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人體病原真菌隱球菌生殖,菌絲生長及致病分子機制之探討

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一、中文摘要

隱球菌(*Cryptococcus neoformans*)為一伺機性人體病原真菌，對免疫缺陷之族群，具致命性真菌性腦膜炎之威脅。隱球菌病原性之研究顯示，該菌主要致病因子包括，莢膜(capsule)、黑色素(melanin)、能生長於 37℃或更高之溫度，以及其交配型基因位點(mating type locus)。隱球菌交配型 *α* 基因位點已被選殖出，其大小超過 100 kb，其上包含 20 個以上之基因，其中包含三個費洛蒙基因。研究費洛蒙基因突變株發現，在交配型 *α* 的菌株中，可能存有費洛蒙自我調控之機轉(autocrine signaling loop)存在，並可能為交配型 *α* 的菌株具較高病原性之成因。本計畫之目的，乃在進一步瞭解隱球菌血清型 D 型菌之費洛蒙反應機制，探討交配型基因位點與致病機制及其他生理調控之關係，主要分為兩個方向，一為找出費洛蒙反應機制之下游基因 *STE6*，以進一步確認費洛蒙自我調控之機轉與有性生殖、致病性之關係。二為針對隱球菌血清型 D 型菌之 G 蛋白質複合體 β 次單元，進行其生理角色之探討。

為了進一步探討此自我調控機轉之作用機制，吾人在隱球菌之基因體序列中，找到了一個和 *S. cerevisiae* **a** 費洛蒙外送蛋白基因 *STE6* 之同源基因。分析 *STE6* 基因突變株之性狀顯示，在隱球菌的兩種不同交配型細胞中，缺乏此費洛蒙傳輸蛋白會明顯降低其有性生殖之效率。但若以兩不同交配型之突變株細胞進行交配時，則其有性生殖之過程將完全阻絕，無法完成。而令人意料的，交配型α *ste6* 突變株仍然保有進行 haploid fruiting 之功能。因此，吾人推測在交配型α細胞中，費洛蒙自我調控之機轉(autocrine signaling loop)，MFα費洛蒙分子不需要經由 STE6 外泌至細胞外，在刺激交配型α細胞進行 haploid fruiting 或其他生理反應；換言之，MFα費洛蒙可能經由細胞內未知標的蛋白之作用，在交配型α細胞內引起對 MFα 費洛蒙之反應。此外，在探討 G 蛋白之功能方面，G 蛋白α與 β次單元基因亦於隱球菌基因體中找到，經由突變株性狀之分析顯示，該二基因應為費洛蒙反應上游 G 蛋白複合體之次單元，G 蛋白α次單元基因 *GPA3* 負向調控隱球有性生殖及 haploid fruiting 之反應；G 蛋白 β次單元基因 GPB1 為有性生殖之正向調控因子。

關鍵詞：隱球菌、費洛蒙反應機制、費洛蒙傳費、洛蒙傳送蛋白質、G 蛋白質α及β次單元

Abstract

Cryptococcus neoformans, an opportunistic human fungal pathogen, causes the life-threatening meningoencephalitis mainly in individuals with compromised immune functions. Studies of the pathogenesis in *C. neoformans* have revealed several important virulence factors such as

capsule, melanin, ability to grow at 37 ℃ or higher temperature, and interestingly the mating type locus. The *MAT*α locus of *C. neoformans* has been identified and characterized. It has unusual large size of over 100 kb and contains more than 20 genes. Three copies of pheromone precursor genes are identified in the *MAT*α locus. Characterization of pheromone gene triple deletion mutant strains suggested an autocrine signaling loop may function and contribute to the virulence of the *MAT*α cells (Shen *et al*., 2002). The purpose of this proposal is to characterize the components in the pheromone response pathway of *C. neoformans* serotype D strain and further address how mating type locus regulates the virulence and how the autocrine signaling loop functions in *C. neoformans*.

To further address the role of pheromones in the signaling loop, we have identified the *STE6* homologue, the *S. cerevisiae* a-factor exporter, in the *C. neoformans* genome and begun to dissect its function. By disrupting the *STE6*, we found that *ste6* mutants in either *MAT*α or *MAT*a background showed partially impaired mating function. However, when *ste6 MAT*α and *MAT*a mutants bilaterally cross with each other, the mating process was completely abolished. Surprisingly, *MAT*α *ste6* mutant does not exhibit a defect in haploid fruiting, which implicates that the pheromone molecules could induce the signaling response intracellularly in *MAT*α cells. We conclude that Ste6 is evolutionally conserved for mating and is not required for monokaryotic fruiting in *C. neoformans*. Additionally, we have also characterized the α and β subunits of heterotrimeric G protein complex. Phenotypic characterization of respective mutants suggests that they possibly form the G protein complex functioning in the upstream of the pheromone response pathway. G protein α subunit gene, *GPA3*, negatively regulates mating and haploid fruiting in *C. neoformans*. G protein β subunit gene, *GPB1*, positively regulates mating in *C. neoformans*.

Keywords: *Cryptococcus neoformans*,
pheromone response pathway,
pheromone,
pheromone transporter,
heterotrimeric GTP binding
protein α and ß subunit

二、緣由與目的

Infectious diseases have long been serious concerns over human public health. In the past two decades, human infections caused by the opportunistic fungal pathogens have dramatically increased due to the emerging number of individuals with immune compromised function. The impaired immune functions among these individuals were the consequences of human immuno-deficiency virus infection, cytotoxic chemotherapy for malignancies, immuno-suppression for organ transplantation, and long-term steroid treatment (Mitchell and Perfect, 1995).

