Published in final edited form as:
Fungal Genet Biol. 2011 November ; 48(11): 1056–1061. doi:10.1016/j.fgb.2011.07.005.

Application of the systematic "DAmP" approach to create a partially defective *C. albicans* mutant

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Abstract

An understanding of gene function often relies upon creating multiple kinds of alleles. Functional analysis in *Candida albicans*, a major fungal pathogen, has generally included characterization of mutant strains with insertion or deletion alleles and over-expression alleles. Here we use in *C. albicans* another type of allele that has been employed effectively in the model yeast *Saccharomyces cerevisiae*, a "Decreased Abundance by mRNA Perturbation" (DAmP) allele (Yan et al., 2008). DAmP alleles are created systematically through replacement of 3′ noncoding regions with nonfunctional heterologous sequences, and thus are broadly applicable. We used a DAmP allele to probe the function of Sun41, a surface protein with roles in cell wall integrity, cell-cell adherence, hyphal formation, and biofilm formation that has been suggested as a possible therapeutic target (Firon et al., 2007; Hiller et al., 2007; Norice et al., 2007). A *SUN41-DAmP* allele results in approximately 10-fold reduced levels of *SUN41* RNA, and yields intermediate phenotypes in most assays. We report that a *sun41Δ/Δ* mutant is defective in biofilm formation *in vivo*, and that the *SUN41-DAmP* allele complements that defect. This finding argues that Sun41 may not be an ideal therapeutic target for biofilm inhibition, since a 90% decrease in activity has little effect on biofilm formation *in vivo*. We anticipate that DAmP alleles of *C. albicans* genes will be informative for analysis of other prospective drug targets, including essential genes.

Keywords

Candida albicans; DAmP; Biofilm; *SUN41*

1. Introduction

The fungus *Candida albicans* is of interest due to its pathogenic potential as well as its biological properties (Pfaller and Diekema, 2007; Rex et al., 2000). It lacks a facile "forward genetics" system, in part because it is a diploid that lacks a complete sexual cycle (Alby et al., 2009; Epp et al., 2010; Soll et al., 2003). However, targeted insertion or deletion mutant strains have enabled gene function analysis (Blankenship et al. 2010; Epp et al., 2010; Homann et al., 2009; Nobile and Mitchell, 2005). There are also many examples where valuable functional information has come from reduced gene expression in insertion/deletion heterozygotes, or increased gene expression from promoter replacement strains (Fu

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et al., 2008; Nobile and Mitchell, 2005; Xu et al., 2007; Zhao et al., 2005) (Care et al., 1999; Oh et al.; Roemer et al., 2003; Uhl et al., 2003). However, there remain cases in which these gene expression levels may not fully inform or test functional hypotheses. For example, a heterozygous insertion/deletion mutant may not have a discernible phenotype, while a homozygous insertion/deletion mutant may have too severe a phenotype – lethality or pleiotropy – to permit a detailed functional interpretation.

In this report we have implemented a systematic strategy to create hypomorphs, or alleles with reduced activity. The strategy is to create a "Decreased Abundance by mRNA Perturbation" (DAmP) allele (Muhlrad and Parker, 1999; Yan et al., 2008). DAmP alleles are created through replacement of 3′ noncoding regions with nonfunctional heterologous sequences, and thus are applicable to almost all genes. The altered 3′ regions lack polyadenylation signals or other sequences that stabilize the mRNA, thus causing reduced mRNA accumulation (Muhlrad and Parker, 1999). Such alleles have been useful for large scale studies in *S. cerevisiae* (Yan et al., 2008), and we anticipate that they will be useful for *C. albicans*.

