Unique Aspects of Gene Expression during Candida albicans Mating and Possible G1 Dependency

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Mating in Candida albicans and Saccharomyces cerevisiae shares several fundamental characteristics, including similar key regulators harbored by the mating-type locus (25), a similar dependency of fusion on mating type (24, 34, 36), similar pheromone-induced shmooing (5, 35, 43), activation of a number of common mating-associated genes (5, 35), similar repression of haploid-specific genes by repressor homologs of complex loci (Mat1p-Mat2p and Mat1p-Mat2p, respectively) (56), a similar central role for the pheromone-activated mitogen-activated protein (MAP) kinase pathway (11, 37), and similar cytological steps in the fusion process (34). This impressive list of similarities suggests that the intensively studied mating process of S. cerevisiae should serve as a paradigm for understanding C. albicans mating. However, the list of fundamental differences is almost as impressive. First, while the alternative a or ρ mating genes are expressed at the MAT locus and conserved at two unexpressed loci (HML and HMR) in an elegant cassette system for mating-type switching in S. cerevisiae (20), they are harbored at a single heterozygous locus in diploid C. albicans, which must undergo homozygosis for mating-type expression (25). Second, while a-specific genes in S. cerevisiae are expressed by default, they are positively regulated by Mta2p in C. albicans (56). Third, while MAT-haploid strains are immediately mating competent in S. cerevisiae, MTL-homozygous strains of C. albicans are not; they must first undergo a complex phenotypic switch from white to opaque (34, 39) that has no counterpart in S. cerevisiae mating and then find mating partners. Furthermore, skin facilitates C. albicans mating (27), which involves the formation of very long, hypha-like conjugation tubes, much longer than those that have been described for S. cerevisiae. Hence, to employ effectively the information collected for the mating process of S. cerevisiae to understand the mating process of C. albicans, we must first distinguish those processes that are similar from those that are dissimilar.

Here we have taken advantage of the high frequency of conjugation tube formation obtained in mating mixtures of opaque a/a and α/α cells or α-pheromone-activated a/a cells derived from saturation phase cultures (16, 34) to identify 56 up-regulated and 30 down-regulated genes, employing an oligonucleotide-based microarray and Northern analyses. Regulation of genes were identified that are involved in mating, hypha formation, switching, and a number of other diverse functions. By combining these results with data from earlier transcript profiling studies of a cells treated with α-pheromone (5, 35), a more comprehensive profile is presented of mating-associated genes regulated similarly and dissimilarly in C. albicans and S. cerevisiae. In addition, a number of genes associated with filamentation and switching were found to be uniquely regulated during C. albicans mating. Finally, a unique group of genes was identified that is expressed similarly in white and opaque cells in the exponential phase of a growth culture, selectively down-regulated in opaque, but not white, cells in saturation phase, and then selectively up-regulated by pheromone. The observations that only opaque cells are mating competent, that this...
new class of genes is selectively down-regulated in saturation phase cultures of opaque but not white cells, and that saturation phase facilitates mating led us to hypothesize that entry into saturation phase may be requisite to pheromone induction and mating. Tests of this hypothesis, however, suggested that opaque cells, whether in the exponential or saturation phase, must simply be in G0 of the cell cycle to respond to pheromone and that the response includes G1 arrest.

MATERIALS AND METHODS

Strain maintenance. The unrelated C. albicans MTL-homozygous strains WO-1 (a/a) (48) and P37005 (a/a) (33) were stored in 20% glycerol at −80°C. For experimental purposes, cells were plated on agar containing Lee’s medium (30) modified as described by Bedell and Soll (3) (modified Lee’s medium). This agar was supplemented with 5 µg/ml phloxine B to differentially stain opaque-phase colonies and sectors red (2). The S. cerevisiae strains MGD353-13D and MGD353-4H4, generous gifts from Brian Rymond at the University of Kentucky, were similarly stored in 20% glycerol at −80°C.

Preparation of microarrays and Northern analyses. Samples from C. albicans mating cultures were prepared according to methods previously described (34) but on a larger scale. In brief, opaque cells from phenotypically homogeneous 7-day colonies of both mating types were individually inoculated into 250 ml of modified Lee’s medium and grown at 25°C for 48 h to saturation phase in a water bath rotary shaker at 250 rpm. Cells were then pelleted and resuspended in modified Lee’s medium containing 37.5 mg/ml phloxine B to differentially stain opaque-(a/a) and white-(α/α) cells. The a/a or α/α cells was pelleted and the cells resuspended in 10 ml of fresh growth medium in 50-ml Falcon tubes. In the case of mating mixtures, 5 ml each of stationary-phase cultures of a/a or α/α cells was mixed, pellet was resuspended, and resuspended in 10 ml of fresh medium in 50-ml Falcon tubes. Cell cultures were incubated at 25°C in a water bath rotary shaker at 250 rpm. Samples were collected from homogeneous cultures at 3.5 h and from mating mixtures at 3.5 and 7.5 h of incubation. Before samples were collected for microarray analysis, they were examined microscopically for shmooing and fusion. Only mating mixtures at 3.5 h containing over 60% cells with unconstricted evaginations (shmoos) or conjugation tubes and only mating mixtures at 7.5 h containing over 85% shmooed cells, of which 25% had fused, were used for microarray analyses. Three independent preparations of each sample were used for microarray analyses. For preparing pheromone-treated samples, opaque P37005 (a/a) cells from 7-day colonies were inoculated into 250 ml of modified Lee’s medium and grown at 25°C for 48 h to stationary phase in a water bath rotary shaker at 250 rpm. Before the cells were treated with α-pheromone, 10-ml portions of cells were resuspended and resuspended in 10 ml of fresh modified Lee’s medium. Chemically synthesized α-pheromone was then added to a final concentration of 3 µM (35), and the cell culture was incubated at 25°C in a water bath rotary shaker at 250 rpm.

Microarray analysis. C. albicans Array-Ready Genome Oligo set version 1.1 (QIAGEN, Valencia, CA) was resuspended at a concentration of 20 µM in 150 mM sodium phosphate buffer (pH 8.5). Oligonucleotides were printed in duplicate blocks on Code Link slides (Amersham Biosciences) at Microarrays, Inc. (Nashville, TN). DNA coupling and postcoupling processing of printed microarray slides were done according to the manufacturer’s instructions (Amersham Biosciences, Piscataway, NJ). The methods for labeling probes and hybridization to microarrays were adapted from protocols developed by The Institute for Genomic Research (TIGR, http://pga.tigr.org/protocols.html). Total RNA was prepared using RNeasy (QIAGEN) according to the manufacturer’s suggested protocol. Each sample started with a 10-min, 70°C incubation of 10 µg total RNA, Arthroideus thalassinus Spot Report spiking controls (Strategene, La Jolla, CA), and 2 µl random hexamer primers (3 mg/ml) (Invitrogen, Carlsbad, CA). RNA samples were reverse transcribed with 400 U of Superscript II reverse transcriptase (Invitrogen) in the presence of deoxycytidine triphosphates (aminoethyl-dUTP and deoxycytidine triphosphates [4:1]). Samples were purified according to the TIGR protocol (22) and coupled to Cy5 or Cy3 (Amersham Biosciences) in the presence of 0.05 M Na2CO3, pH 9.0. cDNA samples were purified postcoupling according to the TIGR protocol. Cy dye-labeled cDNA samples were resuspended in hybridization buffer (10× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.2% sodium dodecyl sulfate) and applied to microarray slides. Prehybridized slides were blocked in buffer containing 5× SSC, 0.1% sodium dodecyl sulfate, and 1% bovine serum albumin (Sigma, St. Louis, MO) for 45 min at 37°C. Hybridizations were performed for 20 h in a 37°C water bath in the dark. Three independent biological replicates were performed for each hybridization comparison. Slides were washed and dried by centrifugation and immediately scanned using a GenePix 4000B scanner (Axon Instruments, Inc., Union City, CA). Images were processed using the software package GenePix Pro 4.0 (Axon Instruments, Inc.) to gather the raw fluorescence intensities of the Cy3 and Cy5 values of each spot.