Cryptococcosis is a pulmonary, systemic, or meningitic infection caused by the haploid basidiomycetous yeast, *Cryptococcus neoformans*. This fungus has drawn lots of attention due to several reasons. First, the incidence of cryptococcal infection has increased significantly due to the reasons mentioned above. Second, life-long medical treatment is required for AIDS patients who have cryptococcal meningitis. Finally, the drug resistance to the currently used antifungal compounds has developed among *C. neoformans* isolates (Alspaugh *et al.*, 1998). Therefore, studying the pathways regulating the pathogenesis in *C. neoformans* will provide the opportunity to identify the novel antifungal target for practical therapeutic use.

In addition to the practical significances, ease manipulation and well developed genetic tools have led *C. neoformans* to a model system for studying pathogenesis and other basic physiological processes in pathogenic fungi. *C. neoformans* is a heterothallic basidiomycete with a bipolar mating system, *MAT*α and *MAT*a (Kwon-Chung, 1975; Alspaugh *et al.*, 2000). Under nitrogen limitation condition, strains

of opposite mating type produce conjugation tubes and fuse to form filamentous dikaryon with fused clamp connections. The tips of the filaments differentiate to form basidium, where nuclear fusion and meiosis occur, and finally four long chains of basidiospores are produced. In addition to nutritional signal, mating is also mediated via pheromone molecules (Shen *et al,* 2002). An asexual fruiting process, termed haploid or monokaryotic fruiting, occurs mostly in the *MAT*α strains in response to nitrogen starvation and severe desiccation (Wickes *et al.*, 1996). This process occurs in the absence of *MAT*a mating partner, but can be greatly enhanced by the adjacent *MAT*a cells (Wang *et al.*, 2000). This process has been suggested to possibly contribute to the prevalence of *MAT*α strains in the natural and clinical isolates (Wickes *et al.*, 1996; Kwon-Chung and Bennett, 1978).

Studies of the pathogenesis in *C. neoformans* reveal several important factors: melanin formation and capsule production (Kwon-Chung and Rhodes, 1986), the ability to grow at 37 ℃ or higher temperature (Odom *et al.*, 1997), and mating type locus (Kwon-Chung *et al.,* 1992). The linkage of mating type α locus to the disease is particularly interesting. In addition that *MAT*α strains are more prevalent than *MAT*a strains in the environment, *MAT*α strains are more virulent than congenic *MAT*a strains in a murine model of systemic cryptococcal infection (Kwon-Chung *et al.,* 1992).

Following these interesting observations, mating type α locus was identified (Moore and Edman, 1993). Initial isolation of genomic regions containing the *MAT*α locus was done through difference cloning, and a fragment encoding the mating pheromone precursor gene was identified. Subsequent characterization of the flanking genomic region has expanded the locus to an over 100 kb genomic contig and more than 20 genes have been identified (Karos *et al.,* 2000; Lengeler *et al.*, 2002). Sequence analysis of the *MAT*α locus found components including genes involved in the pheromone response pathway such as *STE12α*, *STE11α, STE20α*, pheromone receptor gene, and three copies of pheromone precursor genes (Karos *et al.,* 2000; Lengeler *et al.*, 2002; Shen *et al,* 2002).

C. neoformans pheromone gene, *MFα1*, was found to stimulate conjugation tube formation when transformed into *MAT*a cells (Moore and Edman, 1993). The predicted *C. neoformans* MFα1, MFα2, and MFα3, are similar to yeast *S. cerevisiae* a mating factor with a conserved CAAX prenylation motif at the carboxy-terminus (Moore and Edman, 1993; Davidson *et al.*, 2000; Shen *et al,* 2002). To determine the roles of *MFα* pheromone genes in *C. neoformans,* these genes are disrupted in serotype D strains. Phenotypic characterization of the triple pheromone deletion strains found that pheromones are not absolutely required for mating in *C. neoformans*. More surprisingly, deletion of all three pheromone genes also results a haploid fruiting defect. The reconstituted strain with the wild-type copy of *MFα1* or overexpression of the *MFα1* gene enhances haploid fruiting in the triple deletion or wild-type strains. These results suggested that the MFα mating pheromone regulates the haploid fruiting possibly via an autocrine signaling pathway (Shen *et al,* 2002). This signaling loop may explain why the *MAT*α strains prevail in the environment and why the *MAT*α strains are inherently more virulent than the *MAT*a congenic strains. It will be interesting to further dissect this autocrine signaling loop, and determine whether secretion of the pheromone molecules is required for the induction. If this is the case, what receptor is targeted by the loop.

