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

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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 *MAT*α and most other strains, such as BG2, being *MAT*a. 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 α*1* and α*2* genes and, more surprisingly, through differential splicing of the a*1* transcript.

The comparative study of reproductive cycles in fungi reveals varied and often surprising situations. Even quite 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 teleomorph 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 asexual *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 (*HML*α and *HMR***a** 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 **a***1* transcript.

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. WO 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. SPO medium contained 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. SLAD medium was prepared with 0.17% (wt/vol) yeast nitrogen base without amino acids (Difco), 2% (wt/vol) glucose, and 2% (wt/vol) Bacto agar. McClary'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. Malt (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,

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TABLE 1. Descriptions of strains used in this work

Species	Strain	Description	Parent strain	Genotype[a]	Reference or source[b]
S. cerevisiae	FY23			*MAT***a** *ura3-52 trp1Δ63 leu2Δ1*	Winston et al. (30)
	FY73			*MAT*α *ura3-52 his3Δ200*	Winston et al. (30)
	FY1679	FY23 × FY73		*MAT***a**/α *ura3-52/ura3-52 trp1Δ63/+ leu2Δ1/+ his3Δ200/+*	Thierry et al. (27)
	J5	HO^+		*HML*α *MAT*α *HMR*α *HO leu2 his4 thr4 lys2*	Kindly donated to us by Amar Klar
	BY1408	*sst1*Δ **a**	BY4741	*MAT***a** *ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 sst1*Δ::*KANMX*	Euroscarf collection
	BY11408	*sst1*Δ α	BY4742	*MAT*α *ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 sst1*Δ::*KANMX*	Euroscarf collection
	BY6055	*sst2*Δ **a**	BY4741	*MAT***a** *ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 sst2*Δ::*KANMX*	Euroscarf collection
	BY16055	*sst2*Δ α	BY4742	*MAT*α *ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 sst2*Δ::*KANMX*	Euroscarf collection
	BY5645	*ste2*Δ **a**	BY4741	*MAT***a** *ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 ste2*Δ::*KANMX*	Euroscarf collection
C. glabrata	CBS138	Wild type		*MAT*α	Reference strain (ATCC 2001)
	BG2	Wild type		*MAT***a**	Fidel et al. (9)
	ΔH1	CBS138 *his3*Δ	2001U	*MAT*α CAGL0I03080gΔ CAGL0L02937gΔ(−254 to +777)::Cg*URA3*	Weig et al. (29)
	M2	Wild type		*MAT***a**	Our unpublished data
	M3	Wild type		*MAT***a**	Our unpublished data
	M4	Wild type		*MAT*α	Our unpublished data
	M5	Wild type		*MAT*α	Our unpublished data
	HM100a	CBS138 *ura3*Δ		*MAT*α CAGL0I03080gΔ::*KANMX*	This work
	HM106a	CBS138 *trp1*Δ	HM100a	*MAT*α CAGL0I03080gΔ::*KANMX* CAGL0C04092gΔ::Sc*URA3*	This work
	HM102a	CBS138 *HMR*Δ	HM100a	*MAT*α CAGL0I03080gΔ::*KANMX* Cg*HMRa*Δ::Sc*URA3*	This work
	HM103a	CBS138 *sst2*Δ	HM100a	*MAT*α CAGL0I03080gΔ::*KANMX* CAGL0H00374gΔ::Sc*URA3*	This work
	BG14	BG2 *ura3*Δ	BG2	*MAT***a** CAGL0I03080gΔ(−85 to +932)::Tn*903 neo*^{R}	Cormack and Falkow (5)
	HM107a	BG2 *trp1*Δ	BG14	*MAT***a** CAGL0I03080gΔ(−85 to +932)::Tn*903 neo*^{R} CAGL0C04092gΔ::Sc*URA3*	This work
	BG87	BG2 *ura3*Δ *his3*Δ	BG14	*MAT***a** CAGL0L02937gΔ(1 to +631) CAGL0I03080gΔ(−85 to +932)::Tn*903 neo*^{R}	Cormack and Falkow (5)
	HM104a	BG14 *sst2*Δ	BG14	*MAT***a** CAGL0I03080gΔ(−85 to +932)::Tn*903 neo*^{R} CAGL0H00374gΔ::Sc*URA3*	This work
	HM105a	BG87 *sst2*Δ	BG87	*MAT***a** CAGL0L02937gΔ(1 to +631) CAGL0I03080gΔ(−85 to +932)::Tn*903 neo*^{R} *sst2*Δ::Sc*URA3*	This work

[a] An Sc prefix on a gene indicates *S. cerevisiae*; a Cg prefix indicates *C. 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. glabrata* and *S. 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 (RQ1 RNase-free DNase; Promega) and extracted with phenol-chloroform before being subjected to reverse transcription. Reverse transcriptase (RT) Superscript II (catalog no. 18064-014; Invitrogen) was used according to the manufacturer's recommendations. RNasin (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 (~10 ng) of cDNA 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. 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. glabrata* **a**-factor or α-factor or of *S. cerevisiae* α-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 $5 \cdot 10^4$ cells.

For pheromone expression experiments, cells from *C. glabrata* or *S. 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, $5 \cdot 10^4$ cells of the tester strain were spread on the plate.

Pictures were taken after 2 days of growth at 30°C.

