Supplementary data of

The role of *Candida albicans FAR1* in regulation of pheromone-mediated mating, gene expression and cell cycle arrest

By Pierre Côte and Malcolm Whiteway*.

* Corresponding author:
Dr. Malcolm Whiteway
Genetics Group,
Biotechnology Research Institute,
National Research Council of Canada,
6100 Royalmount Ave., Montreal, Quebec,
Canada H4P 2R2
Tel: 001-514-496-6146
Fax: 001-514-496-6213
Email: malcolm.whiteway@cnrc-nrc.gc.ca

Cloning, deletion and reintroduction of *FAR1*.

The complete coding sequence of orf19.7105 was deleted from Ca29, a 3294 derivative strain, using a sequential cassette-blaster strategy (Figure S1; Dignard and Whiteway, 2006). The first allele was replaced by the *URA3* marker, creating strain PCa002 and the second allele was replaced by the *SAT1* marker, creating Δ*far1* (PCa007). This was done by homologous recombination using five hundred bases upstream and downstream of *ORF19.7105*. In a first step, *ORF19.7105* and the -500/+500 base pair surrounding sequence was amplified using primers 5-Far1_PstI and 3-Far1_KpnI (Table S1) and cloned into PstI/KpnI cut pBluescript to create pFar1. An inverted PCR reaction on pFar1 with primers 5NotI500Far1/3XhoI500Far1 was used to remove the coding part of pFar1 and insert two new restriction sites for selection marker insertion. The linearized, PCR amplified plasmid, p500-Far1, was digested with NotI/XhoI, and gel purified with a QIAquick gel purification kit (catalog no. 28706; QIAGEN, Valencia, CAN). The nutrient marker *URA3* or the nourseothricin resistance marker *SAT1* were then cloned into

p500-Far1, creating p500Far1-URA3 and p500Far1-SAT1. Excision of the disruption cassette was done by cutting the p500Far1-marker construct with SacI/PstI and the desired fragment was purified using a QIAquick gel purification kit. Transformation to create $FAR1^{+/-}$ and Δ*far1* was done as noted above. Removal of the *URA3* marker was done selecting intrachromosomal recombinants on plates containing uridine at 50μg/ml and 0.1% (wt/vol) 5'-fluoroorotic acid to create $\Delta far1^{-ura3}$. Reintroduction of the *FAR1* gene copy was done by subcloning the *URA3* marker into pFar1 (creating pFar1-URA3) and transforming this plasmid, linearized by PmeI to target *FAR1* to its own locus, into $\Delta far1^{-ura3}$ to create $FAR1^{rest}$. Reintroduction of the *SST2* gene was done using p1374 (Dignard and Whiteway, 2006) linearized by BglII and integrated at the *RPS1* locus, creating $\Delta far1^{+SST2}$. Overexpression of *FAR1* was achieved using the actin promoter fused to the *FAR1* coding gene, transformed into the *RPS1* locus and selected on ura minus plates. We first PCR amplified and subcloned the coding part of *FAR1* from pFar1 into CIpACT1-cyc using primers 5Far1_MluI/3Far1_SalI, to create pFar1-ACT1pr. Linearization of the plasmid was done using StuI and the plasmid was introduced at the *RPS1* locus of 3294 creating $FAR1^{OP}$ (PCa034).

Liquid cell cycle arrest (growth curve)

To assay cell cycle arrest in cells grown in liquid medium, cells were grown in the presence of various concentrations of pheromone and culture density was recorded by a spectrophotometer. Opaque selected cells were first grown in liquid for 24 hours at 24°C with shaking, then diluted to an OD_{600nm} of 0.5 in fresh SC media. A 96 well plate with flat bottom (COSTAR plate 3595, Corning) was prepared with 180μl of fresh SC media containing α-factor at various concentrations (1, 0.5, 0.1, 0.05, 0.01, 0.005 and 0μg/ml). Cells were then added to each well to final concentration of 0.05 OD_{600nm}. As control, a white form of each strain was also treated with 1μg/ml of α-factor (data not shown). Each tested strain is done in duplicate and the

average curve was analyzed. Measurements were done at OD_{600nm} using a SAFIRE plate reader (Tecan) with the inside chamber temperature maintained between 24.5 and 25.5°C during the 24 hour experiment. The cultures were examined microscopically after growth to ensure the population maintained the opaque state.

