DEPARTAMENTO DE MICROBIOLOGÍA Y ECOLOGÍA

AISLAMIENTO Y CARACTERIZACIÓN DE ABG1, UN GEN ESENCIAL DEL HONGO PATÓGENO OPORTUNISTA Candida albicans

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"AISLAMIENTO Y CARACTERIZACIÓN DE *ABG1*, UN GEN ESENCIAL DEL HONGO PATÓGENO OPORTUNISTA C*andida albicans*"

TESIS DOCTORAL presentada por:

Verónica Veses Jiménez

Valencia, Mayo 2005

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Don José Pedro Martínez García, Catedrático de la Universitat de València y Don Manuel Casanova Monroig, Profesor titular de la Universitat de València,

CERTIFICAN:

Que el trabajo presentado por la Lda. **Verónica Veses Jiménez**, titulado *"***Aislamiento y caracterización de *ABG1*, un gen esencial del hongo patógeno oportunista *Candida albicans***"**, ha sido realizado en la Unidad Docente de Microbiología del Departamento de Microbiología y Ecología de la Universitat de València, bajo nuestra dirección y asesoramiento.

Concluido el trabajo experimental y bibliográfico, autorizamos la presentación de esta Tesis Doctoral, para que sea juzgada por el tribunal correspondiente.

Valencia, Mayo de 2005

José Pedro Martínez García Manuel Casanova Monroig

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A mis padres, Vicente y Mª Esther,
a mi hermana Mª Carmen, y a mi familia.

β-ME:	beta-mercaptoetanol
BSA:	bovine serum albumine/ albúmina de suero bovina
Cfu/ufc:	colony forming units/ unidades formadoras de colonias
CFW:	Calcofluor White
CR/RC:	Congo Red/ Rojo Congo
DAPI:	4´,6-diamidino-2-phenylindole
DNAc/ADNc:	complementary DNA/ ADN complementario
DEPC:	diethyl pyrocarbonate/dietil pirocarbonato
DMSO:	dimethyl sulphoxide/dimetil sulfóxido
DTT:	dithiothreitol/ditiotreitol
dNTPs:	deoxy nucleotide triphosphate/deoxinucleótido trifosfato
EDTA:	ethylendiaminetetraacetic/ácido etilendiamintetraacético
FOA:	5-fluoroorotic acid/ácido 5-fluoroorótico
g:	gram/gramo
x g:	gravity units/gravedades
HEPES:	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HRP:	horseradish peroxidase/ peroxidasa de rábano picante
Kb:	kilobase
kDa:	kilodalton
MIC/CMI:	minimum inhibitory concentration/ concentración miníma inhibitoria
min:	minute/minuto
ml:	millilitre/ mililitro
mM:	milimolar
MCS:	multicloning site/ sitio múltiple de clonaje
OD/DO:	optical density/densidad óptica
ORF:	open reading frame/marco abierto de lectura
PAb:	polyclonal antibody/anticuerpo policlonal
PBS:	phosphate buffer saline/ tampón fosfato salino
PBST:	PBS plus Tween-20/ PBS con Tween-20
PEG:	polyethilene glycol/polietilenglicol
pfu:	plates forming units/unidades formadoras de calvas
PMSF:	phenyl methyl sulfonyl fluoride/fluoruro de fenilsulfonilmetilo
RNAm:	messenger RNA/ARN mensajero
rpm:	revolutions per minute/revoluciones por minuto
SDS:	sodium dodecyl sulfate/dodecilsulfato sódico
Tris:	Tris(hydroxymethyl)aminomethane/Tris(hidroximetil)aminometano
TBS:	Tris buffer saline/ tampón tris salino
TTBS:	TBS plus Tween-20/ TBS con Tween-20
UV:	ultraviolet/ultravioleta
V:	volts/voltios
v/v:	volume/volume/ volumen/volumen
w/v:	weight/volume/ peso/volumen

ÍNDICE

I. INTRODUCTION

I.1. General biology of *Candida albicans*.

Candida albicans is a pleomorphic diploid fungus, which presents the ability to grow under a variety of morphological forms that include unicellular budding yeast, also known as blastospore, true hypha, and pseudohyphal forms (**Fig. I.1**). *C. albicans* also can form chlamydospores, that is to say, asexual spores that develop on pseudohyphal support cells and appear under unfavourable environmental conditions (**Sudbery *et al.*, 2004; Kurtz *et al.*, 1990**).

Fungi are classified on the basis of their ability to reproduce sexually, asexually, or by a combination of both. Thus, *C. albicans* has traditionally been classified in the *Deuteromycota* (fungi imperfecti) since the sexual phase of this fungus is unknown, although recent reports described the presence of mating-type-like orthologs (*MTL*) in *C. albicans* of both of the *Saccharomyces cerevisiae* mating-type genes (*MAT*), a and α (**Hull and Johnson, 1999; Magee and Magee, 2000**). Therefore, *C. albicans* has been now classified as follows:

Phylum	*Ascomycota*
Subphylum	*Ascomycotina*
Class	*Ascomycetes*
Order	*Saccharomycetales*
Family	*Saccharomycetaceae*
Genus	*Candida*

This fungus is both a commensal and opportunistic pathogen of humans, that is carried by a large proportion of the population on the mucosal surfaces of the gastrointestinal and urogenital tract without clinical symptoms, but by contrast to other numerous commensals, *C. albicans* has the ability to colonize and invade host tissues when the host immune systems are weak or when the competing flora is eliminated (**Calderone, 2002; Berman and Sudbery, 2002**). In this context *C. albicans* is the major fungal pathogen in humans (**Calderone, 2002**), and represents the fourth most common agent of microbial lethal septicaemia in immunocompromised patients (**Edmon *et al.*, 1999**).

Principal features of the cellular biology of *C. albicans* as an eukaryotic diploid cell include the arrangement of the genetic material, with a size of about 32 Mb (**Chu *et al.*, 1993**), organized in eight pairs of homologous chromosomes (**Magee, 1993**), the presence of a cell wall, composed basically by mannoproteins, β-glucans and chitin (**Chaffin *et al.*, 1998**; **Martínez *et al.*, 1998; Lopez-Ribot *et al.*, 2004**), and located outside the plasma membrane, whose structure is quite similar to mammalian plasma membranes, that differs in having the nonpolar sterol ergosterol, rather than cholesterol (**Mishra *et al.*, 1992**). Several antifungal approaches are precisely based on this difference, by using agents that interfere with the synthesis of ergosterol, such as the azoles, or that binds to ergosterol thereby perturbing the membrane function to the point of causing leakage of cellular content, such as amphotericin B, an agent that belongs to the family of polyenes.

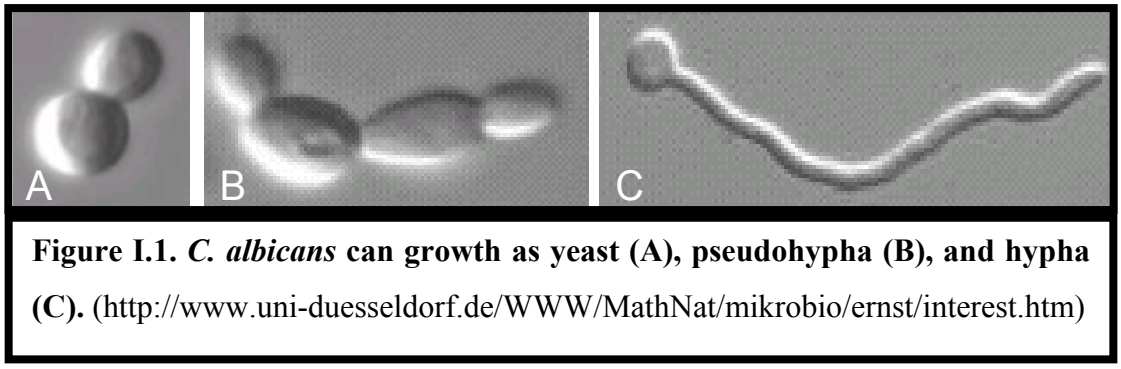

Figure I.1. *C. albicans* can growth as yeast (A), pseudohypha (B), and hypha (C). (http://www.uni-duesseldorf.de/WWW/MathNat/mikrobio/ernst/interest.htm)

The cell wall has also been the focus of study of numerous research groups, since most of the biological functions related to the biology, pathogenicity and virulence in *C. albicans*, reside in this structure (**Calderone, 1993; Calderone and Braun, 1991; Chaffin *et al.*, 1998; Hostetter, 1994; López-Ribot *et al.*, 2004; Martínez *et al.*, 1998; Naglik *et al.*, 2003**).

The whole *C. albicans* genome has been recently sequenced and a partial annotation of this genome is available at the Stanford Genome Technology Center and at the European Candida Database web sites (http://Candida.stanford.edu/btComb.html; http://genolist.pasteur.fr/CandidaDB/). This information has made much easier the identification of novel genes. In this context, *C. albicans* genes that are essential for cell growth and have no homologues in humans, are considered especially promising to discover new potential anti-fungal targets, because they are less likely to cause the

negative side effects that are associated with most anti-fungal therapies. Despite the new molecular tools that can be used to have a deeper knowledge of this pathogenic fungus, the lack of a sexual cycle and the unconventional *C. albicans* codon usage – *C. albicans* translates the CUG codon as serine, rather than the "universal" leucine (**Santos *et al.*, 1993**) – makes difficult genetic manipulations and the use of heterologous genes as an expression reporters.

I.2. *C. albicans* as a pathogenic agent.

Candidiasis is the most common opportunistic fungal infection, and it can be caused by different *Candida* species. Although *C. albicans* is the major responsible for candidiasis, during the last years the frequency with which non-*C. albicans Candida* species (NCAC) have been recovered from sites of infection, has increased significantly (**Pfaller *et al.*, 1999**), and species such as *C. glabrata*, the second species after *C. albicans* isolated from oral candidasis (**Luo and Samaranayake, 2002**), *C. dubliniensis*, the second most common isolated NCAC species in HIV-infected individuals (**Schoofs *et al.*, 1998**), and other species, as *C. tropicalis*, *C. parapsilosis* and *C. krusei* are also responsible of a number of candidiasis cases whose appearance frequency is continuously increasing.

As an opportunistic pathogen *C. albicans* may establish an infection when the immunologic and constitutive host defenses are compromised. Predisposing factors for candidiasis include AIDS, low birth weight, diabetes, and other immunosupressive conditions, such as neutropenics.

I.2.1 Types of candidiasis.

Candidiasis can range from superficial disorders to invasive infections, in general in immunocompromised hosts, and usually are classified into two big groups as follows:

- **Superficial candidiasis,** which may involve the epidermal and mucosal surfaces, including those of the oral cavity, pharynx, oesophagus, intestines, urinary bladder, and vagina. Main manifestations of superficial candidiasis include thrush, a superficial infection of one or more epithelial surfaces (**Schwebke and Hillier, 1996**), oropharyngeal candidiasis (OPC), which occurs in approximately 70% of patients with AIDS, vulvovaginitis, being estimated that 70 % of all women (with or without AIDS) will experience at least one episode of vaginitis caused by *Candida* spp and 20% will experience recurrent disease (**Fidel *et al.*, 1999**), and *Candida* intertrigo. Recurrent *Candida* infections of mucous membranes, skin and nails can lead to chronic mucocutaneous candidiasis (**Ruhnke, 2002**).

- **Systemic candidiasis,** which may affect the kidneys, liver, spleen, brain, eyes, heart, and other tissues. The principal risk factors predisposing to deeply invasive candidiasis are protracted courses of broad spectrum antibiotics, cytotoxic chemotherapy, corticosteroids, VIH and vascular catheters (**Kao *et al.*, 1999**). Systemic candidiasis are the fourth-leading cause of nosocomial infections in the USA and elsewhere in the world, and in those patients with candidemia-isolation of *Candida* spp. from at least one blood culture specimen the attributable mortality rate is 35% (**Wenzel, 1995**).

I.2.2. Treatment of candidiasis.

The massive emergence of fungal diseases associated with AIDS in the 1980s and the rising frequency of fatal mycoses associated with increasing use of immunosuppressive medical therapies since the 1970s stimulated research directed towards the discovery of novel antifungal agents. Because *C. albicans* is an eukaryotic organism and therefore share many of its biological processes with human cells, most antifungal drugs cause deleterious side effects and, at the doses used, are fungistatic rather than fungicidal. For that reason, an important goal in antifungal drug discovery is the identification of new suitable fungal targets with no mammalian homologies. All the

antifungal agents currently available are classified, according with their specific mode of action as follows:

- **Griseofulvin**. It is the earliest inhibitory agent specific of fungal species. The precise mechanism of action is still unknown; however it is supposed to interfere with microtubule assembly (**Develoux, 2001**).
- **Flucytosine**. It works through conversion to 5-fluorouracil within target cells. Fluorouracil becomes incorporated into RNA, causing premature chain termination, and it inhibits DNA synthesis through effects on thymidylate synthase. This antifungal agent is only suitable for those fungal species that possess the target enzymes of this compound, which are cytosine permease, cytosine deaminase, and uracil phosphoribosyl transferase, as in the case of *Candida* species and *Criptococcus neoformans* (**Odds *et al.*, 2003**).
- **Amphotericin B**. This antifungal agent acts by binding to ergosterol, the principal sterol in fungal membranes, thereby perturbing membrane function to the point of causing leakage of cellular contents. Despite of the conformational difference between cholesterol and ergosterol, and the major binding affinity of amphotericin B for this last one, this antifungal agent has potential toxicity for mammalian cells, so a variety of reformulated versions of the agent have been introduced in the last years, to overcome amphotericin B nephrotoxicity by slowing the rate at which the compound is delivered to the kidneys (**Dupont, 2002**).
- **Azoles**. Imidazoles and triazoles are the largest class of antifungal agents of clinical use. Their target is the cytocrome P450, which catalyses 14α-demethylation of lanosterol in the sterol synthesis pathway at the endoplasmic reticulum. Thus, azoles cause ergosterol depletion at the fungal plasma membrane with the subsequent alteration of its permeability and fluidity (**Kelly *et al.*, 1997**).
- **Other sterol synthesis inhibitors**. There are two other classes of antifungal agents that acts at this same pathway, allylamines, which act by inhibition of squalene epoxidase, with fungicidal consequences in many filamentous fungi

but not so much effective in pathogenic yeasts, and morpholines, affecting two different enzymes in the ergosterol pathway, Erg24p and Erg2p.

- **Equinocandins**. Their target is the complex of proteins responsible for synthesis of cell wall β-1,3 glucan polysaccharides, and the component to which equinocandins bind is Fsk1p, although the complete mechanism of the action still remains unknown (**Van den Bossche, 2002**). Caspofungin was the first approved agent from this new class of antifungal agents (**Kartsonis *et al.*, 2003**) and has been recently licensed for the treatment of aspergillosis and candidal infections (**Espinel-Ingroff, 2003**). Two new equinocandins, anidulafungin and micafungin, are currently undergoing phase III clinical trials (**Espinel-Ingroff, 2003**).
- **Sordarins.** These antifungal agents inhibit protein synthesis by blocking the function of fungal translation elongation factor 2 (*EFT2*). Sordarins were discovered time ago, and the specific target of these agents was described in 1998, although they are not developed for clinical use yet (**Odds, 2001**).

I.3. Virulence factors.

In primary pathogens, such as enteric bacteria, the proteins encoded by genes on the pathogenicity islands are defined as virulence factors. In *C. albicans*, an opportunistic pathogen, virulence factors can be defined in different ways, as "all traits required to establish disease", or "these factors that interact directly with mammalian host cells", and also as "the components of a pathogen that damages the host" (**Furman and Ahearn, 1983; Odds *et al.*, 2001; Casadevall and Pirofski, 2001**).

In order to establish an infection, opportunistic pathogens have to evade the immune system, survive and divide in the host environment and spread to new tissues. The factors that *C. albicans* expresses contributing to its pathogenicity include host recognition biomolecules (adhesins), the ability to switch between yeast, hyphal and pseudohyphal morphologies, secreted aspartyl proteases and phospholipases, the phenotypic switching, and biofilm formation (**Calderone and Fonzi, 2001**).

I.3.1. Adhesins.

Adherence of *C. albicans* to host cells is seen as an essential early step in the establishment of disease. Those biomolecules that promote the adherence of *C. albicans* to host cells or host-cell ligands, called adhesins, are next described:

- **Alsp1 and Als5p.** Agglutinin-like sequence (ALS) family is consisted of several glycosylated proteins with three common features, a conserved 5' domain, a central domain containing 108 pbs tandem repeats, and a serine-threonine-rich 3' domain, with homology to the *S. cerevisiae* α-agglutinin protein that is required for cell-cell recognition during mating (**Hoyer, 2001**). Alsp1 and Alsp5 have an adhesion function to human bucal epithelial cells (HBEC) and fibronectin, and Als1p is also important for the adherence of *C. albicans* to the oral mucosa during the early stage of the infection (**Kamai *et al.*, 2002**).

- **Hwp1p.** This protein encodes an outer surface mannoprotein that is believed to be oriented with its amino-terminal domain surface-exposed and the carboxyl terminus most probably covalently integrated with cell wall β-glucan. The amino-terminal sequence of Hwp1p was found to resemble mammalian transglutaminase substrates suggesting that Hwp1p is involved in the formation of stable complexes between germ tubes (initial projections observed when *Candida* switches from yeast form to hyphal growth) and HBEC (**Staab *et al.*, 1999**).

- **Int1p.** This protein is similar to vertebrate leukocyte adhesins, which bind extracellular matrix proteins and induce morphologic changes in response to extracellular signals. Knockout strains of *C. albicans* in *INT1* are less virulent, and adhere 40% less to an epithelial cell line (**Calderone, 1998**).

- **Mnt1p, Pmt1p and Pmt6p.** These three proteins are involved in mannan synthesis, and mannan is a major constituent of the fungal cell wall. Deletion of one of the genes encoding for these proteins results in less adhesion and virulence in the

mutant strains, thus suggesting a possible role of Mnt1p, Pmt1p and Pmt6p in host recognition (**Calderone and Fonzi, 2001**).

I.3.2. Proteinases secretion.

The secreted aspartyl proteinases (SAP) and phospholipases (PL) are two rather large families of *C. albicans* enzymes, some of which have been associated with virulence (**Calderone and Fonzi, 2001**). SAPs degrade many human proteins at lesion sites, such as albumin, haemoglobin, keratin, and secretory immunoglobulin A (**Hube *et al.*, 1998**). Nine different SAP genes (*SAP1-9*) have been identified in *C. albicans*, and their proteolytic activity has been associated with tissue invasion (**Ruchel *et al.*, 1991**).

Phospholipases hydrolize one or more ester linkages of glycerophospholipids. According to the different and specific ester bond cleaved, these enzymes have been classified into phospholipases A, B, C, and D (**Mukherjee and Ghannoum, 2002**). PL are supposed to be a virulence factor since cells producing less PL are less virulent than strains producing high PL levels in a murine model, and also because *C. albicans* strains isolated from blood produced higher level of PL than commensal strains.

I.3.3. Dimorphism.

Dimorphism refers to the transition between unicellular yeast cells and a filamentous growth form. *C. albicans* is able to switch reversibly from yeast cells to either pseudohyphal or hyphal growth, and this property has been often considered to be a component of the repertoire of factors influencing virulence in this fungus (**Calderone and Fonzi, 2001; Gow, 2002; Lo *et al.*, 1997; Odds *et al.*, 2001**). Hyphal forms are invasive, and this property could promote tissue penetration during the early stages of infection whereas the yeast form might be more suited for dissemination in the bloodstream **(Sudbery *et al.*, 2004**).

Besides of virulence implication, morphogenic processes constitute themselves a complex differentiation process, which require regulation of the cell cycle to enable cell shape to be modulated while the nuclear cycle is maintained. Thus, this issue will be subject to an extensive review in **section I.4 of this chapter**.

I.3.4. Phenotypic switching.

The basic mechanism of phenotypic switching is not clear, although is known that *C. albicans* can switch among different phenotypes (smooth, rough, star, stippled, hat, irregular wrinkle, and fuzzy) at high frequency (**Slutsky *et al.*, 1985; Soll, 1992a,b; 1997; 2001**). Of all the switch phenotypes described, the most studied is the white-opaque system in strain WO-1, in which smooth, white colonies, switch to flat, grey colonies (opaque). The role of phenotypic switching in the virulence of this organism remains to be elucidated, although it has been observed that opaque cells colonize the skin in a cutaneous model more than white-phase cells, but are less virulent in a systemic animal model (**Kvaal *et al.*, 1999; Soll, 2001**).

I.3.5. Biofilm formation.

Microorganisms form biofilms on a variety of implanted medical devices, resulting in biofilm-associated infections that constitute a significant public health problem. Although bacterial biofilms have been characterized in great detail (**O'Toole *et al.*, 2000**), the study of medically relevant fungal biofilms has only recently come the forefront. *Candida* species, particularly *C. albicans*, readily form biofilms, consortia of cells that coexist as an organized community with a complex three-dimensional architecture that is enveloped within an exopolysaccharide matrix, attached to a solid sustratum (**Chandra *et al.*, 2001**; **Douglas, 2003**). *Candida* biofilms play a growing role in human medicine, since the majority of manifestations of candidiasis at both mucosal and systemic sites are associated in one way or another with the formation of biofilms on inert or biological surfaces (**Cannon and Chaffin, 1999**; **Crump and Collignon, 2000**). In addition, biofilms have gained notoriety from their ability to resist the most commonly used antifungal agents (**Bachman *et al.*, 2003**; **Baillie and Douglas, 1999**; **Chandra *et al.*, 2001**; **Kuhn *et al.*, 2002**; **Mukherjee *et al.*, 2003**; **Ramage *et al.*, 2001a/b, 2002**). The capacity of *C. albicans* to switch from a yeast morphology to a hyphal morphology, which is one of the major virulence determinants of this fungal species (**Lo *et al.*, 1997**; **Saville *et al.*, 2003**), is a key factor for *C. albicans* biofilm development (**Baillie and Douglas, 1999**).

I.4. Morphogenesis.

Morphogenesis has been a focus of research in *C. albicans* because virulence is associated with the ability to switch between the yeast and hyphal morphologies (**see section I.3.3 of this chapter**). *C. albicans* is able to develop single spherical cells including yeast cells and chlamydospores, as well as elongated cells developing into multicellular true hyphae or pseudohyphae. Thus, the term dimorphism, which traditionally is reserved for the yeast-true hypha interconversion, in a more general sense designates the main theme of *C. albicans* and possibly fungal morphogenesis in general (**Ernst, 2000**). Budding-yeast cells can be induced to form true hypha, which grow by continuous apical extension followed by septation. Pseudohyphae grow differently from true hyphae, by unipolar budding: buds develop into elongated cells, which remain attached to mother cells. Chlamydospores are thick-walled spherical cells and develop on pseudohyphal support cells (**Joshi *et al.*, 1993; Montazeri and Hedrick, 1984**). Finally, in some forms of spontaneous phenotypic switching (**Soll, 1992a,b; 1997; 2001**), enlarged elongated cells arise, which have a different appearance to yeast and pseudohyphal cells, and which do not form extensive filaments (opaque phenotype) (**Fig. I.2**).

Traditionally, the pseudohyphal state has been considered interchangeable with hyphae forms (**Odds, 1988; Gow, 1994; Gow, 1997; Berman and Sudbery, 2002**), since both are elongated. Moreover, pseudohyphae have been considered to represent an intermediate morphological state between yeast and true hyphal growth forms (**Odds, 1985**). Recently, evidence has accumulated to show that pseudohyphae exhibit similarities to yeast, thus suggesting that the differences found between hyphae and pseudohyphae are enough to consider that they are distinct morphological states (**Sudbery *et al.*, 2004**). The most fundamental differences between hyphae and pseudohyphae are related to the organization of their cell cycle, although there are several features that can be useful to distinguish between them (**Table I.1**).

Several environmental factors can induce yeast cells to form hyphae and pseudohyphae through several signal-transduction pathways. This probably reflects the variety of microenvironments in which *C. albicans* must survive in vivo (**Berman and Sudbery, 2002**). In the **Table I.2**, the effect of environmental conditions and /or culture conditions that induce growth of each *C. albicans* morphological forms is shown.

The signalling pathways that transduce environmental signals into morphological switching have been extensively studied. At least two of these pathways also act in *S. cerevisiae* to initiate pseudohyphal growth. First, the signalling pathway based on cAMP and protein Kinase A (PKA) targets the CaEfg1p transcription factor. Second, a mitogen-activated protein (MAP) kinase-based module, which in *S. cerevisiae* is also used as transducer for the mating pheromone response, targets the CaCph1 transcription factor. In addition, two other positive pathways that control morphological transitions have been described in *C. albicans*: a pH response, which is mediated by a pathway that activates the Rim101p transcription factor, and a response to embedding in a solid matrix, which is mediated by a pathway that activates the Czf1p transcription factor.

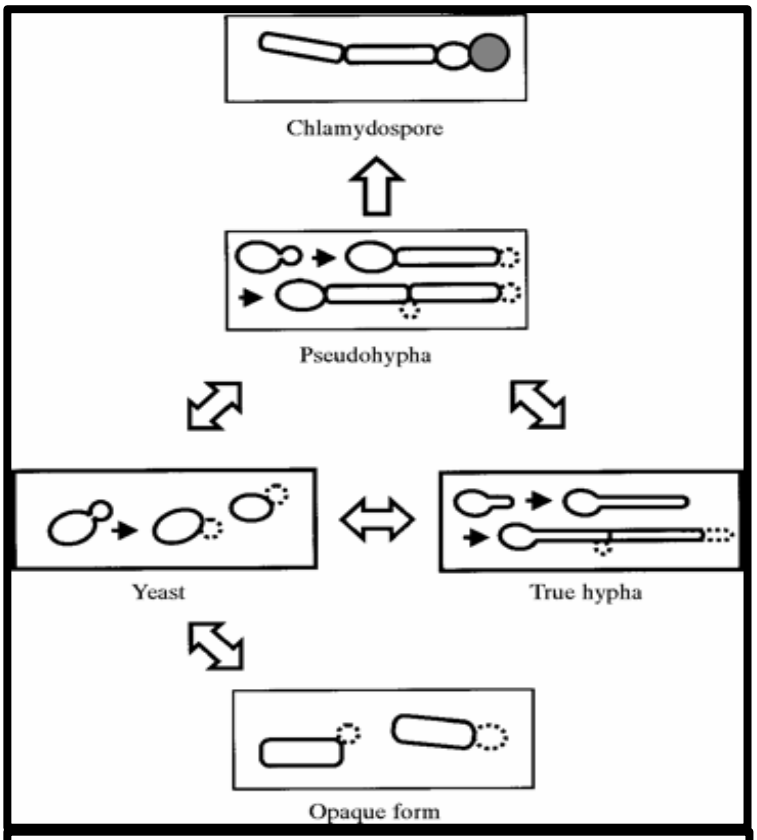

Figure I.2. Morphological forms of *C. albicans* (Ernst, 2000).

Lastly, two negative pathways act through the transcriptional regulators Tup1p and Rpg1p respectively (**Berman and Sudbery, 2002**). Thus, hyphal and pseudohyphal development depends on several factors, the nature, number and intensity of environmental signals, and the activity of signalling pathways including key transcription factors (cellular-response machinery). Many signalling pathways or regulators have been found to regulate filamentation in one or many of the *in vitro* hypha-inducing conditions (**Liu, 2001**).

Table I.1. Characteristics that distinguish *C. albicans* pseudohyphal and hyphal cells (Sudbery *et al.*, 2004).

Criterion	Pseudohypha	Hypha
Shape	Sides not parallel Minimum width ≥ 2.8 μm Constrictions at mother-bud neck and subsequent septal junctions	Sides parallel Width ~ 2 μm No constrictions at septal junctions
Septa	First septum at mother-bud neck	First septum within germ tube
Septins	Septin ring at mother-bud neck	Septin ring within germ tube
Nuclear division	Across mother-bud neck	Totally within germ tube
Mature filaments	Highly branched with cell cycle synchrony	Less branched Apical cells continue growth and division Subapical cells are delayed in G1 Large vacuoles in subcellular compartments

Table I.2. Environmental and/or culture conditions that induce growing of each *C. albicans* morphological form (Sudbery *et al.*, 2004).

Yeast	Pseudohyphae	Hyphae	Other filament-inducing conditions
Growth below 30ºC	pH 6.0, 35ºC	Serum, > 34ºC	Spider medium
pH 4.0	High phosphate	pH 7.0, 37ºC	Mouse kidneys
Cell density > 10^6 cells/ml	Nitrogen-limited growth on solid medium (SLAD)	Lee' s medium, 37ºC N-acetylglucosamine	Engulfment by macrophages Growth in agar matrix Iron deprivation

I.4.1. The mitogen-activated protein kinase pathway.

A transcription factor, Cph1p, which is homologous to the Ste12p transcription factor that regulates mating and pseudohyphal growth in *S. cerevisiae*, has been identified in *C. albicans* (**Liu *et al.*, 1994; Lorenz *et al.*, 2000**). In *S. cerevisiae*, Ste12p is regulated by a conserved MAP kinase cascade. Many components of this pathway show a high degree of sequence conservation with components of morphogenetic MAP kinase (MAPK) signalling pathway in *C. albicans* (**Table I.3**). The cascade consists of the kinases Cst20p (homologous to the p21-activated kinase Ste20p), Hst7p (homologous to the MAP kinase kinase Ste7p), and Cek1p (homologous to the Fus3p and Kss1p MAP kinases) (**Clark *et al.*, 1995; Köhler and Fink, 1996; Leberer *et al.*, 1996; Whiteway *et al.*, 1992**). Null mutants in any of the genes in the MAPK cascade or in the transcription factor Cph1p confer a hyphal defect on solid medium in response to many inducing conditions, as solid Spider medium (**Liu, 2001**), although they have a normal hyphal development in response to serum, proline, or N-acetilglucosamine (**Ernst, 2000; Leberer *et al.*, 1996; Liu *et al.*, 1994**). MAPK mutants do not display identical virulence phenotypes in systemic infections, since *cst20/cst20* and *cek1/cek1* mutants showed reduced virulence, whereas *hst7/hst7* and *cph1/cph1* mutants did not (**Csank *et al.*, 1998; Leberer *et al.*, 1996; Lo *et al.*, 1997**).

Table I.3. Conserved MAPK pathway that promotes pseudohyphal morphogenesis in *S. cerevisiae* and hyphal morphogenesis in *C. albicans* (Brown, 2002).

MAPK pathway component	*S. cerevisiae*	*C. albicans*
G protein	Cdc42	Cdc42
Regulatory kinase	Ste20	Cst20
MAPKKK	Ste11	?
MAPKK	Ste7	Hst7
MAPK	Kss1	Cek1
Transcription factor	Tec1 Ste12	? Cph1

I.4.2. The cAMP-dependent protein kinase A pathway.

The cAMP-dependent protein kinase A (PKA) pathway plays an important role in filamentation in *S. cerevisiae*, *C. albicans* and other fungi (**Lengeler *et al.*, 2000**). Added cAMP has been reported to induce hyphae in *C. albicans* (**Niimi *et al.*, 1980; Sabie and Gadd, 1992; Niimi, 1996**), suggesting that a cAMP pathway controls morphogenesis as it controls pseudohyphal development in *S. cerevisiae* (**Gimeno *et al.*, 1992; Mosch *et al.*, 1996; Robertson and Fink, 1998; Pan and Heitman, 1999**). In *S. cerevisiae*, as in *C. albicans*, filamentation is reduced but no abolished in mutants with defects in the mating/filamentation MAPK pathway. Filamentation is partially restored by overexpression of *PHD1*, which specifies a transcriptional factor with homologs in several other fungi, including Efg1p in *C. albicans* (**Gimeno and Fink, 1994**). This results suggests that Phd1p and its homolog Efg1p may act either downstream of the mating/filamentation MAPK cascade or in a second filamentation pathway. *Cph1/Efg1* deletions abolished filamentous growth under all induction conditions tested (**Lo *et al.*, 1997**), thus suggesting that Efg1p lies downstream in a second different pathway, independent from MAPK pathway (**Rademacher *et al.*, 1998**).

Efg1 is a conserved basic helix-loop-helix-type protein, which is a strong regulator of morphogenetic processes in *C. albicans* since it influences not only yeast-hypha interconversions (**Stoldt *et al.*, 1997; Lo *et al.*, 1997**), but also regulates phenotypic switching and chlamydospore formation of this pathogen (**Sonneborn *et al.*, 1999a,b**). *Efg1* null mutants presented a complete block of hyphal formation under serum or N-acetilglucosamine hyphal induction conditions (**Stoldt *et al.*, 1997; Lo *et al.*, 1997**). On the other hand, hyphal induction under microaerophilic conditions is not defective at all in homozygous *efg1* mutants, but rather appears stimulated (**Sonneborn *et al.*, 1999b; Riggle *et al.*, 1999; Brown *et al.*, 1999**). In fact, depending on environmental conditions *efg1* is an activator and repressor of morphogenesis (**Stoldt *et al.*, 1997**).

Efg1p pathway includes a cAMP signalling module that is conserved in other fungi (**Ernst, 2000**). *C. albicans* homologues of Ras (*RAS1*), adenyl cyclase (*CYR1*), and Protein Kinase A (*TPK2*) have been identified by genome sequencing, strongly suggesting the existence of a Ras-cAMP pathway (**Brown and Gow, 1999**). Current models suggest that Ras1p can activate both the MAPK and cAMP pathways (**Lengeler *et al.*, 2000**).

I.4.3. pH response pathway.

C. albicans has a conserved pH-response pathway. The transcription factor Rim101p (*PRR2*) mediates pH response throught its proteolysis, which is regulated by Rim8p (*PRR1*) and Rim20p (**Porta *et al.*, 1999; Ramon *et al.*, 1999; Davis *et al.*, 2000**). Rim101p is required for alkaline-induced filamentation, the expression of alkaline-responsive genes such as *PHR1*, and the repression of the alkaline-repressed *PHR2*. Mutations on *RIM101* cause filamentation and the expression of alkaline-induced genes in acidic media (**El Barkani *et al.*, 2000**). Efg1p is required for the Rim101p-induced filamentation, but not for the expression of alkaline-induced genes.

I.4.4. Embedded/microaerophilic conditions.

Growth of wild-type colonies embedded in agar stimulates filamentation, and mostly true hyphae were produced. The putative transcription factor Czf1p is probably an important element of the alternative pathway of filamentation in *C. albicans* (**Brown *et al.*, 1999**). There is no direct homologue of Czf1p in the genome of *S. cerevisiae*. Homozygous *czf1* null mutants produce hyphal filaments normally under standard induction conditions, but they are defective in hyphal development when embedded in agar. This defective phenotype occurs only during embedding in certain media, such as complex medium containing sucrose or galactose as carbon sources at 25ºC, but not at 37ºC or in media containing strong inducers including serum and N-acetilglucosamine (**Ernst, 2000**). These characteristics suggest that factors other than Czf1p contribute to filamentation under embedded conditions. Interestingly, an *efg1/efg1* mutant is

hyperfilamentous when grown in matrix, indicating that Efg1p represses filamentation under these conditions (**Giusani *et al.*, 2002**).

I.4.5. Tup1p.

TUP1 encodes a transcriptional repressor that negatively controls filamentous growth in *C. albicans*. Like the well-characterized repressor complex Tup1p-Ssn6p of *S. cerevisiae*, CaTup1p is likely to be part of a transcriptional repressor complex that is brought to promoters by sequence-specific DNA-binding proteins. The *tup1/tup1* deletion in *C. albicans* results in a constitutive hyphal growth phenotype under all conditions tested (**Braun and Johnson, 1997)**. Several genes repressed by Tup1p (*RBT*s) have been identified in *C. albicans* (**Braun *et al.*, 2000**). Many of them are induced during the yeast-hypha transition, but some *RBT*s are not regulated during filamentation, indicating that the regulation of filamentation is not the sole function of Tup1p. This also suggests that Tup1p itself is not regulated during filamentous growth; rather, its associated DNA-binding proteins are likely to be regulated (**Braun *et al.*, 2000).** Transcript profiling of *RBT*s in single, double and triple mutans of *TUP1*, *EFG1* and *CPH1* suggests that each gene represents a separate pathway in the transcriptional regulation of filamentation (**Braun and Johnson, 2000).**

In addition, although the Tup1p protein has been shown to be epistatic to the transcription factor Cph1p, genetic evidence indicates that it does not lie downstream of either Cph1p or Efg1p, but rather in an independent pathway that regulates filamentation (**Braun *et al.*, 2000; Sharkley *et al.*, 1999).** Furthermore, a *cph1/cph1*, *efg1/efg1*, *tup1/tup1* triple mutant, retains the ability to filament in response to environmental signals, thus suggesting the existence of another filamentous growth pathway (**Braun and Johnson, 2000**).

Hyperfilamentous *tup1/tup1* mutant strain has a virulence defect, which is consistent with the finding that a *cph1/cph1* mutant strain also exhibits enhaced hyphal formation and impaired virulence (**Braun and Johnson, 2000**). These findings support a model in which both the yeast form and the filamentous form are required to maintain a stable infection.

In *S. cerevisiae*, the ScTup1p-ScSsn6p complex is targeted to different sets of promoters by different sequence-specific DNA binding proteins (**Smith and Johnson, 2000**). Two more repressors of hyphal development, CaNrg1p and CaRfg1p have been described (**Kadosh and Johnson, 2001; Murad *et al.*, 2001**). These two genes might target the CaTup1p repressor complex to distinct sets of *C. albicans* genes to repress their expression.

I.4.6. Rbf1p.

The protein Rbf1p (RPG-box-binding factor 1) has been reported to be a putative transcription factor in *C. albicans* (**Ishii *et al.*, 1997**). Rbf1p, which binds to a specific DNA sequence including the *C. albicans* chromosomal telomere, has two glutamine-rich regions characteristic of transcription factors, showed transcriptional activation capability in *S. cerevisiae* cells, and was predominantly localized in nuclei. The double disruption of this gene induced filamentous growth in all the conditions tested (**Aoki *et al.*, 1998**).

I.4.7. Hyphal-specific gene transcription.

Several genes have been identified whose expression is induced during hyphal induction. Such genes are listed next, and most of them encode either cell-wall proteins or secreted proteins:

- ***HWP1***: encodes a cell-wall protein that can serve as a target for mammalian transglutaminases to form covalent attachments between *C. albicans* and host epithelial cells (**Staab *et al.*, 1999**).

- ***ECE1***: its expression is detected after 30 minutes of hyphal induction, however null mutants for this gene display a normal hyphal development (**Birse *et al.*, 1993**).

- ***HYR1***: encodes a non-essential cell wall glycoprotein, and null mutants for this gene also display a normal hyphal development (**Bailey *et al.*, 1996**).

- ***ALS3*** and ***ALS8***: these two genes encode glycosylated proteins from the agglutinin-like sequence (ALS) family (**Hoyer *et al.*, 1998**).

- ***RBT1*** and ***RBT4***: these two genes repressed by *TUP1* encode a cell-wall protein and a secreted protein, respectively (**Braun *et al.*, 2000).**

I.5. The fungal vacuole.

The fungal vacuole is the counterpart of the mammalian lysosome, and plays an important role in pH- and ion-homeostasis, and works as storage compartment for ions and amino acids (**Jones *et al.*, 1997**). Furthermore, the vacuole contains a large number of unspecific hydrolases and numerous endo- and exoproteinases (**Jones *et al.*, 1997; Van den Hazel *et al.*, 1996)**. Accordingly, besides the cytosolic proteasome, the vacuole is the main site of intracellular proteolysis (**Thumm and Wolf, 1998**). In this context, the best characterized vacuolar hydrolases are listed in **Table I.4**. While functions of vacuoles are not essential for vegetative growth, they are of increased importance, and often essential, for survival during periods of stress such as starvation or growth at elevated temperatures (**Banta *et al.*, 1988; Takeshige *et al.*, 1992**). Moreover, vacuole may play a central role during differentiation processes. Thus, *S. cerevisiae* mutants defective in vacuolar hydrolase activity are unable to undergo the process of sporulation (**Zubenco and Jones, 1981**). Vacuolar functions and correlation with several aspects of cell cycle and differentiation processes of *C. albicans* such as the yeast-hypha transition are an important part of this project, so this topic will be subjected to an extensive analysis in the next sections of this chapter.

I.5.1. Vacuole acidification.