TABLE 2. Description of oligonucleotide primers used in this work

Type of expts	Oligonucleotide name	Sequence (5′–3′)	Primer pair target or use
RT-PCR	HM_RT_alpha1_F	CTGTTAACTAAACCGAACAA	α*1* of *C. glabrata*
	HM_RT_alpha1_R	ATGGGCCATTATATAATTTAC	
	HM_RT_a1_F	GTTTACAGATATTGCAATCG	**a***1* of *C. glabrata*
	HM_RT_a1_R	TGTTTAGATCTTTCGATCTCT	
	HM_RT_act1_F	GCTGTCTTGTCTCTATACTCC	*ACT1* of *C. glabrata*
	HM_RT_act1_R	CTAGAATAGAACCACCGATC	
	HM_RT_a1Scer_R	AAACTCTTACTTGAAGTGGAG	**a***1* of *S. cerevisiae*
	HM_RT_a1Scer_F	GGATGATATTTGTAGTATGGC	
	HM_RT_alpha1Scer_F	CTTCAATATTATTCGACCACT	α*1* of *S. cerevisiae*
	HM_RT_alpha1Scer_R	CCAAATGTACAAACACATCT	
	HM_YCR097_a1_F	TGTAGTATGGCGGAAAAC	First intron of **a***1* of *S. cerevisiae*
	HM_YCR097_a1_R	TTGTGTAATGTATGTTGCTC	
	HM_YCR097_a1Ter_F	AAGAGAAGAGCCCAAAGG	Second intron of **a***1* of *S. cerevisiae*
	HM_YCR097_a1Ter_R	ATTTAGATCTCATACGTTTA	
	HM_CG_a1_F	GCAATCGGTTAATTCTAATACTC	First intron of **a***1* of *C. glabrata*
	HM_CG_a1_R	CTGTTTGGGTAAATCTATGTTG	
	HM_CG_a1Ter_F	ATCACCACCAATCAAGTTC	Second intron of **a***1* of *C. glabrata*
	HM_CG_a1Ter_R	TCACGATTGTTTAGATCTTTC	
qRT-PCR	HM_YCR040_alpha1_F	TGTCTTGTCTTCTCTGCTC	α*1* of *S. cerevisiae*
	HM_YCR040_alpha1_R	ATTATTCGTCAACCACTCTAC	
	HM_YCR097_a1Bis_F	AAGAGAAGAGCCCAAAGG	**a***1* of *S. cerevisiae*
	HM_YCR097_a1Bis_R	CTTGAAGTGGAGTAATGCC	
	HM_YCR096_a2_F	CAAAAGAAAATGTCCGAATAC	**a***2* of *S. cerevisiae*
	HM_YCR096_a2_R	TGAATGCGAGATAAACTGG	
	HM_YCR039_alpha2_F	ATTTACCTAAGTTACCAGAGAG	α*2* of *S. cerevisiae*
	HM_YCR039_alpha2_R	AGTAGTGAGTTGAGATGTTG	
	HM_YFL026_STE2_F	AGCACCATTAACTACACTTC	*STE2* of *S. cerevisiae*
	HM_YFL026_STE2_R	CCACATCTGACACCAAAC	
	HM_YKL178_STE3_F	ACAAGTCAGCAATAATAGGG	*STE3* of *S. cerevisiae*
	HM_YKL178_STE3_R	CCGCATCTACAATACACG	
	HM_YDL227_HO_F	GACATTGAAGTTAGAGATTTGG	*HO* of *S. cerevisiae*
	HM_YDL227_HO_R	ACAGCAGGAGTTACAAGG	
	HM_YFL039_ACT1_F	CGCTGCTCAATCTTCTTC	*ACT1* of *S. cerevisiae*
	HM_YFL039_ACT1_R	AATCTTTCGTTACCAATAGTG	
	HM_CG_alpha1_F	ATTAGAGAAGAAAGGATGAAAC	α*1* of *C. glabrata*
	HM_CG_alpha1_R	GATGGAAGTATTGAAGTGATG	
	HM_CG_a1Bis_F	AACATATACAACTAGGAAGCC	**a***1* of *C. glabrata*
	HM_CG_a1Bis_R	TTGAACTTGATTGGTGGTG	
	HM_CG_a2_F	GGACCCACAAGAGATAGG	**a***2* of *C. glabrata*
	HM_CG_a2_R	CGATAGGAAGCGGCATAG	
	HM_ CG_alpha2_F	TCAAGAATTAGTATTACGCATC	α*2* of *C. glabrata*
	HM_ CG_alpha2_R	AACTGGAACACAATGATATAAG	
	HM_CG_STE2_F	TTGCCATTATCCTCTATCTG	*STE2* of *C. glabrata*
	HM_CG_STE2_R	ACCCATTGCTCTTATTCG	

Continued on following page

TABLE 2—*Continued*

Type of expts	Oligonucleotide name	Sequence (5′–3′)	Primer pair target or use
	HM_CG_STE3_F HM_CG_STE3_R	GTCTTATGTATTGAACGATTGTG CCGAGTGCTGATGGAATG	*STE3* of *C. glabrata*
	HM_ CG_HO_F HM_ CG_HO_R	GCCAAAGAATCATCACAAG GCATCCAGATACTCAAGG	*HO* of *C. glabrata*
	HM_CG_ACT1_F HM_CG_ACT1_R	AGTTGCTGCTTTAGTTATTG CTTGGTGTCTTGGTCTAC	*ACT1* of *C. glabrata*
Strain construction	HM_CgSST2_pUR_F HM_CgSST2_pUR_R	CGGGATCCTTAAATGATCTGATCCGAGG GGGGTACCAACTGACTCAATACAGCATC	Cloning in pUR for construction of *sst2*Δ strains HM103a, HM104a, and HM105a
	HM_CgSST2_pRA_F HM_CgSST2_pRA_R	CGGGATCCTTAGGCGGTTTCTTCATATG GGGGTACCAGATTAGACAGAAATCAAGC	Cloning in pRA for construction of *sst2*Δ strains HM103a, HM104a, and HM105a
	HM_CgHMR_pUR_F HM_CgHMR_pUR_R	CGGGATCCAGGTATGGCTGATGATCAGGA GGGGTACCATTTTGATTTTCCGGGACAA	Cloning in pUR for construction of *HMR*Δ strain HM102a
	HM_CgHMR_pRA_F HM_CgHMR_pRA_R	CGGGATCCGCTTGCAATCAAAGTGTTCTG GGGGTACCGCCACTACAAATGAGTTCGTG	Cloning in pRA for construction of *HMR*Δ strain HM102a
	HM-CgURA3_pKA_F HM-CgURA3_pKA_R	CGGGATCCTTAAAGCTATAGAATCGGCGGGA GGGGTACCAGAAATAACTAAGCTCGCGTGGC	Cloning in pKA for construction of *ura3*Δ strain HM100a
	HM-CgURA3_pAN_F HM-CgURA3_pAN_R	CGGGATCCGTCATTTTCGGAACAATCCGATT GGGGTACCTTTGAATTCAGCGAGTTAGCGG	Cloning in pAN for construction of *ura3*Δ strain HM100a

RESULTS

There is no report of the mating of *C. glabrata* in the literature. We have, ourselves, tried mating *MAT***a** strains descending from BG2 with *MAT*α 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*.