Evidence from the study of *C. neoformans STE12α* homologue suggested the linkage between the mating type and virulence in *C. neoformans* serotype D strains (Wickes *et al.*, 1997; Chang *et al.,* 2000). Overexpression of the *C. neoformans STE12α* stimulates haploid fruiting and the expression of *CNLAC1*, which is involved in the production of melanin (Wickes *et al.*, 1997). Gene disruption analysis of *STE12α* in both serotype A and D strains had drawn the conclusions that STE12α plays a minor role in mating but is required for haploid fruiting

(Yue *et al.*, 1999; Chang *et al.,* 2000). Reporter gene analysis indicated that STE12α might also involve in regulating virulence (Chang *et al.,* 2000). Virulence studies of the serotype D wild-type strains and *ste12α* mutants showed that deletion of *STE12α* results the low mortality rate and small *in vivo* capsule size. On the other hand, serotype A strains lacking of *STE12α* are fully virulent (Yue *et al.*, 1999). These studies suggested that the mechanisms regulating the virulence in *C. neoformans* serotype A and serotype D strains may be quite divergent. The argument was further supported by the comparison of a G protein α subunit, GPA1 and components in the cAMP-PKA signaling pathway among *C. neoformans* serotype A and serotype D strains (Allen *et al.,* unpub. results; D'souza *et al.,* 2001).

In additional to the mating type specific components, pheromone response elements present in both mating types were also identified (Wang *et al.*, 2000). The gene encoding a heterotrimeric Guanine nucleotide binding protein β subunit, *GPB1*, was cloned in *C. neoformans* serotype A strain. Disruption of *GPB1* results a sterile phenotype which suggestd its active role in mating. This is analogous to the role of *STE4* in *S. cerevisiae*. Additionally, *gpb1* mutants also exhibit a haploid fruiting defect. Overexpression of the presumptive downstream components, a conserved MAP kinase *CPK1* or *STE12α,* in the pheromone response pathway suppressed mating or haploid fruiting defect respectively. These evidences support the model that these components function in the same signaling pathway (Wang *et al.*, 2000).

Besides *GPB1*, the heterotrimeric G protein α subunits, *GPA1*, *GPA2*, and *GPA3*, were also identified and characterized (Alspaugh *et al.*, 1997; Allen *et al.,* unpub. results, Shen *et al.,* unpub. results). GPA1 functions upstream of the cAMP-PKA pathway and regulates capsule and melanin production, mating and virulence (Alspaugh *et al.*, 1997). GPA1 plays an important role in regulating mating and virulence in *C. neoforman* serotype A strain; however, the homologue in serotype D strains appears to play a very minor role in these processes (Allen *et al.,* unpub. results).

Study of another G protein α subunit, GPA3, has revealed interesting findings. Deletion of *GPA3* in *C. neoforman* serotype D strains results a dramatically enhanced haploid fruiting phenotype in response to severe nitrogen and water deprivation after 3 to 5 days (Shen *et al.,* unpub. results). This result suggests that GPA3 might form a heterotrimeric G protein complex with GPB1 and function in the upstream of the conserved MAP kinase module. The lack of GPA3 in the G protein complex results in the constitutive activation of the pathway by releasing the active GPB1 in response to nitrogen starvation and desiccation. Experiments such as two-hybrid assay or co-immunoprecipitation assay are required to demonstrate the physical interaction between GPA3 and GPB1. It will be also interesting to determine the virulence of *gpa3* mutant. Additionally, the constitutively active *gpa3* mutant will be useful to set up a differential screen to isolate other downstream targets of the pathways involved in haploid fruiting, mating, and possibly virulence.

The proposed grant is to further characterize the pheromone response pathway in *C. neoforman* serotype D strain. In this study, we will first identify the *C. neoforman* Ste6, a homologue of *S. cerevisiae* **a**-factor transporter, in the pheromone response pathway to further dissect the novel autocrine signaling loop and determine if secretion of MFα pheromone molecules is required for the activation of the pathway. We will also determine the physiological roles of *GPB1* homologue in the serotype D strain and compare with its correspondence in the serotype A strain.

三、結果與討論

1. Identification and characterization of the *C. neoformans STE6* homologue

Based on the typical characteristics of the *C. neoformans* MFα and MFa, it is tenable to suppose that a homologue of *S. cerevisiae* STE6 pheromone transporter responsible for the secretion of these

signaling molecules may exist in *C. neoformans*. Partial sequence of contig cneo0011005.c81 in the *C. neoformans* genome database was found to have similarity with *S. cerevisiae* Ste6p (26% identity and 47% similarity). BLAST searches of the putative *C. neoformans* STE6 homologue in the GenBank found several members of ABC transporter superfamily in various organisms. Genomic sequence analysis revealed that the gene has a predicted modular architecture, which is distinct among the ABC superfamily. The predicted coding region is about 5.3-kb. Analysis of the cDNA sequence revealed the presence of 7 exons and 6 introns within the gene and the predicted number of amino acid residues is 1655. PCR and Southern blot analysis revealed that this gene is present in both mating type cells. The *C. neoformans* STE6 has two homologous halves; each contains one membrane-spanning domain (MSD) and one nucleotide-binding domain (NBD). The four core domains are in a single polypeptide with a "forward" order MSD1-NBD1-MSD2-NBD2. The NBDs are the most conserved regions in ABC transporters and several conserved motifs are located within this region. The results of the amino acid sequence alignment of these motifs in NBD1 and NBD2.