One of the most remarkable features of the vacuole is its acidification, which is carried out by the vacuolar proton pumping ATPase (V-ATPase). In yeast, the V-ATPase functions to acidify the vacuole by driving the translocation of protons into the lumen of the vacuole. The hydrolysis of cytosolic ATP by the V-ATPase is required for the movement of protons from the cytosol into the interior of the vacuole. The proton gradient generated across the yeast vacuolar membrane by the V-ATPase in yeast is utilized by other membrane-bound transporters to drive the accumulation of ions and small molecules, amino acids and metabolites into the vacuole (**Jones *et al.*, 1997**). The

V-ATPase can be functionally divided into two structurally distinct domains; the catalytic V_1 subcomplex and the proton-translocating V_0 subcomplex. Subunits comprising the catalytic V_1 subcomplex of peripherally associated proteins are localized on the cytosolic face of the vacuolar membrane. The V_1 subcomplex is assembled onto the V_o subcomplex, which is composed of both peripheral and integral membrane proteins, resulting in the formation of a functional enzyme complex (**Fig. I.4**) (**Graham *et al.*, 2000**). Fourteen genes encoding subunits of the enzyme complex have been described and designated VMA (for vacuolar membrane ATPase), and are listed in **Table I.5**, along with three additional factors that have been found to be required for V-ATPase assembly process, althought they are not part of the final V-ATPase.

The pH and electrical potential differences across the vacuolar membrane generated by the V-ATPase are required to drive amino acid and ion transport. The primary mechanism for transport of storage molecules into the vacuole appears to rely on a proton antiport system. In fact, H^+/amino acid antiport systems are present for arginine, arginine-lysine, histidine, phenylalanine-tryptophan, tyrosine, glutamine-asparagine, and isoleucine-leucine. An additional arginine-histidine exchange mechanism that utilizes the chemical potential of the histidine concentration was also detected (**Sato *et al.*, 1984a,b**). The vacuolar uptake of many ions is proposed to occur also by H^+ antiport, and this contributes to the cytosolic ion concentration regulation, which is essential for the cell, since some ions, such as Sr^{2+}, Co^{2+} and Pb^{2+}, are potentially toxic and must be removed from the cytosol. Other physiologically useful ions including Ca^{2+}, Mg^{2+}, and Zn^{2+} may become harmful at excess concentrations, and also precise control of ion concentrations must be maintained if the ions are to be useful in the regulatory processes (**Cornelius and Nakashima, 1987; Raguzzi *et al.*, 1988; White and Gadd, 1986**). In this context the vacuole plays an important role in calcium homeostasis. Two genes, *PMC1* and *VCX1/HUM1*, involved in vacuolar calcium transport have been identified. Pmc1p has homology to calcium-ATPases and is needed for growth on media containing high concentrations of calcium. The vacuolar Ca^{2+}/ H^+ exchanger Vcx1p/Hum1p is postulated to act as a high capacity, low affinity calcium-transporter (**Cunningham and Fink, 1994, 1996; Miseta *et al.*, 1999; Pozos *et al.*, 1996**). Cells lacking a functional V-ATPase are viable, but show an increased sensitivity to calcium ions (**Thumm, 2000**).

The vacuole also stores phosphate both as phosphate and polyphosphate. Polyphosphate is proposed to function as calcium counterions. Since hydrolysis of polyphosphate leads to the release of protons, a role of polyphosphate in pH regulation has also been suggested (**Castro *et al.*, 1995, 1999**; **Dunn, 1994**).

Table I.4. Vacuolar hydrolases (Klionsky *et al.*, 1990)

Name of the protein	Abbreviation	Location
Carboxypeptidase Y	CPY	Soluble
Proteinase A	PrA	Soluble
Proteinase B	PrB	Soluble
Aminopeptidase I	API	Soluble
Trehalase		Soluble
α-Mannosidase		Membrane-associated
Dipeptidyl aminopeptidase B	DPAP B	Membrane-associated
Alkaline phosphatase	ALP	Membrane-associated

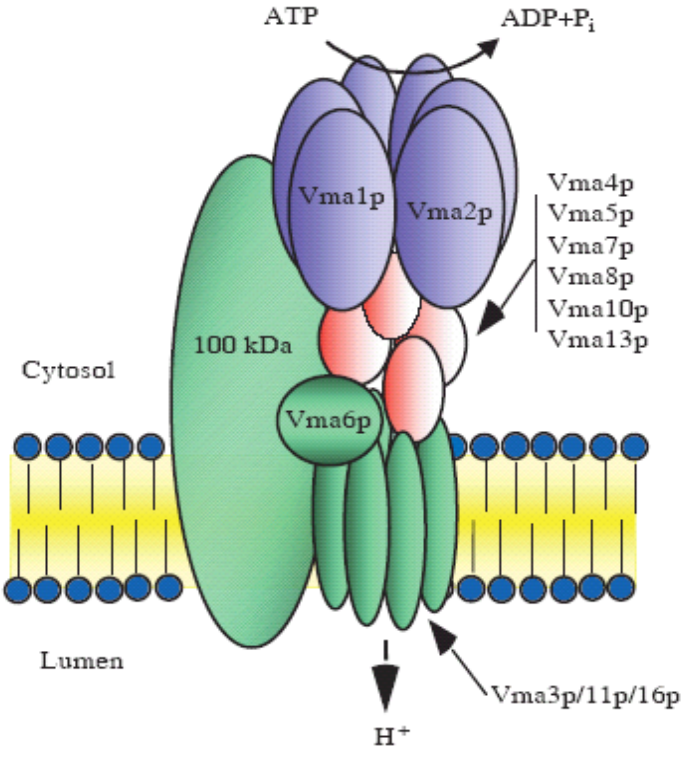

Figure I.4. Proposed structure of the yeast V-ATPase (Graham *et al.*, 2000).

Table I.5. Yeast genes required for V-ATPase subunit structure and assembly (Graham *et al.*, 2000)

Gene	Subunit	Molecular mass (kDa)	Integral or peripheral	Proposed function
V_1				
VMA1	A	69	peripheral	ATP-binding catalytic subunit
VMA2	B	60	peripheral	ATP-binding subunit
VMA4	E	27	peripheral	Interacts with Vma10p
VMA5	C	42	peripheral	Holds together the catalytic and membrane sectors of the V-ATPase
VMA7	F	14	peripheral	Interacts with Vma8p
VMA8	D	32	peripheral	Interacts with Vma7p
VMA10	G	16	peripheral	Required to stabilize Vma4p
VMA13	H	54	peripheral	Activation of the V-ATPase
Vo				
VPH1	a	100	integral	Vacuole-specific Vo subunit
VMA3	c	16.5	integral	Proton-translocating proteolipid
VMA6	d	36	peripheral	Hydrophilic Vo subunit
VMA11	c’	17	integral	Proteolipid similar to Vma3p
VMA16	c’’	23	integral	Proteolipid similar to Vma3p
STV1 (isoform)		100	integral	Similar to Vph1p, non-vacuolar localization
Assembly factors				
VMA12		25	integral	Required for assembly
VMA21		8.5	integral	Hydrophobic protein, required for assembly
VMA22		21	peripheral	Hydrophilic protein, required for assembly

I.5.2. Protein transport pathways to the yeast vacuole.

In yeast cells several different transport pathways converge upon the vacuole (**Fig. I.5**). Newly synthesized proteins, such as vacuole resident proteases, are transported through the secretory pathway to the endoplasmic reticulum (ER) lumen or membrane, pass from the ER through the Golgi apparatus and in the late Golgi are diverted from the secretory pathway (Vps pathway). Plasma membrane and cell surface proteins targeted for degradation are delivered to the vacuole by endocytosis. This two pathways overlap at the stage of the multivesicular body (MVB), also known as the prevacuolar compartment or late endosome. Subsets of proteins that transit to the vacuole via the secretory pathway, including alkaline phosphatase (ALP), use a partially alternate route, referred as the ALP pathway, which bypasses the endosome or prevacuolar compartment. Hydrolase aminopeptidase I (API) is supplied to the vacuole from the cytoplasm by cytoplasm-to-vacuole targeting (Cvt) overlapping with autophagy, which nonselective delivers cytosolic proteins and organelles to the vacuole for degradation and recycling. About 50% of vacuolar material is transferred from mother to the daughter cell in the process of vacuolar inheritance. Thus, the main classes of genes required for vacuole function and protein sorting in yeast include *VAM* (*v*acuolar *m*orphology), *VMA* (*v*acuolar *m*embrane *A*TPase), *VPS* (*v*acuolar *p*rotein *s*orting), *VAC* (*vac*uole inheritance), *APG* (*a*utophagocytosis), *END* (*e*ndocytosis), and *VID* (*v*acuolar *i*mport and *d*egradation). This last one has been described few years ago, when fructose-1,6-biphosphatase (FBPase) has been showed to be delivered to the vacuole for degradation in a novel type of vesicles called *v*acuole *i*mport and *d*egradation vesicles (*VID* pathway) (**Hoffman and Chiang, 1996**).

I.5.2.1. Vacuolar protein sorting (Vps) pathway.

Most soluble and integral membrane proteins of the yeast vacuole studied to date enter the endoplasmic reticulum and traverse the secretory pathway through the Golgi complex. The most thoroughly studied vacuolar protein is carboxypeptidase Y (CPY), which travel trough the early stages of the secretory pathway. CPY is synthesized as an inactive precursor, preproCPY, which is translocated into the lumen of the ER, where

its signal sequence is removed by a signal peptidase and it undergoes N-linked glycosilation to become the 67-kDa form of the protein (p1). By means of vesicular trafficking, in a *SEC*-dependent manner, CPY is transported to and subsequently through the Golgi apparatus, where it receives further oligosaccharide modifications to produce the 69-kDa Golgi modified form of the zymogen (p2). In the last definable subcompartment of the Golgi apparatus p2 CPY is diverted away from the secretory pathway through a receptor-mediated process that leads to its delivery to the vacuole, where is cleaved by vacuolar proteases into its active, mature, 61-kDa form (mCPY) (**Bryant and Stevens, 1998**). The vacuolar protein sorting mutants (*vps*) are arranged in six groups, attending to the vacuolar morphology and other criteria, which are showed in **Table I.6**. A good correlation between morphological phenotype and extend of the CPY sorting defect was observed (**Raymond *et al.*, 1992**).

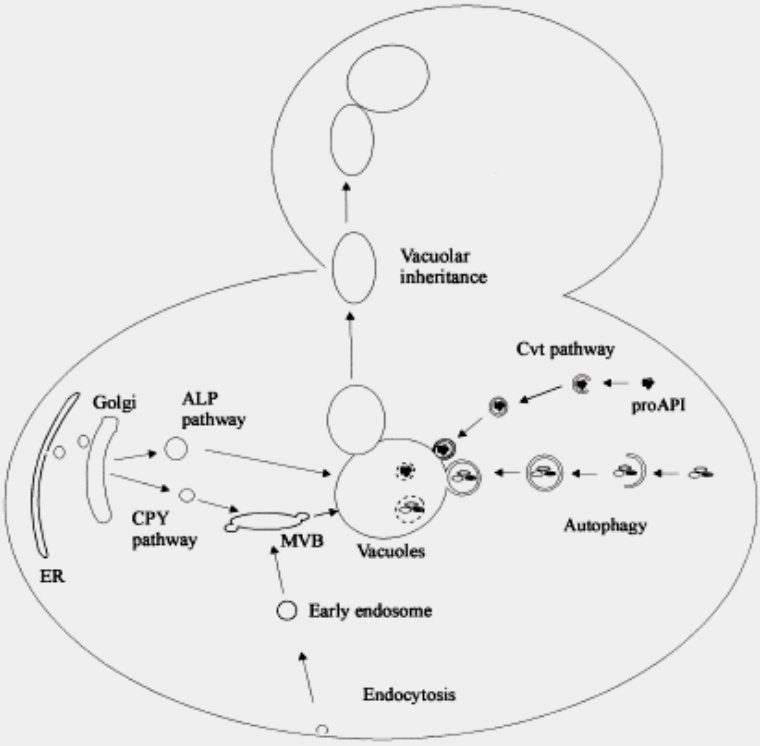

Figure I.5. Multiple routes of transport to the yeast vacuole (Kucharczyk and Ryka, 2001)

Table I.6. Morphological classification of *vps* mutants (adapted from Raymond *et al.*, 1992)

Class	Phenotypes of mutants
A	Wild-type or slightly perturbed vacuoles
B	Fragmented vacuoles, mislocalization of vacuolar proteins
C	Lack of organised vacuole, dispersed intracellular localisation of vacuolar membrane proteins, defective CPY sorting.
D	Defects in vacuolar inheritance and acidification, ALP acidified prevacuolar compartment, ALP localised to vacuole.
E	Vacuoles similar to wild-type, exaggerated and acidified prevacuolar compartment, ALP localised to vacuole
F	Large central vacuoles surrounded by small vacuole-like compartments, severe CPY sorting defects.

I.5.2.2. The ALP pathway.

Unlike most other vacuolar proteins that travel through the secretory pathway, the type II integral vacuolar membrane, protein-repressible alkaline phosphatase (ALP) (encoded by *PHO8*) is consistently delivered to the vacuole in cells that are blocked in the Vps pathway through a route that does not involve its transport to the cell surface and subsequent endocytosis (**Cowles *et al.*, 1997; Piper *et al.*, 1997**). ALP is localized to the vacuole in *vps1* (**Nothwehr *et al.*, 1995**), *vps15* (**Herman *et al.*, 1991**; **Raymond *et al.*, 1992**), *chc1* (**Seeger and Payne, 1992**), *vps*8 (**Horazdovsky *et al.*, 1996**), and *vps45* (**Raymond *et al.*, 1992**) mutant cells, suggesting that ALP may use a vacuolar targeting machinery that is distinct from that required by CPY and other vacuolar proteases.

I.5.2.3. Cytoplasm to vacuole targeting (Cvt) pathway.

Aminopeptidase I was initially characterized as a vacuolar protein that transits through the secretory pathway. Subsequent analyses, however, demonstrate that precursor API (prApi) is not glycosilated, does not reside within compartments of the secretory pathway, and its relatively unaffected by mutants that block transit from the endoplasmic reticulum or Golgi complex. These data indicate that this resident hydrolase uses an alternate targeting mechanism (**Klionsky *et al.*, 1992; Klionsky and**

Ohsumi, 1999), the Cvt pathway. The signal used by API that allows it to be directed to the vacuole and sorted away from other cytoplasmic proteins has not been characterized yet (**Harding *et al.* 1995).** To identify components of the machinery involved in the Cvt pathway, **Harding *et al.*** (1995) used a genetic screen relying on the accumulation of precursor API to identify mutants defective in the cytoplasm-to-vacuole targeting of API. These mutants, designated as *cvt*, fall into eight complementation groups, six of which define novel genes (*cvt1*, *2*, *3*, *5*, *6*, and *7*). These six groups exhibit a specific defect in maturation of API, but do not have a significant effect on vacuolar protein targeting through the secretory pathway. The *cvt* screen also identified *cvt4* and *cvt8* mutants, which were after identified as *vps39* and *vps41*, respectively, since both of them presented defects in the maturation of multiple vacuolar proteases, and have altered vacuolar morphology (**Harding *et al.* 1995**). This observation prompted a systematic screening of *vps* mutants. The discovery that *vps1*, *vps8*, *vps15*, *vps16*, *vps17*, *vps18*, and *vps26* mutants all show defects in API processing suggests that there is overlap between the machinery used by the *vps* pathway taken by carboxipeptidase Y and the Cvt pathway (**Bryant and Stevens, 1998**).

I.5.2.4. Autophagy.

Autophagy is the primary delivery pathway used to transport proteins and organelles for degradation. During nutrient stress, massive protein turnover occurs in the vacuole, resulting in the destruction of nearly half of the total cellular protein in a 4 hours period. This dramatic turnover of cellular material cannot be accommodated by the proteasome and, in general, the vacuole mediates bulk turnover of components, while the cytoplasmic ubiquitin/proteasome system is responsible for selective turnover of proteins. At a morphological level, autophagy can be differentiated into two modes. Macroautophagy involves the formation of double-membrane vesicles, which are distinct from the vacuole. In contrast, microautophagy proceeds by vacuolar membrane-mediated engulfing. In yeast, autophagy appears to occur primarily by macroautophagy, based on electron microscopic studies of nitrogen-starved cells (**Baba *et al.*, 1994**). Thus, macroautophagy is a starvation-induced, non-specific bulk flow transport pathway that delivers cytosolic material and even whole organelles such as

mitochondria to the vacuole for degradation (**Baba *et al.*, 1994; Egner *et al.*, 1993; Takeshige *et al.*, 1992; Thumm *et al.*, 1994**). This process starts with the formation of autophagosomes, double-membrane layered vesicles that enclose parts of the cytosol and even the whole mitochondria (**Baba *et al.*, 1994; Takeshige *et al.*, 1992).** After reaching the vacuole, the outer membrane layer of autophagosomes fuses with the vacuolar membrane and monolayered autophagic vesicles are released into the vacuolar lumen. Inside the vacuole, these autophagic vesicles are finally broken down together with their cytosolic content. The degradation of autophagic vesicles depends on active vacuolar proteinases, especially endoproteinase B (**Takeshige *et al.*, 1992).**

Genetic screenings for yeast mutants defective in autophagy have been performed by screening for an inability to degrade cytoplasmic proteins during starvation (*aut* mutants), or by selecting for an increased sensitivity to starvation (*apg* mutants). Most of the autophagy mutants, with a few exceptions, share typical phenotypes. They growth wild-type-like on rich media and other vesicle-mediated protein transport pathways including secretion, fluid phase endocytosis, and vacuolar protein localization are not influenced. Vacuolar acidification, monitored by the accumulation of quinacrine, appears to be wild-type-like. These mutants exhibit starvation-related phenotypes, such as decreased viability under starvation conditions, inability to accomplish degradation of cytoplasmic marker proteins during nutrient limitation, and in most cases, the lack of accumulation of autophagic bodies in the vacuole in the presence of phenyl-methyl-sulphonyl-fluoride (PMSF) which acts a serine protease inhibitor and under nutrient stress conditions (**Thumm, 2000**). Furthermore, nearly all autophagy mutant strains are impaired in vacuolar uptake and maturation of proaminopeptidase I (**Harding *et al.*, 1996**), and a significant genetic overlap between the *aut* , *apg* and the *cvt* mutant strains has been detected.

I.5.2.5. Endocytosis.

Endocytosis is the uptake of extracellular and plasma membrane material from the cell surface into the cell. Two distinct modes of endocytosis operate in yeast, a constitutive (ligand-independent) mechanism and a ligand-dependent mechanism. In both cases, receptors follow the same intracellular itinerary from the cell surface to the

vacuole, where they are degraded (**Bryant and Stevens, 1998**). The endocytosis starts with internalization from the plasma membrane. The well documented internalization signal in yeast is ubiquitination. Multiubiquitination of substrates is generally required for the recognition and degradation of cytosolic, endoplasmic reticulum and nuclear proteins by proteasome, whereas the presence of a single or a few ubiquitins (with a different ubiquitin linkage) per plasma membrane proteins leads to the recognition by the endocytic machinery and targeting toward the vacuole for degradation (**Kucharczyk and Rytka, 2001**)

I.5.3. Vacuolar inheritance.

Cells do not synthesize subcellular organelles de novo, instead, they build them upon material inherited from mother cells. Cytological studies of mammalian cells have demonstrated vesiculation of nuclear, ER, and Golgi membranes at the beginning of mitosis and subsequent fusion of these vesicles to reconstitute these organelles after cytokinesis (**Warren, 1993**; **Warren and Wickner, 1996**). While not all organelles in *S. cerevisiae* follow this pattern of inheritance, yeast cells inherit vacuoles from the mother through a mechanism that involves the projection of vesicles and tubules, known as segregation structures, from the mother cell vacuole into the growing daughter cell. As with the inheritance of other organelles, vacuolar inheritance is coordinated with the cell cycle (**Warren and Wickner, 1996**). Several screens were employed to identify mutants defective in vacuole inheritance (*vac*) (**Gomes de Mesquita *et al.*, 1996**; **Shaw and Wickner, 1991**; **Wang *et al.*, 1996**; **Weisman *et al.*, 1990**). Based on differences in vacuole morphology, they were subdivided into several classes (**Wang *et al.*, 1996**) and further subdivided on the basis of the normal or defective localization of CPY. This classification is showed in **Table I.7**. The class I *vac* mutants are defined as mutants with a relatively normal vacuole morphology that consists of a multilobed vacuole. Mutants in this class have defects in proteins required to move the vacuole. Analysis of these mutants led to the discovery that the vacuole movement is actin based and that the class V myosin, Myo2p, serves as the motor (**Weisman *et al.*, 1990**). The class II *vac* mutants have relatively enlarged vacuoles. The *vps* class D mutants missort CPY; they are defective in vacuole biogenesis and, in addition, have a vacuole inheritance defect

and present class II vacuole morphology. The class III *vac* mutants have exceptionally enlarged vacuoles and are defective in retrograde traffic out of the vacuole. In addition, they are defective in vacuole acidification and are likely defective in ion transport. The corresponding wild-type genes are required for synthesis of the signalling lipid, phosphatidylinositol 3, 5-biphosphate (**Weisman, 2003**).

Table I.7. Classification of vacuole inheritance mutants (from Weisman *et al.*, 1990).

Classification	Vacuolar morphology	Mutants
Class I: non *vps*	Normal	*act1, pfy1, myo2, vac8, vac9, vac10, vac16, vac17, vac19*
Class II: non *vps*	Enlarged vacuole, arrested segregation structure.	*vac5, vac6, vac11, vac12*
Class II: *vps* (class D)	Enlarged vacuole, arrested segregation structure.	*vps3, pep12/vps6, vps8, vps9, pep7/vps19/vac1, vps21/ypt51, vps45*
Class III: non *vps*	Vacuole membrane fission and acidification defects	*fab1, vac7, vac14, fig4*
Class III: *vps*	Vacuole membrane fission and acidification defects	*vps15, vps34*

I.5.4. Molecular basis of vacuole inheritance.

During each cell cycle the yeast vacuole actively partitions between mother and daughter cells. Vacuole movement is actin based and myosin serves as the motor (**Hill *et al.*, 1996**). Organelle movement is achieved through Myo2p and its interaction with Vac17p and Vac8p, proteins that are part of the vacuole-specific Myo2p receptor (**Fig. I.6**). Both the spatial and temporal control of vacuole distribution is achieved by regulating the appearance and disappearance of the transport complex specifically through changes in Vac17p. *VAC17* is unique among the known vacuole inheritance genes because both *VAC17* mRNA and protein levels oscillate with the cell cycle (**Spellman *et al.*, 1998; Tang *et al.*, 2003**).

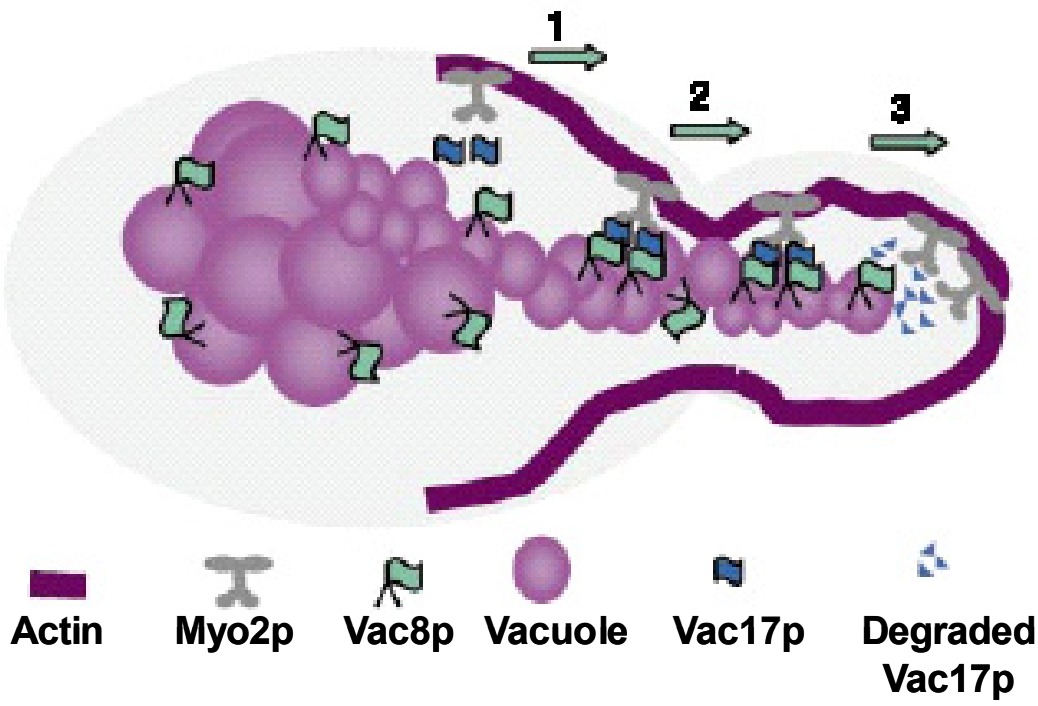

Figure I.6. Model for Vac17p function in vacuolar inheritance (Tang *et al.*, 2003).

The cell cycle-dependent change in Vac17p levels is due to both synthesis and turnover. Vac17p binds simultaneously to Myo2p and Vac8p, a vacuolar membrane protein, and the transport complex, Myo2p-Vac17p-Vac8p, moves the vacuole to the daughter cells, and is then disrupted through the degradation of Vac17p, since it contains a PEST sequence, a signal for rapid protein turnover. *VAC8* sequence encodes 11 armadillo (Arm) repeats. Arm repeats are thought to mediate protein-protein interactions (**Wang *et al.*, 1998**). In addition to its role in vacuole inheritance, Vac8p is required for three additional, distinct vacuole-related processes. The *vac8* mutant has no detectable effect in endocytosis or targeting proteins to the vacuole via the Vps pathway (**Wang *et al.*, 1996**). However *vac8* is defective in the Cvt pathway, since is part of a large complex required for closure of cytoplasm to vacuole targeting (Cvt) vesicles (**Scott *et al.*, 2000**; **Wang *et al.*, 1998**).

By analogy to other armadillo domain proteins, Vac8p is thought to dock various factors at the vacuole membrane (**Pan *et al.*, 2000**). Consequently, two-hybrid and copurification assays were performed, demonstrating that Vac8p form a complex with Nvj1p, an integral membrane protein of the nuclear envelope.

Vac8p-Nvj1p complexes have been localized at the interface between the nucleus and vacuole (**Pan *et al.*, 2000**). This interface, the nuclear-vacuole junction, may play an important role in the microautophagy of damaged nuclear membranes (**Roberts *et al.*, 2003**).

Lastly, Vac8p is required for homotypic vacuole fusion processes, which yield from one to three vacuoles per yeast cell at steady state (**Wang *et al.*, 2001**). It has been proposed that Vac8p may bind the fusion machinery through its armadillo repeats and that its palmitoylation brings this machinery to a specialized lipid domain that facilitates bilayer mixing (**Wang *et al.*, 2001**).

I.6. Correlation between cell cycle progression and vacuole inheritance in hyphal forms of *C. albicans*.

C. albicans is a pleomorphic fungal pathogen, so its cell cycle must be regulated to enable cell shape to be modulated while the nuclear cycle is maintained. In addition the cell cycle can be altered to enable new growth forms to be propagated. For example, cytokinesis, the process of division of the daughter cell at the end of the cell cycle, may be altered or inhibited to permit filamentous cell development. *C. albicans* yeast cell cycle progression appears to be very similar to the process in *S. cerevisiae* yeast cells, with at least 4 major cell cycle-dependent processes or cycles that occur in a discrete order: the budding cycle (directed localization and relocalization of the actin cytoskeleton and secretory pathway components), the DNA replication and the chromosome cycle, the spindle pole body and microtubule cycle and lastly the assembly of a septin ring and the subsequent septation at the ring level.

Pseudohyphal growth is characterized by elongated budded cells that remain attached to one another in a branching pattern. As described in **section I.4 of this chapter**, pseudohyphae have been considered to represent an intermediate state between yeast and true hyphal growth forms. However, recent work has demonstrated that there are significant differences between yeast and pseudohyphal cells (**Sudbery, 2001; Sudbery *et al.*, 2004**).

Septin ring placement and actin organization in pseudohyphae resembles that seen in yeast cells, while the processes of DNA replication and spindle formation have not been carefully analyzed in pseudohyphal cells (**Berman and Gow, 2004**).

Pseudohyphal cells tend to bud in a "unipolar distal" budding pattern, with the first bud formed at the opposite end of the cell from the birth scar and subsequent buds forming at sites adjacent to this first bud. In addition, most pseudohyphal cells remain attached to one another following cytokinesis. This attachment is likely due to the lower levels of chitinase expressed in cells with a pseudohyphal-like morphology (**Bensen *et al.*, 2002**).

During mycelial growth, vegetative growth is achieved by elongation and branching of hyphal filaments. Germ tube emergence is accompanied by a rapid increase of the vacuolar volume in the mother yeast cell (**Barelle *et al.*, 2003; Gow and Gooday, 1982**) but the cellular cytoplasmic volume remains relatively constant while the overall cell volume increases linearly. The remaining cell space in these expanding cells is occupied by an expanding vacuole or vacuoles. The vacuolation process has been reported to be a general feature of the growth of the *C. albicans* mycelial form (**Gow and Gooday, 1987; Gow *et al.*, 1986**) and of a number of other fungi that also exhibit extensive vacuolation during hyphal growth (**Steinberg *et al.*, 1998**). Formation of secondary germ tubes from mother cells or of branches from sub-apical compartments occurs only after regeneration of the cytoplasm at the expense of the vacuole. Recently, correlation between vacuolar inheritance and modulation of the cell cycle of *C. albicans* during true hyphal growth has been described (**Barelle *et al.*, 2003**). While vacuolar volume of mother and daughter cells of *C. albicans* at cytokinesis in yeast and pseudohyphal forms is similar (**Berman and Gow, 2004**; **Yokohama and Takeo, 1983**) hyphal cell division is asymmetric and cytoplasm is partitioned predominantly to the proximal apical cell and most vacuole is inherited by the subapical mother yeast cell or hyphal intercalary compartment (**Barelle *et al.*, 2003**).

Accordingly, the concept of size-regulated cell cycle control has been refined to propose that it is the cytoplasmic volume minus vacuolar volume (and that of other organelles) rather than total cell volume that is relevant in cell size-regulated control of the eukaryotic cell cycle (**Barelle *et al.*, 2003**). This hypothesis predicts that mutations in genes that influence normal vacuolar inheritance will in turn influence the branching

frequency of *C. albicans* hyphal forms. In this context, how organelle biogenesis and inheritance is linked to cell division is poorly understood in fungi, but new genes involved in vacuolar biogenesis and inheritance (*CLN3*, *VPS34*, *ABG1*) have been involved in cell cycle progression (**Han *et al.*, 2003; Bruckmann *et al.*, 2001)**. In this context, repression of *ABG1*, a novel and essential gene of *C. albicans*, whose molecular and functional characterization is described in the present Ph. D. Thesis, leads to an increased frequency of division in hyphal cells, indicated by a higher number of branches in hyphae, in the mutants for *ABG1* with respect to the parental strain. As it will be shown latter in greater detail, *ABG1* codes for a vacuolar protein. Moreover, known genes belonging to the cyclin family have been involved in vacuolar biogenesis, as Cln3p, a G1 cyclin described in *S. cerevisiae*, which controls initiation of cell division and also vacuolar biogenesis and segregation (**Han *et al.*, 2003**).

Besides of these reports, it has been also described that vacuole protein-sorting pathways are also influent in the cell cycle progression and in the yeast-to-hypha transition, since deletion of *CaVPS34* gene caused disturbance of normal nuclear migration, which suggests that in the *CaVPS34* mutant the cell-size mediated control process of cell division is affected (**Bruckmann *et al.*, 2001**), and *vps11*Δ mutants are defective in the yeast-hypha transition, thus suggesting that Vps11p-mediated trafficking steps are necessary to support the rapid emergence and extension of the germ tube from the parent yeast cell (**Palmer *et al.*, 2003**).

I.7. Antecedents and aim of this work.

The present project belongs within the general research line carried out in our group during the last five years and that has been focused on the characterization of new genes, particularly those that are preferentially expressed during the yeast-to-mycelium transition in *C. albicans* which, as above stated, is one of the virulence traits described for this opportunistic fungal pathogen. In this context, immunoscreening of a *C. albicans* cDNA library in λ phage with a polyclonal antibody (PAb anti-gt [**Casanova *et al*., 1989**]; **see Materials and Methods chapter**) that specifically recognized cell wall protein and glycoprotein components of the germ tubes (gt), resulted in the isolation of 11 immunoreactive clones whose characteristics are shown in **Table III.1 (see Results chapter)**.

As indicated in **Table III.1**, some of the isolated clones contained sequences that corresponded to genes with known function previously characterized whereas some clones were simply duplicates of other clones also immunodetected with PAb anti-gt in this previous step. However, two clones that were designated as clone 1 and clone 4 contained sequences for genes that code for proteins of unknown function in *C. albicans*, and a third clone (clone 11) exhibited a high homology with the gene coding for the so called Antigen 2 of *Coccidioides immitis* that might have potential applications for the immunodiagnostic and the prevention of infections caused by this pathogenic fungal species (**Dugger *et al*., 1996; Jiang *et al*., 1999**). Consequently, it was considered of interest to carry out the molecular and functional characterization of *C. albicans* genes that corresponded to the sequences present in these three clones.

Further analysis of clone 1 resulted in the isolation of a gene encoding a 134 kDa lysine/glutamic acid-rich protein, which was consequently designated as *KER1* (GenBank accession number AF337555). The nucleotide and deduced amino acid sequences of this gene displayed no significant homology with any other known sequence. *KER1* coded for a protein that appear to be located at the plasma membrane level but that also is involved in cell wall composition and/or structure and may be important in host-fungus interactions (**Galán, 2003; Galán *et al.*, 2004**).

On the other hand, further analysis of clone 11 resulted in the isolation of a new *C. albicans* gene that was designated *CaHPB1* for *C. albicans h*ydro*p*ho*b*in (GenBank accession number AF307520), coding for a 25 kDa protein (Hpb1p) that exhibited high homology with other partially characterized cell wall proteins of this fungus such as Rbt5p and Csa1p/Wap1p (**Braun *et al.*, 2000**). *In silico* analysis of Hpb1p sequence revealed the presence of a 115 amino acids region containing 8 cysteine residues located in highly preserved positions, as well as the presence of a signal peptide for secretion and a possible GPI motif, characteristics that were also present in Rbt5p and Csa1p/Wap1p species. From the study carried out in our group it was concluded that Hpb1p may represent a class II hydrophobin-like protein (**Pedrós, 2003**), a particular family of secretory proteins, that have already been characterized in several ascomycetes and basidiomycetes fungal species (**de Vries *et al*., 1993; Wessels, 1994**) but not in *C. albicans* until now (**Pedrós, 2003**). It has to be stressed that hydrophobins are among the most surface active biomolecules known (**Wösten and Vocht, 2000**), fulfill a broad spectrum of functions in fungal growth and development (**Kershaw and Talbot, 1998; Wessels, 1997; Wösten *et al*., 1999**), and are interesting candidates for use in a wide range of medical and technical applications (**Scholtmeijer *et al*., 2001; Wösten, 2001**).

The aim of the present Ph. D. Thesis was the molecular and functional characterization of the *C. albicans* gene that corresponded to the sequence present in clone 4. Functional characterization included the subcellular localization of the corresponding gene product, analysis of the gene expression pattern, and generation of a double knock out strain for this gene. This strain was further used to study possible defects in the morphology, cell wall structure and/or composition, and changes in the response to different environmental stress conditions as a consequence of the loss of the gene function. Finally, in order to learn more about the biological function of this gene in living fungal cells, a transcriptional profiling analysis of the knock out mutant strain, and an analysis of the biological partners of the gene product encoded by this novel gene was also planned and attempted.

II. MATERIALS AND METHODS

II.1. Fungal and bacterial strains

Characteristics of the fungal and bacterial strains used in this work are described in **Table II.1** and **II.2**, respectively.

Table II.1. *C. albicans* strains.

Strain	Parental strain	Genotype	Source or reference
26555		Wild-type	ATCC[a]
SC5314		Wild-type	**Gillum *et al.*, 1984**
CAI4	SC5314	*ura3Δ::λimm434/ ura3Δ::λimm434*	**Fonzi and Irwin, 1993**
CAI4-*URA3*	CAI4	*ura3Δ*::λ*imm434/ ura3Δ*::λ*imm434*, *RPS10::URA3*	This work[b]
WO-1			**Soll *et al.*, 1987**
CJ1	CAI4	*ura3Δ::λimm434/ ura3 Δ::λimm434; abg1Δ::URA3-dp1200/ ABG1*	This work
CJ2	CJ1	*ura3Δ::λimm434/ ura3Δ::λimm434; abg1Δ::dp1200/ ABG1*	This work
CV1	CAI4	*ura3Δ::λimm434/ ura3Δ::λimm434*; *abg1Δ::hisG-URA3-hisG/ ABG1*	This work
CV2	CV1	*ura3Δ::λimm434/ ura3Δ::λimm434*; *abg1Δ::hisG/ ABG1*	This work
CV3	CV2	*ura3Δ::λimm434/ ura3Δ::λimm434; abg1Δ::hisG/ abg1Δ:URA3-MET3-ABG1*	This work
CGA4	CAI4	CAI4 derivative, p*ACT-ABG1-GFP*	This work
T1	CAI4	CAI4 derivative, p*ACT-ABG1*-C-ZZ	This work

(a) ATCC-American Type Culture Collection

(b) CAI4-*URA3* strain was kindly provided by Dr. Gwyneth Bertram, School of Medical Science, University of Aberdeen.

Table II.2. *E. coli* strains

Strain	Genotype	Use in this work	Reference
Y1090	*Sup*F, *hsd*R, *ara*D139, Δ*lon*, Δ*lac*U169, *rps*L *trpC22::Tn10*(*tet*^{r}), pMC9	Immunological screening of the expression library and propagation of λgt11	**Young and Davis, 1983**
DH5α	F$^-$ ϕ80*lac*ZΔM15, Δ(*lac*ZYA-*arg*F), U169, *deo*R, *rec*A1, *end*A1, *hsd*R17 (r_K^-, m_K^+), *pho*A, *sup*E44, λ$^-$*thi*-1, *gyr*A96, *rel*A1	plasmid propagation	**Hanahan, 1983**

II.2 Growth conditions.

II.2.1 Fungal strains.

C. albicans yeast cells were routinely grown in YPD, YNB or Lee's (**Lee *et al.*, 1975**) media and incubated at 28º C with shaking. Media were supplemented with uridine (25 μg/ml) when required. To repress *MET3* promoter, *C. albicans* strains were grown in YNB medium with 5 mM methionine and 2 mM cysteine. For sensitivity and phenotypic assays, plates containing solid YPD, YNB or Lee's medium plus 2 mM methionine and 0.5 mM cysteine were used. Solid plates were prepared by adding 1.5% agar to the medium before autoclaving. Composition of the different culture media employed are next described: YPD (1% yeast extract; 2% peptone; 2% glucose); YNB (0.67% Yeast nitrogen base without amino acids [Difco]; 2% Glucose); Lee's medium (0.25% K_2HPO_4; 0.02% $MgSO_4$; 0.5% $(NH_4)_2SO_4$; 0.5% NaCl; 1.25% glucose; 0.05% proline; 0.0001% biotine). The final pH of Lee's medium was adjusted to 7-7.5.

Germ tube formation in *C. albicans* was induced by incubation in serum (**Gow and Gooday, 1982**) or by the starvation method (**Casanova *et al*., 1989**). In the first case *C. albicans* cells were grown to stationary phase in YNB liquid medium supplemented with 5 mM methionine and 2 mM cysteine. Cells were harvested by centrifugation at 3,000 *x* g for 10 min, washed twice in sterile distilled water and resuspended in sterile distilled water at a final concentration of 2 x 10^7 cells/ml. This cellular suspension was added to poly-L-lysine-coated microscope slides (**Crombie *et al.*, 1990**) placed in Petri dishes and incubated 20 min at room temperature to allow the adhesion of the cells to the slide surface. Then the microscope slides were washed twice with sterile distilled water, and placed in new Petri dishes containing 10% (v/v) foetal calf serum (Sigma, UK) in water plus amino acids prewarmed at 37ºC and incubated to allow hyphal development.

For starvation-stimulated dimorphism cells were grown in Lee's medium at 28ºC overnight. Cells were next collected by centrifugation, washed twice in sterile distilled water and resuspended in sterile glass-distilled water at a final concentration of 2 x 10^7 cells/ml. This cellular suspension was incubated at 28ºC with shaking (200 rpm) for 3 h and then subjected to a starvation period at 4ºC for 48 h. After this period cells were collected by centrifugation and resuspended in Lee's medium, prewarmed at 37ºC to a final cell density of 2 x 10^7 cells/ml. To obtain yeast cells under similar culture medium composition conditions, incubation was performed at 28ºC.

II.2.2 Bacterial strains.

