The Asexual Yeast *Candida glabrata* Maintains Distinct a and α Haploid Mating Types

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Received 20 December 2007/Accepted 8 March 2008

The genome of the type strain of *Candida glabrata* (CBS138, ATCC 2001) contains homologs of most of the genes involved in mating in *Saccharomyces cerevisiae*, starting with the mating pheromone and receptor genes. Only haploid cells are ever isolated, but *C. glabrata* strains of both mating types are commonly found, the type strain being *MATα* and most other strains, starting as BG2, being *MATa*. No sexual cycle has been documented for this species. In order to understand which steps of the mating pathway are defective, we have analyzed the expression of homologs of some of the key genes involved as well as the production of mating pheromones and the organism’s sensitivity to artificial pheromones. We show that cells of opposite mating types express both pheromone receptor genes and are insensitive to pheromones. Nonetheless, cells maintain specificity through regulation of the α1 and α2 genes and, more surprisingly, through differential splicing of the α1 transcript.

The comparative study of reproductive cycles in fungi reveals varied and often surprising situations. Even closely related species, such as *Saccharomyces cerevisiae* and *Candida glabrata*, exhibit very different modes of reproduction; the former is a self-fertile, fully sexual species, while the latter has no apparent sexual phase and reproduces clonally. “Candida" species that lack a telomorph are distributed over the whole hemiascomycete phylogenetic tree (15), illustrating both the plasticity of the trait observed and the lack of simple relationships between taxonomic nomenclature and phylogenomics.

The type strain of *Candida glabrata*, CBS138, was sequenced in 2004 through the Genolevures 2 sequencing effort that included four hemiascomycetes of various degrees of relatedness to the model yeast *S. cerevisiae* (6). This confirmed that assexual *C. glabrata* is phylogenetically very distant from *Candida albicans* and close to *S. cerevisiae*. Because of the relatedness of *C. glabrata* to the extensively studied model yeast, we have examined whether known mating pathways from *S. cerevisiae* exist in *C. glabrata*.

In *S. cerevisiae*, the *MAT* locus encodes master transcription factors that regulate three categories of genes: a-specific genes, α-specific genes, and haploidy-specific genes, involved in pheromone expression and sensing, the transduction cascade, and the inhibition of meiosis, respectively (12, 13). The *S. cerevisiae* genome also contains two additional loci, *HML* and *HMR*, harboring, respectively, the α and a genetic information, which are transcriptionally silenced.

Many genes involved in mating are conserved between the two species (26, 31); in particular, the *MAT* cassettes and the *MAT*-like cassettes (*HMLα* and *HMRα* homologs) have the same overall structure and encode the same proteins in both yeasts. A notable difference is that in *S. cerevisiae*, the three cassettes are on the same chromosome, whereas in *C. glabrata*, *MAT* and the *HML*-like cassette are on chromosome II and the *HMR*-like cassette is on chromosome V (7). Cassettes are also inverted with respect to the telomeres between the two species, and examination of other yeasts has shown that the inversion took place in *S. cerevisiae* (20).

In this work, we show that there are several apparent defects in the mating pathway of *C. glabrata* compared to *S. cerevisiae*'s. Cells, nonetheless, maintain some mating type identity through differential expression of α genes and, more surprisingly, differential splicing of the α1 transcript.

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**MATERIALS AND METHODS**

**Yeast media.**YPD medium was prepared with 1% (wt/vol) Bacto peptone (Difco), 1% (wt/vol) Bacto yeast extract (Difco), 2% (wt/vol) glucose, and 2% (wt/vol) Bacto agar, when needed. Selective SC medium was prepared with 2% (wt/vol) glucose, 2% (wt/vol) Bacto agar, and 0.67% yeast nitrogen base without amino acids (Difco) but supplemented with a mix of all amino acids, uracil, and adenine, with omission of nutrients whose prototrophy was selected for, when needed. WD medium was prepared with 0.67% (wt/vol) yeast nitrogen base without amino acids (Difco), 2% (wt/vol) glucose, and 2% (wt/vol) Bacto agar. Gorodkowa medium was prepared with 1% (wt/vol) Bacto peptone (Difco), 0.5% NaCl, 0.1% (wt/vol) glucose, and 2% (wt/vol) Bacto agar. Gorodkowa medium was prepared with 1% (wt/vol) Bacto peptone (Difco), 0.5% NaCl, 0.1% (wt/vol) glucose, and 2% (wt/vol) Bacto agar. McCleary’s acetate medium was prepared with 0.25% (wt/vol) Bacto yeast extract (Difco), 1% KO acetate, 0.1% (wt/vol) glucose, and 2% (wt/vol) Bacto agar. Gorodkowa medium was prepared with 1% (wt/vol) Bacto peptone (Difco), 0.5% NaCl, 0.1% (wt/vol) glucose, and 2% (wt/vol) Bacto agar. McCleary’s acetate medium was prepared with 0.25% (wt/vol) Bacto yeast extract (Difco), 0.98% (wt/vol) potassium acetate, 0.07% (wt/vol) MgSO\(_4\), 0.1% (wt/vol) glucose, and 1.5% (wt/vol) Bacto agar. V8 medium was prepared with 20% (vol/vol) V8 juice, 0.3% (wt/vol) CaCO\(_3\), and 1.5% (wt/vol) Bacto agar, and its pH was adjusted to 6.8 or 5.5 with NaOH. Bacto potato dextrose agar (Difco) medium contained 20% (vol/vol) potato infusion, 2% (wt/vol) glucose, and 1.5% Bacto agar. Mal (Difco) medium contained 3% (wt/vol) malt extract and 1.5% (wt/vol) Bacto agar.

