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Escola de Ciências

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Paracoccidioides brasiliensis: **Study of the sexual cycle**

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Paracoccidioides brasiliensis: **Estudo do ciclo sexual.**

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Trabalho efectuado sob a orientação do
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co-orientação da
Doutora Paula Gonçalves
e da
Doutora Maria João Sousa

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Declaração

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Paracoccidioides brasiliensis: Study of the sexual cycle.

The thermodimorphic fungal pathogen *Paracoccidioides brasiliensis* is the etiological agent of paracoccidioidomycosis, one of the most prevalent systemic mycoses endemic in Latin America, occurring mainly in Brazil, Colombia and Venezuela. The morphological transformation of *P. brasiliensis* is characterized by the existence of two different morphological forms: a mycelium/conidial form that is present at environmental temperatures (below 25°C); and a multiple budding yeast form, present at temperatures of the mammalian host (37°C).

The sexual cycle in *P. brasiliensis* has not been observed in nature or laboratory conditions. Nevertheless, in the present study, we detected low expression levels of mating-related genes, such as α-pheromone, α and **a**-pheromone receptors (*PREB* and *PREA*), and heterothallic mating loci (*MAT1-1* and *MAT1-2*), in yeast and mycelial forms, and verified that heterothallic strains of opposite mating-types are able to express α-pheromone, and both pheromone receptors. In order to further evaluate the functional activity of mating-related genes, particularly α-pheromone and its cognate receptor (PreB), we took advantage of the heterologous expression of these *P. brasiliensis* genes in the corresponding *Saccharomyces cerevisiae* null mutants. Through several functional tests, including cell cycle arrest and *shmoo* formation, we showed that *S. cerevisiae* strains heterologously expressing *PREB* respond to synthetic α-pheromone of *P. brasiliensis*. In addition, mating ability of *S. cerevisiae* non-fertile strains was restored by the expression of *PREB* or α-pheromone in the corresponding null mutants. In general, this study demonstrates novel evidences for the existence of a functional mating signaling system in *P. brasiliensis*.

Paracoccidioides brasiliensis: Estudo do ciclo sexual.

O fungo patogénico termodimórfico *Paracoccidioides brasiliensis* é o agente etiológico da paracoccidioidomicose, uma das mais prevalentes micoses sistémicas, endémica da América Latina, ocorrendo principalmente no Brasil, Colômbia e Venezuela. A transformação morfológica de *P. brasiliensis* é caracterizada pela existência de duas formas distintas: a temperaturas ambientais (25°C) existe sob a forma de micélio/conídeo; e a temperaturas do hospedeiro (37°C) sob forma de levedura.

Apesar de ainda não ter sido observado a existência de ciclo sexual em *P. brasiliensis,* no presente estudo detetámos níveis de expressão de genes relacionados com a reprodução sexuada em diversos fungo, tais como, feromona-α, recetor da feromona α e **a** (*PREB* e *PREA*) e ainda o *MAT* locus (*MAT1-1* e *MAT1-2*). Verificámos que estirpes heterotálicas, de tipos de acasalamento opostos, têm a capacidade de expressar a feromona-α e ambos os recetores. De forma a avaliar a atividade funcional de genes relacionados com a reprodução sexuada, particularmente a feromona-α e respetivo recetor (PreB), procedemos à expressão destes genes de *P. brasiliensis* em estirpes mutantes de *Saccharomyces cerevisiae*. Através de vários testes funcionais, incluindo paragem de ciclo celular e formação de *shmoos,* mostrámos que a estirpe de *S. cerevisiae* que expressa *PREB* tem a capacidade de responder à feromona-α sintética de *P. brasiliensis*. Para além disso, a capacidade de acasalamento de estirpes de *S. cerevisiae* não férteis foi restabelecida pela expressão heteróloga da feromona-α e *PREB*. Este estudo demonstra novas evidências para a existência de um sistema de sinalização de acasalamento funcional em *P. brasiliensis*.

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Chapter I - Introduction

1.1. *Paracoccidioides brasiliensis*

1.1.1. Fungal pathogens and thermal dimorphism

Worldwide over a hundred thousand fungal species are known, though only a limited number are related to human disease [1]. This is thought to be due to the high temperature of the mammalian body, which is considered a barrier against infections by fungi, as most of them have a higher growth rate at environmental temperatures [2]. For several human pathogenic fungi, the abrupt temperature change between the environment (below 25°C) and human body (37°C) is characterized by a morphological transition between yeast and mycelium form, called thermodimorphism, being this ability considered an important virulence factor [1].

The morphological form that the fungus displays at environmental or host temperature can vary according to the fungal species (Figure 1). Important fungal pathogens that show such dimorphic behavior include *Candida albicans*, *Sporothrix schenkii*, *Penicillium marnefii*, *Coccidioides immitis* and the closely related *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis* [3-5].

1.1.2. *P. brasiliensis* and paracoccidioidomycosis

The thermodimorphic fungal pathogen *P. brasiliensis* is the etiological agent of paracoccidioidomycosis (PCM), one of the most prevalent systemic mycoses endemic in Latin America, occurring mainly in Brazil, Colombia and Venezuela [6]. It is estimated that about 10 million people may be infected, though only 2% develop the disease [6, 7]. The morphological transformation of *P. brasiliensis* is reflected by the existence of two different morphological forms: a mycelium/conidial form that is present at environmental temperatures (below 25°C) ; and a multiple budding yeast form, present at temperatures of the mammalian host (37°C) (Figure 1) [1].
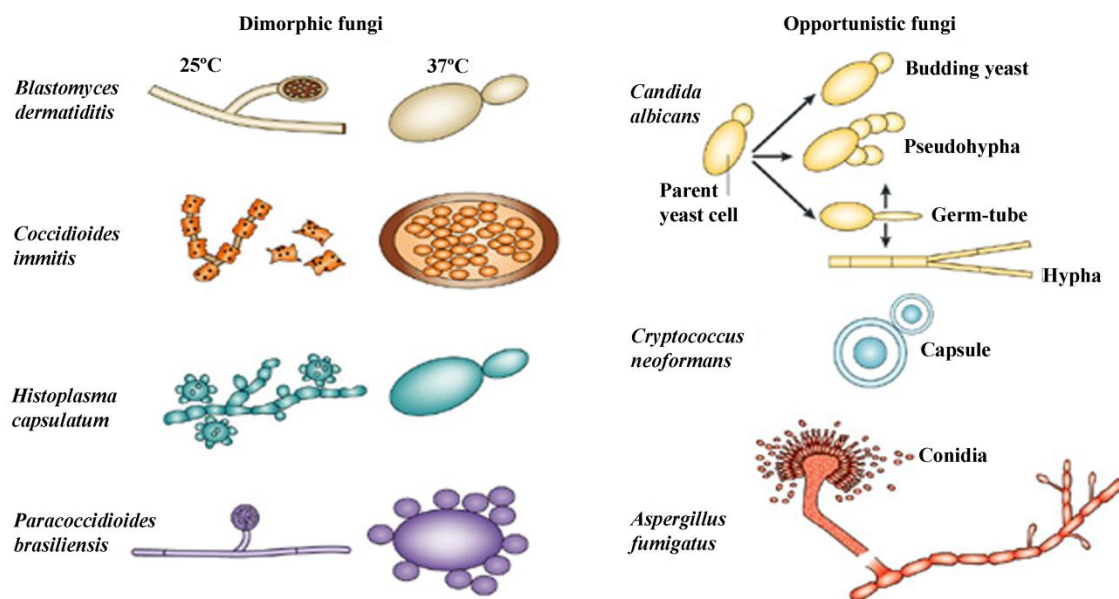
Figure 1 – Dimorphic fungi – Morphological forms at different temperatures of different fungus species [8].

It is believed that the infection starts by inhalation of airborne conidia derived from the mycelial form. When the conidia reach the epithelial cells of the lung, the morphological switch to the yeast form occurs and yeast cells start to spread into the blood system, leading to disseminated infection (Figure 2) [6, 9]. After the inhalation of conidia, the infected individuals can present two main clinical forms of PCM, the acute or sub-acute form (juvenile type) and the chronic form (adult type). The juvenile type of PCM develops within weeks or months after contact with the fungus and is more severe, leading to higher rates of mortality. In contrast, the adult type of PCM can be in latency for several years and accounts to more than 90% of the cases [10].

PCM occurs mainly in rural populations and is more common in males. The mechanisms underlying this gender susceptibility is thought to be related with hormonal regulation of *P. brasiliensis* form switch. *In vitro* studies have shown that female hormones such as oestrogens block the conidia- or mycelium-to-yeast transition, probably via a cytosolic steroid-binding protein [11, 12]. *In vivo* studies with mice corroborate these results, and revealed that 96h after conidia inoculation, the transition to yeast is accomplished in lungs of the male mice, but not in female mice [13]. On the other hand, Pinzan et al. have recently shown that the interference exerted by sexual hormones in the immune response against *P. brasiliensis* impact the differences in the clinical incidence and progression of PCM between males and females [13].
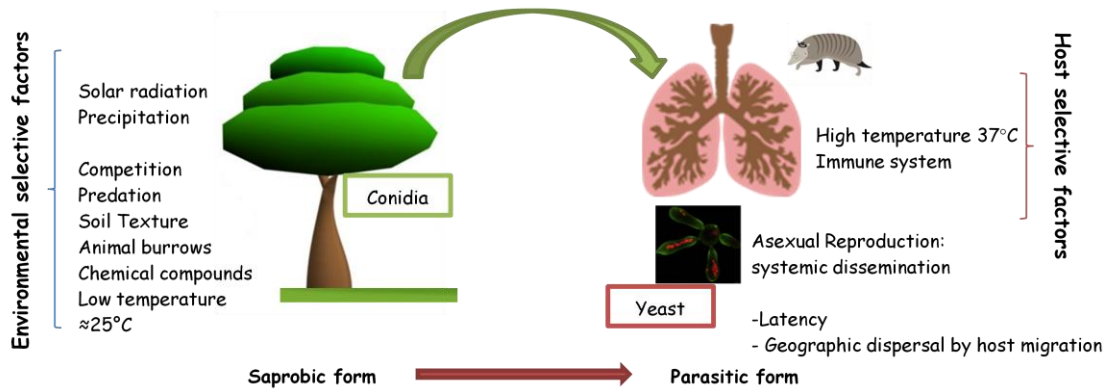
Figure 2 – Hypothetical biological cycle of *P. brasiliensis* (adapted from [14]). In this picture are highlighted some selective forces that may be involved in the supposed ecological niches in hosts and in saprobic environments.

1.1.3. Ecological habitats of *P. brasiliensis* isolates

For a long time, studies involving *P. brasiliensis* were made using clinical isolates, since the isolation of this pathogen from the environment is difficult to achieve [15]. In fact, PCM has a prolonged latency period, which together with the absence of outbreaks makes it difficult to determine the exact habitat of *P. brasiliensis* [14, 16]. The fungus has been repeatedly isolated from nine-banded armadillo (*Dasypus novemcinctus*) in Brazil, being present in 75-100% of animals captured in endemic PCM areas [15-17]. Despite this high incidence in the species *D. novemcinctus*, independent of age or gender, the animals do not show visible signs of PCM disease [17]. The fungus has been identified, by PCR in soil samples collected from armadillos burrow and faeces of naturally infected armadillos. However, until now it has not been possible to isolate *P. brasiliensis* from its saprobic form [17]. The maintenance of a parasitic and saprobic form could contribute to sexual reproduction, since *P. brasiliensis* was found in restricted and well defined areas, which increases the probability to encounter individuals of the same pathogenic fungal species [17]. Bagagli et al. [17] collected some data suggesting that *P. brasiliensis* strains, isolated from armadillos, exhibit a wider spectrum of genotypes when compared to clinical isolates, in part because the same animal can acquire multiple infections with distinct strains belonging to different genotypes.

1.1.4. Phylogeny, cryptic speciation and genome ploidy of *P. brasiliensis*

In classic systematics, *P. brasiliensis* was included in the anamorphic phylum Deuteromycota and in Hyphomycetes class, due to the absence of a traceable sexual phase [1]. However, morphological and phylogenetic studies together with the development of molecular tools made it possible to place *P. brasiliensis* in the phylum Ascomycota, inside the order Onygenales, family *Onygenaceae sensu lato* [1, 17]. More recently, a new family was proposed (Ajellomycetaceae), distinct from *Onygenaceae sensu lato*, which includes the genera *Blastomyces*, *Histoplasma*, *Emmonsia*, and *Paracoccidioides* [14, 17, 18].

Furthermore, Matute *et. al* [19], performed a study where through the analysis of eight regions from five nuclear coding genes, they found that *P. brasiliensis* is stratified in at least three distinct species: S1 (species 1 from Brazil, Argentina, Paraguay, Peru and Venezuela), PS2 (phylogenetic species 2 from Brazil and Venezuela) and PS3 (phylogenetic species from Colombia). Recently, Teixeira et al. [20] showed that 17 genotypically similar isolates, including strain Pb01, were distinct from the three cryptic species previously described and proposed the new "Pb01-like-cluster" as a new species (*Paracoccicioides lutzii*).

Both mycelia and yeast form of *P. brasiliensis* are characterized by a multinucleate nature, while conidia, the supposedly infectious form, comprise only a single nucleus [21]. The knowledge on genetic composition as well as on the mechanisms involved in dimorphism and virulence of *P. brasiliensis* is limited, which is partly due to the lack of a known teleomorphic (sexual) phase and few (cyto)genetic tools for this fungus [7, 21].

The genome characterization and chromosomal mapping of *P. brasiliensis* were previously accessed by distinct approaches [7, 22, 23]. Pulse field gel electrophoresis (PFGE), allowed to determine the size of the fungus genome, initially estimated to be approximately 23-31 Mb. PFGE analysis also revealed the presence of 4-5 chromosomes (2-10 Mb) [22, 23]. However, when these results were compared to the ones obtained by microfluorometry (45.7 to 60.9 Mb), suggested the possibility of the existence of haploid and diploid (or aneuploid) isolates of the fungus [21, 23]. Later on, Almeida et al. [21] evaluated ploidy and genome size of *P. brasiliensis*, by a flow cytometry (FCM) protocol [24]. They reported a genome size ranging from 26.3 ± 0.1 to 35.5 ± 0.2 Mb per uninucleated yeast cell. Concerning the ploidy of *P. brasiliensis*, they showed a ploidy

ratio between 1.0 and 1.1, which means that the analyzed isolates present a haploid, or at least aneuploid, DNA content. No association was detected between genome size/ploidy and the clinical-epidemiological features of the studied isolates; however, additional studies of a higher number of isolates are needed [21].

Total genome sequences of three *P. brasiliensis* strains (Pb01, Pb03, and Pb18) that were recently published by the Broad Institute of Harvard and MIT (http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html) indicated that the two *P. brasiliensis* genomes of Pb18 and Pb03 are similar in size (30.0 Mb and 29.1 Mb respectively), while the *P. lutzii* (Pb01-like) genome is nearly 3 Mb larger at 32.9 Mb [25].

1.2. Sexual reproduction in fungi

1.2.1. General aspects of sexual reproduction in fungi

In eukaryotes it's established that sexual reproduction allows for genetic recombination. The maintenance of the sexual cycle is essential to purge the genome of deleterious mutations, and for the generation of genetic variation, where the combination of beneficial genes in sexual offspring from two parents increases fitness in novel or changing ecological niches [26-28].

Although in some eukaryotes, including many pathogenic fungi, a sexual cycle was not yet established, many fungi are known to undergo sexual reproduction, and diverse patterns of sexual recombination occur throughout the five fungal phyla Ascomycota, Basidiomycota, Chytridiomycota, Mucoromycotina and Microsporidia [28]. Moreover, with the increasing number of completed fungal genome sequences it becomes clear that many of these fungi once thought asexual, actually have retained the genetic machinery for sexual reproduction [29].

It is well established that fungal sexual development is orchestrated by transcription factors encoded by a genetic locus called the mating-type or *MAT* locus, which determines the sex of the fungus [30]. Mating communication between two haploid cells is achieved by the production of small peptide pheromones from one cell, that are sensed by specific receptors on the surface of the opposite cell [31]. Fungi exhibit either of two mating patterns: self-fertile (homothallic) fungi in which sexual reproduction can occur between genetically identical cells without the need of a mating partner, or cells of one mating-type have the ability to undergo mating-type switch; or self-sterile (heterothallic) fungi in which mating occurs only when a cell encounters an opposite mating-type partner [30, 32, 33].

The fungal subkingdom Dikarya, encompassing the phyla Ascomycota and Basidiomycota, includes the most common human fungal pathogens, such as *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* [34]. An important difference between these two phyla lies in the mating-type composition, as fungi belonging to the phylum Ascomycota have just two mating-types, while the ones that belong to the phylum Basidiomycota can have multiple mating-types [31]. Another important difference is the type of sexual reproduction structures produced by both phyla. The sexual progeny from Ascomycetes is characterized by the formation of spores

(ascospores) enclosed within an ascus. In the subphylum Pezizomycotina the ascocarp can be defined as cleistothecium, perithecium, apothecium and ascostroma, depending on the structure [28, 35]. In contrast to the ascomycetes, the sexual spores of basidiomycetes are exposed to the air on the surface of basidia [28]. The genetic details concerning sexual reproduction in these two phyla will be further discussed in the next sections.

1.2.1.1. Genetics of the mating in *Saccharomyces cerevisiae*

S. cerevisiae grows mostly as yeast and can exist either as a haploid or diploid cell, though the predominant ploidy isolated from nature is diploid [28]. In the sexual cycle of *S. cerevisiae*, there are haploid cells with two different mating-types, *MAT***a** and *MAT*α, which can mate to form diploid **a**/α cells (Figure 3) [28, 36]. Each haploid mating-type cell secretes a small mating peptide pheromone that signals the opposite cell for mating: **a** cells produce **a**-factor and α cells produce α-factor. Each type of pheromone is sensed by specific receptors on the opposite mating-type cell: **a** cells encode the α-receptor (*STE2*) and α cells encode the **a**-receptor (*STE3*). After pheromone recognition, the cells develop projections called *shmoos*, characterized by a polarized growth toward a mating partner, ultimately leading to cell fusion, followed by nuclear fusion (Figure 3). The efficiency of the cell fusion requires cell synchronization, which is achieved by a cell cycle arrest in the G1 phase [28, 37, 38].

After the mating process the resulting diploid cell loses the capacity to mate, as expression of haploid-specific genes necessary for sexual fusion, are repressed. These diploid cells however are capable to undergo meiosis and sporulation (Figure 3) [33, 39].

The expression of mating-specific genes in *S. cerevisiae* is regulated by three transcription factors encoded at the *MAT* locus. The *MAT***a** locus encodes **a**1, an HD2 class homeodomain transcription factor, while the *MAT*α locus encodes α1, an alpha box transcription factor, and α2, an HD1 class homeodomain transcription factor [28, 39, 40]. The expression of **a** cell-specific genes is regulated only by MCM1, a transcription factor that belongs to the MADS box family. MCM1 alone binds to the P box of **a**-specific upstream regulatory sequences (URSs) and promotes the transcription of **a**-cell specific genes. In contrast, for the transcription of α cell-specific genes, the presence of α1 is necessary, which together with MCM1 binds to the PQ box of α-specific URSs. The α2 transcription factor binds to two α2 binding sites that flank the P box of **a**-specific URSs, repressing the transcription of **a** cell-specific genes (Figure 4) [39, 40].
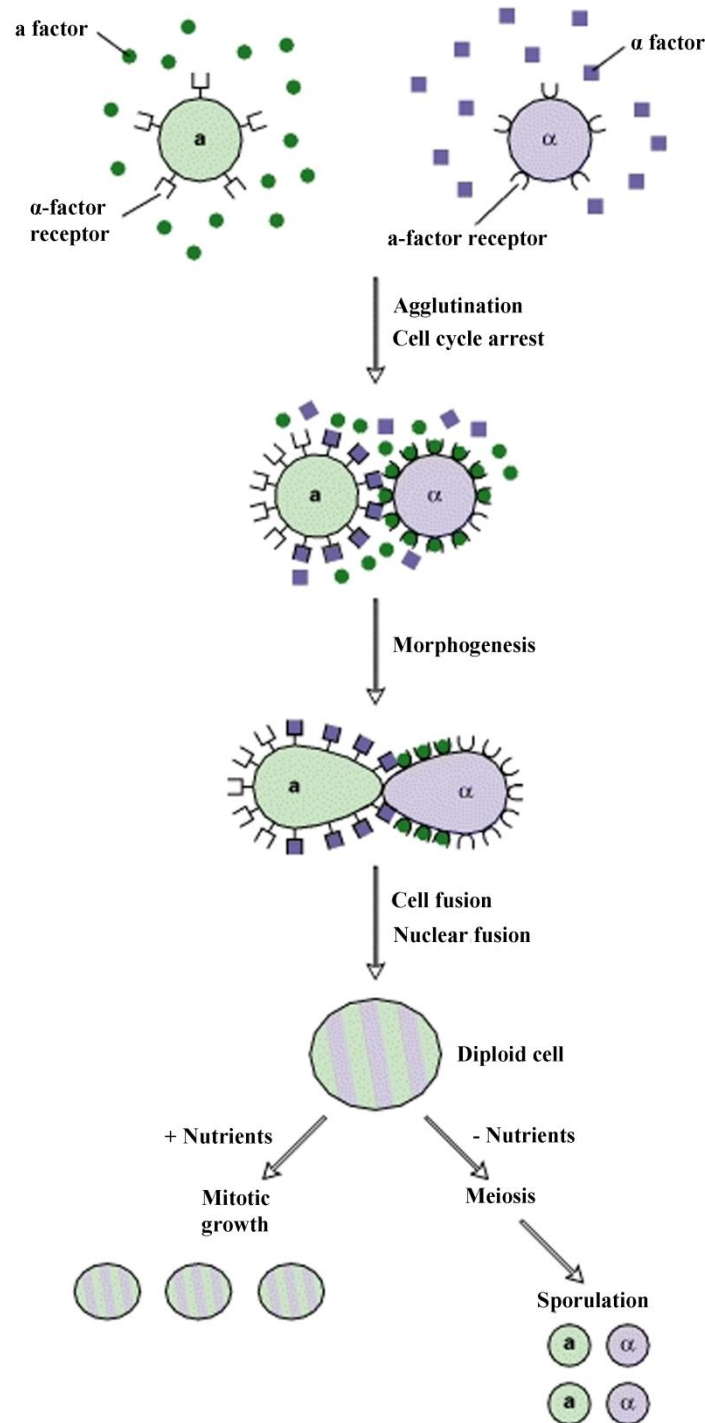
Figure 3 – Mating in *S. cerevisiae*. Two haploid cells secrete pheromone, sensed by the opposite mating-type. The cells develop *shmoos* and the cell/nuclear fusion occurs, resulting in the production of diploid cells. While in the presence of nutrients the diploid cell divides by mitosis, in the absence of nutrients, cells will undergo meiosis, forming four haploid cells [41].

