Sex-Induced Silencing Operates During Opposite-Sex and Unisexual Reproduction in *Cryptococcus neoformans*

Xuying Wang, Sabrina Darwiche, and Joseph Heitman¹

Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, North Carolina 27710

ABSTRACT *Cryptococcus neoformans* is a human fungal pathogen that undergoes a dimorphic transition from yeast to hyphae during **a**- α opposite-sex mating and α - α unisexual reproduction (same-sex mating). Infectious spores are generated during both processes. We previously identified a sex-induced silencing (SIS) pathway in the *C. neoformans* serotype A var. *grubii* lineage, in which tandem transgene arrays trigger RNAi-dependent gene silencing at a high frequency during **a**- α opposite-sex mating, but at an ~250-fold lower frequency during asexual mitotic vegetative growth. Here we report that SIS also operates during α - α unisexual reproduction. A self-fertile strain containing either *SXI2a-URA5* or *NEO-URA5* transgene arrays exhibited an elevated silencing frequency during solo and unisexual mating compared with mitotic vegetative growth. We also found that SIS operates at a similar efficiency on transgene arrays of the same copy number during either α - α unisexual reproduction or **a**- α opposite-sex mating. *URA5*-derived small RNAs were detected in the silenced progeny of α - α unisexual reproduction and RNAi core components were required, providing evidence that SIS induced by same-sex mating is also mediated by RNAi via sequence-specific small RNAs. In addition, our data show that the SIS RNAi pathway also operates to defend the genome via squelching transposon activity during same-sex mating as it does during opposite-sex mating. Taken together, our results confirm that SIS is conserved between the divergent *C. neoformans* serotype A and serotype D cryptic sibling species.

COMPLEX genomes have evolved sophisticated mechanisms to sense the presence, and to control the behavior of genomic invaders such as transposable elements and viruses. Among a panoply of diverse strategies, homology-dependent gene silencing (HDGS) is a ubiquitous phenomenon among fungi, plants, and animals (Cogoni 2001). HDGS, also known as cosuppression, was first found to be triggered by transgenes, viruses, or unusual duplications of endogenous genes in both plants and fungi (Cogoni *et al.* 1996; English *et al.* 1996; Bingham 1997; Cogoni and Macino 1999a). Although a broad variety of HDGS phenomena are known, all of these gene silencing processes are based on the recognition of nucleic acid sequence homology, followed by inactivation of homologous sequences. Gene silencing can occur at a transcriptional or post-transcriptional level involving

sequence-specific mRNA degradation. The most studied and best-characterized HDGS phenomenon in fungi is a process known as quelling, which occurs in *Neurospora crassa* vegetative tissue (Cogoni *et al.* 1996). Quelling is an RNAi-mediated silencing mechanism that post-transcriptionally inactivates duplicated sequences, in many cases resulting from introduction of transgenes. In quelling, the silenced loci can act in *trans*, leading to silencing of all homologous genes throughout the genome. The core RNAi components, including Argonaute, Dicer-like proteins, and RNA-dependent RNA polymerase (RdRP), are all required for quelling (Cogoni and Macino 1999b; Catalanotto *et al.* 2002; Catalanotto 2004; Fulci and Macino 2007).

Sexual reproduction is associated with a high frequency of recombination, which enables species to adapt to diverse environments. However, sexual reproduction can also be beneficial to transposon propagation because meiosis can enable transposon exchange between genomes. In addition, meiosis can lead to heterologous chromosome alignment and recombination or translocation due to the presence of ectopic copies of preexisting transposons (Jinks-Robertson and Petes 1986; Bourc'his and Bestor 2004; Kelly and

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¹Corresponding author: Department of Molecular Genetics and Microbiology, Room 322 CARL Bldg., Box 3546 Research Dr., Duke University Medical Center, Durham, NC 27710. E-mail: heitm001@duke.edu

Aramayo 2007; Ni *et al.* 2011). Thus, transposon control is especially critical during sexual reproduction. Many organisms have evolved specific sex-related host defense mechanisms that are activated during the sexual cycle to promote genome integrity.

The filamentous fungus *Neurospora crassa* is an exemplary model that has developed a number of complex defenses to preserve its genome integrity at different stages of the sexual cycle, including repeat-induced point mutation (RIP) and meiotic silencing of unpaired DNA (MSUD) (Ruiz *et al.* 1998; Borkovich *et al.* 2004; Galagan and Selker 2004; Kelly and Aramayo 2007). Like quelling, RIP is also a homology-based process that acts at the premeiotic stage of sexual development (Selker *et al.* 1987). RIP efficiently detects and inactivates repetitive DNA elements present in haploid genomes destined to participate in meiosis and operates by introducing C:G to T:A mutations into both copies of the duplicated sequences (Cambareri *et al.* 1989; Selker 2002). Up to 30% of the GC base pairs in duplicated sequences can be altered to AT pairs via RIP after a single sexual cycle (Cambareri *et al.* 1991). In contrast to RIP, MSUD occurs during meiosis and detects the unsuccessful pairing of discrete DNA regions during homolog pairing (Shiu *et al.* 2001). An RNAi-mediated silencing mechanism is triggered and post-transcriptionally silences all genes contained in the loop of unpaired DNA region (Lee *et al.* 2003; Fulci and Macino 2007). In this case, MSUD can silence ectopic sequences and newly transposed elements that are present as only a single copy during meiosis. Thus, MSUD serves as a complementary mechanism to RIP, and functions to protect the organism against any transposons that escape RIP. The presence of this powerful genome defense system in *N. crassa* has shaped its genome into one with a very low content of repetitive sequences (Galagan *et al.* 2003; Borkovich *et al.* 2004).

In addition to *N. crassa*, other fungi also exhibit different meiotic silencing mechanisms. Methylation induced premeiotically (MIP) in *Ascobolus immersus* and *Coprinopsis cinerea* is another HDGS phenomenon that is closely related to RIP (Rhounim *et al.* 1992; Barry *et al.* 1993; Freedman and Pukkila 1993). Like RIP, MIP also takes place premeiotically and silences repeated DNA sequences. MIP leads to methylation of C residues within both repeated sequences, but in contrast to RIP, no sequence changes occur (Faugeron 2000; Cogoni 2001). Furthermore, a novel sex induced silencing (SIS) phenomenon has been reported in the human fungal pathogen *Cryptococcus neoformans* (Wang *et al.* 2010). A tandem multicopy transgene insertion triggered a homology-based gene silencing process termed SIS that occurs at ~250-fold higher frequency during the sexual cycle compared to vegetative mitotic growth. SIS is mediated by an RNAi pathway and the increased silencing frequency during sex appears to be linked to an increased abundance of the RNAi pathway elements during sexual reproduction. The RNAi-mediated SIS pathway also functions to squelch transposon activity during the sexual cycle, thus serving as a genome

defense mechanism during meiosis (Wang *et al.* 2010). Transgenes are also repressed via a mitotic-induced silencing pathway (MIS) similar to quelling (Wang *et al.* 2012).