Analysis of pheromone regulated gene datasets (Venn diagram).

Pheromone regulated gene datasets created under different experimental conditions were used in a comparison with the current study (Bennett and Johnson, 2006; Tsong *et al.*, 2006). Gene datasets were extracted from each study and analyzed as mentioned by their authors. For each dataset, we have eliminated genes not conserved in Candida ORF19/20 genome annotation (based on CGD website; Candida genome ORF19/20, release of 25 Oct. 2006) as well as duplicated genes. In the present up-regulated comparison, we did not include the dataset of Bennett et al. (Bennett *et al.*, 2003) because of the absence of a reliable down-regulated dataset even if, as indicated by Bennett and Johnson (2006), the both studies could be considered as complementary. Thus, in the analysis of unique-to-*FAR1*^{OP} gene set, we have removed the one already mentioned by Bennett et al. (2003). Venn diagram of A) the up-regulated and B) down-regulated set genes. The complete list of genes, respecting the diagram color code, can be found respectively in Table S3 and S4 (excel format).

Associated gene ontology analysis (GO search)

GO associated terms of the *FAR1*^{OP} pheromone-modulated sets of genes were analyzed by the CGD Gene Ontology Term Finder using the default setting (http://www.candidagenome.org/cgi-bin/GO/goTermFinder; Candida genome ORF19/20 release, 25 oct. 2006). Suspicious genes were removed from the analysis. Two pheromone modulated genes, BAR1 (Schaefer *et al.*, 2007) and MFA1 (Dignard *et al.*, 2007), were added to the GO

table (Table S5) but were not included in the calculation of the *P*-value because their associated GO-term was not updated yet in CGD.

FACS analysis

Opaque cells were pre-grown in liquid SC media at 24°C with shaking for 24h, then used to inoculate 50ml of fresh SC media; at 0.5OD the α- mating pheromone was added to the culture (to a final concentration of 1μg/ml). Samples were taken before splitting and after two hours of treatment. Cells are then ethanol fixed and stained by Propidium Iodide (PI; Molecular Probes, Eugene, Oregon, USA]. Briefly, 3ml of cells were washed (2 times) with an excess of PBS and finally resuspended in 1ml of PBS; 95% of ethanol was then added for a final concentration of 70% and kept at 4°C (overnight). Cells were then washed (2 times) with an excess of PBS, resuspended in 1ml of PBS and treated by RNase A (10μg/ml) for 1hour at 37°C. PI was added directly into the preparation at a final concentration of 5% and kept in dark at least 1 hour. Cells were counted by a Beckman-Coulter XL-MCL^{TM}; The laser excitation source is an Argon Ion laser 15mW. 50,000 cells were counted for each assay. Signal gating was done one Forward Scatter signal (FS, cell size) and Side Scatter signal (SS, granularity). Signals from doublet and aggregates were discounted by FL3 (red fluorescence from PI) peak signal and linear signal for single cells analysis using optics filters (645nm dichotic Long pass and 620nm band-pass). Beckmand-Coulter EXPO 32^{TM} software was used for result analysis.

Table S1. List of the primers used in this study.

Primer	Sequence
5Far1_screen	ccactttgcaagaactagtagctgcaacttttg
3Far1_screen	ctttgtggtttggactctttgtgaagttttgatttgatc
3Far1_screen2400	gtaaggagggattatgtcaa
5-Far1_PstI	aaaactgcagccaatgcattggaataatgtcacccaatccttggacctcacc
3-Far1_KpnI	cggggtaccgtagactctgcttttgtagtttctggtg
5NotI500Far1	aaggaaaaaagcggccgctttgtgctggtgcaaccatcatcgtgtcg
3XhoI500Far1	gctcgaggtctaacactttaagtggtagagaaatttctac
5Far1_MluI	cgcgacgcgtcgatgcgcaaactgttccactctcctaaaaagagaacttcc
3Far1_SalI	gccgctatggccgacgtcgacgcgtttcgcttttatcatgtctgcacgacacgatgatggttgcaccag

Table S2. List of plasmids used in this study.