The diploid **a**/α cells formed during mating do not produce any kind of receptor or pheromone, since α2 in combination with **a**1 block the α1 expression and consequently

the expression of α cell-specific genes (Figure 4). The heterodimer **a**1-α2 also represses haploid-specific genes, which includes, among others, genes necessary for mating (such as *STE4*, *STE5* and *STE12*), and RME1, a protein responsible for the inhibition of meiosis [31, 39, 40].
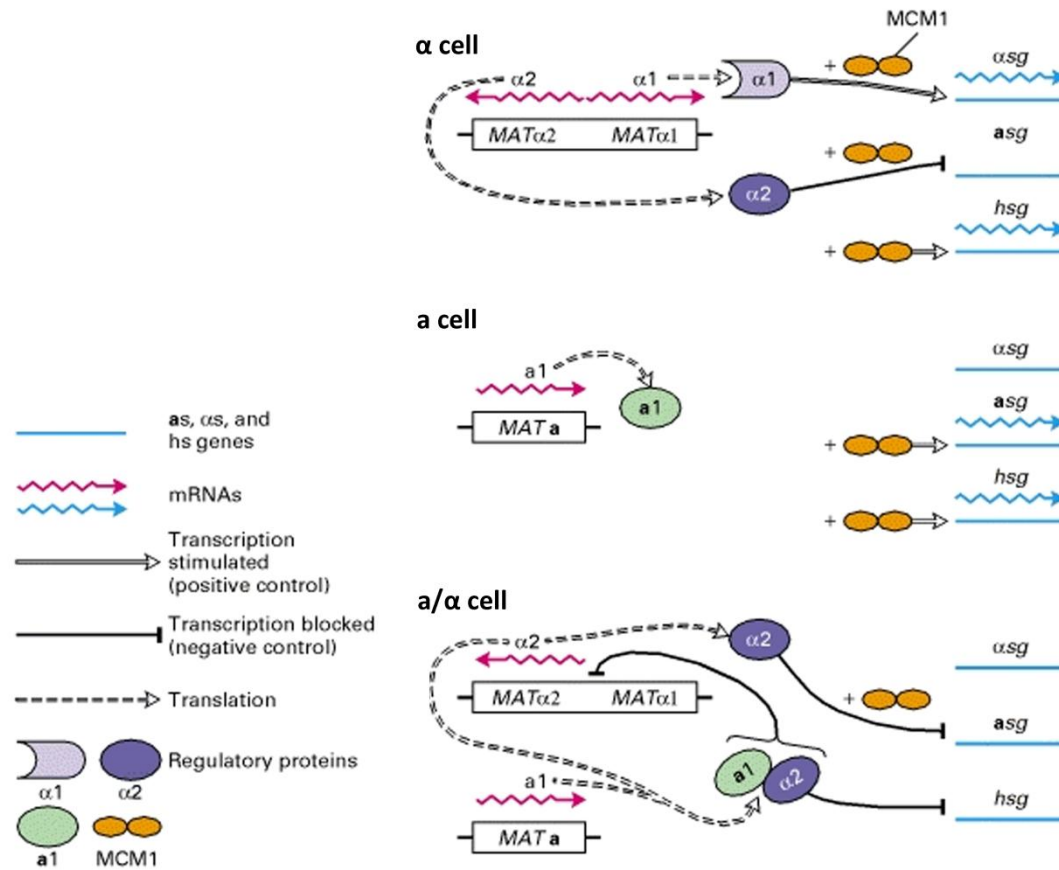
Figure 4 – Regulation of cell-type specific genes. Transcription factors encoded by each *MAT* locus act with MCM1 transcription factor to regulate the expression of **a**sg (a specific genes/mRNAs), αsg (α specific genes/mRNAa), and hsg (haploid specific genes/mRNAss) [41].

Some strains of *S. cerevisiae* are homothallic, which means that a single haploid cell can switch mating-type and undergo a self-fertile sexual cycle. As referred above, the yeast cell type is determined by the expression of specific genes at the *MAT* locus; however in *S. cerevisiae* there are also two silenced loci called *HML*α and *HMR***a** that contain non-transcribed copies of the **a** or α genes, respectively. Recombination between *MAT***a** and *HML*α or *MAT*α and *HMR***a** results in a mating-type switch [28, 39, 40]. The *ho*mothallic switching endonuclease (HO) is the enzyme responsible for this process, and is expressed only in haploid cells, particularly in mother cells [28, 36]. The gene conversion process is

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initiated by the cleavage of a double-stranded DNA, promoted by the HO endonuclease [39, 42]. The repair of the DNA break allows the copy of information from HML or HMR to *MAT*, allowing mating between two strains initially of the same mating-type [39]. *S. cerevisiae* has been used as a model for the study of mating in other fungi, but recent studies on human fungal pathogens showed novel mating paradigms that differ substantially from the *S. cerevisiae* model [29].

1.2.1.2. Mating regulation in *Candida albicans*

Candida species are the most common human fungal pathogens, with *C. albicans* being responsible for about half of all *Candida* infections. *C. albicans* is a commensal organism in the gastrointestinal tract, oral and vaginal mucosa, and is present in about 70% of healthy individuals [43]. However, it is an opportunistic fungus, that has the ability to cause both local mucosa infections and systemic infections, in particular in immunocompromised individuals [44].

C. albicans belongs to the phylum Ascomycota, Saccharomycetaceae family [43], and in this sense is closely related to *S. cerevisiae*. *C. albicans* exists naturally as a diploid yeast and for more than 100 years was considered an asexual organism [45]. Hull and Johnson [46], describe mating-type like (*MTL*) loci, homologous to the *MAT* loci present in the yeast *S. cerevisiae*. *MTL* loci encode transcriptional regulators, similar to those in *S. cerevisiae*; **a**1, α1, α2, and an additional regulator **a**2, an HMG domain protein, also present in *S. cerevisiae*, but have no known function in sexual reproduction (Figure 5) [28, 47]. Both idiomorphs, *MTL***a** and *MTL*α, also encodes three additional pairs of genes, poly(A) polymerase (*PAP***a** and *PAP*α), phosphatidylinositol 4-kinase (*PIK***a** and *PIK*α), and oxysterol binding protein (*OBP***a** and *OBP*α), that are absent in *S. cerevisiae* and have no known function in mating [28, 34, 44]. In *C. albicans*, the transcription of α-specific genes is regulated by α1, and transcription of **a**-specific genes, contrary to what happens in *S. cerevisiae*, is activated by **a**2. The **a**1-α2 heterodimer inhibits the transcription of **a**- and α-specific genes for mating, and consequently, **a**/α diploids cells are unable to mate [28, 44].

Hull et al. [48] by the disruption of the genes of one *MTL* loci from a diploid **a**/α laboratory strain, SC5314, constructed both **a**- and α-type strains, demonstrating that *C. albicans* is able to mate *in vivo*. Magee and Magee [49], constructed **a**/**a** and α/α homozygous strains (equivalent to *S. cerevisiae* **a** and α cells, respectively), by the loss of

one copy of chromosome 5 (which contains *MTL* loci), and demonstrated that *C. albicans* can also mate *in vitro*, although at low efficiency rates.
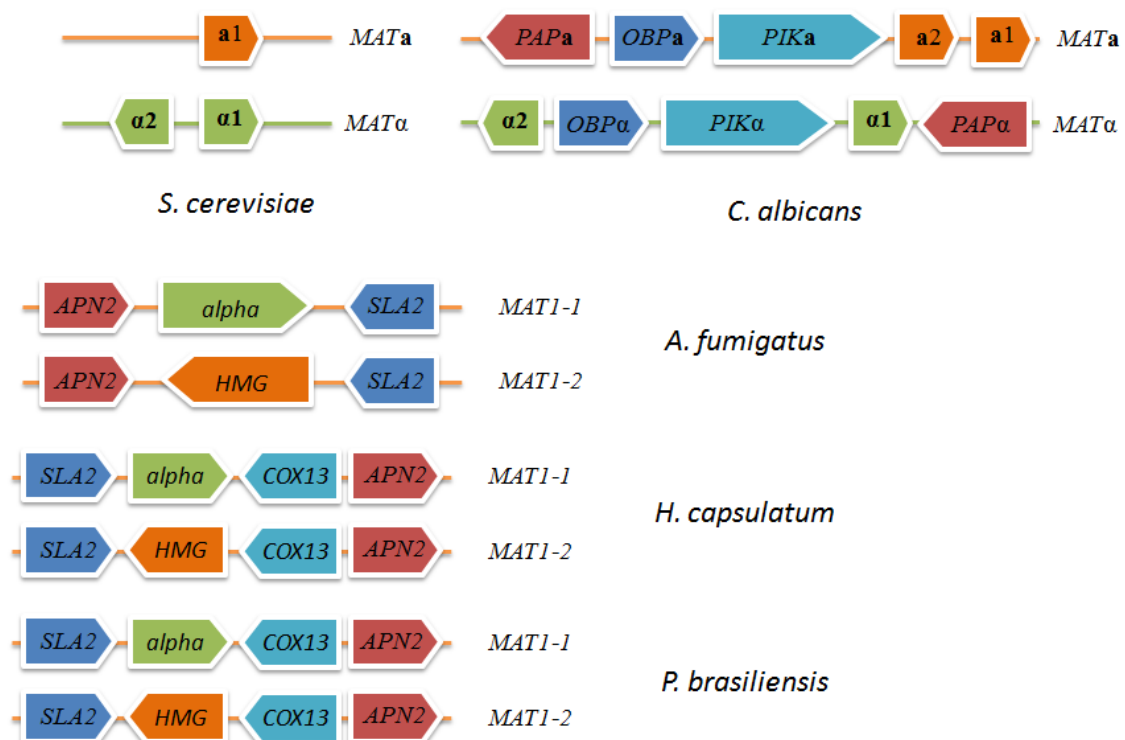
Figure 5 – Organization of *MAT* locus from different Ascomycetes species.

Subsequent analyses by Miller and Johnson [50] showed that *C. albicans* switch from white to the opaque form to become mating competent. Opaque-form **a** and α cells mate about 10^6 times more efficiently than white-form cells [50]. White-form cells exhibit a round shape and dome-shaped white colonies, while opaque-form cells are elongated and form flatter and darker colonies [47, 51]. Besides the morphological differences between these two types of cells, they also show differences in virulence. White-form cells are more virulent in systemic infections while opaque-form cells are more efficient in colonization of the skin [52]. As described above, *C. albicans* is usually present in the gastrointestinal tract, oral and vaginal mucosa (at 37°C). However, opaque-form cells were also able to mate on the skin of baby mice, at a temperature of 31.5°C similar to the human skin. The fact that *C. albicans* opaque-form cells are transiently present on the human skin suggests that they can undergo mating and then either return to the same individual or be transmitted to a new host [28].

This phenotypic transition is negatively regulated by the a1/α2 heterodimer, which blocks diploid cells from mating, unless they undergo homozygosis to yield a/a or α/α cell types.

After the mating between two diploid strains, a tetraploid strain is produced. However, contrary to what is known in *S. cerevisiae*, instead of meiosis, the process responsible for generating 2N cells from 4N cells is a parasexual cycle, involving the loss of a chromosome [29, 45].

Microscopy analysis showed that, when opaque **a** and α cells are mixed, they communicate with each other through pheromone signaling. It was already reported the identification of a pheromone present only in α cells [44]. By homology to *S. cerevisiae* it was possible to identify in *C. albicans* Ste2 (α-pheromone receptor) and Ste3 (**a**-pheromone receptor) [44, 53]. Dignard et al. [54], were able to identify and characterize *MF***a**1, the gene responsible to encode **a**-pheromone.

It was shown that opaque **a** cells, when in contact with α peptide, form mating projections, and a quantitative polymerase chain reaction showed that, *MF***a** and *MF*α were highly induced, contrary to what happens in *S. cerevisiae* [44, 55].

Alby et al. [55], reported that **a** cells produce both **a** and α pheromones, and when Bar1 (a protease present at **a** cells and responsible for the degradation of α pheromone) is absent, **a** cells produce and respond to α pheromone by an autocrine signaling. The **a**-**a** same-sex mating can also be stimulated when a minority of α cells is present as a source of α pheromone.

Taken together, these results indicated that *C. albicans* is also capable of homothallic mating between cells of the same mating-type, but that *C. albicans* strains do not undergo mating-type switching as is the case for *S. cerevisiae*.

1.2.1.3. Mating in *Aspergillus fumigatus*

The genus *Aspergillus* represents some of the most common fungi found in the environment, with only a small number of species related to human disease [34]. *A. fumigatus* is an opportunistic human pathogen, that belongs to the phylum Ascomycota, family Eurotiales [34]. It can cause potentially lethal invasive infections in immunocompromised individuals [56]. Like all the other *Aspergillus* species, it produces conidia that correspond to the infectious propagules, that when released into the atmosphere can be inhaled by the host [28, 29, 57].

In filamentous fungi, like *A. fumigatus*, the classification of the single mating-type locus is *MAT1*. Turgeon and Yoder [58] proposed a standard *MAT* nomenclature in which the two idiomorphs are designated by *MAT1-1* and *MAT1-2*, highly divergent in sequence.

MAT1-1 and *MAT1-2* are distinguished from each other by the presence a characteristic alpha box motif, and a single ORF encoding a HMG gene, respectively (Figure 5).

Analysis of the completed *A. fumigatus* genome sequence showed the presence of a homologue of the *MAT1-2* gene, which encodes a HMG-type protein showing high similarity with mating-type proteins of other filamentous ascomycetes that undergo sexual reproduction [57, 59]. However, *MAT1-1* idiomorphs were not identified. Other mating-related genes were also found including a α pheromone precursor gene *ppgA* (highly homologues to *Neurospora crassa* and *Sordaria macrospora* pheromone precursor gene *ppg1*) as well as α and **a**-pheromone receptor genes homologous to those from *S. cerevisiae*. [56, 58] The genome organization of the *MAT* locus showed a conserved synteny with the *MAT* locus of *A. nidulans*, an *Aspergillus* species with a defined sexual cycle [60].

More recently, Paoletti et al. [56] demonstrated the existence of a *MAT1-1* idiomorph. By performing a multiplex-PCR assay, they revealed the presence of *MAT1-1* and *MAT1-2* in similar proportions, on 290 clinical and environmental isolates. In this study, they demonstrate that the expression of pheromone receptors genes (*PREA* – **a**-pheromone receptor; and *PREB* – α-pheromone receptor) had no clear difference between the two mating isolates. In contrast, the expression levels of α-pheromone precursor were higher in the *MAT1-1* isolates.

O'Gorman et al. [61] demonstrated that *A. fumigatus* can undergo sexual reproduction under certain conditions. They obtained mature cleistothecia after 6 months of incubation at 30°C in the dark.

The findings concerning the sexual reproduction in *A. fumigatus* are very important to understand the biology and evolution of the species, and gives new insights into the elucidation of sexual cycles of other fungi thought to be asexual.

1.2.1.4. Mating in *Histoplasma capsulatum*

Histoplasma capsulatum is a dimorphic fungus that produces airborne conidia or hyphal fragments, which are reverted to the pathogenic yeast form at host temperature [62]. Once inhaled and hosted in the lung it can cause acute pulmonary disease and, in some cases, disseminated disease and death [63]. *H. capsulatum* can be found worldwide and its saprobic phase is associated with soil enriched with guano of bat and bird species,

specially of starlings (*Stumus vulgaris*) [62]. *H. capsulatum* belongs to the phylum Ascomycota, order Onygenales, and the new family Ajellomycetaceae.

Kwong-Chung [64] defined phenotypically, two mating-types of *H. capsulatum*, designated by (+) and (–). Mating between two opposite mating-type strains was confirmed, under laboratory conditions, using the mycelial form of fresh isolates [64]. An increase on pathogenicity of *H. capsulatum* seems to be related to the strains of the (–) mating-type. Samples from patients with acute pulmonary disease showed a higher frequency of strains with (–) mating-type, while samples from patients with disseminated disease and environmental samples had a balanced ratio of both mating-types [65, 66].

Recently, Bubnick et al. [63] correlated the differences of mating-types defined by phenotype with genotypic mating-types designation. Through comparative analysis of the *H. capsulatum* genome sequence available at two websites and *MAT* loci already identified in other Ascomycetes, they identified predicted *MAT1-1* and *MAT1-2* idiomorphs (Figure 5). The strains G217B and WU24, contained a region with high sequence identity and high similarity to the *Aspergillus nidulans* α1 region (found previously), designated by *MAT1-1-1* [63, 67].

Since *H. capsulatum* is a heterothallic fungus and the strain G186AR did not show sequence similarity with *A. nidulans* α1 region, they performed a search for *MAT1-2* idiomorph in its genome. In ascomycetes, the *MAT* idiomorphic regions are flanked by regions of homology (Figure 5). In this sense, performing a BLASTN analysis they found a region, flanked by regions with more than 95% of sequence similarity when compared to flanked regions from *MAT1-1* sequence. The *MAT1-2* idiomorph encode a predicted HMG DNA-binding domain. Based on this information they designated the mating-type of the strain G186AR as *MAT1-2*. T-3-1 is a known phenotypic (–) mating-type strain that contains the *MAT1-2* idiomorph of the mating locus. By performing mating assays they could not obtain asci or ascospores, nevertheless they observed structures associated with the formation of the ascocarp [63, 64].

Laskowski et al. found homologous sequences of *STE2* (α pheromone receptor) and *STE3* (**a** pheromone receptor). They showed that strain G217B (*MAT1-1*) express *STE2*, but not *STE3*, which could indicate that strains of *MAT1-1* mating-type respond to α pheromone [68]. They were also able to identify a putative pheromone gene, designated *ppg1*, but an **a** pheromone gene was not yet identified in *H. capsulatum*.

1.2.1.5. Mating in *Cryptococcus neoformans*

In basidiomycetes the sexual reproduction is also orchestrated by the *MAT* locus, however, while ascomycetes are bipolar (two mating-types), more than 50% of basidiomycetes are tetrapolar. Fungi with a tetrapolar system need two unlinked chromosomal loci, with differences in both alleles, to complete a sexual cycle. The *MAT* locus can be multiallelic, leading to thousands of different mating-types in some mushroom fungi [28, 69].

In basidiomycetes, after the recognition of two compatible mating-types and consequent cell fusion, the nuclear fusion is delayed. These fungi are able to have stable dikaryotic hyphae, which correspond to their predominant vegetative phase [28, 31].

Basidiomycota phyla include the specie *Cryptococcus neoformans*, which is one of the most common human fungal pathogen [34].

Cryptococcus neoformans is an opportunistic fungus, responsible for most cases of meningoencephalitis in immunocompromised patients [69]. It can be found in pigeon guano and trees and the infection begins with the inhalation of airborne propagules that can colonize the host respiratory tract and after can spread to other body sites, specially the brain. However, the infection is usually asymptomatic and it can be either cleared or latent until the immune system of the host is compromised [70].

C. neoformans belongs to the phylum Basidiomycota, being more related to mushroom fungi and *Ustilago maydis* (plant pathogen) than to ascomycetes, like *S. cerevisiae* and many other common fungal pathogens, including *C. albicans*, *A. fumigatus*, and *H. capsulatum* [71]. It is a dimorphic haploid fungus that during the vegetative growth and infection proliferates as budding yeast, and switches to hyphae during mating [72].

Sexual reproduction in *C. neoformans* is related to its virulence, since it is necessary for the production of spores, its infectious form [29]. The sexual cycle in *C. neoformans* is known for more than 30 years, and starts in response to nutritional limitations [64, 73]. It has a bipolar mating system, controlled by two opposite mating-types, *MAT***a** and *MAT*α. Contrarily to ascomycetes, *C. neoformans MAT* locus encodes several mating-type specific genes, namely pheromone/pheromone receptor and homeodomain genes [69, 73, 74].

In *C. neoformans* three copies of the alpha pheromone gene were identified (*MFα1*, *MFα2*, and *MFα3*) [74, 75]. The same study reported a *MAT*α locus with a size of approximately 50kb, being one of the largest *MAT* loci among fungi. Moreover, they

reported that the *MATα* locus from *C. neoformans* encodes a putative pheromone receptor gene for *MF***a** pheromone, designated by *CPRα*, which is located adjacent to *STE12α*. This gene showed a high degree of homology to pheromone receptors identified on other basidiomycetes fungi, such as *Coprinus cinereus*, *Ustilago maydis*, and *Schizophyllum commune*. Subsequently, Chung et al. [76] isolated and characterized the *CPRα* gene, showing that this gene plays an important role in mating. More recently, Chang et al. [77] identified and characterized a putative pheromone receptor from a *MAT***a** strain of *C. neoformans*, designated by *CPR***a**, which is located adjacent to *STE12***a**. In order to understand the role of *CPR***a**, they performed mating assays, and the results suggested that *CPR***a** is involved in the mating pathway. They also demonstrated that the expression of at least one of the pheromone receptors in either mating-type is required for *C. neoformans* undergo sexual reproduction [77].

*MAT***a** specific pheromones were identified and characterized by McClelland et al. [78]. The putative *C. neoformans* pheromone genes identified were designated by *MF***a***1*, *MF***a***2*, and *MF***a***3*. The characterization of *MF***a***1* showed that seems to be structurally similar to the **a**-factor from *S. cerevisiae* and present conserved amino acid regions to *MFα1*.

The *MATα* and *MAT***a** loci from *C. neoformans*, in addition to pheromone and pheromone receptors, also encode homeodomain (HD) factors that have been shown to be important in the control of cell identity and sexual development [79, 80]. Hull et al. [80] identified, in *MATα* cells, an α specific HD factor, named Sex inducer 1α (Sxi1α), and observed that the induction of the sexual development requires at least one **a**-specific component, however, they were not able to find it. More recently [79], using a combination of molecular genetics and bioinformatics, they identified an HD factor present at *MAT* locus from **a** cells, designated by Sxi2**a**. Moreover, the same study showed that the direct interaction between these two HD factors is essential to regulate the transcription of mating-related genes and to the induction of sexual reproduction.

Several studies correlated the mating-type with the virulence of *C. neoformans*. The *MATα* idiomorph has a predominance of >95% in the *C. neoformans* population and it has been shown that α cells are more virulent than **a** cells [29, 73, 81].

1.2.2. Biogenesis of the mating pheromones

In *S. cerevisiae*, the cells signal each other through the production of mating pheromones (**a** and α-factor) in order to stimulate the signal transduction pathway that leads to mating (detailed in the previous chapters). Before being secreted from the cell, both pheromones are generated from larger precursors, which are subsequently modified by posttranslational modifications and proteolysis to their mature form [82]. Despite their functional equivalence role in the cell response to mating, pheromones exhibit quite dissimilar biosynthesis.

S. cerevisiae mature α pheromone is a peptide of 13 amino acids (WHWLGLKPGQPMY), generated from larger precursors encoded by two genes, *MFα1* and *MFα2*, which contain tandem repeats of the mature α-pheromone [83-85]. In this sense, while *MFα1* contains four α-factor repeats, encoding a precursor protein with 165 amino acids, *MFα2* contains only two copies of the pheromone, encoding a protein with 120 amino acids [83, 84]. The precursor proteins are composed at the N-terminal, by a signal sequence followed by a proregion containing three recognition sites for N-glycosylation, and at the C-terminal, by the region containing the α-pheromone repeats separated from each other by connecting regions (Figure 6) [86-88]. The precursor proteins are translocated to the endoplasmic reticulum where the secretion signal is cleaved, producing the α pro-pheromone. The pro-region is extensively glycosylated and then, the α pro-pheromone is transported to the Golgi apparatus, where three proteolytic steps occur [86]. First, the Kex2 protease removes the proregion, by cleaving after the conserved lysine-arginine (KR) residues. Kex1 and Ste13 complete the maturation process by removing the connecting regions (Figure 6) [89, 90]. The mature α pheromone is then exported via the classical secretory pathway.

S. cerevisiae mature **a** pheromone is a peptide of 12 amino acids, which results from a posttranslational maturation of a precursor protein encoded by two genes, *MF**a**1* and *MF**a**2*. The precursor proteins encoded by these two genes have 36 and 38 amino acids length, respectively, containing a N-terminal extension, a conserved C-terminal CAAX motif (C =cysteine, A = aliphatic amino acid, X = any amino acid) and a single mature **a**-pheromone sequence. Following synthesis, the processing of the **a** propheromone starts in the cytosol, by the farnesylation of a cysteine residue, in the CAAX motif, by Rma1 and Ram2 proteins. Subsequently, the action of Rce1 or Ste24 mediates the proteolytic

cleavage of AAX residues. Afterwards occurs the final step in modification of CAAX motif, which corresponds to the methylation of the cysteine, mediated by Ste14. The processing of the N-terminal starts with a proteolytic event, performed by Ste24, that removes the first seven residues, followed by the action of Axl1 that cleaves the others fourteen residues. The export of the mature **a** pheromone is mediated by the Ste6 transporter [87, 91, 92].