C. neoformans grows as a haploid budding yeast with a bipolar mating system that involves **a** and α mating types (Idnurm *et al.* 2005). Spores can be produced via two distinct pathways: **a**- α opposite sex mating and α - α unisexual reproduction. Monokaryotic fruiting, once thought to be mitotic and asexual, has been demonstrated to be a modified form of sexual reproduction occurring between strains of the same mating type and is most commonly observed in α strains but can also occur with **a** isolates (Fraser *et al.* 2005; Lin *et al.* 2005, 2007). Because α isolates predominate (>95%) in most naturally occurring *C. neoformans* populations, and the α mating type is linked to virulence (Kwon-Chung and Bennett 1978; Nielsen *et al.* 2005; Lin *et al.* 2006; Lin 2009), the discovery of α - α unisexual reproduction (same-sex mating) suggests how infectious spores can be produced by this almost unisexual pathogen. Although many similarities are shared between opposite-sex and same-sex mating, ongoing studies comparing the underlying mechanisms provide insight into how same-sex mating can contribute in unique ways to the fitness of these pathogenic fungi (Lee *et al.* 2010; Wang and Lin 2011).

SIS was first identified during the **a**- α opposite-sex mating cycle (Wang *et al.* 2010). In this study, we found that a transgene-induced RNAi-dependent sex-induced silencing pathway also operates during α - α unisexual reproduction and that the silencing frequency during opposite-sex mating and same-sex mating is comparable. More importantly, we observed that transposons were more active during the α - α unisexual cycle in the *rdp1* mutants compared to wild type, leading to elevated transposition and mutation similar to our findings during **a**- α mating of RNAi mutants. Our results suggest that during α - α same-sex mating as well as **a**- α opposite-sex mating, SIS is deployed as a genome defense mechanism to protect the genome during meiosis.

Materials and Methods

Strains and media

Strains and plasmids used in this study are listed in [Supporting Information, Table S1](#). Yeast cells were grown and maintained on yeast extract-peptone-dextrose (YPD) medium. Synthetic dextrose (SD) medium lacking uracil or containing 5-FOA (1 g/liter) were used to test whether isolates of interest are auxotrophic for uracil. Mating of *C. neoformans* was conducted on 5% V8 juice agar medium (pH = 7) in the dark as previously described (Hull *et al.* 2002). Spores generated by either unisexual or opposite-sex mating were isolated by micromanipulation as described (Hsueh *et al.* 2006). The transformed strains containing the *SXI2a-URA5* or the *NEO-URA5* transgene were obtained by biolistic transformation with plasmid DNA (Toffaletti *et al.* 1993). PCR with primers JOHE23492 (CCTGCTTCGTTTAACTCCATGGCAA),

JOHE23493 (CCAACGAGCTTCGCTTCAGGAGAG), JOHE26870 (GGAAGCCGGTCTTGTCGA TCAGG), and JOHE26871 (CTCGATGGGACCGGTGCTTAAGGC) were used to screen for the presence of the *SXI2a-URA5* or the *NEO-URA5* transgene in the genome. The transformed strains used in this study were subjected to mitotic stability examination as described before (Varma *et al.* 1992; Toffaletti *et al.* 1993) to confirm integration into the genome.

Real-time PCR

Quantitative real-time PCR was performed with primers specific to the actin gene *ACT1* and *URA5* (Wang *et al.* 2010) to determine the copy number of the *SXI2a-URA5* and the *NEO-URA5* transgenes. DNA isolated from the wild-type strain JEC20 or XL280, the transformed strains, and the progeny were used as templates. PCR was conducted with Brilliant SYBR Green QPCR Master mix (Stratagene) and the relative quantity of the *URA5* gene determined by the $\Delta\Delta C_t$ method according to the following equations: (Ct value means the threshold cycle) $\Delta C_t = C_t(\text{target}) - C_t(\text{normalize})$ and $\Delta\Delta C_t = \Delta C_t(\text{experimental}) - \Delta C_t(\text{control})$. Comparative expression level = $2^{-\Delta\Delta C_t}$.

Gene disruption

A standard overlap PCR approach (Fraser *et al.* 2003) was used to disrupt the *RDP1* gene in strains XW73 and XW139 with the *NAT* dominant selectable marker. The overlap PCR products were introduced into the genome by biolistic transformation (Davidson *et al.* 2000). Primers that were used for amplification of the ~1 kb of 5' and 3' flanking regions of the *RDP1* disruption cassette are described in Janbon *et al.* 2010. Transformants were initially screened by PCR and Southern blot analyses were then conducted to identify a single integration at the desired locus.

Detection of siRNAs by Northern blot

To detect siRNAs, total RNA from strains of interest was extracted with TRIzol and RNAs of low molecular weight were prepared by precipitating high molecular weight RNAs in the presence of 0.5 M NaCl and 5% polyethylene glycol (MW8000) and recovering the supernatant (Catalanotto *et al.* 2002). Small RNA-enriched fractions were then separated in 15% TBE-urea gels, followed by Northern blot. DNA oligonucleotides of 21 nt and 25 nt served as size markers. After electrophoresis, RNA was transferred to Hybond-N⁺ nylon membrane, and UV cross-linked. Single-stranded RNA probes were *in vitro* transcribed in the presence of ³²P-labeled UTP with the MAXIsript T7 kit (Ambion), and hydrolyzed to smaller size (~30–50 nt) with 80 mM sodium bicarbonate and 120 mM sodium carbonate (Catalanotto *et al.* 2002). After hydrolysis, the RNA probes were added into the hybridization solution and hybridized overnight at 50°.

Measurement of mitotic silencing frequency

To measure the mitotic silencing frequency in a given strain, 10 independent colonies were inoculated into 5-ml YPD

overnight cultures. Cells were then harvested, washed, and plated on both YPD and 5-FOA media at various dilutions. Colony numbers formed on 5-FOA medium and on YPD medium were counted from each individual culture and the silencing frequency was determined based on the counted colony numbers. The final reported silencing frequency is the mean of the 10 individual silencing frequencies.

Transposon trapping and determining the frequency of FK506-resistant mutants

The FKBP12 encoding gene, *FRR1*, was used as a transposon trap as described in previous studies (Cruz *et al.* 1999; Wang *et al.* 2010). Ten independent colonies from wild type (XL280) and from two independent *rdp1* mutants (XW150 and XW151) were inoculated into 5-ml overnight cultures in YPD medium. Cells were then harvested, washed, and plated on both YPD and YPD with FK506 (1 µg/ml) medium (37°) at various dilutions. To measure spontaneous FK506 resistance rate due to trapped transposons or mutations, the number of colonies that formed on YPD medium were counted and compared with those formed on YPD with FK506 (37°). The final reported FK506 resistance rates are the mean of the 10 individual resistance rates.