Escherichia coli strains were routinely grown on LB (1% tryptone; 0.5% yeast extract; 0.5% NaCl; Luria-Bertani medium; **Miller, 1972**) agar medium at 37ºC or in LB liquid medium with shaking (200 rpm). For selection and propagation of plasmids, LB medium was supplemented with a stock solution of 0.1 g/ml filter sterilized ampicillin to a final concentration of 100 μg/ml. For Y1090 strain LB medium was supplemented with 0.2 mM maltose and 10 mM $MgSO_4$.

II.3 Plasmids.

The pUC19 plasmid (**Messing, 1983**) was used to facilitate DNA subcloning. It contained the β-lactamase gene conferring resistance to ampicillin, as a selectable marker, and a fragment of DNA derived from the *lac* operon of *E. coli* (*lac*Z) that codes for the amino-terminal fragment of β-galactosidase, which allows the selection of blue-white colonies.

The pEMBLYe23 plasmid (**Baldari and Cesareni, 1985**) was used to construct the genomic library used in this work. This vector is suitable for transformation in both bacterial and yeast cells. Principal features of this plasmid include the β-lactamase and β-galactosidase genes, and the *URA3* gene of *S. cerevisiae* that can be used as a selectable marker.

Plasmids pBB510 (**Perez-Martín *et al.*, 1999**) and pDDB57 (**Wilson *et al.*, 2000**) were respectively used for URA-blaster and PCR-based gene disruption experiments in *C. albicans*. Plasmid pBB510 derived from pMB7 (**Fonzi and Irwin, 1993**) and contained the *hisG::URA3::hisG* cassette, consisting of the *C. albicans URA3* gene flanked by direct repeats of the *Salmonella typhimurium hisG* gene, while pDDB57 derived from pRS315 (**Sikorski and Hieter, 1989**) and contained the *URA3*-dp1200 cassette, consisting of the *C. albicans URA3* gene flanked by a 200 bp segment of *C. albicans URA3* 3´sequences. These two plasmids share the β-lactamase gene.

The pCaDis plasmid (**Care *et al.*, 1999**) was used after the first copy of a selected gene has been deleted and a Ura^- host regenerated in the first step of the URA-blaster procedure. It allows the second copy of the gene to be placed under the control of the *MET3* promoter. Principal features of this plasmid include the β-lactamase gene, the *URA3* gene of *S. cerevisiae* that can be used as a selectable marker and the *MET3* promoter that allows conditional expression of a selected gene, according to the presence or absence of either methionine or cysteine in concentrations in excess of 1 mM.

Plasmid pAG1 (kindly donated by Esperanza López, Universidad de Salamanca, Spain) was used to construct a recombinant protein by cloning the open reading frame of *ABG1* gene in frame with the yeast-enhanced green fluorescent protein (**Cormack *et al.*, 1997**).

The plasmid pACT-C-ZZ was also used to generate a recombinant protein by cloning the *ABG1* gene ORF in frame with the protein A from *Staphylococcus aureus* (**Blackwell *et al.*, 2003**). This plasmid is based on the *URA3*-containing plasmid CIp10 (**Murad *et al.*, 2000**) and allows the addition of carboxy terminal protein A tag that could be removed post-translationally by treatment with the tobacco etch virus (TEV) protease. Plasmids pAG1 and pACT-C-ZZ share the *ACT* gene promoter, which allows the expression of the recombinant polypeptides obtained at basal levels, the β-lactamase gene and the *URA3* gene of *C. albicans*.

The CIp10 plasmid was used to reintegrate the *URA3* gene in the CAI4 strain of *C. albicans* in the *RPS10* gene locus (**Murad *et al.*, 2000**). The plasmids constructed in this work are fully described in **Table II.3**.

II.4. Isolation of cDNA clones.

II.4.1. Immunoscreening of a cDNA library with PAb anti-gt.

The cDNA library used in this work was kindly provided by Dr. LaJean Chaffin (Texas Tech University, Health Science Center, Lubbock, Texas, USA). This library was constructed with the cDNAs synthesized from a mRNA population of *C. albicans* strain ATCC26555 obtained after 30 min of germ tube induction via starvation. The cDNA population was cloned into the unique site of the expression vector λgt11. If the cDNAs are inserted in the correct orientation and reading frame, a fusion protein is produced consisting of β-galactosidase sequences linked to polypeptide sequences encoded by the cloned DNA. Bacteriophage plaques can then be screened for the presence of material that reacts with antisera specific for the protein of interest, in this case with a germ-tube specific polyclonal antibody (PAb anti-gt), previously obtained by our group (**Casanova *et al.*, 1989**) at a 1:1,000 dilution.

Table II.3. Plasmids constructed in this work.

Plasmid	Vector	Details
pUCC4	pUC19	*ABG1 Bam*HI-*Hin*dIII section digested and inserted in the *Bam*HI-*Hin*dIII digested multicloning site (MCS) of pUC19.
p510VV	pBB510	700 bp *Hin*dIII-*Pst*I fragment from 5´ *ABG1* upstream non-coding region and a 500 bp *Bgl*II-*Kpn*I fragment from *ABG1* 3´ downstream non-coding region cloned into the *Hin*dIII-*Pst*I site and into the *Bgl*II-*Kpn*I site of pBB510.
pMD	pCaDis	200 bp *Bam*HI-*Pst*I PCR product from *ABG1* ORF digested and inserted in the *Bam*HI-*Pst*I site of pCaDis.
pAAG	pAG1	*Bam*HI-*Sma*I PCR product containing the entire *ABG1* ORF digested and inserted in the *Bam*HI-*Sma*I site of pAG1.
pTABG	p-ACT-C-ZZ	*Hin*dIII-*Sal*I PCR product containing the entire *ABG1* ORF digested and inserted in the *Hin*dIII-*Sal*I site of pACT-C-ZZ.

All the reagents used for the immunological screening are listed in **Table II.4**. Several 150-mm plates containing 30,000 recombinants each, were analysed following the method described by **Sambrook *et al.*, (1989)** as follows. Aliquots of 0.1 ml of SM buffer containing 105 plate forming units (pfu) of the expression library in the λ bacteriophage were added to 0.15 ml of culture of *E. coli* strain Y1090 grown for 12-16 h in LB medium supplemented with 0.2 % maltose and 10 mM MgSO4, and this suspension was incubated for 20 minutes at 37ºC to allow the phage to adsorb to the bacteria. Then, 10 ml of molten top LB agar were added to the cell suspension and the mixture was immediately poured onto LB agar plates. These plates were incubated for 3 h at 42ºC until the lysis was visible and were then overlayed with 100 mM IPTG-impregnated nitrocellulose filters. The plates were incubated again overnight at 37ºC.

Following this incubation, the plates were cooled at room temperature, the filters were then marked in asymmetric locations, peeled off from the plates and immersed in TTBS buffer for 30 min at room temperature. After this, the filters were submerged in blocking buffer for 30 min, washed 4 times in TTBS and incubated with the PAb anti-gt at room temperature for at least 3 h. After this incubation, the filters were washed again 4 times in TTBS and the antigen-antibody complex was detected by using a goat anti-rabbit second antibody coupled to horseradish peroxidase (HRP) diluted 1:2,000. After washing the filters 4 times in TTBS, antigen-antibody-HRP-antibody complexes were located using the chromogenic substrate 4-chloro-α,1-naphtol. The filters were incubated in the developing solution and when the purple colour was developed at the site of antigen-antibody complexes, the reaction was stopped by washing the filters in distilled water.

Positive plaques were identified in the plates and a plug of agar from the area containing the positive plaque was removed using a Pasteur pipette and transferred to 1 ml of SM buffer containing two drops of chloroform. The bacteriophage particles were eluted from the agar plug for several hours. A second screen was then utilized at a lower density (3,000 plaques per each 90-mm plate), in order to obtain a single plaque containing a homogeneus population of immunopositive recombinant bacteriophages.

Table II.4. Reagents used in the cDNA library immunoscreening.

Solution	**Composition**
SM buffer	100 mM NaCl; 1 mM MgSO4; 20 mM Tris-HCl pH 7.5; 0.01% gelatine.
TBS buffer	10mM Tris-HCl pH 8.0; 150 mM NaCl.
TTBS buffer	TBS + 0.05% Tween 20.
Molten top LB agar	LB media with 0.6% agar.
Blocking buffer	TTBS buffer + 3% BSA.
PAb anti-gt solution	TTBS buffer +1% BSA + PAb anti-gt 1:1,000 dilution.
2nd antibody solution	TTBS + 1% BSA + goat anti-rabbit antibody HRP 1:2,000.
Developing solution	A: 60 mg 4-chloro-α,1-naphtol + 20 ml ice-cold methanol B: 100 ml TBS buffer + 60 μl H_2O_2.

II.4.2. Liquid amplification of the immunopositive recombinant bacteriophages.

Bacterial strain *E. coli* Y1090 was infected as described before (**see section II.4.1 in this chapter**) and 0.25 ml aliquots of the cell/phage suspension were grown in 5 ml LB medium, supplemented with maltose and magnesium, for about 6 h until cell lysis was visible. Once the lysis was observed, 250 µl of chloroform were added to the culture and incubation was continued for another 10 min. The suspension was then centrifuged to remove cellular debris and dimethyl sulphoxide (DMSO) was added to the supernatant lysate to a 7% (v/v) final concentration. These lysates were then stored at -20ºC.

II.4.3. Determination of the titer of a phage lysate.

In order to determine the titer of a phage lysate, several serial dilutions of the lysate were prepared in SM buffer. A volume of 0.1 ml from each dilution was incubated with 0.15 ml of an *E. coli* strain Y1090 culture and the resulting suspension was plated onto LB agar. After overnight incubation at 37ºC, the plate containing between 30 and 300 plaques was counted. From this calculation, the number of pfu in the original lysate was determined as number of plaques x (1/dilution) x 10.

II.5. DNA purification.

II.5.1. Plasmid DNA purification.

Minipreparations of plasmid DNA were obtained by the alkaline-lysis method originally described by **Birnboin and Doly (1979)** with some modifications. The method consists of growing overnight cultures of *E. coli* in 5 ml of LB-ampicillin medium, at 37ºC. Aliquots of 1.5-3 ml from these cultures were centrifuged and the bacterial pellet was resuspended in 200 µl of GTE buffer (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0).

Three-hundred µl of a 0.2 N NaOH solution containing 1% SDS were added in order to lyse the cells, and the mixture was incubated on ice for 5 min. Then, the solution was neutralized by adding 300 µl of 3.0 M potassium acetate, pH 4.8. Cellular debris were removed by centrifuging at 10,000 rpm for 10 min and RNAse A (10 µg/µl) was added to the supernatant to reach a final concentration of 20 µg/ml and incubated at 37ºC for 20 min. The supernatant was extracted twice with 400 µl of chloroform and the DNA in the aqueous phase was precipitated with 100% isopropanol. After pelleting the DNA by centrifuging at 10,000 rpm for 10 min, the DNA pellet was washed with ethanol 70% and air dried. The plasmid DNA was then precipitated with NaCl at a final concentration of 0.3 M and 2 volumes of absolute ethanol, centrifuged at 10,000 g for 10 min, washed with ethanol 70%, air dried, and resuspended in a proper volume of water or TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

When large amounts of plasmid DNA were required the same method but scaled-up to 100 ml of culture was used. Alternatively the Qiagen spin miniprep kit (Qiagen Sciences, USA) or the Real Turbo Miniprep kit (Durviz, Spain) were used following manufacturer's instructions.

II.5.2. Purification of genomic DNA from *C. albicans*.

The method used for isolation of *C. albicans* DNA was adapted from **Hoffman and Winston (1987)**. Cells were grown overnight in 10 ml of YPD liquid medium, harvested by centrifugation at 3,000 rpm for 5 min, washed with distilled water and pelleted by a second centrifugation step. Cells were resuspended in 0.2 ml of disruption buffer (2 % Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8), containing 0.2 ml phenol/chloroform, and 0.3 g of 425-600 microns-diameter glass beads (Sigma) were added to the resulting cell suspension. Tubes were vortexed until complete cell breakage, and then 0.2 ml of TE buffer, pH 7.4, were added and samples were mixed by inversion. Cell debris were pelleted by centrifugation at 10,000 rpm for 5 min and the resulting aqueous phase transferred to a new tube to which 1 ml ethanol was added and mixed by inversion. Nucleic acid was pelleted by centrifugation at 10,000 rpm for 10 min and resuspended in 0.4 ml of TE buffer with 100 µg of RNase A (10 µg/µl) followed by incubation at 37°C for 5 min.

DNA was precipitated with 0.3 M (final concentration) NaCl and two volumes of ethanol. The DNA was washed with 70% ethanol, dried and resuspended in 50 µl of TE buffer. Samples were stored at 4ºC until used.

II.6. DNA amplification by the polymerase chain reaction (PCR).

Typical PCR amplification reactions were done in 50 µl aliquots containing 5 µl of 10x PCR buffer (supplied by the manufacturer), 3.5 µl of a 50 mM $MgCl_2$ solution, 1 µl from a mixture of 0.2 mM deoxynucleoside triphosphates (Amersham, UK), 1 µl from a 0.2 mM stock solutions of each oligonucleotide primer, template DNA (from 1 ng to 1 µg), and 2 U of Taq DNA polymerase (Ecogen [Spain] or Qiagen). Amplification reactions were carried out in a thermocycler (Progene, Techne, Cambridge, UK) with the following steps:

- Initial melting step at 94ºC for 15 min.
- 30 cycles of 94ºC for 1 min, 55-65ºC (annealing step; temperature varied depending of the melting temperature of the primers) for 1 min, and 72ºC for 1-2 min (extension step; time varied depending of the size of the PCR product)
- Final extension step at 72ºC for 7 min.

When high fidelity DNA amplification was required, the Expand High Fidelity PCR System (Roche, Germany) was used according to manufacturer's instructions. This system is composed of a unique enzyme mix containing thermostable Taq DNA and two DNA polymerases designed to give PCR products with high yield, high fidelity and high specificity from plasmidic and genomic DNA. Oligonucleotide primers used in this work were purchased from Sigma-Genosys (UK). Primer sequences and relevant uses of each primer are shown in **Table II.5**.

Table II.5. Primers used in this work.

Primer	Sequence (5´→ 3´)	Purpose
λgt11F	GGTGGCGGACGACTCCTGGAGCCCG	PCR amplification of cDNA clones
λgt11R	TTGACACCAGACCAACTGGTAATG	PCR amplification of cDNA clones
FC4	GAAAGGATCCAATTTTGGCTCG	*ABG1* cloning into pUC19
RC4	TGCTAAGCTTTGGTGACTTAGCGTGG	*ABG1* cloning into pUC19
FP4	GCGCGGATCCATGGTTTCACTTTCTAATTT	pMD and pAAG construction; Northern analysis
RP4	GCGCGAATTCTTATTTTTATCAAAATCAATTTG	Northern analysis
C4SF	CTTCAAATGCACAATGCC	*ABG1* sequencing Southern analysis
C4SR	GGCGAGGATTGGGAAAAG	*ABG1* sequencing Southern analysis
SFR	CGGCATCATTTTCATTGTCA	*ABG1* sequencing
SFF	CTGATACTGCACCAATTATC	*ABG1* sequencing
PF1F	GCGCAAGCTTCTGGATAAATTGGAAATGAAGG	p510V construction
PR1F	GCGCCTGCAGTATCTCTAGTGCTCCGTATA	p510V construction
PF2F	GCGCAGATCTCTGGACTTGAATTGTTATTC	p510V construction Southern analysis
PR2F	GCGCGGTACCTCTTCCGCTCAGCAAATAAACAA	p510V construction Southern analysis
M4F	AGTAGCAAAAGAGATAAACAAAGTGACAATGAAA ATGATGCCGAAATCGAACAAGAAATTGCATTTTTT AACCAATAGGCCG	PCR–gene disruption
M4R	AGTATCGAAAATGCTGACAGGGTCTGACTCCTCGG AAGCTAAGGATTCAGCACCTTCAAAATTGTGTGGA ATTGTGAGCGGATA	PCR–gene disruption
PORF	GCGCCTGCAGCTTTCCTTAATAATTTCATCT	pMD construction
FGFP	CCCGGGGCACTAGAGATAGCAATG	pAAG construction
RGFP	CCCGGGTTCATTTTTTTTATC	pAAG construction
PAF	TTCTTATTTGCTTTCGGTCGC	Strain construction
TAPF	GCGCAAGCTTATGGTTTCACTTTCTAATTTATTAGT	pTABG construction
TAPR	GCGCGTCGACTTCATTTTTTTTATCAAAATCAAT	pTABG construction
CIP10	GATATCGAATTCACGCGTAG	Strain construction
RP10	GTACATTCCTACTCCGTTCG	Strain construction
M13F	GTAAAACGACGGCCAGT	Plasmid sequencing
M13R	CAGGAAACAGCTATGACC	Plasmid sequencing
HisG1R	GCGCGTGGCGATGCACATGGTC	Strain construction
HisG2F	GCGCGGCGGTTGAGTAGCTCT	Strain construction

II.7. Agarose gel electrophoresis.

DNA fragments were separated by electrophoresis in agarose gel. Samples were mixed (1:5, v/v) with a 10x Bromophenol blue loading dye solution (0.083% bromophenol blue, 0.42% xylene-cyanol, 50% glycerol) and loaded onto 0.7-1.5% (according to the size of the DNA fragments to be separated) agarose gels made in TAE buffer (40 mM Tris base, 20 mM glacial acetic acid, 1mM EDTA) and electrophoresed at 60-90 V (constant voltage). Molecular weight markers (*Eco*RI-*Hin*dIII restricted λ DNA, Roche) were run in parallel to determine the size of the DNA fragments, and after the electrophoresis gels were stained with a solution of ethidium bromide (0.5 mg/ml) and then photographed under UV ilumination (360 nm) using a trans-illuminator coupled to a TDI data processor (Tecnología para Diagnóstico e Investigación; Adislab, Valencia Spain)

II.8. Purification of DNA fragments from agarose gels.

DNA fragments were separated by agarose gel electrophoresis and, after visualization by ethidium bromide staining, the band of interest was excised using a sterile scalpel and the Agarose Gel DNA Extraction Kit (Roche) was used to recover the DNA from the gel according to manufacturer's instructions.

II.9. Purification of RNA.

II.9.1. General remarks on handling RNA.

RNA is prone to degradation with environmental RNases. In order to prevent RNase contamination from hands or dust particles, gloves were always worn when working with RNA. Disposable sterile plastic ware material was preferentially used. Glassware used for RNA work was prepared by rinsing with RNase-free water and heated at 180°C for 4 h. Solutions were made up in RNase-free glassware with RNase-free water, treated with 0.2% (v/v) diethyl pyrocarbonate (DEPC) (Sigma) with vigorous shaking, incubated for 12 h at 37ºC and autoclaved. Electrophoresis tanks and combs were soaked overnight in 1% (w/v) SDS and rinsed thoroughly with autoclaved Milli-Q water. RNA samples were divided into aliquots that were stored at -70°C and kept on ice when thawed.

II.9.2. Isolation of total RNA from *C. albicans*.

Total RNA was purified from yeast and mycelial forms of *C. albicans* using the RNeasy Kit (Qiagen) according to manufacturer's instructions. Cells were grown to stationary phase in 10 ml of growth media (**see section II.2 in this chapter**). One ml of a culture of *C. albicans* cells was added to 100 ml of appropriated growth medium and incubated for gene induction analysis. When the OD_{600} of the culture reached the optimum growth stage, usually between 0.6 to 1.0, cells from 100 ml aliquots were pelleted by centrifugation at 3,000 *x* g for 5 min. Supernatants were removed and cell pellets were flash-frozen in liquid nitrogen.

The sample was immediately grinded thoroughly with a mortar and pestle, or alternatively stored at -70°C. Powder and liquid nitrogen was decanted into an RNase-free, liquid-nitrogen-cooled, 2 ml microcentrifuge tube. Six hundred µl of RLT buffer (containing β-mercaptoethanol) and 600 µl of 70% ethanol were added and the sample was homogenized by pipetting. The mix was added to an RNeasy mini column placed in a 2 ml collection tube, and centrifuged at 8,000 *x* g for 30 s. The flow through was discarded and 700 µl of RW1 buffer were added to the column and the sample was again centrifuged at 8,000 *x* g for 30 s. The flow through was discarded and the column was placed into a new 2 ml collection tube. Five hundred µl of RPE buffer were added and the sample was then centrifuged at 8,000 *x* g for 30 s. This step was repeated twice and then the column was allowed to dry by a final centrifugation step.

The RNeasy column was transferred to a new 2 ml collection tube, 50 µl RNase-free water were added and the column was centrifuged at 8,000 *x* g for 30 s to elute the RNA. Two microlitres of each sample were analyzed by agarose gel electrophoresis to confirm the integrity of RNA. RNA was quantified by UV spectrophotometry as described in **section II.10 of this same chapter**.

II.10. DNA and RNA quantification.

Quantification of DNA was carried out by the measurement of spectrophotometric absorbance at 260 nm (OD_{260}) of DNA samples diluted in sterile distilled water in a Spectrophotometer Lightwave S2000 (WPA; Cambridge, UK).

DNA concentration was calculated on the basis that 1 absorbance unit (AU) at 260 nm is equivalent to 50 μg/ml double stranded DNA. When RNA concentration was calculated 1 absorbance unit was assumed to correspond to 40 μg/ml of RNA. When concentration of a DNA sample was required, the DNA was precipitated by adding NaCl (0.3M, final concentration) and two volumes of cold ethanol. The sample was incubated at -20ºC for 1-2 hours and then centrifuged at 10.000 *x* g for 15 min. Supernatant was washed with 70% (v/v) cold ethanol and air-dried. DNA was resuspended usually in sterile distilled water.

II.11. Restriction enzyme digestion of DNA.

Restriction endonucleases were purchased from Roche Molecular Biochemicals or New England Biolabs, and digestion reactions were performed according to manufacturer's instructions which are summarized in **Table II.6**.

Table II.6. Experimental conditions for DNA digestion with restriction enzymes.

Reaction component	Plasmidic DNA digestion	Genomic DNA digestion
10 x restriction endonuclease buffer (supplied by the manufacturer)	2 μl	10 μl
DNA sample	1-3 μg	20 – 25 μg
Restriction endonuclease	10 U	10 U
Distilled sterile water	Up to 20 μl	Up to 100 μl

When recommended by the manufacturer, bovine serum albumin (BSA, 100 μg/ml, final concentration) was added to the reaction. Digestion reactions were incubated for 2 h at 37ºC for plasmidic DNA digestion or overnight at 37ºC when digesting genomic DNA.

II.12. Ligation of DNA fragments.

Ligation reactions were carried out to subcloning restricted fragments of DNA in plasmid vectors at an insert:vector ratio of 5:1. T4 DNA ligase (Roche) was used to ligate “sticky” and also blunt ended fragments, in a 20 μl volume reaction, containing

the appropriate amounts of insert and vector DNAs, 2 µl of 10 x ligase buffer (supplied by the manufacturer), 1 µl of T4 DNA ligase (1U/µl) and water up to final volume of 20 µl. The ligation mixture was incubated at 4º or 16º overnight.

II.13. Non-radioactive labelling of DNA probes.

DNA labelled probes were generated by random primed incorporation of digoxigenin-labelled deoxyuridine-triphosphate using the Roche Dig DNA Labelling and Detection kit in which the dUTP was linked via a spacer-arm to the steroid hapten digoxigenin (DIG-dUTP). The DNA was labelled by using Klenow enzyme and dNTP labelling mixture following manufacturer's recommendations or PCR amplified by incorporation of DIG-dUTP adding DIG DNA Labelling Mix (Roche) to a 1/10 final concentration of the PCR mix, quantified, and directly used as probe.

II.14. Immobilization of DNA and hybridization analysis.

II.14.1. Transfer of DNA from agarose gels to nylon membranes (Southern blotting).

Southern blot analysis was carried out as described by **Southern (1975)** with the modifications made by **Sambrook *et al.*, (1989)** and **Ausubel *et al.*, (1992)**. Restriction endonuclease digested genomic DNA was subjected to agarose gel electrophoresis as described in **section II.7 of this same chapter.** DNA was depurinated by immersion of the gel in 0.25 M HCl for 30 min, thus reducing the size of the DNA since small fragments are more easily transferred. After this treatment, the DNA was denatured by soaking the gel in 0.5 M NaOH containing 1.5 M NaCl for 30 min. The gel was then rinsed for 30 min with 0.5 M Tris-HCl (pH 7.0), with 1.5 M NaCl, to neutralizate the pH.

DNA was transferred to a Hybond-N positively charged membrane (Roche) by capillary transfer using 20x SSC (0.3 M trisodium citrate with 3M NaCl, pH 7.0) as transfer buffer for 16-24 h. After transfer, the filter was rinsed in 2x SSC to remove excess of salts. DNA was fixed to the membrane by baking at 120ºC for 30 min.

II.14.2. DNA/DNA hybridization and detection.

The hybridization was done according to the protocol described in the DIG DNA Labelling and Detection Kit (Roche). In this method, the filter was prehybridized with 25 ml of hybridization buffer (SSC 5x, 0.1% [w/v] N-laurylsarcosine, 0.02% [w/v] SDS, 2% blocking reagent [supplied by the manufacturer]), for 2 h at 68ºC in a hybridization oven. Next, the solution was replaced with 10-15 ml of hybridization buffer containing freshly heat-denatured labelled DNA and the filters were incubated in this solution overnight at 68ºC. The following day, the filters were washed twice for 5 min at room temperature with a 2x SSC solution containing 0.1% SDS and twice for 15 min at 68ºC with 0.1x SSC plus 0.1% SDS. Filters were then used for detection of hybridised DNA following the manufacturer's instructions. The hybrids were detected by chemiluminiscence using CSPD® (Roche) and processed in a Kodak X-OMAT processor (Eastman Kodak Company, USA).

II.15. Immobilization of RNA and hybridization analysis.

II.15.1. Agarose gel electrophoresis of RNA.

RNA molecules were separated in denaturing agarose gels containing 1.2% agarose, 1x MOPS and 18% formaldehyde. About 10 μg of total RNA were added to a tube containing 2 μl of 10x MOPS, 10 μl of formamide, 3.5 μl of formaldehyde and water to a final volume of 20 μl. Then, 1 μl of an ethidium bromide solution (1μg/ml) was also added to the samples which were incubated at 65ºC for 15 min and subsequently chilled on ice. Loading buffer (1/6 volume) was added before loading the samples onto the gel. A solution of 1x MOPS and 2.75% formaldehyde was used as electrophoresis buffer.

II.15.2. Transfer of RNA from agarose gels to nylon membranes (Northern blotting).

Before the transfer of RNA from the agarose gel to a membrane it was necessary to rinse the gel in DEPC-treated water to remove the formaldehyde. The gel was then soaked in 0.05M NaOH to partially hydrolyze the RNA and improve the efficiency of

transfer. After this treatment, the gel was rinsed again with DEPC-treated water and soaked for 45 min in the transfer buffer (SSC, 10x). The RNA was transferred to a positively-charged nylon membrane (Roche) by capillary diffusion for 16-24 h. After treatment the filter was rinsed in SSC (2x) to remove excess of salts. RNA was fixed to the membrane by heating at 120ºC for 30 min.

II.15.3. RNA/DNA hybridization and detection.

The hybridization was carried out following the instructions given in the same Roche kit used for DNA/DNA hybridizations. The filters were incubated in 25 ml of hybridization solution (50% formamide, 5x SSC, 50 mM K_2PO_4 at pH 7.2, containing 0.1% lauroylsarcosine, 7% SDS, 2% blocking reagent, 50 μg/ml of calf thymus DNA) for 2 hours at 50ºC.

The filters were then hybridized with 5 ml of the same solution containing freshly heated-denatured DIG-labelled DNA, overnight at 50ºC in a hybridization oven. After this, the filters were washed twice in 100 ml of 2x SSC buffer, containing 0.1% SDS, for 5 min at room temperature and twice in 100 ml of 0.2% SSC, with 0.1% SDS for 20 minutes at 65ºC. The hybrids RNA/DNA were detected by chemiluminiscence using CSPD® (Roche) and processed in a Kodak X-OMAT processor (Eastman Kodak Company, USA).

II.16. DNA sequencing.

Sequencing was carried out by the dideoxy-chain termination method of **Sanger *et al*. (1977)** with an Applied Biosystem model 370A automated sequencer following the instructions of the *Taq* DyDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Universal forward and reverse primers (Promega) were used to sequence the various subclones constructed as well as the synthetic oligonucleotide primers deduced from the sequence (Sigma-Genosys).

Sequencing was performed by the faculty internal sequencing service of the Servicio Central de Soporte a la Investigación Experimental (SCSIE) from the University of Valencia.

II.17. Screening of a genomic library.

A genomic library of *C. albicans* (strain WO-1) constructed in the plasmid vector pEMBLYe23 (kindly provided by Dr. B.B. Magee, University of Minnesota, USA) was used to retrieve a genomic clone when screened with a 500 pb DNA fragment from the downstream non-coding region amplified with primers PF2F and PF2R by PCR (**see Table II.5**).

Four 150-mm plates of LB-ampicillin agar, each containing approximately 10,000 colonies, were screened following the methods of **Grunstein and Hogness (1975)** and **Hanahan and Meselson (1983)**. A nitrocellulose filter was placed on the agar surface of each plate for 5 min and, after labelling the position and orientation of the filters on the plates, the filters were carefully removed from the plate, air-dried, placed colony side up on a stack of three sheets of Whatman 3MM paper that were saturated with denaturing solution (0.5 M NaOH, 1.5 M NaCl), and incubated for 5 min. The filters were then transferred to a similarly prepared stack of three sheets of 3MM paper saturated in neutralizing solution (0.5 M Tris-HCl, pH 7.0, 1.5 M NaCl). After 5 min, the filters were transferred to a stack of 3MM paper saturated in 6x SSC solution for 2 min. The filters were then air-dried, and baked at 120ºC for 30 min. The DNA hybridization and detection was performed as described in **section II.14.2 of this same chapter.**

II.18. Transformation of *E. coli*.

II.18.1. Preparation of competent cells.

Competent DH5α cells were prepared by following the method developed by **Mandel and Higa (1970)**, based on soaking the cells in a cold $CaCl_2$ solution. From an overnight culture of *E. coli* in LB medium, a 0.5 ml aliquot was inoculated into a flask containing 100 ml of LB medium prewarmed at 37ºC, and the culture was incubated with shaking until the OD at 600 nm reached a value between 0.5-0.6. Cells were collected at 7,000 rpm for 10 min and resuspended in 20 ml of cold 50 mM $CaCl_2$ solution and kept on ice overnight. Cells were then collected by centrifugation as indicated before, resuspended in 2 ml of the 50 mM $CaCl_2$ solution and used immediately in 200 µl aliquots to transformation experiments.

II.18.2. Transformation of competent *E. coli* cells.

To transform *E. coli*, 200 µl of competent cells prepared as indicated above (see the previous section), or Subcloning Efficiency® DH5α™ Competent Cells (Invitrogen, USA) were added to the ligation mixture (**see section II.12 in this same chapter**) and kept on ice for 30 minutes. Cells were then heat shocked for 1 min at 42ºC, placed on ice for 2 min, suspended up to 1 ml in LB medium and incubated for 1 hour at 37ºC. Cells were pelleted by centrifugation at 3,000 *x* g for 3 min and resuspended in LB medium. Aliquots of cell suspensions were spread onto LB plates supplemented with ampicillin (100 µg/ml). When the white-blue selection was possible (**see section II.3 in this same chapter**) to distinguish the colonies transformed with recombinant plasmids from the ones transformed with the plasmid itself, 100 µl of 100mM IPTG and 20 µl of X-Gal solution (50 mg/ml) were added to the plates. Colonies were subcultured onto LB agar supplemented with ampicillin for further investigation.

II.19. Transformation of *C. albicans*.

C. albicans transformation protocol was carried out accordingly to the lithium acetate procedure described by **Gietz *et al*. (1995)** with some modifications. Cells were grown in 100 ml of YPD medium supplemented with uridine (25 µg/ml) to reach an OD_{600} from 1.0 to 3.0. After that, cells were pelleted by centrifuging at 3,000 *x* g for 5 min and washed once in 20 ml of sterile LiOAc/TE solution (5 ml of 1M LiOAc, pH 7.5; 5 ml of 10x TE, pH 7.5; 40 ml distilled water) and resuspended in this solution to about 3 times the volume of the pellet (1-2 ml). To 100 µl aliquots of competent cells, 10 µl of a freshly cooked (over 95 ºC; over 5 min) calf thymus DNA carrier (Sigma) solution (10 mg/ml) and 10-50 µg of transforming DNA fragment were added, mixed thoroughly and added to 700 µl of polyetilenglycol (PEG) mix (21 g of PEG 3350 (Sigma); 30 ml of distilled water; 5 ml of 1M LiOAc, pH 7.5; 5 ml 10x TE buffer, pH 7.5). The mixture was incubated for 3 h at 30ºC with vigorous shaking and heat shocked at 42 ºC for 45 minutes in a water bath.

Finally, cells were pelleted by brief (10-30 sec) centrifugation, resuspended in 200 µl of YPD and plated directly onto selective YNB plates and incubated at 30ºC for

at least 3 days. Spontaneous Ura^- derivates from an Ura^+ independent clone were selected on YNB medium containing 5'-fluoroorotic acid (5'-FOA) to a final concentration of 0.1% (w/v) and 25 μg/ml uridine, and used then in the second step of the disruption procedures.

II.20. Solubilization of cell components from *C. albicans*.

Different subcellular fractions (cell wall extracts, membranes, cytosol, and microsomal particulate material; see below) were obtained following the protocol described by **Lopez-Ribot *et al*. (1994)**. Cells grown in liquid Lee's medium overnight at 30ºC, were collected by centrifugation (4,000 *x* g, 5 min) and washed twice with chilled 10 mM Tris-HCl buffer, pH 7.2, containing 1 mM PMSF (phenylmethylsulfonyl fluoride) (buffer A) and broken by vigorous shaking in the presence of glass beads (425-600 microns; Sigma). The procedure resulted in complete cell breakage as assessed by examination of the preparation in a phase-contrast microscope. The cell walls were sedimented (4,000 *x* g for 10 min) from the whole cell-free homogenate, washed four times with chilled buffer A, then boiled for 5 min with 2% SDS in glass-distilled water to remove non covalently bound proteins, and finally washed four more times with buffer A. After sedimentation, the purified cell walls were digested in buffer A containing 0.5 mg/ml of Zymoliase 20T (ICN Biomedicals, USA) for 3 h at 28ºC. After treatment, the wall residue was removed by centrifugation (3,000 *x* g, 30 min) and discarded. The solubilized material was concentrated by freeze-drying. The supernatant fluid obtained subsequently to cell breakage (after cell wall sedimentation) was centrifuged at 40,000 *x* g for 40 minutes to obtain a mixed membrane fraction (P40), and the resulting supernatant was then centrifuged at 100,000 *x* g for 1 hour to obtain a microsomal fraction (P100). The supernatant obtained (cytosolic fraction) was kept. All samples were stored at –20ºC until further analysis.

II.21. Determination of protein and total sugar content in the samples.

Protein concentration was determined by the method of **Lowry *et al*. (1951)**, using BSA as standard. Total sugar content was measured according to the procedure described by **Dubois *et al* (1956)**, with glucose as the reference standard.

II.22. SDS polyacrilamide gel protein electrophoresis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions was performed basically as described by **Laemmli (1970)** using linear gels. Gels were run in a vertical Hoeffer cell (Tall Mighty Small model). Polyacrilamide linear (12%) resolving gels (with a acrylamide:bisacrylamide ratio of 30:0.2) were prepared in 0.37 M Tris-HCl buffer, pH 8.8, with 0.15% SDS. The stacking gel contained 6% acrylamide (30:0.2 of acrylamide:bisacrylamide) in 0.25 M Tris-HCl buffer, pH 6.8, containing 0.2% SDS). In all cases before loading the samples 1/2-1/5 volume of a loading buffer (0.5 M Tris-HCl buffer, pH 6.8, containing 35% glycerol, 7% SDS, 0.035% bromophenol blue, 14% β-ME) was added to the samples which were then boiled in a water bath for 10 minutes. Commercial molecular weight markers (Invitrogen) were run in parallel in order to determine the size of unknown polypeptides. The electrophoresis buffer was 25 mM Tris-HCl buffer, pH 8.3, containing 0.2 M glycine and 0.1% SDS). Electrophoretic run was performed at a constant voltage (100 volts).

II.23. Protein staining on polyacrilamide gels: Coomasie Blue staining.

In order to visualize the electrophoretically separated polypeptides in the polyacrilamide gels, gels were stained for 45 min with a solution containing 0.2% Coomassie Blue G-250 (Sigma) in a mixture of methanol:acetic acid:water (40:10:50) at room temperature with gentle shaking, followed by destaining in methanol:acetic acid:water (10:10:80) at room temperature to remove the blue background.

II.24. Western immunoblotting.

Proteins separated by SDS-PAGE were transferred (Western blotting) to a nitrocellulose membrane (BioRad, USA). The membrane was placed in contact with the linear polyacrylamide gel and sandwiched between two layers of 3MM (Whatman) paper and two sponges soaked with transfer buffer (25 mM Tris, 0.2 M glycine, pH 8.3, 20% methanol). The resulting sandwich was encased within a plastic cassette and placed vertically into a Western blot electrophoresis tank (BioRad) containing transfer buffer. Western transfer was achieved by applying a constant voltage (100 volts). Blotted polypeptides were immunodetected according to the following protocol. After transfer, membranes were blocked in a solution containing 3% BSA in TBS buffer for 1 h at 37 ºC. Blocked membranes were incubated in an antibody solution overnight at room temperature using the primary specific antibodies (PAb anti-gt and PAb anti-Abg1p; see below) diluted (1:1,000 and 1:500 respectively) in 0.01 M Tris-HCl buffer (pH 7.4), containing 0.9% NaCl, 0.05% Tween 20, and 3% BSA as a blocking agent. Peroxidase-conjugated secondary antibodies (Bio-Rad) were used at 1:2,000 dilution. After washing the filters 4 times in TTBS, immunoreactive species were detected by the enhanced chemiluminiscent (ECL) method from Amersham Biosciences, following the manufacturer's instructions.

II.25. Polyclonal antibodies.

An antibody recognizing Abg1p protein was obtained from Sigma-Genosys, by using a synthetic peptide selected from the deduced amino acid sequence from *ABG1* gene. PAb anti-Abg1p was raised against a 16-mer residue (ISSKRDKQSDNENDAC) derived from the N-terminal domain of Abg1p. This peptide was chosen because it was likely to be in a hydrophilic, surface-exposed region of the mature, folded protein as predicted by secondary-structure algorithms (**Hopp and Woods, 1981**). Titer of antibodies was assessed by E.L.I.S.A. Sera samples were stored in small aliquots at -20ºC until further use.

The germ tube specific polyclonal antibody (PAb anti-gt) against purified walls from mycelial cells of *C. albicans* was obtained as described previously (**Casanova *et al*., 1989**).

II.26. Enzyme-linked-immunosorbent assay (E.L.I.S.A).

Enzyme-linked immunosorbent assays were carried out as described by **Voller *et al*. (1980)** with some modifications. The assays were performed in microtiter polystyrene plates (Nunc, Denmark).

Wells in the microtiter plates were coated with different amounts of the antigen dissolved in coating buffer (50 µl per well). After this, the plates were incubated at 4ºC for 12-14 h and then washed three times with PBST buffer (0.01M phosphate buffer saline pH 7.4, containing 0.15M sodium chloride [PBS] plus 0.05% Tween 20). The plates were then incubated with a blocking solution (200 µl per well) for 1 h at 37ºC. Next, after washing the wells 3 times with PBST buffer, 50 µl of the solutions containing different concentrations of the antibody to be tested were added to each well. The plates were incubated at 37ºC for 1 h and then washed 3 times with PBST buffer.

Next, 50 µl per well of goat anti-rabbit immunoglobulins coupled to horseradish peroxidase (HRP, diluted 1:1,000) were added and the plates were incubated at 37ºC for 1 h. The wells were subsequently washed and the antigen-antibody-HRP antibody complexes were detected by adding to each well 50 µl of peroxidase substrate and incubating in dark for 10 min. The reaction was stopped by adding 25 µl per well of 3M H_2SO_4. The colour intensity was measured at 492 nm in a Labsystem Multiskan MS Version 8.0 Spectrophotometer. Composition of the different solutions and buffers used for E.L.I.S.A. are listed in **Table II.7**.

Table II.7. Reagents used in E.L.I.S.A.

Solution	Composition
Coating buffer	30 mM sodium carbonate pH 9.6
Blocking solution	3% BSA in PBS buffer
1st Ab solution	1% BSA in PBST buffer
2nd Ab solution	1% BSA in PBST buffer
Peroxidase substrate	20 mg o-phenilendiamine (Sigma); 12.2 ml 0.1 M citric acid; 12.8 ml 0.2 M Na_2HPO_4; 25 ml H_2O; 20µl H_2O_2

II.27. Sensitivity testing.