**Yeast strains.** Strains are described in Table 1. Deletion mutants of *C. glabrata* were obtained by cotransformation of the split-marker vectors (8) pKA, pAN (for the construction of the *ura3* strain HM100a from wild-type CBS138), pUR,
Southern blot analysis (data not shown). The gene were cloned into the BamHI/KpnI sites. Constructions were controlled by which appropriate PCR fragments upstream and downstream of the targeted medium plates. Patches on complete medium plates, collected, and mixed in a patch on plates qPCR was then performed in triplicate on 10-fold dilutions of the cDNA. RT-PCR experiments.

of DNase I and phenol-chloroform extraction was performed as described for the remaining DNA in the preparation, RNAs were first treated with DNase I (RQ1 avoid RNA degradation.

Species Strain Description Parent strain Genotype Reference or source

\( S. \text{cerevisiae} \)
FY23 MATa ura3-52 tryp1Ada3 leu2Δ1
FY73 MATa ura3-52 his3Δ200
FY1679 FY23 × FY73 MATa(ur3-52/ur3-52 tryp1Ada3/+
J5 HO\(^+\) HMLa MATa HMRa HO leu2 his4 thr4 lys2
BY1408 sst1Δ a BY471 MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 sst1Δ::KANMX
BY11408 sst1Δ a BY472 MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 sst1Δ::KANMX
BY6055 sst2Δ a BY471 MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 sst2Δ::KANMX
BY16055 sst2Δ a BY472 MATa ura3Δ0 leu2Δ0 his3Δ1 lyC. glabrata CBS138 Wild type MATa

\( C. \text{glabrata} \)
BG2 Wild type MATa
ΔH1 CBS138 his3Δ CBS138 his3Δ 2001U MATa CAGLJ03080gΔ CAGLJ02937gΔ (−254 to +777):CgURA3

\( \text{M} \)
M2 Wild type MATa
M3 Wild type MATa
M4 Wild type MATa
M5 Wild type MATa
HM100a CBS138 ura3Δ CBS138 ura3Δ 2001U MATa CAGLJ03080gΔ CAGLJ02937gΔ (−254 to +777):CgURA3
HM106a CBS138 trp1Δ HM100a CAGLJ04092gΔ:ScURA3
HM102a CBS138 HMRΔ HM100a MATa CAGLJ03080gΔ:KANMX
HM103a CBS138 sst2Δ HM100a MATa CAGLJ03080gΔ:KANMX
BG14 BG2 ura3Δ BG2 MATa CAGLJ03080gΔ (−85 to +932):Tn903 neo\(^R\)
HM107a BG2 trp1Δ BG14 CAGLJ03080gΔ (−85 to +932):Tn903 neo\(^R\)
BG87 BG2 ura3Δ his3Δ BG14 CAGLJ02937gΔ (1 to +631)
HM104a BG14 sst2Δ BG14 CAGLJ02937gΔ (1 to +631)
HM105a BG87 sst2Δ BG87 CAGLJ03080gΔ (−85 to +932):Tn903 neo\(^R\)

\( ^a \) An Sc prefix on a gene indicates \( S. \text{cerevisiae}; \) a Cg prefix indicates \( C. \text{glabrata}. \)

\( ^b \) Our unpublished data, data of H. Muller, C. Hennequin, B. Dujon, and C. Fairhead.

and pRA (for the construction of HMR and sst2 deletants of ura3 strains), in which appropriate PCR fragments upstream and downstream of the targeted gene were cloned into the BamHI/KpnI sites. Constructions were controlled by Southern blot analysis (data not shown).

Mating assays. Cells from strains of opposite mating types were grown as patches on complete medium plates, collected, and mixed in a patch on plates with various media. After 4 days at 30°C, cells were collected and streaked on WO medium plates and incubated at 30°C. Plates were examined regularly during 1 week, and potential diploid cells were streaked a second time on WO medium plates.

RNA extraction. RNA from \( C. \text{glabrata} \) and \( S. \text{cerevisiae} \) was prepared as described previously (3), by using hot phenol extraction after glass bead cell lysis of mid-log phase cultures.

RT-PCR. Four micrograms of total RNA was used per reaction. To avoid any remaining DNA in the preparation, RNAs were first treated with DNase I (BQi RNase-free DNase; Promega) and extracted with phenol-chloroform before being subjected to reverse transcription. Reverse transcriptase (RT) Superscript II (catalog no. 18084-014; Invitrogen) was used according to the manufacturer's recommendations. RNAs (Promega) was added to all reaction mixtures to avoid RNA degradation.

qRT-PCR. Quantitative RT-PCR (qRT-PCR) experiments were performed using an Abgene ABsolute MAX 2-Step qRT-PCR Sybr green kit. The first step of DNase I and phenol-chloroform extraction was performed as described for the RT-PCR experiments.