TABLE 3. Mating experiments performed[a]

*MAT***a** parent	Phenotype	*MAT*α parent	Phenotype
BG14	Ura$^-$	HM101a	Leu$^-$
		HM106a	Trp$^-$
		ΔH1	His$^-$
HM107a	Trp$^-$	HM100a	Ura$^-$
		HM101a	Leu$^-$
		ΔH1	His$^-$

[a] Ura$^-$ BG14 and Trp$^-$ HM107a were crossed with the indicated *MAT*α parent strains.

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 **a***1*, α*1*, and α*2*, respectively, at the *MAT* locus. We have not examined the **a***2* gene given the absence of any known role for **a***2* 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 **a***2* 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 *STE3* (YKL178C) encoding the **a**-factor receptor (7). Finally, we examined the expression of the 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 **a***1* and α*1* 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 **a***1* genes that contain two introns in *S. cerevisiae* and *C. glabrata* (20, 26), primers amplified a fragment containing the first

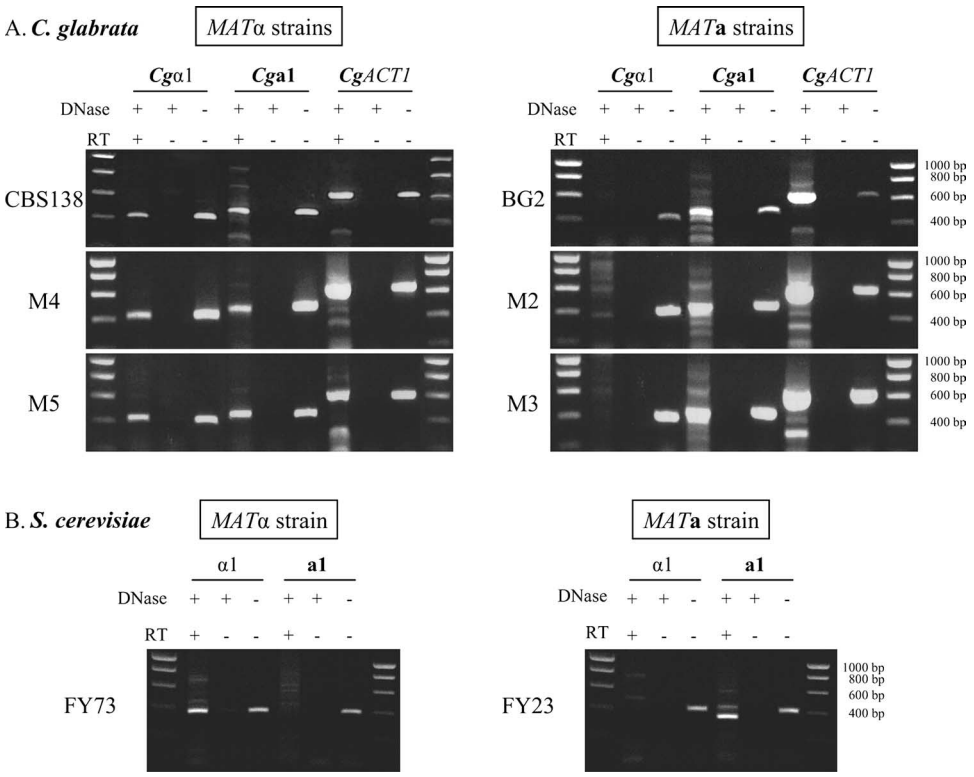

FIG. 1. RT-PCR experiments with *MAT***a** and *MAT*α cassettes of *C. glabrata* (A) and *S. cerevisiae* (B). Mating type is shown at the top, and the gene studied is shown above the gels; for *C. glabrata*, ***Cg****ACT1* is CAGL0K12694g, ***Cg*****a1** is CAGL0E00341g, and ***Cg***α1 is CAGL0B01243g. The presence or absence of DNase and RT is indicated by + or −, respectively. Strain names are at the left of gels. Molecular size markers are shown on the right.

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 *MAT*α gene α*1* is expressed in all *MAT*α strains and not in *MAT***a** strains. In contrast, the **a***1* gene is expressed in both *MAT***a** and *MAT*α strains. In the latter, this transcript must arise from the expression of the *HMR***a** locus, since the *MAT* and *HML* loci contain α-type information. We conclude that *HMR***a** 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 *MAT*α strain with the *HMR***a** locus deleted, strain HM102a. Further analyses include this strain. Control experiments with *S. cerevisiae* show that, as expected, there is clear-cut mating type-specific expression of the **a***1* and α*1* genes (Fig. 1B) (13). The size of the major PCR band on cDNA from the **a***1* 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 **a***1* cDNAs have the same size as the PCR band from genomic DNA, suggesting a splicing defect. More-explicit experiments on **a***1* splicing are described below.

In the qRT-PCR experiments (Fig. 2), we examined the expression of **a***1*, α*1*, and α*2* 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*, **a***1* is expressed at similar levels in both the *MAT***a** strain and the *MAT*α strain, confirming the results of the above-described RT-PCR experiments. As expected, deletion of the *HMR* cassette in strain HM102a results in the absence of the **a***1* transcript (Fig. 2A). Neither *STE2* nor *STE3* homologs display mating type specificity, as they are expressed at similar levels in *MAT***a** and *MAT*α strains. In contrast, the expression of α*1* and α*2* is mating type specific in *C. glabrata*; they are expressed, respectively, 500-fold and 1,200-fold more in *MAT*α cells than in *MAT***a** cells. Deletion of the *HMR* cassette does not interfere with the expression of these two genes. Control experiments show that **a***1*, α*1*, α*2*, *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 a*1* introns. Previous experiments led us to hypothesize that **a***1* may be nonfunctional in *C. glabrata*; we observed concomitant expression of **a***1*, α*1*, and α*2* in *MAT*α cells, a situation that could not occur in *S. cerevisiae* because the **a***1*-α*2* heterodimer represses the expression of α*1* and the deletion of *HMR***a** in *MAT*α cells has no effect on the expression of α*1*, α*2*, *STE2*, and *STE3*. The fact that we observed unspliced forms of **a***1* prompted us to examine this in more detail. Indeed, in *S. cerevi-*