For sensitivity assays, plates containing solid (1.5% agar) YNB medium plus 2 mM methionine and 0.5 mM cysteine were used. Stock solutions of the different agents to be tested were prepared, filter sterilized and added to pre-warmed medium at an appropriate range of concentrations (**see Table II.8**). The control plates contained media from the same batch as the sensitivity testing plates. Five µl samples from serial suspensions of a *C. albicans* stationary phase culture, containing from 10^6 to 10^2 cells/ml were spotted onto the agar surface, allowed to dry and incubated at 28ºC and 37ºC.

Table II.8. Agents used in sensitivity tests.

Agent	Stock solution	Final concentration tested
Congo Red (Sigma)	10 mg/ml	50, 100 and 200 µg/ml
Calcofluor White (Sigma)	10 mg/ml	125 µg/ml
SDS (Sigma)	10 % (w/v)	0.025%
Zymoliase 20T (ICN)	10 mg/ml	5 and 10 µg/ml
Sodium Chloride (Panreac)	4 M	1 M
Calcium Chloride (Sigma)	2 M	0.2 M
Lithium Chloride (Sigma)	3.5 M	35 mM
Caffeine (Sigma)	200 mM	12 mM and 20 mM
H_2O_2 30 % (v/v) (Sigma)	---	6 mM
Glycerol (JTBaker)	---	2.5 M

II.28. Cell staining procedures.

II.28.1. DAPI staining.

Nuclei staining of yeast and hyphal cells was carried out by using the fluorescent dye DAPI (4´,6-diamidino-2-phenylindole). This dye was described by **Williamson and Fennell** in 1975. Appropriate culture volumes containing about 10^8 cells were centrifuged to sediment the cells. Pelleted cells were resuspended in 50 µl of water and permeabilized in 95% (v/v) ethanol for 5 min and stained with 2 µl of a DAPI stock solution (1 mg/ml in water). After adding the dye, the sample was incubated for 20 min in the dark. Cells were washed several times with water and resuspended at a final concentration of 10^8 cells/ml. Drops (10 µl) from this cell suspension were placed on a glass slide, covered with a cover glass slip, and examined with a Nikon Eclipse E800 microscope, equipped for epifluorescence.

II.28.2. Calcofluor White staining.

Chitin localization was assessed by Calcofluor White staining as described previously (**Pringle, 1991**) with some modifications. Briefly, cells were pelleted from aliquots of a liquid culture media by centrifugation, resuspended in a Calcofluor White solution to give a final concentration of 100 µg/ml and incubated for 20 min in the dark. After staining, cells were washed several times with water and resuspended at a final concentration of 10^8 cells/ml. Microscopy observations were performed as described in the previous section.

II.28.3. FM4-64 staining.

Vacuoles of yeast and hyphal cells were stained with the vital stain FM4-64 (Molecular Probes, USA) as described by **Vida and Emr (1995)**. Cells were grown overnight until early exponential phase. FM4-64 dye was added to 1 ml of culture to give a final concentration of 20 µM. Cells were incubated for 30 additional min in the dark with shaking and after washing them several times in water vacuoles were visualized by using a Nikon Eclipse E800 microscope equipped for epifluorescence.

II.29 Transcription profiling analysis with DNA microarrays.

II.29.1. Isolation of total RNA (Trizol® procedure).

Cells were grown to stationary phase in 10 ml of growth medium. One ml of this *C. albicans* culture was added to 100 ml of the appropriate growth medium and incubated for gene induction analysis. When the OD_{600} of this subculture reached the optimum growth point, usually between 0.6 to 1.0, cells (100 ml of culture) were pelleted by centrifugation at 3,000 *x* g for 5 min. Supernatants were removed and cell pellets were resuspended in the remaining small volume of supernatant, and then small drops of this cell suspension were released into liquid nitrogen. The frozen cell drops were transferred to a Teflon vessel with a 7 mm tungsten carbide bead (both pre-cooled in liquid nitrogen) and shaked in a Micro-Dismembrator for 2 min at 1,200 rpm. After shaking, the resulting powder was transferred into an Eppendorf tube, immediately resuspended in 2 ml of Trizol® and vortexed for 1 min. Samples were then incubated for 5 min at room temperature (to allow dissociation of nucleoprotein complexes), and subsequently centrifuged for 10 min at 12,000 *x* g. The supernatant obtained was transferred to a new Eppendorf tube and 0.4 volumes of chloroform were added, followed by shaking of samples by hand for 15 s. Samples were incubated 10 minutes at room temperature and then centrifuged for 5 min at 12,000 *x* g. The top layer was transferred into a new Eppendorf, 0.5 volumes of isopropanol were added (to precipitate DNA) and samples were incubated 15 min at room temperature, and subsequently centrifuged for 10 min at 12,000 *x* g. The supernatant was discarded and the pellet was washed with 1 ml ice-cold 70 % ethanol and centrifuged for 10 min at 12,000 *x* g again. The pellet was allowed to air dry and then resuspended in 500 µl of DEPC-treated water and 500 µl of LiCl buffer, and incubated at -20ºC overnight. The following day, samples were centrifuged for 30 min at 12,000 *x* g, and pellets were washed twice with 1 ml of ice-cold 70% ethanol, and allowed to air dry for 15 min. Finally pellets were resuspended in 50 µl of DEPC-water, and the RNA concentration determined (**see section II.10 of this chapter**).

II.29.2. Preparation of labelled cDNA probes.

Synthesis of cDNA was carried out by a direct labelling reaction of reverse transcription. RNA samples were thawed in ice. To a total amount of 15 µg of RNA, 1 µl of RNAsin (Promega) and DEPC-treated water up to 20 µl were added. This mix was incubated at 42ºC while the labelling master mix reaction was prepared in an ice bath by mixing the reagents described in the **Table II.9**.

Table II.9. Reagents used in the cDNA labelling reaction.

Reagent	Quantity
5x First-strand buffer	8 µl
C. albicans specific primer mix (Eurogentec, Belgium)	1 µl
Oligo dT (12-18) (0.5 µg/µl)	1 µl
dNTP's – dCTP (10 mM)	1 µl
dCTP (1 mM)	1 µl
Cy3 or Cy5 (1 mM) (CyDye™ Fluorescent nucleotides, Amersham)	1,5 µl
DTT (0.1 M)	4 µl

The RNA solution was added to the labelling master mix and subsequently incubated for 5 min at 65ºC, and for 5 min at 42ºC, trying to keep the samples hidden from light as much as possible. Then, 2 µl of Superscript reverse transcriptase (Invitrogen) were added and the samples were incubated for 2 h at 42ºC. To stop the reaction, 5 µl of 50 mM EDTA, pH 8,0, and 2 µl 10 N of NaOH were added and samples were incubated for 20 min at 65ºC. Four µl of 5M acetic acid were added to the mixture after the final incubation step.

II.29.3. Probe clean-up, quantification and concentration.

cDNA probes were cleaned-up by using the Qia-quick PCR purification kit (Qiagen) according to the manufacturer's instructions. Quantification of the probes was assessed by determining the frequency of incorporation (f.o.i) according to the formula:

f.o.i.= pmol of dye incorporating x 324.5/ amount of cDNA (ng), where, pmol of dye incorporating (Cy3)= OD_{550} x (total volume of the probe [µl])/ 0.15; pmol of dye incorporating (Cy5)= OD_{650} x (total volume of the probe [µl])/ 0.25; and amount of probe: OD_{260} x 37 x total volume of the probe (µl)= ng of probe.

Probe concentration was performed prior to hybridization by applying the sample to an Amicon cell using a microcon-30 filter (Amicon).

II.29.4. Hybridization of glass slide microarrays.

For hybridization of a glass slide microarray, 5 µl of each Cy3 and Cy5 cDNA probes were mixed and 5 µl of heat-denatured salmon-sperm DNA (10 mg/ml) were added. The mix was then incubated for 2 min at 95ºC and quick chilled on ice. 40 µl of hybridization buffer (Eurogentec) were added to the probes prior to injection in the glass slide (covered with an appropriate coverslip (Lifterslips 25x44 mm, Erie Scientific). Microarrays were then incubated overnight in a Hybridization chamber (Corning) at 42ºC. The following day, the coverslip was removed by dipping the array in 0.2x SSC containing 0.1 % SDS and washing for 5 min with occasional agitation at room temperature. Then the slide was rinsed with 0.2x SSC for 5 min with occasional agitation at room temperature, and spin-dried at 500 rpm for 5 min.

II.29.5. Data analysis.

Scanning of the microarrays were performed in a GenPix scanner (Axon Instruments, USA) and statistical analysis of the data were carried out with the software GeneSpring, taking into consideration those genes that were 1.5-fold overexpressed or repressed in the mutant strain with respect to the parental strain.

II.30. Protein A-tagging and purification of native macromolecular complexes from *C. albicans*.

Tagging of Abg1p protein was achieved by using a new vector, pACT-C-ZZ (**see section II.3 of this chapter**), and protein A-tagged native complexes were purified on IgG-Sepharose and eluted intact from the resin by treatment with the tobacco etch virus (TEV) protease (Invitrogen, California, USA). Cells were grown in YPD medium (2 liters) overnight at 30ºC. The following day cells were collected by centrifugation (4,000 *x* g, 5 min), washed twice in water, and resuspended in one volume (in ml), equal to the weight (g) of the cell pellet, of buffer A (0.15M potassium acetate [KOAc], pH 7.4; 20 mM Hepes-KOH, pH 7.4; 2 mM magnesium acetate [MgOAc]; 1 mM EDTA; 1 mM EGTA; 1mM PMSF; 2mM DTT; 2μM pepstatin; 1 tablet of protease inhibitors per 50 ml of buffer solution) pre-warmed at 37ºC. Distinct buffer A formulations, differing only in the concentration of KOAc, were used when indicated. Then, the cellular suspension was transferred to a bead-beater (Biospec), 1/3 filled with glass beads (0.5mm), and with the outer chamber of the bead beater filled with ice. Cells were lysed using eight pulses of 1 min each, with pauses of 2 min between pulses, and the cellular lysate was transferred to 50 ml tubes and centrifuged at 4,000 *x* g for 10 min. The supernatant was removed and kept on ice. The pellet was resuspended in 0.5 volumes of buffer A and subjected to a second round of homogenization in the bead beater. The homogenate was centrifuged again, and the resulting supernatant was pooled with the first supernatant obtained. Then, a stock solution of 4M KOAc was added to the supernatants to give a final concentration of 0.5M, assuming that the initial concentration of the sample was 0.1M. The sample was then incubated overnight in batch at 4ºC with 0.5 ml of IgG-Sepharose per 50 ml of lysate.

The next day, the resin was packed into a Poly-Prep Chromatography column (Bio-Rad), and washed with 30 ml of buffer A (0.5M KOAc plus 14% glycerol) and after with 30 ml of buffer A (0.15M KOAc plus 14% glycerol). All washing were done at 4ºC. The column was placed at room temperature and 1 ml of buffer A (0.15M KOAc) containing 200 U of TEV protease, pre-warmed at 37ºC was added, and the sample was incubated at room temperature for 1 hour.

After the digestion with the protease the protein A-tagged complex was eluted with 2 ml of a 0.5M KOAc solution. The eluate was dialized overnight against distilled water and freeze-dried prior to be subjected to SDS-PAGE and subsequent peptide mass fingerprinting analysis. SDS-PAGE was carried out as described in section **II. 22 of this chapter**. Peptide mass fingerprinting analysis was done in the laboratories of Dr. Ángel Domínguez Olavarri (Universidad de Salamanca, Spain), and Dra. Concha Gil García (Universidad Complutense de Madrid, Spain)

II.31. Sensitivity to antifungal agents.

Etest® strips (AB BIODISK, Solna, Sweden) were used to evaluate the antifungal susceptibility of the *Abg1*Δ mutants compared with a control strain. Isolated colonies of the different strains from overnight cultures on Sabouraud dextrose agar plates were resuspended in a 0.85% solution of NaCl to get a final cell density corresponding to 0.5 in the McFarland scale of optical density. A sterile swab was dipped into the inoculum suspension and pressed against the inside of the tube. Then, the entire surface of plates with RPMI 1640 agar was swabbed, rotating 90º in three directions. The excess of moisture was allowed to absorb for about 10-15 min prior to apply the Etest® strips. Amphotericin B, Fluconazol, Itraconazol, and Voriconazol strips were applied into the inoculated agar surface, ensuring that the MIC scale was facing upward and also that the whole length of the strip was in full contact with the agar surface. Once the strips were applied, plates were incubated at 35ºC.

The MIC value was read 48 h after inoculation, and was determined by measuring the point of intersection between the inhibition zone edge and the Etest® strips. For amphotericin B and itraconazol the point of complete inhibition of growth was used.

For fluconazol and voriconazol, the MIC was read at the first point of significant inhibition or marked decrease in growth density, using the principle of 80% inhibition to visually select the end point. Sensitivity studies were kindly performed by Dra Emilia Cantón, from Unidad de Microbiología Experimental, Centro de investigación, Hospital Universitario La Fe, Valencia (Spain).

Interpretation of results was achieved by following the next guidelines (provided by the manufacturer), which uses strain *C. albicans* ATCC90028 as reference:

Antifungal	**Quality control (48h) MIC (µg/ml)**
Amphotericin B	0.125-0.5
Fluconazol	0.125-0.5
Voriconazol	0.004-0.016
Itraconazol	0.064-0.25

III. RESULTADOS

III.1. Aislamiento y caracterización de un clon de ADNc que codifica para un péptido inmunorreactivo con el anticuerpo policlonal PAb anti-gt.

III.1.1. Inmunorrastreo de una genoteca de expresión con el anticuerpo policlonal PAb anti-gt.

Dentro del objetivo general perseguido por nuestro grupo de investigación consistente en el aislamiento de genes de *C. albicans* que codificaran proteínas relacionadas con la transición morfológica en este hongo, se procedió a realizar el inmunorrastreo de una genoteca de expresión mediante el uso de un anticuerpo policlonal específico de la forma micelial de *C. albicans* (PAb anti-gt).

El anticuerpo policlonal (**sección II.25 de Materiales y Métodos**) fue obtenido tras inocular paredes purificadas de células en forma micelial de *C. albicans* a conejos. El suero obtenido se adsorbió a células levaduriformes no viables de *C. albicans* con la finalidad de eliminar aquellos anticuerpos que reconocieran antígenos presentes también en las paredes de las levaduras (**Casanova *et al*., 1989**).

La genoteca de ADNc se obtuvo a partir de ARNm procedente de células de *C.albicans* ATCC 26555 después de inducir durante 30 minutos la formación de tubos germinativos por choque térmico de células sometidas a ayuno metabólico según el método descrito por **Casanova *et al*.,** (**1989**).

El inmunorrastreo de la genoteca de expresión con el PAb anti-gt (**sección II.4.1 de Materiales y Métodos**) condujo al aislamiento de once clones inmunorreactivos frente a dicho anticuerpo. La reactividad de estos clones se confirmó realizando un segundo inmunorrastreo, empleando esta vez títulos más bajos de la genoteca (30-50 ufc/placa). Utilizando los cebadores universales "forward" y "reverse" del fago λgt11 (**Tabla II.5**) se amplificaron por PCR los insertos contenidos en los once clones, que fueron posteriormente separados electroforéticamente en un gel de agarosa al 0.8 % que se muestra en la **Fig. III.1**. Los once clones aislados dieron lugar a un único fragmento, tras su amplificación por PCR con los cebadores universales del fago λgt11, excepto el clon 7, que dio lugar a dos fragmentos, uno de 200 pb y otro de 800 pb, escogiéndose el de mayor tamaño para su posterior análisis.

III.1.2. Análisis de los clones inmunorreactivos frente al PAb anti-gt.

Los insertos de ADNc contenidos en los once clones inmunorreactivos fueron secuenciados utilizando los cebadores universales "forward" y "reverse" del fago λgt11. Las secuencias obtenidas fueron analizadas en las bases de datos de *C. albicans* mediante los programas BLASTN y TBLAST, analizando la localización de las secuencias en los contigs en la página http://www.sequences.stanford.edu8080/bncontigs.htm/ y las posibles homologías en la página http://alces.med.umn.edu/gbsearch/ybc.htm/. Los resultados obtenidos tras la consulta en las bases de datos se muestran en la **Tabla III.1**. La búsqueda de las secuencias de los clones recombinantes inmunorreactivos en las bases de datos mencionadas anteriormente dio como resultado la identificación de cinco de los once clones como genes ya caracterizados, mientras que las secuencias de los clones 1, 4 y 11 correspondían a posibles genes todavía no identificados en *C. albicans*.

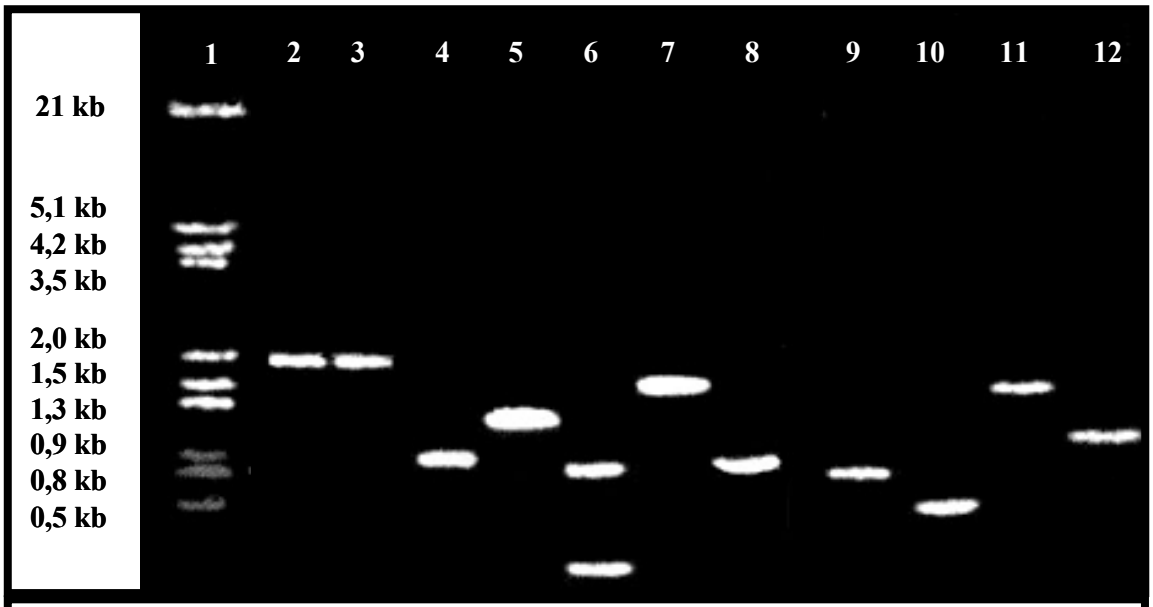

Figura III.1. Amplificación por PCR de los once clones recombinantes inmunorreactivos frente al PAb anti-gt. Los insertos de los clones recombinantes fueron amplificados por PCR mediante el uso de los cebadores universales del fago λgt11. En la calle 1 se muestra ADN del fago λgt11 digerido con *Eco*RI y *Hind*III, que se utilizó como marcador de peso molecular.

Tabla III.1. Características de los clones recombinantes inmunorreactivos frente al PAb anti-gt.

Clon	Calle en la Fig. III.1	Tamaño del inserto	Secuencia con mayor homología	Posible asignación	Contig
Clon 1	2	1.8 kb	384037g10.S1.Seq	ninguna	4-3030
Clon 1'	3	1.8 kb	384037g10.S1.Seq	Idéntico clon 1	4-3030
Clon 2	4	0,95 kb	385020H07.x1.Seq	*NAG1* (*G6PDH*)	4-2984
Clon 4	5	1.2 kb	396005H10.S1.Seq	ninguna	4-3095
Clon 7	6	0,8 kb	396058C08.S1.Seq	*PFK1*	5-3141
Clon 7'	7	1.5 kb	YSAENO1A	*ENO1*	5-2440
Clon 10	8	0,9 kb	AF149421	*TSA1*	5-3237
Clon 11	9	0,83 kb	38501H10.X1:Seq	Ag2 *Coccidiodes immitis*	4-1570
Clon 14	10	0,55 kb	AF149421	Idéntico clon 10	5-3237
Clon 15	11	1,5 kb	YSAENO1A	Idéntico clon 7'	5-2440
Clon 15'	12	1 kb	384024A12.S1.Seq	*THI5*	5-3546

El ADNc contenido en el clon 4 presentó una identidad del 99% con una secuencia presente en el contig 4-3095. La secuenciación con los cebadores universales del fago λgt11 mostró que este clon contenía una pauta abierta de lectura incompleta, presente en el citado contig. Las secuencias obtenidas tras secuenciar con los cebadores directo y reverso se muestran continuación:

- Cebador "forward" λgt11:

5'GGAATTCGCGGCCGCACGGAGCACTAGAGATAGCAATGGTTCACTTTCTAATTT
ATTAGTGTGTTTGGGATTAGTAGTAGTTCCAACTACCCTTGCATTTTCTGATACTG
CACCAATTATCATTCATTCCAACAACCAGGATGCGCTTGAAAGCAACCATAAATAT
ATCACAAAATTTGTTGATGTTAAATCAAAAGTGGATGAAATCATTAAGGAAAGCTG
TAGTGACAAGAACAGCAAGTTATTTATTTATCAAATCGAGGATTTAAGTATTGAAT
CTGATTATTCTTGGTTGTTGAACAAAGGGAGCTATCCTAATGTATTATATCATGAA
AAAAATGAAGCCAACTACGATTTTAATGGTAAATGTGAAAAAAAGGAATCTGGTAC
GGTTCAATTTAGTGTTTTGAAGACTGGCTCATTGCAAGAGTATACGAAAAGCATAA
AGAAAACAAAAACGAGGTATTTATTATCCAAGTATTACCTAAGTTCACCAGTGGTC
ATTCATTAAAGGCTATTAAGGAAAAAATGTACAACTTATACAATGATGAAGATATT
GTGATCAGTGGCAAAAGAGATAAACAAAGTGACAATGAAAATGATGCCGAATCGAA
CAAGAAATTGACGTGATTTGAAGTTGCTGAATCCTTACTTCCGAGGAGTCAGACCC
TGCAGCATTTCGATCTG 3'

- Cebador "reverse" λgt11:

5′CGGCGCTCAGCTGGAATTCGCGGCCGCTTTTTTTTTCCTTATAAAATAATTCCT
TATGTATATTAAAAAAAAAAAGAAANCAANCAGGGAANGCGAATNCTTTTCTNGGT
CCTAACAGTTCTTTAGTATNCGTTCTTTTTTTTTCNGNCTATAGNGGGGAANANAA
CANCAATTCANGNCCANAANCCAAAAGAAACCTTTTTCTTTATTCATTTTTTTNAT
CAAAANNAATTNGTTTTTCAANCGNGGNNTAAGGGATTNCCAANCTANAAAGCCNN
CNCAAANCNTNGGCCAAAAAANCCAANAAAAANCCNGAAAGGNNGATNCCNGNCCA
AATCCCGGANGNAAAAAANGGATNTTTNGNAAAAAAATNATCAGGNTTAACTNCNG
GCCCNTTNTTATTTNGNTTATNAACANCGAAAAGGCNGACNGGGGNNGACTCCNCG
GAAGCTAAGGATTCNGCAACTTCAAAACCNCNCCCAATTTCTNGTNCNGATTTNGG
CANCATTTNCATTGGCNCTTNGNTAATCCCTTTGGCCCCNGANCACAAAATCTNCA
TCATTGGATAAGG 3′

El clon 4, que contenía un inserto de 1,2 kb, fue seleccionado para el presente trabajo ya que presentó un marco abierto de lectura incompleto, que codificaba para un péptido putativo de 288 aminoácidos, del cual se desconoce la función en *C. albicans* y que no presentaba homología con genes de mamíferos, lo que hacía en principio interesante la caracterización molecular y funcional de este péptido putativo para este hongo patógeno oportunista.

El contig 4-3095 (posteriormente designado 5-3295) está constituido por 7051 pb, mostrándose a continuación un fragmento de 1900 pb, que incluye la pauta abierta de lectura contenida en el clon 4:

2401 agaaattagactctcgatgagatgggcaagtttttcttttatgaagaga
2451 ccatccctctaggtaaaccactggtatataacctatacaattaaaagaac
2501 caagtaataacaatggtatctgtgaataagagcaagaaactgactagtat
2551 aaagcaaactaaccctaatactgaaaaaaagaagactgccaaagtggtga
2601 tagaagaggaagagcaagaccaatccaattcatctagtcaagaagtggaa
2651 agtgaagatgaaagtgattcagaattggaagtagcagataatgatgattt
2701 agatcaggaaataggatctgatgatgaagttaatattgcagatgcttcat
2751 catccgaagggtcggacgcaagcgaagatgaaaatgaaaatgaagaagag
2801 gattttccaaaactaaagaagagaaaaacaaagtctacagaagacgggtc
2851 tgaatcttttgctgatgccttgaactctattgttaactcaaaactaaaag
2901 catacgatagaaaggatccaattttggctcgaaataaagtcactttaaag
2951 aagttagaactggataaattggaaatgaaggccaagagagccttattgca
3001 agaaaagaaagtgttacatgacaacgccagagttaaaaacttgttgccaa
3051 caagtaatgaaccggagaaagttcgtcaagtgattgaaaaggaaaaggca
3101 ttgaaaaaagttgcacaacgtggtgttgtcagattgtttaatgcagtgtt
3151 atccacgcaaatcaaaaccaatcaagaagtcaataaggagaagttgggac
3201 aaaccaaaaaggaagaaataatgaatgaagtatctaaaaacaaattcttg
3251 gatctaattgcagctgctggtaatgaataatatagattgttgatttgtat