Plasmid	Main insert	Accession number or reference
pFAR1	FAR1 ORF, with 500bp up and down	this study
p500Far1-URA3	Far1 disruptive cassette with URA3 selection marker	this study
p500Far1-SAT1	Far1 disruptive cassette with SAT1 selection marker	this study
pFar1-URA3	Far1 restore cassette with URA3 selection marker	this study
p1374	Reintroduction of SST2 with URA3 selection marker	Dignard et al., 2006
pACT1	Actin promoter with URA3 selection marker	Nicholls et al., 2004
pFar1-ACT1pr	Ectopic expression of FAR1 (under the actin promoter).	this study

Table S3. *FAR1*^{OP} up-regulated set of genes

See associated excel file

Table S4. *FAR1*^{OP} down-regulated set of genes

See associated excel file

Table S5. Associated gene ontology (GO) table

See associated excel file

Figure S1. Disruption of *FAR1*.

(A) Two step blaster method, for disruption of *FAR1* (*ORF19.7105*). The thick black bar represents genomic DNA at the *FAR1* locus; grey rectangles the *FAR1* coding sequence; white boxes the sequences used for recombination (500bp length, upstream and downstream of *FAR1*). The first allele of *FAR1* was replaced by the *URA3* marker (PCa002) and the second by the *SAT1* marker (creating Δ*far1*, PCa007). A ura3- version of Δ*far1* was created after chromosomal recombination selected on a 5'FOA plate (PCa012). A plasmid with a wild-type version of *FAR1*, plus the selective marker *URA3*, was reintroduced at the *FAR1* locus (PCa024). Red arrows represent orientation and approximate position of oligonucleotides used for PCR analysis and confirmation of disruptions. Blue arrows represent oligonucleotides used for PCR analysis in the case of PCa024, where using the two outside primers was not possible. The number on the right of each DNA molecule represents the expected PCR product size. (B) Confirmation of disruption by PCR and visualization on an agarose gel. Amplification by PCR goes from –550 to +530bp around *FAR1* ORF for all strains, except for PCa024 (-550 to +500). Strains used: Δ*sst2*$^{\Delta ura3}$ (Ca29), *FAR1*$^{+/-\Delta sst2}$ (PCa002), Δ*far1*$^{\Delta sst2}$ (PCa007), Δ*far1*$^{\Delta sst2\Delta ura3}$ (PCa012) and *FAR1*$^{\text{rest }\Delta sst2}$ (PCa024).

Figure S2. Influence of the mating pheromone on cellular growth rate.

Cell division of Δ*far1* strains in the presence of α-factor. The pheromone sensitive strain Δ*sst2* when grown is the presence of α-factor has a slower growth rate. The pheromone influence on Δ*sst2* growth is visible up to 0.005µg/ml (green curve) while at lower concentrations, the growth curve of the induced and non-induced cells (NI) are similar. 3294, the wild-type reference strain, is slightly influenced at 1 µg/ml but no differences were detected at lower pheromone

concentrations (data not shown). *FAR1* deleted strains (*FAR1*$^{+/-}$, Δ*far1* or *FAR1*^{rest}) are not influenced in their growth by the presence of pheromone even with the highest concentration (1µg/ml and non-induced curve are presented, respectively red and blue). Opaque cells are pre-grown for 24 hours at 24°C, then diluted into fresh SC media to a final concentration of 0.05 OD (see Material and Methods). The opaque form for each tested strains was microscopically verified before and after the experiment. The graphic color pattern is conserved among strains. NI, Non-Induced. Strains used: WT (3294), Δ*sst2*$^{\Delta ura3}$ (Ca29), *FAR1*$^{+/-\Delta sst2}$ (PCa002), Δ*far1*$^{\Delta sst2}$ (PCa007) and *FAR1*$^{rest\ \Delta sst2}$ (PCa024).