To measure the spontaneous FK506 resistance rate during same-sex mating, wild type (XL280) and two independent *rdp1* mutants (XW150 and XW151) were solo cultured on V8 pH = 7 medium for 2 weeks. Spores were isolated from the edges of mating cultures and plated on both YPD and YPD with FK506 (1 µg/ml) medium (37°) at various dilutions. The FK506 resistance rate was determined as described above. The same procedure was repeated five times and the final reported FK506 resistance rates from progeny are the mean of the five individual resistance rates.

To analyze the transposons inserted into the *FRR1* gene in the FK506-resistant isolates, sequence analysis was done as follows. First, genomic DNA was isolated and used as a template for PCR amplifications for the *FRR1* gene with primers JOHE38889 (ACGGACAAAAACGACAGACC) and JOHE38990 (AGAATGCCGGTATCAACGAC). The resulting PCR products larger than the wild-type *FRR1* gene were subjected to DNA sequencing. Primers JOHE38893 (TATCCTCACCACCTCTC GTCC), JOHE38894 (GTCGAGAGCTTCACTAAG), JOHE39000 (CCGTTCTGTCAGTGCCATCAC), and JOHE39005 (TTGACGAA GAGAACATCGTC) were used for sequencing. The identified transposon sequences were submitted to GenBank with accession nos. KC440172, KC440173, KC440174, KC440175, KC440176, and KC469619.

Results

Tandem copies of the *SXI2a-URA5* allele are silenced during mating in the serotype D strain

Previously, we observed a sex-induced silencing phenomenon that is triggered by a tandem multicopy insertion of

a *SXI2a-URA5* transgene during α - α opposite-sex mating in the *C. neoformans* serotype A var. *grubii* lineage (Wang *et al.* 2010). Same-sex mating that has been observed under laboratory conditions is mostly found in the *C. neoformans* serotype D lineage and rarely in serotype A (Lin *et al.* 2005, 2006; Bui *et al.* 2008). To test whether SIS operates in the *C. neoformans* serotype D var. *neoformans* lineage, especially during same-sex reproduction, we first utilized a plasmid containing the serotype A *SXI2a-URA5* transgene that had successfully triggered silencing in the serotype A strains (Wang *et al.* 2010). This plasmid was introduced into a recipient strain XL280 *ura5* (var. *neoformans*, serotype D) that undergoes robust α - α mating during solo culture on V8 medium (Figure 1A). This approach generated isolates with multiple copies of the transgene. Integration of the transgene at the *ura5* locus was first confirmed by PCR and Southern analysis. The copy number of the *SXI2a-URA5* transgene was then analyzed by real-time PCR with primers specific to *URA5* and the *ACT1* actin gene as a control. One isolate (XW73, *ura5 SXI2a-URA5*) contained ~ 12 copies of the *URA5* gene, indicating that 11 transgene copies and one endogenous copy are present in this strain (Figure 1B). This transgene strain was then solo cultured on V8 medium in the dark for 2 weeks at room temperature to induce sexual reproduction. As shown in Figure 1C, abundant hyphal growth with production of basidia and spores was observed. Basidiospores were isolated by microdissection and examined for silencing of the *URA5* gene on SD-uracil and 5-FOA media. Among 19 progeny tested, 5 of them ($\sim 25\%$) were found to be uracil auxotrophic. All of the progeny, including both those that were *Ura*⁺ and those that were *ura*⁻, inherited the *SXI2a-URA5* transgene based on PCR analysis (Figure S1 and Figure 1D).

Transgene copy number changes or genomic rearrangements were excluded as causes of the loss of transgene expression as follows. First, to examine whether the transgene copy number had been altered in the *ura*⁻ progeny, quantitative real-time PCR was conducted to compare the copy number of the *SXI2a-URA5* transgene in the *ura*⁻ and *Ura*⁺ progeny. Both the *ura*⁻ and *Ura*⁺ progeny contained ~ 11 –13 copies of the transgene (Figure 2A), indicating that the transgenes are stably inherited during solo-sexual reproduction. Second, Southern analysis was performed with a *URA5* probe to define the genomic configuration of the *ura*⁻ and *Ura*⁺ strains at the *SXI2a-URA5* locus. As shown in Figure 2, B and C, identical Southern patterns were observed in parental strain XW73 and its *ura*⁻ progeny. Thus, no rearrangement has occurred at the transgene locus during solo-sexual reproduction, thus excluding this as another possible explanation for extinction of expression of the transgene. Furthermore, when compared with the signal derived from the endogenous *URA5* locus (~ 7.5 kb), the signal from the introduced *SXI2a-URA5* transgene allele (~ 3.8 kb) was much more intense. This finding is consistent with the observation that multiple copies of the transgene were inserted at *ura5*, resulting in a tandem repeat configuration (Figure 2B).

Figure 1 Silencing of the *SXI2a-URA5* transgene during solo-sexual mating. (A) Schematic illustration of the *SXI2a-URA5* transgene introduced into the hyperfilamentous recipient α strain XL280 *ura5*. (B) Quantitative real-time PCR revealed that ~ 12 copies of the *URA5* gene (1-copy endogenous *URA5* gene and 11-copy *SXI2a-URA5* transgene) are present in the genome of the transformed strain XW73. (C) Hyphal growth of strain XW73 bearing the *SXI2a-URA5* transgene after 2 weeks culture on V8 medium in the dark (left). A basidium with four long basidiospore chains produced by strain XW73 (right). Bar, 10 μ m. (D) Basidiospores were isolated from sexual reproduction, and 5 among a total of 19 f1 progeny were auxotrophic for uracil (see Figure S1). XL280 *ura5*, *ura5 SXI2a-URA5* (XW73), one *Ura*⁺ progeny, and *ura*⁻ progeny strains were grown on YPD and SD medium lacking uracil. PCR analysis showed that the *SXI2a-URA5* transgene is still present in the uracil-auxotrophic progeny.

To investigate whether efficient silencing of the *URA5* transgene array is linked to the solo-sexual mating cycle, we examined the silencing frequency of the transgene during vegetative mitotic growth. Spontaneous 5-FOA resistance was measured after the parental transgene array strain XW73 was grown on rich YPD medium. Approximately 3–7 in every 10,000 isolates exhibited a 5-FOA resistant (*ura*⁻) phenotype, demonstrating a much lower mitotic silencing frequency ($\sim 0.05\%$) compared to meiotic progeny ($\sim 25\%$). Multiple copies of the transgene are required for silencing, as no silencing was observed in a strain containing only one copy of the transgene (Table 1). Both meiotic and mitotic silencing of the transgene were found to be unstable, and reversion of *ura*⁻ to *Ura*⁺ occurred frequently after serial passage on nonselective YPD medium.

