國立交通大學

生物科技學系

博 士 論 文

探討剔除 *CaENO1*、*CaREP5* 及 *CaREP6* 對白色念珠菌之影響

Effects of *CaENO1*, *CaREP5* and *CaREP6* Null Mutations in *Candida albicans*

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中 華 民 國 一 O 三 年 一 月

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誌謝

2006 年 8 月 7 日，下過午後雷陣雨的天空映著彩虹，第一次來到竹南國家衛生研究院，心想，那是個美麗的預兆，從此一路承蒙大家的照顧。

感謝幫忙寫入學考試推薦函的師長，感謝交通大學的所有師長，感謝擔任口試委員的老師們撥冗指導，感謝系辦的熱心，感謝實驗室的學弟妹，感謝在國家衛生研究院的朋友，感謝展旺生命科技公司的朋友們，感謝萬能科技大學、臺中科技大學和中華科技大學的同事和學生，感謝家人，感謝陳文輝老師和師母一路相挺，最感謝楊昀良老師和羅秀容老師，時而嚴師；時而益友，陪伴我完成學業，成就豐富的人生。非常多的恩澤相隨，非常多的感恩於心，請容我當面一一道謝和與道別。

口試前的忐忑在看到一篇文章後漸漸平靜，聖嚴法師說：「沒有人一生下來就是大學畢業，或是具有博士學位，大家都是從不行變成行，從無能變成能，從無知變成知。如果凡事都認為自己不行，輕易否定自己的能力，卻不盡心學習，那就是不負責任。」平實的踏穩腳步，無懼的向前邁進，期待前方盡是精彩。

甲午年戊辰月 風城

探討剔除 *CaENO1*、*CaREP5* 及 *CaREP6* 對白色念珠菌之影響

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摘要

Enolase（2-phospho-D-glycerate hydrolase）是一個組成醣解途徑的酵素，在演化過程中具有高度保留性，在 *Candida albicans* 中，它是 *ENO1* 的基因產物。此基因（*CaENO1*）的表現受到調控致病力的 *EFG1* 的影響。因此，本研究利用 *SAT1* cassette 進行基因剔除以了解 *CaENO1* 的相關功能，發現葡萄糖或果糖會抑制 *Caeno1*/*Caeno1* 同基因合子突變株的生長及發芽管之形成，突變株也無法在不含胺基酸的 Difco-yeast nitrogen base 培養基中生長。在相關的藥物感受性試驗中發現，*Caeno1*/*Caeno1* 雙套基因突變株影響 amphotericin B、miconazole 和 NaCl 的感受性。因此 *CaENO1* 除了醣類代謝的機制，也涉及發芽管形成、藥物感受性與細胞間的離子滲透；另外，在小鼠體內試驗結果發現突變株失去致病力，這些研究結果將可能有助於於開創新一代的抗真菌製劑。

另外，先前實驗室經由在啤酒酵母菌（*Saccharomyces cerevisiae*）所進行的 library screening 發現在啤酒酵母菌中 *CaREP5*（Regulator of Efflux

Pump）和 *CaREP6* 能增加 *CDR1p-lacZ* 的 β-galactosidase 酵素活性。*CDR1* 是一個抗藥基因，先前報導指出它也跟 *CaENO1* 一樣，涉及白色念珠菌的致病機制。本研究主要在白色念珠菌中針對 *CaREP5* 和 *CaREP6* 的基因遺傳學和功能性探討，希望藉此進一步了解抗藥性的調控機制。結果在測試的條件下，null 突變株的表現型並無顯著改變。

Effects of *CaENO1*, *CaREP5* and *CaREP6* Null Mutations in *Candida albicans*

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ABSTRACT

Enolase (2-phospho-D-glycerate hydrolase) is an enzymatic component of the glycolytic pathway and has been well conserved throughout evolution. It is encoded by *CaENO1* in *Candida albicans*, the most frequently isolated human fungal pathogen. The protein product can also be found on the cell surface and bind host plasminogen in association with tissue invasion. In order to understand the role other than that in glycolytic pathway, *CaENO1* was subjected to mutagenesis analysis by the construction of null mutants via gene-replacement with the *SAT1* flipping cassette. Strains lacking *CaENO1* were not able to grow on glucose or fructose and it also failed to grow on Difco-yeast nitrogen base medium without amino acid. It was also observed that null mutations affected the susceptibility to amphotericin B and miconazole, in addition to the resistance to NaCl stress. Hence, *CaENO1* was involved in drug susceptibility in addition to its role in carbon utilization. And it may also be involved in regulation of cell osmolarity or ion channels. Furthermore, the *CaENO1* null mutant was avirulent when tested in a mouse model for systemic infection, and also exhibited defective hyphal formation. These results may help to design new and more effective antifungal agents.