Figure S3. Comparison between different pheromone response profiles.

Venn diagram of the similarities among various pheromone response profiles. The complete ORF genome of *C. albicans* (6891 ORFs) was used (based on the ORF19 annotation of the genome). The complete list of genes up-regulated (A) and down-regulated (B) could be found in associated tables S3 and S4. Similar color codes (white common to all, light-blue Tsong/this study, purple Bennett/this study and blue this study) were used in the table.

Figure S1

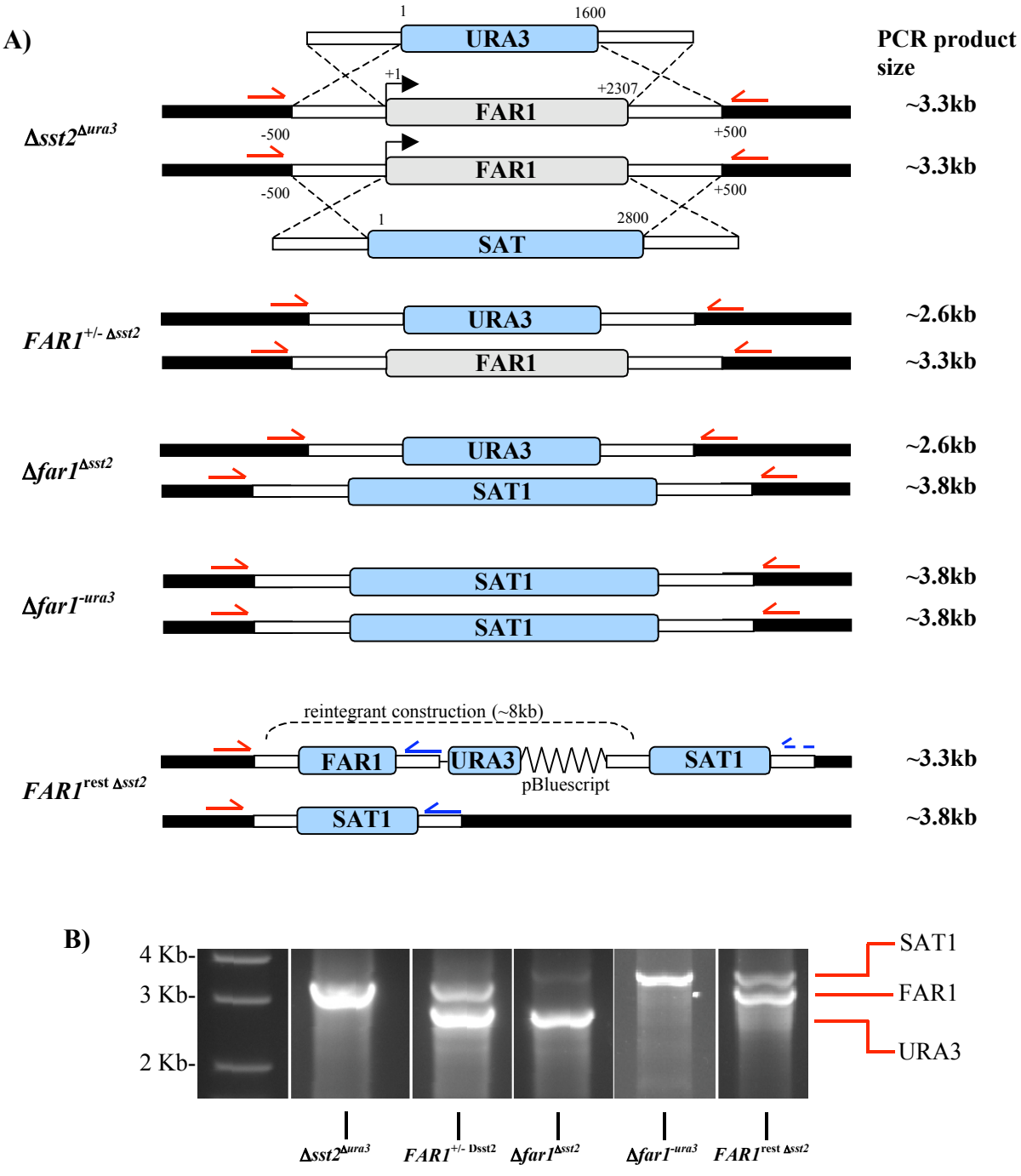

A)
PCR product size
1
1600
URA3
+1
+2307
FAR1
-500
+500
FAR1
-500
+500
1
2800
SAT
Δsst2Δura3
~3.3kb
~3.3kb
FAR1+/- Δsst2
URA3
FAR1
~2.6kb
~3.3kb
Δfar1Δsst2
URA3
SAT1
~2.6kb
~3.8kb
Δfar1-ura3
SAT1
SAT1
~3.8kb
~3.8kb
reintegrant construction (~8kb)
FAR1
URA3
SAT1
pBluescript
SAT1
FAR1rest Δsst2
~3.3kb
~3.8kb
B)
4 Kb-
3 Kb-
2 Kb-
SAT1
FAR1
URA3
Δsst2Δura3
FAR1+/- Dsst2
Δfar1Δsst2
Δfar1-ura3
FAR1rest Δsst2