Taken together, our results show that multiple copies of the *SXI2a-URA5* transgene are silenced during both vegetative mitotic growth and meiotic growth, while the frequency is much higher in the latter process, suggesting SIS also operates in the *C. neoformans* serotype D var. *neoformans* lineage and during solo-sexual mating.

Previously, we have established a link between the RNAi pathway and SIS in the *C. neoformans* serotype A var. *grubii* lineage. In this study, XW73 is derived from the *C. neoformans* serotype D var. *neoformans* strain, which also contains genes encoding Argonaute, Dicer, and RdRP. Whole genome and genetic studies have identified two Argonaute genes (*AGO1* and *AGO2*), two Dicer genes (*DCR1* and *DCR2*), and one *RDPI* gene encoding an RdRP in the serotype D strains JEC21 and B3501A (Loftus *et al.* 2005; Janbon *et al.* 2010; Wang *et al.* 2010). To verify that SIS in solo-

sexual mating is also dependent on an RNAi pathway, we deleted the only *RDP1* gene in the JEC21 derived strain XW73. Two independent deletion mutants were obtained and analyzed. We first examined the silencing frequency of the progeny produced from strain XW73 bearing the *rdp1* deletion. Among 39 meiotic progeny (from mutant 1) and 41 meiotic progeny (from mutant 2) that were analyzed, no *ura*⁻ isolate was observed (0/39 and 0/41, <2.5%) (Figure S2), indicating that Rdp1 is required for SIS in solo-sexual mating. Moreover, we found that the *rdp1* deletion also abolished the silencing during vegetative mitotic growth (<0.00001%) (Table 1).

Because another distinguishing feature of RNAi is the generation of siRNA from dsRNA, we next examined the presence of siRNA in the *URA5* silenced strains. RNA was extracted from *ura*⁻ silenced progeny and analyzed by

Table 1 Multiple copies of the *SXI2a-URA5* transgenes are silenced by unisexual reproduction

	<i>ura5</i> <i>SXI2a-URA5</i>	<i>ura5</i> <i>SXI2a-URA5</i>	<i>ura5</i> <i>SXI2a-URA5</i> <i>rdp1Δ</i>	<i>ura5</i> <i>SXI2a-URA5</i> <i>rdp1Δ</i>	<i>ura5</i> <i>SXI2a-URA5</i>
Transgene copy number	~11	~11	~11	~11	1
Growth condition	YPD	V8	YPD	V8	YPD
Silencing frequency (%)	0.05	~25	<0.00001	<2.5	<0.00001

YPD, yeast colonies generated by mitotic growth; V8, dissected spores produced by meiotic sexual reproduction.

Northern hybridization. When probed with a ^{32}P -labeled sense *URA5* transcript, abundant siRNAs ~ 22 nt in size were detected in the silenced progeny, but not in the original XL280 *ura5* strain or the parental XW73 strain bearing the *SXI2a-URA5* transgene (Figure 2D). The small RNAs are antisense transcripts of *URA5*, suggesting that they are derived from a dsRNA precursor and function as a *trans*-acting factor during silencing. We also noted a difference in abundance of the accumulated small RNAs among different *ura*[−] progeny. We hypothesize that those isolates with less abundant siRNAs are attributable to some cells in the population that have reverted to a *Ura*⁺ phenotype during growth in non-selective YPD medium and thus lack siRNAs. This can be reflected by our observation that some of the *ura*[−] progeny exhibited a modest slow growth phenotype on YPD medium at 30° similar to strain XL280 *ura5*, which can be overcome by adding uracil to the YPD medium, indicating these progeny may have a stronger silencing phenotype than other *ura*[−] progeny. In addition, to examine whether the silencing effect is restricted to *URA5* or spreads into its neighboring gene *RPS8* that encodes a ribosomal protein, we also probed the blot with a *RPS8* sense probe but failed to detect any *RPS8* siRNAs.

Construction of *a* and α strains with a *NEO-URA5* transgene array

Unisexual mating and opposite-sex mating share similar inducing stimuli and elements of the pheromone response pathway (Hsueh *et al.* 2009; Lin *et al.* 2010; Wang and Lin 2011). However, a key difference is that the homeodomain cell identity determinants *Sxi1 α* and *Sxi2a* are only required for postfusion steps during opposite-sex mating and are dispensable for unisexual reproduction (Hull *et al.* 2002, 2005; Lin *et al.* 2005). We have observed that a serotype A *SXI2a-URA5* transgene array triggered silencing during solo-sexual mating of a serotype D strain (XW73). We also found that a wild-type serotype A *SXI2a* gene is able to complement mating defects of a serotype D *sxi2a* Δ mutant strain (Figure S3). Thus, a key question is whether the *Sxi2a* protein produced from the transgene in strain XW73 leads to a signaling cascade more like that operating during opposite-sex mating and possibly thereby enabling *a*- α sex-induced silencing. To test this possibility, we utilized a plasmid bearing a *NEO-URA5* transgene to generate strains containing multiple copies of this transgene.

As shown in Figure 3A, we first introduced the *NEO-URA5* plasmid into the serotype D strain JEC34 (JEC20 *ura5*). Integration of the *NEO-URA5* transgene at the *ura5* locus was confirmed by PCR and Southern analysis. As described above, the hybridization signals derived from the *NEO-URA5* transgene and the endogenous *ura5* allele were detected individually by Southern blot (Figure 3B). The transformed strain (XW105) exhibited two hybridization signals, one at ~ 8.5 kb and the other more intense one at ~ 3.8 kb, suggesting that multiple copies of the transgenes have been integrated into the genome. Compared with multiple hybridization bands

from other transformants reflecting additional ectopic integration events, the simpler Southern pattern from strain XW105 indicated that the transgenes in this strain are only located at the *ura5* locus and are arranged as tandem repeats (Figure 3B). We then crossed the *a* strain XW105 bearing the *NEO-URA5* transgene with the α strain XL280 (hyperfilamentous) to obtain a hyperfilamentous f1 α progeny (XW139) carrying the same transgene array (Figure 3C). Quantitative PCR confirmed that the *URA5-NEO* transgene is present in the same copy number in the XW105 *a* and XW139 α strains. In total, four copies of the *URA5* gene reflect a three-copy transgene and the one endogenous *ura5* gene (Figure 3D). Additionally, based on stability assays conducted following repeated mass transfer of cells grown under nonselective growth conditions, the transgenes in all of the strains used in this study were stably inherited during mitotic growth.