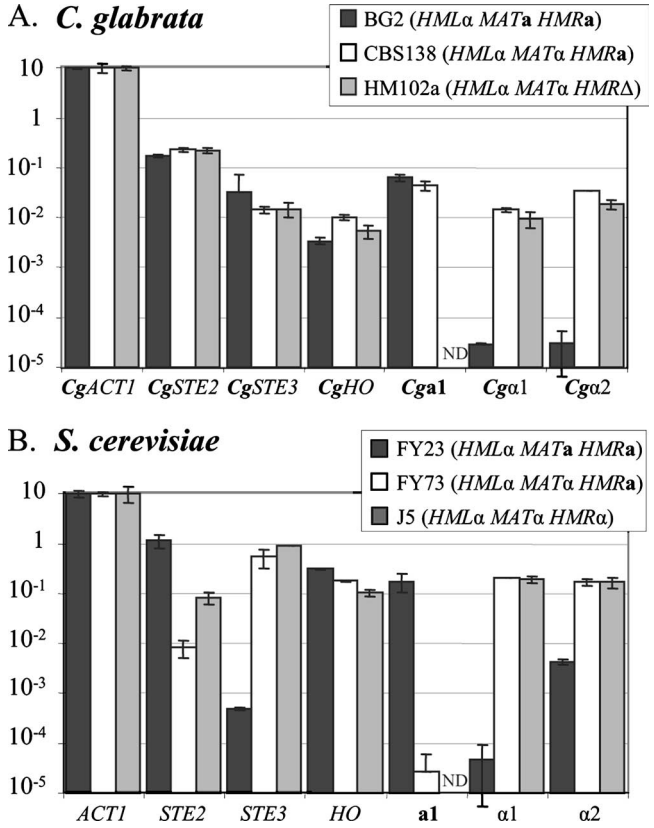

FIG. 2. qRT-PCR experiments with several genes related to mating. Histograms of transcript quantity per cell relative to the quantity of the actin transcript (fixed at 10 transcripts per cell [21]) in *C. glabrata* (A) and *S. cerevisiae* (B). Names of genes are indicated below the histograms; for *C. glabrata*, ***Cg****ACT1* is CAGL0K12694g, ***Cg****STE2* is CAGL0K12430g, ***Cg****STE3* is CAGL0M08184g, ***Cg****HO* is CAGL0G05423g, ***Cg*****a1** is CAGL 0E00341g, ***Cg***α1 is CAGL0B01243g, and ***Cg***α2 is CAGL0B01265g. The *y* axis is in the logarithmic scale. In each case, three different strains were analyzed as indicated in the boxes. ND, not detectable (a qRT-PCR experiment was performed, but since there is no corresponding sequence in the genome, no product was detected, as expected).

siae, when the **a***1* transcript is not spliced, the **a**1 protein is not produced (22). The **a***1* 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 MAT***a** 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 *MAT***a** cells (BG2, M2, M3). In *C. glabrata MAT*α 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 *MAT***a** cells, the doubly spliced form of the **a***1* 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 **a***1* 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 **a***1* transcript is partially spliced in the *MAT***a** strains examined, while it is not spliced at detectable levels in the *MAT*α 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 MAT***a** and *MAT*α strains express both **a**- 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 **a**-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 **a**-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 *MAT***a** 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 *sst2* mutants 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, *ss1*, and *sst2 MAT***a** *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 MAT***a** cells. The synthetic **a**-factor deduced from the genome of *C. glabrata* has no activity on wild-type *S. cerevisiae MAT*α 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 MAT*α cells. The strain of *C. glabrata* with *HMR***a** 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

