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CHARACTERIZATION OF HWP2 A PUTATIVE GPI ANCHORED *CANDIDA ALBICANS* CELL SURFACE PROTEIN

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SEPTEMBER 2007

CHARACTERIZATION OF HWP2 A PUTATIVE GPI ANCHORED *CANDIDA ALBICANS* CELL SURFACE PROTEIN

By

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A thesis submitted in partial fulfillment of the requirement for the degree of

MASTER OF SCIENCE

Molecular Biology

Lebanese American University

September 2007

Under the supervision of Dr. Roy Khalaf

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September 2007

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Thesis approval Form (Annex III)

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Thesis Title: **CHARACTERIZATION OF HWP2 A PUTATIVE GPI ANCHORED *CANDIDA ALBICANS* CELL SURFACE PROTEIN.**

Program : MS in Molecular Biology

Division/Dept : Natural Sciences Division

School : **School of Arts and Sciences**

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Date 24/9/07

Characterization of Hwp2 a putative GPI anchored *Candida albicans* cell surface protein

Abstract

The yeast *Candida albicans* is one of the leading fungal pathogens causing death in immunocompromised individuals. Various factors are thought to be responsible for virulence, such as lipases, proteases and adhesins. Many of these factors are glycosylphosphatidylinositol (GPI) anchored cell surface antigenic determinant proteins responsible for pathogenicity. Hwp2 is a putative GPI anchored protein. The purpose of this study is to characterize the role of Hwp2 as far as filamentation on solid and liquid media, virulence in a mouse model of disseminated candidiasis, drug resistance to six widely used antifungal agents, and white opaque switching, by creating a homozygous null *hwp2* strain. It was observed that an *hwp2Δ* strain was highly filamentation deficient on corn meal and sabouraud solid agar media but not on most liquid media tested, with 100% serum being a minor exception. Furthermore the mutant strain was slightly reduced in virulence compared to the wild strain since all mice infected with the control strain died after 6 days of injection compared with 11 days for the mutant. No discrepancy between the control and mutant was observed as far as drug resistance using the E-test method, or white opaque switching. These results indicate a possible role for Hwp2 in adhesion and invasiveness. Finally aligning the Hwp2 sequence with its two closest homologues, Hwp1 and Rbt1 revealed a small conserved region of high homology found twice in each protein that might reflect a possible sequence necessary for structural integrity.

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Acknowledgment

"Patience and perseverance are the key to success"

With this title above I would like to thank every single person who collaborated with me so this work could be possible.

Dr. Roy Khalaf, friend and advisor, that aided me during the whole process and kept an easy going profile that helped immensely during stress times.

Members of the committee, Dr. Constantine Daher and Dr. Sima Tokajian for their directions and valuable comments.

My friend Leila Dib with whom I spent countless hours working in the laboratory.

Mr. Jean Karam for helping me with my mice experiment and for his good company in other tasks during my MS years.

For the lab personal, Miss Helena Bou Farah for always being present to help whenever is needed.

And to all my LAU fellow colleges that where the true gift of my degree.

Finally my special appreciation goes to my family that supported me during all my years of studies, and for God who is the true effector of all good deeds throughout my life.

Chapter I

Introduction

Candida albicans is an imperfect yeast with a commensal relationship to a large number of animals (Rippon, 1982; Shepherd *et al.*, 1985; Scherer and Magee, 1990). On the advents of AIDS, *C. albicans* became a serious problem as it turns into an opportunistic pathogen for the immunocompromised individuals (Braun *et al.*, 2005). *Candida* infection can be superficial as in oropharyngeal candidiasis in AIDS patients, with shallow levels of tissue invasion that affect different oral areas including hard and soft palates, tongue, buccal mucosa, and floor of the mouth (Fidel, 2006). It is manifested as reddened patches (erythematous) or white curd-like lesions (pseudomembranous) (thrush) (Fidel, 2006). A more serious and deadly type of *Candida* infection is systemic or disseminated candidiasis that spreads over and invades the internal organs traveling through the blood. One example of systemic candidiasis is the newborn systemic candidiasis, with *C. albicans* as the major effector of Candidiasis (Rao and Ali, 2005). It affects about 20% of neonates weighing less than 1000 g (also called very low-birth-weight babies) (VLBW) (Baley *et al.*, 1992; Stoll *et al.*, 1992; Rao and Ali, 2005). The sources of candidiasis in those babies are often endogenous following colonization of the babies with fungi (Rao and Ali, 2005). The timeline risk factor ranges from the first week to the fourth week (Huang *et al.*, 1992). Disseminated candidiasis presents unspecific and diverse symptoms that include temperature instability, refusal of feeds, respiratory

distress, abdominal distension, apnea, lethargy, bradycardia, decreased perfusion or seizures (Rao and Ali, 2005). Organ damage is common in neonates' systemic candidiasis and includes kidneys, brain, lungs, eyes, liver, spleen, bones and joints (Benjamin *et al.*, 2000).

Statistics on systemic candidiasis or *Candida* blood-stream infections (BSI) reflect its importance as a major cause of morbidity and mortality in hospitalized patients (Warnock, 2007). In the United States, *Candida* species ranks as the fourth most common cause of hospital acquired BSI (Warnock, 2007). In numbers, it is 8 infections per 10000 hospital discharges in the United States, and 2-5 infections per 10000 hospital discharges in Europe (Warnock, 2007). In one study conducted in Baltimore and the state of Connecticut, the attributed mortality rate to candidemia ranged around 19 to 24% depending on age (Morgan *et al.*, 2005). Almost 95% of *Candida* BSI worldwide are caused by either *C. albicans*, *C. galabrata*, *C. parapsilosis*, *C. tropicalis*, or *C. krusei*. *C. albicans* is the predominant cause of candidemia worldwide with a varied frequency related to geographic setting, and demographics of the population studied. In North America, it accounted for 45-55% of *Candida* BSI during the 1990s. In recent reports in Brazil and Japan, *C. albicans* accounted for 40% of the *Candida* BSI while in two Nordic countries it was the cause of 70% of candidemia during the 1990s (Warnock, 2007) .

Depending on the milieu on which it grows, *C. albicans* shifts reversibly from yeast to hyphae morphology including several intermediate pseudohyphal forms (Brown and Gow, 1999) (Fig. 1.1). True hyphal filaments are unconstricted cylindrical projections that develop from germ tubes of blastospores arising during the first cell cycle. They have parallel sides along their length, and present no constriction at the neck of the mother cell,

as opposed to pseudohyphae that have constrictions. True hyphal forms are found in many patients suffering from candidiasis and are thought to be necessary for infection and virulence (Sudbery *et al.*, 2004).

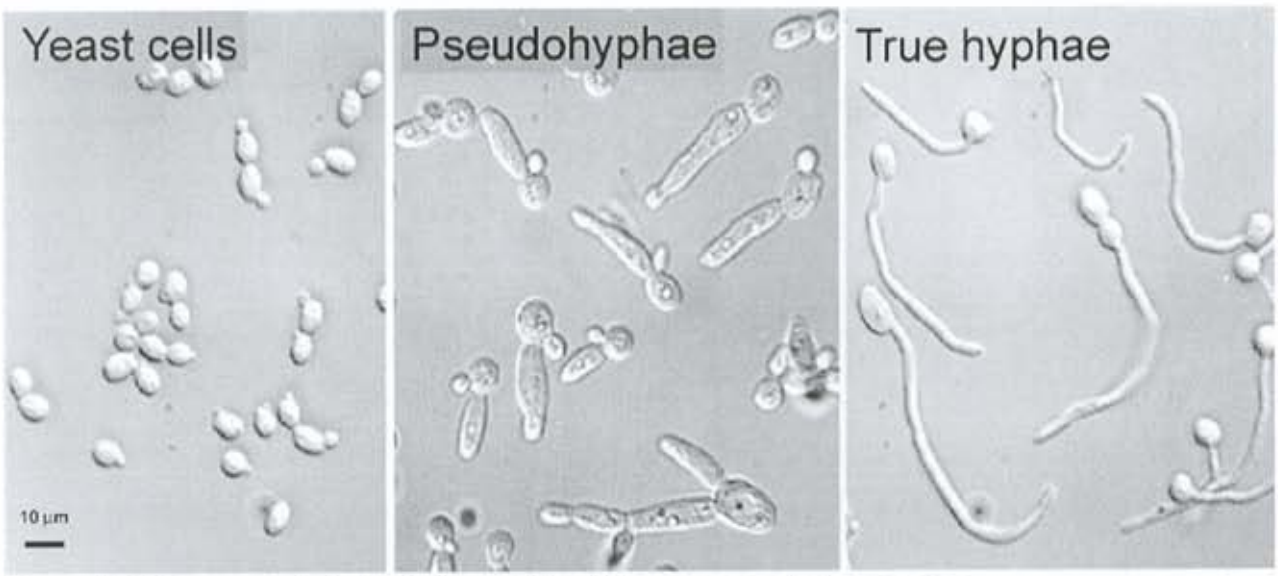

Figure 1.1: Three morphological stages of *C. albicans* represented yeast cells, pseudohyphae and true hyphae.
(figure provided by **www.usask.ca/biology/kaminskyj/collab.html)**

The success of *C. albicans* as a pathogen is related in part to its polymorphism and adhesive capability as strains blocked in one of the above mentioned morphologies proved to be non-virulent. Under *in vitro* conditions, *C. albicans* hyphae formation can be triggered by several media and conditions. The most powerful trigger is liquid serum at human body temperature (37 °C). In serum, the hyphae formation may be attributed to several independent signaling pathways where at least two known inducers of hyphae formation, *N*-acetylglucosamine (GlcNAc) and proline, are found. Those products are generated by degradation of serum glycoproteins (Ernst, 2000). Certain media are also

able to induce filamentation under defined conditions. The medium M199, a synthetic medium supplemented with Earle's salts, L-Glutamine and HEPES buffer, is able to induce filamentation at 37 °C under basic pH 7.5-8, whereas no induction occurs under acidic pH 4 (Sigma-Aldrich, Germany). Medium M199 was first formulated to study nutritional needs of chick embryo fibroblasts (Morgan *et al.*, 1950). Lee's medium, a synthetic medium provided with amino acids, biotin, inorganic salts and glucose induces filamentation under normal inducing conditions (37 °C) and allow yeast proliferation at room temperature (25 °C) (Lee *et al.*, 1975). Under starvation, filamentation occurs under normally non-inducing conditions. Sabouraud medium is a rich medium supplemented with dextrose on which *C. albicans* grows as a yeast at 30°C, it has a low pH favorable for growth of deramtophytes fungi, and with slight inhibitory effects for bacterial contaminants (Ajelo *et al.*, 1963; Kwon-Chung and Bennett, 1992; Reisner *et al.*, 1999). However, upon prolonged incubation *C. albicans* begins to filament and invades the surrounding agar (Cullen and Sprague, 2000).

A peculiar morphological state of *C. albicans* is chlamydospore formation. Chlamydospores are thick-walled spherical cells that are produced on pseudohyphal support cells. Production of chlamydospores relies on growth on a medium that is low in oxygen and in nutrients. The ideal media for that is corn meal agar with Tween 80 or Rice extracts agar with Tween 80 (Montazeri & Hedrick, 1984; Kirsch *et al.*, 1990; Joshi *et al.*, 1993).

The ability of tissue invasion by *C. albicans* has been shown to include epithelial and endothelial cells. For epithelial invasion, *C. albicans* produces hyphae that are expressing certain cell wall proteins for tight anchorage (Kumamoto and Vinces, 2005). The

presence of adhesins such as *HWP1* (Hyphal Wall Protein 1) is needed for this task (Staab *et al.*, 1999). Endothelial invasion is done probably by internalization of yeast cells by endothelial cells. This might arise as expression of certain critical gene (s) that allow attachment of the yeast cells to the correct host cells receptors (Phan *et al.*, 2005). Inside the endothelial cells, the yeast cells filament to breach the endothelium (Kumamoto and Vinces, 2005).