2. Disruption of the *C. neoformans STE6* in the *MAT*α and *MAT*a cells.

To determine the function of this putative *S. cerevisiae* Ste6p homologue, the *C. neoformans STE6* was disrupted by homologous recombination. The *ste6::URA5* disruption allele was introduced by biolistic transformation into the *ura5* strain JEC43 (*MAT*α). Transformants were selected on synthetic medium lacking uracil and containing 1 M sorbitol and then screened by the presumptive impaired mating phenotype on V8 medium in crosses to the *MAT*a tester strain JEC20. Four isolates with apparent reduction of filamentation were obtained among the 64 transformants selected. Genomic DNA of the four putative deletion strains was extracted and PCRs were conducted to confirm that the four isolates impaired in mating all lacked the wild-type *STE6* and contained only the *ste6::URA5* disrupted allele. Southern blot analysis with a flanking 1.5-kb fragment of the *ste6::URA5* construct confirmed the gene replacement by the reduction of the hybridization signal from 7.9-kb to a 6.1-kb fragment among all 4 isolates with restriction enzyme *Cla*I digestion (Fig. 1 and data not shown). To generate a *MAT*a *ste6* mutant, the *MAT*α *ste6* was crossed to a *ura5* strain JEC34 (*MAT*a) on V8 medium and incubated for more than one month. Progeny were isolated by sectioning the agar block with filaments into sterile water and the suspension was spread onto synthetic medium lacking uracil. Single colonies were picked and screened with mating assay and PCR analysis. Approximately half of the progeny were *MAT*a strains with the *ste6::URA5* allele. Two strains were confirmed by Southern blot analysis and selected for subsequent analysis. The *MAT*α *ste6* and *MAT*a*ste6* strains with the reconstitution of the wild type fragment of *STE6* gene were also confirmed by Southern blot analysis (Fig. 1).

3. *ste6* mutant strains are impaired in mating

In *C. neoformans*, coculture of *MAT*α and *MAT*a strains on V8 or SLAD medium results in cell fusion, filamentation, basidia formation, nuclear fusion, meiosis and sporulation. Therefore, observation of the filamentation and sporulation can serve as a morphological assay of mating in *C. neoformans*. To determine the role of *C. neoformans STE6* gene in mating, the *ste6* mutant strains were crossed with wild type tester strain JEC20 (a) or JEC21 (α) on V8 medium (Fig. 2). As prediction, either the *MAT*α*ste6* or *MAT*a*ste6* mutant strains dramatically reduced but apparently is still capable of forming mating filaments on mating medium when crossed with JEC20 or JEC21, although different degrees of filamentation were observed in the mutants with different mating type backgrounds. Reconstitution of the wild-type *STE6* gene in the α *ste6* or a *ste6* mutant strain restored the mating efficiency to the wild type level (Fig.

2). This is in contrast to the case in *S. cerevisiae*, the *Δste6* mutant is completely sterile. The phenotype of the *ste6* mutant, however, is comparable with the phenotype of the *C. neoformans mfα1,2,3* triple deletion mutant, in which mating filaments, basidia and basidiospores were still produced (Shen *et al.,* 2002). In the bilateral crosses (α *ste6* X a *ste6*) the mating behavior was completely abolished (Fig. 2). These findings implicate that the STE6 plays an important role in mating in both *MAT*α and *MAT*a cells. Furthermore, since we think that *ste6* mutants might be impaired in the courtship stage of the mating process, fusion assays were performed to test this hypothesis. Compared to the wild-type cells, the fusion efficiency of the *MAT*a*ste6* mutant or *MAT*α *ste6* was reduced to less than 1% after 24 hour incubation. The *MAT*a*ste6* mutant exhibited decreased fusion efficiency to about 1.5% after 48 hour period, and the *MAT*α *ste6* mutant retained about 40.7% of the fusion efficiency. This result is consistent with the filamentation phenotypes observed on V8 medium and suggests that *ste6* mutants are partially impaired in the fusion step. Taken together, Ste6 functions bilaterally and is required but not essential for mating in *C. neoformans.*

4. *ste6* mutant strains fail to secrete pheromone molecules in the confrontation assays

Confrontation assays have been established to examine the capabilities of the cells to secrete and sense pheromone molecules (Shen *et al*., 2002; Wang *et al*., 2000). Confronting *MAT*α cells and *MAT*a cells without contact on filament agar induce the morphological changes in response to the opposite mating type. Filamentations at the edges of the *MAT*α cells and the presence of swollen *MAT*a cells are typically observed. This phenomenon has been connected to the secretion of the pheromone molecules. Cells respond to the pheromones secreted from the opposite mating type cells by undergoing these morphological changes. Therefore, this assay could also be applied in analyzing the pheromone secretion of the strains interested. Typical morphological responses of the *MAT*α and *MAT*a cells have been observed in the wild-type confronting pairs. In the pairs of the α *ste6* and a wild-type cells or a *ste6* and α wild-type cells, however, the wild-type cells failed to respond and undergo such morphogenesis. The reconstituted strains, both *MAT*α and *MAT*a, can fully restore the ability to trigger the morphogenesis on the opposite mating type cells (Fig. 3). The results indicated that the *ste6* mutants are unable to secrete out the pheromones to induce the morphological changes on the opposite mating type cells.