DNase-free total RNA (0.8 μg) was used for reverse transcription to obtain cDNA. qPCR was then performed in triplicate on 10-fold dilutions of the cDNA solution. Standard curves were obtained by PCR with serial dilutions of DNA of known concentrations. In each well, 12.5 μl of Sybr green was added to 5 μl (30 ng) of DNA or DNA and 8 pmol of the two specific primers (Table 2) in a final volume of 25 μl. Specific primers for each gene were designed using the Beacon Designer software, v. 4.

The PCR program was 14 min at 95°C for initial denaturation and enzyme activation followed by 40 cycles of denaturation (30 s at 95°C) and of hybridization/elongation (30 s at 55°C), and a final step of 1 min at 95°C. The melting curve started at 55°C with 0.5°C increments every 10 s for 80 cycles. qPCRs were run on an iQ5 real-time PCR detection system (Bio-Rad) and analyzed with the iCycler software.

Sensitivity to pheromones and pheromone expression. Synthetic pheromones (Eurogentec SA, Seraing, Belgium, and NeoMPS SA, Strasbourg, France) of \( C. \text{glabrata} \) were synthesized as explained below and in the legend to Fig. 4. For sensitivity assays, 50 μl of a 200-μg/ml solution of \( C. \text{glabrata} \) a-factor or α-factor or of \( S. \text{cerevisiae} \) a-factor (Sigma-Aldrich Inc., St. Louis, MO) was spotted on plates with SC medium without tryptophan (SC-W) or SC medium without adenine (SC-ade) and, when dry, covered with a cell lawn containing 5 \( \times \) 10⁴ cells.

For pheromone expression experiments, cells from \( C. \text{glabrata} \) or \( S. \text{cerevisiae} \) were grown in YPD overnight at 30°C until the end of log phase. Cultures were centrifuged, and supernatants were filtered on 0.22-μm nitrocellulose. Fifty microliters of filtered medium was then spotted directly on SC medium-W or SC medium-ade plates. After the spot was allowed to dry, 5 \( \times \) 10⁴ cells of the tester strain were spread on the plate.

Pictures were taken after 2 days of growth at 30°C.
| Type of expts | Oligonucleotide name | Sequence (5'–3') | Primer pair target or use |
|--------------|----------------------|-----------------|----------------------------|
| RT-PCR       | HM_RT_alpha1_F       | CTGTTAACTAAAACGAACAA | α1 of C. glabrata          |
|              | HM_RT_alpha1_R       | ATGGGGGCTATTATATAATTTAC |                          |
|              | HM_RT_a1_F           | GTTTAGATTTTGCAATCG  | α1 of C. glabrata          |
|              | HM_RT_a1_R           | TTTTAGATTTTGCAATCTC | ACT1 of C. glabrata        |
|              | HM_RT_act1_F         | GCTTGGTTGCTCTATATCC |                          |
|              | HM_RT_act1_R         | TCTAAGAAGAACCACCGATC |                          |
|              | HM_RT_a1Scer_R       | AAACTCTTCTGGAATGGAAG | α1 of S. cerevisiae        |
|              | HM_RT_a1Scer_F       | GGAATTGGTTAATGTGATG | α1 of S. cerevisiae        |
|              | HM_RT_alphascer1_F   | CTTGCAATTATTCGACCCT | α1 of S. cerevisiae        |
|              | HM_RT_alphascer1_R   | CCAATATGCAACCACCATCT |                          |
|              | HM_YCR097_a1_F       | TGTCTTGTCTCTCTCTGC | First intron of α1 of S. cerevisiae |
|              | HM_YCR097_a1_R       | ATTTATCAGGTTATGATGTC |                          |
|              | HM_YCR097_a1Ter_F    | AAGAGAAGAGCCAAAAGG | Second intron of α1 of S. cerevisiae |
|              | HM_YCR097_a1Ter_R    | ATTTTAGATCTCATACTTA |                          |
|              | HM_CG_a1_F           | GCAATCGGTTAATTCGACT |                          |
|              | HM_CG_a1_R           | TGAATGCGGATATACGGTG |                          |
|              | HM_CG_a1Ter_F        | ATCACCAACATCAAGTTC | Second intron of α1 of C. glabrata |
|              | HM_CG_a1Ter_R        | TCACATGTTGGTAATACGG |                          |
| qRT-PCR      | HM_YCR040_alpha1_F   | TGTCTTGTCTCTCTCTGC | α1 of S. cerevisiae        |
|              | HM_YCR040_alpha1_R   | TTATTTGTCGACACCACCTTAC |                          |
|              | HM_YCR097_a1Bis_F    | AAGAGAAGAGCCAAAAGG | α1 of S. cerevisiae        |
|              | HM_YCR097_a1Bis_R    | CTGGAAAGTTGGAATGTC | α1 of S. cerevisiae        |
|              | HM_YCR096_a2_F       | CAAAGAAGAGATTCGCAATAC | a2 of S. cerevisiae |
|              | HM_YCR096_a2_R       | TGAATGCGGAGAATACGG | a2 of S. cerevisiae        |
|              | HM_YCR039_alpha2_F   | ATTTACCTAAGTTACCAGAGAG | STE2 of S. cerevisiae |
|              | HM_YCR039_alpha2_R   | ATTTATCAGGTTATGATGTC |                          |
|              | HM_YFL026_STE2_F     | AGCCACATTAATACACTTACCTCT |                          |
|              | HM_YFL026_STE2_R     | CACCATGTCGACACCAAC |                          |
|              | HM_YKL178_STE3_F     | ACAAGTCAAGAATAATAGGG | STE3 of S. cerevisiae |
|              | HM_YKL178_STE3_R     | CCGGATCTCAATACAG |                          |
|              | HM_YDL227_HO_F       | GACATTGAAGATGAGATTTTC | HO of S. cerevisiae |
|              | HM_YDL227_HO_R       | ACAGCAGGATTACAAG |                          |
|              | HM_YFL039_ACT1_F     | CGTCTTGCTATCTCTTC | ACT1 of S. cerevisiae |
|              | HM_YFL039_ACT1_R     | AATTTTCTGTTAATAGTG | α1 of C. glabrata |
|              | HM_CG_alpha1_F       | ATTAGAGAAGAAAGATGGAAC | α1 of C. glabrata |
|              | HM_CG_alpha1_R       | GATGAAGATTGGAATGATG | α1 of C. glabrata |
|              | HM_CG_a1Bis_F        | AACATATACAACTAGGAAGCC | a2 of C. glabrata |
|              | HM_CG_a1Bis_R        | TTTAATCTGATTGATGATG | a2 of C. glabrata |
|              | HM_CG_a2_F           | GGACCCCAAGAGATAGG | α2 of C. glabrata |
|              | HM_CG_a2_R           | CGATAGGAAAGCAGCATAG | α2 of C. glabrata |
|              | HM_CG_alpha2_F       | TCAAGAATTTGATTACGCACT |                          |
|              | HM_CG_alpha2_R       | AACTGGAACAAATGATATAAG |                          |
|              | HM_CG_STE2_F         | TTGCCATTATCTCTATCTG | STE2 of C. glabrata |
|              | HM_CG_STE2_R         | ACCCATGGCTCTATTTCC |                          |