1.1 White/Opaque switching

C. albicans has, in addition to the hyphae-yeast switching phenotype, a second reversible switching phenotype called white-opaque switching. The morphology of the colonies in either cell morphologies is distinguished by size, shape, and color. In the "white" phenotype, cells form colonies which are hemispherical and white. In the "opaque" phenotype, cells form colonies which are flatter, larger, and grey, or opaque. White cells are similar in size, shape, and budding pattern to cells of common laboratory strains. In contrast, opaque cells are elongate, or bean shaped, and three folds the volume and twice the mass of white cells. In addition, opaque cells differ from white cells in their generation time, in their sensitivity to high and low temperatures, and in their incapacity to filament (Slutsky *et al.*, 1986).

The white-opaque switching was identified in the *C. albicans* strain WO-1 that is used as a control in white-opaque switching testing on lee's Medium (Slutsky *et al.*, 1986). An easy way of distinguishing white opaque cells is the addition of phloxine B to the agar medium renders opaque colonies red, while white colonies appear pink (Anderson and Soll, 1987).

Mating and white-opaque switching are closely related in *C. albicans*. Mating is controlled by the Mating Type Locus *MTL*. There are two mating types loci alleles in a common strain, the *MTLa1* on one chromosome and the *MTLα1* and *MTLα2* in the other (Hull and Johnson, 1999). Engineered homozygous strains, *MTL***a** and *MTL* α (a/- and α/-, respectively), are able to switch to the opaque form to mate *in vivo* and *in vitro* at very low estimated frequencies (Hull *et al.*, 2000; Magee and Magee, 2000). Normal heterozygous strains for the *MTL* locus are not able to mate or undergo white/opaque transition, while homozygous mutant derivatives and natural occurring homozygous strains do (Miller and Johnson, 2002; Lockhart *at al.*, 2002).

The opaque phenotype is sensitive to temperature and as such upon a temperature shift from 25°C to ≥37°C; opaque phase cells convert en masse to the white phase growth form after two cell doublings (Rikkerink *et al.*, 1988; Srikantha and Soll, 1993). As a result, skin which is below 37°C tend to be an ideal place for opaque cell proliferation and for mating since only opaque cells are able to mate.

Virulence is favored for the strains heterozygous for the *MTL* locus (a/α) that are naturally of the white morphology. In *in vivo* mice injection, an a/α strain was far more virulent than either an a/a or an α/α homozygous strain. Furthermore, in a co-injection of the three mentioned strains, the a/α had a competitive advantage at the time of extreme host morbidity or death (Lockhart *at al.*, 2005).

1.2 Antifungal susceptibility

Being a human pathogen, several antifungal agents are used to treat *Candida* infections, the most common being Amphotericin B, followed by azoles and caspofungin (Kerridge, 1985; Ghannoum and Rice, 1999, Letscher-Bru and Herbrecht, 2003).

For more than 30 years, from the mid 1950's till late 1980's, Amphotericin B was the only antifungal agents used in treatment of systemic fungal infections although it is known for its nephrotoxicity (Oura *et al.*, 1955-1956; Ghannoum and Rice, 1999). Amphotericin B is a polyene that interacts with the membrane sterols of *C. albicans* and other fungi leading to the production of aqueous pores. Leakage of cytoplasmic fluids lead to cell death. Resistance to Amphoterecin B is due to the reduction of the ergosterol target from the cell membrane, or by replacement of the ergosterol by 3-oxo or 3-hydroxy sterol (Hamilton-Miller, 1972; Ghannoum and Rice, 1999).

Azoles entered in use by the late 1980's as safer and less toxic antifungal agents compared to Amphoterecin B. Azoles and its derivatves (Fluconazole, Itraconazole, Ketoconazole and Voriconazole) act on inhibiting cytochrome P-450 that leads to accumulation of ergosterol derivates and depletion of ergosterol. These effects lead to a compromised plasma membrane in both function and structure. Resistance to azoles comprises changes in both the cytochrome P-450 and in the pumping effectivness of the cell. Increased numbers of cytochrome P-450 and changes in its binding affinity to azoles confer it part of the resistance (Ghannoum and Rice, 1999). The other part is due to increased production of *C. albicans* efflux pumps (Major Facilitator Super family (MFS)) and the ATP-binding cassette super family (ABC)) leading to rapid removal of the azoles (Rogers and Barkers, 2003; Karababa *et al.*, 2004).

As a third class of antifungal drugs, caspofungin acts on inhibiting the enzyme β-(1,3)-glucan synthetase found on the cell wall of *C. albicans.* It binds to intracellular UDP-glucose preventing its catalysis into β-(1,3)-glucan by Gsl1 the catalytic membrane subunit of D-glucan (Letscher-Bru and Herbrecht, 2003). The result is disruption of the cell wall leading to osmotic stress, lysis, and death of the *C. albicans* (Fung *et al.*, 2002). Mechanisms of resistance to Caspofungin are not well known (Bruno *et al.*, 2006).

1.3 Filamentation pathways in *Candida albicans*

Virulence genes and hyphae specific genes are controlled by common regulators. Theses regulators belong to distinct pathways and their absence has a differential impact upon virulence, and morphology.

Filamentation in *C. albicans* is regulated by more than one pathway (Fig. 1.2). Such pathways are convergent in activating the same sets of hyphae-specific genes as shown in DNA array studies (Fig. 1.3) (Lane *et al.*, 2001b). There is a primary pathway, the cyclic AMP/protein kinase A (cAMP/PKA) pathway that has Efg1 as the key transcription regulatory factor so far (Stoldt *et al.*, 1997). Δ*efg1* strains are highly defective in filamentous growth under most inducing conditions including serum at 37 °C (Lo *et al.*, 1997; Stoldt *et al.*, 1997). In addition to its role in filamentation, Efg1 is an activator of chlamydospore formation (Sonneborn *et al.*, 1999).

The other two known pathways have a less striking role in regulating filamentous growth. The Mitogen activated protein (MAP) kinase pathway has the transcription regulatory factor Cph1 as the activating component. A Δ*cph*1 strain has been shown to affect filamentation hyphae specific genes transcription under certain conditions (Liu *et al.*,

1994; Lane *et al.*, 2001b). A *C. albicans* strain double deleted for the two filamentation regulatory genes, *EFG1* and *CPH1,* is locked in the yeast form and is avirulent in a mouse model (Lo *et al.*, 1997).

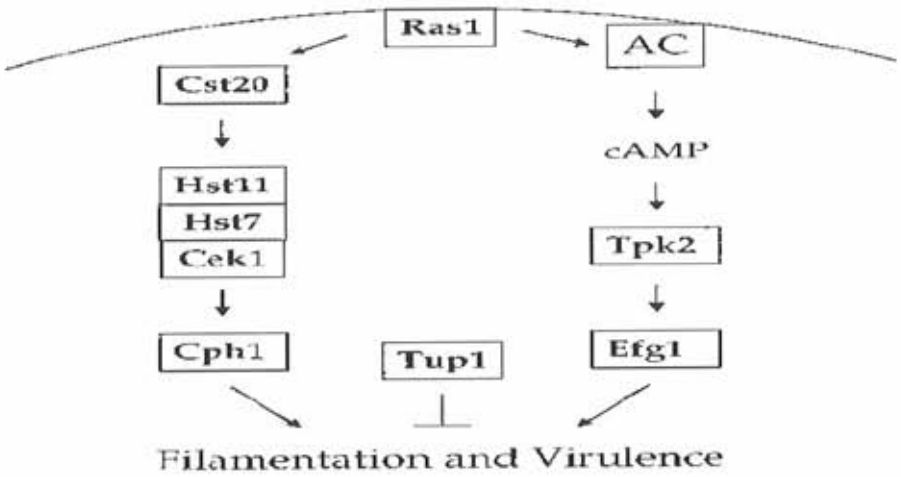

Figure 1.2: " Signaling cascades regulating virulence of C. albicans. Two parallel signaling cascades involving a MAP kinase and a cAMP/PKA signaling pathways regulate filamentation and virulence of this human pathogen. AC. adenylylcyclase (Lengeler *et al.*, 2000).

Another pathway is dependent on the transcription regulatory factor Cph2. Cph2 does not activate directly most of the transcription of hyphae specific genes. It does so by activating the transcription of another the transcription regulatory factor *TEC1* (Fig. 1.3). A Δ*cph2* is defective in filamentation under liquid and solid Lee's media (Lane *et al.*, 2001a).

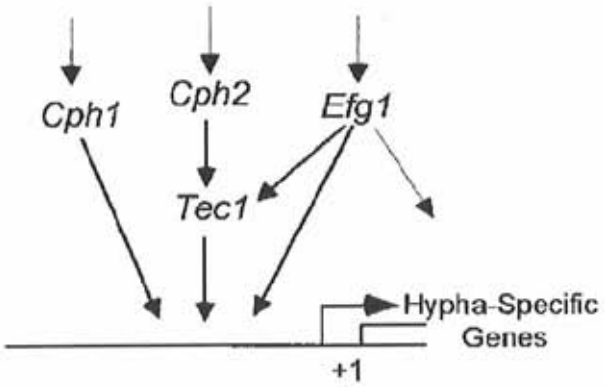

Figure 1.3: "Multiple pathways converge at different points to regulate differentially expressed genes" (lane *et al.*, 2001b).

Another activating pathways for filamentation may still be present and as an example the *WAP1* and *RBT5* (a GPI-anchored cell wall protein) genes (Braun and Johnson, 2000; Braun *et al.*, 2000). These genes are still induced in a Δ*efg1* Δ*cph1* strain in serum medium, which indicates the presence of a fourth inducing pathway (Braun and Johnson, 2000).

1.4 Filamentation repression pathways

Repression of Filamentation in *C. albicans* converges mainly on the transcriptional regulator *TUP1* (Braun *et al.*, 1997; Braun *et al.*, 2000), (Fig. 1.2). Tup1 in *C. albicans* has a general repressing function on hyphae specific genes and thus represses filamentation (Braun *et al.*, 1997; Braun *et al.*, 2000).

In a DNA microarray describing the derepression profile of the hyphae-specific genes, 61 hyphal specific genes were studied for transcriptional control. Tup1 controlled about ½ of these genes, Nrg1 and Rfg1 controlled the majority of the subsets independently or in

conjunction but through Tup1. Interestingly, the other half had no identified repressor yet (Fig. 1.4 C). (Kadosh and Johnson, 2005)

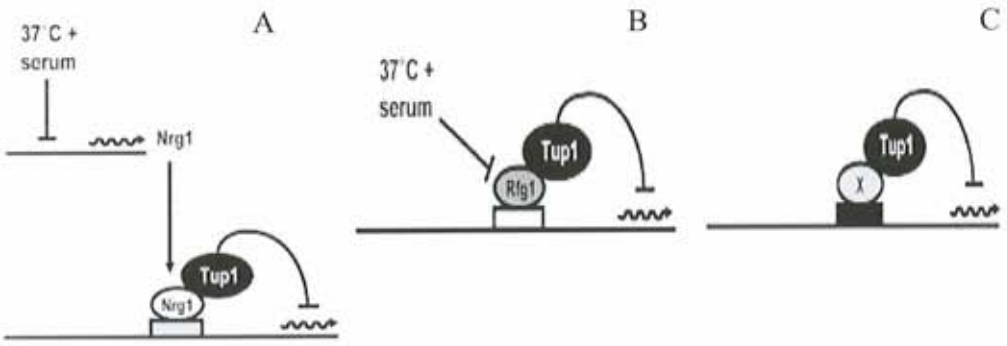

Figure 1.4. Repression of filamentation genes in *C. albicans* by the Rfg1, Nrg1, and Tup1 pathways. A) Rfg1; B) Nrg1; and C) X a yet unidentified DNA-binding protein that binds to the promoters of filament- and virulence-specific target genes and direct transcriptional repression via recruitment of the Tup1 corepressor (Kadosh and Johnson, 2005).