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3301 agattttataggaaattactgaactggatattacaagattgtctttgttg
3351 agtttctctaacgactgcaagtatcttcaaatgcacaatgccttttttatc
3401 atctgttcacttttataaaacttccaaccataataaaatgtttcaatttc
3451 ctgtatataaactatcaacaaattatttttttgaaattctgtccaaactcc
3501 aacaactgggcgaacaacgaggtaacaaaaaaaaaaatcactcctagtaa
3551 actgccgttaaccaatcactgacgccatacatatcattagtatagttata
3601 tacggagcactagagatagcaATGGTTTCACTTTCTAATTTATTAGTGTG
3651 TTTGGGATTAGTAGTAGTTCCAACTACCCTTGCATTTTCTGATACTGCAC
3701 CAATTATCATTCATTCCAACAACCAGGATGCGCTTGAAAGCAACCATAAA
3751 TATATCACAAAATTTGTTGATGTTAAATCAAAAGTAGATGAAATTATTAA
3801 GGAAAGCTGTAGTGACAAAAACAGCAAGTTATTTATTTATCAAATCGAGG
3851 ATTTAAGTATTGAATCTGATTATTCTTGGTTGTTGAACAAAGGGAGCTAT
3901 GCTAATGTATTATATCATGAAAAAAATGAAGCCAACTACGATTTTAATGG
3951 TAAATGTGAAGAAAAGGAATCTGGTACGGTTCAATTTAGTGTTTTGAAGA
4001 CTGGCTCATTGCAAGAGATATACGAAAAGCATAAAGAAAACAAAAACGAG
4051 GTATTTATTATCCAAGTATTACCTAAGTTCACCAGTGGTCATTCATTAAA
4101 GGCTATTAAGGAAAAAATGTACAACTTATACAATGATGAAGATATTGTGA
4151 TTAGTAGCAAAAGAGATAAACAAAGTGACAATGAAAATGATGCCGAAATC
4201 GAACAAGAAATTGAGCGTGATTTTGAAGTTGCTGAATCCTTAGCTTCCGA
4251 GGAGTCAGACCCTGTCAGCATTTTCGATACTGATAAACAAAATAAGAATG
4301 GTACAGTAGTTAAACATGATAATTTATTTACAAAGTATCAATTTTTCACT
4351 TCCGGTATTTGGTCAGGAATCATCATTTCAGGATTTTTATTGGTTATTTT
4401 GTACAATGCTTTGAGTTGGCTTTCTAGTTTGGAAATCACTTATGCCTCGT
4451 TTGAAAAACAAATTGATTTTGATAAAAAAAATGAAtaaagaaaaaggtta
4501 cttttggtttctggacttgaattgttattctattcacctctatagacaga
4551 aaaaaaaagaacgtatactaaagaactgttaggaacaagaaaagtattcg
4601 ctttccttgtttgttttctttttttttttaatatacataaggaattattt
4651 tataaggaactagcttttcgtacttttcccaatcctcgccgtattttgct
4701 ctgcacttcatttcgtctcttacttgacgatggattagcaaggaagcaaa
4751 gtaaataacgtaaaagtaggtcaatggggtttgaaagccagtaggcaaac
4801 accaagataatccaatcaaccagtcacccaaataattaatgtgttgagac
4851 aagccccaccagccttccaccaacaattttgagccagttttagtttggat
4901 gctttttaaatggggcaacttaccttgtctaaaatctgatttttgtttat
4951 ttgctgagcggaagatgtaaaaccctaatgcctgtaatccaacgatcaac
5001 aaacttaatgtccatcctaagttgacttcattgccctttatgctcaaata
5051 tcttgcttgtaaagaataagtccaaggaacccacgctaagtcaccaaagc
5101 ttagcataaaaccaaatccatcggtggttatgtcaatcatcgtcaaacaa
5151 ccttcttcgttcaataccccatcgaatatgtagaatgcttgaagtaagtt
5201 tacaacaatcattgagtctgttacgtagcccaaattatggtactgataat
5251 gcaaacaactcaaatttatcaacaaccataataacatcccggggcgtaac
5301 tcacaaaacaattttatgtcccaactacctattcttgggtttaattcacg
```

Figura III.2. Contig 4-3095. En la figura se muestra una zona de 1900 pb del contig 4-3095 que contiene el marco abierto de lectura (ORF) contenido en el clon 4, de 850 pb, con el codón de inicio (ATG, en verde) en la posición 3653 del contig y el codón de parada (TAA, en rojo) en la posición 4487.

III.1.3. Clonación del gen completo contenido en el Contig 4-3095.

Con la finalidad de aislar el gen completo correspondiente al ADNc contenido en el clon 4 se diseñó un cebador "forward" (FC4; **Tabla II.5 de Materiales y Métodos**) en base a la secuencia proporcionada por el Contig 5-3205, ubicado a 713 pb del codón de inicio del marco abierto de lectura, con una diana de restricción para *Bam*HI y un cebador "reverse" ubicado a 613 pares de bases del codón de terminación, con una diana de restricción para *Hind*III. Posteriormente se amplificó por PCR de alta fidelidad la secuencia contenida entre ambos cebadores usando como molde ADN genómico de la cepa CAI4 de *C. albicans* (**Tabla II.1 de Materiales y Métodos**), obteniéndose un fragmento de 2.200 pares de bases, que contenía todo el marco abierto de lectura más 713 pb de la zona promotora y 613 pb de la zona terminadora. Dicho fragmento se digirió con *Bam*HI y *Hind*III y se ligó en el vector pUC19 digerido con las mismas enzimas de restricción. Con la construcción resultante (**Fig. III.3**) se transformó la cepa DH5α de *E. coli* (**Tabla II.1 de Materiales y Métodos**), obteniéndose varios transformantes, que fueron comprobados por PCR. De entre los mismos se escogió uno, al que se denominó pUC-C4, para secuenciar el inserto clonado.

La secuencia obtenida presentaba un 100% de identidad con la secuencia presente en el Contig 5-3205. En la **Fig. III.3** se muestra el patrón de restricción del plásmido secuenciado. Estudios de interrupción génica (**ver sección III.6 dentro de este capítulo**) permitieron detectar un defecto en la citocinesis que ocasionaba un crecimiento por gemación alterado en las cepas mutantes para este gen, por lo que se precedió a denominarlo *ABG1*, del inglés "***a***ltered ***b***udding ***g***rowth" (crecimiento por gemación alterado).

III.2. Revisión y corrección de la secuencia de *ABG1*.

III.2.1. Modificación de la secuencia de *ABG1* en el ensamblaje 19 del proyecto de secuenciación de *C. albicans* (Universidad de Standford).

En el año 2.002 se publicó la última versión del proyecto de secuenciación de *C. albicans* de la Universidad de Standford, correspondiente al ensamblaje número 19. En esta nueva versión, el contig 5-3205, que incluía la secuencia de *ABG1,* había sido

reestructurado, de forma que la región 5´ no codificante (zona promotora) de *ABG1* estaba ahora contenida en el contig 19-1523. En este nuevo ensamblaje un fragmento de *ABG1* de 366 nucleótidos, que contenía parte del marco abierto de lectura, desaparecía de la base de datos y el último fragmento del gen aparecía ahora contenido en los contigs 19-10122 y 19-20122 (que contenían secuencias similares, pero con algún polimorfismo, por lo que se encontraban ensambladas en dos contigs), codificando para un polipéptido putativo de 189 aminoácidos distinto. La representación esquemática del contig 5-3595 y de su posterior reestructuración en el ensamblaje 19 se muestra en la **Fig. III.4**. Una discrepancia similar para la secuencia de *ABG1* también se encontró en la European *Candida albicans* Database (http://genolist.pasteur.fr/ CandidaDB/).

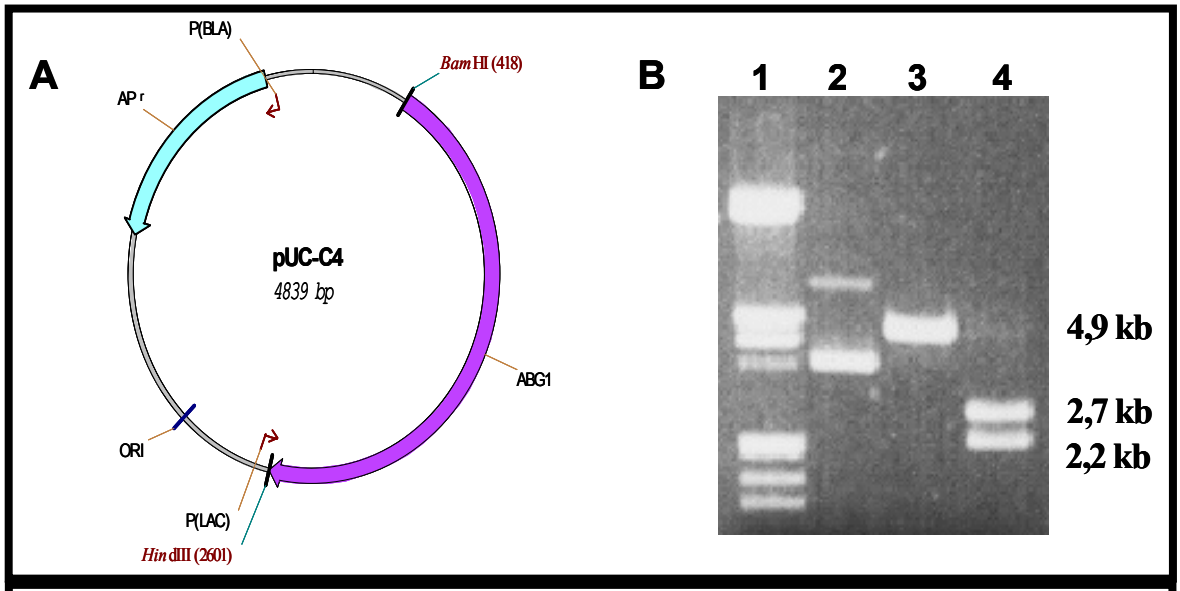

Figura III.3. Clonación de *ABG1*.

A. Plásmido pUC-C4, resultante de la clonación de *ABG1* en pUC19 entre los sitios *Bam*HI y *Hind*III.

B. Patrón de restricción de pUC-C4. Calle 1: ADN del fago λgt11 digerido con *Eco*RI y *Hind*III, utilizado como marcador de peso molecular. Calle 2: plásmido sin digerir. Calle 3: plásmido linearizado con *Eco*RI, que produce un único fragmento de 4,9 kb. Calle 4: plásmido digerido con *Bam*HI y *Hind*III, obteniéndose el vector, de 2,7 kb y el inserto de 2,2 kb.

III.2.2. Rastreo de una genoteca genómica.

Los resultados obtenidos hasta el momento de la publicación del ensamblaje 19 del Proyecto de Secuenciación de *Candida albicans* (inmunorrastreo, clonación y secuenciación del clon genómico de *ABG1*) discrepaban de los últimos datos publicados, indicando un posible error en la actualización de la secuencia de *ABG1* entre la versión quinta y la decimonovena. Con la finalidad de determinar la secuencia real de *ABG1* en el genoma de *C. albicans* se procedió a realizar el inmunorrastreo de una genoteca genómica utilizando como sonda el fragmento 3' no codificante de *ABG1*, amplificado y marcado simultáneamente con digoxigenina mediante el uso de los cebadores FP4 y RP4 (**ver Tabla II.5, del capítulo de Materiales y Métodos**), ya que este fragmento permanecía sin cambios en las versiones quinta y decimonovena del proyecto de secuenciación.

La genoteca estaba construida en el vector pEMBLYe23 con ADN cromosomal de la cepa WO-1 (ver **Tabla II.1, del capítulo de Materiales y Métodos**) de *C. albicans*, clonado entre los sitios *Bam*HI y *Hind*III del vector, con un tamaño medio de inserto de 7 kb. Se rastrearon aproximadamente 40,000 colonias (**sección II.17 de Materiales y Métodos**), aislándose dos clones positivos, que se denominaron pEMBL1 y pEMBL2. La secuenciación mediante el uso de los cebadores universales M13 (ver **Tabla II.5, del capítulo de Materiales y Métodos**) mostró una secuencia idéntica para ambos, con un 100% de identidad con la secuencia de *ABG1* contenida en el ensamblaje número 5 del proyecto de secuenciación.

III.2.3. Análisis del ADN de *C. albicans* mediante Southern.

Con el objeto de corroborar todos los resultados obtenidos tras el inmunorrastreo, la clonación y el rastreo de la genoteca genómica, y establecer con carácter definitivo la secuencia correcta de *ABG1*, se procedió al diseño de un experimento de análisis mediante Southern. Este experimento permitiría determinar el número de copias de *ABG1* en el genoma de *C. albicans*, la ausencia de polimorfismos en la secuencia de este gen entre la cepa CAI4 y una cepa silvestre (SC5314), y también obtener la confirmación de que la secuencia real de *ABG1* era la contenida en el contig 5-3295, mediante la obtención de los fragmentos esperados según el mapa de restricción

proporcionado por el contig 5-3205. En primer lugar se obtuvo ADN cromosomal de las cepas CAI4 y SC5314 (**ver Tabla II.1, del capítulo de Materiales y Métodos**), según el procedimiento descrito en la sección **II.5.2 de Materiales y Métodos**. Los ADNs fueron digeridos con diferentes combinaciones de enzimas de restricción y sometidos a electroforesis en gel de agarosa, transferencia e hibridación con la misma sonda que la empleada en el rastreo de la genoteca genómica. En todos los casos se obtuvo una única banda del tamaño esperado según el patrón de restricción proporcionado por el ensamblaje 5, lo que nos permitió establecer que la secuencia de *ABG1* correcta era la proporcionada en el contig 5-3295, que el mapa de restricción era el mismo tanto en la cepa CAI4 como en la SC5314, y que hay una única secuencia homóloga a este gen en el genoma de *C. albicans*. La secuencia obtenida tras consensuar los resultados de las secuenciaciones del clon inmunorreactivo frente al PAb anti-gt, del clon genómico de *ABG1* introducido en pUC-C4, y de los insertos presentes en pEMBL1 y pEMBL2 fue depositada en GenBank con número de acceso AY193774.

III.3 Análisis informático de la secuencia de *ABG1*.

III.3.1. Análisis de la secuencia nucleotídica de *ABG1*.

El análisis de la secuencia de nucleótidos del contig 5-3295 reveló la presencia de un marco abierto de lectura, de 864 pb, con el codón de inicio (ATG) en la posición 1.140 y el codón de parada (TAA) en la posición 2.004 del contig. La secuencia nucleotídica codificante de *ABG1* presenta un contenido del 36.5% de adenina, 32% de timina, 18,1% de guanina y 13,2% de citosina, resultados que concuerdan con estudios publicados sobre la frecuencia de utilización de bases nitrogenadas por *C. albicans* (**Odds, 1988; Brown *et al.*, 1991**). La frecuencia de utilización de codones también ha sido estudiada en *C. albicans* (**Lloyd and Sharp, 1992**), relacionando el uso de codones sinónimos con el nivel de expresión de los genes, de forma que los genes con bajo nivel de expresión utilizan todos los codones sinónimos de manera aleatoria, mientras que los genes con alto nivel de expresión utilizan preferentemente unos codones frente a otros dentro del grupo de los sinónimos. En ese sentido, el análisis de esta frecuencia en la secuencia codificante de *ABG1*, que se muestra en la **Tabla III.2**, reveló una preferencia de uso mayor para algunos codones, indicando posiblemente que *ABG1* fuese un gen con alto nivel de expresión.

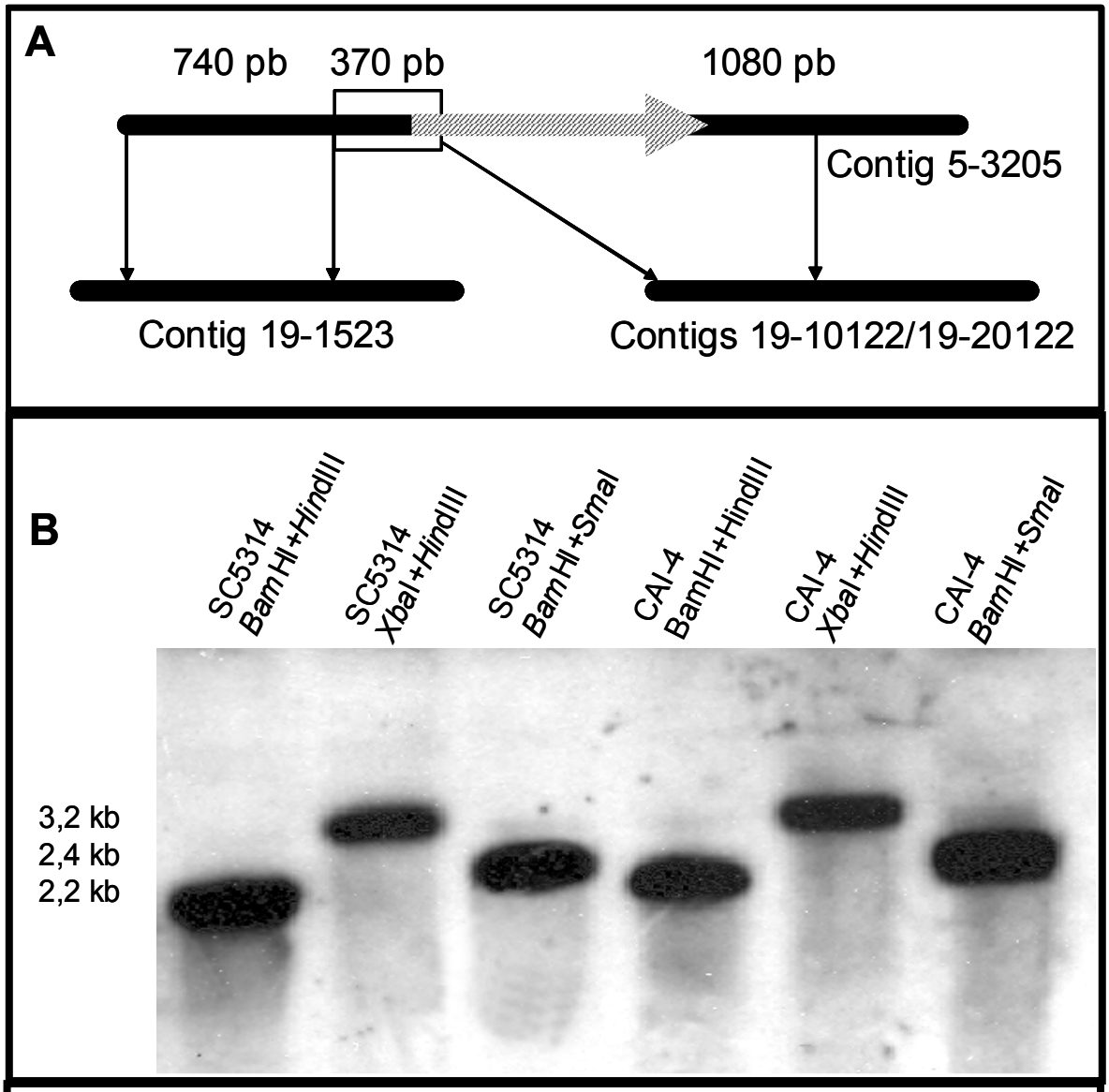

Figura III.4. Revisión de la secuencia de *ABG1*.

A. Diagrama de los Contigs 5-3205, 19-10122, 19-20122 y 19-1523, mostrando la localización del gen *ABG1* (flecha rayada).

B. Análisis por Southern blot de los alelos de *ABG1*. El ADN genómico de las cepas SC5314 y CAI4 fue digerido con *Bam*HI/*Hind*III, *Bam*HI/*Sma*I, y con *Xba*I /*Hind*III. En todos los casos se encontró una única banda coincidente con el tamaño esperado según el mapa de restricción proporcionado por el Contig 5-3205 del *Candida albicans* Sequence Project (Stanford University).

III.3.2. Análisis de la secuencia aminoacídica deducida de *ABG1*.

El marco abierto de lectura presente en el contig 5-3295 codifica para un polipéptido putativo de 288 aminoácidos (Abg1p), que presenta un peso molecular teórico de 32.921 Da, según el análisis de dicho polipéptido realizado con el programa Peptide Mass Calculator. El mismo programa se utilizó para calcular el punto isoeléctrico del polipéptido codificado por *ABG1*, en base al método descrito por **Bjellqvist *et al.* (1993)**, obteniéndose un valor de 4.79 para Abg1p.

La composición de Abg1p se analizó con el programa SAPS (Statistical Analysis of Protein Sequences; **Brendel *et al.*, 1992**), mostrándose en la **Tabla III.3** dicha composición expresada en porcentaje molar.

Tabla III.2. Frecuencia del uso de codones en la zona codificante de *ABG1*.

Codón	AA	N	%	Codón	AA	N	%	Codón	AA	N	%	Codón	AA	N	%
TTT	Phe	12	4,1	TCT	Ser	6	2	TAT	Tyr	7	2,4	TGT	Cys	3	1
TTC	Phe	3	1	TCC	Ser	4	1,3	TAC	Tyr	5	1,7	TGC	Cys	0	0
TTA	Leu	12	4,1	TCA	Ser	7	2,4	TAA	---	1	0,3	TGA	---	0	0
TTG	Leu	9	3,1	TCG	Ser	1	0,3	TAG	---	0	0	TGG	Trp	3	1
CTT	Leu	4	1,3	CCT	Pro	2	0,6	CAT	His	6	2	CGT	Arg	1	0,3
CTC	Leu	0	0	CCC	Pro	0	0	CAC	His	0	0	CGC	Arg	0	0
CTA	Leu	0	0	CCA	Pro	2	0,6	CAA	Gln	9	3,1	CGA	Arg	0	0
CTG	Ser	0	0	CCG	Pro	0	0	CAG	Gln	1	0,3	CGG	Arg	0	0
ATT	Ile	16	5,5	ACT	Thr	6	2	AAT	Asn	12	4,1	AGT	Ser	8	2,7
ATC	Ile	8	2,7	ACC	Thr	2	0,6	AAC	Asn	9	3,1	AGC	Ser	6	2
ATA	Ile	1	0,3	ACA	Thr	3	1	AAA	Lys	18	6,2	AGA	Arg	1	0,3
ATG	MET	2	0,6	ACG	Thr	1	0,3	AAG	Lys	10	3,4	AGG	Arg	0	0
GTT	Val	9	3,1	GCT	Ala	5	1,7	GAT	Asp	17	5,8	GGT	Gly	5	1,7
GTC	Val	1	0,3	GCC	Ala	3	1	GAC	Asp	3	1	GGC	Gly	1	0,3
GTA	Val	7	2,4	GCA	Ala	2	0,6	GAA	Glu	22	7,6	GGA	Gly	3	1
GTG	Val	2	0,6	GCG	Ala	1	0,3	GAG	Glu	6	2	GGG	Gly	1	0,3

El análisis de la secuencia peptídica (**Fig. III.5**) reveló la presencia de un péptido señal entre las posiciones 7-22 y de un posible dominio transmembranal entre las posiciones 241-260. Estos resultados (**Fig. III.5**) se obtuvieron tras analizar la secuencia aminoacídica con el sistema predictivo DAS (Dense Alignment Surface) (**Cserzo *et al.*, 1997**). Para el análisis de dominios conservados presentes en la secuencia de Abg1p se utilizó la base de datos Prosite (**Falquet *et al.*, 2002**). Los principales resultados obtenidos a partir de este análisis se muestran en la **Tabla III.4**.

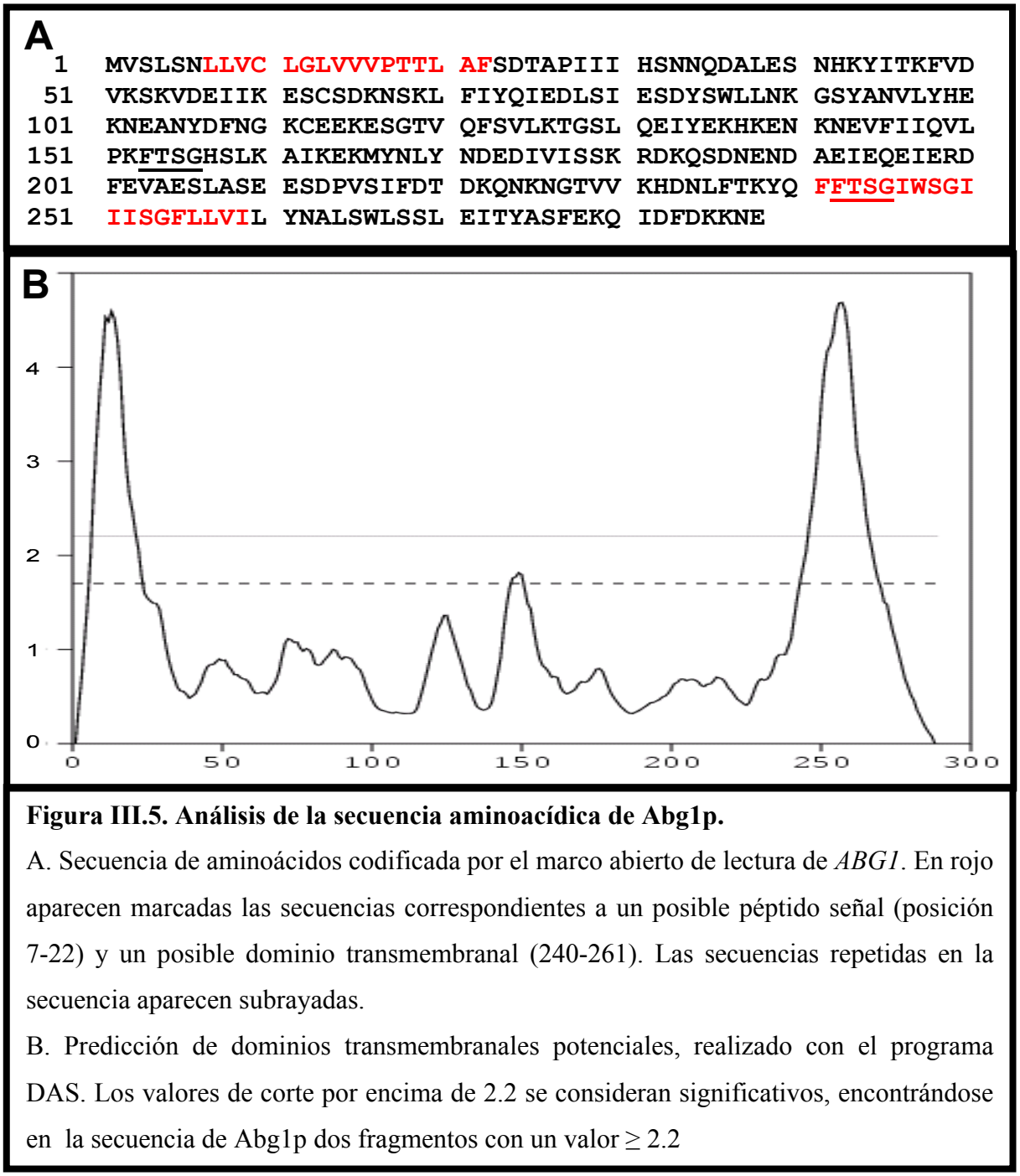

Figura III.5. Análisis de la secuencia aminoacídica de Abg1p.

A. Secuencia de aminoácidos codificada por el marco abierto de lectura de *ABG1*. En rojo aparecen marcadas las secuencias correspondientes a un posible péptido señal (posición 7-22) y un posible dominio transmembranal (240-261). Las secuencias repetidas en la secuencia aparecen subrayadas.

B. Predicción de dominios transmembranales potenciales, realizado con el programa DAS. Los valores de corte por encima de 2.2 se consideran significativos, encontrándose en la secuencia de Abg1p dos fragmentos con un valor ≥ 2.2

Tabla III.3. Composición del polipéptido Abg1p.

Aminoácido	Número de residuos	Porcentaje molar	Aminoácido	Número de residuos	Porcentaje molar
Ala	11	3,8	Met	2	0,7
Cys	3	1,0	Asn	21	7,3
Asp	20	6,9	Pro	4	1,4
Glu	28	9,7	Gln	10	3,5
Phe	15	5,2	Arg	2	0,7
Gly	10	3,5	Ser	32	11,1
His	6	2,1	Thr	12	4,2
Ile	25	8,7	Val	19	6,6
Lys	28	9,7	Trp	3	1,0
Leu	25	8,7	Tyr	12	4,2

Tabla III.4. Presencia de dominios conservados en la secuencia de Abg1p.

Dominio	Localización entre los residuos número	Secuencia
Posible sitio de N- Glicosilación	226-229	NGTV
Posible sitio de fosforilación por Proteína Quinasa C	64-66	SDK
	158-160	SLK
	178-180	SSK
	179-181	SKR
	220-222	TDK
Posible sitio de fosforilación por Caseína Quinasa II	53-56	SKDV
	62-65	SCSD
	129-132	SLQE
	179-182	SKRD
	185-188	SDNE
	216-219	SIFD
	268-271	SSLE
Posible sitio de fosforilación por Tirosina Quinasa	160-167	KAIKEKMY
Posible sitio de N-miristoilación	91-96	GSPYPNV
	245-250	GIWSGI
	249-254	GIIISG

III.3.3. Análisis funcional de la secuencia: búsqueda de homólogos.

La búsqueda de posibles secuencias homólogas a *ABG1* en la base de datos del National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) reveló que este gen no presentaba homología significativa con ninguna secuencia conocida en mamíferos, encontrándose la máxima homología con un hipotético marco abierto de lectura de *S. cerevisiae*, denominado YGR106c (número de acceso NC001139), en base a su localización en la cadena de Crick del cromosoma 7 de esta levadura.

Al realizar el alineamiento de las secuencias proteicas deducidas para *ABG1* y YGR106c se encontró un 27,7% de identidad entre ambas, siendo la parte carboxilo terminal la que presentaba mayor similitud, como se muestra en la **Fig. III.6**. El hipotético marco abierto de lectura de YGR106c codifica un polipéptido de 294 aminoácidos, cuyo análisis mediante el programa DAS (Dense Alignment Surface Program; **Cserzo *et al.*, 1997**), reveló una estructura similar a la encontrada para Abg1p, con péptido señal entre las posiciones 8 y 16, y con un posible dominio transmembranal localizado entre los aminoácidos 222 y 244.

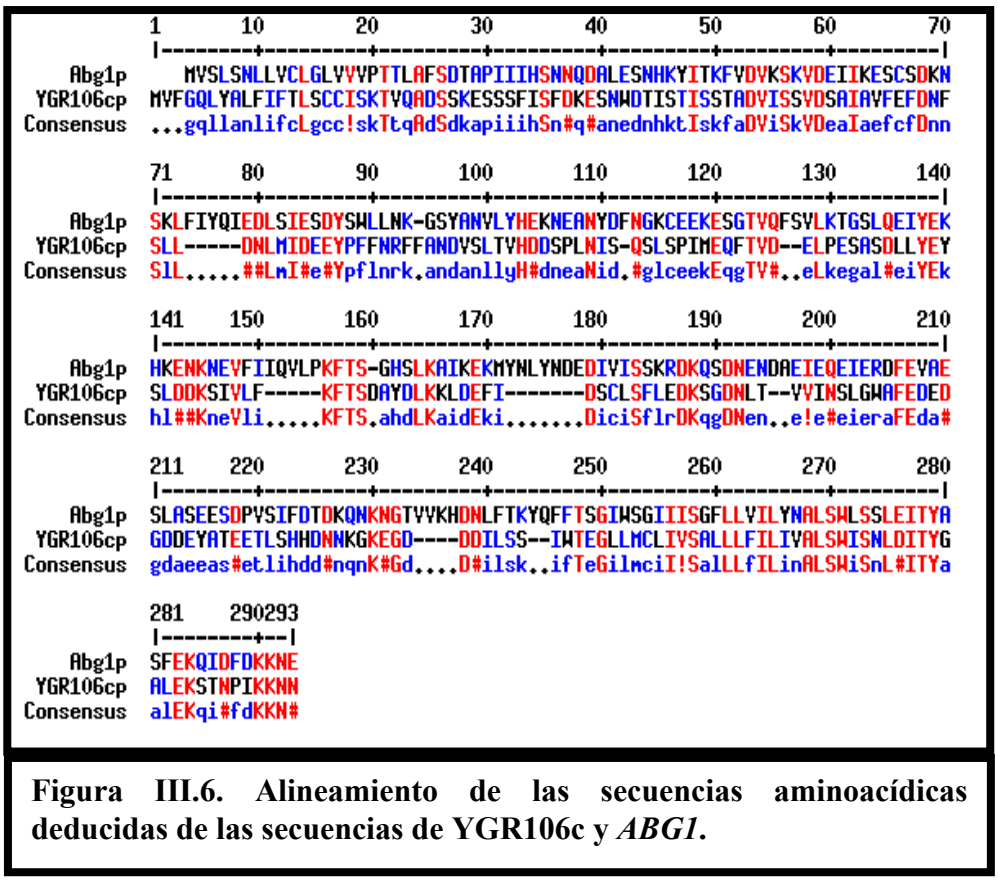

Figura III.6. Alineamiento de las secuencias aminoacídicas deducidas de las secuencias de YGR106c y *ABG1*.

III.4. Estudio de la expresión del gen *ABG1* en *C. albicans*.

Con el objeto de identificar el transcrito codificado por *ABG1* y al mismo tiempo analizar la expresión de este gen durante el proceso de transición morfológica en *C. albicans*, se procedió a obtener el ARN total procedente de células sometidas a reposo metabólico durante 48 horas y posteriormente inoculadas en medio Lee a 28ºC o 37ºC, para obtener células en fase levaduriforme o micelial, respectivamente. Se cosecharon células en fase de ayuno metabólico (tiempo cero) y células incubadas en medio Lee a 37ºC, a los 30, 60 y 120 minutos tras la inoculación y también células incubadas en medio Lee a 28ºC, a los mismos tiempos, para comparar la expresión de *ABG1* entre células creciendo en diferentes condiciones de inducción morfogenética. El ARN total se obtuvo según el procedimiento descrito en **Materiales y Métodos** (**sección II.9.2**) y se utilizó para realizar un análisis mediante Northern blot. Tras cuantificar mediante la determinación de la absorbancia la cantidad de ARN obtenido se utilizaron aproximadamente 10 μg de ARN de cada muestra para realizar la electroforesis en un gel de agarosa-formaldehído. Al finalizar la electroforesis los ARNs se transfirieron por capilaridad a una membrana de nylon, sobre la que se realizó la hibridación.

Para obtener una sonda capaz de reconocer el transcrito de *ABG1* se diseñó una pareja de cebadores (FP4 y RP4; **ver Tabla II.5, del capítulo de Materiales y Métodos**) que permitían amplificar 850 pares de bases correspondientes al marco abierto de lectura completo de *ABG1*. Este fragmento de DNA fue marcado con digoxigenina de forma simultánea a la reacción de amplificación por PCR. El resultado obtenido mediante el análisis por Northern blot se muestra en la **Fig. III.7**, en la que se observa la existencia de un único transcrito para *ABG1* del tamaño esperado (1.300 pares de bases), si se tiene en cuenta que a las 850 pb del marco de lectura hay que sumarle las bases añadidas durante el proceso de transcripción del ARN mensajero, tanto en la adición de la caperuza, como en la poliadenilación de la cola del ARNm.

El transcrito detectado aparece en todas las muestras analizadas, indicando que este gen se expresa durante la transición dimórfica y también en las células recogidas a tiempo cero, tras el ayuno metabólico de 48 horas, pudiendo esto último indicar un posible patrón de expresión constitutiva para *ABG1*.

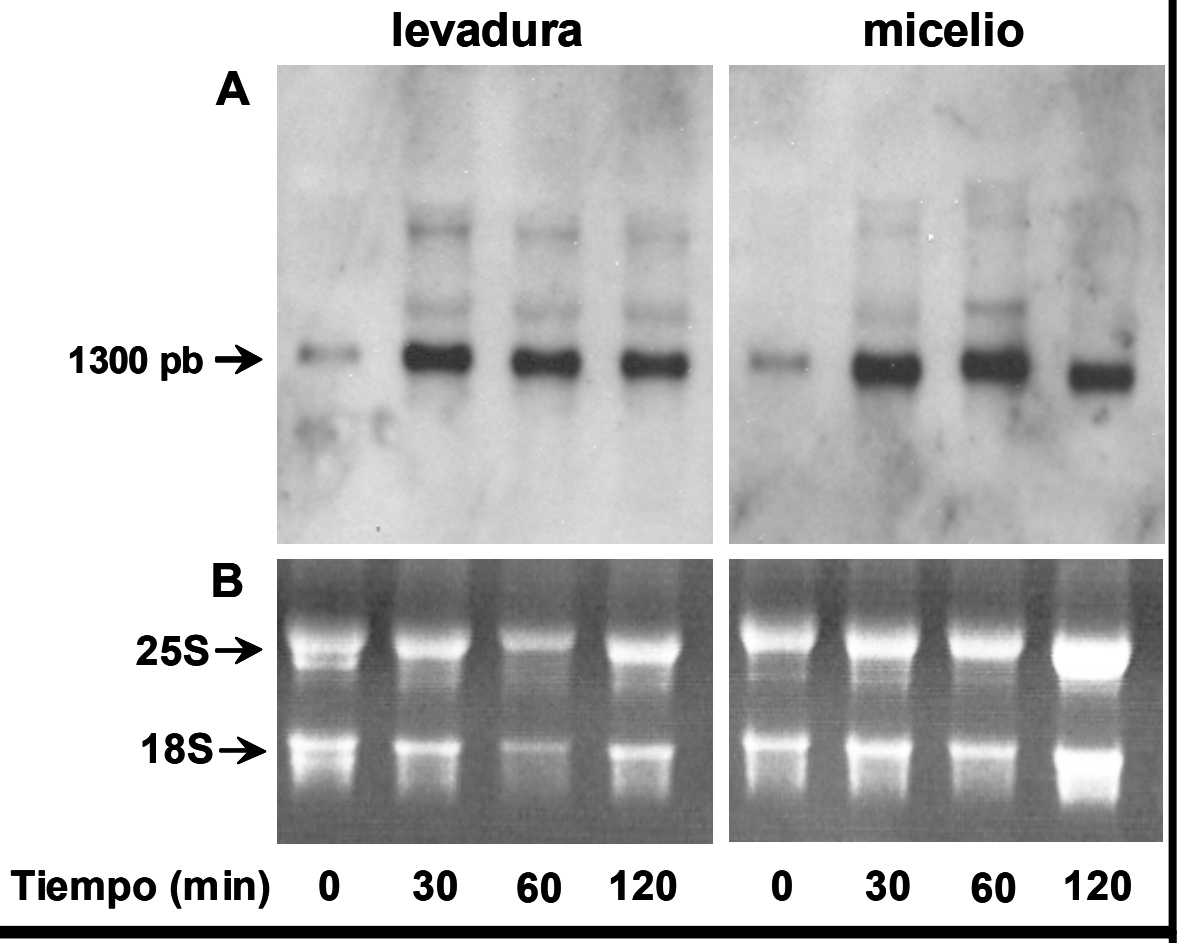

Figura III.7. Análisis de la expresión de *ABG1* mediante Northern blot.

A. El ARN total de las células en fase levaduriforme y micelial se obtuvo a los tiempos indicados y tras realizar la electroforesis, transferencia e hibridación con la sonda correspondiente al ADNc de la zona codificante de *ABG1* se detectó una única banda, de tamaño aproximado de 1.300 pb, presente en todas las muestras analizadas.

B. Los ARNs ribosomales de *C. albicans* se utilizaron como marcador de tamaño, así como para cuantificar el ARN total.

Además de estos resultados, también fue posible detectar mediante Northern la expresión de *ABG1* en todas las condiciones de crecimiento utilizadas, en células creciendo tanto a pH ácido como a pH básico, así como durante la transición morfológica inducida por suero (datos no mostrados), lo que concuerda con el posible patrón de expresión constitutivo sugerido para *ABG1*.

III.5. Localización subcelular de la proteína Abg1p.

III.5.1. Análisis de extractos celulares de *C. albicans*.

Para determinar la localización subcelular del producto del gen *ABG1*, el polipéptido Abg1p, se procedió en primer lugar a analizar mediante técnicas de inmunotransferencia (Western blot) diferentes extractos celulares de la cepa CAI4 de *C. albicans*, utilizando como sonda el anticuerpo policlonal PAb anti-Abg1p generado frente a un dominio fuertemente antigénico situado en el extremo amino-terminal de Abg1p (**ver sección II.25 del capítulo de Materiales y Métodos**).

Con esta finalidad se procedió a inocular células en medio Lee a 30ºC, con objeto de obtener un cultivo de células levaduriformes en fase estacionaria, que fue utilizado para obtener los diferentes extractos celulares, que se describen en la **sección II.20 del capítulo de Materiales y Métodos**. Los diferentes extractos obtenidos fueron analizados mediante SDS-PAGE e inmunotransferencia. Utilizando el anticuerpo policlonal PAb anti-Abg1p como sonda se detectó una especie inmunoreactiva, de 32 kDa, presente en el lisado celular total, así como en el extracto de SDS, y en las fracciones P40 y P100, y que no aparecía en los extractos de Zimoliasa y la fracción citosólica (**Fig. III.8**). Estos resultados sugieren que Abg1p se encontraba asociada a estructuras de membranas celulares, pudiendo tratarse de la membrana plasmática (lo que explicaría su presencia en el extracto de SDS), o bien la membrana de orgánulos internos, esto último sugerido por la presencia de la especie inmunoreactiva frente al PAb anti-Abg1p en las fracciones P40 y P100. Otras especies inmunorreactivas fueron detectadas utilizando como sonda el anticuerpo policlonal PAb anti-Abg1p, aunque con una reactividad bastante débil. Estas especies podrían compartir algún epitopo común con el producto del gen *ABG1* que sería reconocido por el PAb anti-Abg1p.

III.5.2. Localización de Abg1p mediante fusión con la Green Fluorescent Protein (GFP) de *Aequorea victoria*.

Con la finalidad de confirmar los resultados de los experimentos de inmunotransferencia, que sugerían que la especie Abg1p se encuentra asociada a estructuras membranosas subcelulares, y para poder visualizar mediante microscopía óptica la localización subcelular *in vivo* del producto del gen *ABG1*, se procedió a

realizar una construcción que permitiese fusionar el polipéptido Abg1p con la proteína verde fluorescente (GFP) de *A. victoria*. Para construir la proteína de fusión se utilizó el plásmido pAG1 (**ver sección II.3 del capítulo de Materiales y Métodos**) que contenía la yEGFP, una proteína derivada de la GFP pero modificada y optimizada para su uso en *C. albicans* (**Cormack *et al.*, 1997**). La GFP original es una proteína de 238 aminoácidos, que absorbe luz a 395 nm y emite fluorescencia verde a 507 nm, mientras que la yEGFP presenta estas mismas características pero el único codón CTG presente en esta proteína ha sido sustituido por el codón TTG, evitando así los problemas con el sistema no canónico de traducción proteica en *C. albicans* (**Ohama *et al.*, 1993**; **Santos *et al.*, 1993**; **White *et al.*, 1995**).

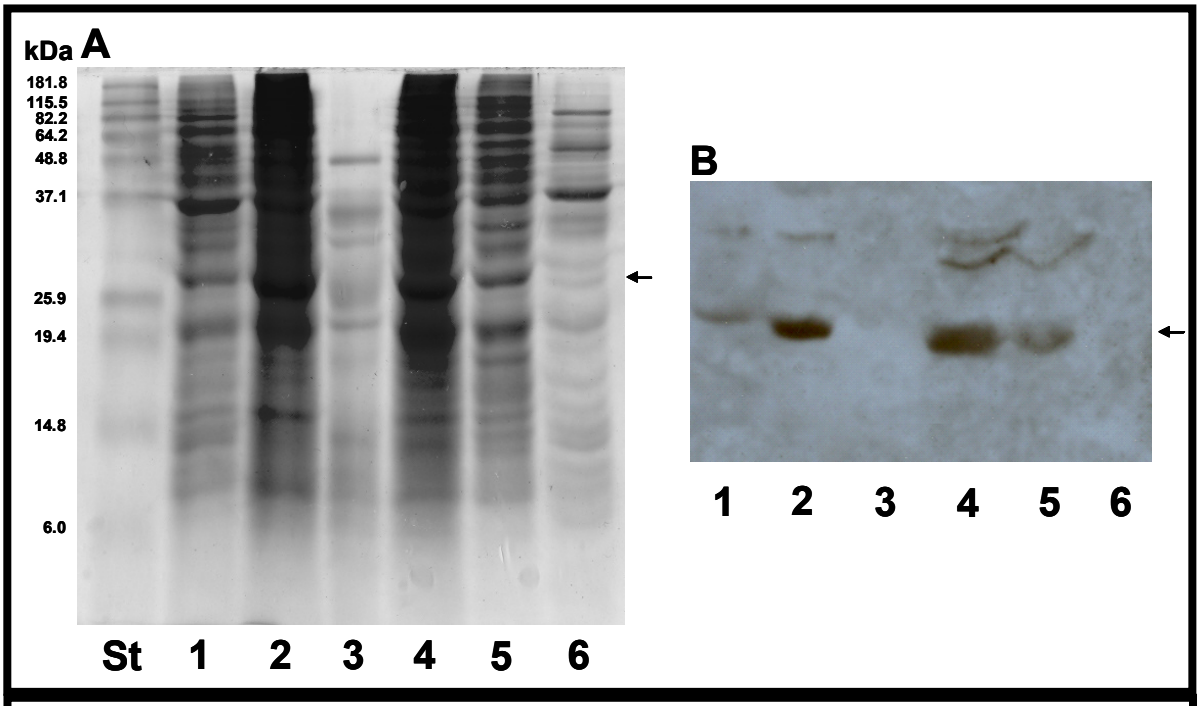

Figura III.8. Análisis mediante inmunotransferencia de extractos celulares.

A. SDS-PAGE y tinción con Coomassie. Lisado celular completo (calle 1), extracto de SDS de paredes celulares (calle 2), paredes celulares digeridas con Zymoliasa (calle 3), fracción P40 (calle 4), fracción P100 (calle 5) y fracción citosólica (calle 6).

B. Detección de especies reactivas frente al PAb anti-Abg1p mediante inmunotransferencia. Las especies proteicas presentes en las muestras (el orden de las calles es el mismo que se indica en el panel A) fueron inmunodetectadas con el PAb anti-Abg1p.

En ambos paneles las flechas señalan la especie proteica que correspondería a Abg1p, de acuerdo con el tamaño teórico establecido para este polipéptido.

La estrategia de construcción consistió en clonar la zona codificante de *ABG1* utilizando el único punto de restricción *Sma*I presente en el plásmido pAG1, generando el vector pAAG, consiguiendo situar así el ORF de *ABG1* bajo el control del promotor constitutivo de la actina, fusionado a su vez por el extremo carboxi terminal con la yEGFP. Este tipo de construcción es preferible a la fusión por el extremo amino terminal, ya que es en esta última zona dónde se suelen encontrar las señales de localización de las proteínas, por lo que la fusión podría conllevar la deslocalización de la proteína recombinante.

Una vez construido este vector se utilizó para transformar, de forma no integrativa, la cepa CAI4 de *C. albicans*, obteniendo numerosos transformantes, que fueron analizados por PCR, seleccionando aquellos en los que se obtuvo un amplificado de 1.034 pb con la pareja de cebadores PAF-RP4 y de 1.134 pb con la pareja PAF-RGFP (**ver Tabla II.5, del capítulo de Materiales y Métodos**), indicativo de la presencia de una secuencia codificante para una proteína de un tamaño correspondiente a la proteína de fusión Abg1p-GFP, situada bajo el control del promotor de la actina.

Se seleccionó uno de los transformantes, CGA-4 (**ver Tabla II.1, del capítulo de Materiales y Métodos**), y con la finalidad de comprobar si la proteína de fusión estaba expresándose se preparó un extracto celular, con el que se realizó un análisis mediante SDS-PAGE e inmunotransferencia, utilizando como sonda un anticuerpo comercial frente a la GFP (Clontech). Se detectó una única especie inmunoreactiva, con un tamaño molecular aproximado de 60.000 Da, coincidente con el tamaño esperado para la proteína de fusión Abg1p-GFP (dato no mostrado).

El transformante CGA-4 se analizó mediante visualización directa de la fluorescencia con un microscopio DMRX (Leica), observándose un patrón de fluorescencia denominado “ring-like”, es decir, en el que la fluorescencia aparece en forma de anillo, recubriendo algún tipo de estructura u orgánulo subcelular (**Fig. III.9**), tanto en células en fase levaduriforme como en forma micelial. Ante estas observaciones se decidió realizar la tinción de estas células con diferentes agentes, con la finalidad de determinar en que tipo de estructura se hallaba localizado el polipéptido recombinante fluorescente. Mediante esta aproximación experimental se encontró que el patrón de fluorescencia encontrado colocalizaba con la fluorescencia obtenida al teñir las células de la cepa CGA-4 con FM4-64, un agente que tiñe específicamente los