Continued on following page
RESULTS

There is no report of the mating of C. glabrata in the literature. We have, ourselves, tried mating MATa strains descending from BG2 with MATa strains descending from CBS138 with different combinations of auxotrophic markers in order to select prototrophic diploid cells, as shown in Table 3. Mating experiments were performed as indicated in Materials and Methods on different media: SPO, V8 at two different pHs, Gorodkowa, McClary’s acetate, SLAD, malt, Bacto potato dextrose agar, and WO. Subsequent streaking on minimal media never allowed the isolation of prototrophic cells. Since no difference was observed between results with different media and since C. glabrata can in fact be considered to be part of the Saccharomyces species complex (6), we decided to examine the functionality of genes involved in mating under standard conditions used for S. cerevisiae.

Expression of mating type-related genes. In order to address the question of mating type expression by haploid C. glabrata cells, we examined the expression of CAGL0E00341g, CAGL0B01243g, and CAGL0B01265g, homologs of the key regulator genes α1, αI, and α2, respectively, at the MAT locus. We have not examined the a2 gene given the absence of any known role for a2 in either haploid or diploid cells in S. cerevisiae and the fact that the gene has no start codon in C. glabrata. It has been speculated that a2 could be a pseudogene of an ancestral gene common to the S. cerevisiae-S. glabrata branch (10). We also examined the homologs of the mating pheromone receptor-encoding genes that are involved in the initial steps of cellular fusion during mating, CAGL0K12430g, a homolog of STE2 (YFL026W) encoding the α-factor receptor, and CAGL0M08184g, a homolog of HO (CAGL0G05423g). In S. cerevisiae, HO is necessary for the completion of the sexual cycle in clonal populations by inducing the formation of cells of opposite mating types within a clone. Since C. glabrata infections are usually monoclonal (1), sexual reproduction may depend on the ability of some cells to switch mating types and fuse with related cells.

We performed RT-PCR on α1 and αI genes in three strains of C. glabrata of the α mating type, CBS138 (sequenced) and M4 and M5 (two isolates from patients), and in three strains of the a mating type, BG2 (a commonly used strain in laboratories) and M2 and M3 (two isolates from patients). For the αI genes that contain two introns in S. cerevisiae and C. glabrata (20, 26), primers amplified a fragment containing the first

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### TABLE 3. Mating experiments performed<sup>a</sup>

| MATa parent | Phenotype | MATa parent | Phenotype |
|-------------|-----------|-------------|-----------|
| BG14        | Ura<sup>-</sup> | HM101a | Leu<sup>-</sup> |
|             |           | HM106a | Trp<sup>-</sup> |
|             |           | ΔH1   | His<sup>-</sup> |
| HM107a      | Trp<sup>-</sup> | HM100a | Ura<sup>-</sup> |
|             |           | HM101a | Leu<sup>-</sup> |
|             |           | ΔH1   | His<sup>-</sup> |