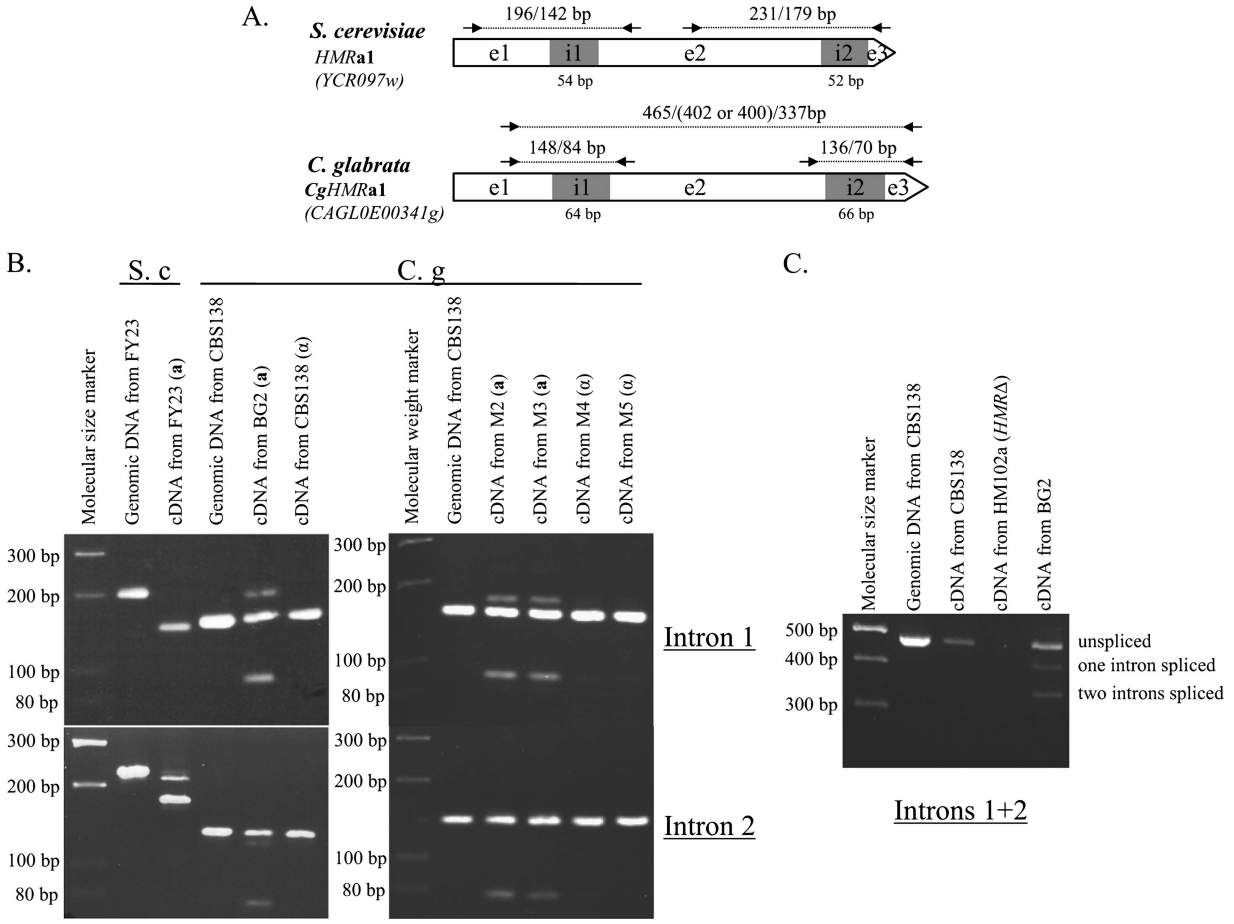

FIG. 3. Splicing of **a***1* introns in *S. cerevisiae* and *C. glabrata.* (A) In representations of genes, white boxes are exons (e1 is exon1, e2 is exon2, and e3 is exon3) and gray boxes are introns (i1 is intron1 and i2 is intron2); their sizes are indicated below the diagrams. Arrows indicate positions of primers relative to introns used in RT-PCR, and the sizes of the PCR products from the cDNAs originating from nonspliced/spliced mRNAs between pairs are noted above the lines for the PCR. (B) RT-PCR experiments to detect splicing of individual introns in *S. cerevisiae* (S. c) and *C. glabrata* (C. g). (C) RT-PCR experiments to detect splicing of both introns in *C. glabrata*.

are sensitive but are themselves insensitive to them. Interspecific sensitivity to α-factor between *S. cerevisiae*, *Saccharomyces kluyveri*, and *Saccharomyces exiguus* has been described previously (14, 18), but the peptidic sequences of α-factors from *C. glabrata* are more diverged from *S. cerevisiae* than are the α-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 α-factor from *C. glabrata* is mediated by the Ste2 receptor, as *ste2* mutants of *S. cerevisiae* do not respond to it or to α-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 sst2* 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 *sst2* 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*α strains and *C. glabrata MAT***a** strains (BG2). The inhibition of the growth of *MAT***a** cells by the *C. glabrata MAT*α 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***a** (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

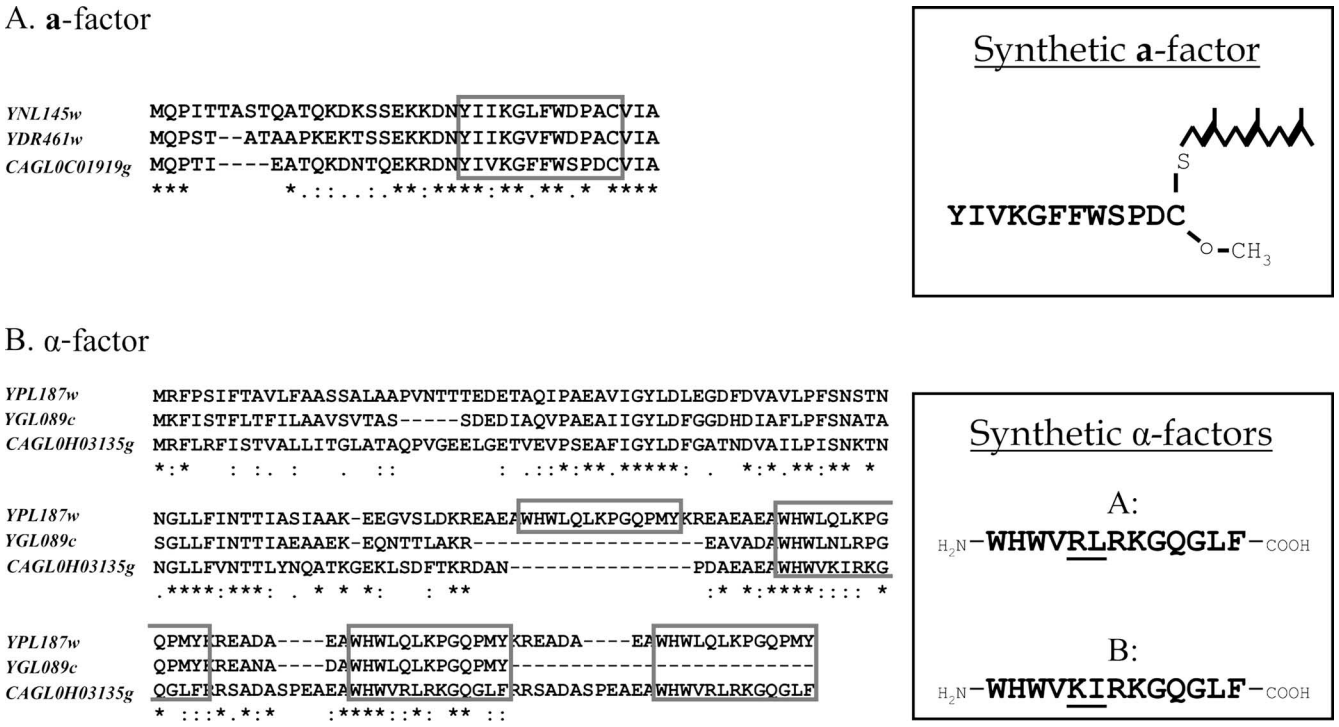

FIG. 4. Mating pheromones. (A) On the left, alignments of sequences of precursors of the **a**-factors of *S. cerevisiae* and *C. glabrata* are shown. The box indicates peptides resulting from precursor proteolysis. On the right, the synthesized form of the **a**-factor of *C. glabrata* is shown. This is modified with a methyl and a farnesyl group at the C terminus. (B) On the left, alignments of sequences of precursors of the α-factors of *S. cerevisiae* and *C. glabrata* are shown. The boxes indicate peptides resulting from precursor proteolysis (forms A and B). On the right, synthesized forms of the α-factors of *C. glabrata* are shown. In the alignments: "*," identical residues; ":," highly similar residues; "." similar residues.

spotted before *sst2 S. cerevisiae* tester cells were plated. No isolate was found to produce pheromones specifically inhibiting the *MAT***a** or *MAT*α *sst2* strains of *S. cerevisiae*, but three unrelated strains (two *MAT***a** strains and one *MAT*α strain) were found to express the same general inhibitor as CBS138 (not shown).