Many of the hyphae specific genes mentioned above are actually cell wall genes that might be necessary for virulence, in combating the immune system of the host or for adherence of the cells to each other and to the host. Subsets of those are the GPI anchored proteins.

1.5 GPI proteins in *Candida albicans*

Glycosylphosphatidylinositol-modified (GPI) proteins are proteins found in lower and higher eukaryotic organisms (Eisenhaber *et al.*, 2001). They are known to incorporate covalently either to the cell wall or to remain attached to the plasma membrane (de Groot *et al.*, 2003). Several functions are attributed to them depending on the organism, such functions may relate to cell wall biosynthesis and cell wall remodeling, determination of

surface hydrophobicity and antigenicity, in addition to adhesion and virulence (Hoyer, 2001; Klis *et al.*, 2001; Sundstrom, 2002).

In *C. albicans* there are two groups of lipid anchored proteins. In the first group we find the amide-linked myristoyl anchors, the (thio)ester-linked fatty acyl anchors and thioester-linked prenyl anchors. The second group is composed exclusively of proteins that possess a C-terminal signal sequence that allows for the linkage to a GPI anchor (Richard and Plaine, 2006). Such anchor can be removed by specific phospholipases turning the protein water-soluble (Griffith and Ryan, 1999). Two features are used in distinguishing GPI proteins: a N-terminal hydrophobic signal sequence for localization to the endoplasmic reticulum (ER); a C-terminal region containing a 9-24 hydrophobic residues for transient attachment to ER, and the ω GPI anchorage site (Richard and Plaine, 2006). The ω site contains 3 regions the ω with small amino acids, ω+1, and ω+2 with critical residues. The linker region between the ω site and the hydrophobic region is composed of 4-19 residues (de Groot *et al.*, 2003) (Fig. 1.5). The ω site is used for cleavage and incorporation of the GPI anchor (Thomas *et al.*, 1990). Replacement of the C-terminal region by the preformed GPI anchor occurs in the ER shortly after protein synthesis. The core constituents of the GPI anchor are: a lipid group for membrane attachment, a myo-inositol, a N-acetyl glucosamine, 3 mannose groups, and a phosphoethanolamine group used to connect the GPI anchor to the protein via an amide linkage (Tiede, *et al.*, 1999). Variety of the anchors has been related to the addition of mannose groups and positioning of side chains like phosphoethanolamine (Richard and Plaine, 2006).

[NSGADC]-[GASVIETKDLF]-[GASV]-X(4, 19)-[FILMVAGPSTCYWN](10)>

ω ↑ ω+1 ω+2

Figure 1.5: GPI algorithm for *C .albicans*. The residues in the square brackets are for possible amino acids at a particular position; number(s) in round brackets stand for (the limits of) the length of a sequence element; X is for any aminoacid; and > indicates the C-terminus of the protein. The GPI anchoring site (ω) is indicated below the algorithm; the arrow marks the cleavage site (de Groot *et al.*, 2003).

The last count of predicted and known function GPI proteins obtained by computer analysis in *C. albicans* is 115. More than 65% (76 proteins) are still of completely unknown function (Richard and Plaine, 2006).

GPI anchor proteins tend to be heterogeneous in function and few of them have been studied in *C. albicans*. The most studied family being the *ALS* (Agglutinin Like Sequence) family (Hoyer, 2001). Such family has very close sequences among its members but presents a high variability among its alleles (60 alleles for 66 different strains for *ALS7* generating 49 different phenotypes (Zhang *et al.*, 2003). Members of the ALS family are cell surface proteins such as Als1 that mediates adherence to endothelial and epithelial cells (Fu *et al.*, 2002). Expression of different Als proteins in *S. cerevisiae* was shown to mediate differential adhesion capability to different media (Sheppard *et al.*, 2004).

Some members of the GPI protein in *C. albicans* have been shown to have roles (some direct others indirect) in cell adhesion and virulence. Twenty eight of the 115 GPI genes have a mutant construct, 15 have been tested and 12 have shown reduced virulence compared to the wild type strain (Richard and Plaine, 2006). Possible roles of GPI proteins in virulence can be attributed to: cell wall biosynthesis and modeling (Phr1, Phr2, Utr2) (Richard and Plaine, 2006), combating oxidative stress (mutant superoxide

dismutase *sod5* has a reduced neutrophil resistance (Fradin *et al.*, 2005)), specific enzymatic functions such as i) *PLB5*, a phospholipase B that when mutated, confers a reduction of *in vivo* organ colonization (Theiss *et al.*, 2006), and ii) *RBT1* an uncharacterized predicted GPI protein that is suspected to interact with and impair the immune system of the host (Braun *et al.*, 2000)), and adherence with representatives of the Als proteins and Hwp1 (Hyphal Wall Protein) having direct interaction with the cells as being GPI-anchored proteins (Staab *et al.*, 1999; Hoyer, 2001).

1.6 Adhesion by Hwp1

One of the most studied GPI anchored proteins is the 634 amino acid long Hwp1 protein. It was named as such because it is activated mainly under hyphal conditions, and its detection on the cell wall through immunofluoresence studies. The protein composition is marked by the presence of 27% proline, 16% glutamine, and 12% aspartate residues. Such residues confer hydrophilic properties to the protein (Staab *et al.*, 1996).

As a cell wall protein, *HWP1* have been shown to interact with mammalian transglutaminase (TGase) and as such to confer adhesion to human buccal epithelial cells. Such adhesion is due to the interaction of the cell wall exposed NH_2-terminal domain of *HWP1* with the TGase (Staab *et al.*, 1999). This occurs since the NH_2 terminus resembles the mammalian TGase substrates particularly the head and central domains of small proline-rich (SPR) proteins (Austin *et al.*, 1996). The mechanism of the cross-linkage of *HWP1* to keratinized epithelial cell is through covalent $N^{\epsilon}(\gamma$-glutamyl)lysine isodipeptide bonds (Staab *et al.*, 1999). Competition assay for the N-terminus using a recombinant

HWP1 lacking the anchoring capability decreased adherence to buccal epithelial cells by 60% adding further proof of the binding capability of *HWP1* (Staab *et al.*, 2004).

The TGase substrate dependent activity is dependent on the GPI anchorage, a C- terminus truncated form of *HWP1* without the GPI anchor site would lead to a cytosol localized product of *HWP1* (Staab *et al.*, 2004).

In biofilm formation, mutant *hwp*1 strains have been unable to form hyphae in catheters as it results in a thin film with only yeast cells without hyphae (Nobile, 2006), treatment with the antifungal agent farnesol have shown that as adherence and biofilm formation decreases so is the concentration of mRNA of *HWP1* in the cytosol (Ramage *et al.*, 2002).

In earlier studies, *HWP1* deletion has been attributed to defects in filamentation and to attenuated virulence in mice (Staab *et al.*, 1999; Tsuchimori *et al.*, 1999). However, this effect was later attributed to the lack of proper expression of the *URA3* (An enzyme in the biosynthetic pathway of uridine) marker that is transformed into the strain at the *HWP1* locus in the *hisG-URA3-hisG* disruption cassette (Fonzi and Irwin, 1993; Sharkey *et al.*, 2005).

The regulation of *HWP1* is dependent on both positive and negative regulation. The positive regulator is the transcription regulatory factor Efg1 on which *HWP1* expression is highly dependent (Sharkey *et al.*, 1999). Negative regulation of *HWP1* is dependent on Tup1 regulation through both DNA binding proteins Nrg1 and Rfg1, although Nrg1 has been shown to play the major role in repression (Sharkey *et al.*, 1999, Braun *et al.*, 2001; Kadosh and Johnson, 2001; Khalaf and Zitomer, 2001; Kadosh and Johnson, 2005).

1.7 *RBT1*, a virulence related gene

RBT1 (Repressed by *TUP1*) is a gene with a sequence translation of 750 amino acids that indicates cell wall localization. It has homology to *HWP1* at its unprocessed NH_2 terminus, which represents a hydrophobic signal sequence, and hydrophobic segments at the COOH terminus. The hydrophobic segments at the COOH terminus are the site of GPI moiety addition (Braun *et al.*, 2000).

RBT1 is expressed under hyphal inducing conditions and has as positive activators the transcription factors *EFG1*, *CPH1*, and *CPH2* and negative regulator the transcription repressor *TUP1* for non-hyphal inducing conditions (Braun and Johnson, 2000; Braun *et al.*, 2000; Lane *et al.*, 2001b). However, unlike *HWP1*, it is not repressed through Nrg1 or Rfg1 but through an unknown DNA binding protein that recruits *TUP1* (Braun *et al.*, 2001).

The deletion of *RBT1* has no effect on the filamentation or a distinguishable morphology *in vitro*. The effect is more evident on virulence as both a heterozygous and a homozygous deletion presents a reduced invasiveness in a rabbit cornea. A *Δrbt1* is also markedly less virulent in mice as it is cleared from the kidney of infected mice unlike the control strain (Braun *et al.*, 2000).

According to Braun *et al.*, (2000), the functions predicted to *RBT1* as a result of the virulence *in vivo* tests are:

1) Interaction with and impairment of immune system components that normally clear *C. albicans*.

2) Providing adhesive interactions that allow the fungal filament to grow into the host tissue and damage it.

RBT1 also shares homology with yet another predicted GPI anchor protein, *HWP2*. Such homologous areas include the NH_2 and the COOH termini related to signaling and to addition of GPI anchorage.

1.8 Hwp2, a hypothetical GPI anchor Protein.

Hyphal Wall Protein 2 (Hwp2) is still a hypothetical protein 908 amino acids long whose RNA transcript (2727 bp) was detected so far only under the filamentous state in *C. albicans*. It shares conserved domains with the well-characterized Hwp1 and Rbt1 proteins mentioned above (Sohn *et al.*, 2003). An alias nomenclature for *HWP2* is *PGA8* (predicted GPI-anchored protein 8) (de Groot *et al.*, 2003). It was identified in an *in-silico* analysis of the *C. albicans* genome using an algorithm used to identify GPI proteins. This algorithm locates the second hydrophobic domain of a GPI protein near the C terminus where cleavage occurs and a GPI anchor is incorporated (Fig. 1.5) (de Groot *et al.*, 2003).

By using the above algorithm, the localized C terminal sequence for *HWP2* was the following with the gap <> representing the putative cleavage site for GPI anchoring, ω position in bold underlined, ω+1 position in bold italics, ω+2 in bold italics underlined, and the 10 final hydrophobic residues in bold :

ISVAVSSAAQSSIAAISSYE**G**<>***TG***NNMKLSFGV**VIAGVAAFAI**. (de Groot *et al.*, 2003).

1.8.1 Regulation of *HWP2* by *EFG1*

The identification of *HWP2* transcription *in vitro* was achieved by DNA microarray analysis using the following strains: Δ*efg1*, Δ*cph1*, Δ*efg1*/Δ*cph1*, under various hyphal and non-hyphal inducing conditions at 37°C, 30°C and 25°C (Sohn *et al.*, 2003).

HWP2 could be detected only under wild type and in a Δ*cph1* strains under hyphae inducing media. A Northern blot analysis confirmed the presence of the transcript of *HWP2* only under hyphae inducing conditions and with the presence of *EFG1*. Theses facts strongly suggest that *HWP2* is transcribed in response to hyphae induction and not to elevated temperature alone. The presence or absence of *CPH1* did not affect the level of transcripts of *HWP2* (Sohn *et al.*, 2003).

Regulation of *HWP2* by *EFG1* is also suggested by the presence of two enhancer elements called E-boxes in *HWP2* promoter (Sohn *et al.*, 2003). Efg1 was shown to be able to bind to E-boxes of hyphae specific genes such as *ALS8* and regulate them (Leng *et al.*, 2001). However, E-boxes are also found upstream of yeast specific genes (e.g. *YWP1*, Yeast Wall Protein), which suggest that additional control is needed for Efg1 to activate selectively either hyphae or yeast specific genes (Sohn *et al.*, 2003).

