

Identification and Characterization of a *Candida albicans* Mating Pheromone

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***Candida albicans*, the most prevalent fungal pathogen of humans, has recently been shown to undergo mating. Here we describe a mating pheromone produced by *C. albicans* α cells and show that the gene which encodes it (*MF* α) is required for α cells, but not *a* cells, to mate. We also identify the receptor for this mating pheromone as the product of the *STE2* gene and show that this gene is required for the mating of *a* cells, but not α cells. Cells of the *a* mating type respond to the α mating pheromone by producing long polarized projections, similar to those observed in bona fide mating mixtures of *C. albicans* *a* and α cells. During this process, transcription of approximately 62 genes is induced. Although some of these genes correspond to those induced in *Saccharomyces cerevisiae* by *S. cerevisiae* α -factor, most are specific to the *C. albicans* pheromone response. The most surprising class encode cell surface and secreted proteins previously implicated in virulence of *C. albicans* in a mouse model of disseminated candidiasis. This observation suggests that aspects of cell-cell communication in mating may have been evolutionarily adopted for host-pathogen interactions in *C. albicans*.**

Candida albicans is the most common fungal pathogen in humans and is responsible for a wide variety of mucosal and systemic infections (9). Two aspects of *C. albicans* biology are examined in this paper. The first is a group of hypha-specific gene products required for full virulence in a disseminated model of candidiasis. These genes include *HWPI* (whose product is required for efficient attachment to epithelial cells) (38), *SAP4*, *SAP5*, and *SAP6* (which encode aspartyl proteases required for tissue invasion) (6, 12), *RBT1* and *ECE1* (which encode cell surface proteins of unknown function), and *RBT4* (which encodes a secreted protein of unknown function) (2). All of these genes encode secreted or membrane-bound proteins, and all are induced when *C. albicans* makes the transition from the blastospore to filamentous forms. On rich laboratory media, *C. albicans* typically grows as single, ellipsoidal cells (blastospores) which divide by budding. However, under a variety of other conditions (for example, in the presence of serum, high temperature, neutral pH, or nutrient-poor media), *C. albicans* assumes a variety of filamentous forms (3, 23, 31, 33, 41). These filamentous forms range from pseudohyphae (in which cells are attached and elongated but still ellipsoidal) to true hyphae (in which cells are attached, highly elongated, and cylindrical). These changes in cell morphology are believed to allow *C. albicans* to rapidly colonize and disseminate in host tissues, facilitating the spread of infection. Although the synthesis of the virulence genes mentioned above is induced when *C. albicans* makes the transition from the blastospore to the filamentous forms of growth, these gene products are not required for filamentous growth per se (3, 23, 31, 33, 41). Rather,

they are believed to endow the filamentous forms with specialized properties related to tissue adhesion and invasion.

The second aspect of *C. albicans* biology relevant to this paper is the recently discovered ability of this organism to undergo sexual mating. Most clinical isolates of *C. albicans* (including the most common laboratory strain, CAF2-1) are the *a*/ α type and are unable to mate. If these strains are converted to *a* and α strains through specific mutations or through chromosome loss, *a* cells can mate with α cells to form tetraploid cells (17, 29). These tetraploids can, in turn, lose chromosomes to produce diploid *a* and α cells, thereby completing a parasexual cycle (1). Naturally occurring *a* and α clinical isolates have also been identified (26), and these, too, can undergo mating (25). However, for *a* and α cells (regardless of their origin) to become mating competent, they must first undergo a heritable, but reversible, transition from the so-called white phase to the opaque phase (30). This switch involves changes in the expression of many genes, including a number of genes implicated in mating (21).

In this paper, we identify the α mating pheromone (α -factor) of *C. albicans* and characterize the response it elicits in recipient *a* cells. The gene encoding α -factor is predicted to encode a precursor protein that is processed into three identical tridecapeptides representing the mature α -factor. We show that a synthetic tridecapeptide of this sequence produces long polarized projections in *C. albicans* *a* cells, but only when those cells were in the opaque (mating-competent) form; those projections had been previously observed in bona fide mating mixtures (25, 30). The tridecapeptide failed to elicit any morphological response in α cells (white or opaque) or in *a*/ α cells. Additional support for this tridecapeptide being an authentic α -factor comes from the demonstration, using gene knockout strains, that the *C. albicans* *STE2* gene, which is homologous to the *Saccharomyces cerevisiae* α -factor receptor gene (15, 19), is

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required for the morphological response of **a** cells to α -factor. To complete the link between the tridecapeptide and mating, we also show that α cells (but not **a** cells) with the α -factor gene (*MF α*) deleted are deficient in mating; likewise, **a** cells (but not α cells) with the gene encoding the α -factor receptor deleted (*STE2*) are mating deficient. Microarray analysis of the transcriptional response of **a** opaque cells to α -factor revealed 62 genes that are induced more than threefold. These genes can be divided into several categories. As expected, one group of *C. albicans* pheromone-induced genes (18 in all) closely resemble genes previously implicated in mating in *S. cerevisiae*. However, the *C. albicans* and *S. cerevisiae* pathways have diverged considerably, and the majority of the pheromone-induced genes in *C. albicans* are specific to that organism. These genes include 18 open reading frames (ORFs) with no close relatives in any sequenced genome (including that of *S. cerevisiae*) and 15 ORFs with at least one close relative but no obvious clues to specific function. The most surprising group of pheromone-induced genes in *C. albicans* are a set of seven cell surface and secreted proteins that were previously shown to be required for virulence in a mouse model of disseminated candidiasis. This observation indicates that *C. albicans* utilizes a number of the same cell surface and secreted proteins for both mating and pathogenesis and suggests an evolutionary link between the two processes.

MATERIALS AND METHODS

Media and pheromone. Media were prepared as described previously (14). α -Factor (synthesized by Genemed Synthesis) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/ml and then brought to a final concentration of 10 mg/ml in 10% DMSO. α -Factor was added to media at a final concentration of 10 μ g/ml unless otherwise noted.

Strains. *C. albicans* CAF2-1 (*URA3 Δ ura3::imm434*) (13), the α mating type strain, CHY247 (*a1::hisG-URA3-hisG*), and the **a** mating type strain, CHY420 (*α 1::hisG-URA3-hisG α 2::hisG*) have been described previously (30). Strains RBY731 (*MTLa/MTLa*) and RBY734 (*MTL α /MTL α*) were derived from strain CAI4 (*Δ ura3::imm434 Δ ura3::imm434*) (13) by growth on sorbose-containing medium for 7 days at 37°C, which selects for loss of one copy of chromosome 5, the chromosome containing the *MTL* locus (18, 29). Following loss of a single copy of chromosome 5, strains were grown on yeast extract-peptone-dextrose, and faster growing colonies were chosen. This step has been proposed to select for duplication of the remaining chromosome 5, resulting in homozygosis of the *MTL* locus (18). These strains will be referred to in this paper as **a/a** or α/α . A *ste2/ste2* strain was constructed by the method of Wilson et al. (42) in strain CAI4. Both alleles of *STE2* were deleted using a construct derived by PCR from pDDB57, and *Ura*⁻ colonies were generated on medium containing 5-fluoroorotic acid. Two completely independent *ste2/ste2* strains were constructed in this way. *MTLa* (MMY566 and MMY567) and *MTL α* (MMY565 and MMY568) strains were then generated from the *ste2/ste2* strains by growth on sorbose-containing medium, as described above. Similarly, *mf α /mf α* strains were constructed using a PCR construct based on pDDB57, *Ura*⁻ colonies were generated on 5-fluoroorotic acid-containing medium, and *MTLa* (MMY563) and *MTL α* (MMY563) derivatives were selected on sorbose-containing medium. Control strains for the quantitative mating assays were CHY257, CHY439, CHY477, and MMY278 (30). The mating type of all mating strains was periodically confirmed by PCR as previously described (30).