2. MATERIALS AND METHODS

2.1 Growth conditions

All *C. albicans* and *S. cerevisiae* strains were passaged in liquid YPD (2% dextrose, 2% Bacto Peptone, 1% yeast extract) at 220 rpm agitation at 30°C. Strains were grown on YPD solid medium (YPD broth, agar) at 30°C. Transformants were selected on synthetic dextrose medium (2% dextrose,0.67% yeast nitrogen base plus ammonium sulfate), to which the necessary auxotrophic supplements were added. Spider medium was prepared as previously described (Blankenship et al. 2010).

2.2 Plasmids and *C. albicans* strains

To create pCTNB4, 2,011 bps of upstream sequence of *SUN41* along with the open reading frame minus the stop codon was amplified by PCR using primers SUN41compl and SUN41 3 tag (Table 1). The pYES2.1 TOPO TA Expression kit (Invitrogen) was used to clone the fragment into a pYES2.1/V5-His-TOPO vector to create pCTNB4 which contains the *CYC1* 3′ UTR sequence 5′
ATCATGTAATTAGTTATGTCACGCTTACATTCACGCCCTCCTCCCACATCCGCTC
TAACCGAAAAGGAAGGAGTTAGACAACCTGAAGTCTAGGTCCCTATTTATTTTT
TTTAATAGTTATGTTAGTATTAAGAACGTTATTTATATTTCAAATTTTTCTTTTTT
TTCTGTACAAACGCGTGTACGCATGTAACATTATACTGAAAACCTTGCTTGAGA
AGGTTTTGGGACGCTCGAAGGCTTTAATTTGCAAGCT 3′ (Norice, 2008). The *SUN41* DAmP strain was created in several sequential steps. First, we PCR-amplified the promoter region, the coding region of *SUN41* and V5 tag from plasmid pCTNB4, with primers pYES2.1-recomb-up and pYES2.1-NOT-recomb-dn (Table 1). The resulting amplicon was inserted by recombination in *S. cerevisiae* into a NotI-digested pDDB78 plasmid (Spreghini et al., 2003) resulting in plasmid pNY100. Following the *SUN41* ORF is vector sequence 5′
GCGGCCGCCACCGCGGTGGAGCTCCAATTCGCCCTATAGTGAGTCGTATTACAA
TTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCA
ACTTAATCGCCTTGCAGCACATCCCCCCTTCGCCAGCTGGCGTAATAGCGAAGA
GGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCG
CGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTG 3′. The NYY100 strain was created by transforming CTN41 with a Nru1-digested pNY100. The complemented *sun41Δ/Δ* + p*SUN41-V5* strain NYY105was created by transforming CTN41 with a NruI-digested pCTNB6.

2.3 RNA purification and quantitative RTPCR

Strains were grown overnight and diluted to an OD_{600} of 0.2 in 50 mL of YPD. Cultures were grown at 30°C with shaking until density reached OD_{600} of ~0.8. Cells were harvested by vacuum filtration and flash frozen in a dry ice/ethanol bath. RNA was extracted using the RiboPure-Yeast kit (Ambion) following the manufacturer's protocol. Cells were beaten with a Next Advance Bullet Blender for 3 minutes at 4°C to ensure complete lysis. 10 μg of the isolated RNA was DNase treated (Ambion), and first-strand cDNA synthesis was performed with the AffinityScript multiple temperature cDNA synthesis kit (Stratagene). A control reaction lacking the reverse transcriptase was performed to ensure absence of DNA contamination. Quantification was then performed through quantitative PCR for *SUN41* and the reference transcript, *TDH3*. All data were normalized to *TDH3*. Primers used for PCR amplification were SUN41 RTforward, SUN41 rtback, JRB227 *(TDH3)*, and JRB228 *(TDH3)*. Sequences are listed in Table 2. Sequences are listed in Table 2. Real-time PCR reactions were prepared and performed on a Biorad iQ5 as previously described (Blankenship et al. 2010).