The presence of two types of pheromones, one prenylated and one unmodified, appears to be conserved amongst the ascomycetes. The presence of unmodified pheromones in several fungi is discussed below.

The mature α pheromone sequence from *C. albicans* has no conservation at the amino acid level compared with α pheromone from *S. cerevisiae*, however, the processing sites of the *C. albicans* pheromone are highly conserved. The conceptually translated protein of *C. albicans* contains a hydrophobic leader sequence, processing sites for the serine proteinase Kex2, and three copies of mature α pheromone. The processing of the α pheromone precursor requires the *KEX2* gene to the *C. albicans* cells become mating competent [53].

The identification of a gene (*ppgA*) encoding a α pheromone precursor from *A. fumigatus* have shown similar flanking regions with *S. cerevisiae* precursor protein. Each one of the two copies of the mature α pheromone has at C-terminal a processing site for the Kex2 protease, the KR motif [57]. These repeats are preceded by the motif XP, which is a substrate for Ste13 in *S. cerevisiae*. At the N-terminal is possible to observe a hydrophobic signal sequence [57]. In addition, the mature nonapeptide pheromone (WCHLPGQGC) is highly similar to other filamentous ascomycetes pheromones [57]. In fact, the presence of several conserved amino acids found in fungal α pheromones overlaps the difficulties related to their small size and dissimilar sequence.

Analyzing the flanking regions of α pheromone locus in *A. fumigatus* it was possible to identify, by genome synteny, a putative α pheromone in *H. capsulatum*. The α pheromone sequence from *H. capsulatum* also show common features to known α pheromones from other fungi [93].
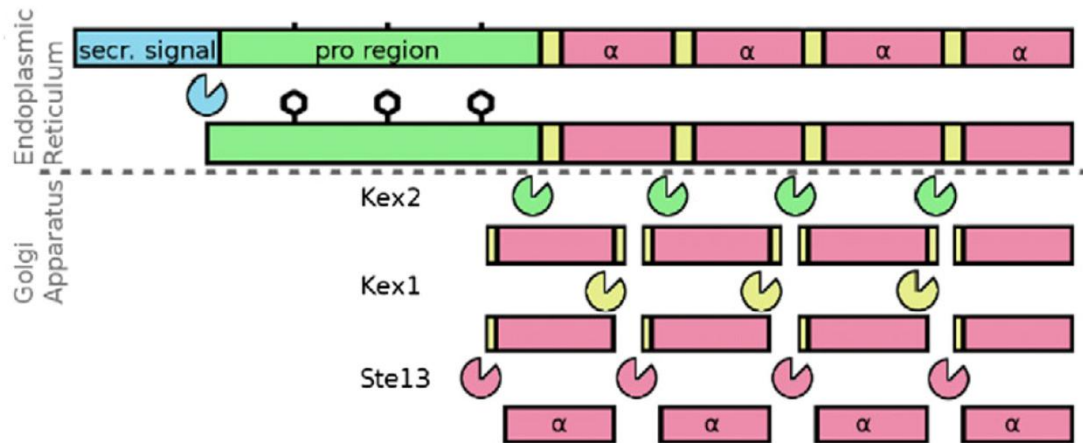
Figure 6 – **Processing of *S. cerevisia*e α mating pheromone.** The α propheromone is produced after the cleavage of the secretion signal. After the glycosylation, the α propheromone is transported from the endoplasmic reticulum to the golgi apparatus where three proteolytic steps leads to the production of the mature α factor [87].

1.2.3. Intracellular mating signaling pathway

The pheromones produced by each mating-type are recognized by one of two specific G protein-coupled transmembrane receptors (GPCRs) – named Ste2 and Ste3 in *S. cerevisiae* - that lead to the activation of a signal transduction cascade that includes a MAPK pathway (Figure 7) [31, 37, 87]. In *S. cerevisiae*, this induction promotes a dissociation of the three subunits of G-protein, G_α(Gpa1), G_β(Ste4) and G_γ(Ste18) [87]. While G_α subunit is responsible for the exchange of GTP for GDP, the $G_{\beta\gamma}$ dimer recruits Ste5 to the plasma membrane, promoting the activation of the MAP kinase cascade and mediating all the physiological responses induced by the pheromone-receptor interaction [40]. Ste5 is a scaffolding protein that has no catalytic domains and that supports Ste11, Ste7, and Fus3 [34, 40]. Ste20, a p21-activated protein kinase, is responsible for the direct phosphorylation and activation of scaffold-bound Ste11. This activation is also assisted by Ste50 that acts as an adaptor of Ste20 to the effector Ste11 [94]. The activated Ste11, a MEK kinase, phosphorylates and activates Ste7, a MAP kinase kinase, which is responsible for the phosphorylation and activation of Fus3, a MAP kinase (MAPK) (Figure 7).

Fus3 has two main roles: activate the expression of mating-related genes and promote cell cycle arrest. Accumulation in the nucleus leads to the inactivation of Dig1 and Dig2,

negative regulators of Ste12, a transcription factor that regulates the expression of mating-related genes, by binding the MCM1 to **a** and α gene-specific promoters [31, 34, 40, 87]. Fus3 also phosphorylate Far1, which has been reported to mediate cell cycle arrest in response to pheromone [95].

MAP kinase modules play a very important role in intracellular signal transduction pathways and are very conserved among diverse eukaryotes, including fungi [96]. Among these conserved MAP kinase pathways is the one involved in the mating response.

Even before the observation of a sexual cycle in *C. albicans* [46], some studies concerning the conserved mating signaling pathway cascade were performed in order to identify orthologs genes to *S. cerevisiae* mating pathway. A putative heterotrimeric G-protein gene, *CAG1*, with high homology to *GPA1* from *S. cerevisiae* was identified and characterized [97]. They performed a mating assay, and concluded that *CAG1* gene complemented the mating defects of *gpa1 S. cerevisiae* mutant.

Interestingly *C. albicans* encodes two homologous genes with sequence identity to *S. cerevisiae FUS3* and *KSS1* (Cek2 and Cek1, respectively). *CEK2* gene was able to complement *S. cerevisiae fus3/kss1* mutant, while the *CEK1* gene was no able to do so. Therefore, it was concluded that *CEK2* is a homolog of *FUS3* from *S. cerevisiae* [98, 99]. The identification of a *STE5* homolog (*CST5*) and its relation with pheromone response were recently published [100, 101].

Clark et al. [102] found a gene, *HST7*, which encodes a structurally similar protein to the *S. cerevisiae* MAP kinase kinase Ste7. Performing mating assays, they observed that Hst7 can efficiently complement the ΔSTE7 mutation. A subsequent study showed that *C. albicans CST20* gene is highly identical to the *S. cerevisiae STE20* [103]. As it was observed for *CAG1* and *HST7*, *CST20* had also the ability to fully complement the mating defect of *S. cerevisiae* Δ*STE20*. The same results were obtained to *CPH1* gene from *C. albicans*, homologous to *STE12* from *S. cerevisiae* [104]. The identification of *FAR1* in *C. albicans* showed that *C. albicans FAR1* plays a central role in the pheromone response [105, 106].

More recently, a study performed by Chen et al. [107] showed that the levels in which Cst20, Hst7, Cek1, Cek2 and Cph1 are required for mating in *C. albicans* are parallel to that of their homologues in the intracellular signaling pathway of *S. cerevisiae* (Figure 7). These findings means that *C. albicans* requires the same MAPK pathway as *S. cerevisiae* to respond to mating pheromones.

A Pheromone response pathway in *S. cerevisiae*

B Described genes in *S. cerevisiae*	C Homologs genes in *C. albicans*	D Homologs genes in *A. fumigatus*	E Homologs genes in *P. brasiliensis*
Ste2	CaSte2	PreB	PreB
Ste3	CaSte3	PreA	PreA
Gpa1	Cag1	GpaA	PbGpa1
Ste4	CaSte4	SfaD	PbSte4
Ste18	CaSte18	GpgA	PbSte18
Ste20	Cst20	AfuSte20	PbSte20
Ste5	Cst5	N.A.	N.A.
Ste50	CaSte50	AfuSte50	PbSte50
Ste11	CaSte11	SteC	PbSte11
Ste7	Hst7	AfuSte7	PbSte7
Kss1	Cek1	MpkB	PbFus3/Kss1
Fus3	Cek2		
Ste12	Cph1	SteA	PbSte12
Far1	CaFar1	N.A.	N.A.

Figure 7 – Intracellular signaling pathway. (A, B) Pheromone response pathway in *S. cerevisiae* (adapted from [34]). (C) Homologous genes of the pheromone response pathway in *C. albicans*. Each of these genes has been functionally characterized for their role in mating. (D) Homologous genes of the pheromone response pathway in *A. fumigatus*. Contrarily to *C. albicans*, the functions in sexual reproduction of most of the genes involved in the pheromone response pathway identified in *A. fumigatus* were not confirmed. (E) Homologous genes of the pheromone response pathway in *P. brasiliensis* obtained by bioinformatics analysis. N.A. – not annotated.

Although there are some studies concerning the identification of genes involved in the intracellular signaling pathways from *A. fumigatus*, their functions in sexual reproduction are not clear. However, genomic analysis performed in *A. fumigatus* have shown genes homologous to *S. cerevisiae* and *Aspergillus nidulans* genes involved in the pheromone responsive pathway.

Liebmann et al. identified a gene encoding a G-protein α subunit, designated by GpaA [108]. The *GPAA* gene sequence is 98% identical to *A. nidulans FADA* [108]. The other two heterotrimeric G-protein components (SfaD [Gβ subunit] and GpgA [Gγ subunit]), a Ste20 homolog, SteC (Ste11 homolog), a Ste7 homolog, MpkB (Fus3 homolog), SteA (Ste12 homolog) were identified in *A. fumigatus* (Figure 7) and show high levels of homology with *A. nidulans* and *S. cerevisiae* orthologs [60].

During the sexual development of *A. nidulans* the expression levels of the subunits of the heterotrimeric G-protein FadA, SfaD, GpgA, SteC, Ste7 equivalent, MpkB and a protein regulator (Ste50 equivalent) were increased [109].

In this sense it can be predicted that some of these genes, important for sexual reproduction in *A. nidulans*, also plays a role in sexual reproduction and pheromone responses of *A. fumigatus*.

The discovery of *C. neoformans* sexual cycle led to an increase effort in the identification of regulatory molecules and mating inducing conditions. The identification of *C. neoformans MAT* locus showed that it harbors more than twenty genes and that many of them are involved in mating [69].

The GPCRs and their cognate heterotrimeric G proteins (Gpa1, Ste4 and Ste18 in *S. cerevisiae*) are responsible to respond to a panoply of extracellular stimuli, including pheromones. A study reported that as a consequence to the cell fate choice between budding yeast growth and asexual or sexual filamentous growth, *C. neoformans* have two Gα protein subunits involved in sexual development [110]. Both Gα subunits, designated by Gpa2 and Gpa3, were identified in the genome sequence and share homology with *S. cerevisiae* Gpa1. They have shown that the principal role of activated form of Gpa3 is to inhibit mating in environments in which there is no mating partner present, and the active form of Gpa2 contributes to the pheromone response that leads to mating [110]. The identified putative *STE18* homolog gene, *GPG2*, in *C. neoformans* [111], is a γ subunit essential for pheromone signaling [110]. A Gβ subunit, Gpb1, was identified and is identical to *S. cerevisiae* Ste4. Wang et al. demonstrated that Gpb1 plays an important role in mating by activating the MAP kinase cascade that leads to conjugation tube formation in both *MAT***a** and *MAT*α cells [112].

Nichols et al. found that *Ste20***a** and *Ste20*α genes, which are located at the *MAT***a** and *MAT*α locus, respectively, play a role in mating since *ste20* mutants are unable to undergo sexual reproduction [113, 114]. It has been already shown that *MAT*α cells are more virulent than *MAT***a** cells and a study showed that a clinical isolate deleted for *STE20*α is less virulent in animal models when compared to wild type [114].

A *S. cerevisiae STE11* homolog gene was identified in the *MAT*α and *MAT***a** locus, and *C. neoformans ste11*α mutants were sterile, as is the case in *S. cerevisiae* [115].

By genomic sequence analysis it was possible to identify *CPK1* and *STE7* genes. Cpk1 revealed a significant identity with Fus3 (52%) and Kss1 (51%) of *S. cerevisiae* and Cek1 (56%) of *C. albicans*. Ste7 revealed a significant identity with Ste7 (39%) of *S. cerevisiae* and Hst7 (37%) of *C. albicans*. Contrary to other genes involved in the intracellular pheromone response (*STE20***a**/α, *STE11***a**/α, and *STE12***a**/α), *CPK1* and *STE7* genes are

not mating-type specific, which means that both are present in *MAT***a** and *MAT*α strains. In addition, *CPK1* and *STE7* gene are not contained within the *MAT* locus [116].

Molecular analysis to the hyphae production in *MAT*α *C. neoformans* allowed the identification of a gene, designated *STE12*α, homolog to *STE12* of *S. cerevisiae* and present only in *MAT*α strains [117]. A *STE12* specific of *MAT***a** strains, named *STE12***a**, was also identified [118]. Both genes are related to the virulence of *C. neoformans* [117, 118].

Concerning *P. brasiliensis* mating intracellular pathway, recent genome annotations of three *Paracoccidioides* isolates showed the presence of conserved mating and meiosis specific genes [25]. However, no tests were performed to confirm the functional homology (Figure 7).

1.2.4. Mating in *Paracoccidioides brasiliensis*

The sexual cycle in *P. brasiliensis* until now has not been described. However, there are some studies that show the presence of *MAT* loci in *P. brasiliensis*. Li et al. [119], identified two *MAT* idiomorphs, *MAT1-1*, which contains an α domain gene, and *MAT1-2*, which contains an HMG domain (Figure 5). Torres et al. [120], identified the mating-type idiomorphs in 71 *P. brasiliensis* isolates from various sources, and explored the basal expression of *MAT* gene in some strains in yeast/mycelial form, and found that their expression is low.

Taking advantage of GenBank database and BLAST tools, the sequence analyzes showed that *MAT1-1* and *MAT1-2* idiomorphs of *P. brasiliensis* show a high homology to homologous genes from *Histoplasma capsulatum* [120]. Synteny analysis also revealed common genome features of the *MAT* locus of *H. capsulatum* and *P. brasiliensis*, where *MAT1-1* and *MAT1-2* are tightly linked with *SLA2*, *COX13*, and *APN2* genes on both species (Figure 5) [119].

Torres et al. performed mating assays with isolates from different mating-types, but they were not able to demonstrate *in vitro* mating [120]. However, equivalent distribution of the two mating-types in *P. brasiliensis* population [120], the presence of mating-related genes on *P. brasiliensis* genome [119] and the fact that some species phylogenetic related

(e.g. *H. capsulatum*) have a defined sexual cycle [28], lead us to consider that maybe *P. brasiliensis* also has the ability to undergo sexual reproduction.

Having in mind all these subjects, the identification and knowledge concerning the *MAT* locus in pathogenic fungi and evolutionary studies are of main importance, not only to understand their pathogenicity but also to elucidate both the ancestral and evolving organization of mating systems [26].

1.3. Aims

The identification of sexual competence in the *Paracoccidioides* genus is an important issue for understanding the ecology and evolution of this fungus. Although the *P. brasiliensis* genome encodes heterothallic mating loci and gene homologues of all mating-signaling pathway components, as referred before, there has been no confirmation for the actual existence of a sexual cycle in this fungus. As it has been shown, mating in most fungi is regulated via the pheromone signaling MAP kinase pathway. We intend to characterize the functionality and activity of the mating pathway components in *P. brasiliensis* through the:

i)   Identification of a α-pheromone encoding gene;

ii)   Evaluation of the basal expression levels of mating-related genes in *P. brasiliensis* isolates of both mating-types in the yeast and mycelial forms;

iii) Functional complementation of *Saccharomyces cerevisiae* null mutants by the heterologous expression *P. brasiliensis* α-pheromone and its respective receptor (PreB).

Using these approaches we hope within this project to provide molecular evidence for the presence of a functional mating system in the *Paracoccidioides* genus. Such data will serve as a basis for further studies aimed at unraveling the basic biological and evolutionary aspects of sexual reproduction mechanisms in *P. brasiliensis*.

Chapter II - Material and Methods

2.1. Strains and culture conditions

Strains of *P. brasiliensis* and *S. cerevisiae* used in this study are listed in Table 1.

For maintenance, *S. cerevisiae* strains were grown at 30°C on rich medium YEPD agar plates (0.5% [w/v] yeast extract, 1% [w/v] peptone, 2% [w/v] glucose, 2% [w/v] agar) or minimal medium dropout YNB agar plates (6,7% [w/v] Yeast Nitrogen Base without amino acids, 2% [w/v] glucose, 2% [w/v] agar), supplemented to meet auxotrophic requirements. For experimental procedures, *S. cerevisiae* strains were grown in YEPD or YNB broth at 26°C and 150rpm.

Table 1 – Strains used in this study.
[a] – Plasmids listed in Table 2.

Organism	Strain	Genotype[a]	Source
S. cerevisiae			
	BY4741	Wild type *MAT*a	EUROSCARF
	BY4742	Wild type *MAT*α	EUROSCARF
	BY4741 ΔSTE2	*MAT*a *STE2*::kanMX4	EUROSCARF
	BY4742 Δ*MF(α)1*	*MAT*α *MF(α)*1::kanMX4	EUROSCARF
	BY4742 Δ*MF(α)2*	*MAT*α *MF(α)*2::kanMX4	EUROSCARF
	BY4741 Δ*GPA1*	*MAT*a *GPA1*::kanMX4	EUROSCARF
	BY4741 Δ*STE4*	*MAT*a *STE4*::kanMX4	EUROSCARF
	AGScα	*MAT*α *MF(α)1*::kanMX4;*MF(α)2*::hph	This study
	AGLPbα	*MAT*α *MF(α)1*::kanMX4;*MF(α)2*::hph pLPbα	This study
	AGMPbα	*MAT*α *MF(α)1*::kanMX4;*MF(α)2*::hph pMPbα	This study
	AGLPreB	*MAT*a *STE2*::kanMX4 pLPreB	This study
	AGLPbGpa1	*MAT*a *GPA1*::kanMX4 pLPbGpa1	This study
	AGLPbSte4	*MAT*a *STE4*::kanMX4 pLPbSte4	This study
P. brasiliensis			
	Pb01	*MAT1-1*	Chronic PCM [20]
	T8B1	*MAT1-1*	Armadillo [121]
	ATCC60855	*MAT1-2*	Chronic PCM [122]
	Pb03	*MAT1-2*	Chronic PCM [123]

P. brasiliensis yeast strains were maintained at 37°C by periodic subculturing on brain heart infusion (BHI) solid media (supplemented with 1% [w/v] glucose, 1.6% [w/v] agar). For subsequent analysis, *P. brasiliensis* strains were grown as yeast cells in BHI broth at

37°C and 200rpm or as mycelium in modified synthetic McVeigh Morton broth (MMcM) [124] at 22°C and 200rpm, respectively.

E.coli strains were grown on LB medium (1% [w/v] tryptone, 0.5% [w/v] yeast extract, 1% [w/v] NaCl) at 37°C, 220rpm.

2.2. Identification of *MAT* loci in *P. brasiliensis* strains

Genomic DNA was isolated from *P. brasiliensis* yeast cells grown in BHI broth for 3 days at 37°C, 200 rpm and harvested by centrifugation (3000xg for 10 min at 4°C). For cellular disruption cells were resuspended in 200μl of lysis buffer (1mM EDTA, 10mM Tris-HCl pH 8.0, 1% SDS, 100mM NaCl) and 200μl phenol/chloroform (1:1), followed by a heat shock treatment for 45 min at 65°C − with intermittent vortexing every 10 min - and freezing for 60 min at -80°C. After centrifugation of samples for 12000xg for 15 min at 4°C, the upper phase was transferred, and an additional chloroform extraction was performed. DNA in the upper phase was precipitated with isopropanol, washed with 70% ethanol and resuspended in water. The genomic DNA was used as a template in a PCR assay to identify *MAT1-1* or *MAT1-2* loci in *P. brasiliensis* strains. PCR was performed in a 20μl reaction volume containing 1x reaction buffer, 2 mM $MgCl_2$, 200μM dNTPs, 200μM of each primer (Supplementary Table S1) and 0.5U DyNAzyme II DNA polymerase (Finnzymes). Thermal cycling conditions were: an initial denaturing step at 94°C for 10 min, followed by 35 cycles at 94°C for 30 sec, 40 sec at the best annealing temperature previous tested and at 72°C for 1min/Kb, and a final elongation cycle at 72°C for 10 min. The amplification products were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide. The mating genotype of each strain was evaluated according to the pairs of primers used.

2.3. *In silico* identification of *P. brasiliensis* mating-related genes

Identification of *P. brasiliensis* mating-related genes was performed using BLAST searches against a genome database of strains Pb01, Pb03 and Pb18, available at the Broad Institute (http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html). In order to identify the α pheromone gene from *P. brasiliensis* we performed

BLASTP and TBLASTN searches in all six reading frames using the protein sequence of the *A. capsulatus* (anamorph *H. capsulatum*) α pheromone. The **a** and α pheromone receptor genes (PreA and PreB, respectively) are annotated at the Broad Institute. The genes involved in the intracellular signaling pathway were obtained by performing a BLASTN using the known sequences of other Ascomycete fungi. All the alignments were obtained by the Bioedit Sequence Alignment Editor. The topology prediction of the α-pheromone receptors were made using TMHMM program and the pictures obtained using TMRPres2D program.

To confirm the *in silico* identified sequences for the *P. brasiliensis* α-pheromone and PreB, these respective transcript sequences were amplified from cDNA (primers – Supplementary Table S1) using a combination of DyNAzyme II and Phusion DNA polymerases (Finnzymes). The PCR products were cloned with TOPO TA Cloning Kit for sequencing (Invitrogen) following the manufacturer's protocol. The plasmid was extracted from *E. coli* strain DH5α using the QIAprep Spin Mniprep Kit (Qiagen) and sequenced at STAB VIDA (Portugal).

2.4. Real-time-PCR analysis of mating gene expression in *P. brasiliensis* strains

Total RNA was extracted from *P. brasiliensis* yeast and mycelial cells using Trizol (Invitrogen) standard method for cellular disruption, complemented with heat shock treatment (45 min at 65°C followed by 60 min at -80°C) and bead-beating using glass beads. Total RNA (10μg) was treated with DNase I (Ambion) and DNA-free total RNA (1μg) reverse transcribed using the DyNAmo™ cDNA Synthesis Kit (Finnzymes). cDNA samples were used as templates in order to measure the basal level expression of the *MAT1-1*, *MAT1-2*, *preA*, *preB* and α pheromone genes in yeast and mycelial cells. Quantitative RT-PCR (qRT-PCR) was performed on the CFX96 Real-Time PCR Detection System (Bio-rad), and qRT-PCR amplification performed using the SsoFast EvaGreen Supermix kit (Bio-rad), according to manufacturer's protocol. The thermal cycling conditions comprised: an enzyme activation step at 95°C for 30sec, followed by 35 cycles at 95°C for 5 sec and at 57°C for 5sec.

All measurements were performed in triplicate and relative expression levels determined using the ΔC_T method [125] versus *TUB2* as a reference gene. The primers used are listed in Supplementary Table S1.