Mating assays of *C. albicans*. Quantitative mating analysis of *C. albicans* strains was performed as described previously (30). Briefly, *Ade*⁻ and *Ura*⁻ mating strains in the opaque phase were grown at 25°C overnight, and approximately 3 \times 10⁷ cells of each strain were mixed and deposited onto 0.8- μ m-pore-size nitrocellulose filters using a Millipore 1225 vacuum sampling manifold. The filters were then grown on the surface of a yeast extract-peptone-dextrose plate containing 55 μ g of adenine per ml and incubated for 6 days at 23°C. Cells were collected from the filters and plated onto *Ade*⁻ *Ura*⁻ media to select for conjugants and onto *Ade*⁻ and *Ura*⁻ plates to monitor each parent population and conjugants.

Construction of *C. albicans* microarray. A *C. albicans* microarray containing ~11,000 spots was constructed by PCR amplification of predicted ORF sequences derived from sequencing the *C. albicans* genome (<http://www-sequence.stanford.edu/group/candida>). Primer design and ORF amplification were performed in collaboration with the laboratory of Gerald Fink, Whitehead Institute, Massachusetts Institute of Technology (MIT). Successful PCR products were ethanol precipitated and printed onto glass slides as previously described (8). Printed slides were rehydrated and snap dried, and the DNA was cross-linked (8). Slides were then blocked with bovine serum albumin by incubation in a solution containing 0.5 M sodium chloride, 0.05 M sodium citrate, 0.1% sodium dodecyl sulfate, and 1% bovine serum albumin for 5 min at room temperature followed by 5 min at 42°C. Slides were rinsed and spun dry in a clinical centrifuge and used within 1 h of processing.

Preparation of cDNA for microarray experiments. Five-milliliter cultures were grown overnight at 23°C and used to inoculate a large overnight culture (4 liters). When the optical density of the culture reached 1.0, a sample of the culture was harvested and frozen (zero time point). To the remainder of the culture, 10% DMSO or α -factor dissolved in 10% DMSO was added (final concentration, 10 μ g/ml). Samples (500 ml) were taken from the α -factor- or mock-treated cultures after 20 min, 1 h, 2 h, or 4 h. The cells were collected by filtration and stored at -80°C.

Total RNA was obtained from cells using a hot phenol protocol as previously described (30). Poly(A) RNA was isolated using the Qiagen Oligotex mRNA isolation mini kit. Each cDNA pool was generated from 4 μ g of poly(A) by first annealing the poly(A) RNA with oligo(dT) (10 μ g) and pdN6 (10 μ g). cDNA was then synthesized using Stratascript reverse transcriptase (Stratagene; 100 units of reverse transcriptase) in reaction mixtures containing 0.5 mM (total) deoxynucleoside triphosphates (aminoallyl-dUTP and deoxynucleoside triphosphates [3:2]). After cDNA synthesis for 2 h at 42°C, the RNA was hydrolyzed with 0.2 N NaOH and 0.1 M EDTA. The reaction was neutralized with 0.33 M Tris (pH 7.4), and the cDNA washed several times with H₂O in a Microcon-30 filter. The cDNA was recovered and dried in a speed-vac.

Coupling of cDNA and hybridization to microarrays. The cDNA was coupled with fluorescent dyes by resuspension in 0.05 M sodium bicarbonate buffer, pH 9.0, and incubation with either Cy3 or Cy5 dye (Amersham Biosciences) for 1 h at room temperature in the dark. Unincorporated dye was removed using a Qiagen PCR purification kit, and the samples were dried in a speed-vac. The samples of cDNA coupled with fluorescent dyes were resuspended in a solution containing 50% formamide, 1 M sodium chloride, 0.1 M sodium citrate, and 0.2% sodium dodecyl sulfate. The appropriate Cy3-cDNA and Cy5-cDNA samples were then mixed and heated for 3 min at 100°C before the samples were applied to the microarray slide. Samples were hybridized for 18 to 24 h at 45°C, and the slides washed, dried, and then analyzed as described below.

Analysis of microarrays. Arrays were scanned on a GenePix 4000 scanner (Axon Instruments, Foster City, Calif.), and data were quantified by using GENEPIX PRO version 3.0 and further processed by using NOMAD (<http://ucsf-nomad.sourceforge.net/>). Pairwise average linkage clustering analysis was performed by using the program CLUSTER and visualized by using TREEVIEW (10). Genes whose expression changed by threefold or more (in two experiments) relative to the zero time point were selected for further analysis. The Saccharomyces Genome Database (<http://www.yeastgenome.org/>) and the Stanford Candida database (<http://sequence-www.stanford.edu/group/candida/index.html>) were used to facilitate further analysis.

RESULTS

Identification and characterization of *C. albicans* α -factor.

We utilized conserved features of the α -factor pheromone family to devise a computer algorithm to search for putative α -factor candidates in the publicly available database of *C. albicans* (<http://www-sequence.stanford.edu/group/candida>). This search revealed one gene (ORF6.4306) with several features characteristic of the α -factor family (Fig. 1). Conceptual translation of this gene produced a hydrophobic leader sequence, consensus Kex2 protease processing sites, and three repeats of a 13-amino-acid sequence, GFRLTNFGYFEPG. These repeats were predicted to be processed into the mature pheromone (Fig. 1). This predicted processing scheme is supported by the observation that the *KEX2* gene is required for