Figure S2

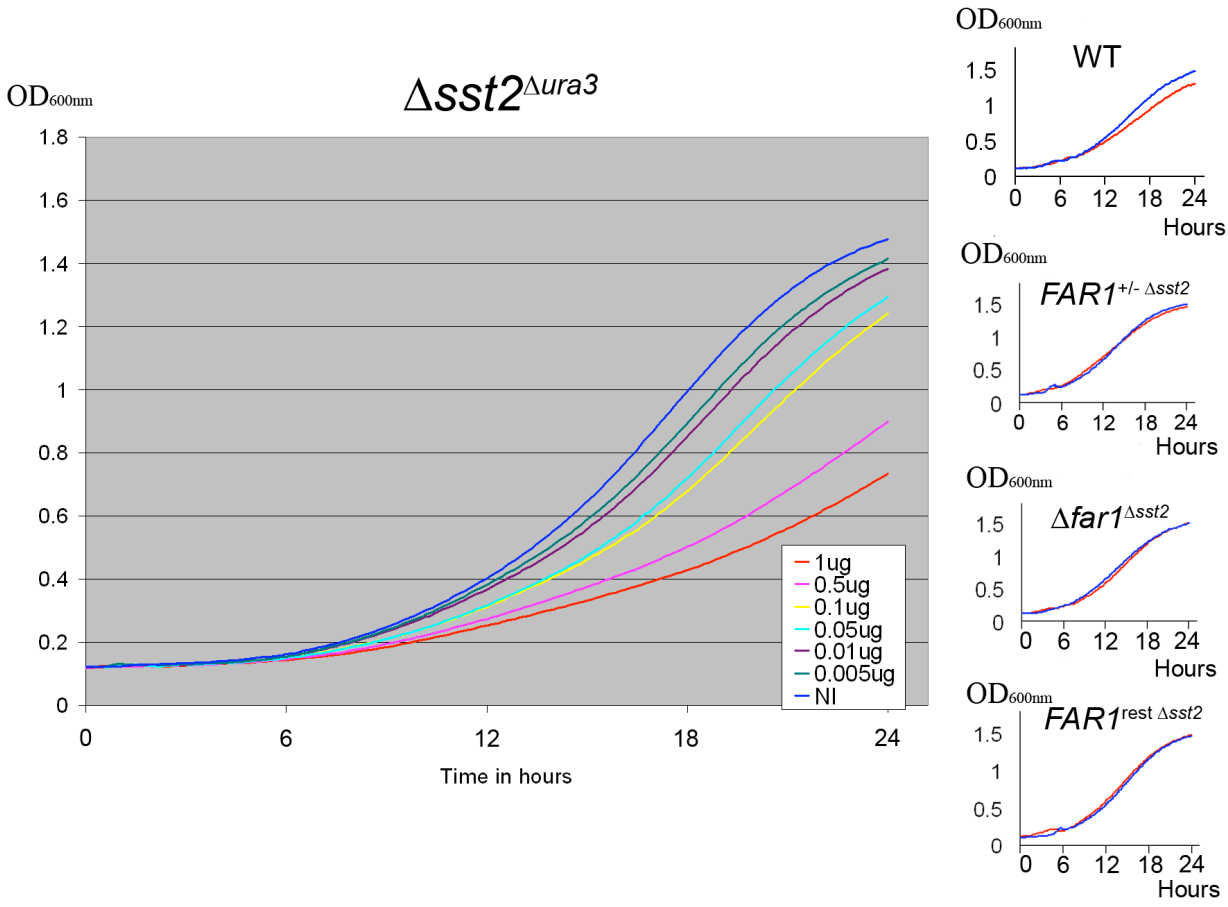