In addition, *CaREP5* (Regulator of Efflux Pump) and *CaREP6* were isolated from *C. albicans* genomic library due to its ability to increase the β-galactosidase activity of $CDR1_{YM990348}$ promoter-*lacZ* fusion construct in *Saccharomyces cerevisiae* in the presence of miconazole. *CDR1* is a drug resistance gene. And just like *CaENO1*, it is known to affect the pathogenesis of *C. albicans*. In this study, these genes were subjected to genetic and functional studies. And the results showed there were no significant differences in the phenotypes between the wild type and the *Carep5*/*Carep5* or the *Carep6*/*Carep6* under the conditions tested.

Table of Contents

List of Tables

List of Figures

List of Appendixes

Abbreviations and Symbols

4NQO	4-nitroquinoline 1-oxide
ABC	ATP-binding cassette
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
CGD	*Candida* Genome Database
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
GlcNAc	N-acetyl-glucosamine
GMS	Grocott Methenamine Silver
H&E	hematoxylin and eosin
mRNA	messenger RNA
MTL	mating type-like
NaCl	sodium chloride
NaDOC	sodium deoxycholate
$NaNO_2$	sodium nitrite
NCCLS	National Committee for Clinical Laboratory Standards
NP-40	octylphenyl-polyethylene glycol
PCR	polymerase chain reaction
RAM	Regulation of *Ace2* and Morphogenesis
REP	Regulator of Efflux Pump
SA	Difco-yeast nitrogen base w/o amino acid, agar
SD	Difco-yeast nitrogen base w/o amino acid, glucose
SDE	Difco-yeast nitrogen base w/o amino acid, glucose, ethanol
SDP	Difco-yeast nitrogen base w/o amino acid, glucose, sodium pyruvate
SE	Difco-yeast nitrogen base w/o amino acid, ethanol
SG	Difco-yeast nitrogen base w/o amino acid, glycerol
SGala	Difco-yeast nitrogen base w/o amino acid, galactose
SG-CSM	Difco-yeast nitrogen base w/o amino acid, glycerol, CSM-HIS-LEU-LYS-TRP-URA
SGE	Difco-yeast nitrogen base w/o amino acid, glycerol, ethanol
SG-Glu	Difco-yeast nitrogen base w/o amino acid, glycerol, glutamic acid
SG-His	Difco-yeast nitrogen base w/o amino acid, glycerol, histidine
SG-Leu	Difco-yeast nitrogen base w/o amino acid, glycerol, leucine
SG-Lys	Difco-yeast nitrogen base w/o amino acid, glycerol, lysine

SG-Trp	Difco-yeast nitrogen base w/o amino acid, glycerol, tryptophan
SG-Ura	Difco-yeast nitrogen base w/o amino acid, glycerol, uracil
SP	Difco-yeast nitrogen base w/o amino acid, sodium pyruvate or Bacto-peptone
SPE	Difco-yeast nitrogen base w/o amino acid, Bacto-peptone, ethanol
SS	Difco-yeast nitrogen base w/o amino acid, serum
SSC	saline-sodium citrate
SY	Difco-yeast nitrogen base w/o amino acid, yeast extract
SYE	Difco-yeast nitrogen base w/o amino acid, yeast extract, ethanol
SYP	Difco-yeast nitrogen base w/o amino acid, yeast extract, Bacto-peptone
SYPE	Difco-yeast nitrogen base w/o amino acid, yeast extract, Bacto-peptone, ethanol
SYPG	Difco-yeast nitrogen base w/o amino acid, yeast extract, Bacto-peptone, glycerol
YEPS	yeast extract-peptone plus 2% sorbose
YP	yeast extract, Bacto-peptone
YPD	yeast extract, Bacto-peptone, glucose
YPDE	yeast extract, Bacto-peptone, glucose, ethanol
YPDG	yeast extract, Bacto-peptone, glucose, glycerol
YPDGE	yeast extract, Bacto-peptone, glucose, glycerol, ethanol
YPDP	yeast extract, Bacto-peptone, glucose, sodium pyruvate
YPE	yeast extract, Bacto-peptone, ethanol
YPFru	yeast extract, Bacto-peptone, fructose
YPG	yeast extract, Bacto-peptone, glycerol
YPGala	yeast extract, Bacto-peptone, galactose
YPGE	yeast extract, Bacto-peptone, glycerol, ethanol
YPP	yeast extract, Bacto-peptone, pyruvate
YPS	yeast extract, Bacto-peptone, serum