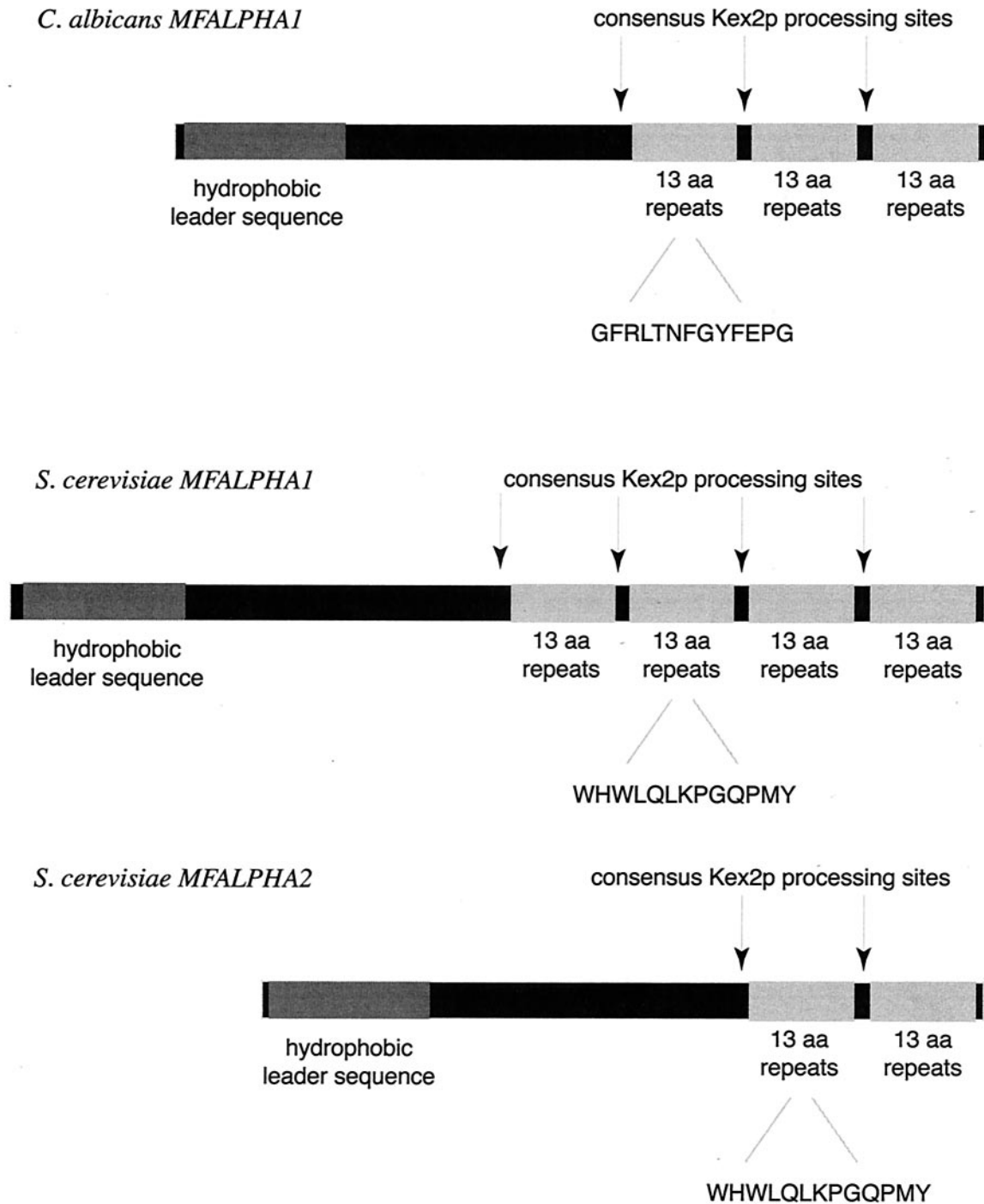


FIG. 1. Schematic diagram of the proposed *C. albicans* α -factor pheromone precursor. The conceptually translated protein consists of a hydrophobic leader sequence and three copies of a 13-amino-acid (aa) sequence. For comparison, the two α -factor genes from *S. cerevisiae* are also shown. The mature *S. cerevisiae* α -factor peptide is formed by several protein processing steps, including cleavage by the Kex2 protease (37).

mating of α cells in *C. albicans* (28); additional processing steps were suggested on the basis of analogy with those of *S. cerevisiae* and should be regarded as provisional. The overall structure of the *C. albicans* gene is similar to that of the *S. cerevisiae* MF α genes, MF α 1 and MF α 2 (Fig. 1), although there is no amino acid sequence similarity between the *S. cerevisiae* ma-

ture α -factor and the predicted *C. albicans* α -factor. Newport et al. (34) had previously noted that ORF6.4306 may encode an α -factor.

To experimentally test the possibility that this gene encodes an α -factor, the GFRLTNFGYFEPG tridecapeptide was synthesized in vitro and added at 10 μ g/ml to *C. albicans* cells of

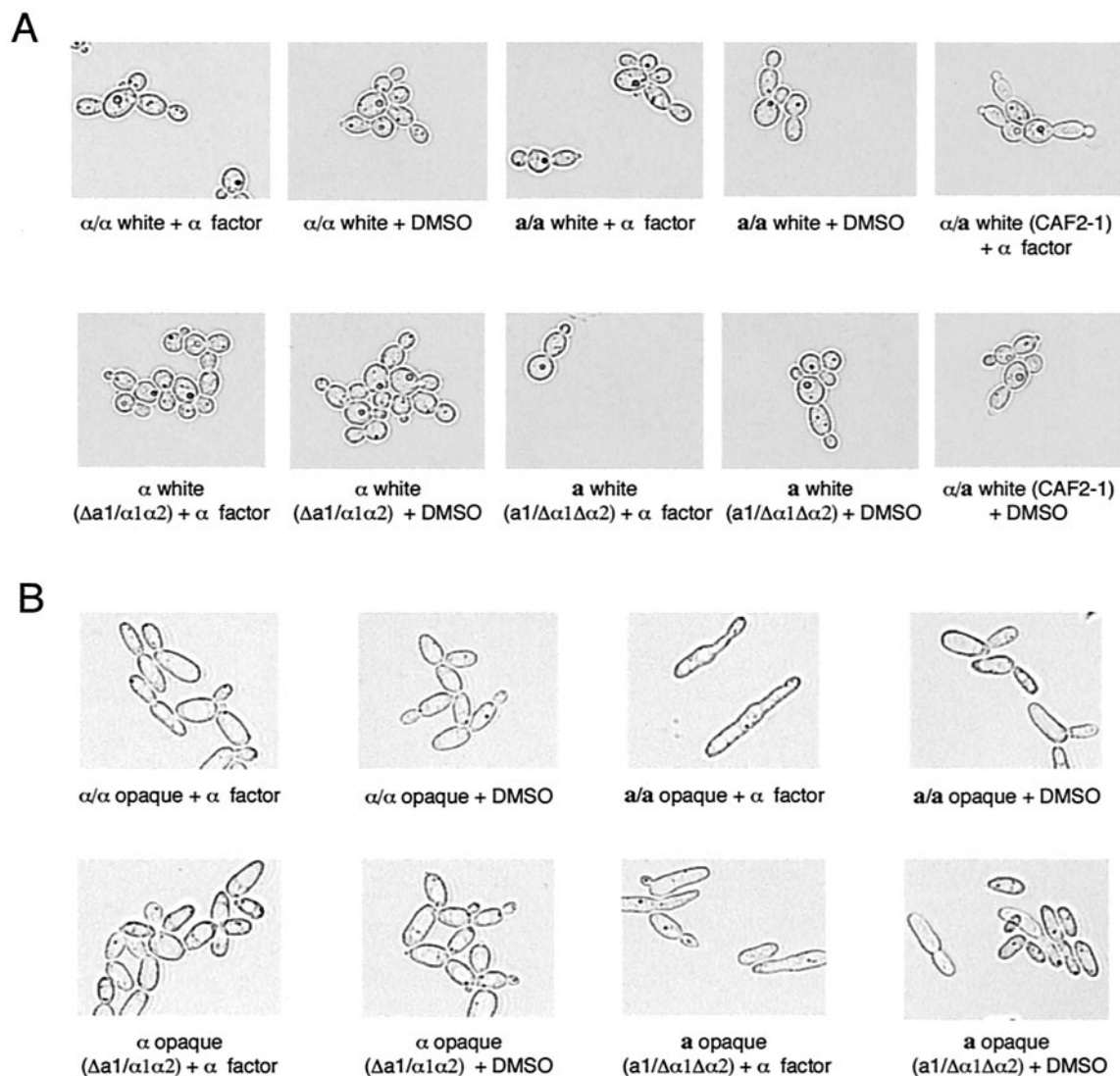


FIG. 2. Response of *C. albicans* cells to synthetic α -factor. *C. albicans* **a** and α cell types in both the white phase (A) and the opaque phase (B) were treated with 10% DMSO or α -factor (10 μ g/ml) dissolved in 10% DMSO. Cells were examined after 4 h of incubation at 25°C. The **a** and α cell types were generated by gene knockouts (e.g., $\Delta a1/\alpha1\alpha2$) or by selection on medium containing sorbose (e.g., *MTL α /MTL α*), as described in Materials and Methods.

the following types: α white, α opaque, **a** white, **a** opaque, and **a/a** white cells (**a/a** cells cannot form opaque cells). The construction of these strains and the isolation of white and opaque forms are described in Materials and Methods. We observed that only **a** opaque cells morphologically responded to the tridecapeptide: they produced long polarized projections which were easily distinguishable from pseudohyphal or other elongated cell types by the absence of a constriction at the site of polarized growth (Fig. 2). These projections, which closely resemble those observed in bona fide mating mixtures of **a** and α opaque cells (25, 30), were first observed after 2 h of peptide exposure (Fig. 3 and Table 1). No change in the response of any of the cell types was detected when the concentration of the peptide was increased 10-fold to 100 μ g/ml: a slight decrease in the response of **a** opaque cells was observed at pep-

tide concentrations of 1 μ g/ml (data not shown). Because the response of *C. albicans* to this tridecapeptide is highly cell type specific, we will hereafter refer to it as α -factor. Additional evidence presented below verifies this initial identification.