compartimentos de la ruta endocítica así como la membrana vacuolar (**Vida and Emr, 1995**), poniendo de manifiesto que la proteína de fusión Abg1-GFP se encontraba asociada a la membrana vacuolar así como la de otras vesículas endocíticas en *C. albicans* (**Fig. III.9**).

III.6. Interrupción del gen *ABG1* en *C. albicans*.

Una vez establecida la localización vacuolar del producto del gen *ABG1* se decidió construir una cepa con las dos copias de dicho gen interrumpidas, con la finalidad de determinar la función del mismo en *C. albicans*. Una vez generado el correspondiente mutante homocigoto nulo, o eventualmente, un mutante condicional (**ver sección III.6.3 dentro de este mismo capítulo**) el estudio fenotípico del mismo se orientó fundamentalmente hacia el análisis de la morfología vacuolar, de acuerdo con los resultados de los experimentos de localización subcelular obtenidos tras los experimentos de inmunotransferencia y fusión con proteína verde fluorescente, aunque el análisis fenotípico incluyó también el estudio de otras características, tales como posibles alteraciones en la morfología celular, en la estructura de la pared celular, y en la respuesta a factores ambientales de estrés, todo ello con la finalidad de caracterizar la función de este nuevo gen en *C. albicans*.

Tradicionalmente la metodología empleada para la construcción de cepas con interrupciones génicas se ha basado en el uso del cassette URA-blaster, adaptado para *C. albicans* por **Fonzi e Irwin (1993)** a partir de la técnica inicial desarrollada para transformaciones en *S. cerevisiae* (**Alani *et al.*, 1987**). Esta técnica se basa en la integración de un cassette, constituido por el gen *URA3* de *C. albicans* flanqueado por dos secuencias homólogas del gen *his*G de *Salmonella typhimurium,* en cada una de las dos copias del gen a disrupcionar, utilizando una cepa de *C. albicans* con auxotrofía para el gen *URA3* (comúnmente la cepa CAI4). Tras integrar el cassette en el primer alelo del gen, el marcador *URA3* se elimina, al incubar las células en un medio suplementado con ácido 5-fluoroorótico (5-FOA), forzando la recombinación homóloga de las secuencias *his*G con la consiguiente pérdida del marcador auxótrofo, puesto que este compuesto se convierte en un metabolito tóxico para aquellas células que no pierdan el marcador *URA3*.

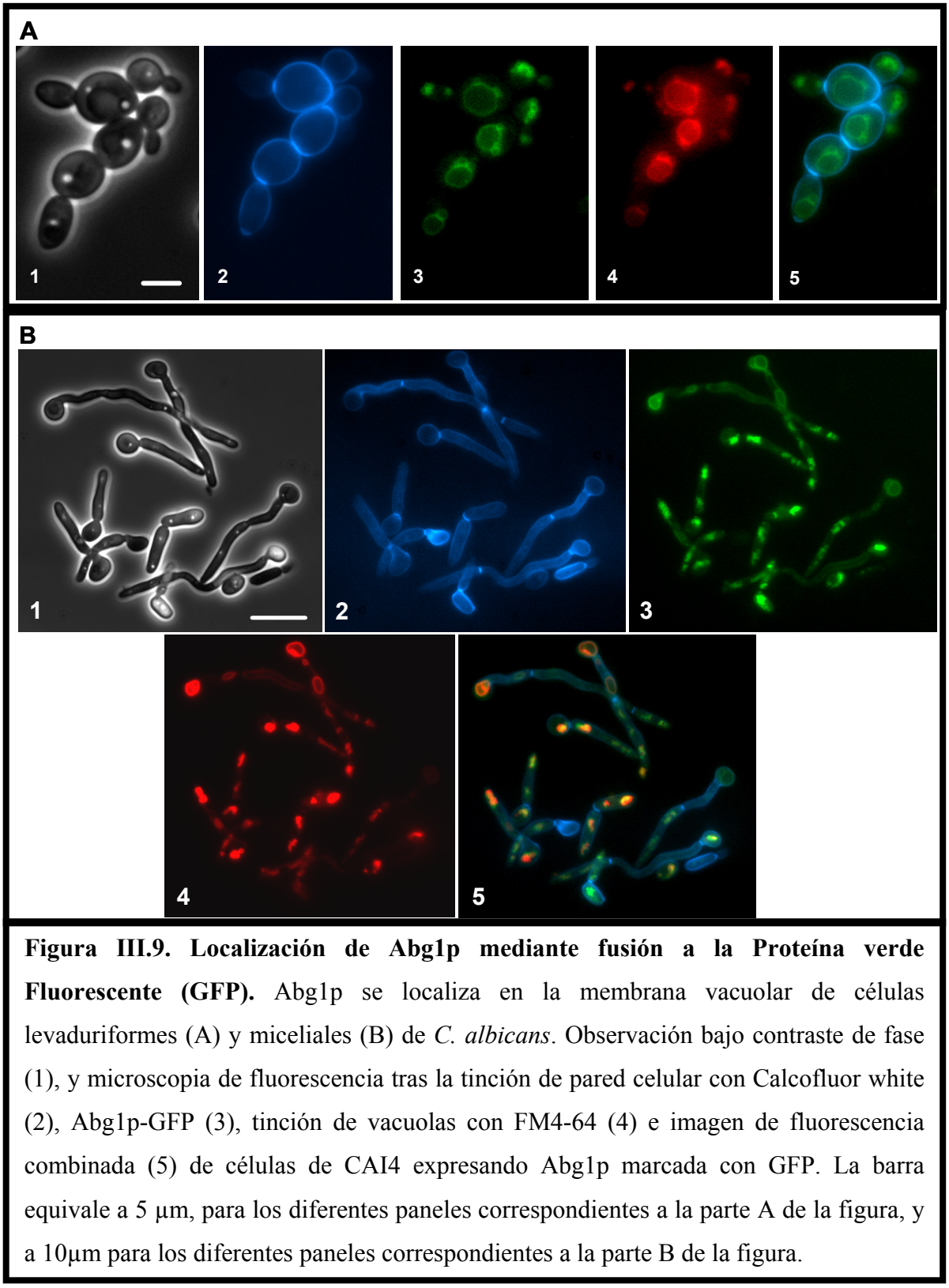

Figura III.9. Localización de Abg1p mediante fusión a la Proteína verde Fluorescente (GFP). Abg1p se localiza en la membrana vacuolar de células levaduriformes (A) y miceliales (B) de *C. albicans*. Observación bajo contraste de fase (1), y microscopia de fluorescencia tras la tinción de pared celular con Calcofluor white (2), Abg1p-GFP (3), tinción de vacuolas con FM4-64 (4) e imagen de fluorescencia combinada (5) de células de CAI4 expresando Abg1p marcada con GFP. La barra equivale a 5 µm, para los diferentes paneles correspondientes a la parte A de la figura, y a 10µm para los diferentes paneles correspondientes a la parte B de la figura.

En un segundo paso se integra de nuevo el cassette de disrupción, seleccionando aquellos transformantes que lo integran en la segunda copia del gen a disrupcionar, y que mantienen la primera copia interrumpida con una copia de *his*G. La integración del cassette se dirige mediante la clonación de secuencias homólogas al gen a disrupcionar (de un tamaño aproximado de 400-500 pb) situadas en los flancos del cassette *his*G-*URA3*-*his*G. Esta clonación requiere la construcción del cassette en un vector adecuado previamente a la disrupción génica en *C. albicans*.

En la actualidad se dispone de nuevas metodologías en las que no se requiere la construcción de un vector previamente, ya que el cassette se obtiene por PCR (**Wilson *et al.*, 2000**). Esto es posible debido a que, en estas técnicas, las zonas homologas al gen, que dirigen la integración del cassette, y el cassette en sí, son de una longitud bastante menor que la empleada en la técnica URA-blaster, lo que permite su amplificación por PCR mediante el diseño de oligonucleótidos mixtos, que contienen las secuencias necesarias para amplificar el cassette completo flanqueado por ambos lados por 60 pb homologas al gen a disrupcionar. A pesar de esto, estas técnicas basadas en la PCR presentan la desventaja de que se pierde especificidad en el proceso de integración del cassette, aumentando la probabilidad de integraciones indeseadas en otros *locus*.

Otras técnicas están basadas en la utilización de cepas con varias auxotrofías (*URA3, HIS3, ARG5* y *ARG6*) (**Negredo *et al.*, 1997**), lo que evita el tener que recuperar el marcador auxotrófico tras la interrupción génica de la primera copia del gen de interés, si bien la existencia de varias auxotrofías en una misma cepa dificulta el posterior uso de los mutantes generados en estudios fenotípicos y de virulencia. Numerosos estudios han puesto de manifiesto que más allá incluso de la presencia del gen *URA3*, la orientación y localización del mismo presenta una elevada influencia en la adherencia y también en la virulencia de *C. albicans* (**Bain *et al.*, 2001**; **Lay *et al.*, 1998**; **Staab and Sundstrom, 2003**). Sin embargo, un reciente estudio (**Brand *et al.*, 2004**) ha puesto de manifiesto que la reintegración del gen *URA3* en el *locus RPS10* restituye los niveles de expresión de este gen hasta niveles normales para cepas que no presentan esta auxotrofía. Por esta razón, en el presente trabajo se utilizó la cepa CAI4-*URA3* de *C. albicans* (**ver Tabla II.1, del capítulo de Materiales y Métodos**) como cepa control para aquellos ensayos llevados a cabo con mutantes Ura^+.

Sin embargo, todas las técnicas anteriormente mencionadas únicamente pueden aplicarse en aquellos genes no esenciales para la viabilidad de *C. albicans*. En el caso de genes esenciales, su interrupción implica letalidad para el hongo, por lo que nunca puede aislarse un mutante nulo para los mismos. A pesar de ello, el estudio de la función de los genes esenciales en *C. albicans* resulta de gran importancia, puesto que constituyen un conjunto de potenciales dianas terapéuticas, lo que ha llevado desarrollo de nuevas herramientas, basadas en el control de la expresión génica (**Care *et al.*, 1999**; **Brown *et al.*, 1996**), que permiten analizar la función del gen en estudio reprimiendo su expresión parcialmente. Estas metodologías se basan en la disrupción de la primera copia del gen mediante la metodología clásica (URA-blaster) y la colocación simultánea de la segunda copia del gen de interés bajo el control de un promotor regulable. Los promotores disponibles más utilizados son el CaMET3p, que se reprime en presencia de metionina y cisteína en el medio de cultivo, y el CaMALp, inducible por maltosa y reprimible por glucosa.

III.6.1. Interrupción génica de *ABG1* mediante el uso del cassette URA-dp1200.

Para llevar a cabo la interrupción de *ABG1* se escogió, en principio, la metodología basada en la obtención del cassette de disrupción por PCR, utilizando el plásmido pDDB57 (**Wilson *et al., 2000***). Este vector contiene el cassette URA-dp1200, constituido por el gen *URA3* con el extremo 3´ del mismo duplicado y clonado en su extremo 5´. Se diseñó una pareja de cebadores (MF4-MR4; **Tabla II.5**) que permitían amplificar el cassette flanqueado por 60 pares de bases por cada lado del marco abierto de lectura de *ABG1*. El amplificado obtenido en la reacción de PCR se utilizó para transformar la cepa CAI4 de *C. albicans*, mediante el protocolo que utiliza acetato de litio (**ver sección II.19 del capítulo de Materiales y Métodos**) obteniéndose 12 transformantes, que se analizaron por Southern, encontrándose que uno de ellos, al que se denominó CJ1, había integrado el cassette en la primera copia de *ABG1*, puesto que esta cepa presentaba una banda de igual tamaño que la presente en la cepa parental, y otra banda, de tamaño superior, correspondiente a una de las copias del gen con el cassette de disrupción insertado en el marco abierto de lectura. La cepa CJ1 se inoculó en placas de YNB-agar suplementadas con 5'-FOA y uridina, y se incubó durante tres

días a 28ºC. Se obtuvieron varios transformantes Ura$^-$, y se analizaron por Southern encontrándose que uno de ellos, al que se denominó CJ2, había perdido el marcador auxotrófico, conservando al mismo tiempo la integración de 600 pares de bases del cassette. Esta cepa se utilizó para realizar una segunda ronda de transformación con el mismo cassette, pero no se consiguió obtener una cepa con los dos alelos interrumpidos, a pesar de que se obtuvieron 120 transformantes, que al ser analizados por PCR con los cebadores C4SF-C4SR (**Tabla II.5**) daban lugar a un amplificado de 1300 pb, del mismo tamaño que el que se obtenía al utilizar la cepa parental, lo que indicaba la existencia de al menos un alelo silvestre de *ABG1*.

Como se ha mencionado en el apartado anterior, esta metodología utiliza 120 pares de bases homólogas al gen para dirigir la integración del cassette, mientras que en la metodología del URA-blaster se utilizan del orden de 1000 pares de bases para dirigir la integración, mejorando considerablemente la eficiencia de transformación, por lo que se decidió intentar de nuevo la interrupción génica pero cambiando la estrategia.

III.6.2. Interrupción génica mediante el uso del cassette URA-blaster.

Para abordar de nuevo la interrupción génica de *ABG1* se procedió en primer lugar a construir el cassette apropiado, utilizando como vector el plásmido pBB510 (**Braun and Johnson, 2000**; **Pérez-Martín *et al.*, 1999**). Se diseñaron dos parejas de cebadores, la primera, PF1F-PR1F (**Tabla II.5**), que permitía amplificar 700 pb de la zona promotora de *ABG1* por PCR, y otra, PF2F-PR2F (**Tabla II.5**), que permitía amplificar 500 pb de la zona terminadora del gen, después del codón de stop TAA. Estos oligonucleótidos incorporan los sitios de corte *Hind*III-*Pst*I, y *Bgl*II-*Kpn*I, respectivamente, lo que facilita su clonación en el vector correspondiente. Una vez obtenidos estos fragmentos se subclonó, en primer lugar, el flanco uno (correspondiente a la zona promotora) en el vector pBB510, y posteriormente el segundo flanco (correspondiente a la zona terminadora), obteniéndose el vector p510V (**Fig. III.10**). El cassette de disrupción para la interrupción se obtuvo digiriendo el vector p510V con *Hind*III y *Kpn*I, que fue seguidamente utilizado para transformar la cepa CAI4 de *C. albicans*, utilizando el protocolo del acetato de litio (**ver sección II.19 del capítulo de Materiales y Métodos**). Se obtuvieron cinco transformantes, cuyo análisis por PCR

mostró que eran todos positivos. Para dicho análisis se utilizaron dos parejas de cebadores distintas (FP4-HisG1R; HisG2F-RP4; **Tabla II.5**), en las que un oligonucleótido de la pareja correspondía a secuencias propias del cassette, y el otro correspondía a la zona del gen *ABG1* externa a la integración del cassette (**Fig. III.10**). Tras comprobar que en todas las cepas transformantes el cassette de disrupción se había integrado en uno de los alelos de *ABG1*, perdiéndose al mismo tiempo la zona correspondiente al marco abierto de lectura en ese alelo, se escogió uno de esas cepas, a la que se denominó CV1, para la recuperación del marcador auxotrófico, dando lugar a la cepa CV2, que fue utilizada a su vez para realizar una segunda ronda de transformación. Tras el análisis de 100 transformantes resultantes de esta segunda ronda, no fue posible obtener una cepa con los dos alelos de *ABG1* interrumpidos.

La imposibilidad de obtener un mutante homozigoto nulo, a pesar de haber utilizado dos metodologías de disrupción distintas, podría deberse a una preferencia de integración del cassette en uno de los alelos, debido a la presencia de la secuencia *hisG*, que proporciona homología entre el primer alelo interrumpido y el ADN exógeno utilizado para transformar, o bien al carácter esencial del gen que se pretendía deleccionar, ya que en ese caso el mutante heterozigoto nulo no es viable, lo que impediría el obtener resultados positivos en una segunda ronda de transformación (**Yesland and Fonzi, 2000**).

III.6.3. Construcción de un mutante condicional para *ABG1*.

Con la finalidad de determinar si la imposibilidad de obtener la doble interrupción génica de *ABG1* se debía al carácter esencial de este gen, o a los fenómenos de preferencia de integración en uno de los alelos, se decidió construir una cepa hemizigota para *ABG1*, derivada de la cepa CV2, obtenida tras disrupcionar una de las copias del gen mediante la técnica del URA-blaster. Mediante el uso del vector pCaDis se conseguiría interrumpir la segunda copia de *ABG1* y al mismo tiempo introducir una copia adicional situada bajo el control del promotor *MET3*. El gen *MET3* de *S. cerevisiae* codifica para la ATP-sulfuroliasa, responsable de catalizar la producción de adenosina5'-fosfosulfato a partir de sulfato inorgánico y ATP.

Esta reacción constituye el primer paso en la asimilación del sulfato inorgánico (**Marzluf, 1997**; **Thomas and Surdin-Kerjan, 1997**). El promotor *MET3* se reprime en presencia de metionina exógena, que es convertida en *S*-adenosil metionina (SAM), compuesto que parece ser el verdadero represor del promotor *MET3* (**Cherest *et al.*, 1985**; **Mountain *et al.*, 1991**; **Thomas *et al.*, 1989**). La biosíntesis de cisteína depende también de los procesos de asimilación de sulfato inorgánico, por lo que la adición de este aminoácido ocasiona también la represión de *MET3*, y algunos autores postulan que sería este compuesto en realidad el verdadero represor del promotor, siendo la acción de la metionina y del SAM dependiente de la interconversión entre los aminoácidos azufrados (**Ono *et al.*, 1996**). Para conseguir la represión génica de *ABG1* se utilizó una combinación de metionina y cisteína a unas concentraciones que oscilaban entre 5 y 2 mM y entre 2.5 y 0.5 mM respectivamente, según se tratara de ocasionar una represión lo mas eficaz posible, para comprobar la viabilidad celular en ausencia de la función de *ABG1* o bien de estudiar los efectos fenotípicos ocasionados por el déficit de expresión de este gen.

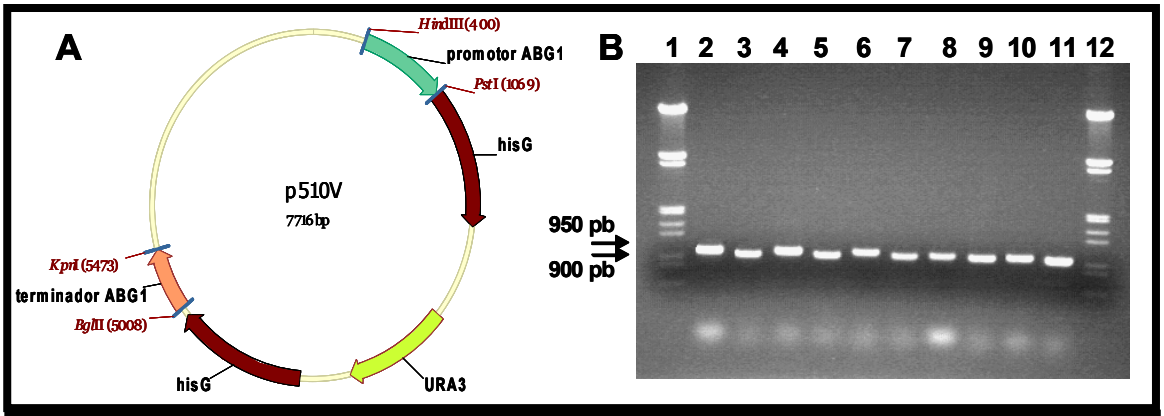

Figura III.10. Interrupción génica de *ABG1* mediante la técnica del URA-blaster.

A. Construcción del plásmido p510V.

B. Comprobación por PCR de los cinco transformantes Ura$^+$ obtenidos en la primera ronda de la interrupción génica. Calles 1 y 12: ADN del fago λgt11 digerido con *Eco*RI y *Hind*III, utilizado como marcador de peso molecular. Los cinco transformantes presentan amplificado con la pareja de cebadores FP4-HisG1R (calles 2, 4, 6, 8 y 10) y con la pareja HisG2F-RP4 (calles 3, 5, 7, 9 y 11) (**Tabla II.5**).

La estrategia de construcción del vector necesario para crear el mutante hemizigoto (**Fig. III.11**) consiste en subclonar el extremo 5' del marco abierto de lectura del gen de interés en el plásmido pCaDis, de forma que el inserto quede tras el promotor *MET3*. Posteriormente se linealiza la construcción utilizando un único sitio de restricción presente en el fragmento del gen clonado y se transforma la cepa heterozigota URA^- con el fragmento lineal de DNA obtenido. El extremo 5' del marco abierto de lectura de *ABG1*, incluyendo el primer triplete ATG, se amplificó por PCR utilizando los oligonucleótidos FP4 y PORF (**Tabla II.5**), con dianas de restricción para *Bam*HI y *Pst*I respectivamente. El fragmento de 200 pb obtenido se digirió con los enzimas de restricción anteriormente mencionados y se ligó en el vector pCaDis, digerido de igual forma.

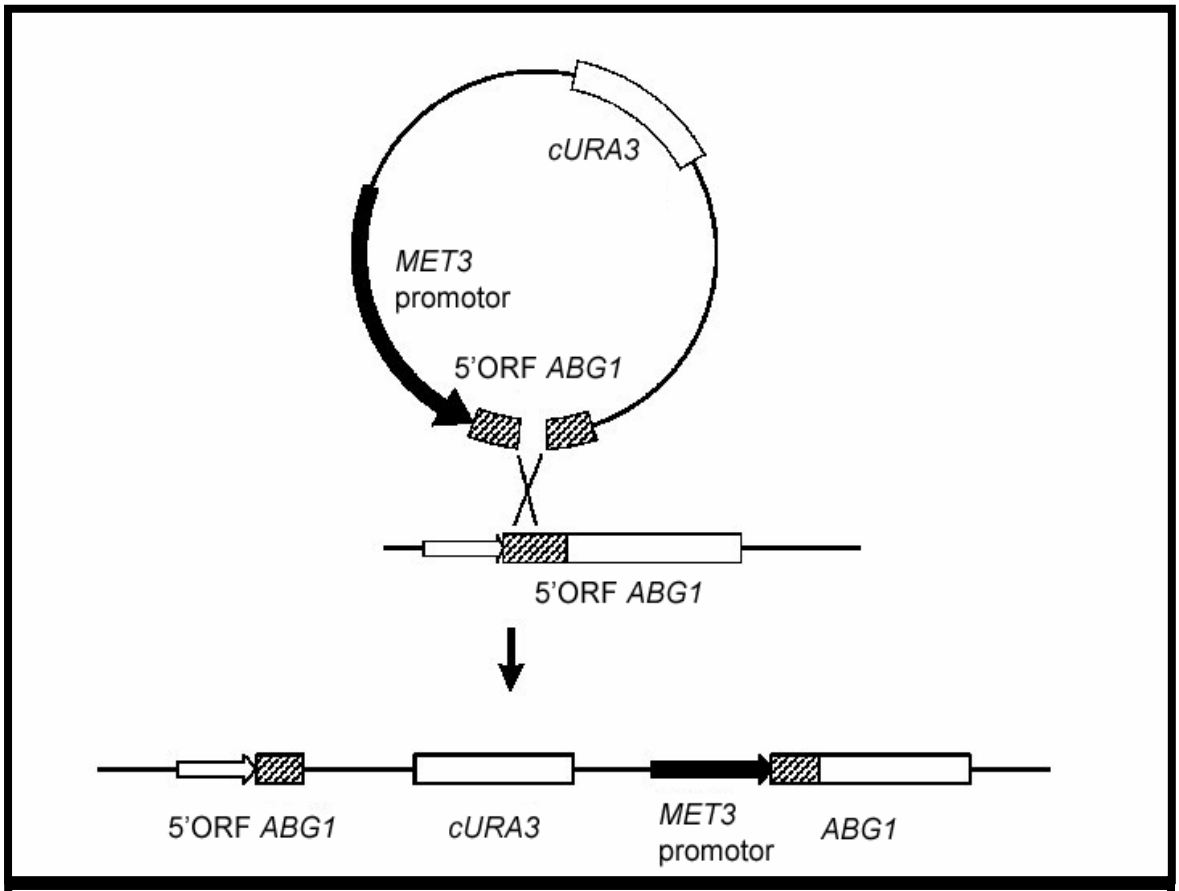

Figura III.11. Estrategia de construcción del mutante condicional CV3.
El uso del vector pCaDis permite la disrupción del segundo alelo del gen de interés y la colocación simultánea de un nuevo alelo situado bajo el control del promotor *MET3*.

La construcción resultante se denominó pMD (**ver sección II.2 del capítulo de Materiales y Métodos**) y se utilizó para transformar la cepa CV2 de *C. albicans*, obtenida en el proceso de interrupción génica (**ver sección III.6.3 del capítulo de Materiales y Métodos**), tras linearizar dicha construcción con el enzima *BsrG*I. Se obtuvieron numerosos transformantes, que fueron analizados por PCR, mediante el uso de un oligonucleótido ubicado en la zona del promotor *MET3* y otro ubicado en la zona 3' no codificante de *ABG1*, y se encontró que uno de ellos, al que se denomino CV3 presentaba amplificado al utilizar esta pareja de oligonucleótidos. Las cepas CV1, CV2 y CV3 se analizaron por Southern (**Fig. III.12**) para comprobar que los procesos integrativos se habían producido de forma adecuada y confirmar los resultados obtenidos por PCR.

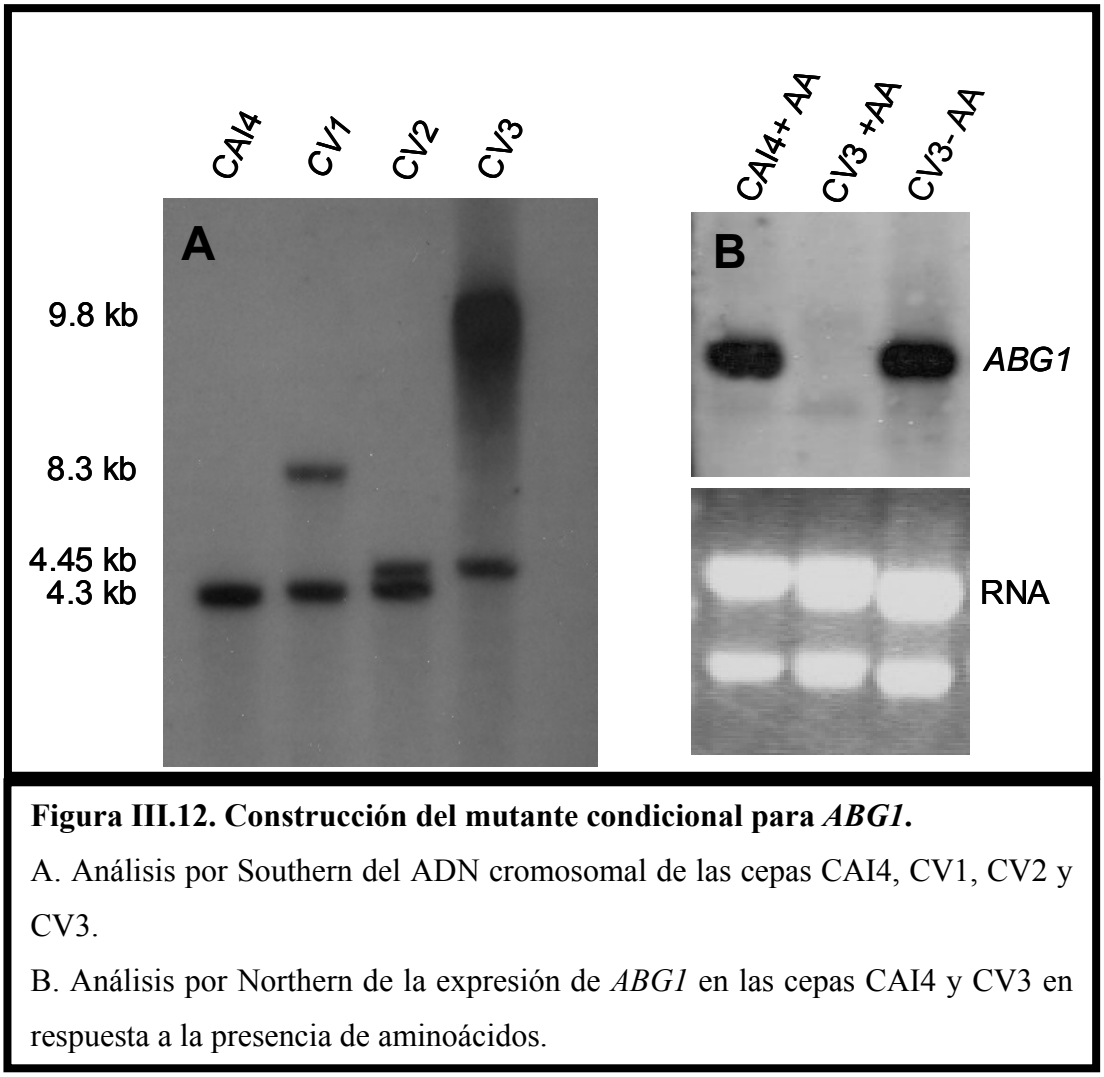

Figura III.12. Construcción del mutante condicional para *ABG1*.

A. Análisis por Southern del ADN cromosomal de las cepas CAI4, CV1, CV2 y CV3.

B. Análisis por Northern de la expresión de *ABG1* en las cepas CAI4 y CV3 en respuesta a la presencia de aminoácidos.

Una vez obtenida la cepa CV3 se procedió a evaluar el efecto de la represión del promotor de *MET3* sobre *ABG1*, y para ello se diseño un experimento de Northern, en el que se analizó la expresión de *ABG1* en presencia y ausencia de metionina y cisteína en la cepa CV3 (**Fig. III.13**). Para ello se cultivó la cepa en medio YNB durante 24 horas y posteriormente células de este cultivo se inocularon a una OD_{600} final de 0.1 en medio YNB suplementado con metionina y cisteína (5 y 2 mM respectivamente).

Después de 4 horas de incubación bajo condiciones de represión no fue posible detectar expresión de *ABG1*. En cambio, el nivel de expresión fue normal cuando las células de la cepa CV3 se inocularon a esa misma densidad óptica en medio YNB sin ambos aminoácidos. Con la finalidad de comprobar que la pérdida de expresión de *ABG1* se debía al control del promotor de *MET3* sobre *ABG*1 y no a un efecto inespecífico causado por la presencia de aminoácidos en el medio de cultivo, se utilizó como control una muestra de RNA procedente de células de la cepa CAI4-*URA3* incubadas durante 24 horas en medio YNB e inoculadas posteriormente en el mismo medio de cultivo suplementado con aminoácidos. En ese último caso se encontró que *ABG1* se expresaba a un nivel similar al observado tras inocular las células de CV3 en medio YNB sin aminoácidos.

El diseño del experimento implicaba la necesidad de inocular las células a baja densidad óptica puesto que algunos autores han descrito que para lograr una represión eficaz del promotor de *MET3* se requiere que la densidad inicial del cultivo sea menor o igual a 0.5, puesto que en caso de inocular más células éstas consumen los aminoácidos presentes en el medio de cultivo rápidamente, disminuyendo así la concentración de los mismos a niveles inferiores a los requeridos para lograr una represión eficaz (**Nishikawa *et al.*, 2002**).

III.6.4. Determinación del carácter esencial de *ABG1*.

Una vez generada la cepa hemizigota *MET3p-ABG1/abg1*Δ*,* denominada CV3, y tras comprobar la correcta integración del promotor así como la funcionalidad del mismo, se procedió a comprobar el efecto de la represión de *ABG1* sobre la viabilidad celular en dicha cepa. Para ello se prepararon placas con agar YNB y placas con agar YNB suplementado con metionina y cisteína (5 y 2 mM respectivamente), en las que se sembró la cepa hemizigota, junto con las heterozigotas Ura^+ y Ura^-, utilizándose como

control la cepa parental CAI4, pero transformada con el plásmido CIp10, lo que permite reintegrar el gen *URA3* en el *locus RPS10*, generando la cepa CAI4-*URA3*, capaz de crecer en medio YNB sin necesidad de añadir uridina y que por tanto permite la comparación a nivel fenotípico con la cepa hemizigota CV3, que es también Ura^+ (**Fig. III.13**).

En las placas que contenían agar YNB sin aminoácidos todas las cepas mostraron un crecimiento similar, a excepción de la cepa CV2, ya que al tratarse de una cepa Ura^- no puede crecer en medio YNB al que no se ha añadido uridina. Sin embargo en las placas de YNB suplementadas con metionina y cisteína se observó un comportamiento similar al observado en las placas de agar YNB, es decir, la cepa CV2 no presentaba crecimiento alguno, y las cepas CAI4-*URA3* y CV1 crecían normalmente, mientras que la cepa CV3 no era capaz de crecer, lo que evidenciaba que *C. albicans* requiere de al menos una copia de *ABG1* funcional para asegurar su viabilidad, lo que demostraba que *ABG1* es un gen esencial para este organismo.

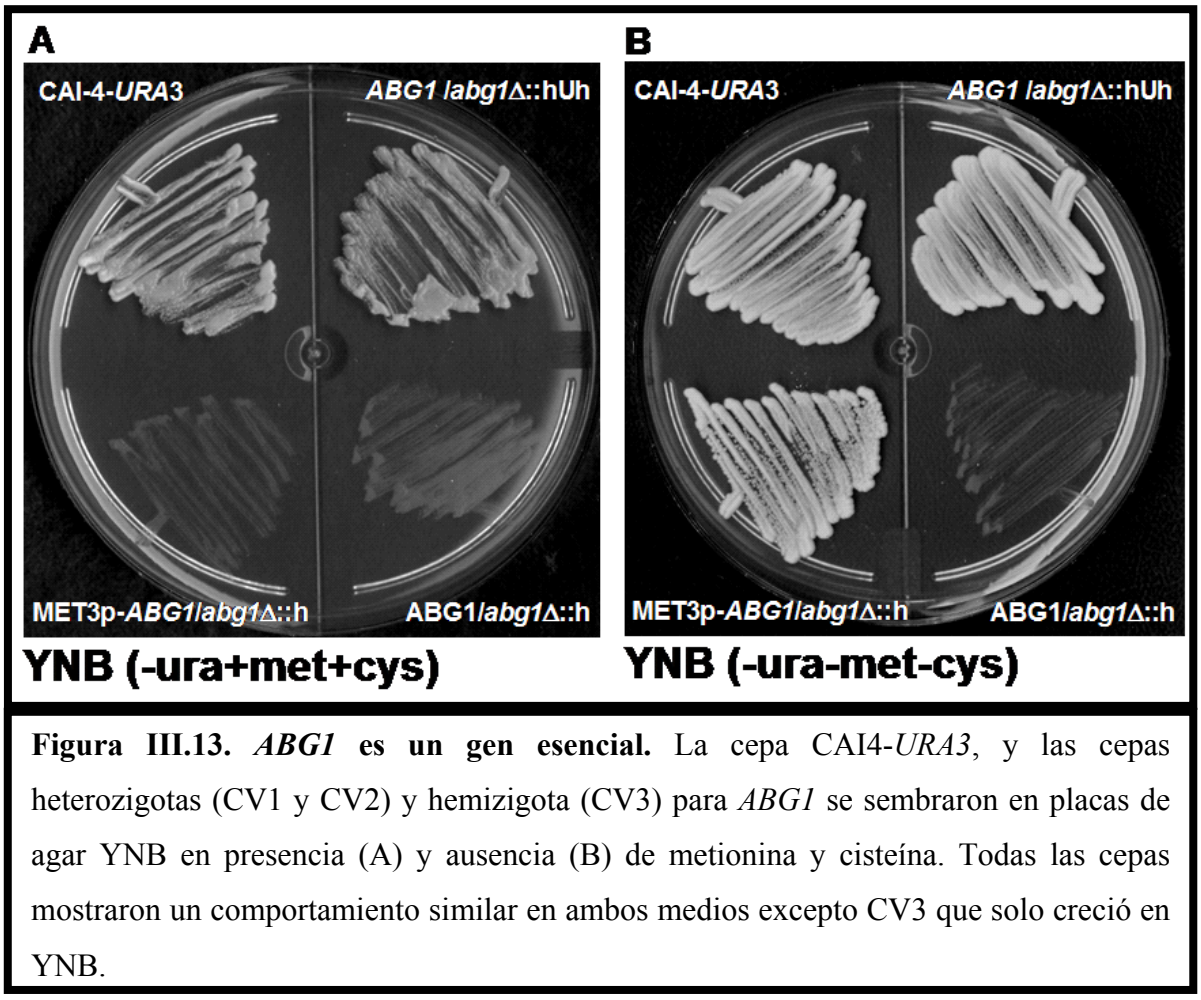

Figura III.13. *ABG1* es un gen esencial. La cepa CAI4-*URA3*, y las cepas heterozigotas (CV1 y CV2) y hemizigota (CV3) para *ABG1* se sembraron en placas de agar YNB en presencia (A) y ausencia (B) de metionina y cisteína. Todas las cepas mostraron un comportamiento similar en ambos medios excepto CV3 que solo creció en YNB.

III.7. Análisis fenotípico del mutante hemizigoto *MET3p-ABG1/abg1*Δ.

El hecho de no disponer de un mutante homozigoto nulo para *ABG1*, al tratarse de un gen esencial, implicaba tener que realizar los estudios fenotípicos en condiciones que permitiesen una represión de *ABG1* en la cepa CV3, pero sin llegar al nivel que inhibiese el crecimiento celular. Esto requería establecer las condiciones, en lo referente a la concentración de aminoácidos, a utilizar en cada prueba fenotípica, teniendo en cuenta, como se ha señalado en secciones anteriores, que los procesos de captación de aminoácidos difieren ligeramente según se trabaje con medios de cultivo líquidos o sólidos, siendo más efectiva en este último caso. En el presente trabajo se utilizó una concentración de metionina/cisteína entre 0.5 mM y 2 mM, cuando se realizaban pruebas de sensibilidad a diferentes compuestos en medio sólido, y entre 2 mM y 5 mM de metionina/cisteína en los experimentos realizados en medio líquido para conseguir una represión mas eficaz.

III.7.1. Análisis del crecimiento en forma levaduriforme.

Durante el crecimiento rutinario de las cepas heterozigotas *ABG1*/*abg1*Δ en medio líquido a 30º C se detectó que al alcanzar las células la fase estacionaria de crecimiento se formaban unas estructuras constituidas por agrupaciones de cadenas de levaduras dispuestas en varias direcciones, formadas habitualmente de tres células, que no se separan y cuyo tamaño decrecía distalmente. La detección de este crecimiento por gemación alterado, ya con un solo alelo interrumpido justificó el denominar a este nuevo gen como *ABG1*, del inglés altered budding growth. Este patrón de crecimiento se repitió en todos los medios de cultivo empleados (Lee, YNB, YPD). Al observar el crecimiento de la cepa hemizigota CV3 se detectó una situación similar, es decir, la formación de cadenas de levaduras de tamaño decreciente, lo que sugería la existencia de un defecto en el proceso de citocinesis, que se manifestaba tanto en ausencia como en presencia de los aminoácidos, aunque en este ultimo caso se apreció una mayor diferencia de tamaño entre las células madre y las hijas, siendo éstas últimas de tamaño bastante menor (**Fig. III.14**).

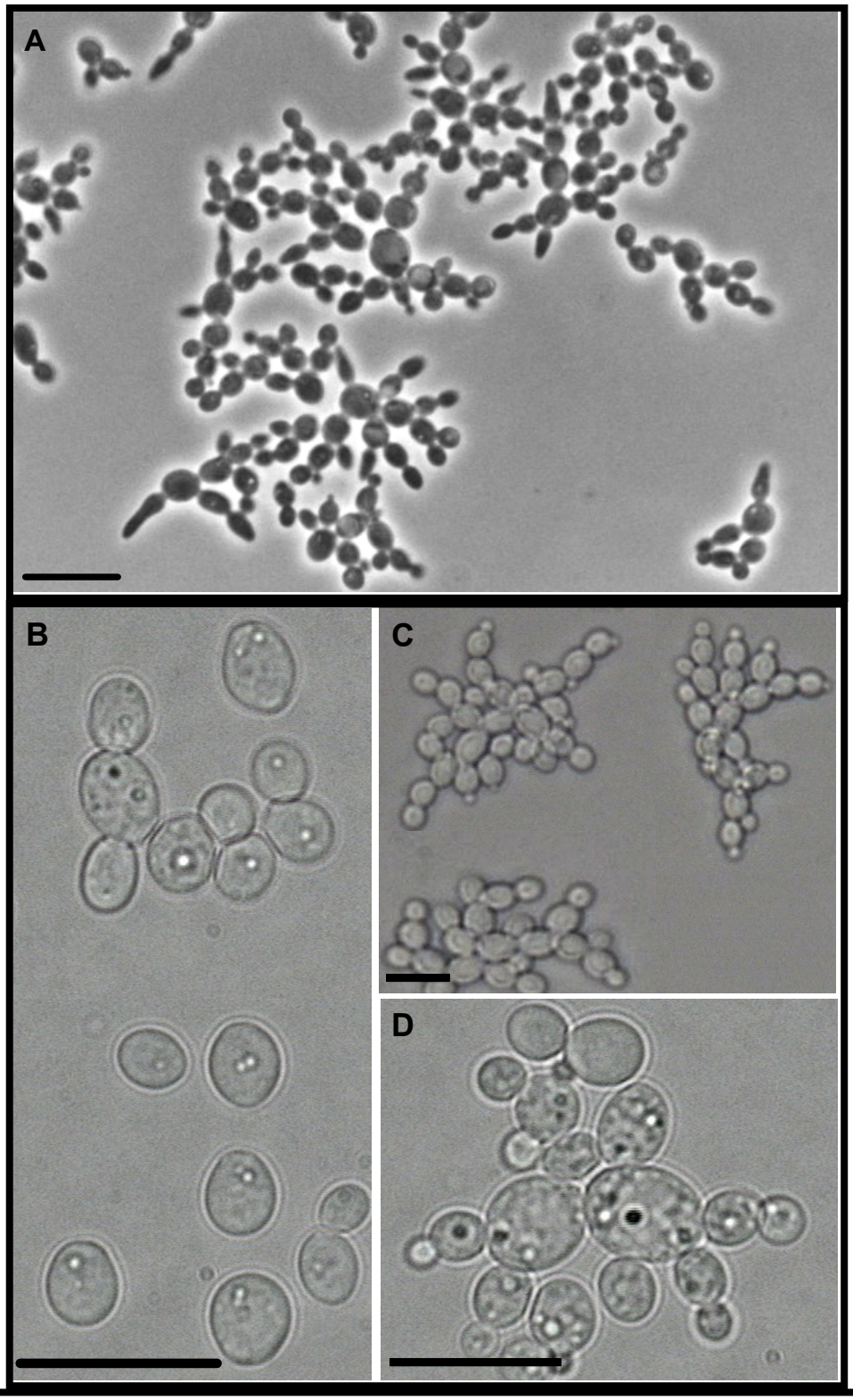

Figura III.14. Observación microscópica de células levaduriformes de *C. albicans*.
(A) Células de la cepa CJ2 crecidas en medio YPD a 30ºC
(B) Células de la cepa CAI4-*URA3* crecidas en medio YNB + metionina + cisteína a 30ºC.
(C, D) Células de la cepa CV3 crecidas en YNB + metionina + cisteína a 30ºC. La barra en cada panel equivale a 10 micras.

La observación mediante microscopía de fluorescencia de la localización, disposición y morfología de los núcleos en las células de la cepa mutante CV3 tras la tinción con DAPI, puso de manifiesto la presencia de núcleos en todas las células de las cadenas de levaduras (**Fig. III.15**), lo que indica que la ausencia de la separación de las células en las cadenas no es consecuencia de un problema en la división y migración de los núcleos.

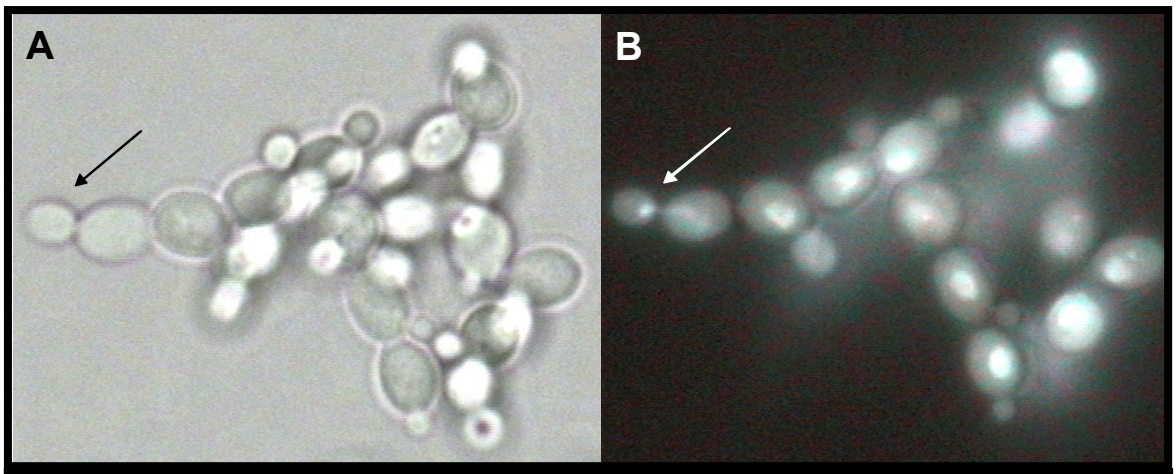

Figura III.15. Visualización de núcleos celulares en el mutante CV3.
A. Observación bajo microscopía de contraste de fases de células levaduriformes de la cepa CV3.
B. Observación bajo microscopía de fluorescencia de la tinción con DAPI de núcleos celulares de células levaduriformes de la cepa CV3. La flecha señala la presencia de núcleo en la célula apical del extremo de la cadena de levaduras.

III.7.2. Análisis del crecimiento en forma micelial.

El estudio del crecimiento en forma micelial en el mutante hemizigoto se realizó en primer lugar con células cultivadas en medio de Lee a 37ºC tras ayuno metabólico (**ver sección II.2.1 del capítulo de Materiales y Métodos**). Células de las cepas CAI4-*URA3* y CV3 incubadas en medio Lee hasta fase estacionaria a 28ºC y posteriormente sometidas a reposo metabólico mediante resuspensión en agua a 4ºC durante 48 horas, fueron inoculadas en medio de Lee precalentado a 37ºC con la finalidad de inducir la transición morfológica levadura-micelio y observar posibles diferencias en el patrón de crecimiento entre ambas cepas.

Transcurridas dos horas desde el momento de la inoculación a 37º C ya fue posible detectar una mayor agregación celular en el cultivo de la cepa CV3 respecto a la cepa parental, con tal intensidad, que impedía incluso visualizar la morfología filamentosa en el mutante (**Fig. III.16, panel B**).

Por ello se decidió modificar el protocolo de inducción de la transición morfológica, haciendo crecer las células en portaobjetos recubiertos con polilisina, polímero que produce la adhesión de las células a la superficie del portaobjetos, permitiendo así el desarrollo del tubo germinativo y la posterior elongación de la hifa en un mismo plano, lo que facilitaba considerablemente la observación microscópica de las preparaciones.