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 α*1* and α*2* are expressed in a mating type-specific manner, in contrast to **a***1*, which is expressed in both mating types, because of a lack of silencing at *HMR***a**, interestingly situated on a different chromosome than *MAT* and *HML*α. Sir1p is responsible for silencing *HML*/*HMR* loci in *S. cerevisiae*; thus, perhaps the absence of a *SIR1* homolog in *C. glabrata* (7) explains this phenomenon. Nonetheless, *HML*α is silenced in *MAT***a** strains, in which we do not observe the expression of the α genes.

Even though the expression of the **a***1* gene is not subject to mating type specificity, experiments suggest that **a***1* is not functional in *MAT*α haploid cells. Moreover, the expression of a functional **a***1* gene in *MAT*α cells would logically lead them to behave as diploid cells, with the risk of their trying to undergo meiosis, a possibly fatal event for a haploid cell. Even though 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 **a***1* and α*2* leads to the repression of α*1* in addition to other haploidy genes. The expression of the three transcription factors **a***1*, α*1*, and α*2* simultaneously in *C. glabrata MAT*α cells thus leads to the hypothesis that **a***1* is not functional. Additionally, the observation that the *HMR***a**Δ *MAT*α strain still expresses α*1*, *STE2*, and *STE3*, like the wild-type *MAT*α strain, strengthens this hypothesis. The fact that we found that the splicing of the **a***1* transcript is detectable only in *MAT***a** cells can explain these observations. In *S. cerevisiae*, it has been shown that the unspliced form of **a***1* is not functional in diploid cells (22). In haploid cells, there is no known role for **a***1*, the *MAT***a** phenotype being the default one. Thus, a partial failure to splice the **a***1* transcript in *MAT***a** cells is not expected to have any effect. In *MAT*α cells, the lack of splicing of the **a***1* 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 **a***1* expression is regulated in

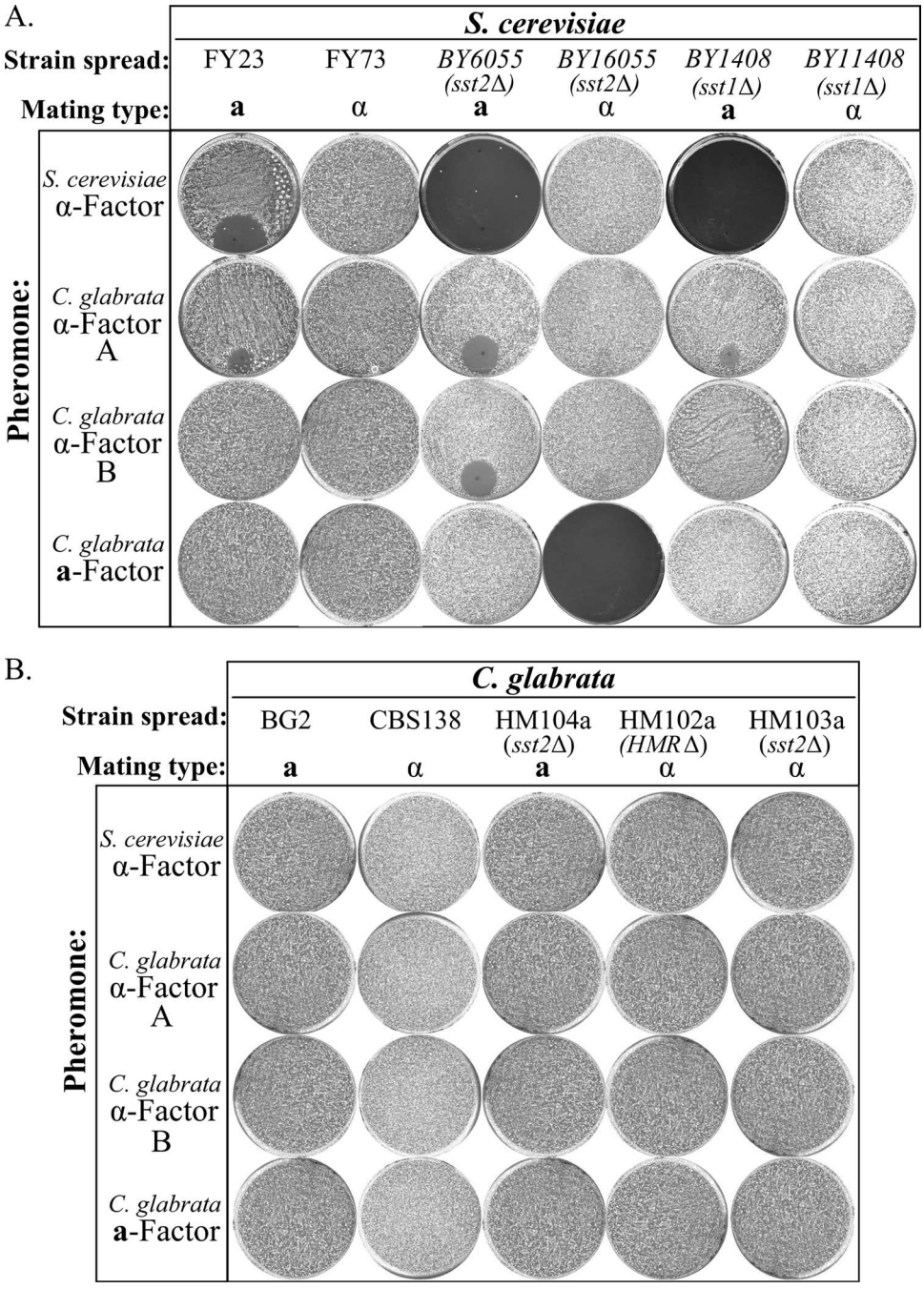

FIG. 5. Effect of synthetic pheromones on *S. cerevisiae* (A) and *C. glabrata* (B) cells. Cell type is indicated at the top, and pheromones are indicated on the left.

some other way, such as splicing. Since there is no sequence difference between *MAT***a***1* and *HMR***a***1* that would explain differences in splicing efficacy (6, 26), the differential splicing must originate either from a general splicing defect in *MAT*α strains or from a mating type-specific mechanism, with both alternatives leading to the inactivation of **a***1*.

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 MAT*α cells, *STE3* is activated by α*1*. In *C. glabrata*, the basal level observed in *MAT***a** 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 α*1*. In *S. cerevisiae MAT*α cells, *STE2* is repressed by the binding of Mcm1 and α*2* on the **a**-specific gene operator. Putative binding sites for Mcm1 and α*2* 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).