As described in Materials and Methods, *C. albicans* **a** cells were constructed both by disrupting genes in the *MTL* (mating type) locus and by homozygosis of the *MTL* locus using sorbose selection. Both types of **a** cells responded to the synthetic α -factor once they were switched to the opaque form. However, the **a/a** strains showed a more pronounced morphological response to α -factor than did the **a/a** $\Delta a1\Delta a2$ strains (Table 1). For example, roughly 20% of the **a/a** $\Delta a1\Delta a2$ strains exhibited polarized projections by 4 h, whereas 50% of the **a/a** strains had projections at the same time point. This difference may be due to a haploinsufficiency effect at the *MTL \mathbf{a}* locus, as hap-

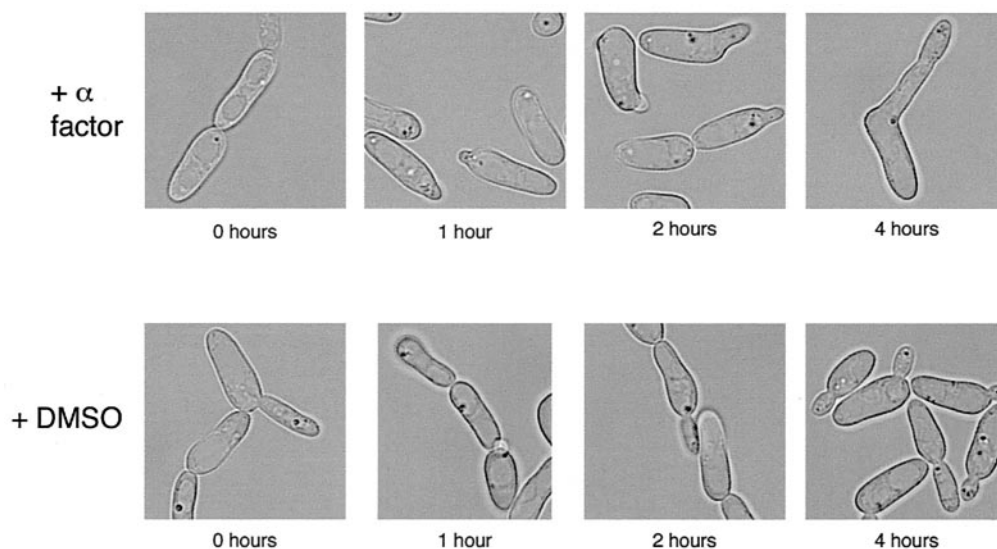


FIG. 3. Time course of the response of **a** cells to α -factor. Cells were treated with α -factor (10 μ g/ml) or mock treated with DMSO and incubated at 25°C. Samples were taken and analyzed for their response to α -factor at 0, 1, 2, and 4 h.

loinsufficiency has been observed for many other genes in *C. albicans* (20, 40).

We also investigated whether the *C. albicans* α -factor caused cell cycle arrest, as has been observed for *S. cerevisiae*. To this end, we performed halo assays by spotting α -factor on filters (at concentrations of 10 mg/ml, 1 mg/ml, and 100 μ g/ml) placed on lawns of **a** opaque cells (approximately 2,000 colonies/plate). We observed no inhibition of cell proliferation by α -factor, as judged by colony size (data not shown). However, colonies of **a** opaque cells exposed to α -factor had a marked wrinkled appearance, and individual cells within the colony exhibited mating projections as described above. In contrast, this same type of experiment performed with *S. cerevisiae* reveals pronounced areas of growth arrest (halos) around the α -factor spots. These observations suggest that *C. albicans*, unlike *S. cerevisiae*, does not undergo prolonged growth arrest

in the presence of α -factor. It is, of course, possible that cell cycle arrest occurs only transiently in *C. albicans*, that it occurs in only a subset of the population, or that the synthetic α -factor is unstable or improperly modified.

MF α is required for α cells to mate. We next determined whether deletion of the *C. albicans* α -factor structural gene (*MF α*) affected the mating efficiency of **a** and α cells. Both alleles of the *MF α* gene were deleted in a *CAI4* strain of *C. albicans*, and **a** and α strains were isolated by selecting for loss of a homolog of chromosome 5 on medium containing sorbose. **a** and α Ura⁻ white strains (either wild-type or *MF α* mutant strains) were switched to opaques and mated with Ade⁻ control mating strains. From the fraction of prototrophs obtained, mating efficiency could be estimated (for details, see Materials and Methods). Deletion of the *MF α* gene from opaque **a** cells did not significantly affect the efficiency of mating of these cells

TABLE 1. Response of *C. albicans* strains to α -factor^a

Strain	Cell type	Genotype	Treatment	No. of cells with projections/total no. of cells	% Cells with projections
CHY257	α white	$\Delta a1/\alpha1\alpha2$	10% DMSO	0/220	<0.5
CHY420	a white	a1 / $\Delta\alpha1\Delta\alpha2$	10% DMSO	0/225	<0.5
RBV731	a/a white	<i>MTLa</i> / <i>MTLa</i>	10% DMSO	2/236	0.8
RBV734	α/α white	<i>MTLα</i> / <i>MTLα</i>	10% DMSO	1/219	0.4
CHY257	α white	$\Delta a1/\alpha1\alpha2$	α -Factor	0/246	<0.5
CHY420	a white	a1 / $\Delta\alpha1\Delta\alpha2$	α -Factor	0/240	<0.5
RBV731	a/a white	<i>MTLa</i> / <i>MTLa</i>	α -Factor	3/235	1.3
RBV734	α/α white	<i>MTLα</i> / <i>MTLα</i>	α -Factor	1/227	0.4
CHY257	α opaque	$\Delta a1/\alpha1\alpha2$	10% DMSO	0/227	<0.5
CHY420	a opaque	a1 / $\Delta\alpha1\Delta\alpha2$	10% DMSO	2/233	0.8
RBV731	a/a opaque	<i>MTLa</i> / <i>MTLa</i>	10% DMSO	1/221	0.4
RBV734	α/α opaque	<i>MTLα</i> / <i>MTLα</i>	10% DMSO	0/219	<0.5
CHY257	α opaque	$\Delta a1/\alpha1\alpha2$	α -Factor	5/217	2.3
CHY420	a opaque	a1 / $\Delta\alpha1\Delta\alpha2$	α -Factor	41/215	19
RBV731	a/a opaque	<i>MTLa</i> / <i>MTLa</i>	α -Factor	121/215	56
RBV734	α/α opaque	<i>MTLα</i> / <i>MTLα</i>	α -Factor	1/221	0.4

^a α -Factor in 10% DMSO was added at a concentration of 10 μ g/ml. Cells were scored for formation of elongated projections 4 h after DMSO or α -factor was added to the cells. This experiment shows that to respond efficiently to α -factor, cells must be of mating type **a** and have been switched to the opaque phase.

TABLE 2. Mating efficiency of *mfα* and *ste2* mutants of *C. albicans*^a

Strain (Ura ⁻)	Mating type	Relevant genotype	Mating efficiency	
			MMY278 a (Ade ⁻)	CHY477 α (Ade ⁻)
CHY439	a	Wild type	<10 ⁻⁸	2.3 × 10 ⁻¹
CHY257	α	Wild type	4.8 × 10 ⁻¹	3.6 × 10 ⁻⁷
MMY564	a	<i>mfα/mfα</i>	<10 ⁻⁸	5.0 × 10 ⁻²
MMY563	α	<i>mfα/mfα</i>	<10 ⁻⁸	<10 ⁻⁸
MMY566	a	<i>ste2/ste2</i>	<10 ⁻⁸	<10 ⁻⁸
MMY565	α	<i>ste2/ste2</i>	1.8 × 10 ⁻¹	<10 ⁻⁸
MMY567	a	<i>ste2/ste2</i>	<10 ⁻⁸	<10 ⁻⁸
MMY568	α	<i>ste2/ste2</i>	1.6 × 10 ⁻¹	5.4 × 10 ⁻⁸

^a Gene knockouts of *MFα* and *STE2* were created, and opaque a and α derivatives (Ura⁻) mated with opaque control mating strains (Ade⁻). The frequency of mating was determined by the number of colonies that grew on Ura⁻ Ade⁻ medium, as described in Materials and Methods.

(Table 2). In contrast, the mating of α cells in which the *MFα* gene had been deleted was drastically lower (more than a millionfold) than that of the parental strain (compare strains MMY564 and MMY563 in Table 2). The *MFα* gene is therefore specifically required for the mating of α cells. This analysis also indicates that *C. albicans* has only one gene encoding a functional α-factor, whereas *S. cerevisiae* has two (*MFα1* and *MFα2*), either of which can support mating (Fig. 1).