5. STE6 is dispensable in haploid fruiting

The MFα pheromone has been shown to regulate haploid fruiting of the *MAT*α cells. The pheromone triple deletion mutant *mfα1,2,3* was found to have a significant defect in haploid fruiting when grown on a nitrogen limiting medium. Overexpression of the *MFα1* pheromone gene enhanced haploid fruiting in the wild-type cells (Shen *et al.,* 2002). To our surprise, the *MAT*α *ste6* mutant was fully capable of undergoing haploid filamentation (Fig. 4). Under nitrogen limitation and desiccation conditions, the *MAT*α*ste6* mutant produced monokaryotic filaments and blastospores to at least the same degree, if not more prolifically than the wild-type *MAT*α cells. Similar observations have also been obtained in the confrontation assay, in which the *MAT*α *ste6* cells filament to a similar extent as the wild-type *MAT*α cells while confronting the wild-type *MAT*α or *MAT*a, and *MAT*α or *MAT*a *ste6* cells (data not shown). These results suggested that *STE6* is not required for haploid filamentation in the *MAT*α *C. neoformans* cells and that the autocrine signaling loop may be triggered intracellularly.

6. *STE6* expression is induced during the mating process

To examine the expression pattern of *STE6*, 6 x 10^7 cells/ml of the *MAT*a JEC20 or the *MAT*α脉JEC21 overnight YPD culture were inoculated onto V8 solid medium respectively or mixed in a 1:1 ratio. Cells

were harvested at 2, 6, 12, 24, and 48 hours post incubation. RNA was extracted and the transcription of *STE6*, *MF*α and *MFa* was examined by northern blot analysis. Hybridization results revealed that *STE6* was expressed at a basal level in response to nutrient limitation in both *MAT*a and *MATα* cells (Fig. 5 and data not shown). Previous studies showed that co-culture of cells of opposite mating type dramatically induces the transcription of *MF*α and *MFa* genes; therefore we further examined the transcription of *STE6* during mating. As shown in Figure 5, co-culture of cells of opposite mating type significantly induced *STE6* transcript at 6 hours post incubation and the expression of the *STE6* returned to the basal level at later time points. Hybridizations with probes for the *MF*α and *MFa* transcripts similarly demonstrated that the highest expression level of pheromone was also at 6 hours post-inoculation (Fig. 5). Thus, the hybridization results suggest that the transcription of pheromones and pheromone transporter genes are highly coordinated and the expression of *STE6* might also be under the control of the pheromone response pathway in *C. neoformans*.

7. Disruption of *C. neoformans GPB1* and characterization of the *gpb1* mutant

C. neoformans serotype D strain *GPB1* gene was identified in the *C. neoformans* genome. To determine the cellular functions of *GPB1* homologue in *C. neoformans* serotype D strains, the *GPB1* gene was replaced with a *gpb1::URA5* mutant allele by biolistic transformation and homologous recombination in the *MATα ura5* strain JEC43. Five transformants, identified by PCR and confirmed by Southern blot, contained the *gpb1* mutant allele and lacked the wild-type locus (Fig. 6). By crossing the *MATα gpb1* mutant with *MAT*a strain, *MAT*a *gpb1* mutants were obtained and verified (Fig. 6).

The *MATα gpb1* and *MAT*a *gpb1*mutant strains were mating impaired but not completely sterile when coincubated with the wild-type strain of opposite mating type on V8 mating medium (Fig. 7). The mating defect of *gpb1* was restored when the wild type copy of *GPB1* was reintroduced. The haploid fruiting of the *MATα gpb1* was unaffected.

A heterotrimeric GTP binding protein *α* subunit, *GPA3*, was also identified and disrupted. *gpa3* mutant maintained similar or better mating ability when compared with the wild-type strain. On the other hand, *gpa3* mutant displayed a dramatic haploid fruiting phenotype. Haploid filaments were produced all over the edge of the colony within a much shorter incubation period. To determine the relationship between *GPA3* and *GPB1*, *gpb1gpa3* double mutant was created. The mating and haploid fruiting phenotypes of *gpb1gpa3* double mutant were similar to those of *gpb1* mutant. Based on the phenotypes of mutant strains, GPA3 and GPB1 are possibly forming the G protein complex functioning in the upstream of the pheromone response pathway. G protein subunit, GPA3, negatively regulats mating and haploid fruiting in *C. neoformans*, whereas G protein β subunit, GPB1, functions downstream of GPA3 and positively regulates mating in the serotype D strain of *C. neoformans*.

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Note:

Parts of this works have been submitted and accepted for publication in 2005 January issue of the Journal "Eukaryotic cells".