***SIS* frequency is comparable during α - α unisexual and *a*- α opposite-sex mating**

We have generated *a* and α strains containing the same copy number of the *NEO-URA5* transgene, which allowed us to examine *SIS* in unisexual mating and also to compare the silencing frequency between α - α unisexual and *a*- α opposite-sex mating. Progeny were isolated by microdissection from both unisexual and opposite-sex mating and analyzed for *URA5* silencing based on growth or absence on SD –uracil and 5-FOA media. When the α transgene array strain XW139 was solo cultured on V8 medium, 4 of a total of 28 progeny (14%) were auxotrophic for uracil (Figure S4 and Figure 4A). In comparison, among 31 progeny isolated from an *a*- α cross between strains XW105 *a* and XW139 α on V8 medium, 4 exhibited a *ura*[−] phenotype (13%) (Figure S5 and Figure 4A). We also noted that the α strain XW139 might undergo some α - α unisexual mating even when cocultured with an *a* strain in the *a*- α cross. To establish that the *ura*[−] progeny obtained are *bona fide* progeny of an *a*- α opposite-sex cross, a mating type-specific PCR amplification assay was performed to determine the mating type of these *ura*[−] progeny using *STE20a*/ α primers established in previous studies (Fraser *et al.* 2004; Li *et al.* 2012). An excess of α progeny would have been interpreted as indicative of a higher level of α - α mating occurring during culture with the *a* partner. As shown in Figure 4B, an equal proportion of *a* and α progeny (1:1) were recovered, providing evidence that they are indeed derived from *a*- α mating. In addition, the *ura*[−] progeny also exhibited a neomycin sensitive (*neo*^s) phenotype, indicating that the *NEO* gene is also silenced. However, the *neo*^s phenotype is much more unstable compared with the *ura*[−] phenotype, which is reflected by the fact that $>50\%$ of cells reverted to neomycin resistance after nonselective growth on YPD medium. In comparison, the reversion rate to *Ura*⁺ of different *ura*[−] isolates was within the range of 1×10^{-4} – 1×10^{-6} .

PCR analysis showed that the *NEO-URA5* transgenes were still present in all of the progeny with a silenced *URA5* gene

Figure 3 Construction of **a** and α strains with a *NEO-URA5* transgene array. (A) Plasmid containing the *NEO-URA5* transgene was introduced into the **a** strain JEC34, which is JEC20 *ura5* by transformation. (B) DNA obtained from individual transformed strains was cleaved with *Xba*I and subjected to Southern analysis. When hybridized with a probe specific for *URA5*, an 8.5-kb product was derived from the endogenous *URA5* and a 3.8-kb product reflects the cleavage product from the *NEO-URA5* transgene. Strain XW105 (indicated by the red arrow), exhibiting the expected hybridization pattern with no ectopic integration and an intense signal from the transgene locus, was selected for further study. (C) **a** strain XW105 bearing the *NEO-URA5* transgene was crossed with α strain XL280 to obtain an α progeny (XW139) that carries the same transgene array and is able to undergo robust unisexual reproduction. (D) Quantitative real-time PCR revealed that four copies of the *URA5* gene are present in strains XW105a and XW139a.

(Figure 4B). We also examined the genomic structures at the *NEO-URA5* locus in these *ura*[−] progeny by Southern analysis with a probe to the *URA5* gene. As shown in Figure 4C, all of the *ura*[−] progeny displayed an identical hybridization pattern compared to the parental transgene array strains XW105 and XW139. The intensity of the signal derived from the transgene indicated that multiple copies of the *NEO-URA5* transgene have been integrated into the genome. Furthermore, quantitative real-time PCR results confirmed that the *ura*[−] progeny still contain the same copy number of the *NEO-URA5* transgene (Figure 4D). In addition, as we observed before in strain XW73 (Figure S2), the silencing occurring during unisexual mating was also abolished when *RDPI* was deleted in the α strain XW139. We examined 29 progeny and 27 progeny from two *rdp1* Δ independent

mutants, and no isolate was *ura*[−] (0/29 and 0/27) (Figure 5 and Figure S6). Northern blot analysis was performed to detect siRNAs in the *ura*[−] progeny. When probed with a sense ³²P-labeled *URA5* transcript, abundant siRNAs were observed in the majority of the *ura*[−] strains (Figure 4E). The *ura*[−] isolates showed various levels of siRNAs, and the ones exhibiting less abundant siRNAs correspond to those with a higher reversion rate to *Ura*⁺. We also probed the same blot with a *NEO* probe, but failed to detect any siRNAs. This could be related to the high reversion rate of the *NEO* silencing phenotype and rapid loss of less abundant siRNAs may lead to the rapid phenotypic reversion.

Taken together, our results showed that the silencing rates during **a**- α opposite-sex mating and α - α unisexual mating are similar (13 vs. 14%). Additionally, both the **a** strain XW105 and the α strain XW139 exhibited a similar low silencing rate ($\sim$ 0.04%) during vegetative mitotic growth on YPD medium (Table 2), demonstrating that the silencing rate increased $\sim$ 350-fold during either α - α unisexual or **a**- α heterosexual reproduction.

Transposons are more active in the *rdp1* mutant strains during α - α same-sex mating

Previously, we have observed that transposons are hyperactive during mating of serotype A *Cryptococcus* strains and that the SIS RNAi pathway plays a critical role in squelching transposon activity during **a**- α opposite-sex mating. We hypothesized that transposon activity would also be suppressed during α - α same-sex mating by SIS.

To address this, the FKBP12 encoding gene, *FRR1*, was used as a transposon trap, as described in previous studies (Cruz *et al.* 1999; Wang *et al.* 2010). FKBP12 is the intracellular receptor for the antifungal drug FK506, which is toxic to *C. neoformans* at 37° when bound to FKBP12 via inhibition of calcineurin (Odom *et al.* 1997). Spores resulting from α - α unisexual reproduction of WT strain XL280 and two independent *rdp1* mutants were germinated on YPD medium with or without FK506 and the rate of spontaneous FK506 resistance was examined. The rate of FK506 resistance during mitotic growth of XL280 and the *rdp1* mutant strains was also assessed. As shown in Table 3, both *rdp1* mutants generated more FK506 resistant isolates than their wild-type parental strain XL280 under both mating and mitotic growth conditions. The progeny obtained from two *rdp1* mutants yielded the highest rate of FK506 resistance (4×10^{-6} or 3×10^{-6}), which is $\sim$ 15–20 times higher than that of meiotic spores produced from the WT strain (2×10^{-7}) and $\sim$ 50–60 times higher than the frequency observed during mitotic growth of the WT strain (6×10^{-8}).