The *C. albicans* *STE2* gene is required for mating and the formation of mating projections in response to α-factor. In *S. cerevisiae*, the *STE2* gene encodes the receptor for α-factor (15, 19). *C. albicans* has a closely related gene (also called *STE2*)

(39), and we tested whether this gene is required for a cells to respond to α-factor. In the experiment shown in Fig. 4, wild-type a opaque cells and *ste2/ste2* a opaque cells (see Materials and Methods for strain construction details) were treated with α-factor for 4 h and analyzed microscopically for morphological changes. The wild-type cells responded as described above, forming mating projections with approximately 50% of cells responding (Fig. 4B). In contrast, the a *ste2/ste2* opaque cells showed no morphological response to α-factor (compare Fig. 4C and D). We note that deletion of the *STE2* gene did not affect the ability of a cells to switch from the white form to the opaque form. Moreover, prior to the addition of α-factor,

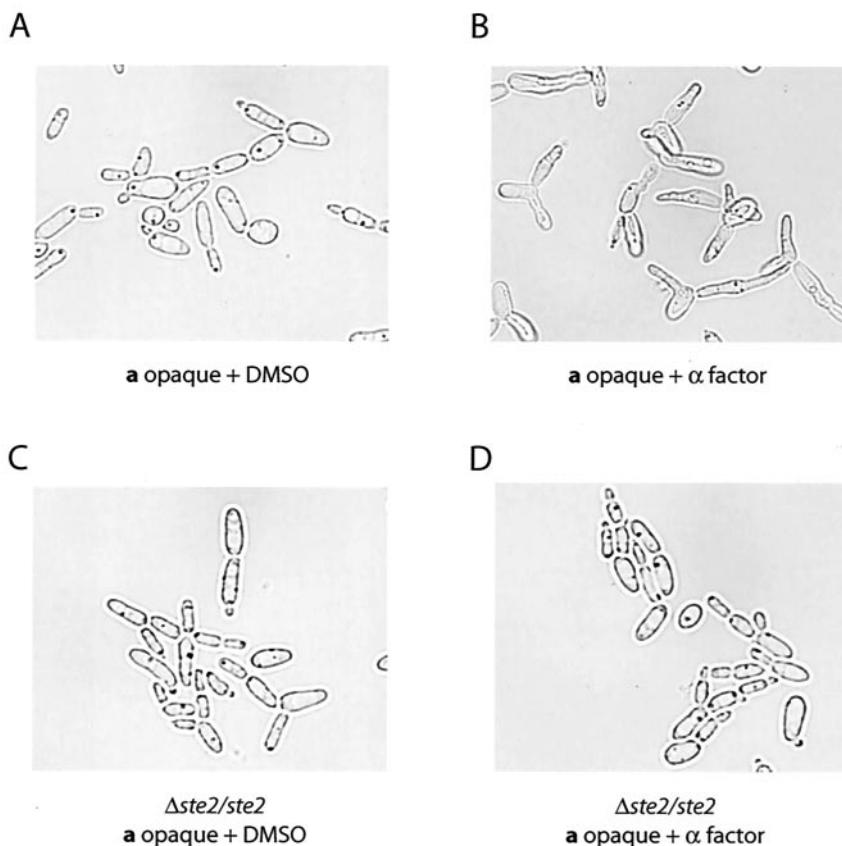


FIG. 4. The *STE2* gene is required for the response of a cells to α-factor. Opaque a cells or opaque *Δste2/Δste2* *MTLa* cells were treated with 10 μg of α-factor per ml and incubated for 4 h at 25°C. The *ste2/ste2* mutant showed no response to α-factor.

wild-type and *ste2/ste2* **a** opaque cells both formed similar elliptical cells characteristic of the opaque phase (Fig. 4A and C). The *STE2* gene in *C. albicans* is therefore not required for formation of opaque cells but is required for **a** opaque cells to form mating projections in response to synthetic α -factor.

We next tested, by constructing gene knockout strains, whether the *STE2* gene was required for mating. When *C. albicans* opaque **a** *ste2/ste2* cells were mated with wild-type α cells, the mating frequency was reduced by more than a millionfold relative to the parental strain (strains MMY566, MMY565, MMY567, and MMY568 [Table 2]). In contrast, deletion of *ste2* from α opaque cells did not significantly affect mating efficiency. These results show that the *C. albicans* *STE2* gene encodes the receptor for α -factor and that this is required for mating only in **a** cells.

Genome-wide expression profiling of the response of *C. albicans* to mating pheromone. To identify the genome-wide changes in transcription that accompany the response to α -factor, *C. albicans* **a** and α opaque strains were treated with α -factor and the transcriptional response was monitored using microarrays. The microarrays used in this analysis contained 11,325 spots representing approximately 6,550 protein-encoding nuclear genes, at least 95% of the estimated number of these genes in the *C. albicans* genome. For this experiment, **a/a** and α/α strains were generated by chromosome loss on sorbose-containing medium, and opaque cells of each mating type were isolated (see Materials and Methods for details on the isolation of forms). Two time course experiments studying the response to α -factor (10 μ g/ml) were performed. In one, samples were taken at 20 min, 1 h, and 4 h, while in the second, samples were taken at 1, 2, and 4 h. Cells were also examined microscopically to confirm that the morphological changes described earlier had taken place; as expected, the **a** opaque cells exhibited polarized growth in response to α -factor (approximately 50% of the population), while the α opaque cells (a control for the experiment) showed no apparent morphological response.

As shown in Fig. 5, the expression of many *C. albicans* genes changes in both **a** and α cells on exposure to DMSO, the solvent used for the synthetic α -factor. However, an obvious cluster of 62 genes was induced only in **a** cells and only when α -factor was present in the DMSO (Fig. 5 and Table 3). Moreover, this set of genes was reproducibly induced during both time course experiments. Although a few genes were induced 1 h after pheromone addition, most show maximal induction after 2 to 4 h. In comparison, the response to α -factor in *S. cerevisiae* has been shown to be much more rapid, with increased expression of many pheromone-responsive genes observed after only 15 min (35). Although there are minor differences in the medium conditions used for the microarray analysis, it appears that the pheromone response occurs more slowly in *C. albicans* than in *S. cerevisiae*.

One large group of genes induced by α -factor (18 of 62) encode proteins of unknown function, with no known close relatives in other organisms, including *S. cerevisiae* (Table 3 and Fig. 6). Another group (15 of 62) includes genes with at least one close relative but no obvious clue to specific function. As will be discussed in more detail below, the remainder of the induced genes include homologs of 18 genes involved in mating in *S. cerevisiae*, several genes previously implicated in the