1.8.2 Regulation of *HWP2* by *TUP1*

The second level of control over *HWP2* is through the Transcriptional repressor of filamentation *TUP1* (Braun *et al.*, 2000). Δ*tup1* strains have been shown to derepress *HWP2* (also known as ORF. 19.3380) and the two hyphae specific genes *HWP1*, and *RBT1* that possess homology domains to *HWP2* (Braun and Johnson, 2000; Braun *et al.*, 2000; Kadosh and Johnson, 2005). *HWP1* is repressed directly by Nrg1 and/or rfg1,

however as for *RBT1*, the DNA binding protein that recruits Tup1 for repression of *HWP2* is not yet known. (Braun *et al.*, 2001; Kadosh and Johnson, 2001; Khalaf and Zitomer, 2001; Kadosh and Johnson, 2005).

1.9 Objectives

In this study, we aim to assess the role of Hwp2 in filamentation, virulence, drug susceptibility and white opaque switching. This will be done through the generation of a null homozygous strain and comparing growth of the mutant to the parental strain in various inducing and non-inducing liquid and agar media. Virulence will be assed in a mouse model of disseminated candidiasis, while antifungal susceptibility to the antifungal agents Amphoterecin B, Caspofungin, Fluconazole, Ketoconazole, Itraconazole, and Voriconazole will be assessed by the E-test method. A possible role of Hwp2 in white/opaque switching will be determined by colony morphology and color. Finally, the aminoacid sequence of Hwp2 will be aligned using the bl2seq tool of the NCBI database and the TCoffee tool provided by the CNRS (Centre Nationale de Recherche Scientifique) database with its closely homologous GPI anchoring proteins Hwp1 and Rbt1. The aim is to identify additional homologous sites that can be conserved domains and or functional domains among the three proteins; and assess the level of homology among the three sequences. Any similarity in virulence phenotype between Hwp2 and Hwp1 or Rbt1 could thus be due to these key conserved domains.

Chapter II

Materials and Methods

2.1 Yeast strain, and cell growth.

The *C. albicans* RM1000 (*ura3Δ::λimm434/ura3Δ::λimm434his1::hisG/his1::hisG)* histidine and uridine auxotroph strain was used in this study; RM100 was kindly donated by Dr. William Fonzi (Georgetown university, Washington D.C, USA) (Negredo *et al.*, 1997).

Cells were maintained and grown on the rich non-selective PDA (HiMedia, India) medium (Kaiser *et al.* 1994). For selective growth, yeast nitrogen base (YNB) synthetic medium (Fluka, Switerzland), was used lacking specific nutrients (Kaiser *et al.* 1994). Photographs were generated with a Sony DSC-S40 digital camera coupled to an Olympus CH 30 light microscope for cellular morphology, and to a Leica Stereozoom 4 dissecting microscope for colony morphology.

2.2 Construction of *HWP2-URA3 and HWP2-HIS1* fragments

2.2.1 PCR

The homozygous *Δhwp2* strain was generated by transforming RM 1000 with hybrid *URA3-HWP2* and *HIS1-HWP2* PCR fragments respectively. The *URA3* deletion allele fragment was generated by using synthetic forward primers that contained *HWP2* sequences from nucleotide position -48 to +60 5'and *URA3* sequences from nucleotide position -413 to -387 3', and a second reverse primer that contained *HWP2* sequences

from nucleotide position +1725 to +1625 5'and *URA3* sequences from nucleotide position + 953 to +928, 3'. Similarly, the *HIS1* deletion allele fragment was generated by forward primer with *HWP2* (-48 to+60 5') +*HIS*1 (-320 to -297 3') and a reverse primer with *HWP2(*+1725 to +1625 5'*)+HIS1(*+926 to +899 3') (Table 2.1 and 2.2).

For amplification of the *URA3* deletion cassette, typical PCR mixtures contained: 50 ng of pABSK2 plasmid DNA carrying the *C. albicans URA3* selectable marker and the ARS3 origin of replication (pABSK2 was kindly donated by Dr. Richard Zitomer, University at Albany, USA), 2 µl of 10mM dNTP, 3µl of 25mM $MgCl_2$, 1µl each of the 60mM forward and reverse *HWP2* primers (the primer sequences are found in Table 2.1), 6µl of the 10x Taq buffer containing $(NH_4)_2S0_4$ and 0.5µl (2.5 units) of Taq polymerase (Fermentas Life Sciences, USA), and 46µl of distilled water. The PCR was performed in a Gene Amp PCR system 9700 (Applied Biosystems) using the following PCR conditions: initial denaturation at 94° C for 5 min, followed by 30 cycles of 45 sec denaturation at 94°C, 50 sec annealing at 52°C, 2 min elongation at 72°C, followed by a final extension step at 72°C for 10 min and a termination step at 4°C for ∞(product size in Table 2.2).

Amplification of *HIS1* allele uses the same procedure using the pLHL plasmid DNA carrying the *HIS1* selectable marker. pLHL was kindly donated by Dr. Alistair Brown (University of Aberdeen, UK) (Dennison *et al.*, 2005) (Tables 2.1 and 2.2).

Table 2.1: PCR primers generated in this study

Primer name	Primer sequence
HWP2 URA3 Deletion Upstream F	**5'CAATACGACACAAGTCTCCGATAGTCATTCG CTTTTATACTTACAAGAATGAGATTTGCAACTA CCCAACTTGCCACACTCGCTTGCTTTATTTTGA CAGCTGAGGCTGGAATTGATTTGGATGGTATA AACGG 3'**
HWP2 URA3 Deletion Downstream R	**5'AAATTGCAAAGGCAGCGACACCAGCAATTAC AACACCAAATGACAATTTCATGTTGTTACCGG TACCTTCATATGAAGAAATAGCAGCAATACTT GATTGAGCAGATCTGTCATGATTTCTAGAAGG ACCAC 3'**
HWP2 Integration Verification primer F	**5' TCAGATCATGCCATACCACTG 3'**
URA3 Integration verification primer R	**5'CGGTCTGGTAAATGATTGACTAAATCCTCC 3'**
HIS1 Integration verification primer R	**5'CGGTCTGGTAAATGATTGACTAAATCC3'**
HWP2 HIS 1 Deletion Upstream F	**5'CAATACGACACAAGTCTCCGATAGTCATTCG CTTTTATACTTACAAGATGAGATTTGCAACTAC CCAACTTGCCACACTCGCTTGCTTTATTTTGAC AGCTGAGGCTCAGAAGTTAGTAGTAACAATTT G 3'**
HWP2 HIS Deletion Downstream R	**5'AAATTGCAAAGGCAGCGACACCAGCAATTAC AACACCAAATGACAATTTCATGTTGTTACCGG TACCTTCATATGAAGAAATAGCAGCAATACTT GATTGAGCAGATATAATATTTATGAGAAACTA TCACTTC 3'**
Actin F primer	**5'GTTGCTGCTTTAATTATCGATAACGGT TCTGG 3'**
Actin R primer	**5'GGTGAACAATGGATGGACCAGATTCGTC 3'**
HWP2 Internal F primer	**5' TGACAGCTGAGGCTACCTTC 3'**
HWP2 Internal R primer	**5' CCAGTGTTGAGTCTACGTCTATC 3'**

F: forward, R: reverse. (Primer sequences obtained online from the Candida genome database CGD www.candidagenome.org).

Table 2.2: Expected PCR product sizes

Product name	Expected size
HWP2 URA3 Deletion fragment	1566 bp
HWP2 HIS1 Deletion fragment	1445 bp
HWP2 URA3 integration verification fragment	757 bp
HWP2 HIS1 integration verification fragment	664 bp
HWP2 internal fragment	827bp
Actin fragment	1102 bp

2.2.2 Gel electrophoresis

The PCR products were visualized by electrophoresis (Pharmacia Biotech Gel Electrophoresis Apparatus GNA-100, Sweden) on a 1.5% agarose gel along with marker XVI (250-3000bp; Roche, Manheim, Germany) to confirm the presence of a product with the expected size.

2.3 Verification of integration by PCR

Verification of correct integration was confirmed by PCR analysis of the *HWP2/hwp2* heterozygous colonies and the *hwp/hwp2* homozygous colonies. Four sets of primers were utilized. The first set involved *HWP2* internal forward primer that hybridized to sequences internal to the *HWP2* alleles at nucleotide position + 46 to +66 5' and *HWP2* internal reverse primer that hybridized to sequences internal to the HWP2 allele at nucleotide position +850 to +873 3'. In the second set of primers, *HWP2* F verification primer hybridized outside the *HWP2* integration site at position -291 to -289 5' and the *URA3* primer hybridized to one end of the *URA3* sequence at nucleotide position +1 to +30. The third set uses the same Forward *HWP2* integration verification primer

mentioned above and a *HIS1* primer that hybridized at nucleotide position +1 to +30. The last set are the actin primers used as a control for the quality of the DNA preparations (Khalaf and Zitomer, 2001). The actin primers hybridizes to the actin sequence at +674 to +706 5' for the forward primer and 1748 to+1776 3'for the reverse primer. (Tables 2.1 and 2.2)

PCR samples were prepared using the same master mix described above and using different DNA samples and primers sets. In all mixtures 50 ng of the tested heterozygous *HWP2/hwp2* or homozygous *hwp2/hwp2* DNA were used. In the integration verification mixtures, 1µl of 60 mM Actin forward and reverse primer were used a DNA quality control in addition to the verification primers. For the Heterozygous transformants, 1µl of the 60mM *HWP2* Integration verification primer, and 1 µl of the 60 mM *URA3* primer were used. For the homozygous transformants, 1µl of the 60mM *HWP2* Integration verification primer, and 1 µl of the 60 mM *URA3* primer were used. For the allele verification deletion for both heterozygous and homozygous strains 1µl of 60mM *HWP2* internal forward and reverse primers were used to verify a homozygous strain from a heterozygous one. (Tables 2.1 and 2.2)

The PCR cycling conditions consisted of initial denaturation at 94˚C for 5 min, followed by 35 cycles of 45 sec denaturation at 94˚C, 50 sec annealing at 52˚C, and 1.45 min elongation at 72˚C, followed by a final extension at 72˚C for 10 min and a termination step at 4˚C for ∞

2.4 DNA purification from agarose gel

DNA was purified from the agarose gel by the Nucleotrap PCR Kit and following the manufacture's protocol (Macherey-Nagel,Germany).

2.5 Transformation Protocol

C. albicans strain RM1000 was transformed with either the *URA3-HWP2* fragment, the *HIS1-HWP2* fragment, or plasmid pABSK2 using a modified protocol of the Alkali Cation Yeast Transformation kit (Q-Biogene, Germany) (Khalaf and Zitomer, 2001). Transformed cells were selected on media containing Yeast Nitrogen Base (YNB) prepared under the manufacture's instructions (Fluka, Switerzland), and supplemented with amino acids as needed.

2.6 DNA Extraction

DNA was extracted following the procedure described by Khalaf, (2001).

2.7 White-opaque switching

HWP2/hwp2 heterozygous, hwp2/hwp2 homozygous, RM1000+pABSK2, and the WO-1 control strains were streaked on Lee's medium and phloxine B (5 μg/ml) agar plates (Slutsky *et al.*, 1986; Anderson and Soll, 1987). The plates were incubated at 35 °C for 4 days and individual colonies were viewed for white-opaque switching. WO-1 was kindly donated by Dr. J. Morschauser (Wurzburg, Germany) (Slutsky *et al.*, 1987).

2.8 Growth on Sabouraud agar.

The *HWP2/hwp2* heterozygous, hwp2/hwp2 homozygous and RM1000+pABSK2 strains were grown on Sabouraud agar+ dextrose (Oxoid, U.K). The strains were plated on the agar and allowed to grow for 14 days at 30 °C. The plates were checked for invasive filamentation.