2.5. Heterologous expression of *P. brasiliensis* mating-related genes in *S. cerevisiae*

2.5.1. Construction of a *MF(α)1/2* double mutant strain of *S. cerevisiae*

For heterologous expression of the *P. brasiliensis* α-pheromone in *S. cerevisiae* a double mutant for both α-pheromones *MF(α)1* and *MF(α)2* genes was constructed in *S. cerevisiae*.

Plasmid pAG34 was digested with restriction enzyme XhoI (FastDigest, Fermentas) for 2h at 37°C, and subsequently transformed in strain BY4742 Δ*MF(α)2*, which has the *MF(α)2* gene substituted by a KanMX cassette. After transformation with linear pAG34, the KanMX cassette in gene *MF(α)2* was substituted by the HPH cassette (hygromycin B) via homologous recombination at the TEF promoter and terminator (AGScα strain). Subsequently, the HPH cassette with *MF(α)2* flanking regions (including TEF promoter and terminator for HPH) was amplified (Primers used are listed in Supplementary Table S1), using the Phusion DNA Polymerase (Finnzymes), and transformed in strain BY4742 Δ*MF(α)1* . Double mutants for the *MF(α)1/2* genes of *S. cerevisiae* were selected on selective plates (hygromycin and/or geneticin) and confirmed by PCR (Primers used are listed in Supplementary Table S1), using DyNAzyme II DNA polymerase (Finnzymes) and genomic DNA as a template, and by growth in medium supplemented with the respective antibiotic.

2.5.2. Construction of heterologous expression plasmids

All vectors reported in this study are listed in Table 2 and were extracted from *E.coli* using the QIAprep Spin Miniprep Kit (Qiagen). The heterologous expression of *P. brasiliensis* mating-related genes was performed using tetracycline-inducible expression vectors, namely pCM189 (low-copy) and pCM190 (multicopy), containing *URA3* as a uracil marker. *P. brasiliensis* α-pheromone and *PREB* genes were amplified from mycelium cDNA, using the Phusion DNA Polymerase (Finnzymes), and cloned by homologous recombination (Primers used are listed in Supplementary Table S1) into the respective vector. The primers used to amplify the sequences had a short sequence homologous to the plasmid. *PREB* was cloned in a low copy vector, designated by

pLPreB. *P. brasiliensis* α-pheromone was cloned in low and multicopy vectors, designated by pLPbα and pMPbα, respectively. The transformants were confirmed by PCR (Primers used are listed in Supplementary Table S1).

Table 2 – Plasmids used in this study.

Plasmid	Construction	Resistance/auxotrophic marker	Source
pAG34	-	Ampicillin; Hygromycin	EUROSCARF
pLPbα	pCM189::Pbα	Ampicillin/Uracil	This study
pMPbα	pCM190::Pbα	Ampicillin/Uracil	This study
pLPreB	pCM189::PreB	Ampicillin/Uracil	This study
pLGpa1	pCM189::PbGpa1	Ampicillin/Uracil	This study
pLSte4	pCM189::PbSte4	Ampicillin/Uracil	This study

2.5.3. *S. cerevisiae* transformation

Transformation of *S. cerevisiae* was performed according to Gietz and Woods [126]. Briefly, the cells were grown overnight and harvested by centrifugation (3min; 13000rpm). Cells were washed, and the followed mix was added: 240μl of polyethylene glycol 3500 (50% w/v); 36μl lithium acetate (1M); 50μl single-stranded DNA (2mg/mL); and 34μl of DNA (0.1 to 1μg) plus water. Subsequently, the sample was incubated at 42°C for 40-75min, centrifuged and the mix removed. In order to recover from the transformation, it was added 600μl of YEPD and the tube was incubated at 26°C (150rpm) for 60-180min. Cells were collected and dissolved in 1mL distilled water for plating on appropriate selective medium. *S. cerevisiae* plasmid transformants were confirmed by PCR, using the DyNAzyme II DNA polymerase (Finnzymes), and by growth in medium supplemented with the resistance marker. For the PCR confirmation a 20μl final volume reaction mixture was defined by reaction buffer 1x, 2 mM $MgCl_2$, 200μM dNTPs, 200μM of each primer (Primers used are listed in Supplementary Table S1) and 0,5 U DyNAzyme II DNA polymerase. The following thermal cycling conditions were used: an initial denaturing step at 94°C for 10 min, followed by 35 cycles at 94°C for 30 sec, 40 sec at the best annealing temperature previous tested and at 72°C for 1min/Kb, and one final elongation cycle at 72°C for 10 min. The amplification products were analyzed by electrophoresis on a 1-1.5% agarose gel stained with ethidium bromide.

2.5.4. Genomic DNA extraction from *S. cerevisiae*

All the transformants obtained by genomic insertion were confirmed by PCR after genomic DNA extraction.

Genomic DNA extraction was performed based on Sambrook and Russell protocol [127]. The cells were grown overnight and harvested by centrifugation (5min; 3000rpm). Cells were washed in deionized water, and resuspended in 200µl of lysis buffer (2% [v/v] Triton-X100, 1% [w/v] SDS, 100mM NaCl, 10mM pH8.0 Tris-Cl, 1mM EDTA), and glass beads and phenol/chloroform/isoamyl alcohol (25:24:1) were also added, before vortexing for 3min. Subsequently, were added 200µl of TE buffer (10mM Tris-Cl pH 7.5; 1mM EDTA) followed by centrifugation for 5min at 13000rpm. The aqueous phase was transferred to a new tube and 1mL of absolute ethanol was added followed by a new centrifugation. The addition of 400µl of TE buffer was followed by the addition of 30µl of RNase (1mg/mL) and incubation at 37°C for 5 min. After the incubation was added 10µl of sodium acetate (3M) and 1mL of absolute ethanol, mixed by inversion. Finally, the tube was centrifuged, the pellet dried at 55°C and eluted in 50µl of water.

To confirm *S. cerevisiae* genomic transformation, the followed PCR reaction was performed, using genomic DNA as a template A 20µl final volume reaction mixture was defined by reaction buffer 1x, 2 mM $MgCl_2$, 200µM dNTPs, 200µM of each primer (Primers used are listed in Supplementary Table S1) and 0,5 U DyNAzyme II DNA polymerase (Finnzymes). The following thermal cycling conditions were used: an initial denaturing step at 94°C for 10 min, followed by 35 cycles at 94°C for 30 sec, 40 sec at the best annealing temperature previous tested and at 72°C for 1min/Kb, and one final elongation cycle at 72°C for 10 min. The amplification products were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide.

In order to analyze the functional ability of *P. brasiliensis* mating-related genes expressed on *S. cerevisiae* null mutants, we used the mature *P. brasiliensis* α-pheromone (Pbα) WCTRPGQGC, synthesized at Metabion (Germany) and synthetic *S. cerevisiae* α-pheromone (Scα) WHWLQLKPGQPMY, obtained from GenScript (USA).

2.5.5. Halo assay

Halo assays to monitor pheromone-induced growth arrest were performed to establish the biological activity of Pbα on a *S. cerevisiae* Ste2 deletion strain expressing the *P. brasiliensis* α-pheromone receptor PreB. The strains BY4741 and AGLPreB were grown in minimal drop out medium YNB (supplemented with the appropriate amino acids) to an optical density at 640nm (OD_{640}) of 1.0 to 1.4. Yeast cells at $1x10^6$ and $5x10^6$ cells were embedded in 3.5mL of soft agar (0.8%), spread on YEPD plates and dried. Subsequently, 10µl of synthetic Pbα or Scα was spotted on and plates incubated for 16-24h at 30°C. Halo development was followed and halo dimensions were measured. Each experimented was repeated a minimum of four times.

2.5.6. *Shmoo* assay

Strains BY4741 and AGLPreB were grown overnight in minimal medium drop out YNB (supplemented with the appropriate amino acids) to an optical density at 640nm (OD_{640}) of 1.0 to 1.4. Cells ($4x10^6$ cells/mL) were resuspended in YEPD and incubated with either 4 or 2µg/mL, Pbα or Scα respectively at 26°C. The percentage of cell-shmooing was determined at different times and cells images were taken on a Zeiss Axioskop equipped with a Carl Zeiss AxioCam (Carl Zeiss, Jena). Shmoo counts were done with 200-300 cells, and each experiment was repeated four times.

2.5.7. Cell cycle analysis by flow cytometry

The strains BY4741 and AGLPreB were grown overnight in minimal drop out medium YNB (supplemented with the appropriate amino acids) to an optical density at 640nm (OD_{640}) of 1.0 to 1.4. The cells ($4x10^6$ cells/mL) were resuspended in YEPD and incubated with either 4 or 2µg/mL, Pbα or Scα respectively at 26°C. Cell cycle analysis was performed at different time points (0h, 30min, 1h30min, 2h, 3h, 4h, 6h and 9h). Cell treatment was adapted from Fortuna et al. protocol [128]. Briefly, cells were fixed by resuspending in 500µl 70% ethanol, and subsequently centrifuged and washed with 1mL of sodium citrate buffer (50mM pH7.5). Next, 850µl of sodium citrate buffer and 125µl of RNase (2mg/mL in Tris-EDTA pH8.0) was added and samples incubated at 50°C for 1h. After the addition of 50µl of proteinase K (20mg/mL) samples were again incubated at 50°C for 1h. The cells were transferred to a cytometry tube and stained overnight at 4°C

with SYBR Green I (1000x, diluted in Tris-EDTA pH8.0). Triton X-100 (0.25% v/v in 50mM sodium citrate buffer pH7.5) was added and the sample was sonicated with three consecutive ultrasound pulses at 40W for 2 sec with an interval of 2 sec between each pulse. The cell cycle analysis was performed by flow cytometry (FCM) on a BD™ LSR II flow cytometer. A minimum of 20000 cells per sample were acquired at low/medium flow rate. Offline data were analyzed with the ModFit LT 3.2 version, a cell cycle analysis software. Each experiment was repeated four times.

2.5.8. Quantitative mating assays

Quantitative mating assays were performed based on the protocol by Guthrie and Fink [129]. The strains whose mating efficiency was determined are listed in Table 3. The cells were grown ON in minimal drop out medium YNB (supplemented with the appropriate amino acids) to an optical density at 640nm (OD_{640}) of 0.5 to 1.0.

Table 3 – Strains used in quantitative mating assay.

S. cerevisiae strains
BY4741 x BY4742
BY4741 x AGLPbα
AGLPreB x BY4742
AGLPreB x AGLPbα
AGLPreB x AGMPbα
BY4741ΔGPA1 x BY4742
BY4741ΔSTE4 x BY4742
AGLPbGpa1 x BY4742
AGLPbSte4 x BY4742

Cells were mixed on a 0.45μm pore membrane (Amersham Hybond-N nylon membranes, GE Healthcare), using a vacuum filtration system (TPP), at different concentrations (1x

corresponds to 1.5x10^7 cells). The filters were placed on the surface of YEPD plates and incubated at 30°C for 5h. The cells were resuspended in sterile water and plated in YNB drop out plates without methionine and lysine, and without methionine/lysine, in order to select diploids, or haploid cells, respectively. Mating efficiency was calculated as:

$$\%mating = \frac{\#Diploids}{\min\{Haploids\}} * 100$$

2.6. Statistical analysis

Data are reported as the mean ± standard error of the mean of at least three independent repetitions of each assay. Data analysis was carried out using the computer software GraphPad Prism®. For comparison between three or more variables, One-way ANOVA was performed with Turkey post test and 95% of confidence interval.

Chapter III – Results and Discussion

The present study focused on the identification and characterization of the putative molecular players for mating in *P. brasiliensis*, taking into account those previously described in other fungi. Although a sexual cycle has not been described in *P. brasiliensis*, *in silico* analysis allowed us to identify most of the mating-related genes. Recent genome annotations of three *Paracoccidioides* isolates confirmed our results for the presence of conserved mating and meiosis specific genes in *P. brasiliensis* [25], while other studies revealed the presence and expression of the two *MAT* idiomorphs [119, 120].

As the identification of the proper conditions for filamentous fungi to undergo sexual reproduction has proven to be difficult, we choose to study the functionality of *P. brasiliensis* mating components in the heterologous expression model *S. cerevisiae*. In particular, this work focused on the characterization of the *P. brasiliensis* α-pheromone and its cognate α-pheromone receptor, PreB, being the primary components for mate-recognition.

3.1. Mating-type analysis of *P. brasiliensis* strains

In filamentous ascomycetes sexual reproduction is regulated by a genetic locus designated mating-type or *MAT* locus, and is classified into heterothallic or homothallic mating-types. Heterothallic fungi harbor a single *MAT* locus encoding one of the two MAT idiomorphs: an idiomorph encoding a transcription factor with a α-box domain (*MAT1-1*) or the idiomorph of a transcription factor with a HMG-box domain (*MAT1-2*) [58].

Table 4 – Mating-type of *P. brasiliensis* strains used in this study.

Strain	Mating-type
Pb01	*MAT1-1*
T8B1	*MAT1-1*
ATCC60855	*MAT1-2*
Pb03	*MAT1-2*

A study performed in 71 *P. brasiliensis* isolates showed that their genome contains either the *MAT1-1* or *MAT1-2* gene, showing high similarity to *MAT* loci of other Ascomycete fungi, and indicated a heterothallic mode of reproduction. In this study we used several *P. brasiliensis* strains (Table 4), for which the presence of either one of the two *MAT* idiomorphs was confirmed by PCR analysis, using *MAT* locus-specific primers [120].

3.2. Pheromone response pathway in *P. brasiliensis*

Mating is initiated by the sensing of pheromones secreted by cells of the opposite mating-type. The pheromones are recognized by G protein-coupled receptors (GPCRs) which are present on the cell surface, and their activation leads to the initiation of a signal transduction cascade that is best characterized in *S. cerevisiae*, but it is highly conserved among fungi (Figure 8).

Taking advantage of bioinformatics tools and the *P. brasiliensis* genome sequences we were able to identify several homologs of the pheromone/mating signal transduction cascade of *S. cerevisiae*. Recently, a study performed by Desjardins confirmed our results and also identified meiosis specific genes [25]. We found that *P. brasiliensis* encodes most of the genes involved in the pheromone response pathway of *S. cerevisiae* (*STE2*, *STE3*, *STE4*, *GPA1*, *STE18*, *STE20*, *STE50*, *STE11*, *STE7*, *FUS3/KSS1*, and *STE12*), with the exception of *STE5* and *FAR1* (Figure 8 - E). Ste5p is a scaffold protein that is responsible for the formation of the MAPK cascade complex, by the binding of Ste11p, Ste7p and Fus3p kinases, and interacts with the plasma membrane via its association with Ste4p. Another function of Ste5p is to limit cross-talk between alternative MAPK signaling pathways as is the case for filamentous growth in *S. cerevisiae* (reviewed in [87]). Far1p has two main functions: (i) in response to pheromone it promotes cell cycle arrest by inhibiting a cyclin-dependent kinase, and (ii) it directs polarized growth along a pheromone gradient towards the mating partner (reviewed in [87]).

In several ascomycetes with known sexual reproduction, including *A. fumigatus*, *A. nidulans* and *H. capsulatum*, Ste5p and Far1p seem to be absent, which would imply that these proteins are dismissible for a functional pheromone-responsive MAPK pathway, or that unidentified proteins are responsible for the formation of the MAPK cascade complex in these fungi. This could apply to the case of *P. brasiliensis* as well.
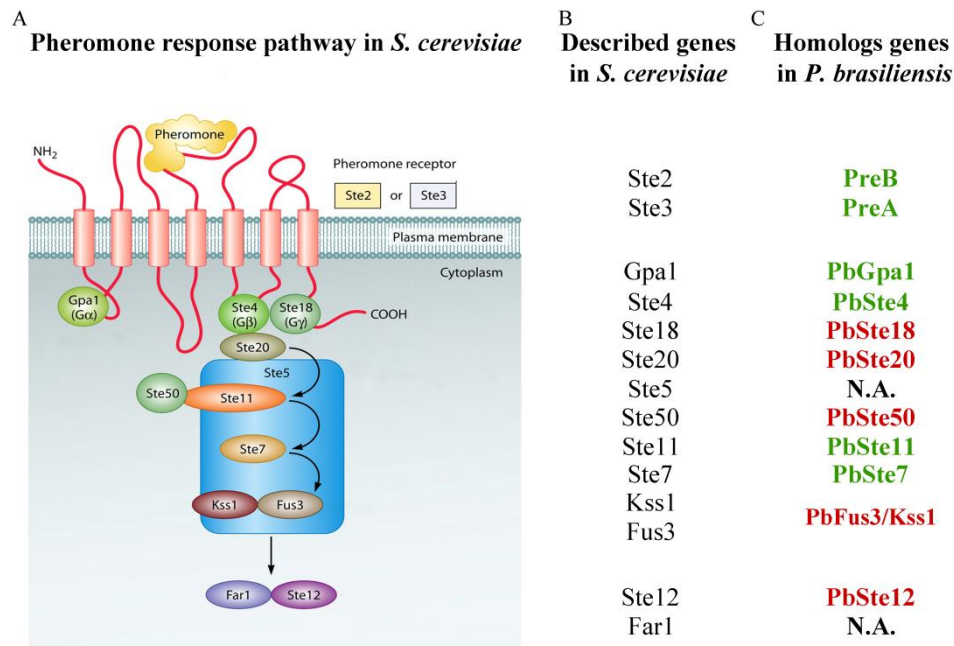
Figure 8 – Homologies among the pheromone response pathway. (A, B) Pheromone response pathway in *S. cerevisiae* (adapted from [34]). (C) Homologous genes of the pheromone response pathway in *P. brasiliensis* obtained by *in silico* analysis. Genes in green - Genes that can be tested for functional complementation by heterologous expression in *S. cerevisiae* null mutants. Genes in red – functional complementation tests using null mutants of *S. cerevisiae* are not possible N.A. – not annotated genes in *P. brasiliensis* database.

Functionality of some *P. brasiliensis* putative mating-related genes was tested using a functional complementation assay in the respective *S. cerevisiae* null mutants (see section 3.4.4.). In this sense, *PbGPA1*, *PbSTE4*, *PREB* and the gene encoding the putative α-pheromone were amplified and expressed in the respective *S. cerevisiae* null mutants in order to test their ability to restore mating (section 3.4.4.). Since *S. cerevisiae* null mutants for Ste12 and Ste18 are not viable and therefore not available at EUROSCARF, these genes could not be tested for their functionality. *P. brasiliensis* homologues of Ste50, Ste20, Fus3 and Kss1 could not be tested since *S. cerevisiae* mutants for these genes are still able to respond to pheromone stimulation and form diploid cells. *PbSTE11* and *PbSTE7* specific amplicons could not be obtained from *P. brasiliensis* cDNA, and were therefore not analyzed. The functionality of the Ste2 pheromone receptor homolog (PreB) was tested in several assays described below. Interestingly both *MAT1-1* and *MAT1-2* strains encode PreB and PreA (**a**-pheromone receptor) as well as the putative α-pheromone.

3.2.1. Identification and characterization of *P. brasiliensis* α-pheromone

In *S. cerevisiae*, as well as in other fungal species, mating starts with the recognition of the mating pheromones by specific receptors on cells of the opposite mating-type [87].

The S. cerevisiae mature α-pheromone is a peptide of 13 amino acids (WHWLGLKPGQPMY), generated from a precursor peptide. This pheromone is encoded by two genes, *MFα1* and *MFα2*, which contain tandem repeats of the mature α-pheromone (four and two repeats, respectively) [83-85]. The identification of a gene encoding the α-pheromone in *C. albicans* showed that the mature peptide contains 13 amino acids (GFRLTNFGYFEPG) and is present in three identical copies in the precursor peptide [53]. More recently, the identification of gene encoding the α-pheromone from *Ajellomyces capsulatus* (anamorph *Histoplasma capsulatum*) showed a single repeat of the mature nonapeptide (WCTRPGQGC).

The identification of a putative gene encoding the α-pheromone of *P. brasiliensis* was achieved by *in silico* comparison with the pheromone of the phylogenetically related fungus *A. capsulatus*. Using the protein sequence of the *A. capsulatus* α-pheromone precursor and performing BLASTP and TBLASTN database searches in all six reading frames against transcripts and genomic sequences it was possible to identify a small ORF encoding a putative α-pheromone in *P. brasiliensis*. In order to confirm this prediction we sequenced the expressed pheromone transcript from cDNA and performed an alignment with the predicted protein sequences (Figure 9) and with the confirmed sequence of the *A. capsulatus* α-pheromone precursor (Figure 10).

The nucleotide composition of the expressed pheromone of *P. brasiliensis* showed a difference of four nucleotides when compared to the prediction (Supplementary Figure S1).
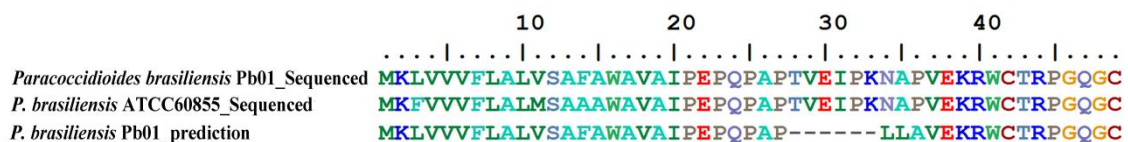
Figure 9 – *P. brasiliensis* α-pheromone precursor. Alignment of the pheromone expressed in *P. brasiliensis* and the predicted sequence for this gene.

The alignment between *A. capsulatus* and *P. brasiliensis* pheromone precursors showed highly conserved regions (Figure 10). The α-pheromone precursor sequence from *P. brasiliensis* showed a 53% identity with the α-pheromone precursor from *A. capsulatus*. However, the mature nonapeptide sequence (WCTRPGQGC) is 100% and 77% identical to the mature α-pheromones of *A. capsulatus* (Figure 10) and *A. fumigatus* (not shown), respectively. Expression levels of the pheromone gene in *P. brasiliensis* strains Pb01, T8B1, ATCC60855 and Pb03 were confirmed by qRT-PCR (see section 3.3.), and pheromone functionality was tested in several assays using a synthetic α-pheromone (Pbα) (see section 3.4.).
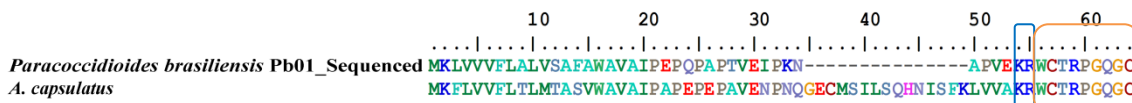
Figure 10 – Alignment of α-pheromone precursor from *P. brasiliensis* and *A. capsulatus*. The orange box corresponds to the mature α-pheromone peptide. The peptide contains conserved WC and GQ residues that are present in other fungal α-pheromones. The *P. brasiliensis* putative α-pheromone gene also encodes a KEX2 recognition sequence (blue box).

3.2.2. Characterization of α-pheromone receptor PreB from *P. brasiliensis*

Mating pheromones are recognized by GPCRs, which in *P. brasiliensis* are designated by PreB (α-pheromone receptor) and PreA (**a**-pheromone receptor). As for strain ATCC60855 the *PREB* sequence was unknown, thus the corresponding transcript was sequenced, as well as the *PREB* sequence from Pb01 strain for confirmation of the transcript predicted by annotation. Sequences were aligned and compared with the predicted transcript sequences available at the Broad Institute (Supplementary Figure S2). Sequencing of *PREB* transcripts showed a 99% identity between *P. brasiliensis* strains Pb01 and ATCC60855, with only four amino acids different (Figure 11). The PreB receptor from both *P. brasiliensis* strains showed a 75%, 44%, and 28% identity with *A. capsulatus*, *A. fumigatus*, and *S. cerevisiae* α-pheromone receptor, respectively.