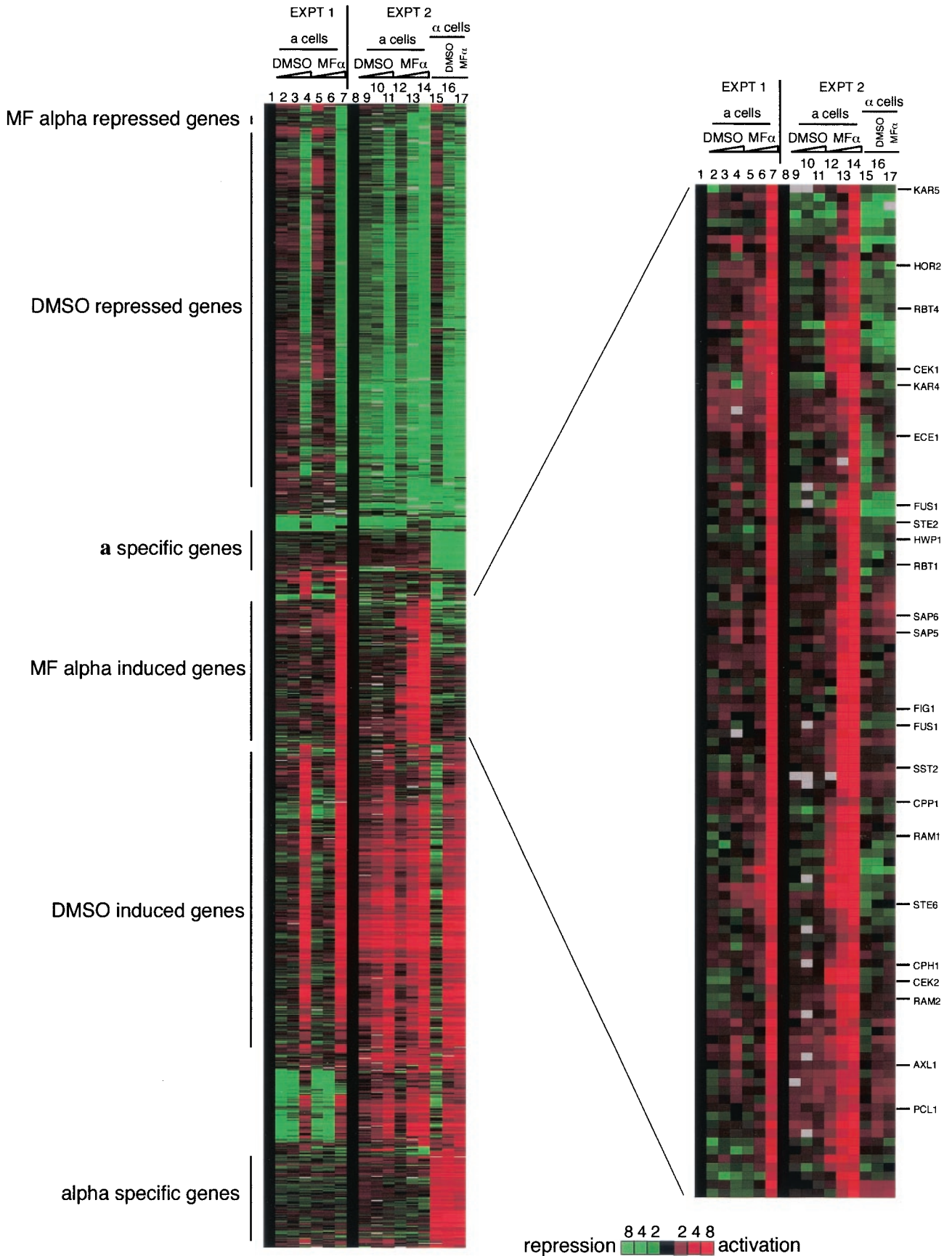
control of filamentous growth in *C. albicans*, and several genes previously shown to be required for virulence in *C. albicans*. In addition to the 62 *C. albicans* genes induced by α -factor, a few genes showed reproducible pheromone-induced repression, and we discuss these first.

Genes repressed by α -factor in *C. albicans*. In *S. cerevisiae*, more than 200 genes are repressed in response to pheromone treatment (35). The large majority of these genes are shut down as part of the cell cycle arrest in G₁, which precedes polarized growth and formation of mating projections. Virtually all gene repression in *S. cerevisiae* is mediated by the Far1 protein, a cyclin-dependent kinase inhibitor (4, 35). In contrast to *S. cerevisiae*, pheromone treatment of *C. albicans* cells resulted in lowered expression of only very few genes. In part, this may reflect the fact that only a fraction (at most 50%) of **a** opaque cells form projections in response to α -factor. Therefore, it might be difficult to observe gene repression that occurs only in a subset of cells. Nonetheless, several genes do show significant repression in the population as a whole, including several that function in DNA replication (*MCM6*, *MCM7*, *PRI*, and *POL5a*). The apparent lack of widespread gene repression supports the earlier observations that *C. albicans* does not appear to undergo whole-scale cell cycle arrest in response to α -factor. It is possible, however, that *C. albicans* transiently arrests DNA replication in response to α -factor.

Induction of mating genes by *C. albicans* α -factor. Fifteen genes are induced in both *C. albicans* and *S. cerevisiae* by α -factor (Table 3). Among these 15 genes are genes that encode the *STE2* α -factor receptor and various components of the mitogen-activated protein (MAP) kinase cascade that mediate the response to α -factor in *S. cerevisiae* (37). Additional genes induced by α -factor in both yeasts include *KAR4* and *KAR5* required for karyogamy after cell fusion, *SST2* and *CPPI* (*MSG5* in *S. cerevisiae*) required for pheromone adaptation, and *AXL1* and *HST6* (*STE6* in *S. cerevisiae*), which are required for **a**-factor processing and export, respectively.

Interestingly, the *RAM1* and *RAM2* genes are induced (seven- and fivefold, respectively) by α -factor in *C. albicans* but not in *S. cerevisiae*. The *S. cerevisiae* gene products do, however, act in the mating pathway by prenylating **a**-factor prior to its export. Similarly, the *KAR9* gene (required for karyogamy) is induced by α -factor in *C. albicans* but not in *S. cerevisiae*. Thus, even for genes likely to be involved in mating in both organisms, the precise induction pattern in response to α -factor differs between *S. cerevisiae* and *C. albicans*. Although **a**-factor has not yet been identified in *C. albicans*, the pheromone-induced transcription of *AXL1*, *HST6*, *RAM1*, and *RAM2*, in combination with the observation that *HST6* mutants of *C. albicans* are deficient in mating (28), strongly support its existence. In all, *C. albicans* α -factor induces 18 genes implicated in mating, and this observation supports the idea that mating is an elaborate process in this organism involving many of the same genes described previously for *S. cerevisiae*.

Overlap between pheromone induction and filamentous growth. A number of *C. albicans* genes required for proper regulation of the blastospore-filament transition have been identified (for reviews, see references 3, 23, 31, 33, and 41), and several of these genes were observed to be significantly up-regulated in **a** opaque cells by α -factor, including *CPH1*, *CEK1*, and *FGR23* (Table 3). As in the case of the mating genes, this



result is not surprising, as a close relationship between mating and filamentous growth has been described for *S. cerevisiae*. While *S. cerevisiae* does not exhibit true hyphal growth, diploid strains grow as pseudohyphae on medium low in nitrogen, and haploid strains undergo invasive growth on rich medium (27). It was discovered that a number of genes in the mating pheromone signaling pathway also function in the signaling pathway for pseudohyphal and invasive growth. These genes include *STE20*, *STE11*, and *STE7* (components of a MAP kinase cascade) and *STE12*, a transcriptional regulator that is a target of this cascade. Some of these same gene products were previously shown to be involved in mating and filamentous growth in *C. albicans* including *CST20* (the *STE20* homolog), *HST7* (the *STE7* homolog), and *CPH1* (the *STE12* homolog) (5, 7, 20, 22, 24, 28, 36). Thus, a signaling pathway required for mating in *C. albicans* (and presumably for pheromone induction) utilizes some of the same signaling components used in filamentous growth.

In addition to the overlap of mating and filamentation signaling pathways in *S. cerevisiae*, there appears to be a functional overlap under certain conditions. For example, *a* cells of *S. cerevisiae* respond to low doses of α -factor by forming filaments similar to pseudohyphal cells and by growing as invasive colonies on agar plates (11, 35). This association makes intuitive sense, as both processes require the formation of polarized outgrowth. Consistent with this idea are the recent observations of Hazan and Liu (16) showing that hyphal formation in *C. albicans* is accompanied by a localization of Cdc42 to the cell tip, a process that requires an intact actin cytoskeleton. Actin-dependent Cdc42 localization to the cell tip is also observed during shmoo formation in *S. cerevisiae* (43), suggesting an important cellular and biological link between these processes. Thus, in both *C. albicans* and *S. cerevisiae*, there seems to be significant overlap between mating and filamentation. The mating and filamentation pathways presumably diverged from a common ancestral pathway but have maintained several components still shared by both. This appears to be particularly true in *C. albicans*, where the elongated mating projections resemble filamentous cells much more so than their *S. cerevisiae* counterparts. In any case, the observation that *C. albicans* α -factor induces transcription of genes previously implicated in the control of filamentous growth supports a large body of evidence that these two processes are closely linked.