2.9 Growth on Cornmeal agar

For chlamydospore formation, the *HWP2/hwp2* heterozygous, hwp2/hwp2 homozygous and RM1000+pABSK2 strains were grown on Cornmeal Agar supplemented with 1% Tween 80 (Surechem, England) that was prepared according to the manufacturer's instructions (HiMedia, India). A sterile blade was used to cut perpendicularly the Cornmeal agar plates with two small cuts facing each other per plate. The 3 strains were inoculated along the edges of the opening and were covered completely with a glass cover slip to create an anaerobic condition (Buckley et al, 1982), incubated in a dark room at room temperature (25^0C), and monitored microscopically over 7 days for the formation of hyphae and chlamydospores.

2.10 Filamentation on liquid hyphae inducing media and FBS

Filamentation capability of the wild type, the *HWP2/hwp2* and the *hwp2/hwp2* strains were tested under different liquid hyphae inducing media; M199 with Earle's salts, L-Glutamine and 150mM HEPES, at pH 4 or buffered to pH 7.5 (Sigma-Aldrich, Germany), Lee's media buffered to pH 6.8 (Lee *et al.*, 1975), 100% Fetal Bovine Serum (FBS, Biowhittaker, Belgium). The heterozygous, the homozygous, and parental strain

were each inoculated into 10 ml of the above media and incubated at 37°C with a shaking speed of 220 rpm. A 15µl sample was taken from each sample after overnight incubation and examined under the microscope for assessment of the phenotype.

2.11 Murine model of Disseminated Candidiasis

RM1000+pABSK2, *HWP2/hwp2* heterozygous, and hwp2/hwp2 homozygous cultures at OD_{600} 0.1 were each inoculated in 10 ml of Potato Dextrose Broth (PDB) (Himedia, India), and incubated at 30°C to OD 0.75. Cells were counted using a hemacytometer, diluted to $3x10^7$ cells/ml, harvested, and washed with Phosphate buffered Saline solution (PBS). Six female BALB/c mice (Lebanese American university stock) weighing 20g to 30g were injected via the lateral tail vein with $6x10^6$ cells (200µl of the $3x10^7$ cells/ml suspension) for each of the tested strains. Mice were housed in a controlled environment (22±2°C, 12 hours of light and 12 hours of dark) having free access to water and mice chow food. The mice were monitored for survival 3 times daily, over a period of 30 days.

2.12 Antifungal agents

The Epsilometer test (E-test) method was used to assess the antifungal susceptibility of *HWP2/hwp2* heterozygous and *hwp2/hwp2* homozygous strain to the parental strain RM1000+pABSK2 on RPMI-1640 medium with L-Glutamine and 165mM MOPS prepared according to the manufacture's instructions (Biowhittaker, Belgium). E-test strips of amphotericin B (AP), voriconazole (VO), fluconazole (FL), ketoconazole (KE), itraconazole (IT), and caspofungin (CS) were supplied by AB Biodisk, Solna, Sweden.

Inoculum preparation with turbidity of 0.5 MacFarlen, inoculation, and application of the strip were performed according to the manufacturer's instructions.

Minimal Inhibitory Concentration (MIC) readings were done after 24-hour incubation at 35°C. As recommended by the manufacturer, amphotericin B MICs were read as the lowest concentration on the E-test strip at which there was 100% inhibition i.e., at the point of intersection of the strip with the border of the elliptical inhibition zone, whereas caspofungin and azole MICs were read either as the least concentration at which there was 80% growth inhibition or where there was a significant decline in growth as described by the manufacture. The quality control strain used was the CLSI reference strain *C. albicans* ATCC 90028. MIC range for ATCC 90028 is provided by the Etest manufacturer (AB Biodisk, Solna, Sweeden).

2.13 Homology assessment

The amino acid sequence of the three genes *HWP2*, *HWP1* and *RBT1* were aligned using the NCBI alignment tool blast2seq available at http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi and the CNRS multiple sequence alignment (MSA) tool Tcoffee available at http://igs-server.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/index.cgi.

Chapter III

Results

3.1 Verification of integration

The *HWP2/hwp2* heterozygous strain was created by transforming a 1566 bp DNA cassette containing a *URA3* marker flanked by 100 bps of upstream and downstream sequences of the *HWP2* ORF (Fig 3.1 A, B). Homologous recombination displaced the wild type *HWP2* allele creating a heterozygous *HWP2/hwp2:URA3* strain (Fig 3.1 C, D). Transformants were plated on selective YNB medium lacking uridine to select for ura+ transformants.

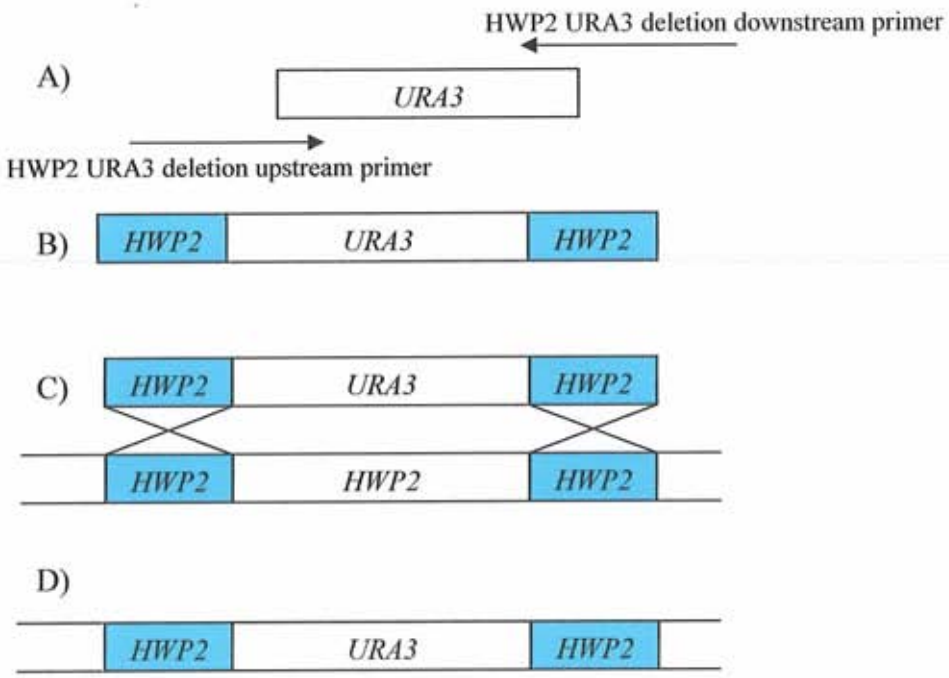

Figure 3.1: Generation and integration of *URA3* cassette. A) Primers used to generate integration cassette; B) Integration Cassette; C) homologous recombination at the 5' and 3' flanking regions occurs at the *HWP2* locus; D) integration of cassette at *HWP2* locus.

The integration was verified by amplifying a 757 bps fragment with the *HWP2* integration through verification primer that hybridized upstream of the integration site and the *URA3* Internal primer that hybridized within the *URA3* locus (Fig 3.2A; Fig 3.3A lane2).

Furthermore to confirm the presence of *HWP2* allele, and thus the heterozygosity of the strain, internal *HWP2* primers were used to amplify an 827bp fragment (Fig. 3.3 B Lane2).

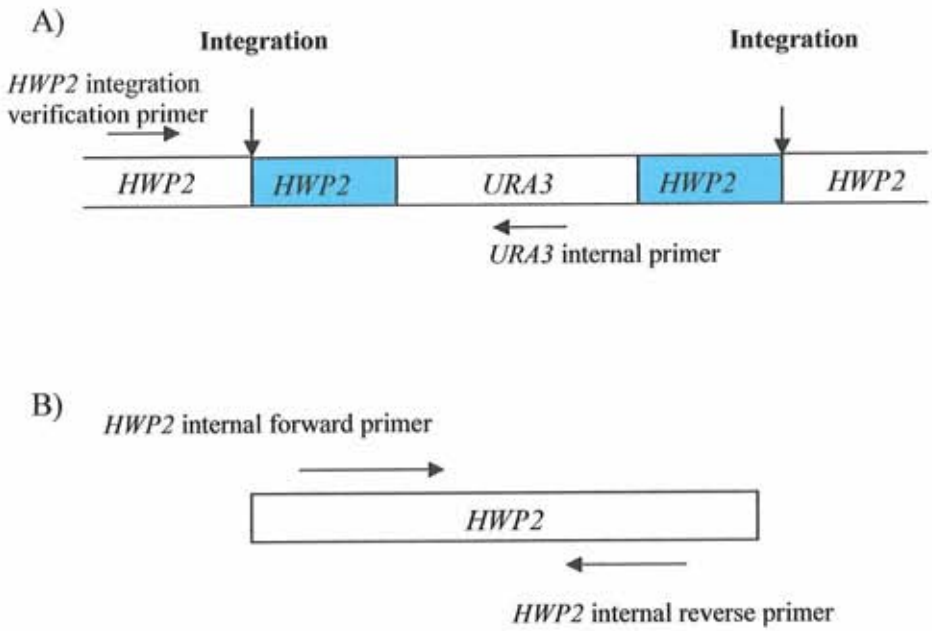

Figure 3.2: Verification of integration. A) *URA3* internal and *HWP2* integration verification primers confirming integration of the *URA* cassette. B) *HWP2* internal primers verifying either heterozygous or homozygous integration.

The Homozygous *hwp2/hwp2* strain was obtained in a similar procedure by transforming the above mentioned heterozygote *URA+* strain with a 1445 bp *HIS1* cassette and plating on YNB medium lacking uridine and histidine to select for transformants. To confirm successful integration, the *HWP2* integration verification primer and an internal *HIS1* primer amplified a 664 bp product (Fig 3.3A lane 1) to confirm the homozygous aspect of the strain produced by

the transformation of the *URA3* and *HIS1* hybrid fragments to the *HWP2* loci respectively, internal *HWP2* primers were used to amplify an 827bp fragment. The fragment was present in the heterozygous strain and absent in the homozygous one (Fig 3.2B; Fig 3.3B lane1, lane2).

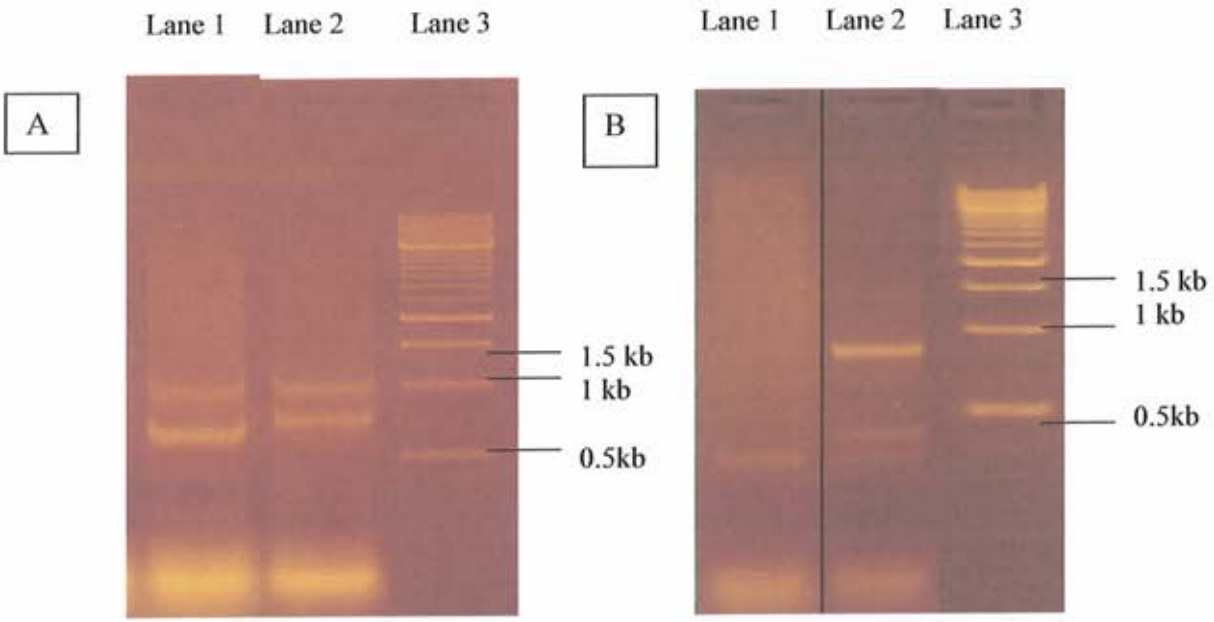

Figure 3.3: Deletion verification:

(A) Integration verification; lane 1: *hwp2/hwp2* homozygous transformant, lower band 664 bp *HIS1* homologous integration verification product, upper band 1.1kb actin control; lane 2: *hwp2/HWP2* heterozygous transformant, lower band 757 bp *URA3* homologous integration verification product, upper band 1.1 kb actin control. Lane3: 0.5kb DNA marker.