Microarray data analysis. To determine an appropriate normalization method, microarray plots, as described by Dudito et al. (18), were used initially to analyze the fluorescence (RG) data. The results of this analysis indicated that a global normalization was appropriate and all raw fluorescence intensities were exponentially transformed prior to fitting the model. The normalization model for this study was \( y_{\text{exp}} = \mu + A_i + D_j + (\alpha \cdot e_{\text{dye}} + e_{\text{spot}}) \), where \( y_{\text{exp}} \) is the signal from spot \( i \) on gene \( j \), \( e_{\text{dye}} \) and target \( k \); is the average signal across all factors; \( A_i \) is the global effect of slide \( i \); \( D_j \) is the global effect of dye level \( j \); \( e_{\text{spot}} \) is the interaction between array and dye; and \( e_{\text{dye}} \) is residual error. The residuals from this model, which are regarded as a crude indicator of relative expression level and are referred to as normalized expression levels, were then subjected to gene-specific models of the form: \( \hat{e}_{i,j} = \mu + A_i + D_j + T_k + e_{i,j} \), where \( A_i \) is r spot effects, \( D_j \) is gene-specific dye k effect, \( T_k \) is expression of gene specifically attributable to target k, and \( e_{i,j} \) is residual error.

The normalization and gene models were fit using PROC MIXED in SAS/STA software version 8 (SAS Institute, Inc., Cary, NC). SAS code for this analysis was based on code available in the Introductory Manual for Mixed Model Analysis of Microarray Data (MANMADA) (2002) (http://statgen.nwu.edu/ggbson/Manual.html). In the normalization model, all effects were treated as random except for the fixed effect of dye. To assess the magnitude and significance of the effects of the cell phases and their interaction on the normalized fluorescence levels, least-squares means were used, the LSMEANS option in SAS (SAS Institute, Inc., 2000) were calculated as described in the MANMADA (2002). The LSMEANS statement calculates the mean value for each target category, adjusted for the other terms in the model. For each pairwise comparison, the difference between the two normalized expression levels and their standard errors are calculated along with the results of t tests (adjusted with Bonferroni’s correction) for the statistical significance of the difference between the two normalized expression levels, standard errors were initially exponentially (base 2) transformed, the resulting difference between expression levels that is observed is interpreted as change (n-fold).

Website containing full transcript datasets. The full transcript data sets can be found in the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo) under the GEO accession number GSE1969.

Northern analysis. Northern analysis was performed as previously described (59). Total RNA from each sample was prepared with the RNeasy kit as recommended by the manufacturer (QIAGEN, Valencia, CA). Five-microgram portions of RNA were separated on a 1.2% agarose-formaldehyde gel, transferred to a Zetabind nylon membrane, and probed with PCR products obtained from PCRs with the primers listed in Table 1. Prehybridization and hybridization were performed by the methods of Church and Gilbert (14). Autoradiography was performed by exposing membrane to Kodak X-Omat film (Eastman Kodak, Rochester, NY) at −80°C with an intensifying screen. All Northern analyses were performed twice with independent preparations.

Quantitative fluorescence microscopy of nuclear DNA. Nuclear DNA content was quantitated using a modification of a protocol by J. A. Huberman and colleagues (http://saturn.roswellpark.org/huberman/Quant Flu Micro/Quant_Flu_Micro.html).

Cells (2.5 × 10⁶) were washed three times with water prior to fixation in 1 ml of 70% ethanol. After 5-min fixation, the ethanol was removed by centrifugation, and the preparation was rinsed three times in 50 mM sodium citrate (SC) and resuspended in 1 ml SC. RNase A (100 µg/ml) (QIAGEN, Valencia, CA) was added and incubated overnight at 37°C. The cells were harvested by centrifugation, rinsed three times in 35 µl SC, and resuspended in 35 µl SC. The cells were mixed in a 1:2 ratio with solution containing 37.5 µM Sytox Green (Molecular Probes, Eugene, OR), 1.5 mM p-phenylenediamine, and 75% glycerol. The cells were distributed in the solution by gentle pipetting, and 2 µl of stained cells was placed on a glass slide and covered with an 18-mm square cover glass to ensure a uniform single-cell layer. The edges of the cover glass were sealed with fingernail polish and the slides incubated overnight at room temperature. Quantitation of Sytox Green-labeled DNA fluorescence was performed using a Bio-Rad Radiance 250MP confocal microscope system on a Nikon TE2000U microscope, with 60× plan apochromat water immersion objective (numerical aperture of 1.2) and 3× digital zoom. LayserSharp 2000 (release 5.2) software was used for image acquisition and fluorescence quantitation. Sytox Green was excited using the 488-nm argon laser line at 3% power, and simultaneously transmitted light differential interference contrast images were generated using the 476-nm argon
TABLE 1. Primers used in this study

| Primer | Sequence |
|--------|----------|
| AKL1F  | 5’TATGGTCATCTTCTAAAAA3’ |
| AKL1R  | 5’TCTTGTTTCTCGTATT3’ |
| CDC55F | 5’ATAATGGCCCTCGTATAA3’ |
| CDC55R | 5’TATGCCTAAATCATCG3’ |
| CPH1F  | 5’TAAGCGCTTAAATACCT3’ |
| CPH1R  | 5’TCTCTGATGTATCTTAC3’ |
| CZF1F  | 5’AACAAAGGTGTCATACC3’ |
| CZF1R  | 5’CAACACAGAAGCTTAT3’ |
| DDR48F | 5’CAAACTGTTTGTGGTCT3’ |
| DDr48R | 5’TATATGCAGAAGATCT3’ |
| ERK25mAIF | 5’TCCCCCGGAAGAAATGAGAATCT3’ |
| ERK25mAIR | 5’TCCCCCGGTAAGCACTGACTA TTCT3’ |
| FANEFG15’ | 5’GGCTGCGAAGTCTCAAGTATC TATA3’ |
| FANEFG13’ | 5’GGCCCGGTCCTTTTCTTTGGCA AGACT3’ |
| GCN4F  | 5’GTAAAGATGCTGTACT3’ |
| GCN4R  | 5’ACAGAGATGATATAGT3’ |
| HAC1F  | 5’ATGGGTTAATCTGTTAT3’ |
| HAC1R  | 5’TCTCTCTGGTGCTAT3’ |
| NRG1F  | 5’AACCTACTATAGGGACCA3’ |
| NRG1R  | 5’TGACACATCTATCTATG3’ |
| OP4SPHR | 5’GGTGGTTGTCCCTCCGGACTAATAA AGTTCCTTT3’ |
| ORF6.8100F | 5’ATCCTATGGGTGCTGTT3’ |
| ORF6.8100R | 5’GAACGTGTTTGTGGTAT3’ |
| RCE1F  | 5’TCAATATGGAAGTGATAC3’ |
| RCE1R  | 5’TCTCATGTAACAAACAC3’ |
| RIM101F | 5’ATGCAGCGTGAACGTC3’ |
| RIM101R | 5’GCCTCAGGCTATCTGAT3’ |
| SAP1F  | 5’TCAATCTTACTTCTCCCTTCCTT AACA3’ |
| SAP1R  | 5’CCAGTACATTAAACAGGTTATAGA CACA3’ |
| TEC15mAIF | 5’TCCCCGGGATTATTGATCGCA AGCT3’ |
| TEC1R  | 5’TCTTGCTTAACCACCCTT3’ |
| STE3F  | 5’ATTCAGATGTAAAAGGACG3’ |
| STE3R  | 5’CCGCTGCACTGTTATACCAGC ATTT3’ |
| STE20F | 5’GCTCAGATATACGGGCTGAC3’ |
| STE20R | 5’CCGCTGCGTCTCCAGATCTA3’ |
| FAR1F  | 5’ATGGTTGCTTGTGGTCAGA3’ |
| FAR1R  | 5’TCCCCGCAATAGAGTTG3’ |