Overlap between the pheromone response and virulence in *C. albicans*. Perhaps the most surprising aspect of the response of *C. albicans* *a* opaque cells to α -factor is the induction of seven genes (*HWPI*, *SAP4*, *SAP5*, *SAP6*, *ECE1*, *RBT1*, and *RBT4*) encoding cell surface or secreted proteins previously shown to be required for full virulence in the mouse tail vein model for disseminated candidiasis (discussed in the introduc-

tion). Although these genes are highly induced during filamentous growth, they are not required for filamentous growth per se, as mutants with these genes deleted still form hyphae and pseudohyphae. Most of these genes do not have clear homologs in *S. cerevisiae* and are therefore thought to define aspects of *C. albicans*-host interactions (3, 23, 31, 33, 41).

HWPI is highly induced (more than 20-fold) when *a* opaque cells were exposed to α -factor. This gene encodes a glycosylphosphatidylinositol (GPI)-modified cell wall protein that is strongly induced during hyphal growth and mediates attachment of *C. albicans* to host epithelial cells (38). Three members of the secreted aspartyl proteinase family of genes, *SAP4*, *SAP5*, and *SAP6*, were also induced (between 9- and 15-fold) by α -factor. (Since these three *SAP* genes have similar nucleotide sequences, it is difficult, due to potential cross-hybridization on the microarray, to rigorously determine each induction ratio.) The three *SAP* genes are differentially transcribed when hyphal growth is induced and are known to contribute to the virulence of *C. albicans* in the mouse model, probably by allowing *C. albicans* to proteolyze tissues during invasion (6, 12). Other genes induced by α -factor include two additional cell wall proteins (*ECE1* and *RBT1*) and one secreted protein (*RBT4*). The functions of these latter proteins are not well understood, but all these proteins are highly induced during filamentous growth and are required for full virulence in the mouse model.

It should be noted that only a subset of *C. albicans* hypha-specific genes was induced by α -factor. Transcription profiling has shown that the expression of 18 genes was increased at least twofold during the transition from yeast form to hyphal cells and induced by addition of serum to the growth medium (32). α -Factor significantly increased the expression of only seven of these genes, namely, the genes discussed above (*HWPI*, *ECE1*, *RBT1*, *RBT4*, *SAP4*, *SAP5*, and *SAP6*). These observations indicate that the genes induced during hyphal growth and upon pheromone treatment overlap but that there are also many genes that are specific to each condition.

DISCUSSION

We have identified an α -factor mating pheromone in *C. albicans* and shown that mating-competent (i.e., opaque) *a* cells respond to it by forming projections characteristic of those observed in bona fide mating reactions. We also show that this response is blocked by mutation of the *STE2* gene, which we identify as the α -factor receptor. The gene encoding the α -factor (*MF α*) is required for mating, but only by *a* cells; likewise, the receptor (*STE2*) is required for mating, but only by *a* cells.

FIG. 5. Microarray analysis of the response of *a* and α opaque cells to α -factor. All spots on the microarray that were induced more than threefold in at least two of the time points are shown. In the first time course experiment (EXPT 1), samples were taken 20 min, 1 h, and 4 h after the addition of DMSO (lanes 2 to 4) or α -factor (lanes 5 to 7) to *a* cells. In the second time course experiment, samples were taken at 1, 2, and 4 h after the addition of DMSO (lanes 9 to 11) or α -factor (lanes 12 to 14). As an additional control, DMSO or α -factor were also added to α cells, and samples were taken after 4 h (lanes 16 and 17, respectively). All samples were normalized to the opaque *a* cells at the zero time point. The array results clustered into six sets of genes: *a*- and α -specific genes (including the *MTL* genes), DMSO-induced or -repressed genes, and α -factor-induced or -repressed genes. The cluster corresponding to α -factor-induced genes is shown enlarged on the right. Selected genes are indicated to the right of the cluster. Note that many genes are represented by multiple spots on the array (the duplicates are not labeled).

TABLE 3. Genes whose transcription is induced more than three-fold in a opaque cells in response to α -factor^a

Gene ^b	ORF6 designation ^c	Fold induction ^d	<i>S. cerevisiae</i> homolog ^e	Induced in <i>S. cerevisiae</i> ^f	Filamentation ^g	Function ^h
ORF6.7473	ORF6.7473	104	<i>YPS3</i>	No		Weak similarity to GPI-anchored aspartyl protease
YMR244w	ORF6.9141	73	YMR244W	No		Unknown
<i>PRM1</i>	ORF6.6562	57	<i>PRM1</i>	Yes		Plasma membrane fusion
<i>FUS1</i>	ORF6.3131	51	<i>FUS1</i>	Yes		Required for cell fusion in mating
ORF6.3434	ORF6.3434	46				Putative lectin binding domain
<i>FIG1</i>	ORF6.3476	41	<i>FIG1</i>	Yes		Involved in cell polarization and cell fusion
<i>POL/TCA3</i>	ORF6.1312	39				Reverse transcriptase
ORF6.4462	ORF6.4462	38				Unknown
<i>FGR23</i>	ORF6.1030	37			Yes	Extracellular α -1,4-glucan
<i>HST6</i>	ORF6.8600	33	<i>STE6</i>	Yes		Transporter of α -factor
<i>SST2</i>	ORF6.2767	25	<i>SST2</i>	Yes		Adaptation to pheromone
<i>RBT1</i>	ORF6.2929	25			Yes	TUP1-regulated, HWP1-related cell wall protein
<i>HWP1</i>	ORF6.4883	22	<i>FLO11</i>	No	Yes	GPI-anchored aspartyl protease
<i>CEK2</i>	ORF6.2854	15	<i>FUS3</i>	Yes		MAP kinase in mating cascade
<i>SAP4</i>	ORF6.3803	15	<i>YPS3</i>	No	Yes	Secreted aspartyl protease
<i>STE2</i>	ORF6.4012	13	<i>STE2</i>	Yes		α -Factor pheromone receptor
<i>RBT4</i>	ORF6.538	12	<i>PRY3</i>	No	Yes	TUP1-regulated, similar to plant pathogenesis proteins
ORF6.5658	ORF6.5658	11	YOL157C	Yes		Unknown
<i>SAP6</i>	ORF6.3624	11	<i>YPS3</i>	No	Yes	Secreted aspartyl protease
<i>GAG</i>	ORF6.3764	10				Retrotransposon
<i>KAR4</i>	ORF6.1096	9	<i>KAR4</i>	Yes		Karyogamy
ORF6.4265	ORF6.4265	9				Unknown
<i>SAP5</i>	ORF6.4427	9	<i>YPS3</i>	No	Yes	Secreted aspartyl protease
<i>SOK1</i>	ORF6.3088	9	<i>SOK1</i>	No		Unknown
ORF6.333	ORF6.333	8				Leucine-rich repeat family protein
<i>ECE1</i>	ORF6.2886	8			Yes	Filament-specific cell wall protein
ORF6.1965	ORF6.1965	7				Weak similarity to bacterial α -1,6-mannanase
ORF6.1768	ORF6.1768	7				Unknown
<i>RAM1</i>	ORF6.3323	7	<i>RAM1</i>	No		Farnesyltransferase that prenylates α -factor
<i>CEK1/ERK1</i>	ORF6.1819	7	<i>KSS1</i>	No	Yes	MAP kinase
<i>DEM1</i>	ORF6.6315	7	<i>DEM1</i>	No		Unknown, weak similarity to pre-tRNA processing
ORF6.5533	ORF6.5533	6				Unknown
<i>MOH1</i>	ORF6.2891	6	<i>MOH1</i>	No		Unknown
ORF6.5572	ORF6.5572	6	<i>PRY3</i>	No		Similarity to plant pathogenesis-related proteins
ORF6.8898	ORF6.8898	6				Leucine-rich repeat family
ORF6.2507	ORF6.2507	6				Unknown
<i>GAS1</i>	ORF6.3873	6	<i>GAS1</i>	No		1,3- β -glucanosyltransferase activity
ORF6.5495	ORF6.5495	6				Unknown
ORF6.3472	ORF6.3472	5				Unknown
ORF6.2403	ORF6.2403	5	YIL130W	No		Unknown, similar to transcription factors
<i>EXG1</i>	ORF6.1982	5	<i>EXG1</i>	No		Glucan 1,3- β -glucosidase activity
YDR124W	ORF6.8915	5	YDR124W	Yes		Unknown
<i>CPH1</i>	ORF6.695	5	<i>STE12</i>	Yes	Yes	Transcription factor
<i>RAM2</i>	ORF6.5223	5	<i>RAM2</i>	No		Farnesyltransferase that prenylates α -factor
ORF6.4292	ORF6.4292	5				Unknown, homoserine kinase motif
<i>RHR2</i>	ORF6.8673	5	<i>RHR2</i>	No		Glycerol-1-phosphatase activity, response to osmotic stress
<i>KAR5</i>	ORF6.2573	5	<i>KAR5</i>	Yes		Component of cytoskeleton
ORF6.4127	ORF6.4127	4	<i>MNN4</i>	No		N-linked glycosylation
YKR043C	ORF6.4339	4	YKR043C	No		Unknown
<i>PCL1</i>	ORF6.1258	4	<i>PCL1</i>	Yes for PCL2		G ₁ /S-specific cyclin