General Introduction

Clinical significance of *Candida albicans*

Candida albicans belongs to Ascomycota, Saccharomycetales and mitosporic Saccharomycetales. It is a polymorphic opportunistic pathogenic fungus (Odds, 1985), which forms symbiotic relationship with mammalian hosts to colonize the gastrointestinal, vulvovaginal, epithelial surfaces, oropharyngeal and oesophageal muscosal of most healthy individuals. The high risk groups for infections are immunosuppressed persons, organ transplant recipients, chemotherapy patients, HIV-infected patients, and low-birth weight infants (Pfaller and Diekema, 2007; Schelenz, 2008). Systemic candidiasis is the major source of morbidity and mortality in the immunocompromised patients. *Candida albicans* perhaps is the most common causal agent of surface infections and life-threatening systemic candidiasis (Fradin et al., 2003). In the clinical context, vulvovaginal candidiasis was quite prevalent among females that above 75% of them were infected once in the life time and some of them experience repeated occurrences of this infection (Sobel, 1997; Sobel et al., 1998; Monroy-Pérez et al., 2012).

Morphogenesis in *Candida albicans*

Candida albicans is a morphological diverse fungus. It has at least four kinds of morphological forms so far, including yeast cells, filamentous hyphae, pseudohyphae, and chlamydospores (Merson-Davies and Odds, 1989; Berman and Sudbery, 2002; Biswas et al., 2007; Whiteway and Bachewich, 2007). The fungus is a diploid pathogen whose life cycle composed of transition between yeast and filamentous hyphal forms. In general, all the cells grow as yeasts

essentially under appropriate nutritional conditions, but several other conditions may induce hyphae growth. The transition from a commensal to a pathogen may involve changes in environmental conditions (Huang, 2012). The reversible morphogenetic conversions triggered by various environmental cues such as serum, pH, temperature, N-acetylglucosamine, osmotic stress, undernourishment were rapid (Hazan et al., 2002; Biswas et al., 2007; Kabie et al., 2012).

Yeast form cells disseminate within hosts easily; on the other hand, hyphal form perhaps responsible for breaching barriers of host defenses to microbial invasion (Hazan et al., 2002). The yeast to hypha switch regulates expression of cell wall proteins (Bailey et al., 1996; Argimón et al., 2007; Walker et al., 2009), and promotes the dissemination and penetration (Gow et al., 2002; Gow et al., 2003). It was the expressions of some adhesins and secreted proteinases as well the coordination with yeast-hypha transition that allow the filamentous form to be involve in adhesion and invasion of the host tissue (Hube et al., 1994; Staab et al., 1996; Argimón et al., 2007). Lo and co-workers provided direct evidence that the ability to change between yeast and hyphal forms was significant for virulence (Lo et al., 1997). Certain studies have shown that *C. albicans* yeast growth pattern mimics *S. cerevisiae*; but long filamentous hyphal growth pattern mimics *Aspergillus nidulans* and *Neurospora crassa* (Irazoqui and Lew, 2004; Whiteway and Bachewich, 2007).

Pathogenicity of *Candida albicans*

Candida albicans was the most common fungal pathogen of humans. Under normal environment, it exists as commensal in various body locations and did not usually cause diseases. But conversion of yeast form to hyphae to invade the deeper tissue and bloodstream could lead to fatal organ infection accompanying a mortality rate approaching 50%. In US hospital patients, the

infection prolonged hospitalization and increased medical expenses (Gow et al., 2002; Casadevall and Pirofski., 2003; Gudlaugsson et al., 2003; Zaoutis et al., 2005; Perlroth et al., 2007; Pfaller and Diekema, 2007). The cellular morphogenesis concerns with the changes in cell wall composition as well as structure, which are relevant to pathogenesis and host immune response (Shibata et al., 2007; Biswas et al., 2007; Gow et al., 2013), as the pathogenicity depends upon the immune status of the hosts.