(B) Deletion verification using internal primers of *HWP2*. Lane 1: *hwp2/hwp2* homozygous transformant with no internal *HWP2* amplification band; lane 2: *hwp2/HWP2* heterozygous transformant with an 827 bp *HWP2* internal fragment. Lane 3: 0.5 kb DNA marker.

3.2 Filamentation on hyphal inducing liquid media and serum

The three strains parental RM1000+pABSK2, heterozygous mutant *HWP2/hwp2*, and homozygous mutant *hwp2/hwp2* were tested on the following liquid inducing media.

3.2.1 FBS

Under hyphae inducing condition serum at 37 °C the parental strain behaved normally producing true hyphae that formed clumps after overnight incubation (Fig. 3.4 A, B). The heterozygous strains did form clumps and did filament well with a large percentage of hyphal cells and some pseudohyphae (Fig.3.4 C, D). The homozygous strain contained a mixture of yeast and hyphal cells. However the percentage of hyphae was less than in the two other strains (Fig. 3.4 E, F).

3.2.2 M199 at pH 7.5 and 4.0

The three strains filamented normally after 12 hours of incubation on liquid M199 pH 7.5 at 37°C (Fig 3.5). At pH 4.0, the three strains grew exclusively in the yeast morphologyat 37 °C (Fig. 3.5).

3.2.3 Lee's medium

On lee's liquid medium no difference was observed at 37°C as all strains formed hyphae after 12 hours incubation (Fig. 3.6).

3.3 Growth on Solid Media.

3.3.1 Corn Meal agar.

Significant differences were observed on corn meal agar media after 4 days incubation. The control RM1000+pABSK2 strain invaded agar producing abundant filaments and

chlamydospores (Fig. 3.7 A). The *HWP2/hwp2* strain was able to filament and produce chlamydospores, however the density of the filaments and the extant of invasiveness was less than the parental strain (Fig. 3.7 B). The *hwp2/hwp2* strain was non invasive and non filamentous in general, with only very few filaments invading agar while the majority of the growth was in yeast form (Fig. 3.7 C).

3.3.2 Sabouraud agar

After three days of incubation at 30 °C, the three strains tested grew to form smooth colonies on Sabouraud with dextrose agar. After 14 days of incubation, morphological differences amongst the 3 strains appeared. The wild type RM1000+pABSK2 invaded agar and formed extensive filaments (Fig. 3.8 A). The *HWP2/hwp2* heterozygote was also able to invade with the extent of invasion similar to the wild type (Fig. 3.8 B). On the other hand, the mutant *hwp2/hwp2* was not able to invade agar (Fig. 3.8 C).

3.4 White opaque switching

All the three strains were plated on Lee's medium with phloxine B for 4 days at 35 °C. No difference in switching was observed amongst the three tested strains (Fig. 3.9 B,C,D). None switched at a rate comparable to the control strain WO-1 (Fig. 3.9 A).

3.5 Murine model of Disseminated Infection

Using the mouse systemic candidiasis model, it was observed that all of the mice infected with $6x10^6$ cells of the three strains died within 11 days (Fig. 3.10). However by day 6, all of the

wild type infected mice died, while both the *HWP2/hwp2* heterozygous strain and the *hwp2/hwp2* homozygous deletion strain had 83% and 67% survival rates (Fig. 3.10).

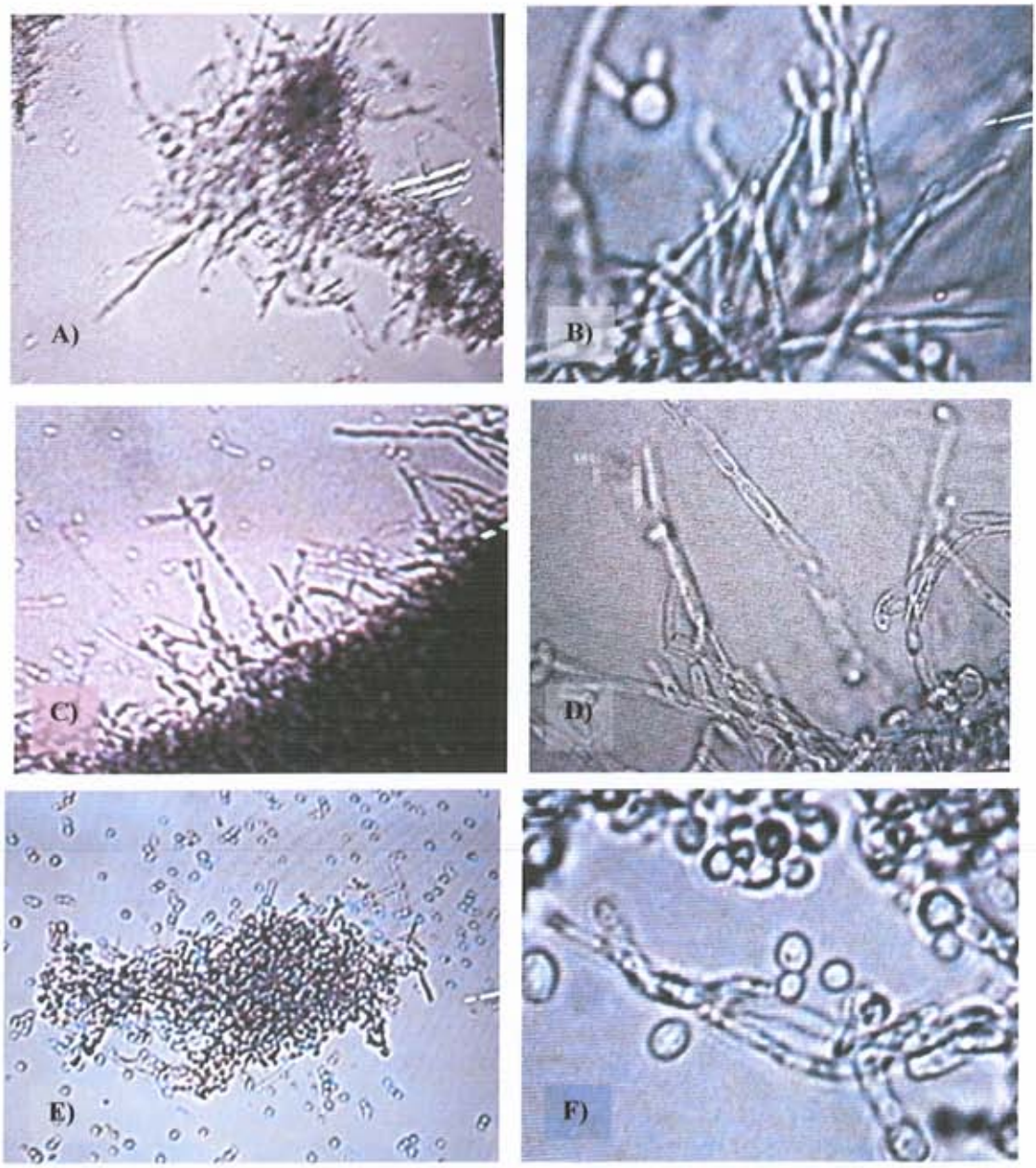

Figure 3.4: Filamentation phenotypes in FBS at 37 °C after 12 hours incubation. (A,B) RM1000+pABSK2: Filamentation induction was normal with production of extensive filaments, (C,D) *HWP2/hwp2* strain: A mixture of mainly hyphal and some pseudohyphal cells , (E,F) *hwp2/hwp2* strain: A majority of yeast cells with presence of hyphae. Magnification 100X (A,C,E), 400X (B,D,F).

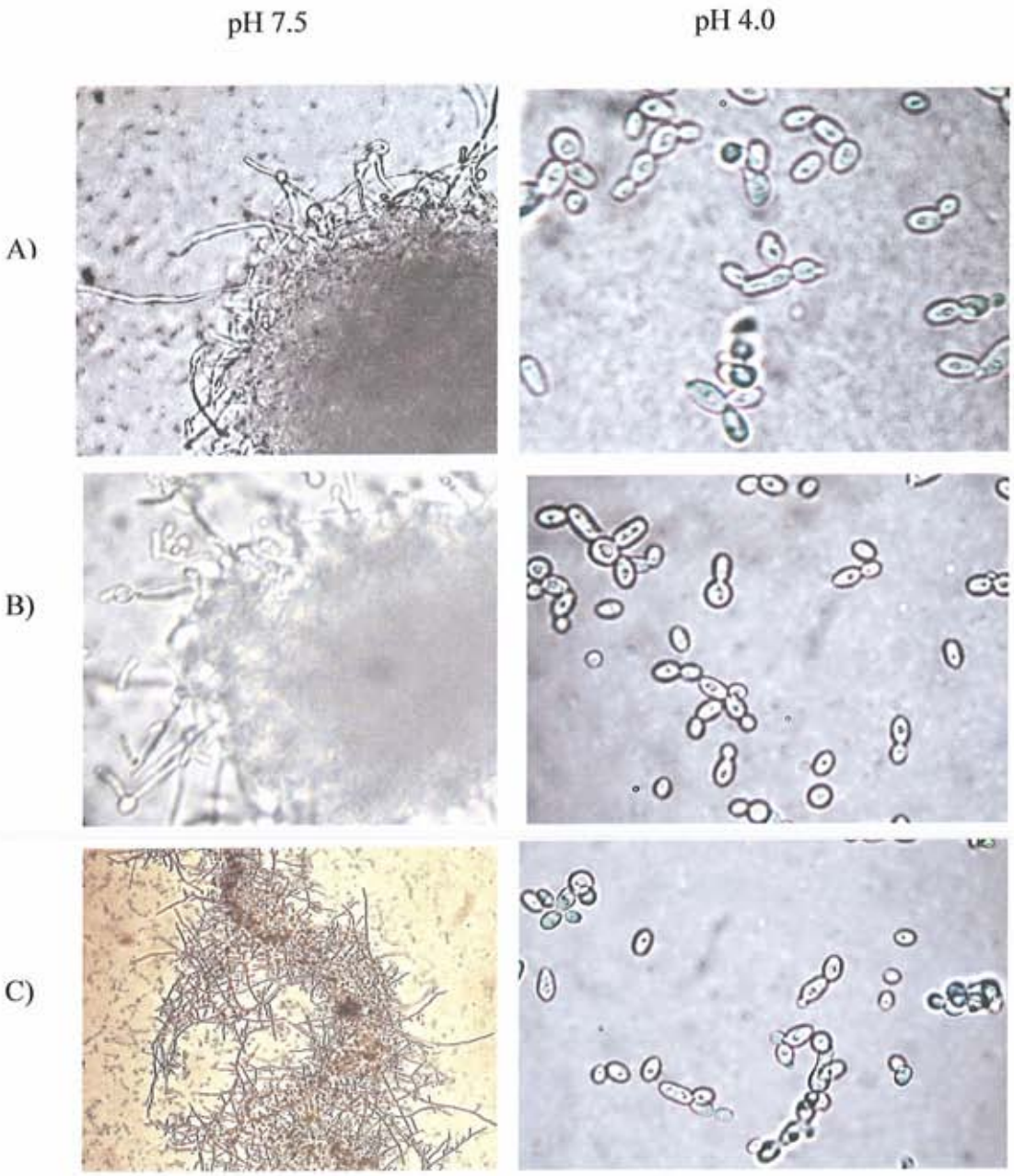

Figure 3.5: Liquid medium M199 at 37°C. A) RM1000+pABSK2, B) *HWP2/hwp2,* and C) *hwp2/hwp2* were tested at pH 7.5 and pH 4.0. All the three strains produced normal filaments at pH 7.5. At pH 4.0 the three strains grew as yeast cells. Magnification of 400X for the pH 4.0 pictures and 100X for the pH 7.5.