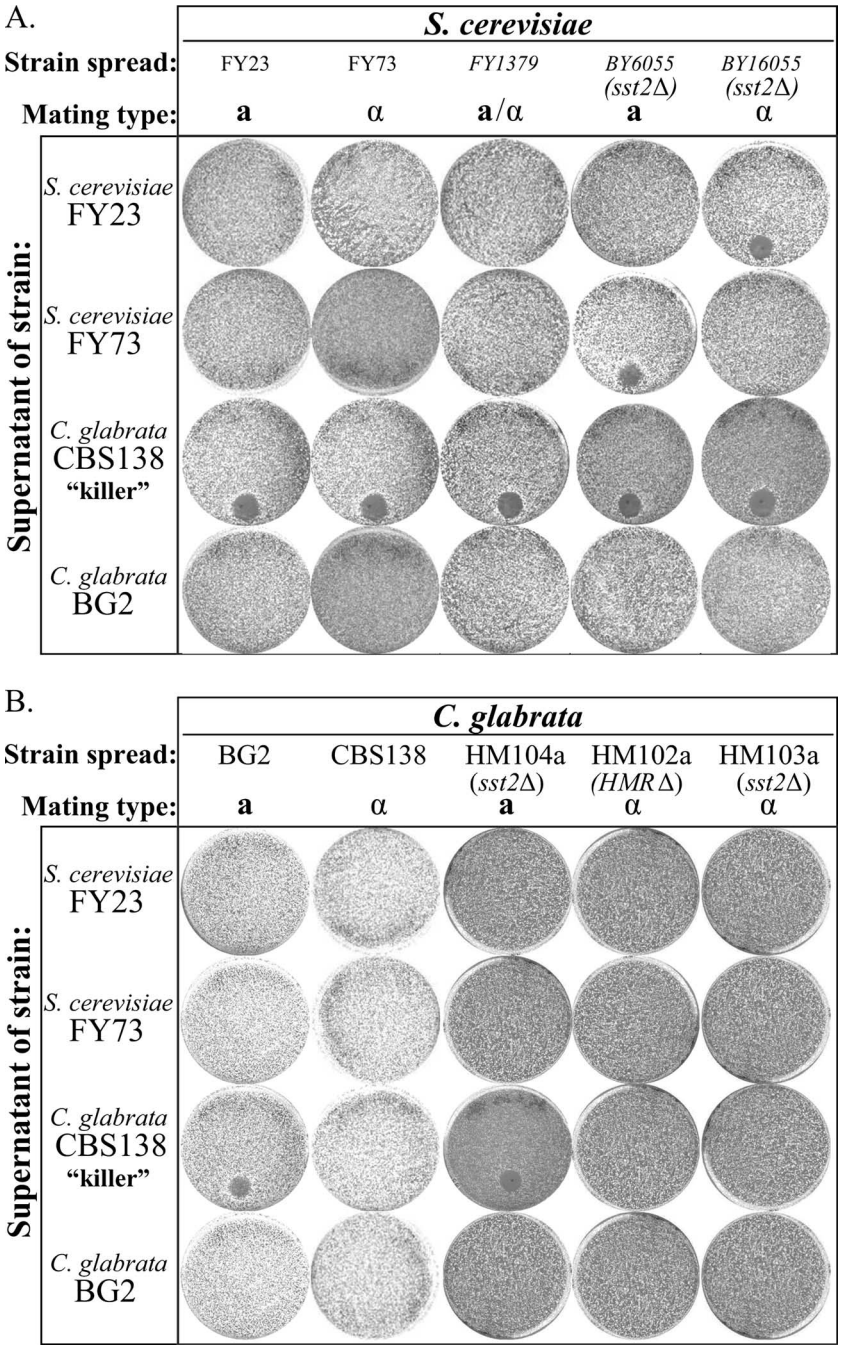

FIG. 6. Effect of culture supernatant on *S. cerevisiae* (A) and *C. glabrata* (B) cells. Cell type is indicated at the top, and the nature of the supernatant is indicated on the left.

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 G_1-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-

anism 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* (TNNNYGCG/ANC/AANT/G) can be identified (23). Indeed, HO from *S. cerevisiae* cuts the *MAT***a** site but not the *MAT*α 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 *MAT***a** to *MAT*α. In *Kluyveromyces lactis*, 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 *MAT***a** sites, not *MAT*α sites. In this case, activity must be infrequent to account for the three- to fourfold predominance of *MAT***a** strains (26; C. Hennequin, H. Muller, B. Dujon, and C. Fairhead, unpublished data). This predominance could also be explained by a better fitness of *MAT***a** strains than that of *MAT*α strains, a possibility that needs to be tested. The second hypothesis is that HO is inactive in *C. glabrata*, and switching from *MAT***a** to *MAT*α is an uncontrolled event that is more likely than *MAT*α-to-*MAT***a** 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 **a***1* 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).

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AUTHOR'S CORRECTION

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

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Volume 7, no. 5, p. 848–858, 2008. Pages 856 and 857: The authors retract Fig. 5 and Fig. 6 from the original version of the article because the same pictures of some of the plates with negative results were mistakenly used at several distinct places in the figures. The correct versions of these figures and their legends are provided below. The conclusions of the paper remain the same.

