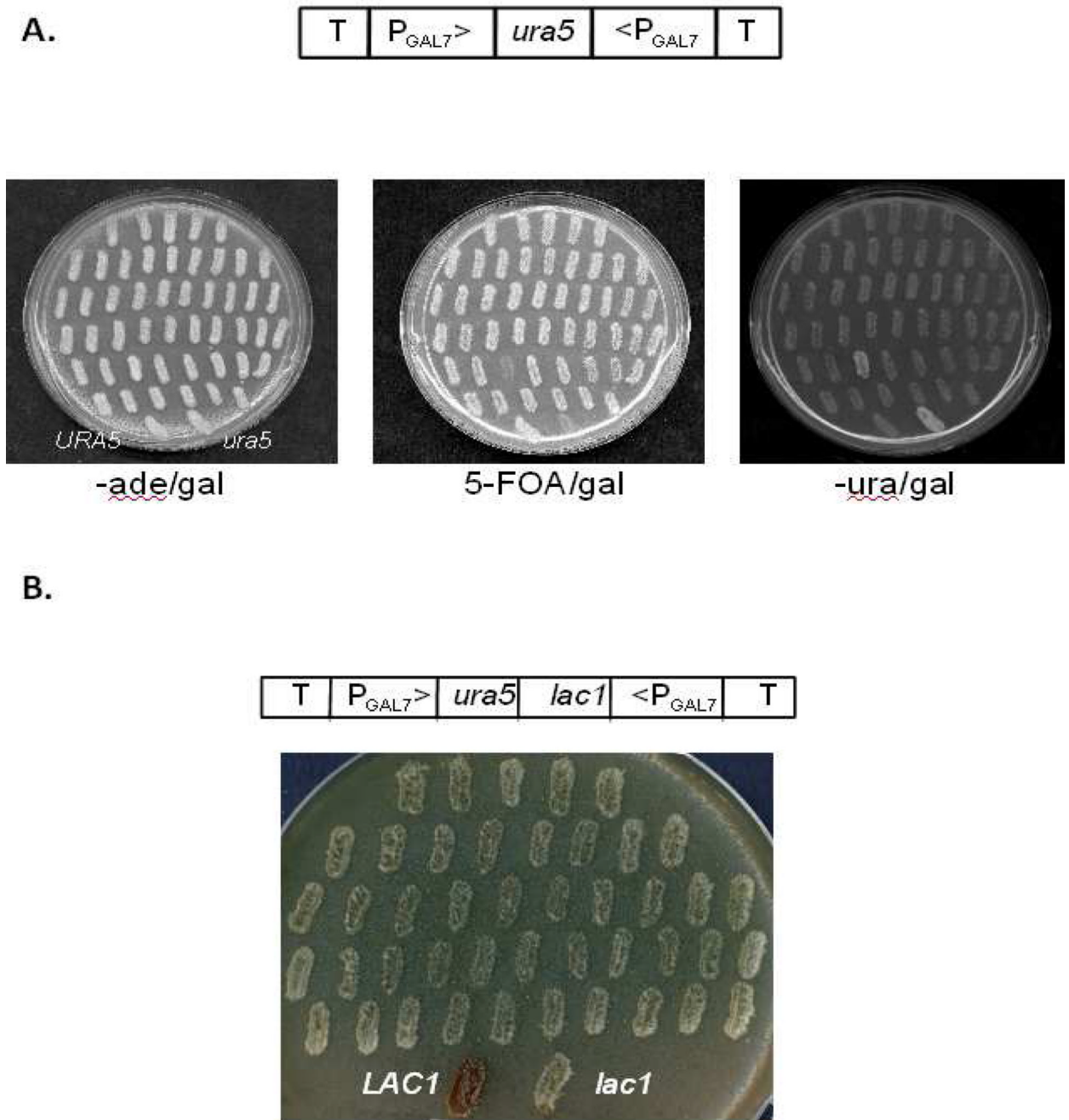
- Boeke JD, Trueheart J, Natsoulis G, Fink GR. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol.* 1987; 154:164–175. [PubMed: 3323810]
- Cox GM, Rude TH, Dykstra CC, Perfect JR. The actin gene from *Cryptococcus neoformans*: structure and phylogenetic analysis. *J Med. Vet. Mycol.* 1995; 33:261–266. [PubMed: 8531025]
- Edman JC, Kwon-Chung KJ. Isolation of the *URA5* gene from *Cryptococcus neoformans* var. *neoformans* and its use as a selective marker for transformation. *Mol Cell Biol.* 1990; 10:4538–4544. [PubMed: 2201894]
- Edman JC. Isolation of telomeric sequences from *Cryptococcus neoformans* and their use in high-efficiency transformation. *Mol Cell Biol.* 1992; 12:2777–2783. [PubMed: 1588969]
- Janbon G, Maeng S, Yang DH, Ko YJ, Jung KW, Moyrand F, Floyd A, Heitman J, Bahn YS. Characterizing the role of RNA silencing components in *Cryptococcus neoformans*. *Fungal Genet. Biol.* 2010; 47:1070–1080.
- Liu H, Cottrell TR, Pierini LM, Goldman WE, Doering TL. RNA interference in the pathogenic fungus *Cryptococcus neoformans*. *Genetics.* 2002; 160:463–470. [PubMed: 11861553]
- Loftus BJ, et al. The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans*. *Science.* 2005; 307:1321–1324. [PubMed: 15653466]
- McDade HC, Cox GM. A new dominant selectable marker for use in *Cryptococcus neoformans*. *Med Mycol.* 2001; 39:151–154. [PubMed: 11270405]
- Nakayashiki H, Kadotani N, Mayama S. Evolution and diversification of RNA silencing proteins in fungi. *J Mol Evol.* 2006; 63:127–135. [PubMed: 16786437]
- Panepinto J, Komperda K, Frases S, Park YD, Djordjevic JT, Casadevall A, Williamson PR. Sec6-dependent sorting of fungal extracellular exosomes and laccase of *Cryptococcus neoformans*. *Mol Microbiol.* 2009; 71:1165–1176. [PubMed: 19210702]
- Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS.* 2009; 23:525–530. [PubMed: 19182676]
- Rapplee CA, Engle JT, Goldman WE. RNA interference in *Histoplasma capsulatum* demonstrates a role for alpha-(1,3)-glucan in virulence. *Mol Microbiol.* 2004; 53:153–165. [PubMed: 15225311]
- Reilly MC, Levery SB, Castle SA, Klutts JS, Doering TL. A novel xylosylphosphotransferase activity discovered in *Cryptococcus neoformans*. *J Biol Chem.* 2009; 284:36118–36127. [PubMed: 19864415]