<sup>a</sup> Ura<sup>-</sup> BG14 and Trp<sup>-</sup> HM107a were crossed with the indicated MATa parent strains.
intron in S. cerevisiae and both introns in C. glabrata. In the three independent C. glabrata strains of each mating type examined by RT-PCR (Fig. 1A), the MATα gene a1 is expressed in all MATα strains and not in MATα strains. In contrast, the α1 gene is expressed in both MATα and MATα strains. In the latter, this transcript must arise from the expression of the HMRα locus, since the MAT and HML loci contain α-type information. We conclude that HMRα on chromosome V is not silenced in C. glabrata. Thus, the type strain expresses genes from both mating types. Reasoning that this might contribute to its apparent infertility, we constructed a CBS138 MATα strain with the HMRα locus deleted, strain HM102α. Further analyses include this strain. Control experiments with S. cerevisiae show that, as expected, there is clear-cut mating type-specific expression of the a1 and α1 genes (Fig. 1B) (13). The size of the major PCR band on cDNA from the a1 transcript reveals the presence or absence of the splicing of its intron (5). In S. cerevisiae, the major band is smaller than the PCR band from genomic DNA, demonstrating splicing of the intron. In C. glabrata, despite the presence of multiple bands, the major PCR bands on a1 cDNAs have the same size as the PCR band from genomic DNA, suggesting a splicing defect. More-explicit experiments on a1 splicing are described below.

In the qRT-PCR experiments (Fig. 2), we examined the expression of a1, α1, and α2 and of STE2, STE3, and HO in S. cerevisiae and their homologs in C. glabrata. As a standard, we used the actin transcript, estimated to occur at around 10 copies per cell in S. cerevisiae (21). Standard S. cerevisiae strains are ho mutants, so we included as a control the J5 strain, which contains a wild-type HO gene and α-type information at MAT, HML, and HMR.

In C. glabrata, a1 is expressed at similar levels in both the MATα strain and the MATα strain, confirming the results of the above-described RT-PCR experiments. As expected, deletion of the HMR cassette in strain HM102α results in the absence of the a1 transcript (Fig. 2A). Neither STE2 nor STE3 homologs display mating type specificity, as they are expressed at similar levels in MATα and MATα strains. In contrast, the expression of α1 and α2 is mating type specific in C. glabrata; they are expressed, respectively, 500-fold and 1,200-fold more in MATα cells than in MATα cells. Deletion of the HMR cassette does not interfere with the expression of these two genes. Control experiments show that a1, α1, α2, STE2, and STE3 exhibit mating type-specific expression in S. cerevisiae (Fig. 2B). The HO gene is transcribed at similar levels in all strains of C. glabrata examined and at levels 20-fold lower than in haploid S. cerevisiae cells, where around one transcript per 10 cells is observed.

Splicing of α1 introns. Previous experiments led us to hypothesize that a1 may be nonfunctional in C. glabrata; we observed concomitant expression of a1, α1, and α2 in MATα cells, a situation that could not occur in S. cerevisiae because the a1-α2 heterodimer represses the expression of a1 and the deletion of HMRα in MATα cells has no effect on the expression of α1, α2, STE2, and STE3. The fact that we observed unspliced forms of a1 prompted us to examine this in more detail. Indeed, in S. cerevisiae...
In the genome, no product was detected, as expected. Analyzed as indicated in the boxes. ND, not detectable (a qRT-PCR axis is in the logarithmic scale. In each case, three different strains were produced (22). The genome contains two genes predicted to encode pheromone precursors. The predicted translation product of CAGL0C01919g contains a 12-amino-acid long peptide similar to the α-factor of S. cerevisiae (Fig. 4A) (7, 19). The predicted translation product of CAGL0H03135g contains three 13-amino-acid-long peptides similar to the α-factor of S. cerevisiae (Fig. 4B) (7, 24). Two peptides are identical, but one differs by 2 amino acids. We used the two different sequences as putative α-factors for C. glabrata, form A and form B. For the synthesis of the artificial pheromones, we assumed that posttranslational modifications that occur in S. cerevisiae also occur in C. glabrata since the genes involved in these processes are conserved (7). These include precursor proteolysis of both pheromones and farnesylation and methylation at the C terminus of the α-factor (4).

We tested C. glabrata mating pheromones on S. cerevisiae cells, and we included sst1 and sst2 deletion mutants of S. cerevisiae because they exhibit greater sensitivity to pheromones (2). The SST1 gene, whose standard name is BAR1, encodes an aspartyl protease secreted into the periplasmic space of MATα cells that inactivates α-factor. Thus, its action is mating type specific, as shown below. SST2 encodes a G protein regulator that is required to prevent receptor-independent signaling of the mating pathway. We constructed sst2 mutants (deletion of CAGL0H00374g) in C. glabrata cells of both mating types to check for increased sensitivity. Null sst2 mutants of S. cerevisiae shmoo constitutively, even in absence of cells of the opposite mating type, and this results in a longer generation time compared to that of the wild type. We observed no constitutive shmooing of cells or on sst2 MATα cells. The synthetic α-factor of C. glabrata when grown in YPD or SC medium.