2.4 Cell wall sensitivity assays

Strains were tested for drug sensitivity as previously described (Bruno et al., 2006). Single colonies were inoculated in YPD and grown overnight at 30°C. Cultures were diluted to an OD_{600} of3 and serially diluted five-fold to an OD_{600} of 0.6, 0.12, 0.024, 4.8×10^{-3}, 9.6×10^{-4}, or 1.9×10^{-4}. Cells were spotted onto YPD, and YPD plus 200 μg/ml Congo red and incubated at 30°C for 48 hours.

2.5 Hyphal growth assays

Strains were grown overnight in YPD liquid medium. The strains were then diluted to an OD_{600} of 0.08 in spider medium at 37°C. The cultures were then incubated at 37°C with shaking at 250 rpm for 4 hours, washed with PBS, and incubated for ten minutes in PBS + 0.1 mg/ml calcoflour white (Sigma) (Watanabe et al., 2005). Samples were visualized using a Zeiss Axio Observer Z.1 microscope and a 63x objective and acquired on a Coolsnap HQ^2 (Photometrics) camera using Axiovision (Zeiss) software. ImageJ was used to process images (Abramoff, 2004).

2.6 *In vitro* biofilm assays

Biofilms were assayed as previously described (Nobile and Mitchell, 2005). Briefly, single colonies were grown overnight in YPD at 30°C. 12-well plates containing siliconesquare substrates pretreated with fetal bovine serum were inoculated with cultures diluted to OD_{600} of 0.5 in 2 ml of Spider medium (1% nutrient broth, 1% D-mannitol, 0.2% K_2HPO_4). The samples were incubated for 90 min at 35-rpm agitation at 37°Cfor adhesion to occur. The squares were then washed with 2 mlof phosphate-buffered saline (PBS) to remove any unadhered cells and transferred to 2 ml of fresh Spider medium. The plates were then incubated for 48 hours at 35-rpm agitation at 37°C. After 48 hours the plates were photographed and analyzed for biofilm growth.

2.7 Biofilm dry mass calculations

Measurements of biofilm dry masses were performed in triplicate on biofilms grown in standard conditions for 48 hrs. Three silicone squares were gently removed to maintain all of the biofilm attached to the squares, vortexed and collected under suction on pre-weighed 0.45 μm nitrocellulose filters (Millipore). Filters were dried for four days and weighed. Final biomass was determined by subtracting the mass of the nitrocellulose filters from the total mass of the filter and cells.

2.8 *In vivo* biofilm model

In vivo biofilms were assayed by a rat central-venous-catheter infection model, as previously described (Andes et al., 2004). Catheters were removed from rats after 24 hours of *C. albicans* infection. The distal 2 cm of catheter material was assayed for biofilm growth and imaged using SEM at 50x and 100X magnification as described previously (Nett et al., 2007).

2.9 Protein extraction and Western blot analysis

Strains were grown overnight and diluted to an OD_{600} 0.2 in YPD and grown to an OD_{600} ~1.0 at 30°C with 220-rpm agitation. Protein extraction was performed on the supernatants of the samples as previously (Brodsky and Schekman, 1993) with minor modification. Briefly, $1/10^{th}$ volume of 0.3% deoxycholate, and $1/10^{th}$ volume of 100% TCA were added to the supernatants. After 30 minutes on ice the samples were pelleted, washed two times with cold equilibrated acetone and dried for 30 minutes. The pellets were dissolved in SDS sample buffer overnight on ice. The samples were boiled for 5 minutes, pelleted and the supernatants were transferred to a new tube.

Gel electrophoresis and western blotting was done as previously described (Laemmli, 1970; Towbin et al., 1979) with minor modifications. The samples were boiled for 2 minutes and then loaded on precast gradient SDS polyacrylamide gel (BioRad). Following transfer to nitrocellulose the blot was inoculated with 1:2000 Anti-V5 primary antibody (Invitrogen) overnight, followed by a 1:5000 HRP-conjugated Goat Anti-Mouse secondary antibody (Invitrogen) for 1 hour. The HRP signal was developed using Supersignal West Pico chemiluminescent substrate (Thermo) and visualized with a Fulifilm LAS-3000 image Reader.