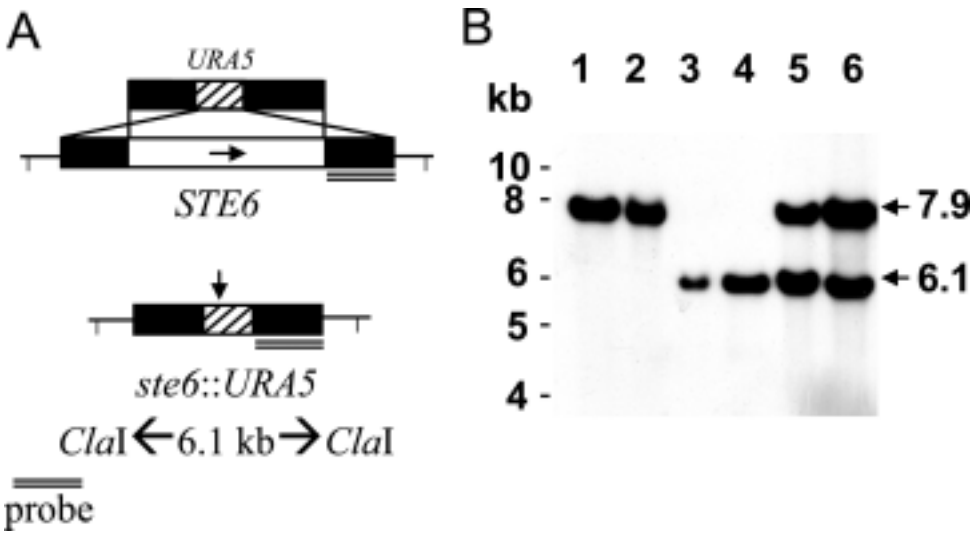


Fig. 1. Construction of the *ste6::URA5* allele and Southern hybridization analysis of wild type, *ste6*, and *STE6* reconstituted strains. (A) The *ste6* deletion allele was created by replacing the *STE6* with the *URA5* selectable marker. (B) Genomic DNA of each strain was digested with ClaI, electrophoresed, blotted and hybridized with a p^{32}-labeled *STE6* fragment indicated. Lane 1: *MAT*α WT; Lane 2: *MAT*a WT; Lane 3: *MAT*α *ste6*; Lane 4: *MAT*a *ste6*; Lane 5: *MAT*α *ste6* + *STE6*; Lane 6: *MAT*a *ste6* + *STE6*.

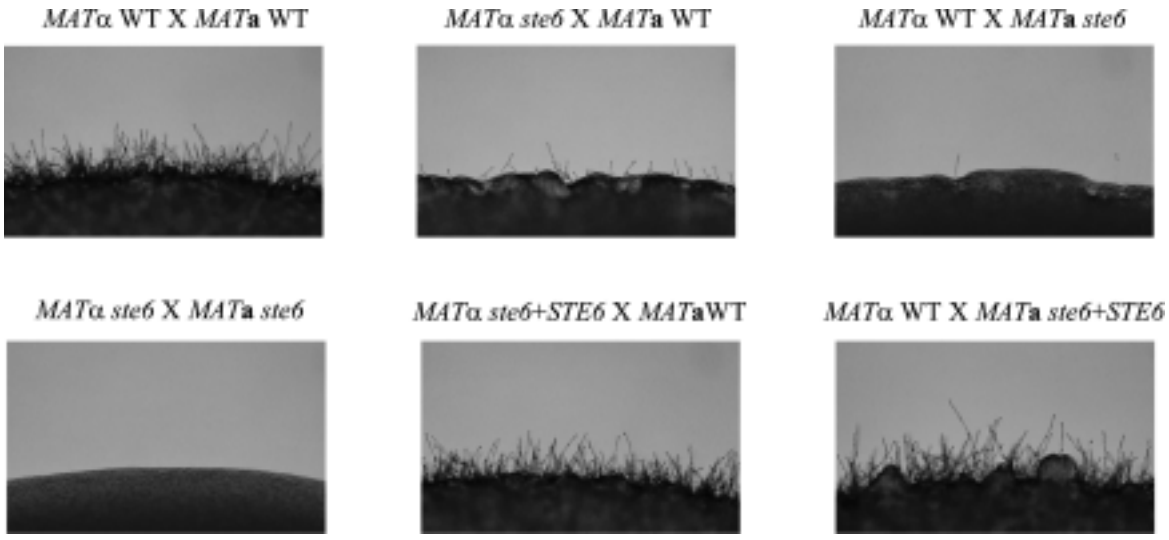


Fig. 2. *STE6* is required but not essential for mating in both mating types of *C. neoformans.* Wild type and *ste6* strains were coincubated with mating partners on SLAD plates in the dark for 30 hours at 26℃. The edges of the mating mixtures were photographed at 100X magnification.