Para este protocolo se utilizó un procedimiento de inducción de la transición morfológica distinto, que se llevó a cabo en presencia de suero, puesto que esta sustancia es el inductor de la miceliación mas potente que se conoce, y porque por las peculiaridades del crecimiento en portaobjetos, el choque térmico no es suficiente para inducir la miceliación. Para llevar a cabo este experimento, se hizo crecer las células sobre portaobjetos recubiertos con polilisina, tal y como se describe en la **sección II.2.1 del capítulo de Materiales y Métodos**, y se procedió a su observación al microscopio, detectándose una mayor frecuencia de ramificación en el mutante condicional (**Fig. III.17**).

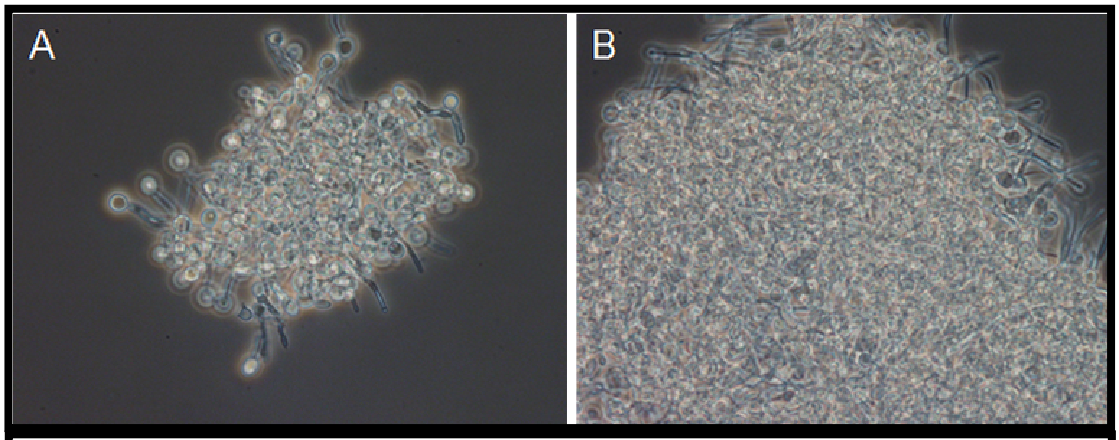

Figura III.16. Observación microscópica de células de *C. albicans* CAI4-*URA3* (A) y CV3 (B) crecidas en medio de Lee (suplementado con metionina y cisteína) a 37ºC.

Con objeto de cuantificar este fenómeno se procedió a determinar la frecuencia con que aparecían ramificaciones en las hifas, así como el número de compartimentos celulares en los que aparecía ramificación. Los experimentos se realizaron por triplicado, utilizando tres replicados biológicos distintos para cada cepa, y los resultados obtenidos fueron validados estadísticamente por ANOVA.

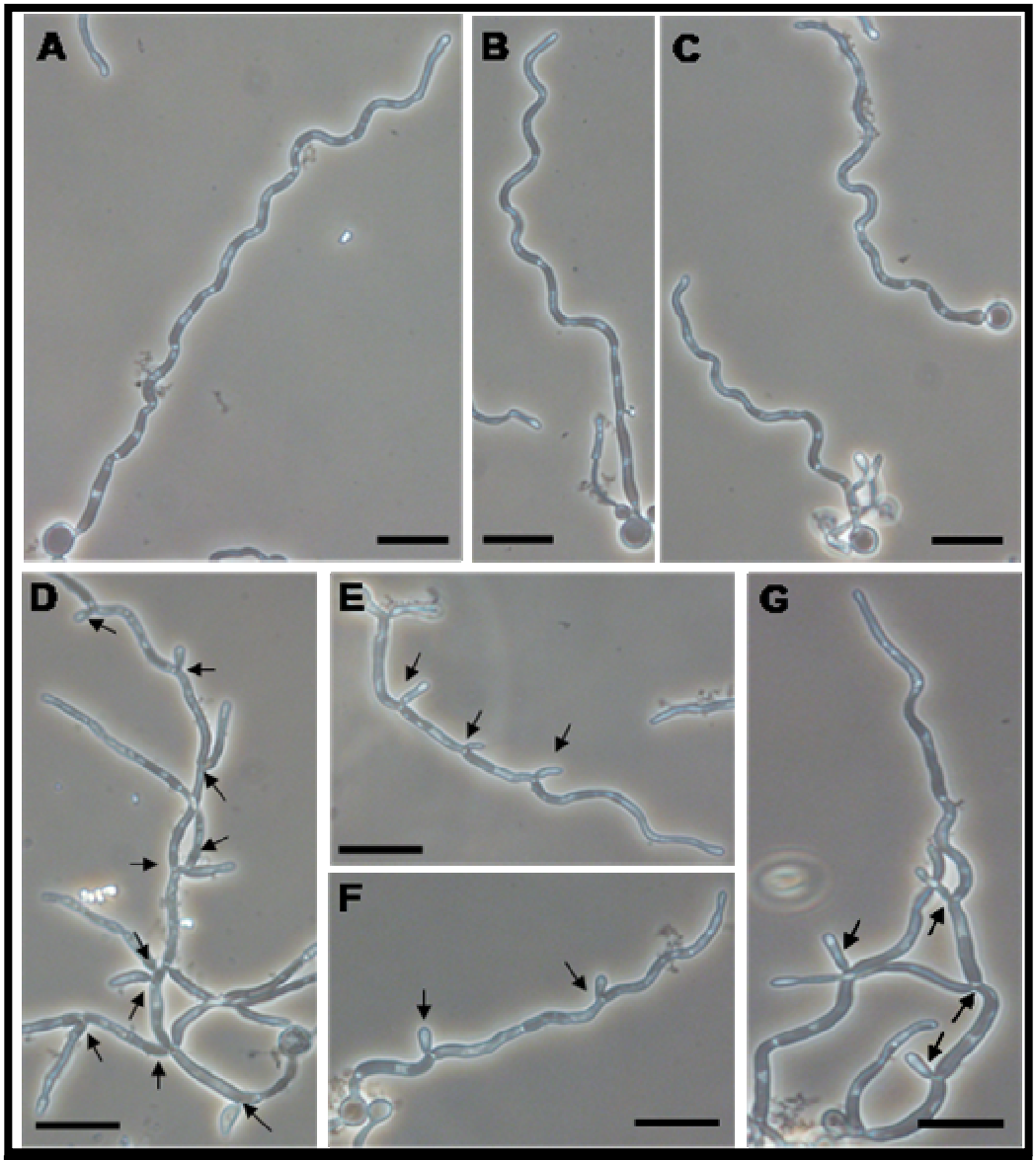

Figura III.17. Observación microscópica de células miceliales de las cepas CAI4-*URA3* (A, B and C) y CV3 (D, E, F and G). La barra en cada panel equivale a 10 µm. Las flechas en los paneles D, E, F y G señalan las ramificaciones en los filamentos miceliales.

Los datos procedentes del análisis de la frecuencia de ramificación se muestran en la **Tabla III.5**. El análisis estadístico de los mismos puso de manifiesto que el 95% ± 5% de las hifas en la cepa CV3 presentaban una o mas ramificaciones, mientras que en el caso de la cepa CAI4-*URA3* el porcentaje de hifas que presentaban ramificación era del 20% ± 5%. En cuanto al porcentaje de compartimentos celulares que presentaban ramificación, la cepa CV3 presentaba ramificación en el 37% ± 11,4% de compartimentos, comparado con unos valores de 6% ± 4% observados para la cepa control.

Tabla III.5. Análisis de la frecuencia de ramificación de las cepas CAI4-*URA3* y CV3, creciendo en forma micelial.

Cepa	Total de hifas	Porcentaje de hifas con alguna ramificación	Total de compartimentos celulares	Compartimentos que presentan ramificación
CAI4-*URA3* replicado nº1	20	20 %	145	10,34%
CAI4-*URA3* replicado nº2	20	25 %	161	6,2 %
CAI4-*URA3* replicado nº3	20	15 %	136	2,2 %
CV3 replicado nº1	33	90 %	159	23,89%
CV3 replicado nº2	20	100 %	222	43,24%
CV3 replicado nº3	20	95 %	206	44,17%

III.7.3. Examen de la morfología vacuolar.

Al estar el producto génico codificado por *ABG1* localizado en la membrana vacuolar, tal como se había puesto de manifiesto mediante los experimentos de fusión génica a GFP (**ver sección III.5 de este mismo capítulo**) se procedió a examinar la morfología de las vacuolas en el mutante condicional CV3, utilizando para ello el colorante vital FM4-64 (**ver sección II.28.3 del capítulo de Materiales y Métodos**).

En el caso de la cepa CAI4-*URA3*, los compartimentos vacuolares ocupan un volumen relativamente grande en el citosol de las células tanto en fase levaduriforme (**Fig. III.18**) como micelial (**Fig. III.19**). En cambio, la observación de los compartimentos vacuolares en las células de la cepa CV3 reveló la presencia de una única vacuola, aparentemente fragmentada en las células levaduriformes (**Fig. III.18**), mientras que en la células creciendo en forma micelial fue posible detectar varias vacuolas en cada compartimento celular, pero más pequeñas que las presentes en la cepa control (**Fig. III.19**).

III.7.4. Análisis de la integridad de la pared celular.

La existencia de un defecto en la citocinesis en las células levaduriformes de las cepas hemizigota y heterozigota para el gen *ABG1* (**ver sección III.7.1 de este mismo capítulo**) sugería una posible implicación del producto de este nuevo gen de *C. albicans* en los procesos de biogénesis de la pared celular fúngica, por lo que se procedió a evaluar la sensibilidad de las células de las cepas CV3 y CV1 a diferentes agentes que interfieren con la construcción de esta estructura celular a diferentes niveles, como el Calcofluor white (CFW), el dodecil sulfato sódico (SDS) y el Rojo Congo. El CFW es un agente que se une a las cadenas nacientes de quitina e impide la unión entre las mismas, impidiendo así la formación de microfibrillas (**Munro *et al.*, 2003**).

El análisis del crecimiento de las cepas CV1 y CV3 en placas de agar YNB conteniendo 125 μg/ml de CFW mostró una hipersensibilidad de las mismas a este compuesto (**Fig. III.20**). Se ha descrito que mutantes con elevados niveles de quitina o con defectos adicionales en la pared celular presentan este tipo de comportamiento (**Ram *et al.*, 1994**).

Los mutantes heterozigoto y hemizigoto para *ABG1* mostraron también hipersensibilidad en su respuesta a Rojo Congo, agente que inhibe el ensamblaje del β-1,3-glucano (**Kopecka and Gabriel, 1992**); sin embargo, presentaron mayor resistencia a la acción del detergente SDS (**Fig. III.20**), lo que sugiere un defecto adicional en la composición química de la pared celular, que ha sido observado ya por otros autores al interrumpir otros genes tanto en *C. albicans* (**Moreno *et al*., 2003**), como en *S. cerevisiae* (**Van der Vaart *et al*., 1995**).

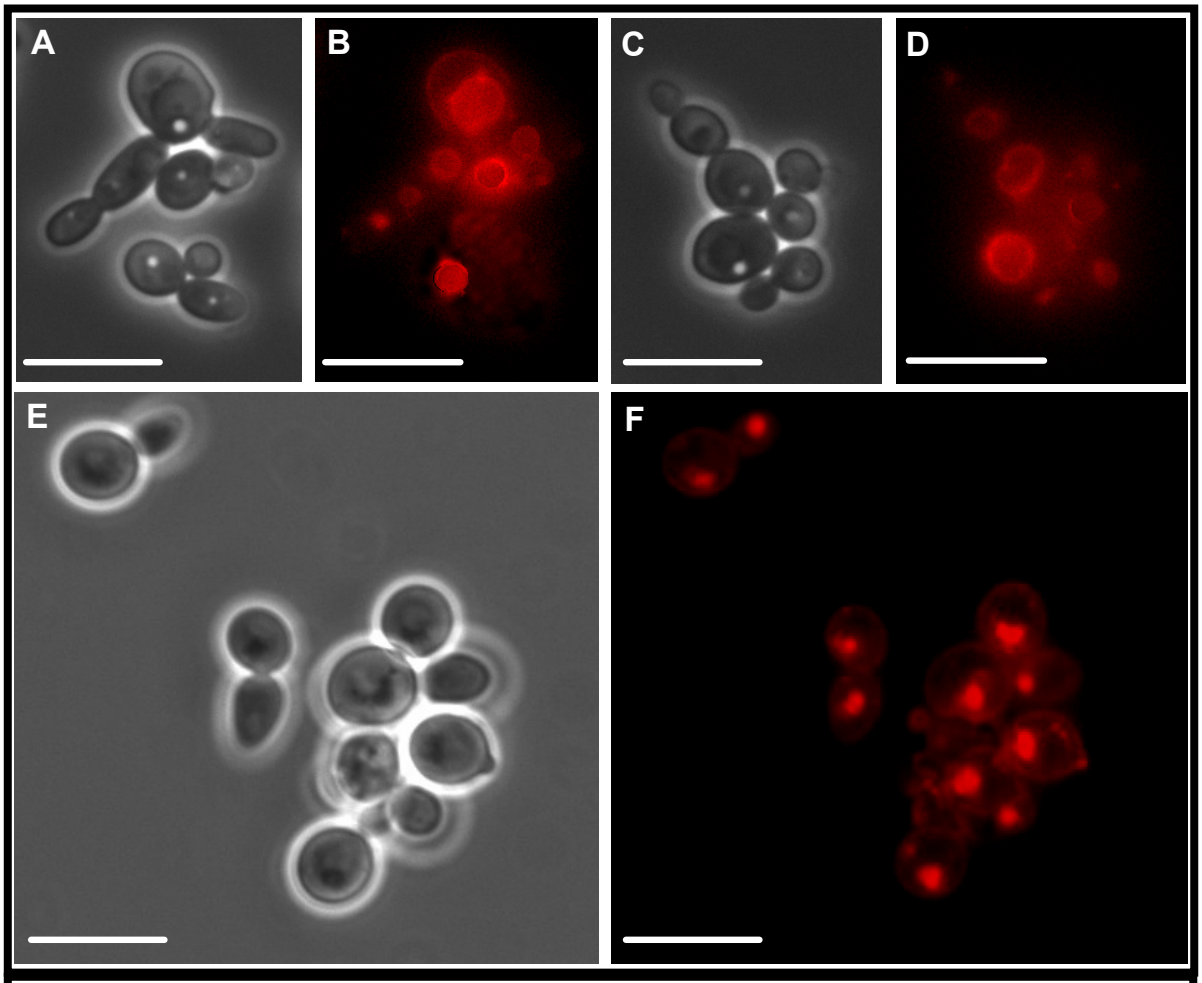

Figura III.18. Examen de la morfología vacuolar en células levaduriformes. Observación bajo microscopía de contraste de fases (A, C y E) y de fluorescencia tras la tinción con FM4-64 (B, D y F) de las células de las cepas CAI4-*URA3* (A-D) y CV3 (E-F). Barra = 10 μm en todos los paneles.

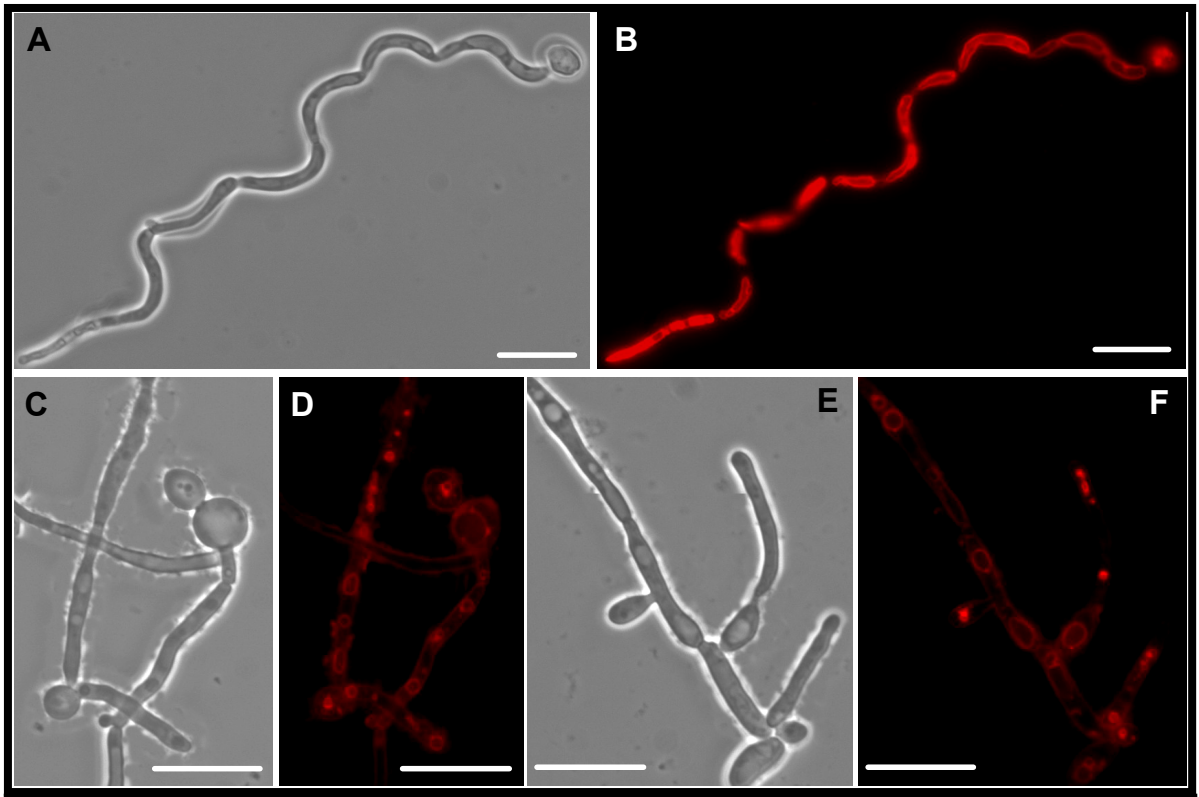

Figura III.19. Examen de la morfología vacuolar en células miceliales. Observación bajo microscopía de contraste de fases (A, C y E) y de fluorescencia tras tinción con FM4-64 (B, D y F) de las cepas CAI4-*URA3* (A y B) y CV3 (C-F). Barra = 10 µm en todos los paneles

III.7.5. Análisis de la respuesta a estrés.

La vacuola fúngica es un orgánulo con funciones relacionadas con la degradación de proteínas celulares, almacenaje de metabolitos celulares y regulación de la homeostasis citoplasmática, lo que implica un papel esencial de la misma para la supervivencia de la célula durante periodos de estrés térmico, osmótico u oxidativo (**Palmer *et al.*, 2003**). En este contexto se procedió a determinar la respuesta de los mutantes heterozigoto (CV1) y condicional (CV3) frente a diferentes situaciones de estrés.

En concreto, se evaluó el crecimiento celular de las cepas CV1 y CV3, comparado con el de la cepa parental CAI4-*URA3* a 37 y 42ºC, así como en situaciones de estrés osmótico (en presencia de cloruro de sodio 1M y glicerol 2.5M), no detectándose diferencia alguna entre el comportamiento de las tres cepas, lo que sugiere que la represión de *ABG1* no modifica la capacidad de respuesta de la vacuola a situaciones de estrés para la célula.

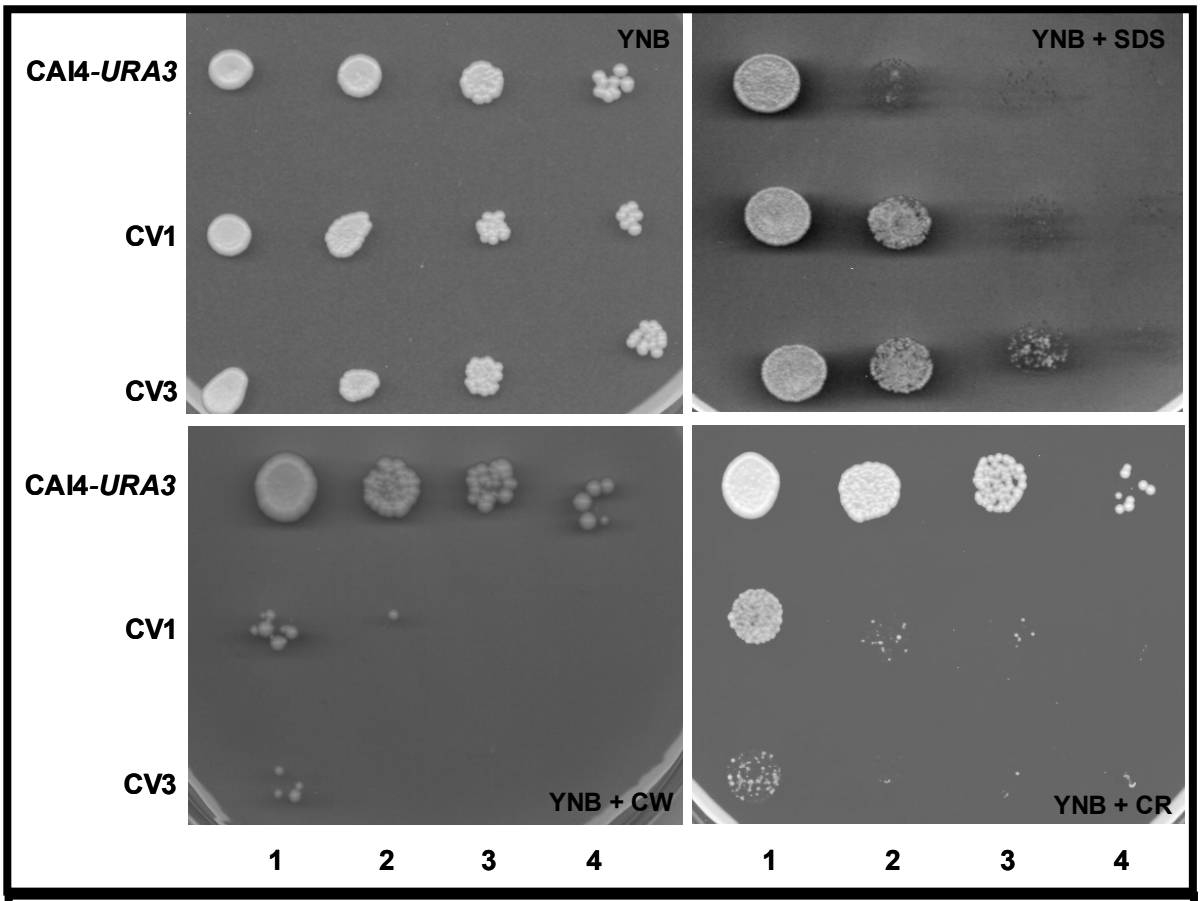

Figura III.20. Sensibilidad a distintos agentes que interfieren en el ensamblaje los componentes de la pared celular. Cinco microlitros de suspensiones celulares seriadas (conteniendo desde 10^6 a 10^3 células/ml), de izquierda a derecha, de las cepas control (CAI4-*URA3*), heterozigota (CV1) y condicional (CV3) fueron inoculadas en la superficie de placas de agar YNB suplementado con aminoácidos y conteniendo SDS (0.025%), Calcofluor White (125 µg/ml) o Rojo Congo (200 µg/ml), respectivamente.

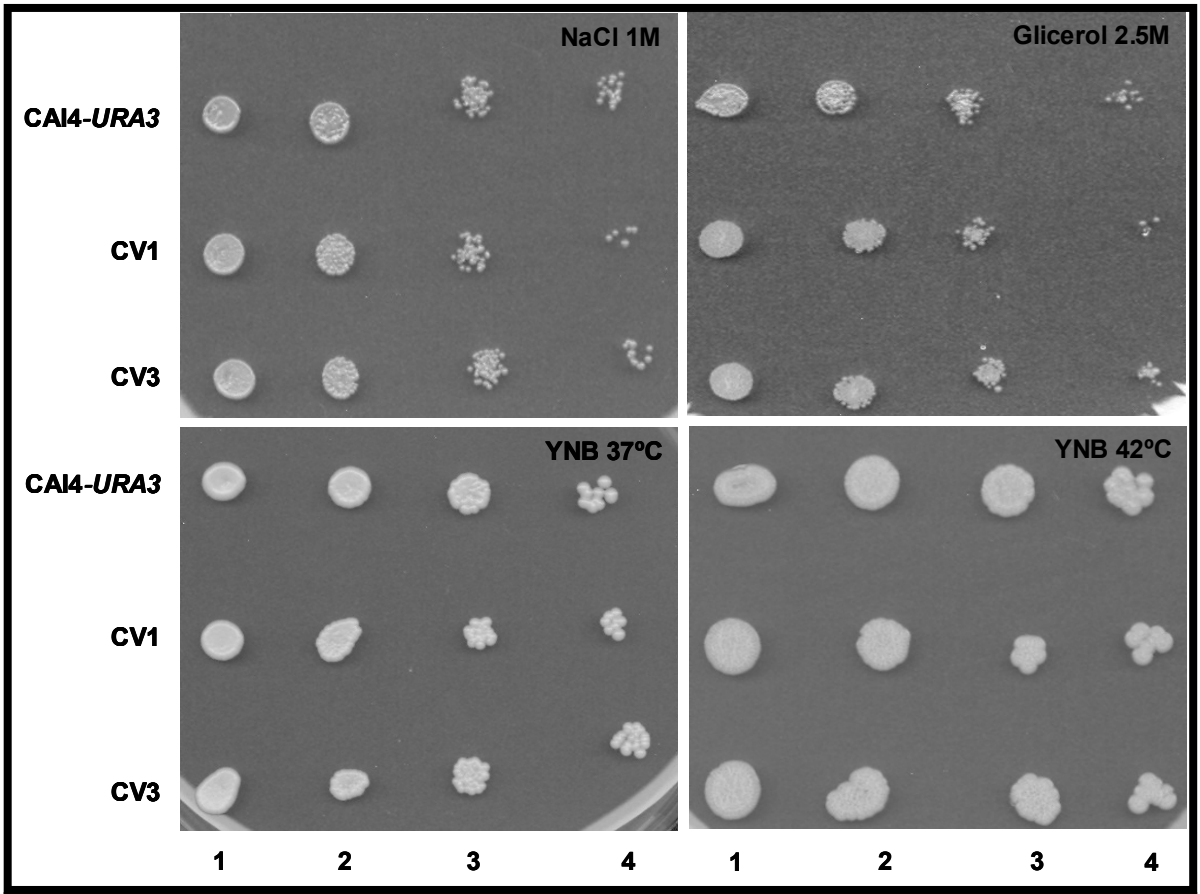

Figura III.21. Análisis de la respuesta a factores de estrés térmico y osmótico.
Cinco microlitros de suspensiones celulares seriadas (conteniendo desde 10^6 a 10^3 células/ml) (calles 1-4, de izquierda a derecha), de las cepas control (CAI4-*URA3*), mutante heterozigota (CV1) y mutante condicional (CV3) fueron inoculadas en la superficie de placas de agar YNB suplementado con NaCl 1M, glicerol 2.5M e incubadas a 28ºC, o en agar YNB e incubadas a 37ºC o 42ºC.

III.7.6. Estudio de sensibilidad a antifúngicos mediante Etest®.

Dentro del conjunto de pruebas encaminadas hacia la caracterización fenotípica del mutante condicional CV3, se procedió a evaluar la sensibilidad de dicha cepa frente a diferentes compuestos con actividad antifúngica, comparándola con la sensibilidad presentada por la cepa de referencia de *C. albicans* (CAI4-*URA3*), utilizada en el presente estudio.

En concreto se realizaron pruebas de sensibilidad, mediante tiras Etest®, frente a itraconazol, fluconazol, voriconazol y anfotericina B, con la cepa mutante condicional (CV3), en placas con agar RPMI 1640, en presencia de metionina y cisteína (5 mM y 2mM respectivamente), y en ausencia de ambos aminoácidos, y con la cepa control (CAI4-*URA3*) en esas mismas condiciones. Tras incubar las placas durante 48 horas se procedió a evaluar los valores de concentración mínima inhibitoria (CMI) del 50% y 80% para los azoles fluconazol y voriconazol y la CMI del 100% en el caso de la anfotericina B e itraconazol, en cada una de las cepas. Los resultados obtenidos se muestran en **la Fig. III.22** y se resumen en la **Tabla III.6**.

Tabla III.6. Sensibilidad frente a antifúngicos de la cepa mutante condicional (CV3) y la cepa control.

Cepa y condiciones de crecimiento	**Fluconazol**		**Voriconazol**		**Itraconazol**	**Anfotericina B**
	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}	MIC_{100}	MIC_{100}
CAI4-*URA3*	0,5	2	0,016	0,125	>32	1,5
CAI4-*URA3* + AA	0,5	2	0,016	0,125	>32	1,5
CV3	0,38	2	0,012	0,032	0.12	1,5
CV3 + AA	0,38	1	0,08	0,047	<0.002	1,5

(+AA: crecimiento en presencia de aminoácidos)

Al comparar la sensibilidad frente a anfotericina B no fue posible detectar diferencias entre los valores de CMI obtenidos en ambas cepas evaluadas, tanto en ausencia como en presencia de los aminoácidos, resultando todas ellas resistentes *in vitro* a este antifúngico. En cambio, al evaluar la sensibilidad frente a los azoles, la cepa mutante condicional mostró unos valores de CMI_{50} y CMI_{80} menores que los que presentó la cepa control cuando ambas crecieron en presencia de aminoácidos, si bien los valores de la CMI_{80} obtenidos para fluconazol y voriconazol indican que todas las cepas analizadas son resistentes *in vitro* a ambos. Al evaluar la sensibilidad frente a itraconazol la cepa parental resultó ser resistente *in vitro* a dicho agente, mientras que la cepa mutante condicional presentaba una CMI_{100} que la situaba como sensible a este compuesto antifúngico.

No se encontraron diferencias entre los valores de CMI obtenidos con la cepa control en función de la presencia o ausencia de metionina y cisteína en el medio de cultivo. En cambio, la cepa mutante condicional creciendo en medio sin aminoácidos presentaba unas CMI intermedias entre los valores obtenidos para la cepa control y los obtenidos para la propia mutante condicional en presencia de los aminoácidos, en el caso del voriconazol y del itraconazol, y unos valores similares a los de la cepa mutante condicional en presencia de los aminoácidos en el caso del fluconazol.

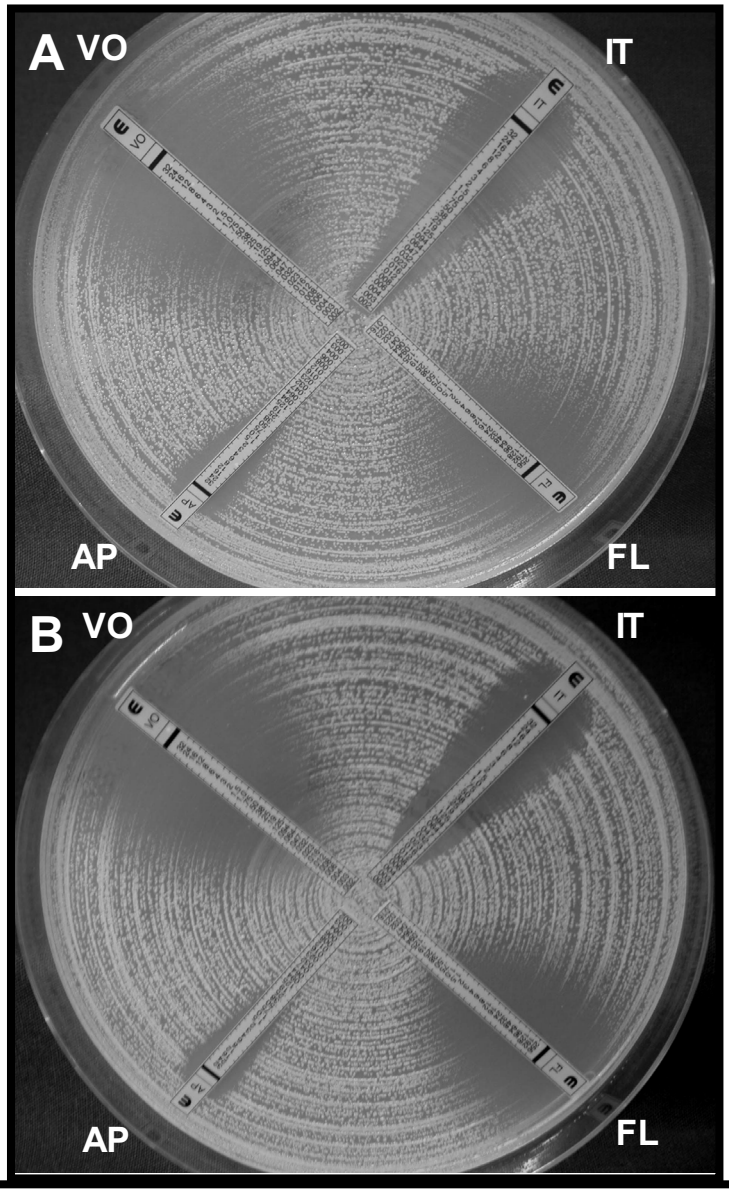

Figura III.22. Sensibilidad in vitro a diferentes agentes antifúngicos.
Determinación mediante tiras Etest® de la sensibilidad de la cepa parental CAI4-*URA3* (A) y de la mutante condicional CV3 (B) a voriconazol (VO), itraconazol (IT), fluconazol (FL) y anfotericina B (AP).

III.8. Perfil transcripcional del mutante condicional CV3.

Con la finalidad de lograr una aproximación lo más precisa posible sobre la función de *ABG1* en *C. albicans*, se decidió llevar a cabo un estudio comparativo del perfil de expresión génico en el mutante condicional CV3 frente a la cepa control CAI4-*URA3*, para detectar los genes expresados diferencialmente en la cepa mutante.

Para estos estudios se utilizaron microarrays de DNA de *C. albicans* fabricados por Eurogentec S.A. en colaboración con el European Galar Fungail Consortium. Estos microarrays contienen "spots" correspondientes a amplificados procedentes de las 6.039 posibles ORFs del genoma de *C. albicans*, por duplicado.

A partir de células de las cepas CAI4-*URA3* y CV3 incubadas hasta fase estacionaria, se aisló el ARN total (**ver sección II.29.1 del capítulo de Materiales y Métodos**). El ARN obtenido se utilizó para sintetizar ADNc mediante una reacción de transcripción inversa en la que se incorporó a las nuevas moléculas sintetizadas los fluoróforos Cy5-dCTP y Cy3-dCTP, empleando el primero para la cepa CAI4-*URA3* y el otro para el mutante CV3 en uno de los microarrays (**Fig. III.23**) y al contrario en el segundo microarray. El intercambio de fluoróforos se realiza de forma rutinaria con la finalidad de equilibrar la emisión de fluorescencia, ya que el fluoróforo Cy3-dCTP emite con mayor eficacia que Cy5-dCTP. Tras realizar la hibridación según el procedimiento descrito en la **sección II.29.4 del capítulo de Materiales y Métodos**, se procedió a leer los microarrays utilizando un lector de barrido GenePix (Axon Instruments), que permite registrar la emisión de fluorescencia por los fluoróforos presentes en el ADNc que se ha hibridado sobre el microarray.

La aplicación del software Genespring permitió el análisis estadístico de la imagen de fluorescencia obtenida tras la lectura de los microarrays, obteniendo así el conjunto de genes que aparecen expresados diferencialmente en la cepa mutante condicional (CV3) respecto a la cepa control (CAI4-*URA3*). Dicho análisis estadístico permitió detectar 8 genes que estaban sobreexpresados en el mutante condicional CV3, y que son los que se muestran en la **Tabla III.7.**

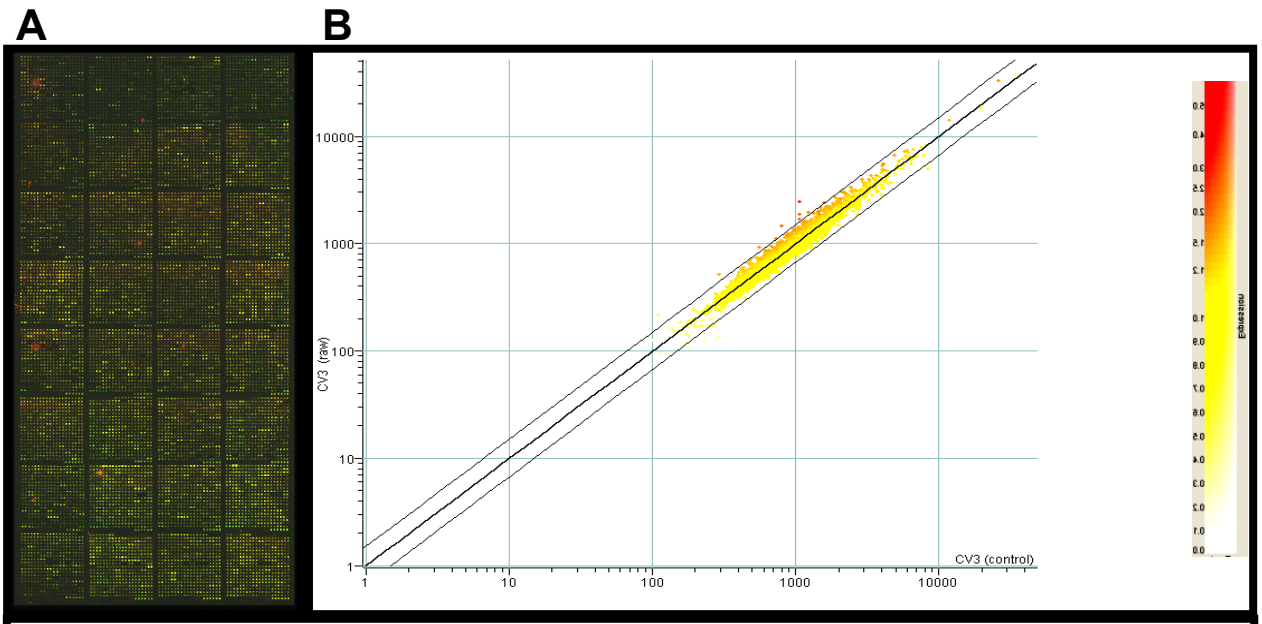

Figura III.23. Análisis del perfil transcripcional del mutante CV3.
A. Imagen de un microarray de *C. albicans* hibridado con ADNc de la cepa parental CAI4-*URA3* (marcado con Cy5) y con ADNc de la cepa mutante condicional CV3 (marcado con Cy3).
B. Gráfica que muestra los niveles de expresión génica de la cepa CV3 en comparación con la cepa control. Los puntos comprendidos entre las líneas superior e inferior representan los genes de CV3 que presentan una expresión similar a los valores de la cepa control. Los puntos que aparecen por encima de la línea superior representan aquellos genes que están sobreexpresados en la cepa CV3.

III.9. Identificación de componentes proteicos que puedan encontrarse interaccionando con Abg1p *in vivo*.

Puesto que tras el análisis fenotípico y la caracterización del perfil transcripcional del mutante CV3, continuaba sin poder determinarse fehacientemente el papel biológico de la especie Abg1p *in vivo*, se procedió a utilizar una aproximación proteómica para establecer la función biológica del producto del gen *ABG1*, llevando a cabo una identificación de las proteínas celulares que pudieran interaccionan con Abg1p *in vivo*. La aproximación empleada consistió en añadir una "etiqueta" a la proteína objeto de estudio que permitiese en base a una determinada característica de dicha etiqueta, por ejemplo, la capacidad de interaccionar con la región Fc de la molécula de la inmunoglobulina G, IgG, si la etiqueta utilizada es la proteína A, como en el caso del

Tabla III.7. Genes sobreexpresados en el mutante condicional CV3.

Expresión en CV3	Gen (Nombre sistemático)	Gen (Nombre común)	Función
2,3	CA0316	*ALS1.3EOC*	Proteína pseudoaglutinina (Agglutinin-like protein)
1,8	CA0413	*ALS12.3F*	Proteína pseudoaglutinina Agglutinin-like protein
1,8	CA1528	*ALS4.3F*	Proteína pseudoaglutinina Agglutinin-like protein
1,7	CA5857	*PCK1*	Fosfoenolpiruvato carboxiquinasa
1,7	CA6079	*RPL18*	Proteína ribosomal (subunidad mayor)
1,6	CA3566	*GUT2*	Glicerol-3-fosfato dehidrogenasa (mitocondrial)
1,6	CA3309	IPF8806	6-fosfofructosa-2-quinasa
1,6	CA2075	*IFE2*	Función desconocida

presente trabajo, separar dicha proteína y las que están interactuando con ella, del resto de proteínas celulares (**Puig *et al.*, 2001; Blackwell *et al.*, 2003**). La probabilidad de aislar las proteínas que se encuentren interaccionando con la de interés aumenta cuanto más suaves son las condiciones de obtención del complejo, es decir, cuando se trabaja en condiciones que se aproximan a las condiciones nativas.

Para el marcaje de Abg1p con la proteína A se utilizó el vector pACT-C-ZZ (**ver sección II.3 del capítulo de Materiales y Métodos**). Este vector esta construido en el plásmido CIp10 y permite la incorporación de la proteína A en el extremo carboxi-terminal de la proteína de interés, separada por una secuencia que contiene una diana de restricción para la proteasa del "tobacco etch virus" (TEV), que sirve para eliminar post-traduccionalmente la proteína A por tratamiento con la proteasa, liberando así las proteínas unidas a la IgG mediante la interacción con la proteína A. La estrategia de marcaje de la proteína Abg1p con la proteína A consistía en clonar el ORF de *ABG1* en el vector pACT-C-ZZ de forma que quedase colocado tras el promotor de la actina y fusionado en fase por el extremo carboxi-terminal con la proteína A, que quedaba separada de la proteína Abg1p por la secuencia de corte para la proteasa (**Fig. III.24**).

Para realizar el marcaje de Abg1p, de acuerdo con la estrategia descrita, se amplificó por PCR el ORF de *ABG1*, utilizando los oligonucleótidos TAPF y TAPR (**ver Tabla II.5 del capítulo de Materiales y Métodos**), que permitían obtener dicho fragmento con las dianas de restricción para *Hind*III y *Sal*I, respectivamente. Tras realizar la digestión con estos enzimas se procedió a clonar el fragmento obtenido en el vector pACT-C-ZZ, digerido de igual forma y se transformó la cepa CAI4 con la construcción resultante de forma no integrativa, generando la cepa T1 de *C. albicans*, que contenía el plásmido pACT-*ABG1*-ZZ-PA, lo que se comprobó por PCR, al obtenerse un amplificado de 1.000 pares de bases con los oligonucleótidos PAF-TAPR. Con la finalidad de comprobar si la proteína de fusión estaba expresándose en este mutante se preparó un extracto celular (**ver sección II.20 del capítulo de Materiales y Métodos**), con el que se realizó una inmunotransferencia, de acuerdo con procedimiento descrito en las **secciones II.22 y II.24 del capítulo de Materiales y Métodos**, utilizando como sonda un anticuerpo comercial frente a la Proteína A (Sigma), detectándose una única especie inmunoreactiva, de aproximadamente 60,000 Da, coincidente con el tamaño esperado para la proteína de fusión Abg1p-PA (**Fig. III.25**).

Tras comprobar que la proteína recombinante se estaba expresando, se procedió a preparar un extracto de la cepa T1, mediante rotura mecánica de las células, obteniendo un lisado celular total, que se incubó durante 16 horas a 4ºC en presencia de IgG-Sepharose, favoreciendo así la unión de Abg1p (junto con las proteínas que estuvieran interaccionando en ese momento a ella) a las partículas de la matriz cromatográfica recubiertas de IgG, a través de la proteína A.

A continuación, se empaquetó la matriz cromatográfica (**ver sección II.30 del capítulo de Materiales y Métodos**) con las proteínas retenidas en una columna de cromatografía y, tras un lavado exhaustivo de la misma, se procedió a un tratamiento con la proteasa del TEV para cortar la secuencia entre Abg1p y la proteína A en el complejo. Las proteínas liberadas tras el tratamiento con la proteasa viral se eluyeron de la columna con tampón acetato potásico 0.5M. El eluido fue analizado mediante SDS-PAGE y tras la tinción con Coomasie blue, se detectaron 6 especies polipeptídicas (**Fig. III.25**), que fueron analizadas mediante MALDI-TOF. Los resultados de este estudio se muestran en la **Tabla III.8**.

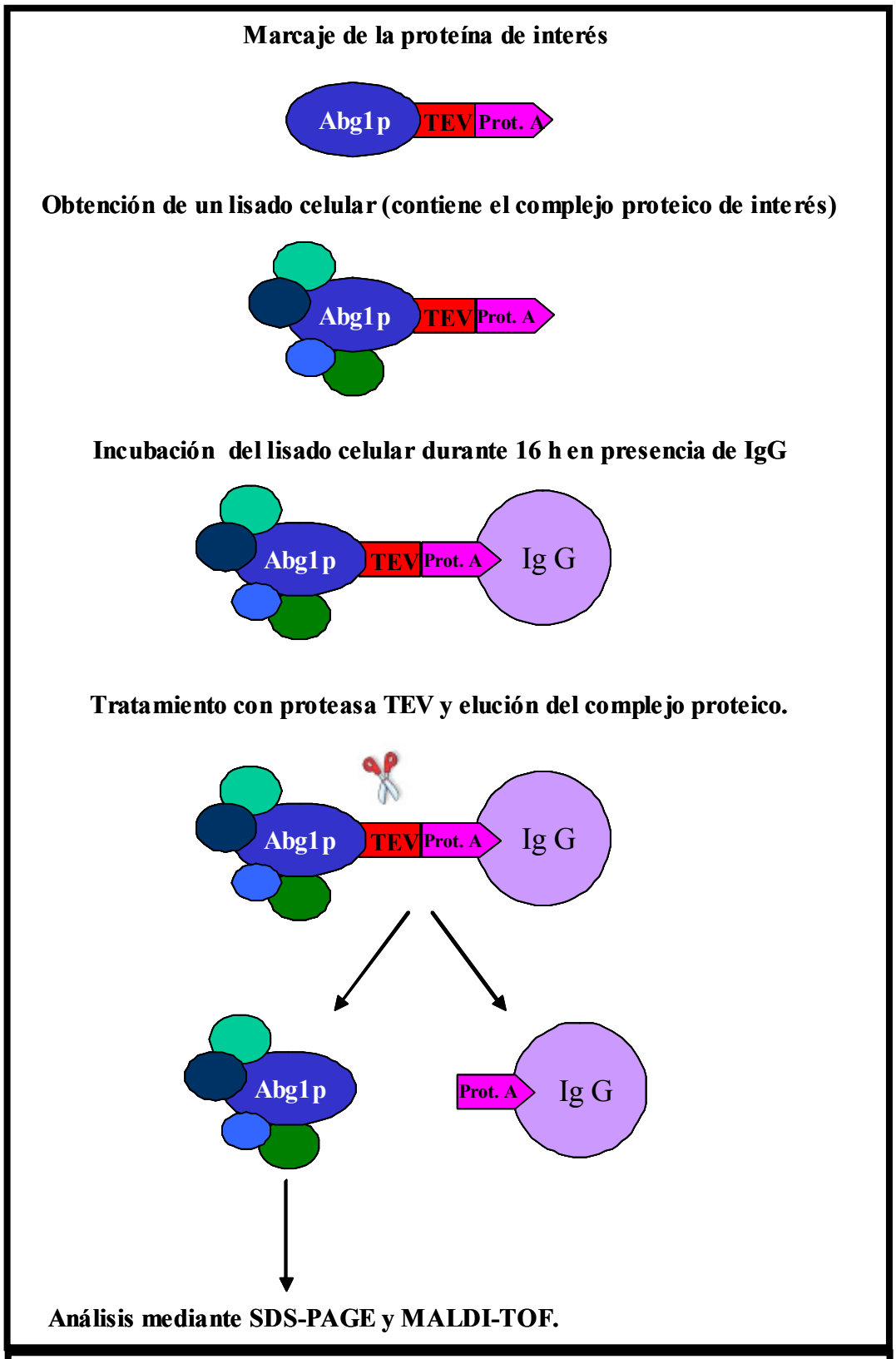

Figura III.24. Representación esquemática del proceso de obtención del complejo proteico de interés.

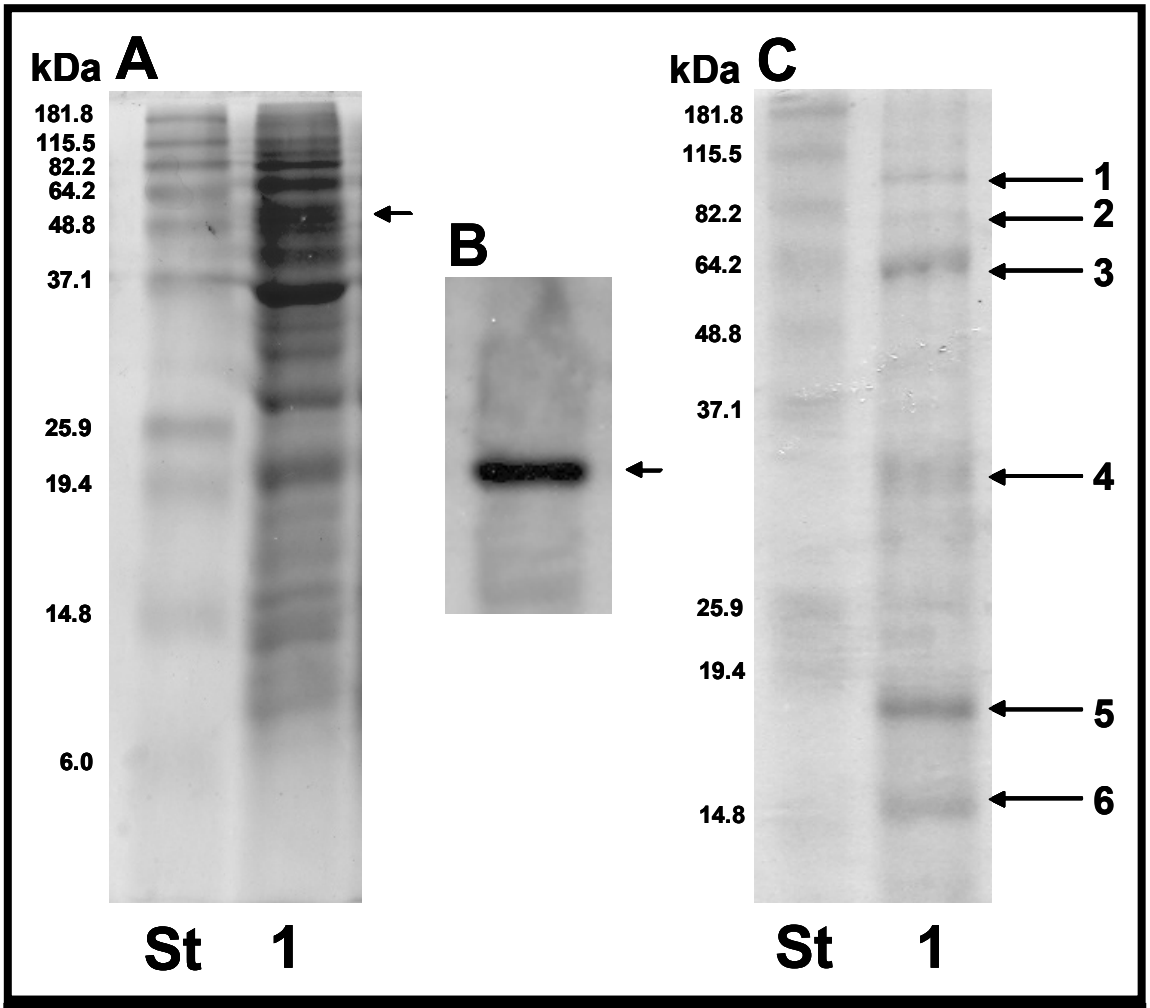

Figura III.25. Análisis mediante electroforesis en geles de poliacrilamida e inmunotransferencia de los extractos celulares de la cepa T1 de *C. albicans*.
A. SDS-PAGE y tinción con Coomasie blue de un lisado celular total (calle 1) de la cepa T1 de *C. albicans*.
B. SDS-PAGE y detección mediante inmunotransferencia (Western blotting) con el PAb anti-proteína A. La muestra sometida a análisis fue el extracto celular total de la cepa T1 de *C. albicans*. Las flechas en los paneles A y B señalan la especie proteica que correspondería al polipéptido recombinante Abg1p-PA, de 60 kDa.
C. SDS-PAGE y tinción con Coomasie blue de las especies proteicas eluidas de la columna de Ig-Sepharose tras el tratamiento con la proteasa del TEV (calle 1). Las flechas señalan la posición de las 6 bandas mayoritarias detectadas. La porción de gel correspondiente a cada banda fue cortada, y los diferentes polipéptidos presentes en cada sección del gel, fueron digeridos con tripsina y los fragmentos resultantes se analizaron mediante MALDI-TOF.
En las calles marcadas con las letras St de los paneles A y C se corrió en paralelo una mezcla de proteínas estándar de peso molecular conocido que se indica en kDa a la izquierda de cada panel.

Tabla III.8. Especies proteicas asociadas a Abg1p.

Número de banda en la Fig. III.24, panel C	Especie proteica	Tamaño molecular (Da)
1	Aminopeptidasa yscII (Ape2p)	107.579
2	Factor de elongación de la transcripción (Eft2p)	93.865
3	Proteína de choque térmico Ssa4p	70.452
3	Proteína de choque térmico Ssa1p	70.199
4	3-hidroxi-metilglutaril coenzima A sintasa (Erg13p)	49.952
5	O-acetilhomoserina O-acetilserina sulfhidrilasa (Met15p)	47.959
5	Enolasa I (Eno1p)	47.203
6	Proteasa del “tobacco etch virus” (TEV)	34.821

IV. DISCUSSION

In the past years, molecular genetic approaches have been used to identify genes encoding hypha-specific cell-surface proteins that may contribute to differences in cell-wall structure or function in *C. albicans* (**Chaffin *et al.*, 1998; Hoyer *et al.*, 1995**; **Staab *et al.*, 1996; Chen *et al.*, 2000**), since it is believed that hyphal cells expressing cell-wall proteins that facilitate adhesion to human tissues are important for tissue invasion, as well as for escape from phagocytosis mediated by neutrophils or macrophages (**Chaffin *et al.*, 1998; Martínez *et al.*, 1998; Berman and Sudbery, 2002**), although it has been recently suggested that is the ability to switch between both morphological forms which is the key factor for *C. albicans* virulence (**Saville *et al.*, 2003; Berman and Sudbery, 2002; Mitchell, 1998**). In this context, genetic manipulations that are carried out easily in *S. cerevisiae* are much more laborious in *C. albicans* because of the lack of a complete sexual cycle in this presumed obligate diploid. However, recent advances in molecular-genetic techniques, together with the availability of the complete *C. albicans* genome sequence, have revolutionized research in this organism. Moreover, recent reports described the presence of mating-type-like orthologs (MLT) in *C. albicans* of both of the *S. cerevisiae* mating-type genes (MAT) a and α (**Hull and Johnson, 1999; Magee and Magee, 2000**), so conventional genetic techniques might soon be available in *C. albicans*.

In this work, immunoscreening of a *C. albicans* cDNA library with a polyclonal antiserum which specifically recognized protein and glyco(manno)protein components of the cell wall of germ tubes (PAb anti-gt; **Casanova *et al.*, 1989**), led to the isolation of a novel gene encoding a putative polypeptide of 288 amino acids, which on the basis of the ***a***ltered ***b***udding ***g***rowth pattern exhibited by the heterozygous and conditional strains for this gene, was designated as *ABG1*. A search for sequence similarities in the current protein databases revealed that there was no obvious homologue of *ABG1* gene product (Abg1p) in mammalian cells, and the best match (27.7% overall identity) was found with a hypothetical ORF from the most related organism *S. cerevisiae* that encodes a putative polypeptide of 293 amino acids.

Analysis of the deduced amino acid sequence from Abg1p showed the presence of a potential transmembranal domain and a signal peptide and the use of a specific PAb anti-Abg1p antibody, allowed the identification in Western immunoblots of a 32.9 kDa immunoreactive polypeptide mostly in a mixed membrane fraction (P40) obtained by

centrifugation at 40,000 x g from a whole homogenate of *C. albicans* cells, but also in microsomal structures subsequently sedimented from the same cell-free homogenate at 100,000 x g (P100 fraction), and in SDS cell wall extracts, thus suggesting that *ABG1* encoded an integral membrane protein rather than a cell wall protein. The fact that antibodies raised against purified cell wall preparations may also cross-react with non-cell wall components released during the isolation and purification processes is not unusual since similar results have been also reported by different authors (**Eroles *et al.*, 1997**; **Galán *et al.*, 2004**). It should be stressed that subcellular fractionation followed by Western blotting analysis was the only suitable procedure available for immunological detection of the product coded by *ABG1* gene, since the presence of a thick wall in *C. albicans* cells represents an unavoidable obstacle (along with the plasma membrane) for immunoglobulins to access to cytosolic space. Hence, subcellular immunolocalization of Abg1p was not a likely alternative, unless an immunoelectronmicroscopy-based procedure would have been employed. However, the complex procedures required to prepare the samples for electron microscopy observations along with the fact that the only immunological probe available in our hands was a polyclonal antibody would have most likely led to spurious artifactual or unspecific findings.

Data available about the possible *S. cerevisiae* homolog (YGR106c) for *ABG1 C. albicans* gene in the *Saccharomyces* Genome database (http://www.yeastgenome .org) showed that the GFP-tagged gene product for YGR106c was found to be located in the yeast vacuolar membrane (**Huh *et al.*, 2003**), although the biological function of YGR106c remained still unknown. Complementation approaches in order to establish whether *ABG1* and YGR106c are true homologs have severe limitations since the *S. cerevisiae ygr106c*Δ mutant has no detectable phenotype and YGR106c ORF contains at least one CUG codon which in *C. albicans* is translated as serine instead of the universal leucine (**Santos *et al.*, 1993**). Abg1p was tagged with the codon optimized GFP (**Cormack *et al.*, 1997**) and was localized in the vacuolar membrane of living *C. albicans* cells, in both yeast and hyphal forms, thus confirming that gene products from both *ABG1* and YGR106c share the characteristic of being located at the vacuolar membrane level, despite the low homology existing between both gene products.