laser line at 10% power. Acquisition parameters, including laser power, pinhole size, and photomultiplier detector gain and black levels, were set by imaging with unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture. A nucleus in a mother cell near the bud neck, with replicated unreplicated DNA (see Results and Discussion) from a yeast cell in exponential-phase culture.

RESULTS AND DISCUSSION

Microarray analysis of gene expression in mating mixtures. For microarray analyses, natural opaque a/a cells of strain P37005 and opaque a/a cells of strain WO-1 were individually grown to saturation phase in liquid medium, in which over 90% of both strains accumulated as unbudded cells (Fig. 1A and B). Although opaque P37005 cells (Fig. 1B) were rounder and larger than opaque WO-1 cells (Fig. 1A), both switched to white at the same frequency, regulated the same repertoire of opaque-specific and white-specific genes, and exhibited the same unique pimples on the cell surface and the same large intracellular vacuole in the opaque phase (33, 35) (data not shown). For microarray analyses, cells were incubated under the following conditions: (i) opaque a/a cells alone, (ii) opaque a/a cells alone, and (iii) a mixture of a/a and a/a cells at equal concentrations. After 3.5 h of incubation, the majority of cells in both a/a and in a/a cultures remained unbudded (Fig. 1C and D). In marked contrast, after 3.5 h the majority (>60%) of cells in mixed a/a and a/a cultures had formed shmoo (Fig. 1E). After 7.5 h of incubation, over 80% had shmooed, and approximately 25% of shmooed cells (approximately 20% of the entire cell population) had fused (Fig. 1F). For analysis, cultures of either opaque a/a cells or opaque a/a cells alone were sampled at 3.5 h. Mating mixtures were sampled at 3.5 and 7.5 h. The microarray comparisons included (i) 3.5-h a/a versus 3.5-h a/a-a/a maturing mixture, (ii) 3.5-h a/a versus 3.5-h a/a-a/a mating mixture, (iii) 3.5-h a/a versus 7.5-h a/a-a/a maturing mixture, and (iv) 3.5-h a/a versus 7.5 h a/a-a/a mating mixture. Each comparison was repeated three times with independent preparations.

A gene was considered up-regulated if one or more of the four comparisons demonstrated an increase of ≥2.0-fold, the remaining comparisons demonstrated increases between 1.1- and 1.9-fold, and the P values adjusted with Bonferroni’s correction were significant. Thus, it included genes that were peaked at 3.5 h or exhibited maxima at 7.5 h. Fifty genes were identified by these criteria as up-regulated. On the basis of known or deduced functions of the C. albicans genes or the S. cerevisiae orthologs, up-regulated genes included the following genes: 7 encoding proteins involved in mating and/or the cell cycle, 7 encoding membrane proteins, 5 encoding cell wall-related proteins, 9 encoding transcription regulators, 3 encoding cytoskeleton-related proteins, 7 encoding proteins involved in energy metabolism, and 12 encoding proteins with other or unknown functions (Table 2). Seven of the 50 genes had been identified by Bennett et al. (5) as up-regulated in a-pheromone-treated opaque a cells derived from log-phase cultures employing a cDNA microarray (Table 2). Orthologs of 13 of the 50 C. albicans genes identified as up-regulated in mating mixtures had also been demonstrated to be up-regulated in S. cerevisiae a cells treated with a-pheromone (Table 2) (46).

A gene was considered to be down-regulated if one or more of the four comparisons demonstrated a decrease of ≥2.0-fold, the remaining comparisons demonstrated decreases between
1.1- and 1.9-fold, and the Bonferroni-adjusted \( P \) values were significant. Thus, this definition included genes that exhibited minima at 3.5 or 7.5 h. Thirty genes were identified by these criteria as down-regulated. On the basis of known or deduced function of the \( \text{C. albicans} \) genes or the \( \text{S. cerevisiae} \) orthologs, down-regulated genes included 3 genes encoding secreted proteinases and lipases, 3 encoding cell surface proteins, 13 encoding proteins involved in energy and metabolism, and 11 encoding proteins involved in other or unknown functions (Table 3). Bennett et al. (5) reported down-regulation of four genes (\( \text{MCM6}, \text{MCM7}, \text{PRI}, \) and \( \text{POL5a} \)) in opaque \( a/a \) cells treated with \( \alpha \)-pheromone, none of which overlapped the 30 down-regulated genes identified in the present study (Table 3). Orthologs of six of the 30 genes identified here as down-