Continued on following page

TABLE 3—Continued

Gene ^b	ORF6 designation ^c	Fold induction ^d	<i>S. cerevisiae</i> homolog ^e	Induced in <i>S. cerevisiae</i> ^f	Filamentation ^g	Function ^h
ORF6.3431	ORF6.3431	4				Unknown
ORF6.5360	ORF6.5360	4				Unknown
ORF6.3939	ORF6.3939	4				Unknown
<i>AXL1</i>	ORF6.8423	4	<i>AXL1</i>	Yes		Bud site selection, pheromone maturation
<i>RSN1</i>	ORF6.5211	4	<i>RSN1</i>	No		Unknown
ORF6.8300	ORF6.8300	4				Unknown
<i>KAR9</i>	ORF6.7998	3	<i>KAR9</i>	No		Karyogamy
<i>MUM2</i>	ORF6.4410	3	<i>MUM2</i>	No		Premeiotic DNA synthesis
<i>CPP1</i>	ORF6.7345	3	<i>MSG5</i>	Yes		Adaptation to pheromone
ORF6.5308	ORF6.5308	3	<i>YHB1</i>	No		Probable flavohemoprotein, response to stress
ORF6.8381	ORF6.8381	3				Unknown
ORF6.8384	ORF6.8384	3				Unknown

^a None of these genes is induced in α opaque cells when treated with α -factor.

^b Gene name, if assigned. If not assigned, gene name based on the closest *S. cerevisiae* homolog.

^c Identification number assigned by the Stanford genome project. These identification numbers are based on assembly 6 available at www-sequence.stanford.edu/group/candida.

^d Fold induction by α -factor.

^e If a closely related *S. cerevisiae* gene exists (Blast score of at least 10^{-6}), its name is given in this column.

^f Induced in *S. cerevisiae* α cells in response to *S. cerevisiae* α -factor (35).

^g Implicated in filamentous growth and/or virulence. Two of the genes implicated in filamentous growth (ORF6.3472 and ORF6.4292) were identified in microarray experiments (D. Kadosh and A. D. Johnson, unpublished observations). *FGR23* was recently shown to be defective in filament formation (40).

^h A brief synopsis of the known or inferred role of the indicated gene.

Full genome transcriptional profiling of the response of α opaque cells to α -factor revealed 62 genes that were specifically induced. Eighteen genes induced by *C. albicans* α -factor had previously been implicated in mating in *S. cerevisiae*, and fifteen of these genes are also induced by *S. cerevisiae* α -factor. Overall, this overlap is not surprising and is consistent with the idea that *C. albicans* and *S. cerevisiae* share a conserved set of mating genes (5, 28, 39). What is perhaps unexpected is how different the sets of pheromone-induced genes are in the two organisms; with the exception of the known mating genes, the

response of α cells to α -factor is highly distinctive for each organism. The largest class of pheromone-induced genes in *C. albicans* are ORFs with no known homologs in any other organism and ORFs with only poorly defined relatives. Clearly, much work remains in understanding mating in *C. albicans*. Given that many of these genes are not present in the genome of *S. cerevisiae*, it is possible that some of these genes function to promote mating of *C. albicans* in the hostile environment of a mammalian host.

Of particular interest are a set of seven *C. albicans* phero-

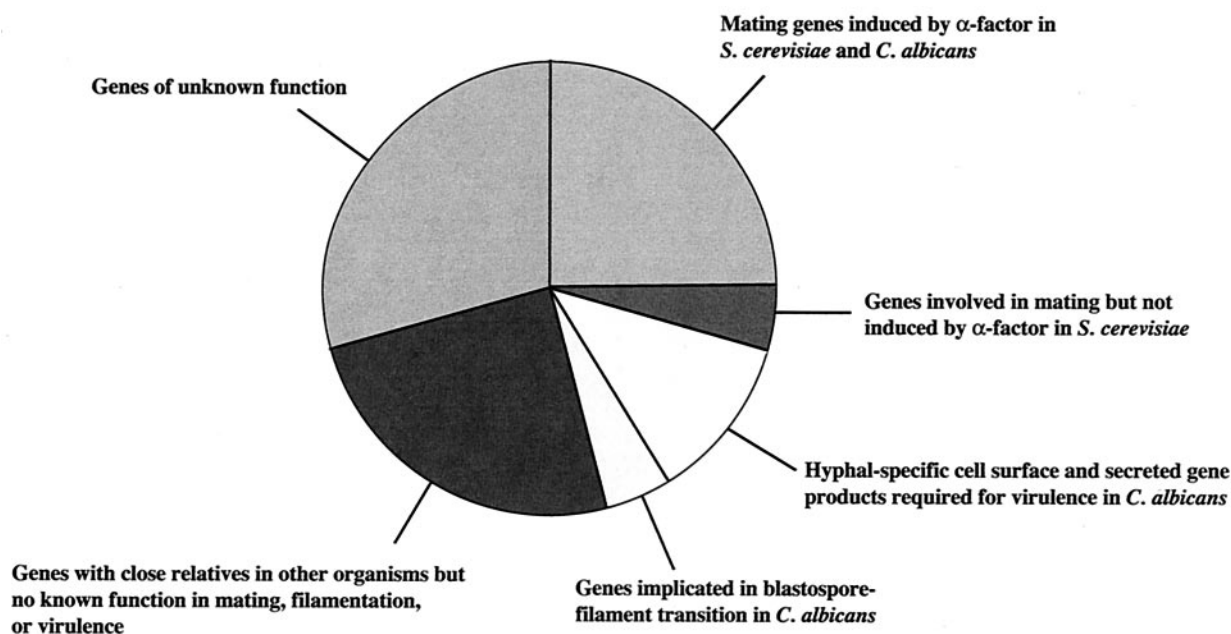


FIG. 6. Pie chart of genes induced by α -factor in *C. albicans* opaque α cells.

mone-induced genes (*HWP1*, *ECE1*, *RBT1*, *RBT4*, *SAP4*, *SAP5*, and *SAP6*) that encode cell surface or secreted proteins. As shown in previous work, each of these genes is required for full virulence of *C. albicans* in a mouse model of disseminated candidiasis (for references, see the introduction). These observations suggest that mating and virulence pathways in *C. albicans* share some of the same gene products to mediate cell-cell interactions. Consistent with this idea, preliminary experiments indicate that deletion of *RBT1* and *HWP1* in both mating partners reduced mating efficiency by a factor of approximately 20 (R. J. Bennett and A. D. Johnson, unpublished observations). (*RBT1* and *HWP1* are closely related proteins, and deletion of a single protein did not significantly affect mating.) It is tempting to speculate that since *S. cerevisiae* and *C. albicans* diverged from a common ancestor some 100 to 200 million years ago, structural components of the mating apparatus were adapted by *C. albicans* for use in interacting with its mammalian host.

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ADDENDUM IN PROOF

Since this paper was accepted, we have found through ongoing annotation efforts that three additional genes have been found that are induced by α -factor in a opaque cells. These are ORF6.2919 (no close homologs), ORF6.2933 (a homolog of *RBT1* and *HWP1*), and ORF6.4306 (*MF α*).

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