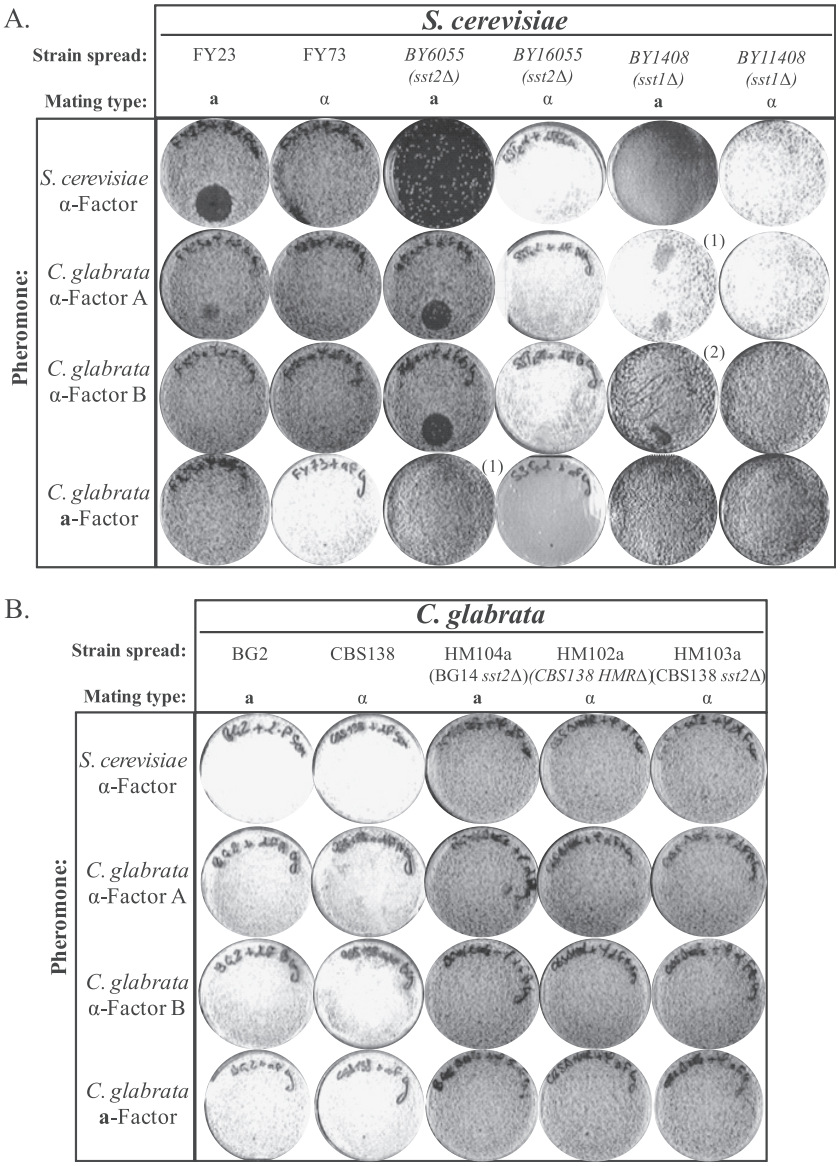

FIG. 5. Effect of synthetic pheromones on *Saccharomyces cerevisiae* (A) and *Candida glabrata* (B) cells. Assays were performed as indicated in Materials and Methods. Cell type is indicated at the top, and pheromones are indicated on the left. [Notes: (i) on this plate, two drops of pheromone solutions were deposited, with the top one corresponding to a higher concentration (1 mg/ml solution); (ii) the halo at the bottom of the plate is due to an artifact during plate handling and does not indicate growth arrest.]

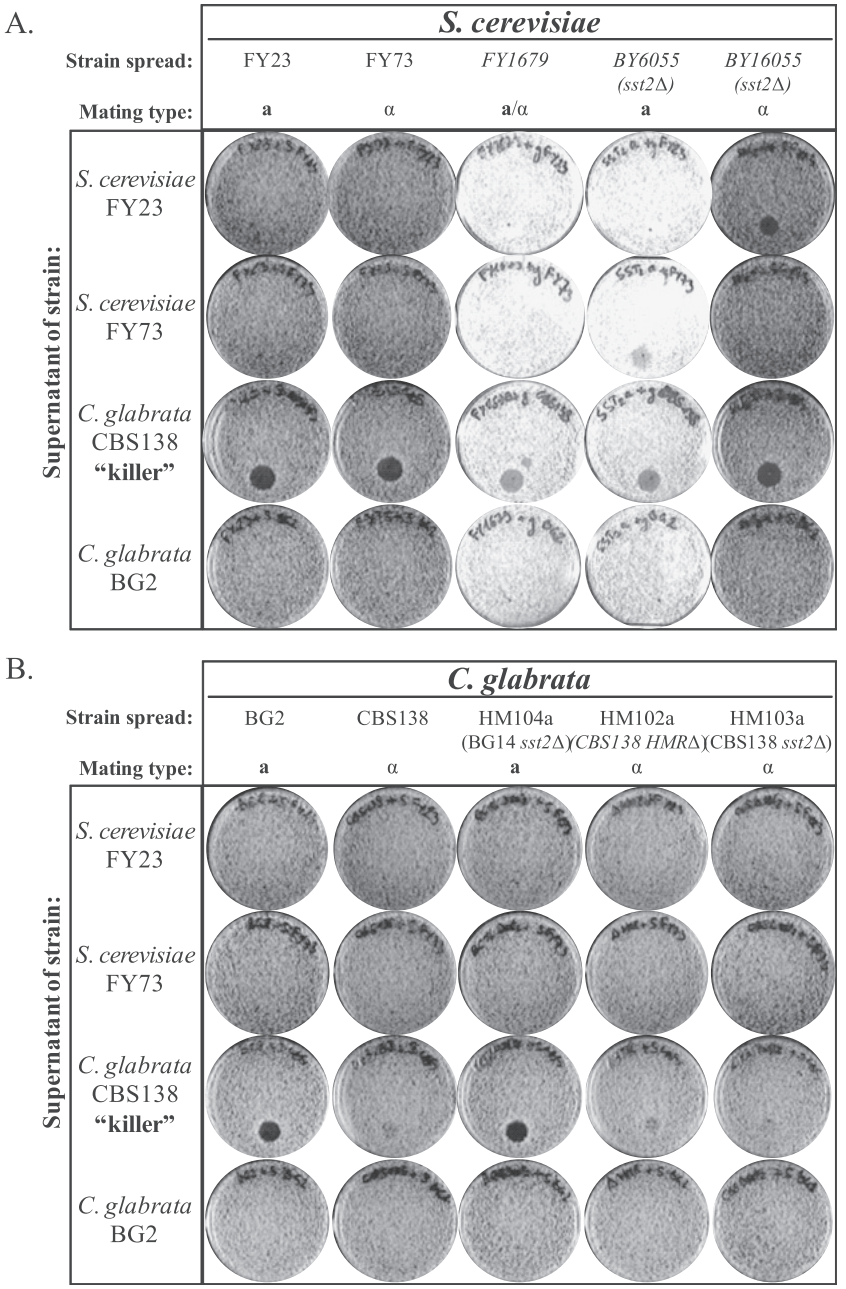

FIG. 6. Effect of culture supernatant on *S. cerevisiae* (A) and *C. glabrata* (B) cells. Assays were performed as indicated in Materials and Methods. Cell type is indicated at the top, and the nature of the supernatant is indicated on the left.