Kinetics of pharmaceutical

Currently available antifungal drugs for clinical treatment can be divided into five main classes including (1) polyenes, (2) azoles, (3) fluorinated pyrimidine, (4) allylamines, and (5) echinocandins.

1. Polyenes

Amphotericin B, amphotercin B lipid complex, amphotericin B cholesteryl sulfate, amphotericin B liposomal, and nystatin are the clinically useful members of this class. The polyene antifungal drugs are derived from fermentation production of *Streptomyces* species. They are a group of macrolide lactones.

The fungicidal effect of the polyenes results from their interaction with the cell membrane sterol, ergosterol. It acts on the ergosterol within the cell membrane of *C. albicans* to form pores or channels so as to change the permeability of the cell membrane. It also leads to the precipitation of potassium ion and other cytoplasmic composition and the oxidative damage, and finally, cell death (Vanden et al., 1994; Sanglard and Bille, 2002).

Amphotericin B has long been used to treat invasive fungal infection. It

provides a similarly broad spectrum of activity. It was isolated by Gold et al. in 1956 and the first patent of amphotericin B was by Dutcher et al. in 1959. Unfortunately, the clinical use is limited by nephrotoxicity or dose-dependent adverse effects.

White et al. have isolated *Candida* strains treated with amphotericin B for long period of time and they have observed obvious reduction of ergosterol content within cell membrane that associated with specificity of drug (White et al., 1998). This led to the later development using amphotericin B as treatment for fungal infection.

Furthermore, nystatin has been used as an antifungal agent more than 50 years, but the clinical usage is hindered by intrinsic toxicity and solubility problems (Larson et al., 2000).

2. Azole antifungal agents

The first therapy of systemic fungal infections began to emerge with the introduction of azole drugs in the 1980s, which develops into the most widely used fungistatic (Chakrabarti, 2011). There are two groups in clinical use: the imidazoles including clotrimazole, econazole, ketoconazole and miconazole, and the triazoles including fluconazole, itraconazole, posaconazole and voriconazole. They inhibit the cytochrome P_{450} 14α-lanosterol demethylase, which is encoded by the *ERG11* gene (White et al., 1998; Lamping et al., 2007). The inhibitory effect of the azoles results from interfering with ergosterol biosynthesis.

3. Fluorinated pyrimidine

The fluorinated pyrimidine analogue flucytosine (5-fluorocytosine) is an antimetabolite type of antifungal drugs. It is activated by deamination within

the target cells to 5-fluorouracil, and causes aberrant on both protein and DNA synthesis (Bennett, 1977; Sanglard and Bille, 2002; Groll et al., 2003; Akins, 2005). Flucytosine in treating infections caused by *Candida* species was effective. However, treatment with flucytosine alone can lead to the development of drug-resistance easily. Hence its usage is limited to combination with other drugs, especially in clinically isolated *Candida* strains which were resistance to azoles (but still sensitive to flucytosine) (Harder and Hermans, 1975; Samra et al., 2010).

4. Allylamines

The allylamines, such as terbinafine, acts by inhibiting squalene epoxidase, an early step in ergosterol biosynthetic pathway (Favre and Ryder, 1996). In *Candida albicans*, some azole-resistant strains due to the over-expression of *CDR1* or *MDR1* are found to have the cross-resistant characteristic to terbinafine, and *CDR1* mutant is hypersusceptible to azoles and terbinafine (Sanglaard et al., 1996; Vanden-Bossche et al., 1998).

5. Echinocandins

Caspofungin is the first one to be licensed for clinic application among such new antifungal agents; in addition, there are micafungin and anidulafungin. They have the functions to inhibit the β-1,3 glucan polysaccharides synthesis on the cell wall of fungi (Kurtz et al., 1996). Until recently, there are very few cases of echinocandin resistance, toxic effects and drug interactions (Denning, 2003), but the cost of treatment usually is expensive.

Drug resistance

Resistance to antifungal agents has been observed in clinical isolates of *C. albicans* and is a major clinical challenge.