In *S. cerevisiae* the domains that contribute to α-pheromone binding and Ste2 activation have been extensively studied, however, they are not fully defined [130, 131]. Using the TMHMM program the protein topology of PreB from *P. brasiliensis* and *A. capsulatus* was predicted (Figure 12).

Figure 11 – Alignment of PreB sequences from *P. brasiliensis* strains Pb01 and ATCC60855. The four amino acids that differ between both sequences are highlighted (orange boxes).

In *S. cerevisiae*, the α-pheromone residues responsible for the receptor activation are located near the N-terminus, while the ones important for receptor binding are located near the C-terminus [132]. According to previous studies, the domains responsible for the α-pheromone binding and the activation of the respective receptor in *S. cerevisiae* are related to transmembrane domains (TM), in which the C terminus of the α-pheromone interacts with TM1 and the N-terminus with TM5-TM6 [131, 133, 134].

The *A. capsulatus* and *P. brasiliensis* mature α-pheromone are identical, and we therefore analyzed the important TMs already identified in *S. cerevisiae*, including the flanking extracellular domains (E), in order to understand if the binding and activation mechanisms could be the same. In this sense, we aligned the different domains of PreB from *A. capsulatus* and *P. brasiliensis MAT1-2* strains that were predicted using the TMHMM program (Figure 12).

The N terminus and the TM1 domains are associated to the binding of α-pheromone to the receptor and the analysis of *A. capsulatus* and *P. brasiliensis* showed a 75% identity between both domains (Figure 13).

Figure 12 – Topology of α-pheromone receptor. (A) PreB from *P. brasiliensis* ATCC60855 strain; (B) *Sc*Ste2 (C) PreB from *A. capsulatus*.
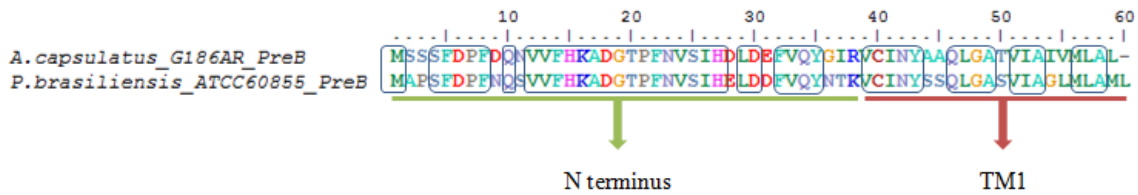
Figure 13 – **Alignment of domains from *A. capsulatus* and *P. brasiliensis* PreB that are known to be related to the pheromone binding in *S. cerevisiae*.** There is a high identity between N terminus and TM1 of both species (blue boxes). TM – transmembrane domain.

The alignment of E2-TM5 and TM6-E3 domains from *A. capsulatus* and *P. brasiliensis*, responsible for the receptor activation in *S. cerevisiae*, showed 89% (Figure 14 – A) and 88% (Figure 14 – B) of identity, respectively. Comparatively, when we do an alignment between *P. brasiliensis* and *S. cerevisiae* for these domains we observe only a very low similarity (Figure 15). These results lead us to consider that the differences between PreB and Ste2 do not allow the interaction of the α-pheromone from *S. cerevisiae* with PreB, and vice-versa. Nevertheless, to avoid any possible interference in the functional tests, both *MF(α)1* and *MF(α)2* genes were deleted from the *S. cerevisiae* BY4742 strain.

After recognition of the pheromone by the GPCRs, the intracellular mating signaling pathway is activated. Clark et al. [135] studied the relevance of the third intracellular loop region of Ste2, since it was shown that this region is important for the mammalian receptors implicated in G protein contact [136]. First, these authors replaced each amino acid one by one by an alanine, analyzed their mating efficiency, and concluded that none of the amino acids in the third loop is absolutely required for mating. However, some of these mutants failed to show growth arrest, which means that they alter the Ste2 signal transduction. Afterwards, the authors constructed Ste2 strains with double site-directed amino acid substitutions and showed that the R233A/F241A double mutants have reduced mating efficiencies and failed to arrest growth, indicating an involvement of these two amino acids in the activation of the intracellular pheromone-signaling pathway.

As referred before, PreB shows a 28% identity with *Sc*Ste2 (Supplementary Figure S2), and by analyzing the predicted topology of both α-pheromone receptors (Figure 12) we aimed to identify conserved domains between both species. Beside these two species we decided to analyze the third intracellular loop of α-pheromone receptor from *C. albicans*

(*Ca*Ste2) as well, as heterologous expression of this receptor previously led to successful functional complementation in *S. cerevisiae* [137].
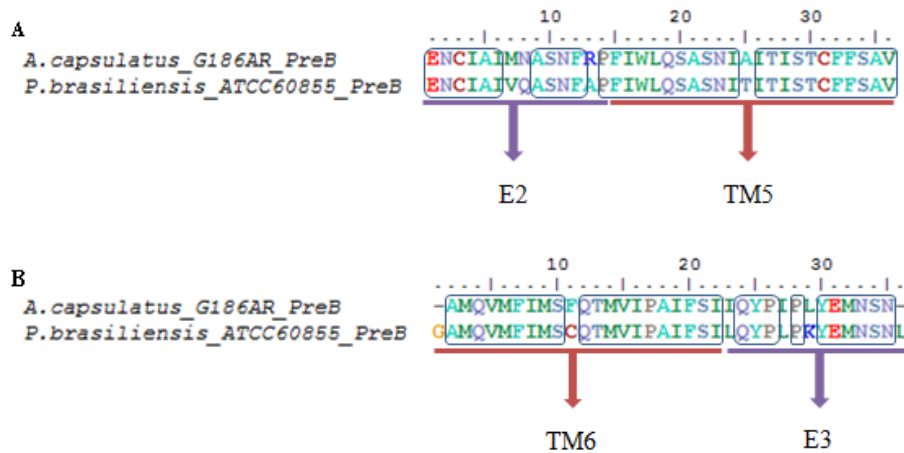
Figure 14 – **Alignment of PreB domains from *A. capsulatus* and *P. brasiliensis* responsible for Ste2 activation in *S. cerevisiae*.** A – E2-TM5. B – TM6-E3. E – Extracellular domain; TM – Transmembrane domain. The identical amino acids are highlighted (blue boxes).

Figure 15 – Alignment of (A) E2TM5 and (B) TM6E3 using *P. brasiliensis* and *S. cerevisiae* sequences. The identical amino acids are highlighted (orange boxes).

The third intracellular loop of *Sc*Ste2 and *Ca*Ste2 are highly similar, with 91% of sequence identity (Figure 16 – A), however similarity between PreB and *Sc*Ste2 is very low (Figure 16 – B). Both amino acids needed for the activation of intracellular mating pathway in *S. cerevisiae* (R233 and F241) are also present and separated by the same number of amino acids in PreB (R223 and F231), though the topology prediction places the phenylalanine residue into the transmembrane domain (Figure 16 – B). The triplet LGL is conserved in all of the three studied species.

Despite these similarities, the differences present can alter the activation of the mating pathway. As reported by Clark et al. [135], mutations in the receptor can: alter the conformation and consequently the binding affinity of the ligand; affect the ability to

bind and activate the G protein; interfere in the number of receptors expressed on the cell surface. In this sense, since our receptor is being expressed in *S. cerevisiae*, the activation of the mating signaling pathway might be altered.
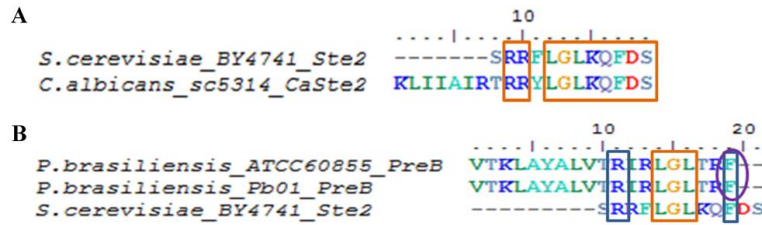
Figure 16 – **Alignment of the third intracellular loop of α-pheromone receptor from different species.** (A) – alignment between *S. cerevisiae* (BY4741) and *C. albicans* (sc5314) third intracellular loop shows a high degree of identity (orange boxes) (B) – alignment between *P. brasiliensis* (ATCC60855 and Pb01) and *S. cerevisiae* (BY4741) shows: the presence of the two amino acids (R and F) involved in the activation of the intracellular pheromone signaling pathway in *S. cerevisiae* (blue boxes); the LGL triplet (orange box); the presence of the phenylalanine (purple box), placed in the transmembrane domain.

3.3. Differential expression of the mating-related genes in *P. brasiliensis* yeast and mycelium

In order to analyze expression levels of the *in silico* identified mating-related genes, a qRT-PCR analysis was performed during yeast and mycelial growth. The expression of *P. brasiliensis* mating-related genes under basal conditions revealed to be low in both yeast and mycelium (Figure 17), however, it has already been shown in other fungi that these expression levels can be increased when the cells are exposed to the respective pheromone [63].

The strains used in this study are heterothallic, which means that they only express one of the two *MAT* idiomorphs. Regarding the *MAT1-1* locus there seems to be a pattern where the yeast form has significantly lower expression levels when compared to the mycelium form (Figure 17 – A). In contrast, the *MAT1-2* expression in strain ATCC60855 was similar in both morphological forms, and in the strain Pb03 was significantly higher in the yeast form (Figure 17 – B).

According to the Turgeon and Yoder nomenclature [58], the *MAT1-1* idiomorph is defined by the presence of an ORF encoding a protein with an alpha box motif, while the *MAT1-2* idiomorph encodes a protein with an HMG motif. In this sense and according to the *S. cerevisiae* model, the strains that harbor the *MAT1-1* locus should

express the α-pheromone and the **a** pheromone receptor, whereas *MAT1-2* strains should express the **a** pheromone and the α-pheromone receptor. However, although the strains used in this study are heterothallic, basal expression levels of the α-pheromone, *PREA* and *PREB* was observed in both mating-types.

The *MAT1-1* strains express both pheromone receptors at low levels and there is no pattern between yeast and mycelial forms (Figure 17 – C and G).

In the case of *MAT1-2* strains, the expression levels of both receptors (PreA and PreB) are significantly higher in the mycelium form (Figure 19 – D and H). Besides that, the *MAT1-2* strains (mycelial form) have higher expression levels of both receptors, when compared to the *MAT1-1* strains.

Previous studies have shown that some heterothallic ascomycetes express pheromone receptors in a mating-type independent manner, as is the case for *A. fumigatus*, *Neurospora crassa* and *Candida glabrata* [56, 138, 139]. Pöggeler and Kück [138] consider that the expression of pheromone receptors in a cell-type-independent manner supports the idea that both pheromone receptors can have evolved new functions, independent of mating, thereby enforcing their expression in both mating-types of heterothallic fungi [140, 141].

In *P. brasiliensis*, the α-pheromone is expressed by both mating-type strains, and with the exception of Pb03, the expression levels are significantly higher in the mycelium form (Figure 17 – E and F). However, expression levels are very low for *MAT1-2* strains and only the *MAT1-1* strain Pb01 show significantly higher levels of expression in mycelium. As the post translational mechanisms, as well as, the role of the *MAT* locus in the activation of mating-related genes are not known, we do not know if the α-pheromone is functional in both mating-type strains.

Despite the presence and expression of *MAT* loci and mating-related genes, these results do not give a confirmation of a sexual life cycle in *P. brasiliensis*. Therefore, in order to assess the functionality of mating-related proteins of *P. brasiliensis*, we performed heterologous expression of these proteins using *S. cerevisiae* null mutants.
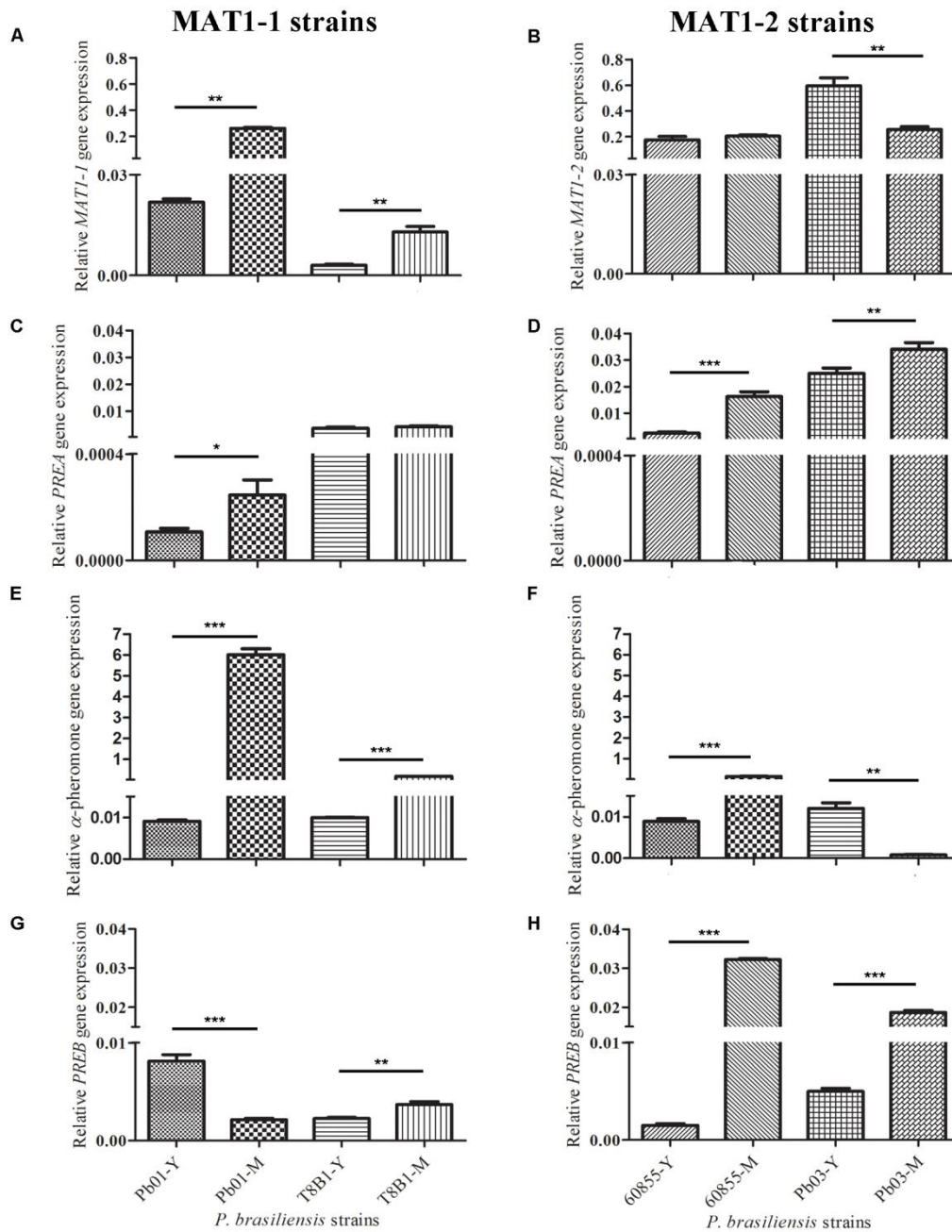
Figure 17 – Gene expression levels of mating-related genes in *P. brasiliensis* strains under basal conditions in both yeast and mycelial forms. A – Expression levels of *MAT1-1* gene; B – Expression levels of *MAT1-2* gene; C, D – Expression levels of *PREA*; E, F – Expression levels of α-pheromone; G, H – Expression levels of *PREB*. Yeast cells were grown in BHI broth supplemented with glucose and mycelium in MMVM synthetic medium. Mating-related genes expression levels obtained by qRT-PCR were normalized to the internal reference TUB2. (Y – yeast; M – mycelium). * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

3.4. Heterologous expression of *P. brasiliensis* mating-related genes in *S. cerevisiae*

Since the molecular and cellular tools for studying the interaction between the pheromone and receptor of *S. cerevisiae* were available, we decided to assess the functionality of *P. brasiliensis* mating-related proteins by heterologous expression in *S. cerevisiae*.

First, we examined the ability of *PREB* to complement a *S. cerevisiae STE2* null mutant and respond to *P. brasiliensis* synthetic α-pheromone (Pbα). In this sense, *PREB* from *P. brasiliensis* was sub-cloned into the plasmid pCM189 (low copy plasmid) to yield the plasmid pLPreB (AGLPreB strain). In this construct PreB is constitutively expressed under the control of a TET-OFF promoter. As referred before (see section 3.2.) the ORF encoding a putative α-pheromone of *P. brasiliensis* was identified and the CDS of the putative α-pheromone was sub-cloned into the plasmids pCM189 (low-copy) and pCM190 (multicopy) to yield the plasmid pLPbα (AGLPbα strain), and pMPbα (AGMPbα strain), respectively. In both constructs, α-pheromone is constitutively expressed under the control of a TET-OFF promoter. All the strain used in the present study are described in Table 1 (See section 2.1.).

3.4.1. Halo assay

The efficiency of the cell fusion during sexual reproduction requires cell synchronization, which is achieved by a cell cycle arrest in the G1 phase [38]. In order to test if the AGLPreB strain responds to Pbα we performed a halo assay (Figure 18). The wild-type *S. cerevisiae* strain BY4741 stimulated with *S. cerevisiae* synthetic α-pheromone (Scα) was used as a control for this method.

Our results show that AGLPreB undergoes Pbα-induced growth arrest with increased halo areas at higher pheromone doses (Figures 18 and 19). We also observed that neither PreB nor *Sc*Ste2 responded to the non-cognate α-pheromone (data not shown). However, as can be seen in Figure 18B turbid halos were observed when compared to the BY4741 control stimulated with Scα (Figure 18 A), which complicated accurate measurements of halo areas for AGLPreB.

We also tested turbidity in halo formation with a lower concentration of cells ($1x10^6$cell/mL), which led to high area halos (Figure 19), but did not reduce turbidity (data not shown).

The presence of turbid halos can be related to an incomplete receptor activation of the intracellular mating pathway or the activation of a specific recovery pathway [142, 143]. In our study it is more plausible that the turbid halo is the consequence of an incomplete receptor activation of the intracellular mating pathway.
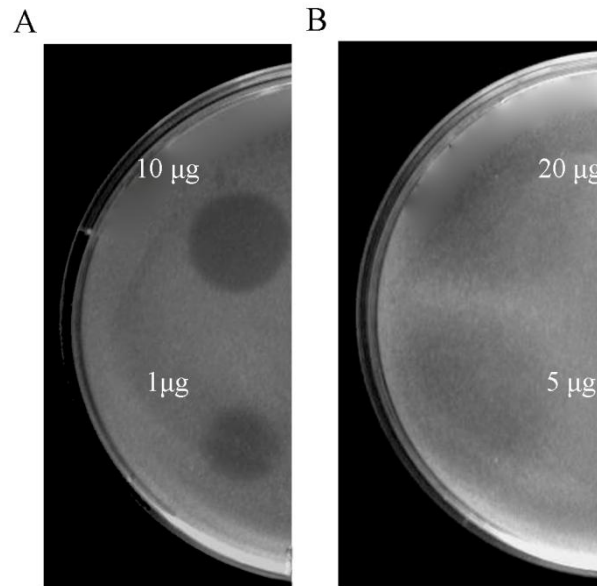
Figure 18 – Halo assay. (A) – BY4741 stimulated with synthetic Scα; B – AGLPreB stimulated with synthetic Pbα. For this assay 5.10^6cell/mL was used.

It has been shown that the third intracellular loop of Ste2p from *S. cerevisiae* is important in the activation of this pathway (see section 3.2.) [135]. The sequence of PreB and Ste2 share only 28% of identity (Supplementary Figure S2) and the predicted third loop shows no similarity. This situation supports the idea that the turbid halos could be a consequence of the incomplete activation of the intracellular mating pathway. However, a study performed by Mayrhofer and Pöggeler [144] showed that Pre2p (pheromone receptor from *Sordaria macrospora*) is functionally homologous to the *S. cerevisiae* Ste2 pheromone receptor, despite the fact that there are no homologies in the amino acid sequence of the predicted third intracellular loop [138].
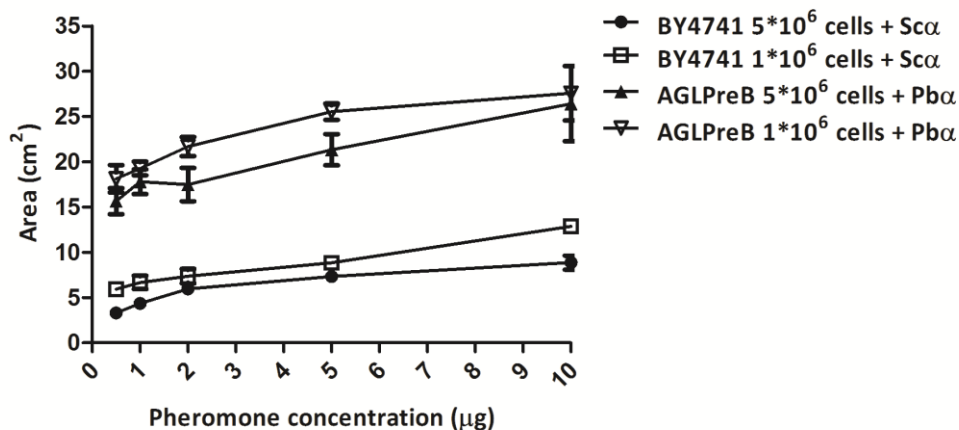
Figure 19 – Halo area quantification. Cells were harvested in the exponential phase and stimulated with different concentrations of synthetic pheromone. Independently of the number of cells used, the BY4741 strain shows a halo area smaller but more clear then AGLPreB strain.

Another study performed by Janiak et al. [137] showed that *Ca*Ste2p, the *C. albicans* homolog of the *S. cerevisiae* α-pheromone receptor Ste2p, was able to restore the ability of a *ste2* mutant *S. cerevisiae* strain to respond to *C. albicans* synthetic pheromone and produce a non turbid halo. Recently, Wendland et al. [145] also showed the formation of clear halos when *S. cerevisiae ste2* mutant cells expressing the α-pheromone receptor of *Ashbya gossypii* were stimulated with the synthetic pheromone of that fungus. Nevertheless, is important to notice that both *Ca*Ste2p and *Ag*Ste2p show higher identity with Ste2p from *S. cerevisiae*, at 36% and 40.1%, respectively.

3.4.2. *Shmoo* assay

After pheromone recognition, yeast cells develop projections called *shmoos*, characterized by a polarized growth toward a mating partner, ultimately leading to cell fusion, followed by nuclear fusion [37]. In order to see if the heterologous strain AGLPreB has the ability to respond to stimulation with synthetic Pbα, a s*hmoo* assay was performed (Figure 20 and 21).