3.0 Results

3.1 Construction of a *SUN41* DAmP strain

We chose to test DAmP strain utility in *C. albicans* with the *SUN41* gene. Sun41 is a surface and secreted protein that is required for several biological processes, such as cell wall integrity and biofilm formation (Firon et al., 2007; Hiller et al., 2007; Norice et al., 2007). We anticipated that a partial defect in Sun41 function, caused by reduced expression, may cause a spectrum of phenotypes that are distinguishable from both wild-type and null mutant phenotypes. In other words, there is a better chance of detecting intermediate activity of an allele if there are several phenotypes to assay rather than just one.

The DAmP allele of *SUN41* was based on a functional complementing construct that also specified a C-terminal V5 epitope tag and included the 3′ untranslated region of the *S. cerevisiae CYC1* gene (Figure 1). The DAmP construct included the *SUN41-V5* coding region, but lacked *CYC1* sequences (Figure 1), so that the 3′ end of the transcript would include vector sequences. For expression and functional assays, the *SUN41-V5* and *SUN41-V5-DAmP* constructs were integrated at the *HIS1* locus in a *sun41Δ/Δ* strain, and were compared to the *sun41Δ/Δ* strain carrying the vector alone integrated at *HIS1*. Quantitative RT-PCR assays indicated that *SUN41* RNA accumulation was reduced approximately 7-fold in the *sun41Δ/Δ* + p*SUN41-V5-DAmP* strain compared to the *sun41Δ/Δ* + p*SUN41-V5* complemented strain, or approximately 10-fold compared to the wild-type *SUN41/SUN41* strain (Figure 2). A western blot indicated that the Sun41 protein accumulation was reduced more than 10-fold in the *sun41Δ/Δ* + p*SUN41-V5-DAmP* strain (Figure 3, lanes 2 and 3) compared to the *sun41Δ/Δ* + p*SUN41-V5* complemented strain (Figure 3, lane 1). These results indicate that the DAmP allele reduces, but does not abolish *SUN41* expression.

3.2 Phenotypic analysis of the *SUN41* DAmP strain

Strains lacking *SUN41* have defects in numerous cell wall-related processes. These processes include sensitivity to cell wall inhibitors, cell aggregation, hyphal morphogenesis, and biofilm formation *in vitro* (Firon et al., 2007; Hiller et al., 2007; Norice et al., 2007). Microscopic examination of the *sun41Δ/Δ* + p*SUN41-V5-DAmP* strain indicated that the *SUN41-V5-DAmP* construct retained some function. Aggregation of yeast-form cells at 30° was substantially reduced in the *sun41Δ/Δ* + p*SUN41-V5-DAmP* strain compared to the *sun41Δ/Δ* mutant strain (Figure 2). In addition, hyphae produced at 37° by the *sun41Δ/Δ* + p*SUN41-V5-DAmP* strain had more parallel walls and a more regular structure than those of the *sun41Δ/Δ* mutant (Figure 2). These observations suggest that the *SUN41-V5-DAmP* allele retains at least partial functional activity.

Assays for sensitivity to the cell wall inhibitor Congo Red revealed that the DAmP allele was not fully functional. We confirmed that the *sun41Δ/Δ* mutant strain was hypersensitive compared to the wild-type and *sun41Δ/Δ* + p*SUN41-V5* complemented strains (Figure 2). However, the *sun41Δ/Δ* + p*SUN41-V5-DAmP* strain displayed sensitivity that was intermediate between the mutant and complemented strain. These results indicate that the *SUN41-V5-DAmP* allele is not fully functional.