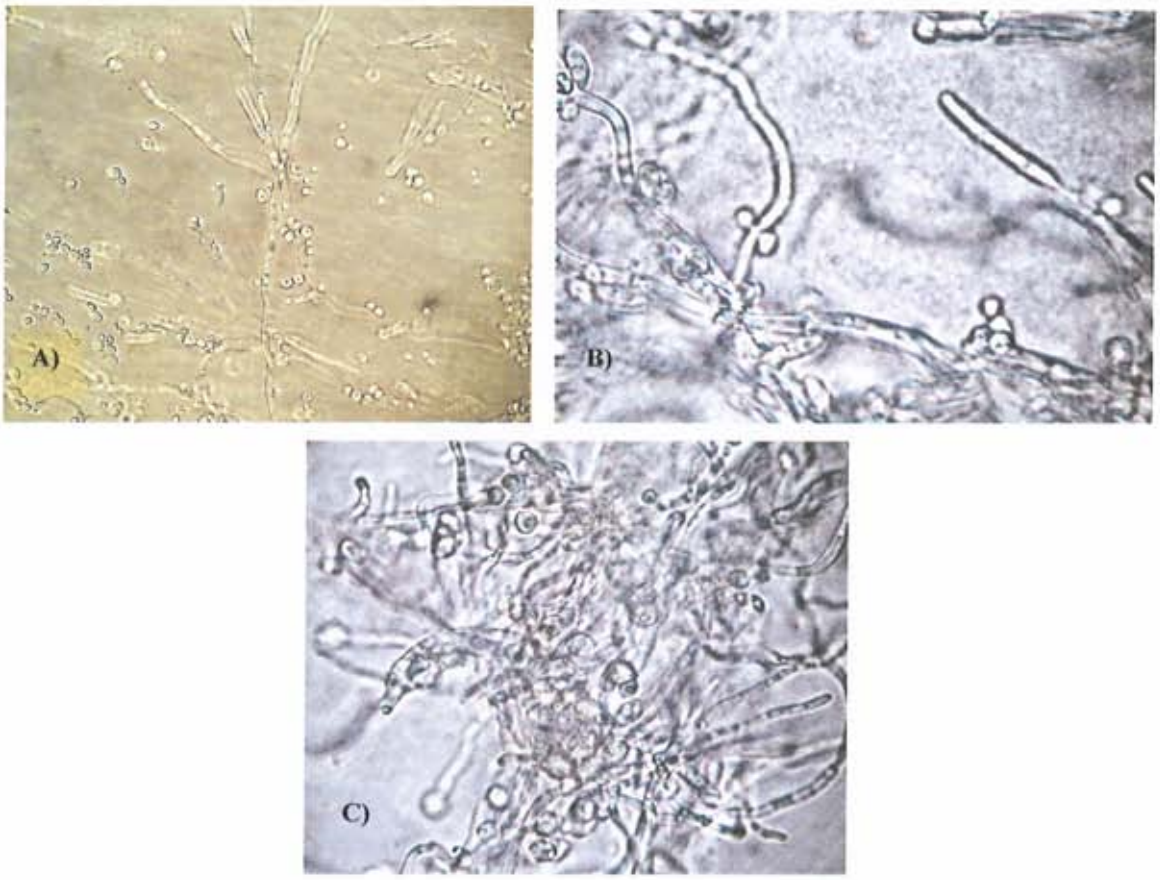

Figure 3.6: Filamentation phenotypes in Lee's liquid medium at 37 °C after 12 hours incubation. The three strain filamented normally producing true hyphae. A) RM1000+pABSK2, B) *HWP2/hwp2*, and C) *hwp2/hwp2*. Magnification: 400X.

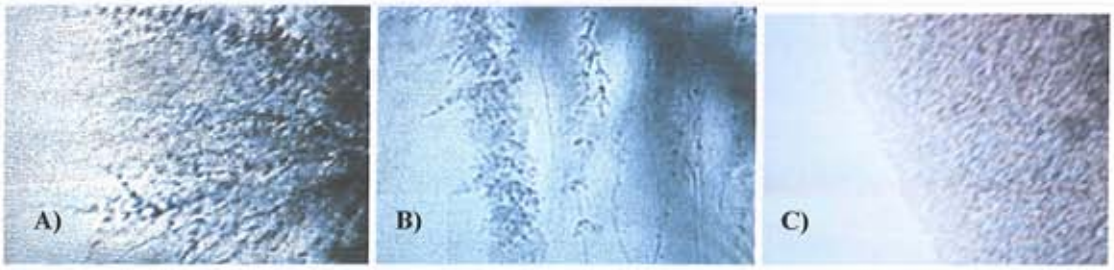

Figure 3.7: Invasiveness and chlamydospore production on corn meal agar. A) RM1000+pABSK2 invaded the agar thoroughly producing a large extent of filaments and chlamydospores; B) *HWP2/hwp2* did invade the agar, however the thickness of invasion after 4 days was less than that for RM1000+pABSK2; C) *hwp2/hwp2* the cells remained in yeast form. Magnification 40X

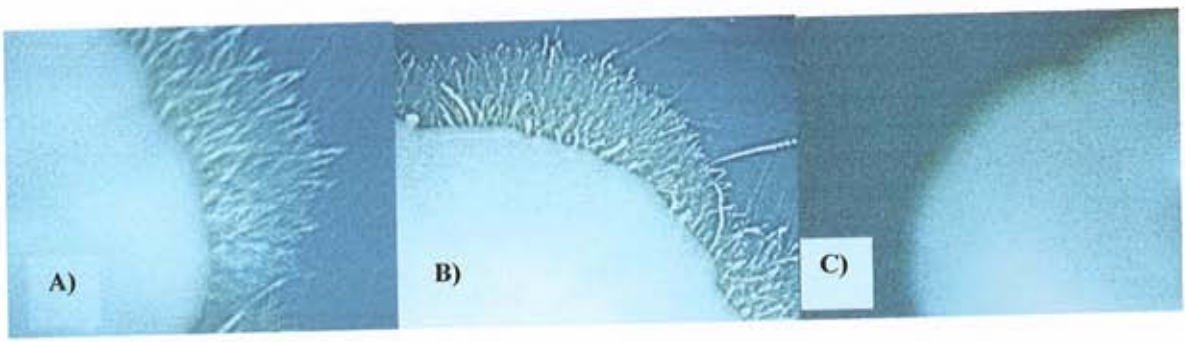

Figure 3.8: Invasiveness on Sabouraud agar after 14 day incubation. A) RM1000+pABSK2 invaded the agar thoroughly producing filaments; B) *HWP2/hwp2* invaded the agar with the extent of filamentation comparable to RM1000+pABSK2; C) The *hwp2/hwp2* mutant strain was unable to produce invading filaments. Magnification 10X.

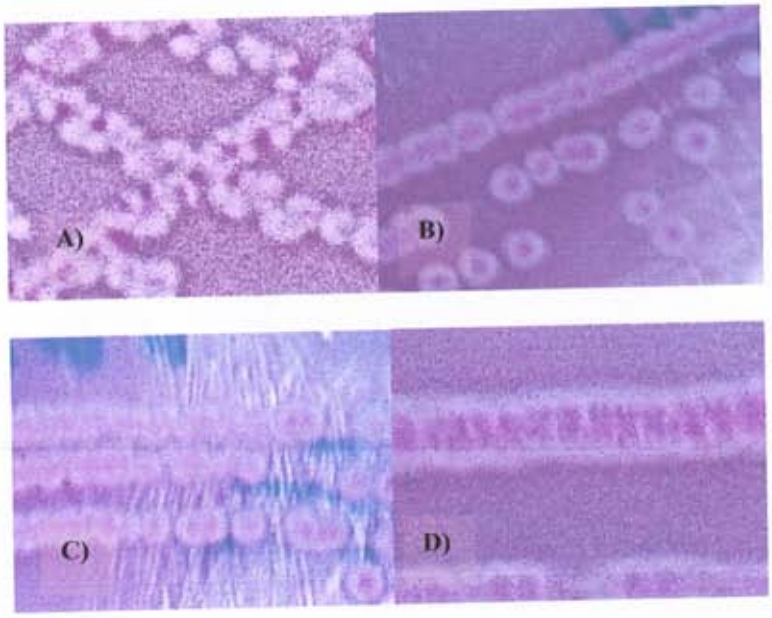

Figure 3.9: White/opaque switching. A) WO-1 control strain with presence of distinct white colonies (pink) and opaque colonies (red); B) RM1000+pABSK2; C) *HWP2/hwp2*; D) *hwp2/hwp2*.

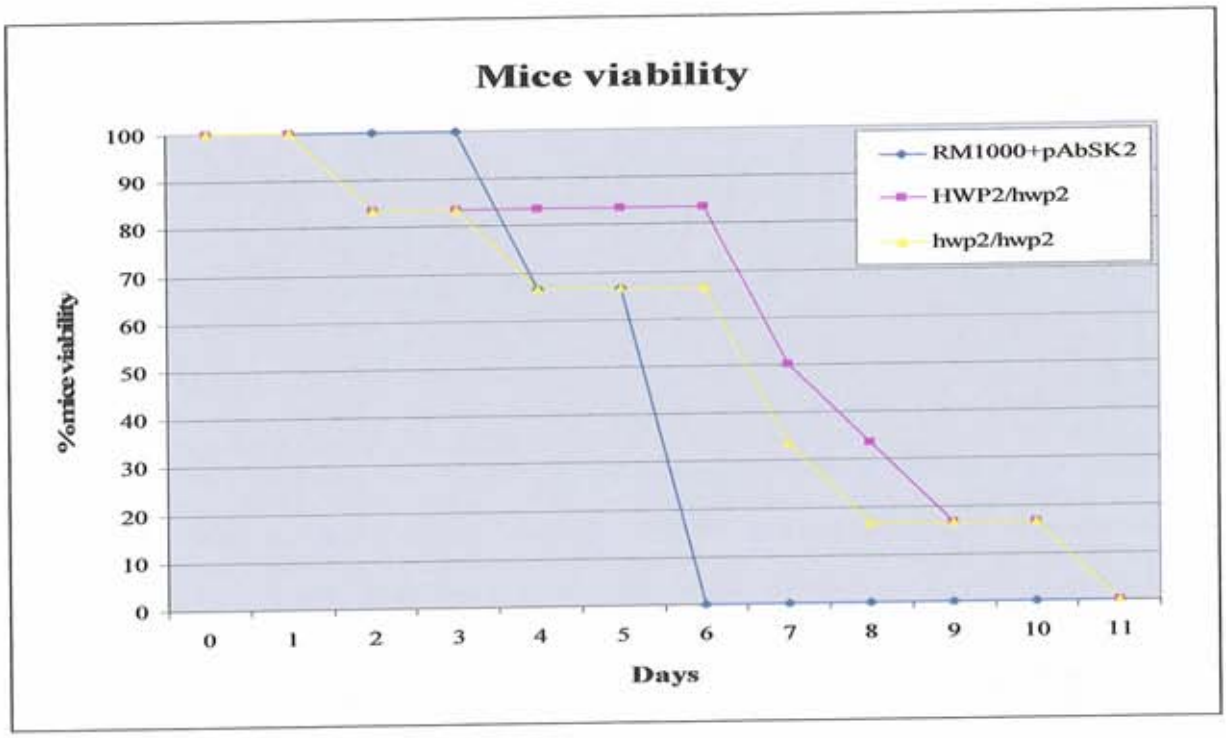

Figure 3.10: Percentage survival curves of mice following intravenous injection with $6x10^6$ cells of RM1000+pABSK2, HWP2/hwp2 and *hwp2/hwp2* (sample size = 6)

3.6 Antifungal drugs

The antifungal susceptibility E-test was done to determine sensitivity of the homozygous, heterozygous and parent strain to Amphotericin B, azoles, and caspofungin on RPMI 1640. After 24 hours of incubation, the MIC values were read at the lowest drug concentration at which there was complete inhibition of growth.