The effect of synthetic pheromones on C. glabrata and S. cerevisiae cells is shown in Fig. 5. Drops of synthetic pheromones were put on minimal medium agar plates, and cells were spread after absorption. As shown, form A of the C. glabrata α-factor is active on wild-type, sst1, and sst2 MATα S. cerevisiae cells. Form B is active only on the hypersensitive S. cerevisiae sst2 cells. Neither form is active on either wild-type or sst2 C. glabrata MATα cells. The synthetic α-factor deduced from the genome of C. glabrata has no activity on wild-type S. cerevisiae MATα cells or on sst1 cells but has a very strong effect on sst2 cells. It has no observable activity on either wild-type or sst2 C. glabrata MATα cells. The strain of C. glabrata with HMRα deleted does not respond to pheromones any more than the original CBS138 strain. Thus, C. glabrata cells are potentially able to produce active pheromones to which S. cerevisiae cells respond differentially.

### Figure 2

![qRT-PCR experiments with several genes related to mating.](image)

**A. C. glabrata**

- BG2 (HMLα MATα HMRα)
- CBS138 (HMLα MATα HMRα)
- HM102a (HMLα MATα HMRα)

**B. S. cerevisiae**

- FY23 (HMLα MATα HMRα)
- FY73 (HMLα MATα HMRα)
- J5 (HMLα MATα HMRα)

**Legend:**

- CgACT1
- CgSTE2
- CgSTE3
- CgHO
- Cga1
- Cgo1
- Cgo2

### Figure 3

**A. C. glabrata**

- C. glabrata MATα cells (FY23), the singly spliced form of the transcript that contains both introns is not spliced, the α1 transcript is not produced (22). The α1 gene from C. glabrata contains two introns, as in S. cerevisiae, and four primer pairs were designed for each species in order to analyze the splicing of both introns (Fig. 3A).

As shown on Fig. 3B, in S. cerevisiae MATα cells (FY23), the size of the only cDNA band amplified around intron 1 corresponds to the spliced transcript; i.e., splicing is total, whereas for intron 2, some unspliced forms remain, although most transcripts are spliced. In C. glabrata cells, for both introns, the major cDNA band has the same size as the genomic DNA band. Minor cDNA bands corresponding to the predicted spliced forms of 84 and 70 bp are observed only in MATα cells (BG2, M2, M3). In C. glabrata MATα cells (CBS138, M4, M5), no spliced form from either intron can be detected. Amplification of the fragment of the transcript that contains both introns with the external primers (Fig. 3C) shows that, in BG2 MATα cells, the doubly spliced form of the α1 transcript exists, although unspliced transcripts are more abundant and transcripts with only one intron spliced also exist. In the latter case, we cannot distinguish between transcripts spliced for intron 1 or for intron 2 because there is only 2 nucleotides’ difference in size between the two introns and therefore between the two transcripts. The band of larger size than that of the unspliced α1 transcript is assumed to be a PCR artifact, as its presence is variable and as larger bands are also sometimes detected in the S. cerevisiae experiments.

In conclusion, in C. glabrata, the α1 transcript is partially spliced in the MATα strains examined, while it is not spliced at detectable levels in the MATα strains examined. Translation of the unspliced transcripts cannot give rise to a functional protein because there are in-frame stop codons in the first intron.

**Response to pheromones.** Since C. glabrata MATα and MATα strains express both α- and α-factor receptor genes (Fig. 2), we wondered whether cells were sensitive to mating pheromones. The C. glabrata genome contains two genes predicted to encode pheromone precursors. The predicted translation product of CAGL0C01919g contains a 12-amino-acid long peptide similar to the α-factor of S. cerevisiae (Fig. 4A) (7, 19). The predicted translation product of CAGL0H03135g contains three 13-amino-acid-long peptides similar to the α-factor of S. cerevisiae (Fig. 4B) (7, 24). Two peptides are identical, but one differs by 2 amino acids. We used the two different sequences as putative α-factors for C. glabrata, form A and form B. For the synthesis of the artificial pheromones, we assumed that posttranslational modifications that occur in S. cerevisiae also occur in C. glabrata since the genes involved in these processes are conserved (7). These include precursor proteolysis of both pheromones and farnesylation and methylation at the C terminus of the α-factor (4).
are sensitive but are themselves insensitive to them. Interspecific sensitivity to \( H9251 \)-factor between \( S. cerevisiae \), \( Saccharomyces kluyveri \), and \( Saccharomyces exiguus \) has been described previously (14, 18), but the peptidic sequences of \( H9251 \)-factors from \( C. glabrata \) are more diverged from \( S. cerevisiae \) than are the \( H9251 \)-factors from \( S. kluyveri \) and \( S. exiguus \) (5 to 6 amino acids conserved out of 13 for \( S. cerevisiae \) versus 7 to 9 amino acids conserved out of 13 for \( S. kluyveri \) and \( S. exiguus \)). Despite this divergence, the sensitivity of \( S. cerevisiae \) to \( H9251 \)-factor from \( C. glabrata \) is mediated by the Ste2 receptor, as ste2 mutants of \( S. cerevisiae \) do not respond to it or to \( H9251 \)-factor from \( S. cerevisiae \) (not shown).

We then asked whether \( C. glabrata \) cells actually produce pheromones, using \( S. cerevisiae \) as a test.