Cells in exponential phase (4.10^6 cell/mL) were incubated with the respective synthetic α-pheromone (Pbα – 4μg/mL; and Scα – 2μg/mL) and the percentage of cells exhibiting *shmoo* projections were counted at different time points (Figure 21). As can be observed, both types of cells respond to the stimuli using the synthetic pheromones (Figure 20). However, neither Ste2 nor PreB respond to the non-cognate alpha-pheromone (data not shown).
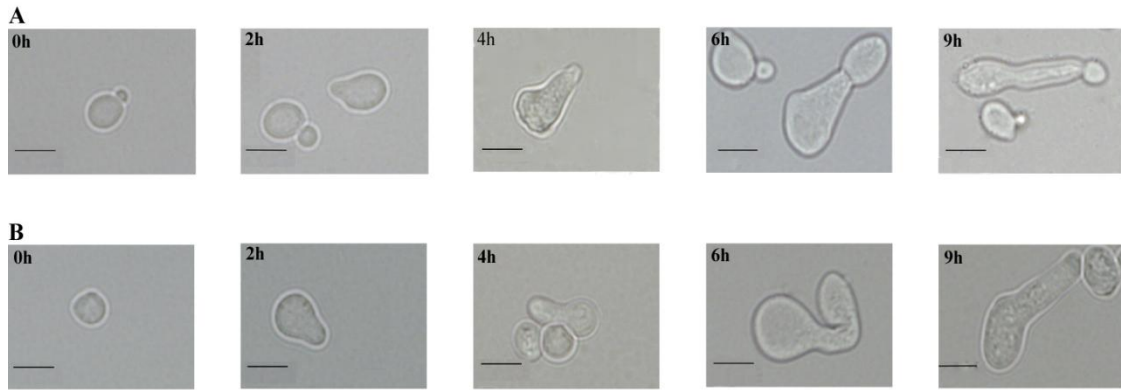
Figure 20 – *Shmoo* formation. Cells in exponential phase (4.10^6 cell/mL) were stimulated with synthetic pheromone: (A) BY4741 with Scα (2μg/mL) or AGLPreB with Pbα (4μg/mL). Yeast cell morphology was registered using a Zeiss Axioskop equipped with a Carl Zeiss AxioCam at indicated time points (0-2-4-6-9 hrs).

In strain BY4741 the percentage of shmoos starts decreasing 6h after stimulation with Scα, while in strain AGLPreB, 6h after stimulation with Pbα we can still observe a slight increase. Both strains showed a decreased in shmoos percentage 9h after stimulation. The differences in the percentage of shmoos are not statistically different. However, even stimulated with the double amount of synthetic pheromone, compared to BY4741, it is possible to observe a lower *shmoo* formation in the AGLPreB strain. It has been shown that *S. cerevisiae* strain expressing *Ca*Ste2p was able to respond to *C. albicans* synthetic α-pheromone, by forming *shmoo* projections [137]. After 3h of incubation with the α-pheromone (10μg/mL), it was observed that ~24% of cells respond to the stimulus. In our results, after 3h we could observe a lower percentage of AGLPreB cells, ~13%, that respond to Pbα. However, it is important to note that we used a lower concentration of α-pheromone and more importantly, *Ca*Ste2 is more similar to Ste2 than PreB (see section 3.2.), which could indicate a stronger intracellular signal. Thus, as it happens in halo assay, this lower *shmoo* formation in AGLPreB strain can be due to the incomplete receptor activation of the intracellular mating pathway.

Taking into account that the non-shmooing cells are slightly larger than cells that were not stimulated with α-pheromone, we inferred that this increased size could indicate arrested cells that did not form *shmoos* [137]. As it can be observed by the *shmoo* pictures (Figure 20), 6h after the stimulation, the cells start having an aberrant morphology. As referred by Throm and Duntze [146], after prolonged exposure to α-pheromone cells may grow up to 30 times of their normal size.
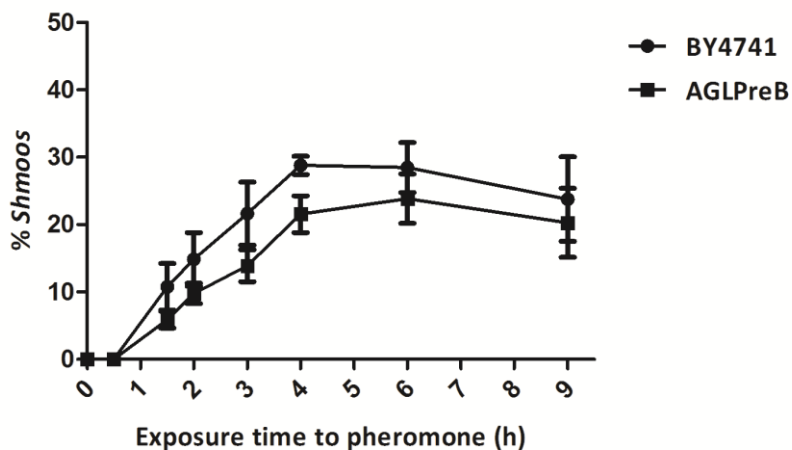
Figure 21 - *Shmoo* assay – Cells in exponential phase (4.10^6 cell/mL) were stimulated with the respective synthetic pheromone (Pbα – 4μg/mL; and Scα – 2μg/mL), harvested at different time points, and the percentage of yeast cells exhibiting *shmoo* projections were counted.

3.4.3.   Cell cycle arrest assay

In order for cell and nuclear fusion to occur between mating partners it is necessary that both cells have the same DNA content. Cell synchronization is possible by the presence of mating pheromones that promote a cell cycle arrest, which starts in the G1 phase, prior to DNA synthesis [39]. Using flow cytometry analysis it is possible to quantify the number of arrested cells, since this method allows us to exactly determine the DNA content of each cell. Cells in G0/G1 and G2/M phase have a uniform DNA content, with the latter having twice as much DNA, and consequently a double intensity in fluorescence, when compared to cells in G0/G1 phase. The analysis was performed on AGLPreB or BY4741 cells in exponential phase (4.10^6 cell/mL) that were stimulated with the respective synthetic α-pheromone (Pbα – 4μg/mL; and Scα – 2μg/mL) and harvested at different time points. As a control we also analyzed the cell cycle of both strains without pheromone treatment. Comparing the percentage of cells in the G0/G1 phase for both experiments (Figure 22) we could observe that the number of BY4741 cells in G0/G1 phase is higher 3h after stimulation, with 63% of cells arrested in G0/G1 phase while in the control this value is only 36% (Figure 23 – B and E). The cells started recovering from division arrest 4 hrs after stimulation, with an increase in the % of cells in S and G2/M phases and a decrease in G0/G1 (Figure 23). At 9 hrs after the stimulus cells had fully recovered from division arrest with an increase in G2/M and a

decrease in G0/G1 phase, corresponding to restore of cell division (Figure 22; Figure 23– E and F).

In contrast, the AGLPreB strain showed a higher decrease in the number of cells in G2/M phase (13% of cells) and an increase in G0/G1 phase (73%) (Figure 24 – E) 2h after synthetic α-pheromone exposure (Figure 22). As for the BY4741 strain, the cells fully recovered from the division arrest at 6h and 9h after the stimulation (Figure 23 – E and F; Figure 24).

When we compare these results with the *shmoo* assay (Figure 21) we can see that for both strains there are huge differences in the percentage of cells that respond to pheromone. The delay and the differences observed in the number of cells responding to the pheromone in *shmoo* assay compared to flow cytometry analysis, can be related to: the specific alterations in protein synthesis required for *shmoo* formation, which takes more time than growth arrest; and the constitutive expression of the PreB that can, for some reason, interfere in the cells response and morphology. It is therefore necessary to perform additional studies to clarify this question.
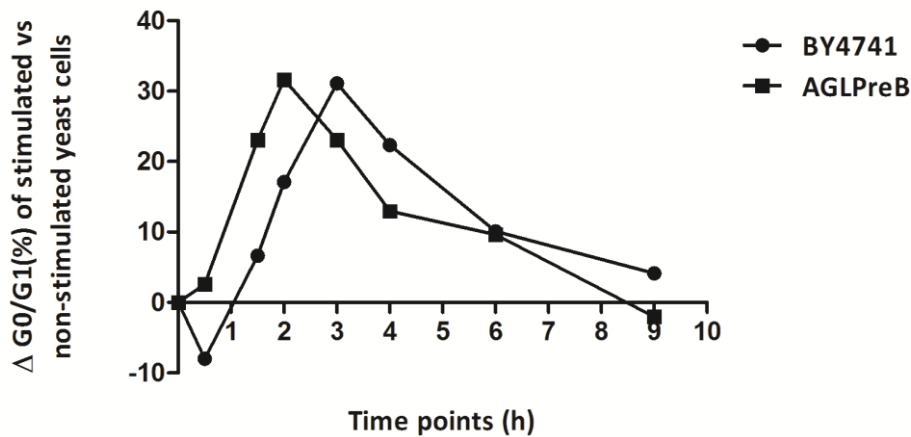
Figure 22 – **Cell cycle arrest analysis**. Percentage of cells in G0/G1 phase upon stimulation with synthetic Pbα or Scα pheromone compared to non-stimulated cells, at different time points.

A previous analysis showed that the concentration of α-pheromone determines the ability of cell to undergo cell cycle arrest and to form *shmoos*. Dose-response curves revealed that for the induction of *shmoo* formation 100-fold-higher concentrations of α-pheromone are needed than for cell cycle arrest [147]. In the present study we used the same amount of pheromone for both experiments, which can also give an explanation

for the differences in the percentage of responsive cells between the *shmoo* assay and cell cycle analysis.

In summary, we showed that heterologous expression of *PREB* from *P. brasiliensis* in a *S. cerevisiae MAT***a** *ste2* mutant and stimulated with Pbα can activate the *S. cerevisiae* intracellular mating pathway, leading to cell cycle arrest and *shmoo* formation.
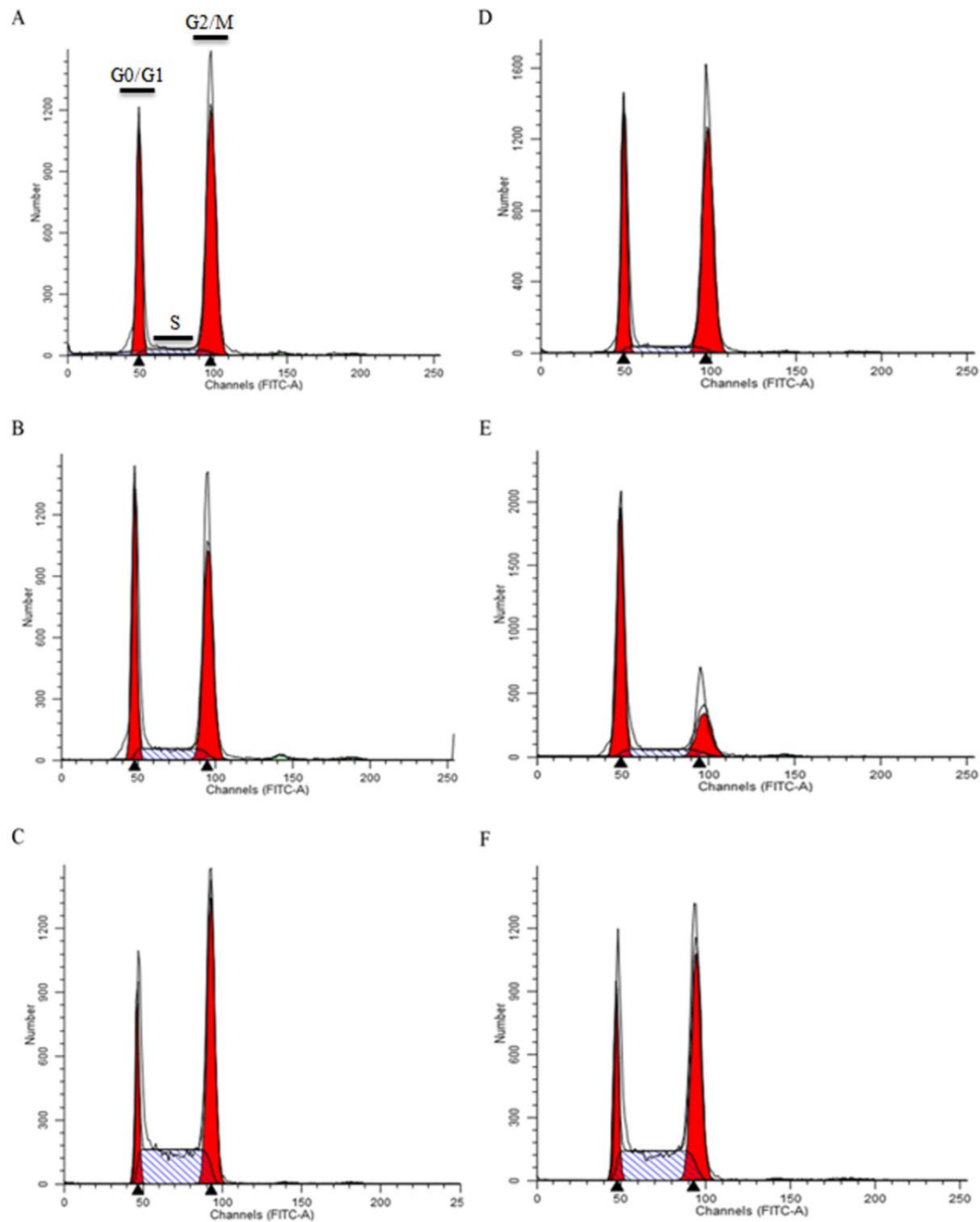
Figure 23 – Representative cell cycle analysis histogram of BY4741 strain. BY4741 cells in exponential phase of growth in YNB medium, supplemented to meet auxotrophic requirements, were subjected to cell cycle analysis. Cell cycle profile presented as the number of cells in each cell cycle phase (G0/G1, S and G2/M) versus fluorescence intensity (corresponding to DNA content). A, B, and C – cell cycle analysis of non-stimulated cells (0h, 3h and 9h, respectively); D, E, F – cell cycle analysis of cells stimulated with synthetic α-pheromone (0h, 3h and 9h, respectively).

60
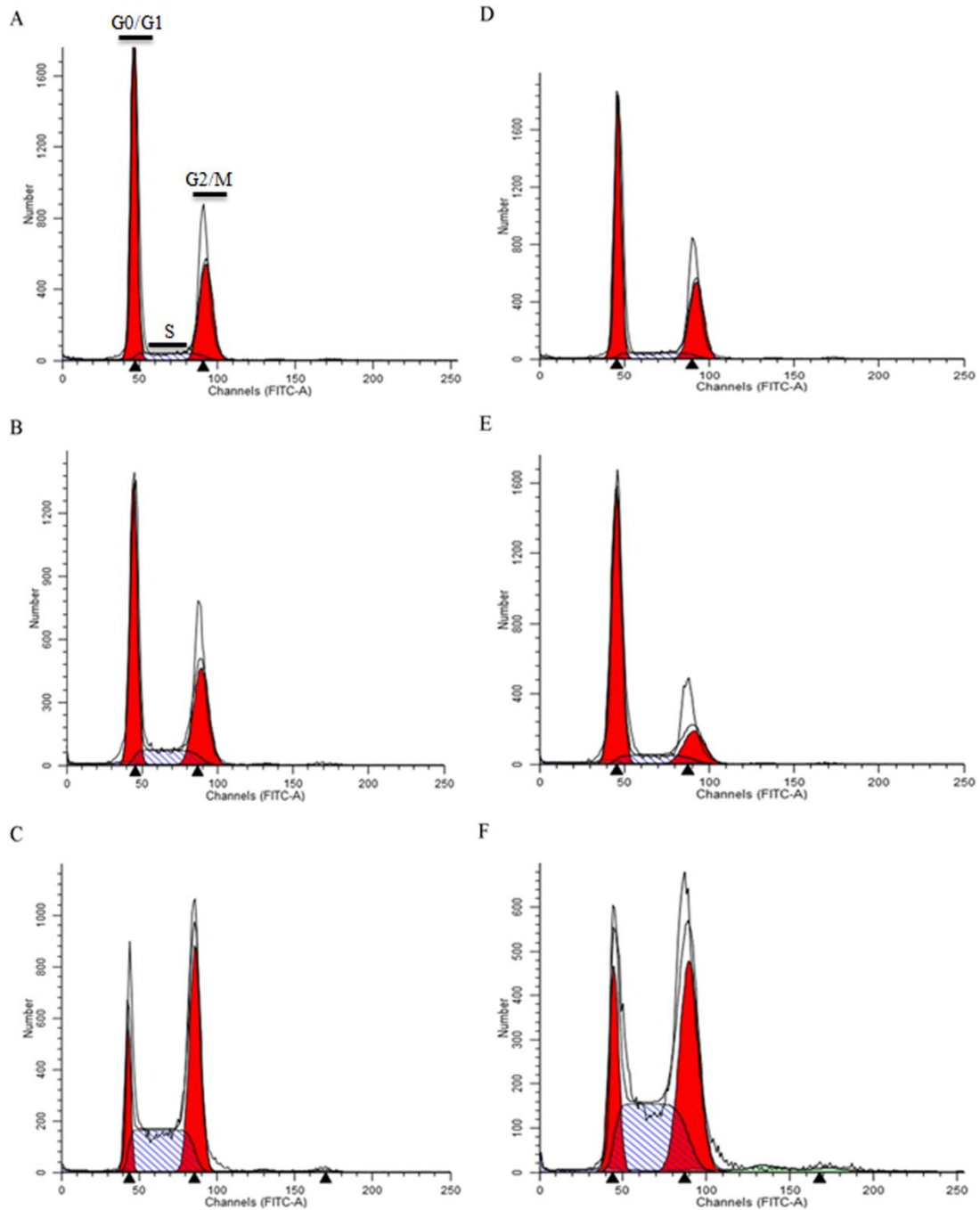
Figure 24 – **Representative cell cycle analysis histogram of AGLPreB strain**. AGLPreB cells in exponential phase of growth in YNB medium, supplemented to meet auxotrophic requirements, were subjected to cell cycle analysis. Cell cycle profile presented as the number of cells in each cell cycle phase (G0/G1, S and G2/M) versus fluorescence intensity (corresponding to DNA content). A, B, and C – cell cycle analysis of non-stimulated cells (0h, 2h and 9h, respectively); D, E, F – cell cycle analysis of cells stimulated with synthetic α-pheromone (0h, 2h and 9h, respectively).

3.4.4. Mating assay

Functionality of the *P. brasiliensis* mating-gene homologues was also tested using a functional complementation assay in the respective *S. cerevisiae* null mutants. *S. cerevisiae* null mutants for Ste12 and Ste18 are not viable, and consequently they are not available at EUROSCARF. *P. brasiliensis* homologues to Ste50, Ste20, Fus3 and Kss1 could not be tested since *S. cerevisiae* mutants for these genes are still able to respond to pheromone stimulation and form diploid cells.

The genes encoding the Ste3 and Ste2 pheromone receptor homologs (PreA and PreB, respectively) as well as *PbGPA1* and *PbSTE4* were amplified and expressed in the respective *S. cerevisiae* null mutants. *PbSTE11* and *PbSTE7* specific amplicons could not be obtained from *P. brasiliensis* cDNA, and were therefore not analyzed.

In order to test the functional complementation of *Pb*Gpa1 and *Pb*Ste4 in *S. cerevisiae* strains Δ*gpa1* and Δ*ste4*, respectively, we performed a mating assay. Our results showed that these two genes do not complement the null mutants of *S. cerevisiae* (data not shown).

Previous experiments allowed us to conclude that the *S. cerevisiae* strain expressing *PREB* can respond to stimulation with exogenous synthetic Pbα. Therefore we continued our studies by testing if strains expressing *PREB* (AGLPreB) and *P. brasiliensis* α-pheromone (AGLPbα and AGMPbα) are able to mate and form diploids. To test this hypothesis we performed mating assays with an incubation time of 5h (Figure 25). Our results showed that Ste2 and PreB recognition of the non-cognate α-pheromone from *P. brasiliensis* and *S. cerevisiae* respectively was not detected. As for the mating assays we used strains deleted in both *MF(α)1* and *MF(α)2*, or deleted in *STE2*, interference of the non-cognate proteins can be excluded.

Analyzing the mating between the wild type strains it can be observed that around 48.8% of the cells form diploids, whereas in our experiments using heterologous strains expressing *P. brasiliensis* α-pheromone and receptor, the efficiency is much lower.
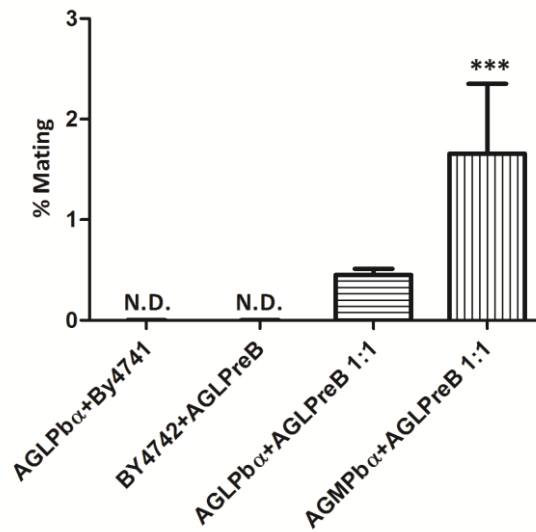
Figure 25 – Quantitative mating assays. Mating efficiency of *S. cerevisiae* null mutants, expressing *P. brasiliensis* α-pheromone (AGLPbα and AGMPbα) and respective receptor (AGLPreB), incubated for 5h.
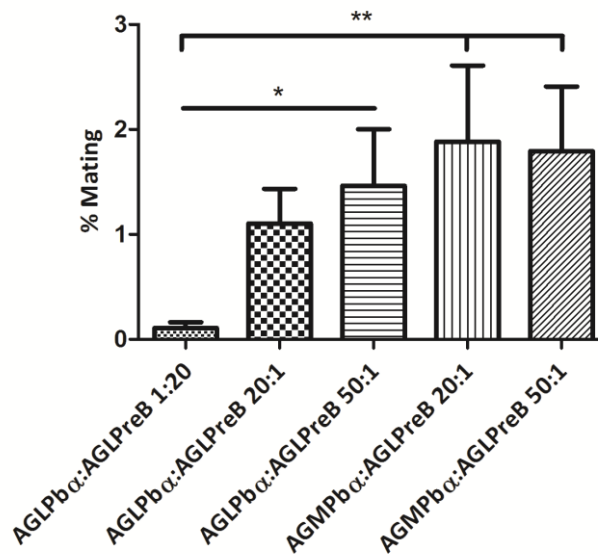
Figure 26 – Quantitative mating assays using different ratios. Mating efficiency of *S. cerevisiae* null mutants, expressing *P. brasiliensis* α-pheromone (AGLPbα and AGMPbα) and respective receptor (AGLPreB) incubated for 5h and crossed at different ratios.

The crossing between AGMPbα and AGLPreB (Ratio 1:1) confer a mating efficiency of 1.66%, while in crosses using AGLPbα the mating ability decreases to 0.45%. When we increase the number of cells expressing *P. brasiliensis* α-pheromone (Ratio 20:1) we

obtained higher percentages of mating with an increase to 1.1% using AGLPbα strain and 1.9% with AGMPbα (Figure 26). However, increasing the number of cells expressing *P. brasiliensis* α-pheromone seems to have a limit on the mating efficiency. Using the AGMPbα (Ratio 50:1) it is possible to observe a slight decrease of the mating efficiency to 1.8%. Interestingly this tendency is not observed when we increase the number of cells expressing PreB (Figure 26), which suggest that the activation of the pheromone response pathway in the heterologous strains is dependent on the concentration of α-pheromone available.

These low mating efficiencies can be explained by the heterologous expression of PreB and *P. brasiliensis* α-pheromone, since: α-pheromone processing and secretion might be hampered; the PreB receptor might be positioned differently in the membrane; there might be poor physical interaction between PreB and the heterotrimeric G protein of *S. cerevisiae* that can lead to a decrease in mating efficiency because of the low activation of the intracellular pheromone response pathway. In fact, we observed no mating restoration when expressing PbGpa1 in a *S. cerevisiae*, which lead us to conclude that the low interaction between PbGpa1 and, Ste2 and Ste4, leads to an inefficient activation of the intracellular mating pathway.