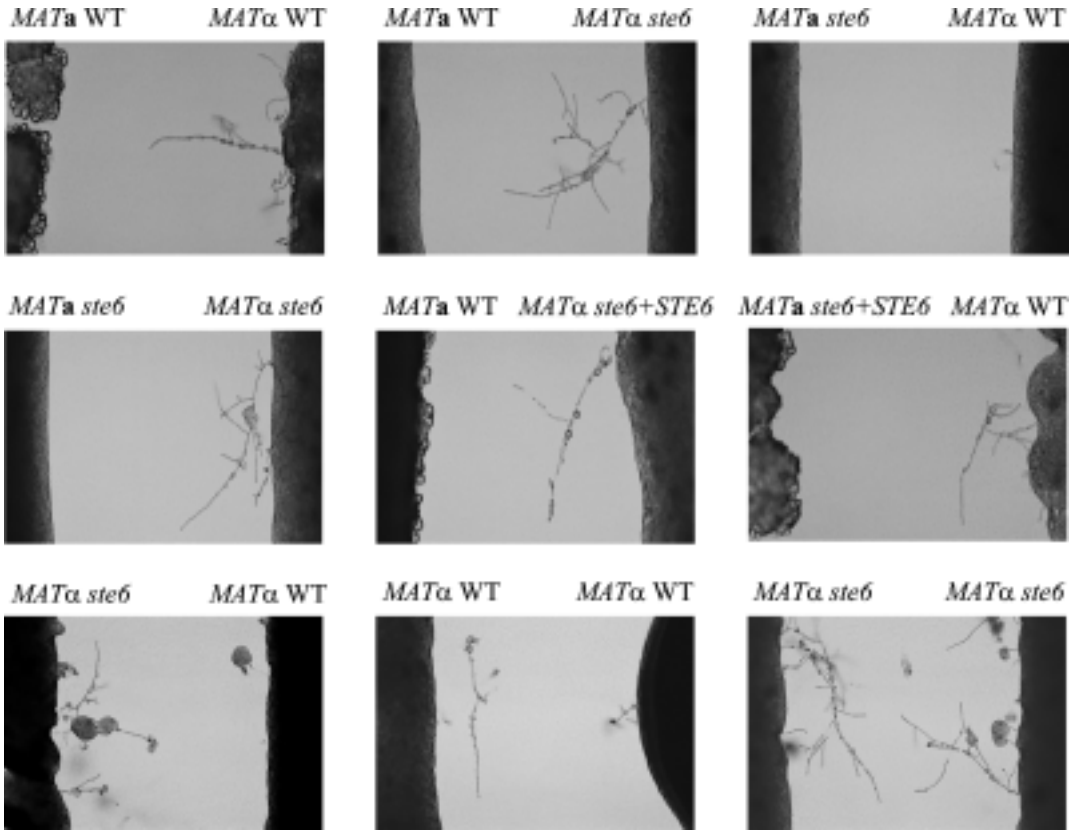


Fig. 3. *ste6* mutant strains fail to secrete pheromone molecules in confrontation assays. Congenic wild type and *ste6* mutant strains of opposite mating types were streaked in parallel on filament agar. Pictures were taken 4 days post incubation at 100X magnification.

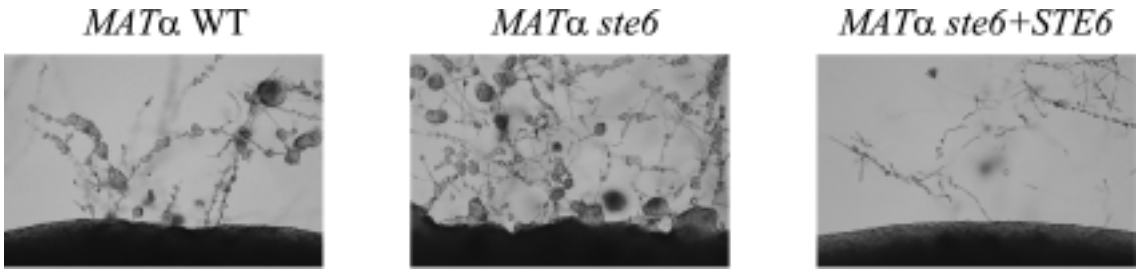


Fig. 4. *STE6* is dispensable for haploid fruiting. Suspensions of the *MAT*α wild type , *MAT*α *ste6* mutant and *MAT*α *ste6* reconstituted cells were spotted onto the filament agar and incubated in the dark at 26℃. The edges of the spots were photographed at 100 X magnification after 12 days.