Candida albicans obtained resistance to azoles through multiple mechanisms, including alteration of the drug target; bypassing pathways, upregulation of multidrug transporters; cellular stress responses; chromosomal aneuploidy or isochromosome, and biofilms (Sanglard et al., 1996; Sanglard et al., 1997; Lamb et al., 2000; Coste et al., 2006; Nobile and Mitchell, 2006; Chakrabarti, 2011; Shapiro et al., 2011). The major mechanisms for the generation of drug resistance were due to the over-expressions of major facilitator superfamily drug pump Mdr1p or ATP-binding cassette pumps Cdr1p or Cdr2p, which in turn lead to the difficulty of accumulation of azoles drug and decreased drug concentration in *C. albicans* (White et al., 1998; Perea et al., 2001; Lamping et al., 2007; Chakrabarti, 2011). Furthermore, the drug target encoded by the *ERG11* gene has been overexpressed and/or point mutated to reduce azoles binding (White et al., 1998; Lamb et al., 2000; Sanglard and Bille, 2002; Akins, 2005). In addition, the sterol composition change within cell membrane also leads to the reduction of permeability of cell membrane for drugs.

The mechanisms for the generation of polyenes resistance were related to the reduced ergosterol at the target sites and increased catalase activity (Loeffler and Stevens, 2003). Moreover, the reason toward echinocandins resistance was correlated with drug target site alteration. Clinically separated flucytosine-resistant strains are usually related to the mutation of uracil phosphoribosyltransferase, which prevent the conversion of 5-fluorouracil into

5-fluorouridine monophosphate (Dodgson et al., 2004).

The capacity of *C. albicans* to acquire resistance to antifungal drugs rapidly, such as amphotericin B, flucytosine, and a series of azoles, arguing that continued development of new antifungals remains an important focus for clinicians and pharmaceutical companies. In my study, I tried to study the functions of three genes, which were potential drug targets.

Chapter I

Study of *CaENO1* Null Mutants on Drug Susceptibility, Hyphal Formation, and Virulence of *Candida albicans*

1.1 Introduction

1.1.1 Feature of *ENO1*

The taxonomic group of *ENO1* exists in eukaryotes, including animals, fungi, plants, diatoms, apicomplexans and bacteria, including proteobacteria, actinobacteria and firmiccutes.

Enolase (2-phospho-D-glycerate hydrolyase; EC 4.2.1.11), encoded by *ENO1*, is a phyllogentically conserved enzyme. It involved in both glycolysis pathway (Fig. 1) and gluconeogenesis anabolic processes (Fig. 2), catalyzes the conversion of 2-phosphoglycerate to give phosphoenolpyruvate and the reverse reaction (Dinovo and Boyer, 1971; Stubbe and Abeles, 1980).

1.1.1.1 *ENO1* in vertebrate

Enolase of vertebrate is a dimer composed of 3 subunits, alpha, beta and gamma. α-Enolase is present in the liver as well as immature organs and localized to cytoplasm and cell membrane, while β-enolase is found in muscle tissue and the γ isozyme is neuronal-specific (Rider and Taylor, 1974; Fletcher et al., 1976; Jørgensen and Centervall, 1982; Cooper et al., 1984; Oliva et al., 1989). α-Enolase is encoded by *ENO1*. It can act as a plasminogen receptor to concentrate plasmin activity on the cell surface (Miles et al., 1991).

Diaz-Ramos et al. have demonstrated that α-enolase regulates the

recruitment of inflammatory cells in tissue, and the formation of satellite-cell-dependent new myofiber (Diaz-Ramos et al., 2012). Interestingly, Wistow et al. showed α-enolase was present in embryonic duck lens, and that the cDNA encodes both α-enolase and lens τ-crystallin structural proteins (Wistow et al., 1988).

Giallongo et al. have addressed that the regulation of human α-enolase in response to heat shock induction and mitogenic stimulation of peripheral blood lymphocytes (Giallongo et al., 1986), and they also found the putative promoter region of α-enolase exist several SP1 binding sites and high content of G+C (Giallongo et al., 1990). The posttranslational modifications of α-enolase related to Alzhemer's disease through multiple functions have been reported, and upregulation of α-enolase has been found in cardiac infarction (Takei et al., 1991; Nakajima et al., 1994; Castegna et al., 2002; Butterfield and Lange, 2009; Diaz-Ramos et al., 2012). α-Enolase detected on the cell surface of cholangiocarcinoma may associate tumor cell invasion (Yonglitthipagon et al., 2012).