Chapter IV – Conclusions and Future Perspectives

Although sexual reproduction has not been reported in *P. brasiliensis*, several genetic features recently identified in this fungus suggested the existence of a functional sexual cycle. These include the presence of heterothallic mating loci that are expressed and show an equivalent distribution between isolates, and the indication that genetic recombination can occur in *P. brasiliensis* [19, 120]. The fungus also encodes all/most components of the pheromone signaling MAP kinase pathway that is conserved and regulates mating in many fungi such as *S. cerevisiae*, *C. albicans* and *C. neoformans* [107, 148]. These observations led us to further study the functionality of these molecular players in the present work. While we identified gene homologues of the pheromone signaling MAP kinase pathway, as well as an α-pheromone gene, we were not able to confirm if this pathway actively regulates mating in *P. brasiliensis*. We showed that *P. brasiliensis* expresses mating-related genes in both mycelial and yeast form in a mating-type independent way. Through several functional tests in a heterologous yeast model, we could confirm that the α-pheromone and the respective receptor (PreB) constitute a functional signaling pair. Specifically, using *S. cerevisiae* null mutants expressing these *P. brasiliensis* mating-related genes we were able to induce cell cycle arrest, *shmoo* formation and restore mating ability.

For a better understanding of *P. brasiliensis* biology and for future genetic studies in this fungus, it is important to, demonstrate that sexual reproduction can occur and identify the regulatory mechanisms involved. In this sense, it is necessary to perform additional studies:

- In our study we were not able to restore mating ability of *S. cerevisiae* null mutants expressing the G-protein PbGpa1. By constructing a double mutant *S. cerevisiae* strain expressing both PreB and PbGpa1 we might be able to assess if the observed mating impairment is related to weak interactions between PbGpa1 and Ste2 or if PbGpa1 is not able to activate the downstream pathway components;

- Since *P. brasiliensis* harbors all the components of the sexual machinery found in *S. cerevisiae* it is important to evaluate the functionality of all mating-related genes using the *S. cerevisiae* model;

- As shown in the present study, *P. brasiliensis* strains express mating-related genes in both mycelial and yeast forms. In order to understand if *P. brasiliensis* has the ability to undergo sexual reproduction it is important to evaluate if expression of genes involved in the intracellular mating pathway can be induced by stimulation with α-pheromone;

- Finally, it is essential to assess the environmental conditions and genetic requirements that promote mating between *P. brasiliensis* strains.

References

1. San-Blas, G. and G. Nino-Vega, *Paracoccidioides brasiliensis: chemical and molecular tools for research on cell walls, antifungals, diagnosis, taxonomy.* Mycopathologia, 2008. **165**(4-5): p. 183-95.
2. Bergman, A. and A. Casadevall, *Mammalian endothermy optimally restricts fungi and metabolic costs.* MBio, 2010. **1**(5).
3. Bottone, E.J., *An Atlas of the Clinical Microbiology of Infectious Diseases: Viral, fungal and Parasitic Agents*, ed. 1st. 2006, Abingdon, UK: Taylor & Francis.
4. Sudbery, P., N. Gow, and J. Berman, *The distinct morphogenic states of Candida albicans.* Trends Microbiol, 2004. **12**(7): p. 317-24.
5. Andrianopoulos, A., *Control of morphogenesis in the human fungal pathogen Penicillium marneffei.* Int J Med Microbiol, 2002. **292**(5-6): p. 331-47.
6. Brummer, E., E. Castaneda, and A. Restrepo, *Paracoccidioidomycosis: an update.* Clin Microbiol Rev, 1993. **6**(2): p. 89-117.
7. Felipe, M.S., et al., *Functional genome of the human pathogenic fungus Paracoccidioides brasiliensis.* FEMS Immunol Med Microbiol, 2005. **45**(3): p. 369-81.
8. Romani, L., *Immunity to fungal infections.* Nat Rev Immunol, 2004. **4**(1): p. 1-23.
9. McEwen, J.G., et al., *Experimental murine paracoccidiodomycosis induced by the inhalation of conidia.* J Med Vet Mycol, 1987. **25**(3): p. 165-75.
10. Borges-Walmsley, M.I., et al., *The pathobiology of Paracoccidioides brasiliensis.* Trends Microbiol, 2002. **10**(2): p. 80-7.
11. Salazar, M.E., A. Restrepo, and D.A. Stevens, *Inhibition by estrogens of conidium-to-yeast conversion in the fungus Paracoccidioides brasiliensis.* Infect Immun, 1988. **56**(3): p. 711-3.
12. Restrepo, A., et al., *Estrogens inhibit mycelium-to-yeast transformation in the fungus Paracoccidioides brasiliensis: implications for resistance of females to paracoccidioidomycosis.* Infect Immun, 1984. **46**(2): p. 346-53.
13. Pinzan, C.F., et al., *Immunological basis for the gender differences in murine Paracoccidioides brasiliensis infection.* PLoS One, 2010. **5**(5): p. e10757.
14. Bagagli, E., et al., *Paracoccidioides brasiliensis: phylogenetic and ecological aspects.* Mycopathologia, 2008. **165**(4-5): p. 197-207.
15. Puccia, R., J.G. McEwen, and P.S. Cisalpino, *Diversity in Paracoccidioides brasiliensis. The PbGP43 gene as a genetic marker.* Mycopathologia, 2008. **165**(4-5): p. 275-87.
16. Richini-Pereira, V.B., et al., *Molecular approaches for eco-epidemiological studies of Paracoccidioides brasiliensis.* Mem Inst Oswaldo Cruz, 2009. **104**(4): p. 636-43.
17. Bagagli, E., et al., *Phylogenetic and evolutionary aspects of Paracoccidioides brasiliensis reveal a long coexistence with animal hosts that explain several biological features of the pathogen.* Infect Genet Evol, 2006. **6**(5): p. 344-51.
18. Untereiner, W.A., et al., *The Ajellomycetaceae, a new family of vertebrate-associated Onygenales.* Mycologia, 2004. **96**(4): p. 812-21.
19. Matute, D.R., et al., *Cryptic speciation and recombination in the fungus Paracoccidioides brasiliensis as revealed by gene genealogies.* Mol Biol Evol, 2006. **23**(1): p. 65-73.
20. Teixeira, M.M., et al., *Phylogenetic analysis reveals a high level of speciation in the Paracoccidioides genus.* Mol Phylogenet Evol, 2009. **52**(2): p. 273-83.

21.   Almeida, A.J., et al., *Genome size and ploidy of Paracoccidioides brasiliensis reveals a haploid DNA content: flow cytometry and GP43 sequence analysis.* Fungal Genet Biol, 2007. **44**(1): p. 25-31.

22.   Cano, M.I., et al., *Electrophoretic karyotypes and genome sizing of the pathogenic fungus Paracoccidioides brasiliensis.* J Clin Microbiol, 1998. **36**(3): p. 742-7.

23.   Feitosa Ldos, S., et al., *Chromosomal polymorphism, syntenic relationships, and ploidy in the pathogenic fungus Paracoccidioides brasiliensis.* Fungal Genet Biol, 2003. **39**(1): p. 60-9.

24.   Almeida, A.J., et al., *New insights into the cell cycle profile of Paracoccidioides brasiliensis.* Fungal Genet Biol, 2006. **43**(6): p. 401-9.

25.   Desjardins, C.A., et al., *Comparative genomic analysis of human fungal pathogens causing paracoccidioidomycosis.* PLoS Genet, 2011. **7**(10): p. e1002345.

26.   Li, W., et al., *Organization and evolutionary trajectory of the mating type (MAT) locus in dermatophyte and dimorphic fungal pathogens.* Eukaryot Cell. **9**(1): p. 46-58.

27.   Kuck, U., *Cryptic sex in fungi.* Fungal Biology Reviews, 2009. **23**: p. 86-90.

28.   Lee, S.C., et al., *The evolution of sex: a perspective from the fungal kingdom.* Microbiol Mol Biol Rev, 2010. **74**(2): p. 298-340.

29.   Heitman, J., *Evolution of eukaryotic microbial pathogens via covert sexual reproduction.* Cell Host Microbe, 2010. **8**(1): p. 86-99.

30.   Lee, S.C., et al., *The evolution of sex: a perspective from the fungal kingdom.* Microbiol Mol Biol Rev. **74**(2): p. 298-340.

31.   Casselton, L.A., *Mate recognition in fungi.* Heredity, 2002. **88**(2): p. 142-7.

32.   Hsueh, Y.P. and J. Heitman, *Orchestration of sexual reproduction and virulence by the fungal mating-type locus.* Curr Opin Microbiol, 2008. **11**(6): p. 517-24.

33.   Goncalves-Sa, J. and A. Murray, *Asymmetry in sexual pheromones is not required for ascomycete mating.* Curr Biol, 2011. **21**(16): p. 1337-46.

34.   Butler, G., *Fungal sex and pathogenesis.* Clin Microbiol Rev, 2010. **23**(1): p. 140-59.

35.   Shiu, P.K. and N.L. Glass, *Cell and nuclear recognition mechanisms mediated by mating type in filamentous ascomycetes.* Curr Opin Microbiol, 2000. **3**(2): p. 183-8.

36.   Katz Ezov, T., et al., *Heterothallism in Saccharomyces cerevisiae isolates from nature: effect of HO locus on the mode of reproduction.* Mol Ecol. **19**(1): p. 121-31.

37.   Gustin, M.C., et al., *MAP kinase pathways in the yeast Saccharomyces cerevisiae.* Microbiol Mol Biol Rev, 1998. **62**(4): p. 1264-300.

38.   Cherkasova, V., D.M. Lyons, and E.A. Elion, *Fus3p and Kss1p control G1 arrest in Saccharomyces cerevisiae through a balance of distinct arrest and proliferative functions that operate in parallel with Far1p.* Genetics, 1999. **151**(3): p. 989-1004.

39.   Herskowitz, I., *Life cycle of the budding yeast Saccharomyces cerevisiae.* Microbiol Rev, 1988. **52**(4): p. 536-53.

40.   Lodish, H., Berk, A., Kaiser, C., A., Krieger, M., Scott, M., P., Bretscher, A., Ploegh, H., Matsudaira, P., *Molecular cell biology*. 6th ed. 2008, New York: W. H. Freeman and Company.

41.   Lodish, H., Berk, A., Zipursky, S., L., Matsudaira, P., Baltimore, D., Darnell, J., *Molecular Cell Biology*, ed. 4th. 2000, New York: W. H. Freeman.

42. Butler, G., et al., *Evolution of the MAT locus and its Ho endonuclease in yeast species.* Proc Natl Acad Sci U S A, 2004. **101**(6): p. 1632-7.

43. Kim, J. and P. Sudbery, *Candida albicans, a major human fungal pathogen.* J Microbiol, 2011. **49**(2): p. 171-7.

44. Bennett, R.J. and A.D. Johnson, *Mating in Candida albicans and the search for a sexual cycle.* Annu Rev Microbiol, 2005. **59**: p. 233-55.

45. Bennett, R.J. and A.D. Johnson, *Completion of a parasexual cycle in Candida albicans by induced chromosome loss in tetraploid strains.* EMBO J, 2003. **22**(10): p. 2505-15.

46. Hull, C.M. and A.D. Johnson, *Identification of a mating type-like locus in the asexual pathogenic yeast Candida albicans.* Science, 1999. **285**(5431): p. 1271-5.

47. Alby, K. and R.J. Bennett, *Sexual reproduction in the Candida clade: cryptic cycles, diverse mechanisms, and alternative functions.* Cell Mol Life Sci, 2010. **67**(19): p. 3275-85.

48. Hull, C.M., R.M. Raisner, and A.D. Johnson, *Evidence for mating of the "asexual" yeast Candida albicans in a mammalian host.* Science, 2000. **289**(5477): p. 307-10.

49. Magee, B.B. and P.T. Magee, *Induction of mating in Candida albicans by construction of MTLa and MTLalpha strains.* Science, 2000. **289**(5477): p. 310-3.

50. Miller, M.G. and A.D. Johnson, *White-opaque switching in Candida albicans is controlled by mating-type locus homeodomain proteins and allows efficient mating.* Cell, 2002. **110**(3): p. 293-302.

51. Whiteway, M. and C. Bachewich, *Morphogenesis in Candida albicans.* Annu Rev Microbiol, 2007. **61**: p. 529-53.

52. Morschhauser, J., *Regulation of white-opaque switching in Candida albicans.* Med Microbiol Immunol, 2010. **199**(3): p. 165-72.

53. Bennett, R.J., et al., *Identification and characterization of a Candida albicans mating pheromone.* Mol Cell Biol, 2003. **23**(22): p. 8189-201.

54. Dignard, D., et al., *Identification and characterization of MFA1, the gene encoding Candida albicans a-factor pheromone.* Eukaryot Cell, 2007. **6**(3): p. 487-94.

55. Alby, K., D. Schaefer, and R.J. Bennett, *Homothallic and heterothallic mating in the opportunistic pathogen Candida albicans.* Nature, 2009. **460**(7257): p. 890-3.

56. Paoletti, M., et al., *Evidence for sexuality in the opportunistic fungal pathogen Aspergillus fumigatus.* Curr Biol, 2005. **15**(13): p. 1242-8.

57. Poggeler, S., *Genomic evidence for mating abilities in the asexual pathogen Aspergillus fumigatus.* Curr Genet, 2002. **42**(3): p. 153-60.

58. Turgeon, B.G. and O.C. Yoder, *Proposed nomenclature for mating type genes of filamentous ascomycetes.* Fungal Genet Biol, 2000. **31**(1): p. 1-5.

59. Varga, J., *Mating type gene homologues in Aspergillus fumigatus.* Microbiology, 2003. **149**(Pt 4): p. 816-9.

60. Galagan, J.E., et al., *Sequencing of Aspergillus nidulans and comparative analysis with A. fumigatus and A. oryzae.* Nature, 2005. **438**(7071): p. 1105-15.

61. O'Gorman, C.M., H.T. Fuller, and P.S. Dyer, *Discovery of a sexual cycle in the opportunistic fungal pathogen Aspergillus fumigatus.* Nature, 2009. **457**(7228): p. 471-4.

62. Kasuga, T., J.W. Taylor, and T.J. White, *Phylogenetic relationships of varieties and geographical groups of the human pathogenic fungus Histoplasma capsulatum Darling.* J Clin Microbiol, 1999. **37**(3): p. 653-63.

63. Bubnick, M. and A.G. Smulian, *The MAT1 locus of Histoplasma capsulatum is responsive in a mating type-specific manner.* Eukaryot Cell, 2007. **6**(4): p. 616-21.

64. Kwon-Chung, K.J., *Studies on Emmonsiella capsulata. I. Heterothallism and development of the ascocarp.* Mycologia, 1973. **65**(1): p. 109-21.

65. Kwon-Chung, K.J., R.J. Weeks, and H.W. Larsh, *Studies on Emmonsiella capsulata (Histoplasma capsulatum). II. Distribution of the two mating types in 13 endemic states of the United States.* Am J Epidemiol, 1974. **99**(1): p. 44-9.

66. Kwon-Chung, K.J., M.S. Bartlett, and L.J. Wheat, *Distribution of the two mating types among Histoplasma capsulatum isolates obtained from an urban histoplasmosis outbreak.* Sabouraudia, 1984. **22**(2): p. 155-7.

67. Dyer, P.S., M. Paoletti, and D.B. Archer, *Genomics reveals sexual secrets of Aspergillus.* Microbiology, 2003. **149**(Pt 9): p. 2301-3.

68. Laskowski, M.C. and A.G. Smulian, *Insertional mutagenesis enables cleistothecial formation in a non-mating strain of Histoplasma capsulatum.* BMC Microbiol, 2010. **10**: p. 49.

69. Lengeler, K.B., et al., *Mating-type locus of Cryptococcus neoformans: a step in the evolution of sex chromosomes.* Eukaryot Cell, 2002. **1**(5): p. 704-18.

70. Lin, X. and J. Heitman, *The biology of the Cryptococcus neoformans species complex.* Annu Rev Microbiol, 2006. **60**: p. 69-105.

71. Hull, C.M. and J. Heitman, *Genetics of Cryptococcus neoformans.* Annu Rev Genet, 2002. **36**: p. 557-615.

72. Kozubowski, L., S.C. Lee, and J. Heitman, *Signalling pathways in the pathogenesis of Cryptococcus.* Cell Microbiol, 2009. **11**(3): p. 370-80.

73. Wang, L. and X. Lin, *Mechanisms of unisexual mating in Cryptococcus neoformans.* Fungal Genet Biol, 2011. **48**(7): p. 651-60.

74. Karos, M., et al., *Mapping of the Cryptococcus neoformans MATalpha locus: presence of mating type-specific mitogen-activated protein kinase cascade homologs.* J Bacteriol, 2000. **182**(21): p. 6222-7.

75. Moore, T.D. and J.C. Edman, *The alpha-mating type locus of Cryptococcus neoformans contains a peptide pheromone gene.* Mol Cell Biol, 1993. **13**(3): p. 1962-70.

76. Chung, S., et al., *Molecular analysis of CPRalpha, a MATalpha-specific pheromone receptor gene of Cryptococcus neoformans.* Eukaryot Cell, 2002. **1**(3): p. 432-9.

77. Chang, Y.C., G.F. Miller, and K.J. Kwon-Chung, *Importance of a developmentally regulated pheromone receptor of Cryptococcus neoformans for virulence.* Infect Immun, 2003. **71**(9): p. 4953-60.

78. McClelland, C.M., et al., *Isolation and characterization of the Cryptococcus neoformans MATa pheromone gene.* Genetics, 2002. **160**(3): p. 935-47.

79. Hull, C.M., M.J. Boily, and J. Heitman, *Sex-specific homeodomain proteins Sxi1alpha and Sxi2a coordinately regulate sexual development in Cryptococcus neoformans.* Eukaryot Cell, 2005. **4**(3): p. 526-35.

80. Hull, C.M., R.C. Davidson, and J. Heitman, *Cell identity and sexual development in Cryptococcus neoformans are controlled by the mating-type-specific homeodomain protein Sxi1alpha.* Genes Dev, 2002. **16**(23): p. 3046-60.

81.   Kwon-Chung, K.J., J.C. Edman, and B.L. Wickes, *Genetic association of mating types and virulence in Cryptococcus neoformans.* Infect Immun, 1992. **60**(2): p. 602-5.

82.   Konopka, J.B. and S. Fields, *The pheromone signal pathway in Saccharomyces cerevisiae.* Antonie Van Leeuwenhoek, 1992. **62**(1-2): p. 95-108.

83.   Singh, A., et al., *Saccharomyces cerevisiae contains two discrete genes coding for the alpha-factor pheromone.* Nucleic Acids Res, 1983. **11**(12): p. 4049-63.

84.   Kurjan, J. and I. Herskowitz, *Structure of a yeast pheromone gene (MF alpha): a putative alpha-factor precursor contains four tandem copies of mature alpha-factor.* Cell, 1982. **30**(3): p. 933-43.

85.   Brake, A.J., D.J. Julius, and J. Thorner, *A functional prepro-alpha-factor gene in Saccharomyces yeasts can contain three, four, or five repeats of the mature pheromone sequence.* Mol Cell Biol, 1983. **3**(8): p. 1440-50.

86.   Waters, M.G., E.A. Evans, and G. Blobel, *Prepro-alpha-factor has a cleavable signal sequence.* J Biol Chem, 1988. **263**(13): p. 6209-14.

87.   Jones, S.K., Jr. and R.J. Bennett, *Fungal mating pheromones: choreographing the dating game.* Fungal Genet Biol, 2011. **48**(7): p. 668-76.

88.   Struck, D.K., W.J. Lennarz, and K. Brew, *Primary structural requirements for the enzymatic formation of the N-glycosidic bond in glycoproteins. Studies with alpha-lactalbumin.* J Biol Chem, 1978. **253**(16): p. 5786-94.

89.   Julius, D., et al., *Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-alpha-factor.* Cell, 1984. **37**(3): p. 1075-89.

90.   Dmochowska, A., et al., *Yeast KEX1 gene encodes a putative protease with a carboxypeptidase B-like function involved in killer toxin and alpha-factor precursor processing.* Cell, 1987. **50**(4): p. 573-84.

91.   Michaelis, S., et al., *Biogenesis of yeast a-factor involves prenylation, methylation and a novel export mechanism.* Antonie Van Leeuwenhoek, 1992. **61**(2): p. 115-7.

92.   Chen, P., et al., *Biogenesis of the Saccharomyces cerevisiae mating pheromone a-factor.* J Cell Biol, 1997. **136**(2): p. 251-69.

93.   Laskowski, M., *Uncovering molecular mechanisms that regulate mating in Histoplasma capsulatum*, in *Department of Pathobiology and Molecular Medicine*. 2009, University of Cincinnati: Cincinnati. p. 165.

94.   Schwartz, M.A. and H.D. Madhani, *Principles of MAP kinase signaling specificity in Saccharomyces cerevisiae.* Annu Rev Genet, 2004. **38**: p. 725-48.

95.   Peter, M., et al., *FAR1 links the signal transduction pathway to the cell cycle machinery in yeast.* Cell, 1993. **73**(4): p. 747-60.

96.   Saito, H., *Regulation of cross-talk in yeast MAPK signaling pathways.* Curr Opin Microbiol, 2010. **13**(6): p. 677-83.

97.   Sadhu, C., et al., *A G-protein alpha subunit from asexual Candida albicans functions in the mating signal transduction pathway of Saccharomyces cerevisiae and is regulated by the a1-alpha 2 repressor.* Mol Cell Biol, 1992. **12**(5): p. 1977-85.

98.   Whiteway, M., D. Dignard, and D.Y. Thomas, *Dominant negative selection of heterologous genes: isolation of Candida albicans genes that interfere with Saccharomyces cerevisiae mating factor-induced cell cycle arrest.* Proc Natl Acad Sci U S A, 1992. **89**(20): p. 9410-4.

99.   Chen, J., Q. Wang, and J.Y. Chen, *CEK2, a Novel MAPK from Candida albicans Complement the Mating Defect of fus3/kss1 Mutant.* Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai), 2000. **32**(3): p. 299-304.

100. Yi, S., et al., *Utilization of the mating scaffold protein in the evolution of a new signal transduction pathway for biofilm development.* MBio, 2011. **2**(1): p. e00237-10.

101. Cote, P., et al., *Evolutionary reshaping of fungal mating pathway scaffold proteins.* MBio, 2011. **2**(1): p. e00230-10.

102. Clark, K.L., et al., *Constitutive activation of the Saccharomyces cerevisiae mating response pathway by a MAP kinase kinase from Candida albicans.* Mol Gen Genet, 1995. **249**(6): p. 609-21.

103. Leberer, E., et al., *Signal transduction through homologs of the Ste20p and Ste7p protein kinases can trigger hyphal formation in the pathogenic fungus Candida albicans.* Proc Natl Acad Sci U S A, 1996. **93**(23): p. 13217-22.

104. Liu, H., J. Kohler, and G.R. Fink, *Suppression of hyphal formation in Candida albicans by mutation of a STE12 homolog.* Science, 1994. **266**(5191): p. 1723-6.

105. Atir-Lande, A., T. Gildor, and D. Kornitzer, *Role for the SCFCDC4 ubiquitin ligase in Candida albicans morphogenesis.* Mol Biol Cell, 2005. **16**(6): p. 2772-85.

106. Cote, P. and M. Whiteway, *The role of Candida albicans FAR1 in regulation of pheromone-mediated mating, gene expression and cell cycle arrest.* Mol Microbiol, 2008. **68**(2): p. 392-404.

107. Chen, J., S. Lane, and H. Liu, *A conserved mitogen-activated protein kinase pathway is required for mating in Candida albicans.* Mol Microbiol, 2002. **46**(5): p. 1335-44.