- Sudarshan S, Davidson RC, Heitman J, Alspaugh JA. Molecular Analysis of the *Cryptococcus neoformans* *ADE2* gene, a selectable marker for transformation and gene disruption. *Fungal Genet Biol.* 1999; 27:36–48. [PubMed: 10413613]
- Tenney AE, Brown RH, Vaske C, Lodge JK, Doering TL, Brent MR. Gene prediction and verification in a compact genome with numerous small introns. *Genome Res.* 2004; 14:2330–2335. [PubMed: 15479946]
- Wickes BL, Edman JC. The *Cryptococcus neoformans* *GAL7* gene and its use as an inducible promoter. *Mol Microbiol.* 1995; 16:1099–1109. [PubMed: 8577246]
- Wickes BL, Edman U, Edman JC. The *Cryptococcus neoformans* *STE12alpha* gene: a putative *Saccharomyces cerevisiae* *STE12* homologue that is mating type specific. *Mol Microbiol.* 1997; 26:951–960. [PubMed: 9426132]



**Figure 1.**

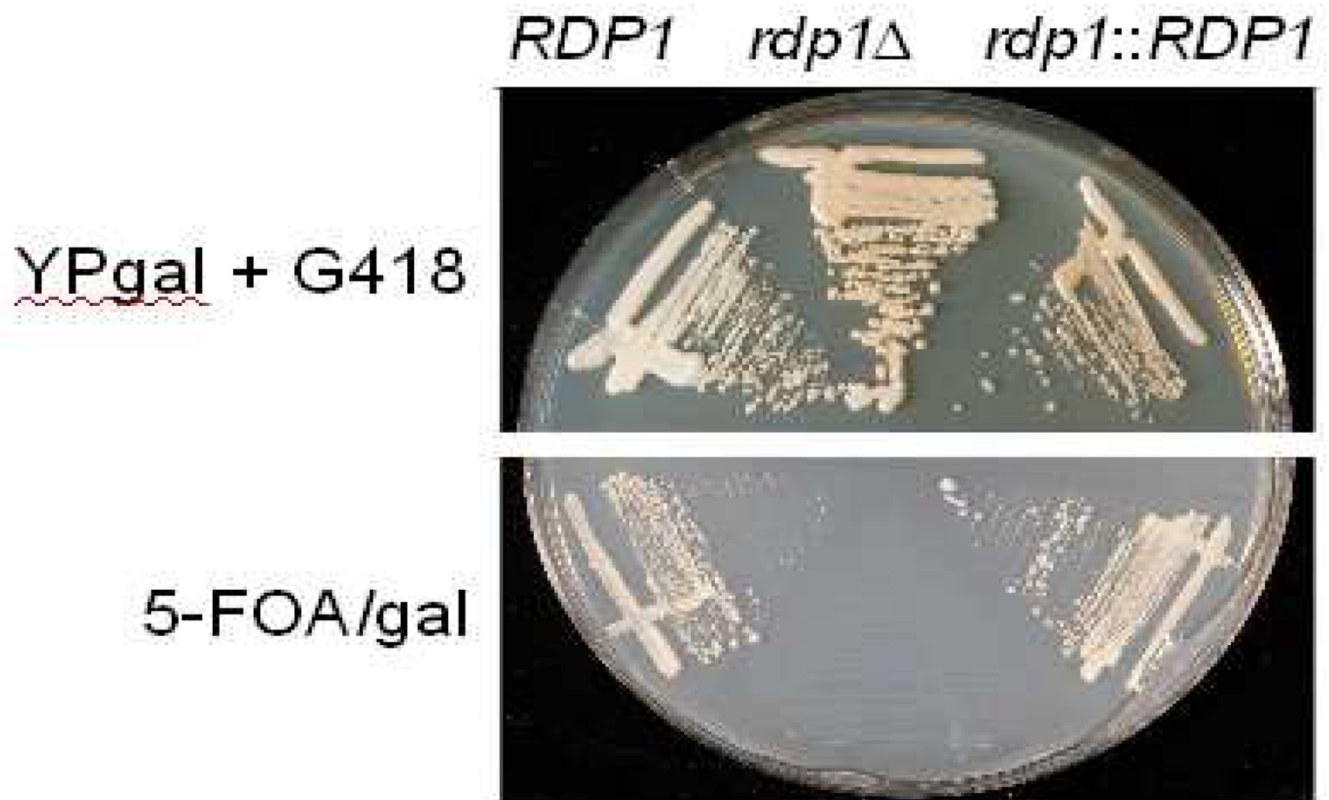
Opposing promoter constructs mediate RNAi. **A.** Schematic view of an RNAi plasmid with major elements shown; see text for details. *TEL*, telomeres; T, terminator; P, promoter; Target, sequence to be silenced. Restriction sites are indicated by vertical lines labeled with the abbreviated enzyme name. **B.** An opposing  $P_{GAL7}$  construct mediates silencing of *ADE2* and *LAC1*. *Left*, A *ura5* mutant (JEC43) was transformed with plasmid pIBB1, shown schematically, and grown on galactose medium lacking uracil (-ura/gal) for 2 days at 30°C. *Right*, a parallel study with plasmid pIBB29, showing a master plate with a random selection of transformants streaked onto niger seed agar with galactose (NSA/gal) and grown for 2 days at 30 °C. Controls streaked at the bottom of the plate are a wild-type strain that melanizes and appears brown (*LAC1*), and a deletion strain that does not melanize and therefore remains white (*lac1*).