FIG. 1. Description of the preparations and cell phenotypes used in transcript profiling studies of opaque-phase \( a/a \) and \( \alpha/\alpha \) mating mixtures. The original 0-h preparations were from saturation phase cultures. Opaque (Op) cells of strain P37005 \( (a/a) \) were consistently larger than those of strain WO-1 \( (\alpha/\alpha) \), but both switched back to white at the same frequency, possessed cell wall pimples, and expressed the same phase-specific genes (data not shown). Small white arrows in panels E and F point to shmoos and fusions, respectively. Cells were resuspended in fresh medium at time zero at the original saturation phase concentration \((3 \times 10^7 \text{ per ml})\). Bars in panels A and D, 5 \( \mu \text{m} \) (all panels at the same magnification).
| ORF | Gene\(^a\) | Pheromone response\(^b\) | Function(s)\(^f\) |
|-----|------------|--------------------------|------------------|
|     |            | \(S. cerevisiae\) | \(C. albicans\) | |
| Mating and cell cycle related |
| orf6.3306 | MFa1 | Induced | Induced | α-Pheromone |
| orf6.3131 | FUS1 | Induced | Induced | Surface protein, cell fusion |
| orf6.2854 | ERK2 | Induced | Induced | MAP kinase |
| orf6.6562 | PRM1 | Induced | Induced | Pheromone-regulated membrane protein |
| orf6.5090 | CDC55 | No | No | Cell cycle protein kinase |
| orf6.7520 | RCE1 | No | No | α-Pheromone maturation |
| orf6.8018 | MPT5 | No | No | Cell cycle reentry |
| Membrane proteins |
| orf6.7231 | RSN1 | No | No | Membrane protein |
| orf6.4763 | DSE4 | No | No | Daughter cell-specific glucanase |
| orf6.1470 | NA | No | No | Membrane-spanning peroxisome |
| orf6.1684 | MDN1 | No | No | Similar to membrane protein YLR106c |
| orf6.6464 | FRP6 | Induced | No | Membrane protein, ammonia production |
| orf6.5714 | YPT7 | No | No | GTP binding protein, endosome-vacuole fusion |
| orf6.1292 | SNX4 | No | No | Nexin-like protein, sorting |
| Cell wall-related proteins |
| orf6.4883 | HWP1 | NA | Induced | Hyphal wall protein |
| orf6.4889 | RBT1 | NA | Induced | Repressed by TUP1, similar to MUC1 |
| orf6.1267 | EMC9 | No | No | Cell wall organization and biogenesis |
| orf6.5738 | MNT2 | No | No | N-myristoyltransferase for wall biosynthesis |
| Transcriptional regulator |
| orf6.5290 | GCN4 | No | No | Amino acid biosynthetic genes |
| orf6.7973 | RTF1 | No | No | DNA binding properties of TBP\(^g\) |
| orf6.9111 | ARG11 | No | No | Mitochondrial amino acid biosynthesis |
| orf6.3349 | RMS1 | No | No | Transcription regulator |
| orf6.7972 | MET28 | No | No | Sulfur metabolism |
| orf6.3395 | HAC1 | No | No | Phospholipid metabolism |
| orf6.7210 | CTAG | No | No | Transcriptional regulator |
| orf6.5049 | TVE7 | Induced | No | Glycolytic gene expression |
| orf6.7119 | SPT5 | No | No | Cell wall organization and biogenesis |
| Cytoskeleton related |
| orf6.8546 | AKL1 | No | No | Serine/threonine protein kinase, actin cytoskeleton |
| orf6.6411 | EN13 | No | No | Recruitment of clathrin, Golgi traffic |
| orf6.7150 | CDC55 | No | No | Protein phosphatase, actin organization, pseudohypha growth |
| Energy and metabolism |
| AF26526 | COX3 | NA | No | Cytochrome oxidase subunit 3 |
| orf6.8673 | RHR2 | No | Induced | α-Glycerol phosphate synthetase, glycerol biosynthesis, stress response |
| orf6.6577 | IDH1 | No | No | Isopentenyl-diphosphate delta-isomerase, ergosterol biosynthesis |
| orf6.4518 | PR03 | No | No | Δ1-Pyrroline-5-carboxylate reductase |
| orf6.9158 | AYR1 | No | No | 1-Acyl dihydroxyacetone phosphate reductase |
| orf6.5186 | DPS1 | No | No | Aspartyl-tRNA synthetase |
| orf6.6334 | OLE1 | No | No | Δ-9-Fatty acid desaturase |
| Other functions |
| orf6.555 | – | NA | No | Similar to RPI08 of Rickettsia prowazekii |
| orf6.1938 | CTF5 | No | No | Minichromosome maintenance |
| orf6.6854 | DDR48 | No | No | Stress response |
| orf6.9095 | – | NA | No | C3HC4-type zinc finger protein, translation termination |
| orf6.1039 | – | NA | No | Lipase, peroxisome biogenesis |
| orf6.2192 | CDC91 | No | No | GPI transamidase subunit |
| Unknown functions |
| orf6.6035 | – | NA | No | Unknown |
| orf6.8658 | – | NA | No | Unknown |
| orf6.7430 | – | NA | No | Unknown |
| orf6.147 | – | NA | No | Unknown |
| orf6.8877 | – | NA | No | Unknown |
| orf6.7824 | – | NA | No | Unknown |

\(^a\) See “Microarray analysis of gene expression in mating mixtures” in Results and Discussion for criteria for considering a gene to be up-regulated.

\(^b\) ORF, open reading frame.

\(^c\) Names of \(C. albicans\) genes were obtained from the \(C. albicans\) genome database (http://www.genolist.pasteur.fr/CandidaDB). In case the genes were not annotated, the names of their \(S. cerevisiae\) orthologs were listed. If an unambiguous \(S. cerevisiae\) ortholog was not identified from the \(S. cerevisiae\) genome database (http://www.yeastgenome.org), the name of the closest ortholog was listed. –, unnamed sequence.

\(^d\) Pheromone response data of \(S. cerevisiae\) were obtained from Roberts et al. (46). Pheromone response data of \(C. albicans\) were obtained from Bennett et al. (5).

\(^e\) Induced, demonstrated induction; No, demonstrated no induction; NA, not available (not reported or tested).

\(^f\) Known or deduced from the \(S. cerevisiae\) ortholog.

\(^g\) TBP, TATA binding protein.

\(^h\) GPI, glycosylphosphatidylinositol.
regulated (ALG8, SLC1, COX11, PFK2, HEM13, and TRM9) had been demonstrated to be down-regulated in S. cerevisiae a cells treated with α-pheromone; however, homologs of five (FLO9, WSC2, LKH1, TH14, and HSP31) had been demonstrated to be up-regulated by α-pheromone (46) (Table 3). The OP4, SAP1, and SAP3 genes, which are all opaque specific, had been previously demonstrated by Northern analysis to be down-regulated in C. albicans opaque a/a cells treated with α-pheromone (35).

Eight of the genes identified as up-regulated (AKL1, CDC55, CAP1, DDR48, GCN4, ERK2, RCE1, and HAC1) and three genes identified as down-regulated (OP4, SAP1, and SAP3) by microarray analysis (Table 2) proved to be similarly regulated when analyzed by Northern blot hybridization (Fig. 2). However, while 7 of 12 genes identified as unchanged by microarray analysis proved to be similarly unchanged by Northern analysis (WH11, EFG1, TEC1, OP6.8100, SLN1, CSH20, and FARI), five proved to be up-regulated (RIM101, CZF1, NRG1, CPH1, and STE3) (Fig. 2; also data not shown).

## Table 3. Microarray analysis identified 30 genes down-regulated during the mating process of C. albicans

| ORF | Gene | Pheromone response in S. cerevisiae | Function(s) |
|-----|------|-----------------------------------|-------------|
|    |      |                                   |             |
| SAPs and LIPs |      |                                   |             |
| orf6.9036 | SAP3 | NA                                | Secreted aspartyl proteinase 3 |
| orf6.4644 | SAP1 | NA                                | Secreted aspartyl proteinase 1 |
| orf6.5210 | LIP2 | NA                                | Secretory lipase |
| Cell surface proteins |      |                                   |             |
| orf6.8640 | FLO9 | Induced                           | Similar to hypheregulated protein HYR1 |
| orf6.1042 | WSC2 | Induced                           | Cell wall integrity, stress response protein |
| orf6.3288 | YWP1 | NA                                | Putative cell wall protein |
| Metabolism |      |                                   |             |
| orf6.3413 | APE2 | No                                | Aminopeptidase |
| orf6.4694 | SUL1 | No                                | High-affinity sulfate transporter |
| orf6.2190 | SAM2 | No                                | S-adenosylmethionine synthetase 2 |
| orf6.5806 | LKH1 | Induced                           | Probable leukotriene A hydrolase |
| orf6.8469 | ALG8 | Repressed                         | Glycosyl transferase |
| orf6.6671 | PUT4 | R/I                               | Proline permease |
| orf6.9051 | TH14 | Induced                           | Thiazole biosynthetic enzyme precursor |
| orf6.6385 | COX11 | Repressed                         | Cytochrome c oxidase assembly protein |
| orf6.4023 | PFK26 | No                                | 6-Phosphofructokinase kinase |
| orf6.4161 | LEU42 | No                                | 2-Isopropylmalalate synthase |
| orf6.5274 | PFK2 | Repressed                         | Phosphofructokinase beta-subunit |
| orf6.1494 | HEM13 | Repressed                         | Oroporoporphynogen III oxidase |
| Other functions |      |                                   |             |
| orf6.6056 | OP4 | NA                                | Opaque-phase-specific protein |
| orf6.8863 | CHS7 | No                                | Probable membrane protein |
| orf6.5830 | TRM9 | Repressed                         | tRNA methyltransferase |
| orf6.4726 | CTF8 | No                                | Putative kinetochore protein |
| orf6.6713 | NA | Induced                           | Probable heat shock protease |
| orf6.6475 | HSP31 | Repressed                         | Heat shock protein |
| orf6.6564 | PSPI | No                                | High-copy suppressor of TS mutant of DNA Pol |
| orf6.6535 | PTC4 | No                                | Ser/Thr protein phosphatase PP2C |
| orf6.8075 | HLI1 | No                                | Tail-anchored ER membrane protein, unknown function |
| Unknown functions |      |                                   |             |
| orf6.3229 | NA |                                   |             |
| orf6.6060 | NA |                                   |             |