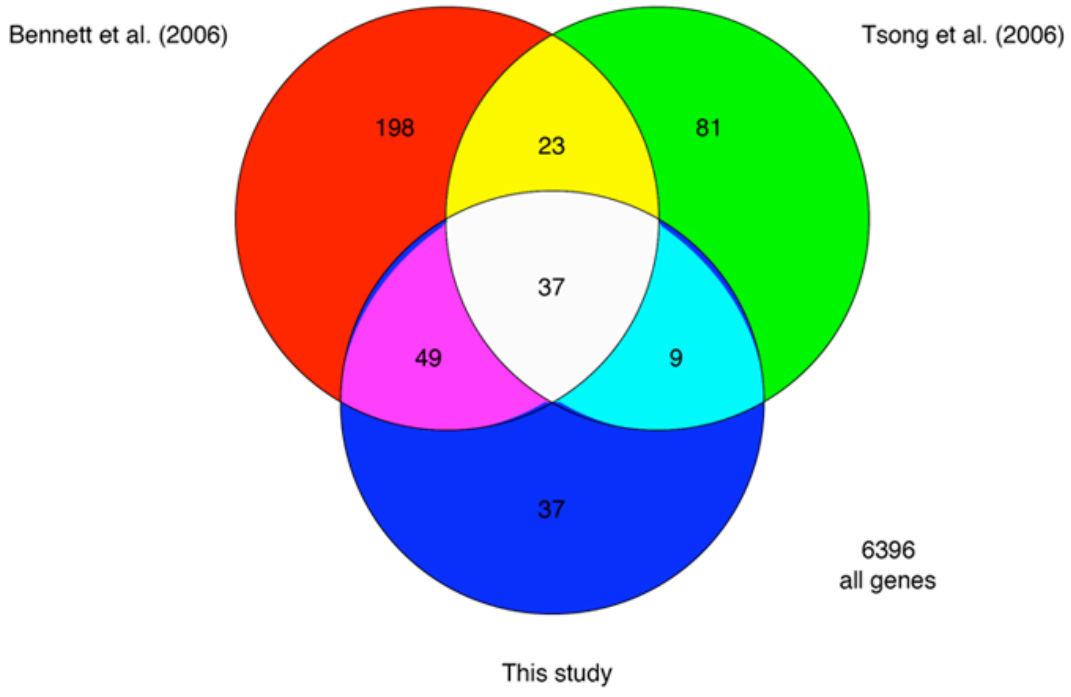
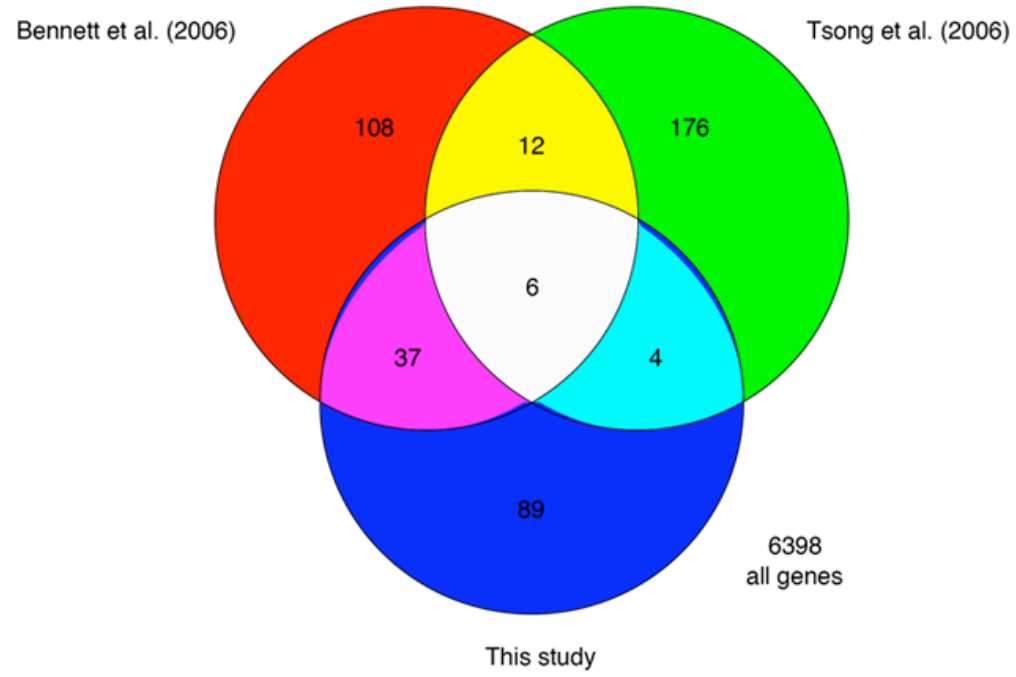