**Figure 2.**

Silencing *URA5* allows 5-FOA selection of cells actively performing RNAi. A. Cells transformed with plasmid pIBB32 containing the RNAi cassette cartooned above were recovered and treated as described in the text before plating to medium with galactose but lacking adenine (*left*) and replica-plating to galactose media with 5-FOA (*middle*) or without uracil (*right*). Cells undergoing effective RNAi grow on 5-FOA and not on medium lacking uracil; this plate was chosen to show the single transformant (of over 100) that did not demonstrate this phenotype. The bottom two streaks on each plate are control strains with normal (*left*) or mutant (*right*) *URA5* genes as indicated in the left panel. B. JEC50 cells were transformed with pIBB69 targeting *LAC1* as cartooned above, pretreated with 5-FOA,

and selected on –ade/gal medium as for the study in Fig. 2A. Transformants were restreaked on NSA/gal and grown for 2 days at 30°C, demonstrating effective silencing by white color matching that of control *lacI* cells (bottom right of the plate). In contrast, the wild-type *LACI* strain is dark brown (bottom left).





**Figure 3.** Deletion of *RDP1* abolishes RNAi silencing. Wild-type *RDP1*, *rdp1Δ*, and *rdp1::RDP1* complemented strains were electroporated with pIBB103 and transformants were grown on either complete media containing galactose and G418 (YPgal + G418) or 5-FOA/gal media at 30°C for 2 days.

**Table 1**

<b>Plasmid</b>	<b>Forward promoter</b>	<b>Reverse promoter</b>	<b>Target gene</b>	<b>Marker gene</b>
pIBB1	<i>GAL7</i>	<i>GAL7</i>	500 bp <i>ADE2</i>	<i>URA5</i>
pIBB5	<i>GAL7</i>	<i>ACT1</i>	500 bp <i>ADE2</i>	<i>URA5</i>
pIBB24	<i>ACT1</i>	<i>ACT1</i>	500 bp <i>ADE2</i>	<i>URA5</i>
pIBB29	<i>GAL7</i>	<i>GAL7</i>	650 bp <i>LAC1</i>	<i>URA5</i>
pIBB32	<i>GAL7</i>	<i>GAL7</i>	610 bp <i>URA5</i>	<i>ADE2</i>
pIBB69	<i>GAL7</i>	<i>GAL7</i>	610 bp <i>URA5</i> + 650 bp <i>LAC1</i>	<i>ADE2</i>
pIBB75	<i>GAL7</i>	<i>GAL7</i>	250 bp <i>URA5</i>	<i>ADE2</i>
pIBB103	<i>GAL7</i>	<i>GAL7</i>	250 bp <i>URA5</i>	<i>neo<sup>R</sup></i>