The MIC values were determined for RM1000+pABSK2, *HWP2/hwp2*, *hwp2/hwp2*, and the quality control strain *C. albicans* ATCC 90028 (Table 3.1). Data presented in Table 3.1 indicate that the MIC values of the *C. albicans* ATCC 90028 were within the control limits (Pfaller *et al.*, 1994). No significant discrepancy amongst the three strains was noted for all six drugs tested.

Table 3.1: MIC determination of RM1000+pABSK2, *HWP2/hwp2*, *hwp2/hwp2*, and ATCC 90028 on RPMI 1640 medium

MIC μg/ml	ATCC 90028	RM1000+pABSK2	*HWP2/hwp2*	*hwp2/hwp2*
AmphotericinB	1.0	0.19	0.25	0.19
Caspofungin	0.094	0.032	0.094	0.094
Fluconazole	1.0	1.0	1.0	0.75
Ketoconazole	0.032	0.016	0.016	0.023
Itraconazole	0.006	0.023	0.032	0.032
Voriconazole	0.016	0.016	0.023	0.016

3.7 Homology assessment

The homology of Hwp2 to Rbt1 and Hwp1, the closest Hwp2 homologues was assessed by pairwise aligning the amino acid sequences of Hwp2 with either Rbt1 or Hwp1. A region in Hwp2 had high homology to 2 sequences of Hwp1 (Table 3.2). This region is in addition to the previously mentioned homologous regions that are found to the NH_2 and the COOH termini that are related to localization and GPI anchoring respectively (Sohn *et al.*, 2003; de Groot *et al.*, 2003).

Homology between Rbt1 and Hwp2 was initially thought to be lower than between Hwp2 and Hwp1 but still it spanned over greater sequence areas generating 7 different alignments (Table 3.3). However upon careful examination the same Hwp2 sequence mentioned above was found to align with high identity to a short stretch of amino acids in Rbt1 (Hwp2: 221-262/ Rbt1: 416-457).

As a control we performed a pairwise alignment of Hwp2 with other GPI proteins such as Plb5 and Als1 and we found no significant alignment amongst them.

By multiple sequence alignment (MSA) using the software Tcoffee, we identified a second region of homology among Hwp1, Hwp2 and Rbt1. This region is approximately 56 amino

acid residues long, and consists of 55% identical amino acids and 79% conserved (Table 3.4, Fig. 3.11).

By combining the result of the pairwise alignment and the MSA we identify an area of homology shared by the three sequences represented by the MSA alignment that is duplicated right upstream for Hwp1 and Rbt1 as revealed by the pairwise alignment of Hwp2 to Hwp1 and Hwp2 to Rbt1, and is duplicated further upstream in Hwp2 (Fig. 3.12).

Table 3.2: Areas of sequence alignment between Hwp2 and Hwp1

Region	Hwp2/Hwp1 amino acid sequences	Identities
1	222-258/313-349	26/37 (70%)
2	222-258/378-414	24/37 (64%)

Table 3.3: Areas of sequence alignment between Hwp2 and Rbt1

Region	Hwp2 / Rbt1 amino acid sequences	Identities
1	405-908/274-750	175/547 (31%)
2	70-574/278-728	143/508 (28%),
3	1-703/1-720	202/745 (27%)
4	156-662/275-726	125/513 (24%),
5	69-294/493-726	68/238 (28%)
6	671-888/268-494	67/237 (28%)
7	717-877/267-429	52/166 (31%)

Table 3.4: MSA high homology region among Hwp2, Rbt1, and Hwp1

Sequence name	Aligned amino acid sequences
Hwp2	695-751
Hwp1	362-418
Rbt1	499-555

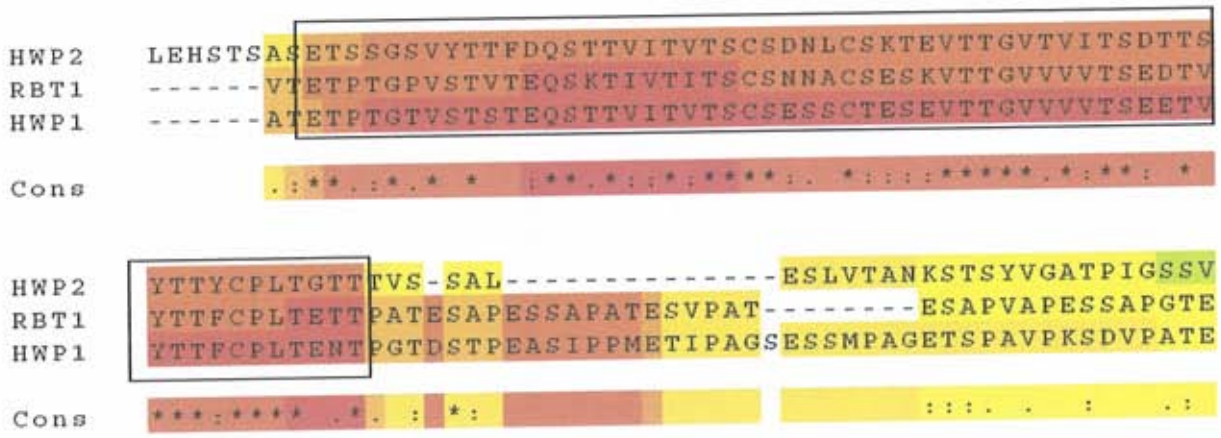

Figure 3.11: MSA of Hwp2, Rbt1, and Hwp1 by Tcoffee; The marked sequence represent the 56 bps region of high homology among the three amino acid sequences. Legend: Cons (Consensus); * (Identity), : (close homology), . (homology).

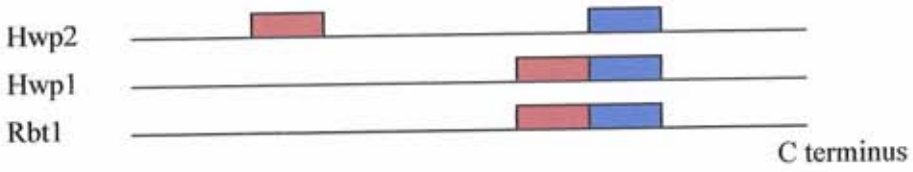

Figure 3.12: Homology amongst Hwp1, Hwp2, and Rbt1 based on both MSA and pairwise alignment. Blue boxes represent the areas that align among the three sequences based on the MSA data. The red boxes are duplications of those sequences that were identified by pairwise alignment. Note that in Hwp1 and Rbt1 the duplications are very close to each other while in Hwp2 the duplication is further upstream. (The figure is not to scale).

Chapter IV

Discussion and Conclusion

The current strategy used to delete the *HWP2* gene was adopted for the sake of obtaining a strain that would be able to express *URA3* in normal quantities. The proper expression of *URA3* in *C. albicans* has been shown to be important for both virulence and filamentation (Kirsch and Whitney, 1991). When using a recyclable deletion cassette for deleting a gene it was shown that the presence of direct repeats such as the *hisG* of the *hisG-URA3-hisG* cassette widely used in gene deletion tend to decrease *URA3* expression and subsequently lead to attenuated virulence and decreased filamentation (Sharkey *et al.*, 2005). In constructing our *HWP2* deletion strain, we used non-recyclable cassettes of *HWP2-URA3-HWP2* and *HWP2-HIS1-HWP2*. This allowed us to obtain a proper *URA3* expression avoiding the artifacts of a phenotype unrelated to *HWP2* deletion. For the same reasons mentioned above, our parental control strain RM1000 was transformed with a *URA3* selectable marker plasmid and used throughout this study.

The use of double verification through PCR for both homologous integration of the cassette, in addition to the presence of an *HWP2* fragment in the genome was also helpful in ruling out any suspected deletion strain that might have duplicated a portion of the chromosome containing one *HWP2* allele. In fact one ura+ his+ deletion strain obtained was determined to have an additional *HWP2* allele and thus ruled out from further analysis.

The phenotype of the transformants was observed on three different liquid media under inducing conditions. No significant discrepancy was observed on Lee's and M199 media

at 37 °C, and a slight discrepancy was observed in serum whereby the percentage of hyphal cells was lower in the deletion strain. Sohn *et al.,* (2003) previously showed that *HWP2* is activated by Efg1 in media containing serum, thus it is not surprising that the *Δhwp2* might have some phenotype in serum. On the other hand, expression of *HWP2* in Lee's and M199 pH 7.5 has not been studied and the possibility that *HWP2* is not expressed under these conditions cannot be ruled out, thus no differences in morphology is to be expected. The filamentation of the homozygous mutant in these two media is due to expression or repression of numerous other genes.

Under non-inducing media such as M199 pH 4.0, no difference in morphology was obtained. This is to be expected since Hwp2 is only turned on under hyphae inducing media.

Rather than filamentation in liquid media, the ability to invade tissues is what account for a good deal of *C .albicans* virulence. We assessed the role of Hwp2 in agar invasion, a condition which might mimic tissue invasion. Interestingly and as opposed to the slight morphology discrepancies observed in serum liquid media, the *hwp2/hwp2* strain was unable to filament on solid media. On Sabouraud solid medium at 30 °C, invasion only occurred on the parental and the heterozygous strains. Such result might seem perplexing at first since Sabouraud and 30 °C are non-inducing conditions, conditions where no Hwp2 expression is expected. However, filamentation was only observed in the wild type and heterozygote after 14 days of incubation where glucose depletion becomes a factor and cells filament and invade agar in search of more hospitable glucose rich environments. Glucose starvation is a fungal-wide filamentation signal since it has been previously shown to be responsible for filamentation of *S. cerevisiae* (Cullen and

Sprague, 2000). On the other hand in *C. albicans* several factors repress filamentation including availability and type of the carbon source (Hayes, 1966). In this case, dextrose depletion might have acted as a positive trigger for filamentation. Under these conditions, Hwp2 is probably expressed and helps the cell invade agar while in the mutant, and in the absence of Hwp2, adhesion and invasion are abolished.

The effect on cornmeal agar was similar to Sabouraud with invasion occurring mostly with the parental and heterozygous strains after 4 days of incubation, with only small invading projections emanating from the majority yeast cells in the deletion strain. Corn meal agar is inherently a glucose poor medium and thus the difference in morphology does not require 2 weeks of glucose depletion to manifest itself.

One of the requirements for formation of filaments and invasion is the ability of yeast cells to change its cell surface enabling the cells to adhere to each other and to invade the agar substratum (Cullen and Sprague, 2002). Adherence is essential for the ability of yeast *C. albicans* to form filaments and invade agar. Relationship of adherence and hyphae formation in *C. albicans* has been previously shown with the surface protein Int1p. A deletion strain for *INT1* is unable to produce hyphae and is non-adherent to epithelial cells (Gale *et al.*, 1998). Hwp2, as a predicted surface protein, was unable to invade Sabouraud agar and remained as yeast form in cornmeal agar with very limited invasion. Since adherence is a precursor to invasivness, these results might imply a role for Hwp2 in adherence to solid media and for hyphal cells to adhere to one another forming long filaments necessary for tissue invasion.

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