**Pheromone production by \( C. glabrata \).** To test pheromone production, \( C. glabrata \) and \( S. cerevisiae \) cell lawns were plated on top of drops of filtered supernatants from late-log-phase cultures of \( C. glabrata \) and \( S. cerevisiae \) strains, as described above. The results (Fig. 6A) show that \( S. cerevisiae \) ste2 mutants exhibit sensitivity to supernatants of cultures from \( S. cerevisiae \) cells of opposite mating types. Due to the limited concentration of pheromones in the supernatant, no effect is observed on wild-type cells. In contrast, no supernatant from \( C. glabrata \) has a mating type-specific inhibitory effect on wild-type or ste2 mutants of \( S. cerevisiae \). However, this assay reveals the inhibitory effect of the CBS138 supernatant on all cell types (see below).

Figure 6B shows that \( C. glabrata \) cells are insensitive to the culture supernatants of both \( S. cerevisiae \) MAT\( a \) and MAT\( \alpha \) strains and \( C. glabrata \) MAT\( a \) strains (BG2). The inhibition of the growth of MAT\( \alpha \) cells by the \( C. glabrata \) MAT\( a \) CBS138 supernatant cannot be taken as a mating type-specific effect, as this supernatant inhibits the growth of mating type cells of \( C. glabrata \) of both types (not shown) (but not of mating type cells of itself or derived strains) and of mating type cells of \( S. cerevisiae \) of both types, as explained above. Thus, strain CBS138 produces an inhibitor of the growth of other strains as the “killer” strains of other yeast species (17). This effect is observed when supernatants of rich medium cultures but not minimal medium cultures are used (not shown).

We then extended this growth inhibition assay to detect pheromone production to a collection of \( C. glabrata \) clinical isolates that we characterized independently, of which 80% are MAT\( \alpha \) (C. Hennequin, H. Muller, B. Dujon, and C. Fairhead, unpublished data). This collection contains 182 isolates that were grown in 96-well plates. Supernatants were filtered and
**A. a-factor**

| Strain   | Sequence          |
|----------|-------------------|
| YNL145w  | MQPITTTAAGTQKDKSSEKDKWNIKGLFWDPCVIA |
| YDR461w  | MQPST---ATAAIPKRSSEKDKWNIKGFVNPDCVIA |
| CAGL0C01919g | MQPTI-----EATQKDNQKEDKNIIVGKFNNPSDCVIA |

**B. α-factor**

| Strain   | Sequence          |
|----------|-------------------|
| YPL187w  | MRPSIFSTAVFLAASSALAPVNTTEDTAQIPAEAVIGYLDLEGFDVAVLPFSNSTN |
| YGL089c  | MKFISTFTFLAILAASVTAS----SDEDIAQVPAEAIYLDLEGFDVAVLPFSNATA |
| CAGL0H03133g | MRFLRFISTVALLGATQEGEEGETVEVPEASAPIYLDGATNDVAILPISNTN |

**DISCUSSION**

The reason why sexual reproduction is so common in living species remains debated, and many species in which clonal propagation is possible get by without it. This is particularly true in the fungal kingdom, where lack of an observed sexual stage is often associated with pathogenic interactions with humans or plants or with obligatory symbiosis with plant species. In this work we have examined the reasons why no teleomorph has been observed in the hemiascomycete _C. glabrata_ despite the presence of genes homologous to those known to be involved in mating (7, 26, 31). We have observed several differences in the mating pathway from that of _S. cerevisiae_.

First, our results show that α1 and α2 are expressed in a mating type-specific manner, in contrast to α1, which is expressed in both mating types, because of a lack of silencing at _HMRα_. Sir1p is responsible for silencing _HMLα/HMR_ loci in _S. cerevisiae_; thus, perhaps the absence of a _SIR1_ homolog in _C. glabrata_ (7) explains this phenomenon. Nonetheless, _HMLα_ is silenced in _MATα_ strains, in which we do not know which conditions could induce meiosis in _C. glabrata_, it is likely that they would be met at some point by cells. This hypothesis is in accordance with the fact that only haploid cells are ever isolated, so that if diploid cells are formed, they must sporulate readily. In _S. cerevisiae_ diploid cells, the coexpression of α1 and α2 leads to the repression of α1 in addition to other haploidy genes. The expression of the three transcription factors α1, α1, and α2 simultaneously in _C. glabrata_ cells thus leads to the hypothesis that α1 is not functional. Additionally, the observation that the _HMRα_ _MATα_ strain still expresses α1, STE2, and STE3, like the wild-type _MATα_ strain, strengthens this hypothesis. The fact that we found that the splicing of the α1 transcript is detectable only in _MATα_ cells can explain these observations. In _S. cerevisiae_, it has been shown that the unspliced form of α1 is not functional in diploid cells (22). In haploid cells, there is no known role for α1, the _MATα_ phenotype being the default one. Thus, a partial failure to splice the α1 transcript in _MATα_ cells is not expected to have any effect. In _MATα_ cells, the lack of splicing of the α1 transcript may be necessary to compensate for the leakage of expression from _HMR_ by functionally inactivating the transcript from the rogue gene. Alternatively, complete silencing may not be necessary because α1 expression is regulated in
some other way, such as splicing. Since there is no sequence difference between MATα1 and HMRα1 that would explain differences in splicing efficacy (6, 26), the differential splicing must originate either from a general splicing defect in MATα/H9251 strains or from a mating type-specific mechanism, with both alternatives leading to the inactivation of α1.