Mutants lacking Sun41 are defective in biofilm formation *in vitro* (Firon et al., 2007; Hiller et al., 2007; Norice et al., 2007). Control assays confirmed that our s*un41Δ/Δ* strain was biofilm-defective, while the wild-type and complemented strains were biofilm-competent (Figure 4). The *sun41Δ/Δ* + p*SUN41-V5-DAmP* strain appeared biofilm-competent (Figure 4). Quantitation of biofilm biomass revealed that the DAmP strain had a slight biofilm defect, so that its phenotype was intermediate between the mutant and complemented strains. These results indicate that substantial biofilm formation proceeds despite a reduction in Sun41 protein levels, but supports the conclusion that the *SUN41-V5-DAmP* allele is not fully functional.

To more clearly define the relevance of these observations to infection, we tested biofilm formation *in vivo* in a rat catheter infection model (Andes et al., 2004). The wild-type and complemented strains produced substantial biofilms *in vivo*, as visualized with scanning electron microscopy (SEM) (Figure 5). The *sun41Δ/Δ* mutant strain produced sparse growth in the catheter, forming what appeared to be small colonies with long emanating hyphae (Figure 5). The DAmP strain produced a substantial biofilm *in vivo* that included both yeast cells and hyphae (Figure 5). The 50x images show the differences in biofilm formation ability among the strains most clearly. The 1000x images show that what growth occurs is fairly normal in appearance, even for the *sun41Δ/Δ* mutant. These results indicate that Sun41 is required for biofilm formation *in vivo* in this model, and that the DAmP allele produces sufficient product to support biofilm formation *in vivo*.

4. Discussion

Loss of Sun41 leads to a constellation of phenotypes (Firon et al., 2007; Hiller et al., 2007; Norice et al., 2007). We report here that a *SUN41* DAmP strain displays partial phenotypic defects compared to a *sun41Δ/Δ* strain. Notably, for *SUN41*, there is no gene dosage effect that distinguishes the wild-type strain, with two alleles, and the complemented strain, with one allele. Thus the *SUN41* DAmP strain provides a unique opportunity to examine the consequences of limiting Sun41 activity.

Our studies here illustrate one useful application of DAmP strains: to evaluate a possible therapeutic target. Sun41 is a good candidate target because it is a surface and secreted protein, hence accessible to external inhibitors. In addition, as we report here, Sun41 is

required for biofilm formation *in vivo* in a catheter infection model, so in principle it could be targeted to eliminate a major source of disseminated infection. However, the *SUN41* DAmP strain produces a considerable biofilm *in vivo*. Therefore, an inhibitor would have to be extremely active to reduce Sun41 function below the threshold required for catheter biofilm formation. It would make sense to test other candidate targets through analysis of DAmP strains or other approaches to permit informed prioritization.

One great value of DAmP alleles is to enable systematic functional analysis of essential genes in *C. albicans*. The goal is not simply to determine whether a gene is essential or not, but to define its functional role. Existing approaches include acute shut-off experiments using fusions to regulated promoters (Backen et al., 2000; Care et al., 1999; Nakayama et al., 2000; Park and Morschhauser, 2005; Roemer et al., 2003) and analysis of heterozygous deletion strains (Oh et al.; Roemer et al., 2003; Uhl et al., 2003; Xu et al., 2007; Zhao et al., 2005). On a case-by-case basis, one can also use characterized mutations in orthologous genes as a model to create temperature-conditional alleles (Devasahayam et al., 2002; Fang and Wang, 2006; Kennedy et al., 2000), or use characterized chemical inhibitors (Bastidas et al., 2009; Cowen and Lindquist, 2005; Zacchi et al., 2010). No single approach is perfect for all genes; there is no “one size fits all” in Genetics! Hence we expect that DAmP strains, which have proven extremely useful in *S. cerevisiae* (Yan et al., 2008), will also prove useful in *C. albicans*.

Acknowledgments

We would like to thank lab members for their advice and discussions. We thank Dr. Carmelle T. Norice for construction of plasmid pCTNB4.