To determine whether the FK506-resistance phenotype is attributable to spontaneous *frr1* point mutations or transposon insertions into this gene, the *FRR1* gene was PCR amplified from genomic DNA isolated from the FK506-resistant progeny derived from the *rdp1* mutants. Among a total of 50 progeny examined, a PCR product of the expected size was obtained from 15 progeny, whereas increased size

Figure 4 SIS frequency is comparable during unisexual mating and opposite-sex mating. (A) *ura⁻* progeny from α - α unisexual mating (4 of 28, 14%, see Figure S4) and from α - α opposite-sex mating (4 of 31, 13%, see Figure S5) were grown on YPD medium with or without neomycin and SD medium without uracil. Strains XW139, XW105, JEC20 *ura5* (JEC34), and XL280 *ura5* were included as controls. (B) Mating type of *ura⁻* progeny derived from a cross between the α and *a* strains XW139 and XW105 was determined by a mating type-specific PCR amplification assay using the *STE20a*/ α gene primers (top). PCR analysis confirmed the presence of the *NEO-URA5* transgene in the uracil-auxotrophic progeny (bottom). (C) *ura⁻* progeny derived from either α - α unisexual or α - α opposite-sex mating were subjected to Southern analysis with a *URA5* probe. The same hybridization pattern was observed in *ura⁻* progeny and in the parental strains XW105 and XW139. (D) Quantitative real-time PCR revealed that the *URA5* gene is present at the same copy number in all of the *ura⁻* progeny. (E) *URA5* siRNAs are present in the *ura⁻* progeny based on hybridization with a *URA5* sense probe.

PCR products were observed from the remaining 35 progeny. A PCR product derived from the *FRR1* locus was obtained from all 50 isolates, excluding deletions across either primer site or translocations. The PCR products larger than wild type from 10 isolates were subjected to DNA sequencing. The results suggested six different transposon trapping events occurred: three DNA transposons, T1, T2, and T3 had been inserted into the *FRR1* open reading frame at different locations or in different orientations (details in Figure 6A). These DNA transposons have been identified in

Cryptococcus from two independent labs, and each contains a transposase gene and terminal inverted repeats (Cruz *et al.* 1999; M. C. Cruz and J. Heitman, unpublished data; Janbon *et al.* 2010). The T2 transposon exists in the genome as a longer (~3.5 kb) and a shorter (~0.8 kb) version, and we found both versions integrated into the *FRR1* gene in different FK506-resistant *rdp1* isolates (Figure 6A).

In accord with the higher transposition rate observed in the progeny from the *rdp1* mutant strains, Northern blot analysis also showed that the genes encoding the T2 and

Figure 5 *rdp1* deletion abolishes silencing of the *NEO-URA5* transgene during α - α unisexual mating. The *RDPI* gene was deleted in the α strain XW139. Twenty-nine progeny were isolated from α - α unisexual mating of the *rdp1* Δ mutant strain (XW211) and examined for silencing of the *URA5* gene by growth on 5-FOA, SD-uracil, and YPD medium (streaked in that order). Strains JEC20 and JEC20 *ura5* (JEC34) were included as controls. All of the plates were incubated at room temperature for 3 days and photographed. No isolates showed a *ura⁻* or 5-FOA-resistant phenotype, other than the *ura5* control strain. See Figure S6 for a second independent *rdp1* Δ mutant with an identical phenotype.

Table 2 SIS frequency is comparable during unisexual mating and opposite-sex mating

	NEO-URA5 MAT α	NEO-URA5 MAT α	NEO-URA5 MAT α x MAT α	NEO-URA5 MAT α
Growth condition	YPD	YPD	V8	V8
Silencing frequency (%)	0.04	0.05	13	14

YPD, yeast colonies generated by mitotic growth; V8, dissected spores produced by meiotic sexual reproduction.

T3 transposases were highly expressed during α - α same-sex mating of the *rdp1* mutants (Figure 6B). The expression levels of these genes were either barely detectable (transposon T2) or reduced (transposon T3) when the *rdp1* mutants were mitotically grown on YPD medium. The WT strain XL280 consistently showed lower transcript levels of the transposase genes compared with the *rdp1* mutants. Taken together, our results suggest that SIS functions during α - α unisexual reproduction to repress transposon activity and thus guards integrity of the genome.

Discussion

SIS was first identified as operating during opposite-sex α - α mating of *C. neoformans* serotype A var. *grubii* strains (Wang *et al.* 2010). Beyond silencing exogenous transgene arrays, SIS also plays a critical role in transposon control during sexual reproduction. This is reflected in our previous observations that progeny derived from RNAi mutants exhibited an increased transposition/mutation rate and abundant Rdp1-dependent siRNAs that mapped to repetitive transposable elements (Wang *et al.* 2010). Thus, SIS serves as a meiotic genome defense mechanism in α - α opposite sex mating of *C. neoformans*.

However, the natural *Cryptococcus* population is almost exclusively of the α mating type (Hull and Heitman 2002), and thus unisexual reproduction may be the predominant route to produce meiotic progeny and spores in nature. In fact, evidence from population genetic analyses has shown that unisexual reproduction occurs naturally and contributes to the population structure (Lin *et al.* 2007, 2009; Bui *et al.* 2008). Thus, uncovering whether SIS occurs during α - α unisexual reproduction is a key question to understand how the *Cryptococcus* genome has been shaped through this unique life cycle.

The results presented in this work demonstrate that SIS also operates during solo and α - α unisexual mating. A sexually precocious strain bearing a tandem multicopy NEO-URA5 transgene, when undergoing unisexual reproduction, exhibited ~350-fold higher silencing frequency (~14%) than during mitotic vegetative growth (~0.04%). Additionally, when this strain was crossed to an α strain containing the same transgene array, a comparable silencing rate was found (Table 2). These findings indicate that SIS operates at a similar efficiency during unisexual reproduction and opposite-sex mating.

The role of RNAi as a mechanism to defend genomes against mobile transposons is broadly conserved among

plants, animals, fungi, and ciliates (Ketting *et al.* 1999; Llave *et al.* 2002; Aravin *et al.* 2003; Drinnenberg *et al.* 2009). It has been shown that the RNAi pathways function to control the mobility of transposons in serotype D *C. neoformans* during vegetative mitotic growth (Janbon *et al.* 2010; Wang *et al.* 2010). In our study, not only did we observe an increased transposition rate and highly expressed transposase genes in the *rdp1* mutant strains lacking the RNA-dependent RNA polymerase required for SIS, we also found that these transposons are more active during same-sex mating than during vegetative growth (Table 3 and Figure 6). This observation is similar to our finding with respect to SIS in opposite-sex mating (Wang *et al.* 2010). These findings lead us to propose that the potential high mutational burden due to hyperactive transposons during mating necessitates a mechanism like SIS, which is much more robust than silencing occurring during vegetative mitotic growth, to defend against transposition and thereby guard genomic integrity of the progeny.