OD600nm
Δsst2Δura3
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
Time in hours
1ug
0.5ug
0.1ug
0.05ug
0.01ug
0.005ug
NI
OD600nm
WT
1.5
1
0.5
0
0 6 12 18 24
Hours
OD600nm
FAR1+/- Δsst2
1.5
1
0.5
0
0 6 12 18 24
Hours
OD600nm
Δfar1Δsst2
1.5
1
0.5
0
0 6 12 18 24
Hours
OD600nm
FAR1rest Δsst2
1.5
1
0.5
0
0 6 12 18 24
Hours

Figure S3

A)


Bennett et al. (2006)
Tsong et al. (2006)
198
23
81
37
49
9
37
6396
all genes
This study

B)


Bennett et al. (2006)
Tsong et al. (2006)
108
12
176
6
37
4
89
6398
all genes
This study

Reference List

Bennett,R.J., and Johnson,A.D. (2006) The role of nutrient regulation and the Gpa2 protein in the mating pheromone response of C. albicans. *Mol Microbiol* **62**: 100-119.

Bennett,R.J., Uhl,M.A., Miller,M.G., and Johnson,A.D. (2003) Identification and characterization of a Candida albicans mating pheromone. *Mol Cell Biol* **23**: 8189-8201.

Dignard,D., El-Naggar,A.L., Logue,M.E., Butler,G., and Whiteway,M. (2007) Identification and characterization of MFA1; the gene encoding Candida albicans a-factor pheromone. *Eukaryot Cell*.

Dignard,D., and Whiteway,M. (2006) SST2, a Regulator of G-Protein Signaling for the Candida albicans Mating Response Pathway. *Eukaryot Cell* **5**: 192-202.

Schaefer,D., Cote,P., Whiteway,M., and Bennett,R.J. (2007) Barrier Activity in Candida albicans Mediates Pheromone Degradation and Promotes Mating. *Eukaryot Cell*.

Tsong,A.E., Tuch,B.B., Li,H., and Johnson,A.D. (2006) Evolution of alternative transcriptional circuits with identical logic. *Nature* **443**: 415-420.