* See “Microarray analysis of gene expression in mating mixtures” in Results and Discussion.
* ORF, open reading frame.
* SAPs and LIPs were obtained from the C. albicans genome database (http://www.genolist.pasteur.fr/CandidaDB). In case the genes were not annotated, the names of their S. cerevisiae orthologs were listed. If an unambiguous S. cerevisiae ortholog was not identified from the S. cerevisiae genome database (http://www.yeastgenome.org), the name of the closest ortholog was listed; –, unnamed sequence.
* Pheromone response data of S. cerevisiae were obtained from Roberts et al. (46). Induced, demonstrated induction; No, demonstrated no induction; NA, not available (not reported or tested); Repressed, demonstrated repression; R/I, repressed/induced.
* TS, mutant of DNA Pol, temperature-sensitive mutant of DNA polymerase.
* ER, endoplasmic reticulum.
cells, while the present study analyzed a/a-a/α mating mixtures.

**Regulation of mating-associated genes.** Bennett et al. (5) demonstrated that α-factor induced a number of mating-associated genes in a/α cells. These included genes encoding proteins involved in receptor-mediated signal transduction (STE2, CEK1, and CEK2), transcription activation (CPH1), pheromone transport and maturation (RAM1 and AXL1), and membrane specialization (PRM1, FUS1, HWP1, FIG1, and RBT1), pheromone adaptation (SS2), and karyogamy (KAR4). Lockhart et al. (35) demonstrated by Northern analysis that α-pheromone induced the STE2, STE4, CAG1, FIG1, HWP1, and KAR4 genes in opaque a/α cells. Here, using both microarray and Northern analyses, we also identified a number of mating-associated genes up-regulated in mating mixtures, including several of the genes previously identified by Bennett et al. (5) and Lockhart et al. (35). Genes previously demonstrated to be up-regulated included CEK2, CPH1, HWP1, FUS1, PRM1, and RBT1 (Table 2 and Fig. 2D). New genes identified as up-regulated included the following: STE3, which encodes the receptor for α-pheromone (21); MFA1, which encodes the α-pheromone (37); RCE1, an ortholog of the S. cerevisiae gene that encodes a CaaX prenyl proteinase involved in apheromone maturation (7); and MPT5, an ortholog of the S. cerevisiae gene that encodes a protein involved in reentry into the mitotic cell cycle after pheromone arrest (12) (Table 2 and Fig. 2D). Northern analysis also revealed that neither CST20, the homolog of S. cerevisiae STE20, which encodes a key kinase that activates the MAP kinase pathway (58), nor the homolog of S. cerevisiae FAR1, which encodes a key kinase inhibitor that blocks cell cycle progression during mating (44), was regulated in C. albicans mating mixtures or a/α cells treated with α-pheromone (Fig. 2E). Both genes were equally expressed in untreated and pheromone-treated opaque a/α cells and in mating mixtures.

The results obtained in this study have been combined with those of Bennett et al. (5) and Lockhart et al. (35) to provide a more comprehensive picture of mating-associated gene regulation during the mating process of C. albicans (Fig. 3). Twenty mating-associated genes have been demonstrated to be up-regulated in α-pheromone-treated opaque a/α cells and/or in mating mixtures of opaque cells (Fig. 3A). Orthologs of all but four of these genes (STE4, CEK1, RCE1, and RAM1) are also up-regulated during S. cerevisiae mating (Fig. 3B). CST20 was constitutively expressed, as was its ortholog in S. cerevisiae STE20 (58), while FAR1, which is pheromone induced in S. cerevisiae (46), was constitutively expressed in C. albicans (Fig. 3B). Hence, while a majority (20 of 25) of mating-associated genes were similarly regulated in C. albicans and S. cerevisiae mating, a minority (5 of 25) were dissimilarly regulated. The latter five genes were dispersed throughout the pheromone-activated program (Fig. 3A) and, hence, provided no clue as to why their regulation differed between the two species.

**Regulation of filamentation-associated genes.** Bennett et al. (5) found that several genes involved in the regulation of filamentation (CPH1, CEK1, and FGR23) and several regulated by filamentation (RBT1, HWP1, SAP4, RBT4, SAP6, SAP5, and ECE1) were up-regulated in a cells treated with α-pheromone. Our microarray analysis also identified three of these genes, CPH1, HWP1 and RBT1, as up-regulated, as well as an addi-
FIG. 3. A profile of mating-associated gene regulation during *Candida albicans* mating (A) and comparison with *Saccharomyces cerevisiae* mating (B). The data used to develop the model in panel A were from this study and studies by Bennett et al. in 2003 (5) and Lockhart et al. in 2003 (35). (A) Up-regulation of a gene is noted by a vertical arrow, color coded according to the source of data. No definitive information exists...
tional gene, DDR48 (29) (Table 2 and Fig. 2C and D). Our results further revealed that YWP1, previously demonstrated to be down-regulated during hypha formation (49), was similarly down-regulated in mating mixtures (Table 3). To investigate further the regulation of filamentation-associated genes during mating, we analyzed by Northern blot hybridization five additional genes, NRG1 (8), EFG1 (55), TEC1 (47), CZF1 (10), and RIM101 (17). Three of these genes, NRG1, CZF1, and RIM101, were up-regulated in mating mixtures and in a/a cells treated with pheromone (Fig. 2C). Together, these results demonstrate that a number of genes associated with filamentation are uniquely up-regulated during C. albicans mating (Table 4).

The filamentation-associated genes regulated by pheromone in opaque C. albicans cells have not been reported to be similarly regulated in S. cerevisiae, primarily because for the majority, there are no S. cerevisiae orthologs (Table 4). Comparison of the cell biology of pheromone-induced shmooing in the two species provided a clue to the reason for the difference. While a cells of S. cerevisiae form relatively short projections in response to pheromone (Fig. 4A through D), C. albicans forms long, hypha-like conjugation tubes (Fig. 4E through H) (34). Since meiosis in S. cerevisiae results in both a and α offspring, fusions can and probably do occur between a and α cells in close proximity. Hence, the mating process of S. cerevisiae may not require long conjugation tubes. In marked contrast, meiosis has not been demonstrated in C. albicans. Because mating types are expressed through MTI homoygosis rather than cassette switching, a and α offspring do not arise in proximity in a single cell lineage as they do in S. cerevisiae. C. albicans conjugation tubes may, therefore, have to travel longer distances to fuse, as has been observed in cell clumps formed in suspension cultures (34) and on skin (27).