Unisexual and opposite-sex mating involve many shared components, including the Cpk1 MAPK signal transduction cascade that responds to pheromones and governs the dimorphic transition in *C. neoformans* (Wang and Lin 2011; Hsueh *et al.* 2009; Lin *et al.* 2010). However, the homeodomain cell identity protein Sxi2a encoded by the α mating type locus is present and functions only during α - α mating but not α - α mating. Sxi2a forms a heterodimer with Sxi1 α and functions to control postfusion hyphal development in α - α opposite-sex mating (Hull *et al.* 2002, 2005). In this study, we have introduced a heterologous serotype A Sxi2a-URA5 transgene into a self-fertile α serotype D strain. This haploid strain XW73, thus may express the Sxi1 α /Sxi2a complex similar to α - α mating and also exhibited RNAi-dependent SIS (Figure 2 and Table 1) when subjected to self-mating. SIS frequency in this case is higher (25 vs. 14%) than what we observed in the α strain

Table 3 Higher FK506-resistance rate in the *rdp1* mutant strains during α - α unisexual mating

	WT	<i>rdp1</i>	<i>rdp1</i>
Progeny produced by meiotic reproduction	2×10^{-7}	4×10^{-6}	3×10^{-6}
	~3	~67	~50
Yeast colonies generated by mitotic growth	6×10^{-8}	6×10^{-7}	9×10^{-7}
	1 ^a	~10	~15

^a FK506-resistance rate from the WT strain during mitotic growth was set as 1, and the resistance rates obtained from the *rdp1* mutant strains were normalized to this.

Figure 6 Transposons are hyperactive in the *rdp1* mutant strains during α - α unisexual reproduction. (A) Schematic illustration of DNA transposon insertions into the *FRR1* open reading frame detected by transposon trapping and DNA sequencing. The wild type and the *rdp1* mutant strains are sensitive to FK506 at 37°. The FK506-resistant progeny resulting from α - α unisexual reproduction of the *rdp1* mutant strain contain a mutant *frr1* gene. Six different transposon trapping events were found: DNA transposon T1 (in XW238 and XW240), T2 (shorter version in XW235 and longer version in XW231 and XW233), and T3 (in XW237) had integrated into the *FRR1* open reading frame at different locations or in different orientations. The arrows above the *frr1* gene represent the orientation of the inserted transposons. (B) Total RNAs were isolated from the indicated strains grown on YPD medium or V8 medium (pH = 7). Northern blot analyses were conducted to analyze the expression levels of the T2 and T3 transposase genes. *GPD1* served as a loading control.

(XW139) containing a *NEO-URA5* transgene array. Given the previous findings that transgene copy number is linked to the silencing efficiency (Wang *et al.* 2010, 2012) together with the observation that similar SIS rates were found in α - α opposite-sex mating and α - α unisexual mating (Table 2), we propose that the higher SIS rate occurring in strain XW73 is attributable to the presence of a higher copy number of the *SXI2a-URA5* transgene (11 copies of *SXI2a-URA5* vs. 3 copies of *NEO-URA5*). Given that silencing occurred in α strains with transgenes both containing or lacking *SXI2a*, we conclude that *SXI2a* is not essential for the SIS pathway, and the shared pheromone-sensing Cpk1 MAPK signal transduction cascade in both unisexual and opposite-sex mating may be involved in triggering SIS.

Our results not only demonstrate that SIS operates during α - α unisexual reproduction, but demonstrate that SIS is conserved among different *Cryptococcus* varieties.

The first SIS phenomenon was found in the *C. neoformans* serotype A variety *grubii* lineage. In this study, we observed that SIS occurs in both opposite-sex and unisexual mating of the serotype D variety *neoformans* strains. We also noted that SIS took place at an ~50% frequency in the serotype A mating as reported before (Wang *et al.* 2010), which is somewhat higher than that in the serotype D mating (14%) induced by similar copies of the *URA5* transgene. We first suspected that the lower efficiency might be due to use of the heterologous serotype A *URA5* promoter and coding sequence in the transgene, resulting in less efficient silencing in serotype D strains. However, after replacing these elements with the serotype D derived sequences, a similar silencing frequency (~13%) was observed in the serotype D mating progeny (data not shown). In addition, the silencing triggered by the *SXI2a-URA5* transgene during vegetative growth also showed a lower frequency in the serotype D strain (0.05%, Table 1) compared with that in the serotype A strain (0.2%) (Wang *et al.* 2010). Furthermore, a similar phenomenon was reported previously that serotype A and serotype D strains exhibited different RNAi silencing efficiencies when responding to the same hairpin RNA construct (Skowrya and Doering 2012). Taken together, we submit that the SIS/RNAi rate in *C. neoformans* may be serotype specific and occurs at an approximately fourfold higher level in serotype A compared to D.

The facts that (1) mutation of *RDP1* blocked SIS and (2) abundant antisense siRNAs mapping to repetitive transgene clusters were identified in the silenced progeny indicate that SIS is an RNAi-dependent silencing pathway. RNAi components are conserved in most of the *Cryptococcus* species, including *C. neoformans* (serotype A and D) and its sibling species *C. gattii* (Loftus *et al.* 2005; Janbon *et al.* 2010; Wang *et al.* 2010). *C. gattii* can be divided into four molecular types (VGI, VGII, VGIII, and VGIV) (Fraser *et al.* 2005; Bovers *et al.* 2008). Strikingly, *C. gattii* VGII strains are the only ones that lack RNAi pathway elements. In the genome of the *C. gattii* VGII strain R265, both Argonaute genes are absent and *RDP1* and *DCR1* are pseudogenes (D'Souza *et al.* 2011; B. Billmyre and J. Heitman, unpublished results). Notably, this RNAi deficient *C. gattii* lineage is the same one responsible for an ongoing expanding disease outbreak on Vancouver Island and the Pacific Northwest. This coincidence brings up a possibility that loss of RNAi could be beneficial under certain circumstances during evolution. Data have been shown to support that same-sexual mating occurring between two closely related VGII α strains, the major Vancouver Island genotype isolates, may contribute to the production of infectious spores (Fraser *et al.* 2005). In this case, the absence of the SIS-RNAi silencing pathway in the same-sex mating between VGII strains may lead to more rapid genetic changes and result in hypervirulent progeny, thus providing a mechanism by which genetic diversity is increased during same sex reproduction within populations. Ongoing studies are being conducted to examine this mode of rapid adaptation in the *C. gattii* VGII strains.

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Sex-Induced Silencing Operates During Opposite-Sex and Unisexual Reproduction in *Cryptococcus neoformans*

Xuying Wang, Sabrina Darwiche, and Joseph Heitman

Figure S1. Silencing of the *SXI2a-URA5* transgene during solo-sexual reproduction. **A)** Strain XW73 (*ura5 SXI2a-URA5*) was solo cultured on V8 medium in the dark for two weeks to induce sexual reproduction. Basidiospores were isolated and examined for silencing of the *URA5* gene by streaking them first on 5-FOA medium and then SD-uracil medium and finally on YPD medium. Strains XL280 and XL280 *ura5* were included as controls. All of the plates were incubated at room temperature for 3 days and photographed. 5 among a total of 19 (~25%) f1 progeny were auxotrophic for uracil (indicated by arrows). **B)** PCR analysis showed that all of the 19 f1 progeny inherited the *SXI2a-URA5* transgene.