1.1.1.2 *ENO1* in plant

The literature pictured enolase in plant as follows. In castor bean (*Ricinus communis* cv. Hale), induction of enolase was unaffected by gibberellin A_3 (González and Delsol, 1981). In tomato, the anaerobic induced was low in enolase activity that may be less in response to anaerobic stress, and enolase is not a heat shock inducible protein (Van der Straeten et al., 1991). In *Arabidopsis thaliana* plants, Van der Straeten et al. suggested a substrate shuttle replaced enolase for glycolysis in chloroplasts (Van der Straeten et al., 1991). Parbhakar et al. suggested *ENO1* might be the only missing enzyme for a complete glycolysis within *Arabidopsis* plastids (Prabhakar et al., 2010). The salt stress induced increase in enolase activity and expression of mRNA, and

enolase transcripts increased during the induction of Crassulacean acid metabolism by temperature and anaerobic stresses in *Mesembryanthemum crystallium* L. (Forsthoefel et al., 1995). Fox et al. suggested that enolase expression may be related during later acclimation periods of anaerobic stress in *Echinochloa phyllopogon*, but higher enolase activity is in response to the anoxic in *Echinochloa crus-pavonis* (Fox et al., 1995). Lal et al. pictured enolase involves in *Zea mays* roots during anaerobic stress (Lal et al., 1998). Voll et al. supposed that reduce enolase activity affects shikimate branch of aromatic amino acid biosynthesis pathway within the plastid in enolase antisense *Nicotiana tabacum* (Voll et al., 2009).

1.1.1.3 *ENO1* in Saccharomycetales

ENO1 in Saccharomycetales has been investigated in Debaryomycetaceae and Dipodascaceae. Among them the *Candida* species includes *C. albicans*, *Candida dubliniensis* and *Candida orthopsilosis* etc. Enolase in *S. cerevisiae* is expressed as isozymes by two genes, *ENO1* and *ENO2* (McAlister and Holland, 1982; Entian et al., 1987).

1.1.1.4 *ENO1* in *Candida albicans*

CaENO1 loci exist on chromosome 1 in *C. albicans* (*Candida* Genome Database; CGD, http://www.candidagenome.org), the systematic name is orf19.395; and the second allele was orf19.8025. The protein sequence of *CaENO1* is 64.7% homologous to the *Homo sapiens* alpha-enolase (Fig. 3), which is highly associated with tumor invasion of cholangiocarcinoma patients (Yonglitthipagon et al., 2012).

The identified *CaENO1* protein sequence within fungal genomes show 98.0% similarity with Cd36_08010 *Candida dubliniensis CD36*, 90.7%

similarity with CTRG_03163 *Candida tropicalis MYA-3404*, 86.2% similarity with CPAR2_207210 *Candida parapsilosis CDC317*, 86.0% similarity with PGUG_04391 *Candida guilliermondii ATCC6260*, 85.6% similarity with CLUG_03897 *Candida Iusitaniae ATCC 42720*, 85.9% similarity with CORT_0A06360 *Candida orthopsilosis Co 90-125*, 77.1% similarity with CAGL0102486g *Candida glabrate CBS138*, 85.8% similarity with LELG_00641 *Lodderomyces elongisporus NRLL YB-4239*, and 84.2% similarity with DEHA2G14058 *Debaryomyces hansenii CBS767*. The Multiple alignment result and phylogenetic tree view align protein sequences of *ENO1* relate to *Homo sapiens* and Saccharomycetales species by COBALT multiple aligment tool showed in Fig. 4 and Fig. 5. The *C. albicans* enolase also showed 76.4% sequence identity with *S. cerevisiae* YGR254W *ENO1*, and 74.5% with YHR174W *ENO2*.

The 1323 bp cDNA encoding 440 amino acids of *CaENO1* was similar to that of *S. cerevisiae*, and both have been found to be the most abundant proteins in the cell of which the expression was controlled by transcriptional modulators of controlling multiple genes (Maitra et al., 1971; Sundstrom and Aliaga, 1992). Sundstrom and Aliaga presented the structural differences between *C. albicans* and *S. cerevisiae*, where the CaEno1 contain no cysteine residues, and a two-amino-acid insertion in the main domain (Sundstrom and Aliaga, 1992).