Regulation of white and opaque-phase-specific genes. It was previously demonstrated by Northern analysis that the opaque-specific genes OP4, SAP1, and SAP3 were down-regulated in cultures of a/a cells treated with α-pheromone (35). Microarray and Northern analyses performed in the present study revealed that these genes were down-regulated in mating mixtures as well (Table 3 and Fig. 2A). Because the Northern blot hybridization signals of these genes were high in a/a and α/α cells prior to mating and negligible in mating mixtures (Fig. 2B), these results further demonstrate that a-pheromone must down-regulate these opaque-specific genes in opaque α/α cells, just as α-pheromone has been shown to down-regulate them in opaque α/a cells (35).

Down-regulation of opaque-specific genes suggested that opaque cells may switch to white in response to pheromone. If this were the case, then one would expect coordinate up-regulation of white-specific genes. Our microarray analysis did not reveal up-regulation of the white-specific genes WH11 (52), EFG1 (53), and TEC1 (R. Zhao, S. R. Lockhart, and D. R. Soll, unpublished observations). Northern analysis further confirmed that these three white-specific genes were not up-regulated during mating or in a/a cells treated with α-pheromone (Fig. 2B). Hence, pheromone does not appear to induce an immediate switch from opaque to white. Rather, opaque-specific genes are independently down-regulated by switching and by pheromones in the mating process. The absence of white-specific gene up-regulation in mating mixtures that contained between 20 to 25% fusants also indicated that a/a-α/α fusion did not immediately cause a switch from opaque to white.

New class of genes regulated by switching, mating, and growth phase. Northern analyses performed to verify mating-induced up-regulation of a number of genes (AKL1, CDC55, DDR48, HCA1, and GCN4) revealed that while these genes

| C. albicans gene | S. cerevisiae ortholog | Response to pheromone in: | Hypha regulation or function in C. albicans |
|------------------|------------------------|---------------------------|---------------------------------------------|
|                  |                        | C. albicans*              | S. cerevisiae                              |
| CPH1             | STE12                  | Up-regulated (B, TS)      | Up-regulated                                |
| CEK1             | KSS1                   | Up-regulated (B)          | Hyphal growth (Liu et al. [32])             |
| FGR23            | −                      | Up-regulated (B)          | Wall growth (Braun et al. [9])              |
| RBT1             | −                      | Up-regulated (B, TS)      | −                                           |
| HWP1             | −                      | Up-regulated (TS)         | Hyphal wall protein (Staab et al. [54])    |
| SAP4             | −                      | Up-regulated (B)          | Up-regulated in hypha (Hube et al. [23])    |
| RBT4             | −                      | Up-regulated (B)          | −                                           |
| SAP6             | −                      | Up-regulated (B)          | Up-regulated in hyphae (Hube et al. [23])   |
| SAP5             | −                      | Up-regulated (B)          | −                                           |
| ECE1             | −                      | Up-regulated (B)          | Hypha-specific expression (Birse et al. [6])|
| DDR48            | DDR48                  | Up-regulated (TS)         | Down-regulated                             |
| CZF1             | −                      | Up-regulated (TS)         | Hypha regulator (Brown et al. [10])         |
| RIM101           | RIM101                 | Up-regulated (TS)         | pH-induced filamentation (Ramot et al. [45])|
| YWP1             | −                      | Down-regulated (TS)       | Bud-specific (Sohn et al. [49])             |

* B. Bennett et al. (5); TS, this study.

b −, no homolog.

for HST11 and HST7 in the MAP kinase cascade. Boxed genes indicate differences in regulation between C. albicans and S. cerevisiae. Note that the scaffold gene STE5, a necessary component of the MAP kinase cascade in S. cerevisiae mating (13), has not been identified in C. albicans. (B) Induction is compared between C. albicans and S. cerevisiae. The latter data are from the study of Roberts et al. in 2000 (46). Induction or no induction is noted by a plus or minus symbol, respectively. n.a., data not available. The dashed line means no homolog. Genes with differences in regulation between C. albicans and S. cerevisiae are shown on light blue background.
were expressed at low levels in opaque cells and up-regulated in mating mixtures upon pheromone treatment, they were expressed at high levels in white cells (Table 2). None of these genes was identified as up-regulated in \( a/a \) cells treated with \( \alpha \)-pheromone by Bennett et al. (5). Northern analyses further revealed that \( CZF1 \), \( RIM101 \), and \( NRG1 \), which encode trans-acting factors involved in filamentation (10, 17, 42), and \( CAP1 \), which encodes a trans-acting factor involved in oxidative stress (1), were also expressed at high levels in white cells, low to negligible levels in opaque cells, and high levels in opaque mating mixtures or in \( a/a \)-treated \( a/a \) cells (Fig. 2C). It was puzzling that none of these nine genes had previously been identified as white specific in a number of diverse screens for white-specific genes (28, 50, 52).

An explanation can be offered on the basis of the growth history of the cells. In the protocol for both the microarray and Northern analyses of mating mixtures in the present study, opaque \( a/a \) and opaque \( \alpha/\alpha \) cells were derived from liquid growth cultures that had entered saturation phase, since we had qualitatively observed that these cells appeared to respond to pheromone and to fuse at higher levels than cells derived from exponential-phase cultures. In previous screens for opaque-specific genes, cells in the exponential phase of growth had consistently been employed (28, 40, 41, 52). In addition, Bennett et al. (5) employed log-phase cells in their microarray analysis of \( \alpha \)-pheromone-induced genes. Therefore, the possibility that differential expression of this newly discovered class of apparently white-specific genes might be limited to saturation phase was entertained. In other words, these genes may be expressed in both white and opaque cells in exponential phase but down-regulated in opaque, but not white, cells that had entered saturation phase. Furthermore, selective down-regulation of these genes could be related to the mating competency of opaque, but not white, cells: i.e., these genes may have to be down-regulated to achieve mating competency. To test these possibilities, we examined expression of two of these genes, \( AKL1 \) and \( CDC55 \), in white cells and in opaque cells in the exponential and saturation phases of a growth culture (Fig. 5A and B, respectively). The transcript levels of the two genes were similarly high in exponential-phase cultures of both white and opaque cells and remained high in saturation phase cultures of white cells (Fig. 5C). However, the transcript levels were low in saturation phase cultures of opaque cells (Fig. 5C). These results demonstrate that down-regulation of this new class of genes occurs only in opaque cells that have entered saturation phase in a growth culture.