Although vacuolar mislocalization of GFP-tagged proteins has been reported (**Kunze *et al.*, 1999**), these species were NH_2-terminal GFP fusion products in contrast with the COOH-terminal fusion protein used in this work, and vacuolar targeting signals are usually within the NH_2-terminal sequences of proteins (**Klionsky *et al.*, 1990**; **Martínez *et al.*, 1999**). Moreover, Abg1p localization at the vacuolar membrane could explain the cross-reaction with the germ-tube specific polyclonal antibody used in the immunoscreening, since the vacuole is a large compartment in the cytoplasm of hyphal cells (**Gow and Gooday, 1982**). For this reason, it is possible that contamination with vacuolar components may occur during the cell wall purification process, and consequently polyclonal antiserum raised towards purified cell walls (PAb anti-gt) may also generate antibodies to vacuolar components.

Construction of a conditional strain for *ABG1* under the control of *MET3* promoter enabled us to demonstrate that this novel gene coding for a vacuolar protein was essential for *C. albicans*, since the growth of the conditional strain was completely inhibited in the presence of methionine and cysteine at levels that allowed the normal growth of the CAI4-*URA3* parental strain. Methionine and cysteine-mediated repression of *ABG1* in the conditional strain was used to study the effects of Abg1p depletion on the morphogenesis and vacuolar morphology of *C. albicans*. Under repressing conditions, yeast cells of the conditional strain exhibited a morphological phenotype characterized by a failure in cytokinesis that lead to the formation of bud chains whose size was decreasing towards the distal apex of the chain. When hyphal growth was induced in the presence of amino acids, normal hyphal induction was observed in the conditional strain but both yeast and hyphal mutant strain cells exhibited an altered vacuolar morphological appearance, as revealed by fluorescence microscopy observations of FM4-64 stained cells. These alterations in the number, morphology and size of the vacuoles in the conditional mutant strain correlated with the vacuolar localization deduced from bioinformatics and immunoblotting analysis and GFP fusion experiments. Also, an altered hyphal vacuolation pattern was observed in the conditional mutant strain, characterized by an increased rate of hyphal branching, when compared with a control strain.

The vacuole is a multifunctional compartment involved in hydrolysis like the mammalian lysosome, and in storage, calcium ion homeostasis and osmoregulation in the case of plant vacuole; in fungal cells, vacuoles are required for compartmentalization of metabolites as well as toxic substances (**Klionsky *et al.*, 1990**; **Teter and Klionsky, 2000**). Known roles suggested for vacuoles in yeast are described as degradation of cellular proteins, cytoplasmic homeostasis and survival during periods of stress (**Banta *et al.*, 1998**; **Takeshige *et al.*, 1992**). In this context, several genes encoding proteins related with vacuolar morphology and/or functions have been characterized. Over the past two decades, genetic screens have identified many genes as being involved in vacuolar morphology (*VAM* genes) and also in the expression of vacuolar functions (*VMA*, *VAC*, and *VPS*) in *S. cerevisiae*.

Nine *VAM* genes have been identified in *S. cerevisiae* and classified into two groups (**Wada *et al.*, 1992**). The class I *vam* mutants (*vam1*, *vam5*, *vam8* and *vam9*) show a few small vesicles that are stained with histochemical markers for the vacuolar compartment, and their growth is hypersensitive to high concentrations of $CaCl_2$ or a temperature of 37ºC. In contrast, the class II *vam* mutants (*vam2*, *vam3*, *vam4, vam6* and *vam7*) contain numerous small vesicles stained with the vacuolar histochemical markers and do not show any apparent growth defects in the presence of $CaCl_2$ or at 37ºC. However, FM4-64 staining of cells of the conditional CV3 strain described in this work, under *ABG1* repression, revealed the presence of a single, smaller and collapsed vacuole in each yeast cell compartment, and the presence of several and smaller vacuoles in hyphal cells, instead of the single, large vacuolar compartment observed in the hyphal cells of the control wild-type cells. Moreover, conditional and heterozygous *ABG1* strains did not show any growth defect in the presence of $CaCl_2$ or at 37ºC.

One of the most remarkable features of the vacuole is its acidification, which is carried out by the vacuolar proton pumping ATPase (V-ATPase). Yeast cells lacking V-ATPase activity due to disruption of a *VMA* gene exhibit a characteristic set of growth phenotypes. The cells lacking V-ATPase activity remain viable but demonstrate increased sensitivity to Ca^{+2} in the growth medium, an inability to grow in medium buffered to neutral pH and an inability to utilize nonfermentable sources of carbon (i.e., glycerol or ethanol) (**Graham *et al.*, 2000**). Since *ABG1* is an essential gene, and, its

repression does not cause any increase in the sensitivity to Ca^{+2} or to glycerol it can be concluded that *ABG1* is not related with V-ATPase function.

Vacuole contains a large number of unspecific hydrolases and numerous endo- and exoproteinases (**Jones *et al.*, 1997; Van den Hazel *et al.*, 1996),** since one of the prominent vacuolar functions is degradation of cellular proteins. In this context, vacuole resident proteases are transported through the secretory pathway to the endoplasmic reticulum (ER) lumen or membrane, pass from the ER through the Golgi apparatus and in the late Golgi are diverted from the secretory pathway (Vps pathway). In *S. cerevisiae*, Vps proteins are involved in vesicular protein/lipid transport from the late Golgi compartment to the vacuole. More than 40 *VPS* genes have been identified in genetic screens of carboxypeptidase Y (the most thoroughly studied vacuolar protein) missorting (**Bankaitis *et al.*, 1986; Robinson *et al.*, 1988; Rothman and Stevens, 1986**).

Recent works have identified two *C. albicans VPS* homologs, *CaVPS34* and *CaVPS11*. In contrast to the accumulation of the specific endocytic dye FM4-64 in the vacuole membrane in *C. albicans* wild-type cells (ring staining pattern), the *CaVPS34* mutant strain exhibited a staining of punctated structures, possibly multivesicular bodies that are scattered all over the cell. In addition, deletion of *CaVPS34* gene caused disturbance of normal nuclear migration, which suggests that in the *CaVPS34* mutant the cell-size mediated control process of cell division is affected (**Bruckmann *et al.*, 2001**). Deletion of *CaVPS11* also resulted in the absence of a vacuolar compartment, and furthermore, *vps11*Δ strains were defective in yeast-to-hypha morphologic transition, thus suggesting that Vps11p-mediated trafficking steps are necessary to support the rapid emergence and extension of the germ tube from the parent yeast cell (**Palmer *et al.*, 2003**). The *Saccharomyces cerevisiae* Genome Deletion Project Consortium has generated a set of yeast deletion strains. All constructed strains have been collected by EUROSCARF (***Euro*pean *S*accharomyces *c*erevisiae *A*rchive for *F*unctional Analysis) and are available to the scientific community. The strain with a deletion in YGR106c has been analyzed, and it does not show any phenotype that could resemble those of the *vps* mutants of *S. cerevisiae* described so far. None of the phenotypes displayed by *C. albicans* mutants for *VAM*, *VMA*, and *VPS* genes resemble the characteristic phenotype exhibited by *ABG1* mutant strains, which appear to be

affected in cytokinesis and branching processes and were not sensitive to osmotic, oxidative and temperature stress.

On the other hand, the mechanism by which a new vacuole is generated is unknown. There are several membrane-trafficking pathways to the vacuole, and each of these pathways may contribute to the formation of a new vacuole. Yeast mutants that lack a vacuole throughout their cell cycle have not been identified because a functional vacuole is essential for yeast viability. The mother cell requires a vacuole before it can produce a bud. Accordingly, in yeast mutants with a defect in vacuolar inheritance, the bud or newly formed daughter rapidly generates a new vacuole. Several screens were employed to identify mutants defective in vacuole inheritance (*vac*) (**Gomes de Mesquita *et al.*, 1996**; **Wang *et al.*, 1996; Weisman *et al.*, 1990**), which on the basis on differences in vacuole morphology, have been subdivided into classes (**Wang *et al.*, 1996**). Interestingly, deletion of *ABG1* causes an altered budding growth, characterized by the formation of bud chains, usually formed by three cells. Thus, depletion of *ABG1* does not result in any vacuolar inheritance defect, since vacuolar compartments were present in daughter cells, although the lack of complete cell division could be due to the lack of functionality of the vacuolar compartments inherited, or maybe to the inability of the daughter cells to generate a functional new vacuole from the vacuolar material inherited from the mother cell.

Loss of *ABG1* function also resulted in several pleiotropic effects on structure and/or composition of the cell wall. Since many of the biological functions related to the biology, pathogenicity and virulence in this microorganism, reside in the cell wall (**Calderone, 1993**; **Calderone and Braun, 1991**; **Chaffin *et al.*, 1998**; **Hostetter, 1994**; **Lopez-Ribot *et al.*, 2004**; **Martínez *et al.*, 1998**; **Naglik *et al.*, 2003**), changes in the cell wall structure and/or composition associated to *ABG1* repression could result in alterations of such functions. Defects in cytokinesis observed in *ABG1* mutant strains may reflect alterations in the cell wall structure and, in this context, the conditional CV3 mutant strain was found to be more sensitive to different cell wall perturbing agents such as Calcofluor White and Congo Red.

However, CV1 and CV3 strains exhibited a lower sensitivity to SDS which suggests that the different component of the cell wall could be in unbalanced relative proportions in the mutant strains. A similar result (increased resistance to SDS) has been reported in the case of *C. albicans* (**Moreno *et al.*, 2003**) and *S. cerevisiae* (**Van der Vaart *et al.*, 1995**) harbouring disruptions for other genes. Secretory pathway could also be affected by *ABG1* repression which in turn may affect, directly or indirectly, cell wall biogenesis. In fact, comparison of the transcriptional profile of *abg1Δ* conditional mutant yeast cells with that of wild-type yeast cells revealed the presence of eight genes whose expression was consistently increased. The three genes which present a higher variation in their expression were *ALS1*, *ALS12*, and *ALS4*, that belong to the Agglutinin-like sequence (ALS) family (**Hoyer, 2001**). Recent data from the transcriptional profiling of *C. albicans* protoplast cells growing under cell-wall regeneration conditions revealed that these three genes are the first ones which are up-regulated during the early stage of cell wall regeneration (**Castillo, L., Martínez, A. I., Garcerá, A., Elorza, M.V., Valentín, E., and Sentandreu, R.; personal communication**), thus suggesting that yeast cells of CV3 conditional strain overexpress the genes involved in the first stage of the cell-wall biogenesis processes, perhaps in response to the cytokinesis defect and the subsequent incomplete cell division process observed as a consequence of *ABG1* repression.

In an attempt to elucidate the role of *ABG1* in the cell biology of *C. albicans*, a post-genomic experimental approach was used. *In vivo*, most gene products play their biological role either as stable components of multi-subunit complexes or interacting temporarily with other factors to carry out their function (**Blackwell *et al.*, 2003**). For that reason, purification of native protein complexes, containing the tagged Abg1p species, was achieved by affinity purification on IgG-Sepharose and eluted intact from the resin by treatment with TEV protease. Results from MALDI-TOF revealed the presence of seven protein species potentially interacting with Abg1p *in vivo*: Ape2p, Eft2p, Ssa4p, Ssa1p, Erg13p, Met15p, and Eno1p. Different authors have reported the presence of enolase (Eno1p) and proteins from the highly conserved 70-kDa stress protein family (Ssa1p) in different purification processes from cell wall extracts of *C. albicans* (**Eroles *et al.*, 1997**).

On the other hand, a subset of protein species that differ in function, molecular weight and pI but that all share the common characteristic of being very abundant in the cell cytoplasm, have been detected repeatedly using the TAP tagging protocol as common background contaminants, including heat shock proteins (Ssa1p, Ssa2p), Tef1p, Eno2p, and ribosomal proteins (**Shevchenko *et al.*, 2002**).

Taking into account that the protein complex containing Abg1p was isolated from a whole cytosolic extract and that a single tag was used in the purification process, detection in such a complex of different proteins which are known to be quite abundant in the cell, like Eft2p, Ssa4p, Ssa1p, Erg13p, Met15p, and Eno1p, was not an unexpected finding. However, two independent research groups have described the interaction between Ssa1p, a cytosolic member of the 70-kDa stress protein family, and the aminopeptidase I (**Silles *et al.*, 2000**; **Satyanarayana *et al.*, 2000**). Six members of the Hsp70 family (Ssa1p, Ssa2p, Ssa3p, Ssa4p, Ssb1p, Ssb2p) are present in the cytoplasm of *S. cerevisiae* (**Werner-Washburne *et al.*, 1987**). Ssa proteins are known to be involved in protein import to organelles and in the assembly of oligomeric protein complexes whereas the Ssb proteins are involved in protein biosynthesis. Work from **Satyanarayana *et al.* (2000)** and **Silles *et al.* (2000)** demonstrated that the constitutively expressed members of the yeast cytoplasmic Ssa subfamily, Ssa1/2p, are involved in the transport of the vacuolar hydrolase aminopeptidase I from the cytoplasm to the vacuole, while **Seguí-Real and collaborators** described that aminopeptidase I transport is made specific and saturable by an unknown receptor that appears to recognize specific transport motifs in the prepro-NH2 extension of the cytosolic precursor of the aminopeptidase I (**Seguí-Real *et al.*, 1995**).

Finally, Ape2p which was also found in the complex eluting from the IgG-Sepharose column along with Abg1p and the other proteins above mentioned is known to present leucyl aminopeptidase activity (**Trumbly and Bradley, 1983**) and plays a role in peptide metabolism in *S. cerevisiae* (**Trumbly and Bradley, 1983**; **Hirsch *et al.*, 1988**). This protein has been found to be located in the cytoplasm and in the periplasmic space (**García-Alvarez *et al.*, 1991**), and also in the mitochondrion (**Sickmann *et al.*, 2003**). Overall, results from protein A-tagging experiments suggested that Ape2p could be also in part targeted to the vacuolar membrane by Ssa1p and Ssa4p, and that Abg1p could act as the specific receptor for the Ape2p in the vacuolar membrane.

On the other hand, how organelle biogenesis and inheritance is linked to cell division is poorly understood in fungi. Organelles must be accurately divided and moved to their proper location within the new cells (**Weisman, 2003**). Therefore, a major goal is the identification and characterization of the cytoskeletal elements and associated proteins required for organelle movement. Second, there must be temporal control of organelle distribution, so organelle growth and division must be coordinated with cell growth and division. Thus, another major goal is the determination of how organelle inheritance is coordinated with the cell cycle.

Loss of Abg1p leads to a defect in yeast cell cytokinesis, causing an altered budding growth and also an increase in branching frequency during hyphal development. The observed alterations in the number and in the morphology and size of vacuoles may be correlated with the increased rate of hyphal branching, which is in agreement with the relationship between vacuolar volume and cell cycle progression as reported by **Barelle *et al.* (2003)**. Results presented in this work about the possible relationship between the cell-size mediated control process and the vacuolar functions, are somehow related with those reported for *CaVPS34* gene in *C. albicans* whose deletion has been showed to cause disturbance of normal nuclear migration, thus suggesting that the cell-size mediated control process of cell division was also affected in the *CaVPS34* mutant (**Bruckmann *et al.*, 2001**).

Interestingly, Cln3p, a G1 cyclin described in *S. cerevisiae*, controls initiation of cell division and also vacuolar biogenesis and segregation (**Han *et al.*, 2003**). Organelle contribution to overall cell size is not usually taken into consideration in studies of cell size control but the results from increased branching in hypha in the CV3 conditional strain cells suggest that vacuoles are playing an essential role in the cell size control. Thus, the CV3 cells have an increased frequency of division, indicated by a higher number of branches in hyphae, respect to the parental strain which correlated with the presence of more but smaller vacuoles in each cellular hyphal compartment when compared with the wild type strain. On the basis of the observed correlation between vacuolation and cell cycle progression (**Barelle *et al.*, 2003**), the concept of size-regulated cell cycle control must be refined, that is to say, the cell size is related to its cytoplasmic volume minus the vacuole (and other organelle) space rather than total cell volume.

ABG1 gene product links vacuole biology with cellular morphogenesis, cell wall biosynthesis and cell cycle progression. All of these processes are intimately associated with virulence traits in *C. albicans*. However, little is known about the role that intracellular organelles, such as the vacuole, play in growth and pathogenicity of *C. albicans*. The vacuole is an important organelle in fungal cells required for compartmentalization of metabolites as well as toxic substances. In this context, it has been recently reported the characterization of the first vacuolar protein belonging to the superfamily of ATP-binding cassette (ABC) proteins, encoded by *MLT1* (**Theiss *et al.*, 2002**). ATP-driven transport proteins belonging to the ABC superfamily perform important functions in cell metabolism and detoxification, and include a wide variety of energy driven efflux pumps with broad range substrate specificities. The only ABC transporters described to date in *C. albicans*, apart from Mlt1p, are the Cdr proteins (Cdr1 to Cdr4) which, with the exception of Cdr3 and Cdr4, are directly involved in multiple drug resistance to antifungal compounds (**Prasad *et al.*, 1995**; **Sanglard *et al.*, 1995**; **1997**; **Franz *et al.*, 1998**). Comparison of antifungal susceptibility *in vitro* between the conditional strain and the control strain reveals an increased sensitivity to azoles in the first one, thus suggesting that the vacuolar defects observed in the *abg1*Δ mutant strains could affect to the ABC transporter (Mlt1p) located in the vacuolar membrane (**Theiss *et al.*, 2002**).

C. albicans genes, especially those that are essential for fungal growth, and that lack homologues in mammalian cells, might be good candidates to be used as specific targets for new antifungal drugs to minimize the negative side effects that are associated with most antifungal therapies currently employed. In this context, Abg1p which is the first essential vacuolar protein described in *C. albicans* and has no homology with human genes, could be regarded as a potential target for new antifungal chemotherapy.

VIII. CONCLUSIONES

1. Mediante el inmunorrastreo de una genoteca de expresión de *Candida albicans* con un anticuerpo policlonal específico de la forma micelial de dicho hongo (PAb anti-gt), se ha aislado, clonado y caracterizado un nuevo gen de este microorganismo. Dicho gen está contenido en el contig 5-3205 del proyecto de secuenciación del genoma de *C. albicans*, y contiene un marco abierto de lectura que codifica para un polipéptido putativo de 288 aminoácidos, con un peso molecular aproximado de 32.921 Da.

2. La represión del gen aislado ocasionó un severo defecto en la morfogénesis de *C. albicans*, caracterizado por la formación de cadenas de levaduras, generalmente de tres elementos, de tamaño decreciente en dirección al extremo apical de la cadena, por lo que el gen fue denominado *ABG1* (del inglés, "***a***ltered ***b***udding ***g***rowth"), en base al fenotipo de crecimiento por gemación alterado observado en las cepas mutantes para dicho gen.

3. En el último ensamblaje (Assembly 19) del *Candida albicans* Sequence Project de la Universidad de Stanford, el contig 5-3205 que contenía la secuencia de *ABG1*, ha sido reestructurado, ya que la zona promotora del gen está contenida en contig 19-1523, parte del marco abierto de lectura así como la región terminadora aparecen en los contigs 19-10122 y 19-20122, y una región de 366 nucleótidos que incluye parte de la zona promotora y la primera porción del marco abierto de lectura del gen, han desaparecido. Este ensamblaje es, obviamente, erróneo y debe corregirse volviendo a la anotación original para *ABG1* contenida en el antiguo contig 5-3205.

4. La secuencia de aminoácidos de Abg1p, el producto del gen *ABG1*, presentó los porcentajes de identidad más significativos con un hipotético marco abierto de lectura existente en otro hongo levaduriforme, *Saccharomyces cerevisiae*, denominado YGR106c. El análisis bioinformático de la secuencia de Abg1p reveló la presencia de un péptido señal entre los aminoácidos 7 y 22 y de un posible dominio transmembranal entre los residuos aminoacídicos 241 y 260, características propias de proteínas transmembranales. Ensayos de inmunotransferencia (Western immunoblotting) de fracciones subcelulares con un anticuerpo policlonal frente a Abg1p, y posteriores estudios de fusión con la proteína verde fluorescente (GFP) de *Aequorea victoria*,

revelaron que Abg1p se localizaba en la membrana vacuolar de *C. albicans*. El patrón de fluorescencia debido a la proteína de fusión Abg1p-GFP colocalizó con el observado tras la tinción con FM4-64, agente fluorescente que tiñe selectivamente la membrana vacuolar y la de las vesículas endocíticas.

5. Con la finalidad de establecer el papel de *ABG1* en la biología de *C. albicans* se procedió a la construcción de un mutante nulo, proceso que no pudo llevarse a cabo a pesar de utilizar dos técnicas de interrupción génica distintas. Se procedió, por tanto, a la construcción de un mutante condicional, en el que la primera copia de *ABG1* estaba interrumpida con el cassette URA-blaster y la segunda copia quedaba situada bajo el control del promotor de *MET3*. Al reprimir la expresión de *ABG1*, lo que se consigue por la acción represora de la metionina y cisteína sobre el promotor de *MET3*, el mutante fue incapaz de crecer, lo que estableció el carácter esencial de *ABG1* para *C. albicans*.

6. Consecuentemente con la localización subcelular de Abg1p, el análisis fenotípico del mutante condicional reveló anomalías en la morfología, tamaño y número de las vacuolas presentes en las células. La represión de *ABG1* en *C. albicans* ocasionó, además, defectos en la división celular. En células levaduriformes estos defectos consistieron en alteraciones en el proceso de citocinesis, mientras que en las células miceliales se detectó un aumento de la frecuencia de división, manifestado como un mayor número de ramificaciones en las hifas.

7. La represión génica de *ABG1* ocasionó una serie de efectos pleiotrópicos tales como una mayor sensibilidad a agentes que interfieren en la biogénesis de la pared celular, tales como Calcofluor White y Rojo congo, así como una mayor resistencia a SDS, lo que sugiere una posible alteración de la estructura y/o composición de la pared celular. También se detectó un incremento en la sensibilidad de la cepa mutante a agentes antifúngicos de la familia de los azoles. Ello podría indicar una alteración en uno de los mecanismos de resistencia a estas drogas, tales como las bombas de flujo, algunas de las cuales se ha descrito que están localizadas en la membrana vacuolar.

8. El análisis del perfil transcripcional del mutante condicional creciendo en condiciones apropiadas para inducir el crecimiento en forma de levadura, permitió detectar la sobre-expresión de un grupo de genes de la familia *ALS* de *C. albicans*, que han sido recientemente implicados en las primeras etapas del proceso de construcción de la pared celular del hongo. Ello sugiere un posible mecanismo de compensación a los defectos en la citocinesis observados en las células levaduriformes como consecuencia de la represión de *ABG1*.

9. La búsqueda de proteínas que interaccionan *in vivo* con Abg1p mediante una técnica de marcaje con Proteína A, puso de manifiesto la posible interacción de Abg1p con una aminopeptidasa (Ape2p) y una proteína de la familia de las proteínas de estrés térmico (Ssa1p). A partir de estas observaciones se puede sugerir un modelo de función para Abg1p en la membrana vacuolar de *C. albicans*, en el que el producto del gen *ABG1* actuaría como receptor de Ape2p, que sería transportada del citosol a la membrana vacuolar mediante su interacción con Ssa1p.

10. En este trabajo se presenta por primera vez, evidencia de la existencia de un producto génico, Abg1p, que interrelaciona la biología vacuolar con la morfogénesis celular, la biogénesis de la pared celular y la progresión del ciclo celular, procesos todos ellos íntimamente asociados con la virulencia de *C. albicans*. Además, Abg1p es la primera proteína vacuolar esencial descrita en este hongo, y no tiene homólogos en células de mamíferos, por lo que representa una diana potencial para el desarrollo de nuevas estrategias terapéuticas.

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