Early studies have shown that there are abundant enolase in the blood of candidiasis patients and it is useful as a marker of internal infection (Walsh et al., 1991). Sundstrom and Aliaga indicated that cell extracts comprised 0.7% and 2.0% of enolase in the yeast and hyphal forms of *C. albicans*, respectively. Further, *CaENO1* is a marker for disseminated candidiasis and may be the antigen to the selective stimulation of host responses when the host encounters fungal infection (Sundstrom and Aliaga, 1992; Sundstrom and Aliaga, 1994). Postlethwait and Sundstrom showed that the carbon sources for propagating the cell perhaps modulate *CaENO1* mRNA levels and suggested enolase or other

glycolytic enzymes might be useful antifungal targets (Postlethwait and Sundstrom, 1995). Martínez-Gomariz et al. showed *CaENO1* expression is reduced after both ethanol and glucose are used in cellular respiration, and *CaENO1* less is abundant in the planktonic yeast cells than in hyphae and biofilms (Martínez-Gomariz et al., 2009). In addition, Shirtliff et al. demonstrated that enolase is down-regulated when *C. albicans* is exposed to farnesol (Shirtliff et al., 2009). Bharucha et al. suggested that *CaENO1* in RAM (Regulation of *Ace2* and Morphogenesis) pathway mutants (*cbk1*/*CBK1 efg1*/*efg1*) was mediated by the cAMP-dependent protein kinase A pathway in *C. albicans* (Bharucha et al., 2011). Ramirez-Garcia et al. showed a higher rate of synthesis of *CaENO1* related to the increase in melanoma cell adhesion *in vitro*, hence, involved in the pro-metastatic effect (Ramirez-Garcia et al., 2013).

1.1.2 Previous works

In *C. albicans*, the *cph1*/*cph1 efg1*/*efg1* mutant strains show defect in filament formation, and are avirulent in a mouse infection model (Lo et al., 1997). Previous studies have screened the expression differences between the wild type strain and *cph1*/*cph1 efg1*/*efg1* double mutant strain by Suppression Subtractive Hybridization (SSH), and efforts have been made to find specific genes associated with filamentous growth or virulence (Kuo, 2002; Hsu, 2004). The levels of *CaENO1* expression are different between *cph1*/*cph1 efg1*/*efg1* double mutant and wild-type parental strains. *CaENO1* has been demonstrated to be required for growth in the presence of glucose, and the *TR-CaENO1*/*Caeno1* strain does not grow on solid media containing glucose (Yang et al., 2006). The tetracycline-controlled expression system is able to control gene expression among variety of eukaryotes cells. In the absence of tetracycline, the target gene is capable of actively expressed by the binding of the tetracycline-regulatable transactivator to tetracycline operator. On the

contrary, the target gene is able to suppress expression by the binding of tetracycline (Resnitzky et al., 1994; Weinmann et al., 1994; Kistner et al., 1996; Baron et al., 1997; Nakayama et al., 1998). However, the role of *CaENO1* in filamentous growth and virulence requires further clarification.

1.1.3 Specific aims of the study

To obtain more information on the regulation of enolase gene expression in *C. albicans*, the gene disruption used *SAT1* flipper cassette that contains the nourseothricin-resistance marker (Reuss et al., 2004) was performed to investigate physiological properties of *CaENO1*. The rationale behind those studies was to determine whether there is any correlation between the knock-out of *CaENO1* and change in cellular characters and virulence of the cells.

Hence, this study would focus on the functional study of *CaENO1*, especially its involvement in growth repercussion and drug susceptibility in *C. albicans*. Upon the completion of constructing the null mutants, germ tube formation assay was conducted to examine the morphology variations. Secondly, the strains were tested on various carbon sources to examine the state of growth and cultured with drugs or chemicals to address the effects of *CaENO1* on drug susceptibility. Finally, the focal point would be on understanding the involvement of *CaENO1* for virulence in mice.

1.2 Materials and Methods

1.2.1 Strains and growth conditions

Candida albicans strains used in this study were listed in Table 1. The *CaENO1* mutant strains were necessarily grown in YPGE medium (1% yeast extract, 2% Bacto-peptone, 3% (v/v) glycerol, 2% (v/v) ethanol) at 30°C.

1.2.2 Construction of the *Caeno1*/*Caeno1* null mutant strain

The oligonucleotides used in the study were listed in Table 2, and the plasmids in Table 3.