**Saturation phase facilitates mating.** The observations that opaque, but not white, is the mating-competent phenotype (33, 39), that a group of pheromone-inducible genes are selectively down-regulated in opaque but not white cells when they enter saturation phase, and that cells at saturation phase mate more efficiently than cells in the exponential phase of growth led us to hypothesize that entrance into saturation phase may be requisite for mating. To test this hypothesis, we first compared the rate of shmooing in low-density mating mixtures derived from either exponential- or saturation phase growth cultures. Cell multiplication continued in the low-density mating mixtures derived from exponential-phase growth cultures (Fig. 6A) and resumed in low-density mating mixtures derived from saturation phase growth cultures. Cell multiplication continued in the low-density mating mixtures derived from exponential-phase growth cultures (Fig. 6A) and resumed in low-density mating mixtures derived from saturation phase growth cultures (Fig. 6B). In the mixture derived from exponential-phase cells, the proportion of unbudded cells was 58% at 0 h and 45% at 8 h. Ten percent of the cell population had shmooed after 8 h (Fig. 6A). In the mating mixture derived from saturation phase cells, the proportions of unbudded cells were 92% at 0 h and 35% at 8 h. Forty percent had shmooed after 8 h (Fig. 6B). In both cases, only unbudded
cells shmooed. These results were consistent with the hypothesis that opaque cells may have to enter saturation phase to be pheromone responsive. The far smaller proportion of cells that shmooed in mixtures derived from exponential-phase cultures could be explained as that proportion of the cell population that had exited the cell division cycle.

In high-density mating mixtures derived from exponential-phase cultures, budding cells, which initially made up approximately 40% of the cell population, divided as the cell population entered saturation phase (Fig. 6C). By 8 h, 40% of the population were unbudded and 50% had shmooed (Fig. 6C). Only unbudded cells had shmooed. In high-density mating mixtures derived from saturation phase cultures, the proportion of unbudded cells at 0 h was 92%, and no further cell multiplication was evident (Fig. 6D). At 8 h, the great majority of unbudded cells had shmooed (Fig. 6D). These results were consistent with the hypothesis that mixtures of opaque a/a and α/α cells in the exponential phase became pheromone responsive as they entered the saturation phase and that the majority of cells in a mating mixture that were in saturation phase were pheromone responsive.

Saturation phase per se, however, is not requisite for pheromone induction. However, because mating mixtures rely upon the endogenous production of pheromone, we considered the alternative possibility that the low proportion of shmooing in exponential-phase mating mixtures may simply be due to low levels of pheromone, rather than to a difference in pheromone responsiveness. If this were the case, the addition of chemically synthesized α-pheromone to low-density a/a cells derived from exponential-phase cultures should increase the proportion of shmooing cells over that observed in mating mixture to which no exogenous pheromone was added. This was observed (Table 5). In exponential-phase cultures of a/a cells to which chemically synthesized α-pheromone was added, 35% of the cell population shmooed after 3 h, a frequency nine times higher than that for a comparable low-density mating mixture that relied on endogenously generated pheromone (Table 5). Shmooing in α-pheromone-treated cultures was restricted to unbudded cells and included 50% of that portion of the population, or roughly a quarter of the entire cell population (Table 5). The addition of α-pheromone to low-density a/a cells derived from saturation phase cultures induced 73% of the cell population to shmoo after 3 h, three times the level of comparable mating mixtures that relied on endogenously generated pheromone (Table 5). These results demonstrate that pheromone was limiting in mating mixtures, but more importantly, they show that approximately half of the unbudded cells in an exponential-phase population are responsive to α-pheromone and that shmooing is, therefore, not an exclusive characteristic of saturation phase cells.

G1 may be a requisite for shmooing. Our results (Table 5), therefore, indicated that approximately half of the unbudded cells in an exponential-phase growth culture, representing between 25% and 35% of the entire cell population, can be induced by pheromone to shmoo, just as the majority of cells in a saturation phase population can be induced. What characteristics do saturation phase cells and half of the unbudded cells in an exponential-phase culture have in common? First, they are both in the unbudded phase of the cell cycle. Second, they may both be in G1 of the cell cycle. Prior observations indicated that in modified Lee’s medium (3), cells entering saturation phase accumulated in G1 of the cell cycle (4, 51). Hence, the requisite for mating competency may not be saturation phase, per se, but simply G1 of the cell cycle. To test this hypothesis, we developed a method for measuring the DNA content of the nucleus of individual cells that could be staged microscopically in the budding cycle. Sytox Green was used to stain DNA, and scanning confocal microscopy was used to quantify staining. Measurements were made from a line scan through the nucleus (Fig. 7A through F, left panels of each pair of panels). The line scan intensities were then plotted as a function of pixel distance (Fig. 7A through F, right panels of each pair of panels). A representative intensity scan through the nucleus of an unbudded saturation phase cell is presented in Fig. 7A. The peak was at 125 pixel intensity units. Peak measurements of pixel intensity for the nuclei of 28 unbudded

**FIG. 5.** The genes in the newly identified group of genes selectively down-regulated in opaque cells and then pheromone induced are expressed at high levels in both log-phase white cells and log-phase opaque cells. (A) Growth kinetics of white-phase WO-1 cells. (B) Growth kinetics of opaque-phase WO-1 cells. In growth kinetics curves of both cell types, the time at which samples were taken from log-phase (LP) and saturation phase (SP) cultures are boxed. (C) Northern analysis of the expression of two genes, AKL1 and CDC55, in white (W) and opaque (O) cells derived from log-phase (LP) and saturation phase (SP) cell cultures.
saturation phase cells, presented in a histogram of ascending values in Fig. 8A (blue), ranged from 100 to 125 pixel intensity units, with a mean ± standard deviation of 113 ± 7 pixel intensity units. Peak measurements of the pixel intensities of 28 nuclei of exponential-phase unbudded cells, presented in a histogram of ascending values in Fig. 8A (red), included two groups. Measurements of the first group, which included the nuclei of 13 cells (46%), ranged from 105 to 125 pixel intensity units.

FIG. 6. Shmoo formation in mating mixtures of opaque α/α (WO-1) and opaque α/α (P37005) cells in suspension is facilitated by deriving cells from saturation phase rather than log-phase growth cultures and resuspending them at a high cell density that inhibits further cell multiplication. (A, B) Opaque α/α and α/α cells derived from log-phase or saturation phase growth cultures, respectively, were mixed in fresh medium at low cell density (2 × 10⁶ per ml). (C, D) Opaque α/α and α/α cells derived from saturation phase growth cultures, respectively, were mixed in fresh medium at high cell density (3 × 10⁷ per ml). The cell concentration and the proportions of unbudded singlets, budding cells, and “shmooing” cells were monitored in each case as a function of time.
units, mimicking the distribution of measurements of the nuclei of saturation phase cells. The mean peak pixel intensity for this group was 113 ± 5, similar to that of saturation phase cells (113 ± 7). Measurements of the second group, which included the nuclei of 15 cells (54%), ranged between 145 and 245 pixel intensity units. The mean peak pixel intensity for the second group was 184 ± 37. The three highest values in the second group (242, 245, and 247 pixel intensity units) were approximately twice the average value of nuclei in the first group (Fig. 8A), suggesting that the range of values in the second group reflected different stages of DNA replication. The peak measurements of nuclei in mother cells with anucleate buds ranged between 240 and 255 pixel intensity units (n = 10). A value of 245 pixel intensity units was, therefore, deemed the replicated state, since it was approximately twice the value of stationary-phase nuclei or nuclei of unbudded exponential-phase cells in

| Condition                        | Cell origin | Proportion of shmoos at 3 h (%) | Fold increase in cell number at 3 h | Proportion of singlets (%) at: | % Change after 3 h | Proportion of budding cells (%) at: | % Change after 3 h |
|----------------------------------|-------------|---------------------------------|-------------------------------------|------------------------------|------------------|-----------------------------------|------------------|
|                                  |             |                                 |                                     |                              |                  |                                   |                  |
| a/a-α/α mating mixture           | Log phase   | 4                               | 2.0                                 | 58                           | 50               | -8                                | 42               | 45               | +3               |
|                                  | Stat. phase | 25                              | 2.4                                 | 92                           | 60               | -30                               | 8                | 15               | +7               |
| α-Pheromone-treated a/a cells    | Log phase   | 35                              | 2.3                                 | 58                           | 44               | -14                               | 42               | 24               | -20              |
|                                  | Stat. phase | 73                              | 1.7                                 | 92                           | 19               | -73                               | 8                | 12               | +4               |

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*Log- and stationary-phase cells were diluted into fresh medium containing 3 × 10⁻⁶ M chemically synthesized α-pheromone (Lockhart et al. [35]) at a final cell density of 2 × 10⁷ per ml.