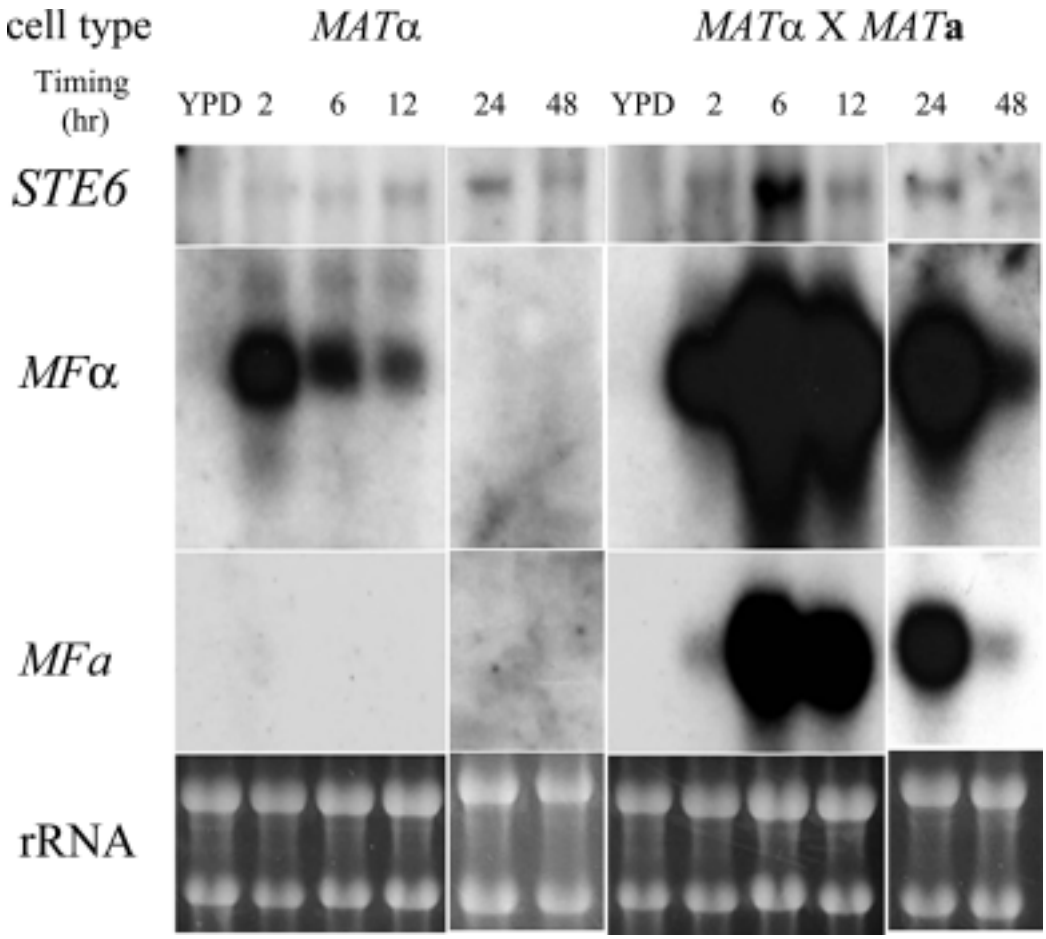


Fig. 5. *STE6* expression is induced during mating. Total RNA was prepared from cells grown on the V8 plates for 0, 2, 6, 12, 24 and 48 hours. Northern blot was hybridized in succession with probes for *STE6*, *MFα* and *MFa*. RNA loading is demonstrated by the ethidium-bromide-stained RNA gel.

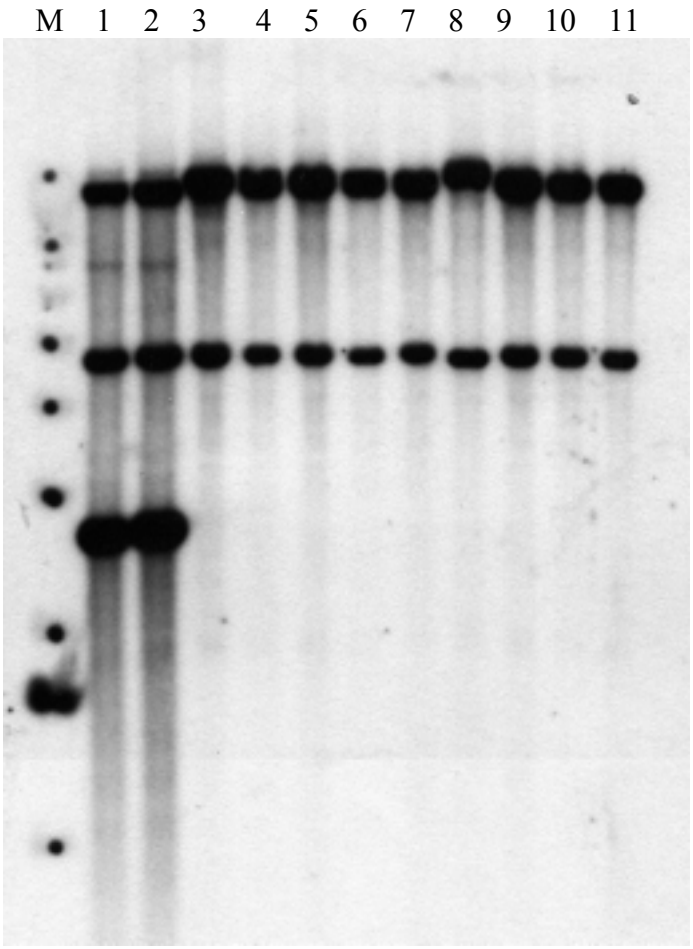
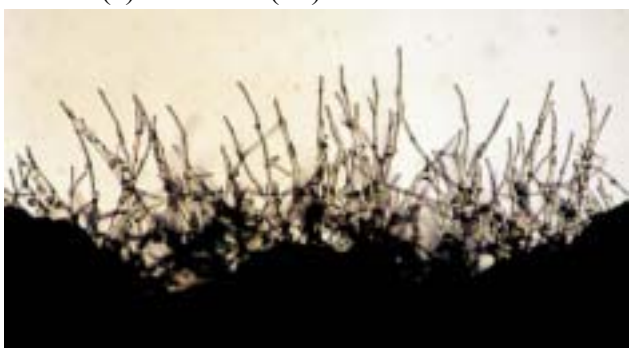


Fig. 6. Disruption of the *GPB1* gene. Genomic DNA of wild-type *MAT*α (lane 1, JEC21) and *MATa* (Lane 2, JEC20) strains, *MAT*α*gpb1* original transformants (Lane 3 to 7), and *MAT*α*gpb1* (Lane 8 and 9) and *MATagpb1* (Lane 10 and 11)progeny in the cross of *MAT*α*gpb1* original transformants and *MATa* strain was digested with *Eco*RI, electrophoresed, and analyzed by Southern hybridization with *GPB1* containing *Pst*I genomic fragment.

JEC20 (a) X JEC21 (α)



JEC20(a) X *gpb1*(α)

gpb1(a) X JEC21 (α)

JEC20 (a) X *gpb1*seroA (α)

Fig. 7. The *MAT αgpb1* and *MATagpb1*mutant strains were mating impaired but not sterile. Wild type and *gpb1* strains were coincubated with mating partners on V8 mating plates in the dark for 3 days at 26℃. The edges of the mating mixtures were photographed at X100 magnification.

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

人體病原真菌隱球菌生殖,菌絲生長及致病分子機制之探討

計畫類別：■ 個別型計畫　　□ 整合型計畫
計畫編號：NSC　92－2311－B－002－094－
執行期間：　　92 年 8 月 1 日至 93 年 7 月 31 日

計畫主持人：沈偉強
共同主持人：
計畫參與人員：薛雁冰

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

本成果報告包括以下應繳交之附件：
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執行單位：臺灣大學植物病理與微生物學系

中　華　民　國　　93　年　12　月　18　日