The pheromone receptor genes STE2 and STE3 (CAGL0K 12430g and CAGL0M08184g) are expressed in both cell types in C. glabrata, while in S. cerevisiae, STE3 is highly regulated and STE2 less tightly regulated. In S. cerevisiae MATα cells, STE3 is activated by α1. In C. glabrata, the basal level observed in MATα cells is higher than in S. cerevisiae (100-fold, if we assume that the actin transcript is expressed at similar levels in both species) and there is no activation by α1. In S. cerevisiae MATα cells, STE2 is repressed by the binding of Mcm1 and α2 on the α-specific gene operator. Putative binding sites for Mcm1 and α2 are found upstream of the homolog of STE2 in C. glabrata, but their spacing is different from S. cerevisiae’s. This suggests that the expression of this gene could be mating type specific under some as-yet-undefined conditions (28; B. Tuch, personal communication).

FIG. 5. Effect of synthetic pheromones on S. cerevisiae (A) and C. glabrata (B) cells. Cell type is indicated at the top, and pheromones are indicated on the left.
The expression of both receptors in C. glabrata does not make the cells sensitive to both pheromones, as our tests with artificial pheromones show. Furthermore, there is no pheromone production detectable in standard laboratory culture in our primitive test. Nonetheless, pheromones synthesized using the genome sequences of C. glabrata are active on S. cerevisiae cells. The genes encoding factors that modify the pheromones are also found in the genome of C. glabrata. Thus, C. glabrata has retained its ability to encode active pheromones but does not respond to them, possibly because the signal cascade leading to the G1-S cell cycle arrest in S. cerevisiae does not properly operate in C. glabrata. This is consistent with the absence of the shmooing of sst2 mutants of C. glabrata. Alternative explanations of the absence of a pheromone response in C. glabrata are that pheromones are expressed under some unknown conditions and that some additional regulation of the signal cascade exists in C. glabrata, allowing for a mating type-specific response to the opposite mating pheromone.

We also show that the HO gene is transcribed in C. glabrata. In S. cerevisiae, the HO endonuclease drives mating type switching by initiating a double-strand break at the MAT locus. This mech-

FIG. 6. Effect of culture supernatant on S. cerevisiae (A) and C. glabrata (B) cells. Cell type is indicated at the top, and the nature of the supernatant is indicated on the left.
amnism allows the creation of cells of opposite mating types in clonal populations, thus facilitating mating under conditions of isolation. Partially degenerate HO sites in *C. glabrata* (7) that match a previously published consensus sequence for the endonuclease from *S. cerevisiae* (TNNNYGGC/ANC/AANT/G) can be identified (23). Indeed, HO from *S. cerevisiae* cuts the MATa site but not the MATα site of *C. glabrata* (our unpublished results). In vivo mating type switching events have been reported to occur in *C. glabrata* (1, 16), always from MATα to MATa. In *Kluyveromyces lactic*us, where the loci are organized as in *C. glabrata* (7), switching is also more likely from a to α than the other way around (11). These observations lead to two hypotheses. The first is that HO from *C. glabrata* is active and has the same specificity as the one from *S. cerevisiae*, so that the endonuclease is able to recognize and cut only MATα sites, not MATa sites. In this case, activity must be infrequent to account for the three- to fourfold predominance of MATα sites (26; C. Hennequin, H. Muller, B. Dujon, and C. Fairhead, unpublished data). This predominance could also be explained by a better fitness of MATα strains than that of MATa strains, a possibility that needs to be tested. The second hypothesis is that HO is inactive in *C. glabrata*, and switching from MATα to MATa is an uncontrolled event that is more likely than MATα-to-MATa switching because of the chromosomal configuration of the cassettes. In the latter case, there would be no active control of switching and therefore potential mating, but with the first hypothesis, the possibility of a yet-unproved active control of switching is open, perhaps in the human body, as suggested previously (25).

In conclusion, although our experiments to mate *C. glabrata* have failed, the facts that (i) so many genes of the mating pathway remain in the genome, (ii) the modes of splicing of the α/α transcript differ between mating types (this study), and (iii) this would have no cause to arise unless some mating/meiosis pathways are at least partially active indicate that it is possible that *C. glabrata* cells mate under some still-to-be-discovered conditions, such as in the human body. This could be followed by a diploid phase that may be transient, allowing for meiotic or pseudo-meiotic recombination to occur, as in *C. albicans* (25).

ACKNOWLEDGMENTS

We thank the Génolevures consortium and members of the Unité de Génétique Moléculaire des Levures, in particular, Bertrand Llorente and Alain Jacquier, for stimulating discussions. We thank Brian Tuch for critical reading of the manuscript and sharing of unpublished information.

This work was supported in part by ACI grants MCI0314, GDR 2354, and ANR GENARISE. B.D. is a member of the Institut Universitaire de France. H.M. is a recipient of a doctoral fellowship of the Ministère de la Recherche through University Paris 6 and of a final-year fellowship from the Fondation pour la Recherche Médicale.

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