Figure S2. *rdp1* mutation abolishes silencing of the *SXI2a-URA5* transgene during solo-sexual reproduction. The sexual progeny produced from strain XW73 bearing the *rdp1Δ* deletion were collected and examined for the silencing frequency as described in the legend to Figure S1. Strains XL280 and XL280 *ura5* were included as controls. Among 39 meiotic progeny that were analyzed, no *ura*- isolate was observed (<2.5%).

Figure S3. Wild-type serotype A *SXI2a* gene complements mating defects of a serotype D *sxi2a* Δ mutant strain. A wild-type serotype D α strain (JEC21 α) was cocultured with three mating partners [wild type serotype D **a** strain (JEC20**a**), a *sxi2a* Δ mutant strain (CHY768 *ura5*) in the JEC20**a** background, or the *sxi2a* Δ mutant strain in which the wild type serotype A allele of *SXI2a* was introduced by transformation (XW219)] on V8 agar medium for 3 days in the dark at 22°C to examine the production of hyphae during mating. Inspection of the wild-type and complemented mating reactions at day=7 revealed basidia and spore chains (data not shown).

Figure S4. Silencing of the *NEO-URA5* transgene during α - α unisexual mating. The α strain XW139 was incubated on V8 medium in the dark for 7 days at room temperature. Progeny resulting from α - α unisexual reproduction were isolated and cultured on 5-FOA, SD-uracil, and YPD media to determine the silencing frequency of the transgene as described above. Strains JEC20 and JEC34 (JEC20 *ura5*) were included as controls. 4 out of 28 (~14%) progeny exhibited a ura- phenotype (indicated by arrows).

Figure S5. Silencing of the *NEO-URA5* transgene during *a*- α opposite-sex mating. The α strain XW139 and the *a* strain XW105 were crossed on V8 medium in the dark for 7 days at room temperature. Strains JEC20 and JEC34 (JEC20 *ura5*) were included as controls. Among 31 progeny obtained from this *a*- α cross, 4 strains were auxotrophic for uracil (~13%) (indicated by arrows).

Figure S6. *rdp1* deletion abolishes silencing of the *NEO-URA5* transgene during α - α unisexual mating. The *RDP1* gene was deleted in the α strain XW139 a second time independently to generate strain XW212 that is a second independent *rdp1* Δ mutant (see also Figure 5 data). 27 progeny were isolated from α - α unisexual mating of the mutant strain (XW212) and cultured on 5-FOA, SD-uracil, and YPD media to determine the silencing frequency of the transgene. Strains JEC20 and JEC34 (JEC20 *ura5*) were included as controls. No isolates showed a *ura*⁻ phenotype (<3.7%), other than the JEC34 control strain.

Table S1. Strains and plasmids used in this study.

Strain	Genotype	Parent	Source/Reference
<i>C. neoformans</i> var. <i>neoformans</i> (serotype D)			
JEC21	<i>MATα</i>		(Kwon-Chung et al. 1992)
XL280	<i>MATα</i>		(Lin et al. 2005)
JEC20	<i>MATa</i>		(Kwon-Chung et al. 1992)
XL280 <i>ura5</i>	<i>MATα ura5</i>	XL280	This study
JEC34	<i>MATa ura5</i>	JEC20	(Moore and Edman 1993)
XW73	<i>MATα ura5 [SXI2a::URA5]₁₁</i>	XL280 <i>ura5</i>	This study
XW120	<i>MATα ura5 [SXI2a::URA5]₁₁ solo-sexual ura-</i>	XW73	This study
XW121	<i>MATα ura5 [SXI2a::URA5]₁₁ solo-sexual ura-</i>	XW73	This study
XW122	<i>MATα ura5 [SXI2a::URA5]₁₁ solo-sexual ura-</i>	XW73	This study
XW123	<i>MATα ura5 [SXI2a::URA5]₁₁ solo-sexual ura-</i>	XW73	This study
XW141	<i>MATα ura5 [SXI2a::URA5]₁₁ rdp1Δ::NAT</i>	XW73	This study
XW142		XW73	This study
XW105	<i>MATa ura5 [NEO::URA5]₃</i>	JEC34	This study
XW139	<i>MATα ura5 [NEO::URA5]₃</i>	XL280 x XW105	This study
XW166	<i>MATα ura5 [NEO::URA5]₃ unisexual ura-</i>	XW139	This study
XW167	<i>MATα ura5 [NEO::URA5]₃ unisexual ura-</i>	XW139	This study
XW168	<i>MATα ura5 [NEO::URA5]₃ unisexual ura-</i>	XW139	This study
XW169	<i>MATα ura5 [NEO::URA5]₃ unisexual ura-</i>	XW139	This study
XW171	<i>MATa ura5 [NEO::URA5]₃ heterosexual ura-</i>	XW139 x XW105	This study
XW172	<i>MATα ura5 [NEO::URA5]₃ heterosexual ura-</i>	XW139 x XW105	This study
XW173	<i>MATa ura5 [NEO::URA5]₃ heterosexual ura-</i>	XW139 x XW105	This study
XW174	<i>MATα ura5 [NEO::URA5]₃ heterosexual ura-</i>	XW139 x XW105	This study
XW211	<i>MATα ura5 [NEO::URA5]₃ rdp1Δ::NAT</i>	XW139	This study
XW212		XW139	This study
NE517	<i>MATα rdp1Δ::NAT</i>	JEC21	(Janbon et al. 2010)
CHY768 <i>ura5</i>	<i>MATa sxi2aΔ::ura5</i>	JEC20	(Hull et al. 2005)

XW219	<i>MATa sxi2aΔ::ura5 SXI2a::URA5</i>	CHY768 <i>ura5</i>	This study
XW150	] <i>MATα rdp1Δ::NAT</i>	XL280	This study
XW151		XL280	This study
XW231	<i>MATα rdp1Δ::NAT frr1-8</i>	XW151	This study
XW233	<i>MATα rdp1Δ::NAT frr1-9</i>	XW151	This study
XW235	<i>MATα rdp1Δ::NAT frr1-4</i>	XW151	This study
XW237	<i>MATα rdp1Δ::NAT frr1-5</i>	XW151	This study
XW238	<i>MATα rdp1Δ::NAT frr1-6</i>	XW151	This study
XW240	<i>MATα rdp1Δ::NAT frr1-7</i>	XW151	This study
Plasmids			
pJF35	<i>SXI2a::URA5 Amp^R</i>		(Hsueh et al. 2008)
pJF36	<i>NEO URA5 Amp^R</i>		This study
pXW12	<i>NEO URA5 Amp^R</i>		This study

] indicates independently derived mutants.

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