108. Liebmann, B., et al., *cAMP signaling in Aspergillus fumigatus is involved in the regulation of the virulence gene pksP and in defense against killing by macrophages.* Mol Genet Genomics, 2003. **269**(3): p. 420-35.

109. Paoletti, M., et al., *Mating type and the genetic basis of self-fertility in the model fungus Aspergillus nidulans.* Curr Biol, 2007. **17**(16): p. 1384-9.

110. Hsueh, Y.P., C. Xue, and J. Heitman, *G protein signaling governing cell fate decisions involves opposing Galpha subunits in Cryptococcus neoformans.* Mol Biol Cell, 2007. **18**(9): p. 3237-49.

111. Palmer, D.A., et al., *Gib2, a novel Gbeta-like/RACK1 homolog, functions as a Gbeta subunit in cAMP signaling and is essential in Cryptococcus neoformans.* J Biol Chem, 2006. **281**(43): p. 32596-605.

112. Wang, P., J.R. Perfect, and J. Heitman, *The G-protein beta subunit GPB1 is required for mating and haploid fruiting in Cryptococcus neoformans.* Mol Cell Biol, 2000. **20**(1): p. 352-62.

113. Lengeler, K.B., et al., *Identification of the MATa mating-type locus of Cryptococcus neoformans reveals a serotype A MATa strain thought to have been extinct.* Proc Natl Acad Sci U S A, 2000. **97**(26): p. 14455-60.

114. Nichols, C.B., J.A. Fraser, and J. Heitman, *PAK kinases Ste20 and Pak1 govern cell polarity at different stages of mating in Cryptococcus neoformans.* Mol Biol Cell, 2004. **15**(10): p. 4476-89.

115. Clarke, D.L., et al., *The Cryptococcus neoformans STE11alpha gene is similar to other fungal mitogen-activated protein kinase kinase kinase (MAPKKK) genes but is mating type specific.* Mol Microbiol, 2001. **40**(1): p. 200-13.

116. Davidson, R.C., et al., *A MAP kinase cascade composed of cell type specific and non-specific elements controls mating and differentiation of the fungal pathogen Cryptococcus neoformans.* Mol Microbiol, 2003. **49**(2): p. 469-85.

117. Chang, Y.C., et al., *Cryptococcus neoformans STE12alpha regulates virulence but is not essential for mating.* J Exp Med, 2000. **191**(5): p. 871-82.

118. Chang, Y.C., L.A. Penoyer, and K.J. Kwon-Chung, *The second STE12 homologue of Cryptococcus neoformans is MATa-specific and plays an important role in virulence.* Proc Natl Acad Sci U S A, 2001. **98**(6): p. 3258-63.

119. Li, W., et al., *Organization and evolutionary trajectory of the mating type (MAT) locus in dermatophyte and dimorphic fungal pathogens.* Eukaryot Cell, 2010. **9**(1): p. 46-58.

120. Torres, I., et al., *Presence and expression of the mating type locus in Paracoccidioides brasiliensis isolates.* Fungal Genet Biol, 2010. **47**(4): p. 373-80.

121. Hebeler-Barbosa, F., M.R. Montenegro, and E. Bagagli, *Virulence profiles of ten Paracoccidioides brasiliensis isolates obtained from armadillos (Dasypus novemcinctus).* Med Mycol, 2003. **41**(2): p. 89-96.

122. Gomez, B.L., et al., *Detection of melanin-like pigments in the dimorphic fungal pathogen Paracoccidioides brasiliensis in vitro and during infection.* Infect Immun, 2001. **69**(9): p. 5760-7.

123. Morais, F.V., et al., *Polymorphism in the gene coding for the immunodominant antigen gp43 from the pathogenic fungus Paracoccidioides brasiliensis.* J Clin Microbiol, 2000. **38**(11): p. 3960-6.

124. Restrepo, A. and B.E. Jimenez, *Growth of Paracoccidioides brasiliensis yeast phase in a chemically defined culture medium.* J Clin Microbiol, 1980. **12**(2): p. 279-81.

125. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.

126. Gietz, R.D. and R.A. Woods, *Yeast transformation by the LiAc/SS Carrier DNA/PEG method.* Methods Mol Biol, 2006. **313**: p. 107-20.

127. Sambrook, J., Russell, D., W., *Molecular cloning: a laboratory manual*, ed. 3th. Vol. 2. 2001, New York: Harbor Laboratory Press, Cold Spring

128. Fortuna, M., et al., *Cell cycle analysis of yeasts.* Curr Protoc Cytom, 2001. **Chapter 11**: p. Unit 11 13.

129. Guthrie, C., and G. R. Fink, *Guide to Yeast Genetics and Molecular and Cell Biology.* Vol. 1. 2004, San Diego: Academic Press.

130. Umanah, G.K., et al., *Identification of residue-to-residue contact between a peptide ligand and its G protein-coupled receptor using periodate-mediated dihydroxyphenylalanine cross-linking and mass spectrometry.* J Biol Chem, 2010. **285**(50): p. 39425-36.

131. Lee, B.K., et al., *Identification of residues of the Saccharomyces cerevisiae G protein-coupled receptor contributing to alpha-factor pheromone binding.* J Biol Chem, 2001. **276**(41): p. 37950-61.

132. Naider, F. and J.M. Becker, *The alpha-factor mating pheromone of Saccharomyces cerevisiae: a model for studying the interaction of peptide hormones and G protein-coupled receptors.* Peptides, 2004. **25**(9): p. 1441-63.

133. Lee, B.K., et al., *Tyr266 in the sixth transmembrane domain of the yeast alpha-factor receptor plays key roles in receptor activation and ligand specificity.* Biochemistry, 2002. **41**(46): p. 13681-9.

134. Ding, F.X., et al., *Study of the binding environment of alpha-factor in its G protein-coupled receptor using fluorescence spectroscopy.* J Pept Res, 2002. **60**(1): p. 65-74.

135. Clark, C.D., T. Palzkill, and D. Botstein, *Systematic mutagenesis of the yeast mating pheromone receptor third intracellular loop.* J Biol Chem, 1994. **269**(12): p. 8831-41.

136. Savarese, T.M. and C.M. Fraser, *In vitro mutagenesis and the search for structure-function relationships among G protein-coupled receptors.* Biochem J, 1992. **283 (Pt 1)**: p. 1-19.

137. Janiak, A.M., et al., *Functional expression of the Candida albicans alpha-factor receptor in Saccharomyces cerevisiae.* Fungal Genet Biol, 2005. **42**(4): p. 328-38.

138. Poggeler, S. and U. Kuck, *Identification of transcriptionally expressed pheromone receptor genes in filamentous ascomycetes.* Gene, 2001. **280**(1-2): p. 9-17.

139. Muller, H., et al., *The asexual yeast Candida glabrata maintains distinct a and alpha haploid mating types.* Eukaryot Cell, 2008. **7**(5): p. 848-58.

140. Brzostowski, J.A. and A.R. Kimmel, *Signaling at zero G: G-protein-independent functions for 7-TM receptors.* Trends Biochem Sci, 2001. **26**(5): p. 291-7.

141. Xue, C., Y.P. Hsueh, and J. Heitman, *Magnificent seven: roles of G protein-coupled receptors in extracellular sensing in fungi.* FEMS Microbiol Rev, 2008. **32**(6): p. 1010-32.

142. Marsh, L., *Substitutions in the hydrophobic core of the alpha-factor receptor of Saccharomyces cerevisiae permit response to Saccharomyces kluyveri alpha-factor and to antagonist.* Mol Cell Biol, 1992. **12**(9): p. 3959-66.

143. Kurjan, J., J.P. Hirsch, and C. Dietzel, *Mutations in the guanine nucleotide-binding domains of a yeast G alpha protein confer a constitutive or uninducible state to the pheromone response pathway.* Genes Dev, 1991. **5**(3): p. 475-83.

144. Mayrhofer, S. and S. Poggeler, *Functional characterization of an alpha-factor-like Sordaria macrospora peptide pheromone and analysis of its interaction with its cognate receptor in Saccharomyces cerevisiae.* Eukaryot Cell, 2005. **4**(4): p. 661-72.

145. Wendland, J., A. Dunkler, and A. Walther, *Characterization of alpha-factor pheromone and pheromone receptor genes of Ashbya gossypii.* FEMS Yeast Res, 2011. **11**(5): p. 418-29.

146. Throm, E. and W. Duntze, *Mating-Type-Dependent Inhibition of Deoxyribonucleic Acid Synthesis in Saccharomyces cerevisiae.* J Bacteriol, 1970. **104**(3): p. 1388-90.

147. Moore, S.A., *Comparison of dose-response curves for alpha factor-induced cell division arrest, agglutination, and projection formation of yeast cells. Implication for the mechanism of alpha factor action.* J Biol Chem, 1983. **258**(22): p. 13849-56.

148. Lengeler, K.B., et al., *Signal transduction cascades regulating fungal development and virulence.* Microbiol Mol Biol Rev, 2000. **64**(4): p. 746-85.

149. Marques, E.R., et al., Identification of genes preferentially expressed in the pathogenic yeast phase of Paracoccidioides brasiliensis, using suppression subtraction hybridization and differential macroarray analysis. Mol Genet Genomics, 2004. 271(6): p. 667-77.

Supplementary data

Supplementary Table S1. Primers used in this study. Sequences for homologous recombination (HR) in the plasmid as well as in *S. cerevisiae* are indicated by shading. Start and stop codons are indicated in bold and italics.

Primer	Sequence 5´-3´	Target	Reference
***MAT* locus confirmation**			
MAT1-1-Fw	GCAATTGTCTATTTCCATCAGT	*MAT1-1*	Torres et al. [120]
MAT1-1-Rev	CTAGATGTCAAGGTACTCGGTA	*MAT1-1*	Torres et al. [120]
MAT1-2-Fw	TTCGACCGTCCACGCCTATCTC	*MAT1-2*	Torres et al. [120]
MAT1-2-Rev	TCATTGCGAAAAGGTGTCAA	*MAT1-2*	Torres et al. [120]
Sequencing			
PREB-Fw	ATGGCACCCTCATTCGACCCCTTC	*PREB*	This study
PREB-Rev	TCAGGCCTTTGTGCCAGCTTC	*PREB*	This study
Pbα-Fw	ATGAAGTTCGTCGTGGTTTTC	*P. brasiliensis* α-pheromone gene	This study
Pbα-Rev	CTAGCAACCCTGACCAGGG	*P. brasiliensis* α-pheromone gene	This study
Homologous recombination			
PREB-HR-Fw	CACTAAATTACCGGATCAATTCGGG***ATG***GCACCCTCATTCGACCCCTTC	pCM189 / *PREB*	This study
PREB-HR-Rev	TCGATGTTAACAGGCCTGTTTAAAC***TCA***GGCCTTTGTGCCAGCTTC	pCM189 / *PREB*	This study
Pbα-HR-Fw	CACTAAATTACCGGATCAATTCGGG***ATG***AAGTTCGTCGTGGTTTTCCTTGC	pCM189-pCM190 / *P. brasiliensis* α-pheromone gene	This study
Pbα-HR-Rev	TCGATGTTAACAGGCCTGTTTAAAC***CTA***GCAACCCTGACCAGGGCGAGTG	pCM189-pCM190 / *P. brasiliensis* α-pheromone gene	This study
PbGpa1-HR-Fw	CACTAAATTACCGGATCAATTCGGG***ATG***GGGTGTGGAATGAGCACCG	pCM189 / *PbGPA1*	This study
PbGpa1-HR-Rev	TCGATGTTAACAGGCCTGTTTAAAC***TCA***TATCAGTCCACAGAGGCG	pCM189 / *PbGPA1*	This study

PbSte4-HR-Fw	CACTAAATTACCGGATCAATTCGGG*ATG*GCGGCCGATTTGAGCGGCGAGC	pCM189 / *PbSTE4*	This study
PbSte4-HR-Rev	TCGATGTTAACAGGCCTGTTTAAAC*TCA*TGTCATCATAGATTTGATG	pCM189 / *PbSTE4*	This study
MFα2-HR-HPH-Fw	GCTAGTGTTCACTTGCTCATTGATGTCCCGCCTCGTCCCCGCCGGGTCACC	*MFα2* flank / HPH	This study
MFα2-HR-HPH-Rev	GGCCAATTATTACTGCTAAAGATAAACTCCAGTATAGCGACCAGCATTCAC	*MFα2* flank / HPH	This study

Gene replacement confirmation

pCM-Conf-Fw	GCATGCATGTGCTCTGTATG	pCM189 / pCM190	This study
pCM-Conf-Rev	TTTCGGTTAGAGCGGATGTG	pCM189 / pCM190	This study
HPH-Conf-Fw	CGCAAGGAATCGGTCAATAC	HPH	This study
HPH-Conf-Rev	AAAGCATCAGCTCATCGAGA	HPH	This study
MFα-Conf-Rev	GTCCGAAAAATTGAAAGTC	*MFα2* flank	This study

Real-time PCR

Pbα-Fw	GTCGTGGTTTTCCTTGCATTG	*P. brasiliensis* α-pheromone gene	This study
Pbα-Rev	TCTCGACGGGCGCATTC	*P. brasiliensis* α-pheromone gene	This study
PREB-Fw	GTGATCCCAGCCATCTTCTC	*PREB*	This study
PREB-Rev	GTAGCCACTGAAGCCCATAG	*PREB*	This study
PREA-Fw	TCCCCAAGAAACATCAGTCC	*PREA*	This study
PREA-Rev	CATGACCATGCTAGAGGGATG	*PREA*	This study
MAT1-1-Fw	CATTCAGAAGCTTCTACTCTAC	*MAT1-1*	This study
MAT1-1-Rev	CCTTCGCAAGGATTGCCCAC	*MAT1-1*	This study
MAT1-2-Fw	AACGACATATCGATACTCCTTG	*MAT1-2*	This study
MAT1-2-Rev	GATAGTAAGGGTGATCTTTG	*MAT1-2*	This study

| Tub2F | AGCCTTGCGTCGGAACATAG | *β-tubulin (TUB2)* | Marques *et al.*, 2004 [149] |
| Tub2R | ACCTCCATCCAGGAACTCTTCA | *β-tubulin (TUB2)* | Marques *et al.*, 2004 [149] |

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                                                          10        20        30        40        50        60        70        80
                                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Paracoccidioides brasiliensis_Pb01_Sequenced  ATGAAGCTCGTTGTGGTTTTCCTTGCATTGGTGTCCGCTTTCGCATGGGCCGTTGCCATCCCAGAACCCCAGCCAGCTCC
P. brasiliensis ATCC60855_Sequenced           ATGAAGTTCGTCGTGGTTTTCCTTGCATTGATGTCCGCTGCCGCGTGGGCCGTTGCCATCCCAGAACCCCAGCCAGCTCC
P. brasiliensis Pb18_Prediction               ATGAAGTTCGTCGTGGTTTTCCTTGCATTGATGTCCGCTGCCGCGTGGGCCGTTGCCATCCCAGAACCCCAGCCAGCTCC
P. brasiliensis Pb03_Prediction               ATGAAGTTCGTCGTGGTTTTCCTTGCATTGATGTCCGCTGCCGCGTGGGCCGTTGCCATCCCAGAACCCCAGCCAGCTCC
P. brasiliensis_Pb01_Prediction               ATGAAGCTCGTTGTGGTTTTCCTTGCATTGGTGTCCGCTTTCGCATGGGCCGTTGCCATCCCAGAACCCCAGCCAGCTCC

                                                          90        100       110       120       130       140       150       160
                                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Paracoccidioides brasiliensis_Pb01_Sequenced  TACCGTTGAGATTCCAAAGAATGCGC----CCGTTGAGAAGCGCTGGTGCACTCGTCCTGGACAGGGTTGCAG------
P. brasiliensis ATCC60855_Sequenced           TACCGTTGAGATTCCAAAGAATGCGC----CCGTCGAGAAGCGCTGGTGCACTCGCCCTGGTCAGGGTTGCTAG-----
P. brasiliensis Pb18_Prediction               TACCGTTGAGATTCCAAAGAATGCGCGTAACCGTCGAGAAGCGCTGGTGCACTCGCCCTGGTCAGGGTTGCTAGTGGAAT
P. brasiliensis Pb03_Prediction               TACCGTTGAGATTCCAAAGAATGCGCGTAACCGTCGAGAAGCGCTGGTGCACTCGCCCTGGTCAGGGTTGCTAGTGGAAT
P. brasiliensis_Pb01_Prediction               TC--------TTCTAG-----------CCGTTGAGAAGCGCTGGTGCACTCGTCCTGGACAGGGTTGCTAG-----

                                                          170       180       190       200       210       220       230       240
                                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Paracoccidioides brasiliensis_Pb01_Sequenced  -----------------------------------------------------------------------------
P. brasiliensis ATCC60855_Sequenced           -----------------------------------------------------------------------------
P. brasiliensis Pb18_Prediction               TCAACACCACAGCGACCATCGACTGCCATGGCAGTCGATTGTGGGGTCCCAATCTTCGGACTCTTCCATCCTCTCCGCTT
P. brasiliensis Pb03_Prediction               TCAACACCACAGCGACCATCGACTGCCATGGCAGTCGATTGTGGGGTCCCAATCTTCGGACTCTTCCATCCTCTCCGCTT
P. brasiliensis_Pb01_Prediction               -----------------------------------------------------------------------------

                                                          250       260       270       280       290       300       310       320
                                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Paracoccidioides brasiliensis_Pb01_Sequenced  -----------------------------------------------------------------------------
P. brasiliensis ATCC60855_Sequenced           -----------------------------------------------------------------------------
P. brasiliensis Pb18_Prediction               CGACCTGGTGCTACGAGCGAGAGGAGAATTCTATCTGCTGTTACGCATCTCGTTTCTAGTGTTCGACTTGTTATCGTTCT
P. brasiliensis Pb03_Prediction               CGACCTGGTGCTACGAGCGAGAGGAGAGCTCTATCTGCTGTTACGCATCTCGTTTCTAGTGTTCGACTTGTTATCGTTCT
P. brasiliensis_Pb01_Prediction               -----------------------------------------------------------------------------

                                                          330       340       350       360       370       380       390       400
                                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Paracoccidioides brasiliensis_Pb01_Sequenced  -----------------------------------------------------------------------------
P. brasiliensis ATCC60855_Sequenced           -----------------------------------------------------------------------------
P. brasiliensis Pb18_Prediction               TAAATACCGAACTTATGCGGATCGCATATTCGAGATATTCACATCGACTTTTTGCTGGCACATTTTAGCGCACGCGGAAT
P. brasiliensis Pb03_Prediction               TAAATACCGAACTTATGCGGATCGCATATTCGAGATATTCACATCGACTTTTTGCTAG-------------------
P. brasiliensis_Pb01_Prediction               -----------------------------------------------------------------------------

                                                          410       420       430       440       450       460       470       480
                                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Paracoccidioides brasiliensis_Pb01_Sequenced  -----------------------------------------------------------------------------
P. brasiliensis ATCC60855_Sequenced           -----------------------------------------------------------------------------
P. brasiliensis Pb18_Prediction               ACAACAATGGCCTCGATATCAATGGAATATTACTGTTCTTCGTTCATGGTAATGCACTTATCCATTCATATGTTATCATT
P. brasiliensis Pb03_Prediction               -----------------------------------------------------------------------------
P. brasiliensis_Pb01_Prediction               -----------------------------------------------------------------------------

                                                          490
                                                   ....|....|....|
Paracoccidioides brasiliensis_Pb01_Sequenced  ----------------
P. brasiliensis ATCC60855_Sequenced           ----------------
P. brasiliensis Pb18_Prediction               GAGGCACATGCATAA
P. brasiliensis Pb03_Prediction               ----------------
P. brasiliensis_Pb01_Prediction               ----------------
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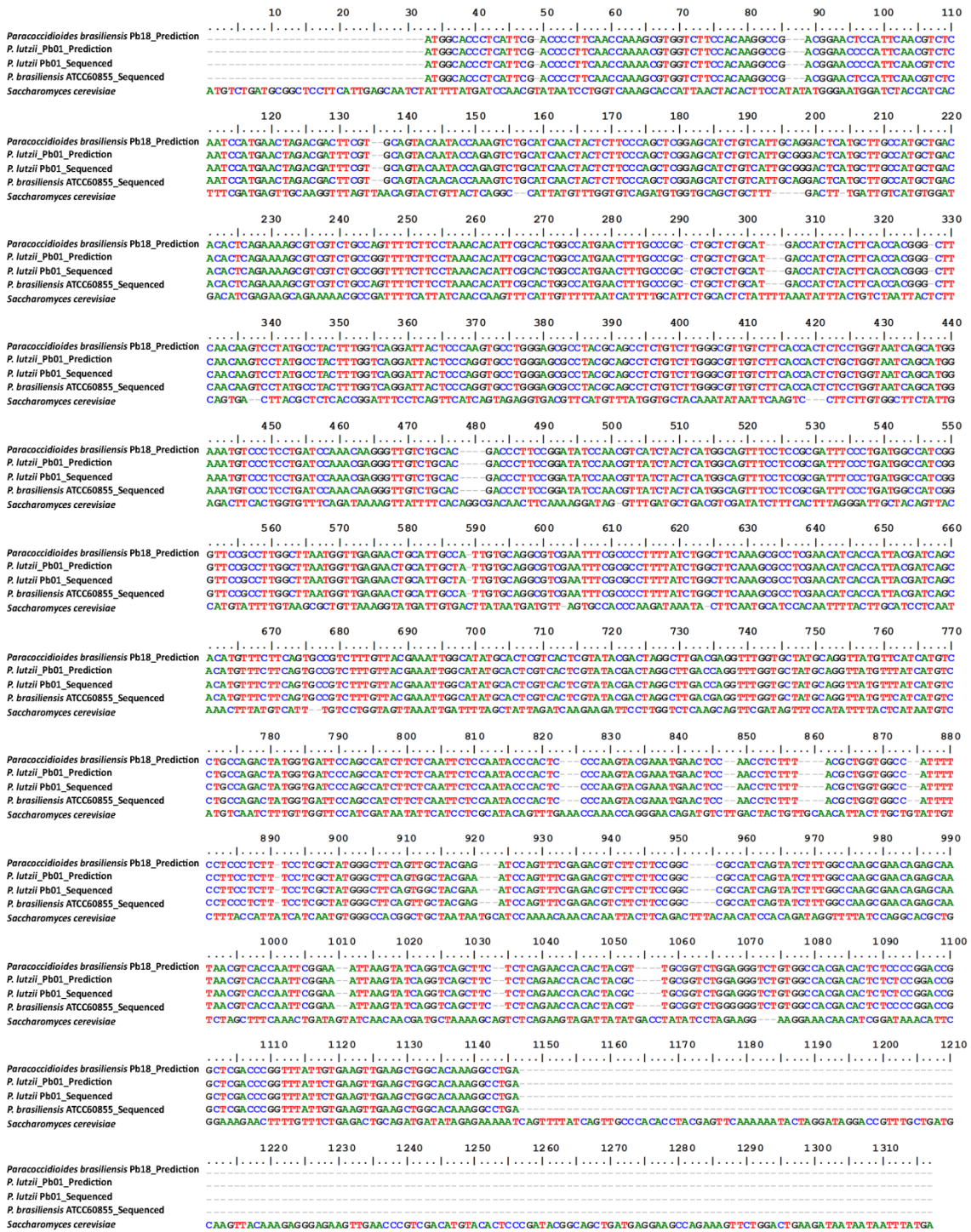
Figure S1 – Sequence of the α-pheromone precursor. Alignment of the sequenced and predicted α-pheromone gene of several *P. brasiliensis* strains.

Figure S2 – Sequence of the α-pheromone receptor. Alignment of sequenced and predicted *PREB* of several *P. brasiliensis* strains versus Sc*STE2*.