Stat., stationary.

FIG. 7. Nuclear DNA was quantitated by staining with Sytox Green and quantitating fluorescence by obtaining line scans of pixel intensity using a laser-scanning confocal microscope. (A) Saturation phase unbudded, unreplicated DNA. (B) Log-phase unbudded cell, unreplicated DNA. (C) Log-phase budded cell, replicated DNA. (D) Log-phase cell undergoing pseudohyphal growth, with a nucleus in the mother cell containing unreplicated DNA (white) and a nucleus in the first daughter cell containing replicated DNA (red). (E, F) Shmoos derived from log-phase cells containing unreplicated DNA. Fluorescence intensity is noted as pixel intensity, plotted along the scan. Bar, 5 μm.
FIG. 8. Histograms of peak fluorescent intensity (pixel intensity) demonstrate that the nuclei of all saturation phase unbudded cells contain unreplicated DNA, half the nuclei of log-phase unbudded cells contain unreplicated DNA and half DNA undergoing replication, and the nuclei of unbudded cells forming conjugation tubes (shmooing) contain unreplicated DNA. (A) Comparison of saturation phase unbudded cells and log-phase unbudded cells. (B) Comparison of log-phase unbudded cells and α-pheromone-induced log-phase shmoo. Cells were treated with α-pheromone for 3.5 to 4.0 h. In each histogram, cells are ordered in ascending DNA content.

the first group (Fig. 8A). A scan of a nucleus of an exponential-phase cell in the first group, with a peak pixel intensity of 124 (unreplicated), is presented in Fig. 7B, and a scan of the nucleus of an exponential-phase cell that had formed an anucleate bud, with peak pixel intensity of 245 (replicated), is presented in Fig. 7C. Scans of an unreplicated nucleus in a mother cell and a replicated nucleus in a daughter cell that had in turn budded in a pseudohyphal sequence are presented in Fig. 7D.

If shmooing occurred exclusively in unbudded cells in G1, in an α-pheromone-treated culture of dividing cells, then the distribution of nuclear DNA intensities of cells that had just shmooed should mimic the distribution of nuclei in saturation phase cells or untreated exponential-phase unbudded cells in the first group (Fig. 8A). That was exactly the result we obtained. The range for 28 α-pheromone-treated exponential-phase cells that had just shmooed was between 100 and 145 pixel intensity units (Fig. 8B). The mean was 120 ± 11 pixel intensity units, very close to the average value of a nucleus with unreplicated DNA (113 ± 5 pixel intensity units) and approximately half that of nuclei with replicated DNA. Examples of scans of the nuclei of representative cells that had shmooed in α-pheromone-treated exponential-phase cultures are presented in Fig. 7E and F. These results indicate that shmooing induced by α-pheromone in a low-density, multiplying culture of cells may occur only in unbudded cells in the G1 phase of the cell cycle.

Pheromone blocks cells in G1. Measurements of the relative DNA content of the nuclei of cells that had shmooed in exponential-phase cultures treated for 4 h with pheromone revealed that in every case nuclear DNA was unreplicated (Fig. 8B). For the majority of these cells, the length of the evagination (conjugation tube) was between one-quarter and one cell diameter. These results suggested not only that cells had to be in G1 to shmoo but also that pheromone blocked cells in G1. To demonstrate this point, we searched for those cells in exponential-phase cultures treated with α-pheromone for 4 h that had formed conjugation tubes greater than two cell diameters and that had not reverted apically to the budding growth form (34). The relative DNA content of the nuclei in these cells, which remained localized in the parent cell body, ranged between 110 and 125 pixel intensity units (n = 10), the same range as the nuclei of the general population of cells that had shmooed. These results indicate that cells must not only be in G1 to respond to α-pheromone but that pheromone then blocks cells in G1 as the conjugation tube grows. In S. cerevisiae, it has been demonstrated that pheromone similarly blocks cells in G1 (57). When the conjugation tubes of C. albicans reverted apically to the budding growth form (34) in 7-h cultures, the relative DNA content of the nucleus ranged between 150 and 240 pixel intensity units (n = 4), indicating that upon reversion, the block is lifted.

Conclusions. The mating system of C. albicans is remarkable not only for the characteristics it shares with S. cerevisiae but also for its unique features. C. albicans, like S. cerevisiae, relies upon pheromones from opposite mating types to induce the formation of conjugation projections, which find each other through chemotropism (34). These tubes then fuse so that the nuclei of opposite mating types can undergo karyogamy and recombination. The pheromone response pathway is similar in the two organisms, as are the genes regulated by the pathway (5, 11, 25a, 37). In addition, we have shown here that C. albicans cells respond to pheromone in G1 and are then blocked in G1 by pheromone, characteristics similar to those of S. cerevisiae. However, there are differences, some of which are related to the incorporation of two developmental programs into the C. albicans mating process. While the majority of mating-related genes analyzed here and in previous studies (5, 35) were similarly regulated in C. albicans and S. cerevisiae, a minority were regulated quite differently. A few of these latter genes, such as STE4 and FAR1, are pivotal in the mating response pathway, so it seems quite unlikely that the differences are without purpose, although that purpose is not obvious. On the other hand, the unique up-regulation of hypha-associated genes correlates with the unique hypha-like conjugation tubes formed by C. albicans. On skin, which facilitates shmooing and fusion, the conjugation tubes have been shown to extend long distances over complex terrain to fuse (27). It seems likely that pheromone activation of filamentation-associated genes and induction of hypha-like conjugation tubes reflect integration of the filamentation program into the
C. albicans mating process, which may have evolved through host-pathogen interactions (38). This may also be at least part of the explanation for why cells must switch from white to opaque to mate and why pheromone down-regulates opaque-phase-specific genes. Cells in the opaque phase selectively colonize skin (26), which facilitates mating (27). Cells in the opaque phase also do not release a white blood cell chemottractant and are, hence, invisible to human polymorphonuclear leukocytes (19). Down-regulation of opaque-specific genes suggests that while the opaque phenotype may be required for the first steps in the mating process, expression of some opaque-specific genes may be incompatible with later steps in the mating process. Finally, it is not obvious why a set of genes identified for the first time in the present study are selectively down-regulated in opaque, but not white, cells entering saturation phase in liquid culture. Since exponential-phase cells can mate, the selective down-regulation of these genes in saturation phase may serve an opaque-related function other than mating. What is clear is that C. albicans, in contrast to S. cerevisiae, has incorporated two developmental programs, switching and hypha formation, into the mating process. These differences may be due to the fact that C. albicans, unlike S. cerevisiae, is a true pathogen that has developed a life cycle that depends upon its interactions with a host.

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