Role of Funding: Our work was funded by NIH Grant R01 AI067703 (APM) and NIH postdoctoral fellowship F32 AIO85521 (JSF). The funding institutes played no role in the design, or implementation of experiments described here.

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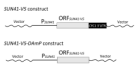

Figure 1. *SUN41-V5* and *SUN41-V5-DAmP* alleles

Complementing and DAmP constructs were created in vector pDDB78 (Spreghini et al., 2003). Each epitope-tagged coding region ($ORF_{SUN41\text{-}V5}$) ends with a stop codon, followed by the 3′ untranslated region from the *S. cerevisiae CYC1* gene (*SUN41-V5*) or vector sequences (*SUN41-V5-DAmP*). The coding regions are preceded by the *SUN41* promoter and 5′ sequences (P_{SUN41}). Vector sequences are bacterial in origin and derive from the plasmid vector. These 3′ sequences are detailed in the Methods section.

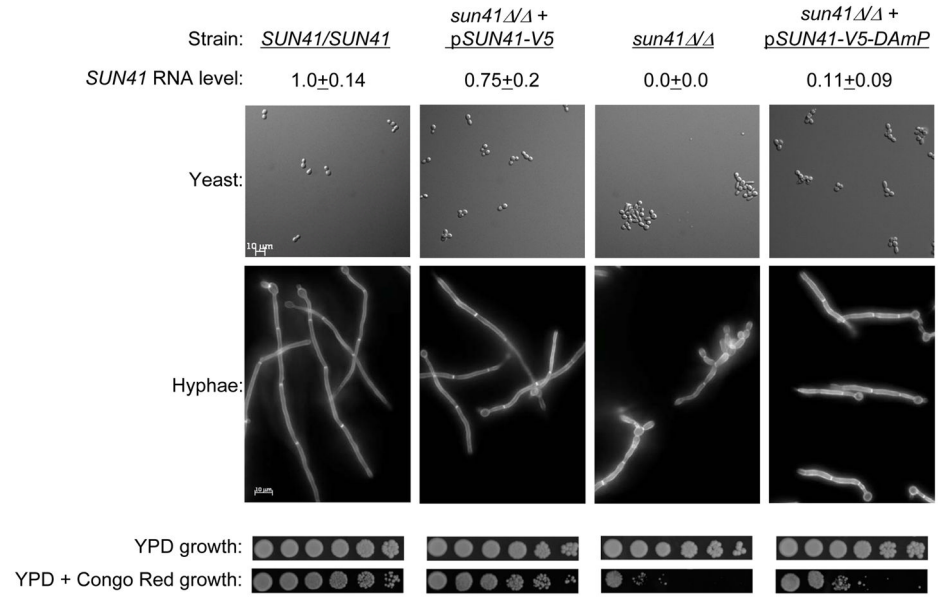

Figure 2. Phenotypic analysis of the *SUN41* DAmP strain

Strains indicated at the top of each column include *SUN41/SUN41* (DAY185), *sun41Δ/Δ* + p*SUN41-V5* (NYY105), *sun41/*(CTN46), and *sun41Δ/Δ* + p*SUN41-V5-DAmP* (NNY100). Levels of *SUN41* RNA in each strain were determined by QRTPCR from YPD cultures, and are expressed as the ratio to control *TDH3* RNA, then normalized to the wild type strain ratio. Yeast were visualized after 8 hr growth to mid-exponential phase in YPD at 30°C. Hyphae were visualized after 90 min growth in Spider medium at 37°C; samples were prepared by washing with PBS and staining for 10 minutes with 0.1 mg/ml of calcofluor white (Watanabe et al., 2005). Growth was compared on YPD and YPD + 200 μg/ml Congo Red after 48 hours at 30°C; overnight cultures were serially diluted five-fold from left to right.

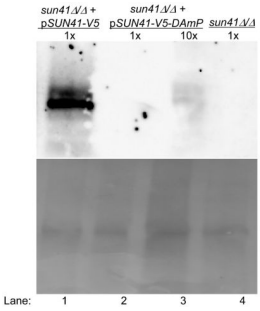

Figure 3. Assay of Sun41 protein accumulation

Strains were grown in YPD to logarithmic phase and supernatant proteins were fractionated by SDS PAGE. Sun41 protein was detected with anti-V5 (upper panel); total protein was detected with Ponceau S (lower panel). Strains are indicated at the top of each lane, including *sun41Δ/Δ* + p*SUN41-V5-DAmP* (NNY100; lane 1), *sun41Δ/Δ* + p*SUN41-V5* (NYY105; lanes 2, 3), and *sun41/*(CTN46; lane 4). Lane 3 was loaded with 10-fold more supernatant than the other lanes. We estimated the size of Sun41-V5 at 50 kDa by comparison with protein size standards.

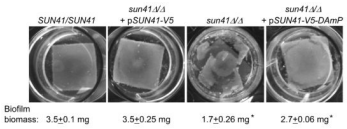

Figure 4. Biofilm formation *in vitro*

Biofilm formation *in vitro* was photographed and measured after 48 hrs for the strains indicated in the Figure 2 legend. Biofilm biomass assays were performed in triplicate. Asterisks indicate measurements that are significantly different from the reference *SUN41/SUN41* strain. Statistical significance (*p*-values) was calculated with a two-tailed *t*-test function in Microsoft Excel.

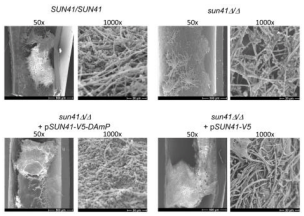

Figure 5. Biofilm formation *in vivo.*

Biofilm formation *in vivo* was assayed at 24 hrs for the strains indicated in the Figure 2 legend. Catheter lumen surfaces were imaged via scanning electron microscopy at 1000x or 50x magnification as indicated.

Table 1

Primers used in this study

Sun41 detect for	TCA ACT GGT GGT TCT GGT
Sun41 detectback0	AGT ACT ACC ACC TCC AAC AAC GGT
pYES2.1 recomb up	GCC AGG GTT TTC CCA GTC ACG TTG TAA AAC GAC GGC CGT AAT ACG ACT CAC TAT AGG G
pYES2.1 NOT recomb dn	GAT ATC GAA TTC CTG CAG CCC GGG GGA TCC ACT AGT TCT AGC GGC CGC AGA AAC TCA ATG GTG ATG GT
SUN41 RTforw	TGC CAA AGT GGT ATG TCC AA
SUN41 rtbak	TTC AAT GGA GCC CAG TTA CC
JRB227	ATC CCA CAA GGA CTG GAG A
JRB228	GCA GAA GCT TTA GCA ACG TG
SUN41compL	CATGTCATCAACAACTGTACTC
SUN41 3 tag	ATTATACAAGACAAAGTCAGC

Table 2

Strains used in the this study

DAY185	*ura3Δ::λimm434 ARG4::URA3::arg4::hisG HIS1::his1::hisG* *ura3Δ::λimm434 arg4::hisG his1::hisG*	(Davis et al., 2002)
NYY100	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS-SUN41-V5-DAmP sun41::ARG4* *ura3Δ::λimm434 arg4::hisG his1::hisG sun41::URA3*	This study
CTN41	*ura3Δ::λimm434 arg4::hisG his1::hisG sun41::ARG4* *ura3Δ::λimm434 arg4::hisG his1::hisG sun41::URA3*	(Norice et al., 2007)
CTN46	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1 sun41::ARG4* *ura3Δ::λimm434 arg4::hisG his1::hisG sun41::URA3*	(Norice et al., 2007)
NYY105	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS-SUN41-V5 sun41::ARG4* *ura3Δ::λimm434 arg4::hisG his1::hisG sun41::URA3*	This study