The sequence of *CaENO1* is based on the information obtained from the *Candida* Genome Database, which provides the sequence of assembly contig4-3031_0010 of *C. albicans*. The *C. albicans* SC5314 genomic DNA was used as a template for polymerase chain reaction (PCR) amplification using the primers HJL00980 (5'-ggtaccATTAAGCCGTGGGTTCTCAA-3') and HJL00981 (5'-ctcgagAAAAAGGGAGAAAAGGAAAGAAA-3'). Amplified fragments contain sequence of *CaENO1* from -578 to -50 (529 bp) upstream of transcription starting site. A fragment containing the downstream sequence of *CaENO1* from +1308 to +1884 was amplified by primers HJL00982 (5'-GGCTTCTCAATTGTAAGTTTGC-3') and HJL00983 (5'-CAGGATCTATTGACGAATTCCA-3') from wild type strain SC5314 genomic DNA. The PCR products were cloned into the pGEM-T Easy plasmid (Promega, Madison, WI, USA). The downstream sequence was

excised from purified plasmid DNA using the *Not*I restriction endonuclease, and the upstream sequence was excised using *Kpn*I and *Xho*I. The upstream and downstream fragments of *CaENO1* were cloned into 5' and 3' respectively of the *SAT1* flipper cassette in plasmid pSFS2A to create LOB317 plasmid. The DNA of LOB317 was subjected to *Kpn*I and *Sac*I digestion to produce the DNA fragment containing the *SAT1* flipper cassette flanked by the upstream and downstream *CaENO1* sequence for the transformation of SC5314. The wild-type SC5314 strain of *C. albicans* was transformed by replacing the corresponding genomic *CaENO1* sequence with the LOB317 *SAT1* flipper through a double cross-over in a 2-step procedure. After transformed with the LOB317 fragment, the transformants were plated onto YPD containing 200 μg/mL of nourseothricin. The nourseothricin-resistant colonies were grown on YP with 2% maltose for 48 hours to induce the FLP recombinase-mediated excision of the *SAT1* flipper cassette to obtain the nourseothricin sensitive, heterozygous *CaENO1*/*Caeno1* mutant strain YLO365 and YLO366.

The homozygous *Caeno1*/*Caeno1* null mutant strain YLO367 and YLO368 were constructed by repeating the procedure following the excision of the second wild-type *CaENO1* allele from the YLO365 and YLO366 strain, except that the selection was performed on YP agar with glycerol and nourseothricin (Fig. 6). The homozygous knock-out mutants of *C. albicans* SC5314 strains are obtained after two rounds of insertion and excision of the *SAT1* cassette by growing in yeast extract with peptone medium plus 2% maltose to generate *Caeno1*/*Caeno1* transformants YLO00367 and YLO00368 (Fig. 7).

The *CaENO1* gene was reintroduced into the *Caeno1*/*Caeno1* null mutant genome to obtain the rescued strain. PCR amplification of *CaENO1* using the HJL980 primer (5'-ggtaccATTAAGCCGTGGGTTCTCAA) and the HJL983

primer (5'-CAGGATCTATTGACGAATTCCA), nucleotide positions -578 to 1884 was conducted, and the PCR product was cloned into the pGEM-T plasmid. The wild-type *CaENO1* gene sequence were excised from pGEM-T as a single fragment using *Kpn*I and *Xho*I, and was cloned into LOB317 through the *Kpn*I and *Xho*I sites, replacing the upstream fragment to generate the LOB318 plasmid. The DNA of LOB318 was cleaved with *Kpn*I and *Sac*I, and the resulting fragment that contained the *CaENO1* and the *SAT1* flipper cassette was transformed into YLO367 and YLO368. The transformants were plated on YPD with nourseothricin. The nourseothricin-resistant colonies were cultured on YP with 2% maltose for 48 hours to induce the FLP-mediated excision of the *SAT1* flipper cassette to generate the *Caeno1*/*Caeno1*::*CaENO1* rescued strain YLO369 and YLO370 (Fig. 8).

1.2.3 Southern blot analysis of *CaENO1* mutants

The genomic DNA from different strains was isolated by MasterPure™ yeast DNA purification kit (Epicentre, catalog nos. MPY80010 and 80200), and digestion with *Acc*I then the digested genomic DNA were resolved in 1.2% agarose gel, next transferred to a nylon membrane. For southern blotting analysis, the DNA fragment between the 1308th and the 1884th nucleotides (the A of the translation initiation site ATG as +1) was used as a probe, which was PCR amplified and DIG-labeled using plasmid LOB318 contain *CaENO1* with a pair of primer HJL00982 and HJL00983. The experiment was performed according to the instruction from the manufacturer.

Southern blot analysis was carried out with the DIG DNA Labeling and Detection Kit (Roche, catalog no. 1 093 657) according to the manufacturer's

recommendations. First, the plasmid DNA fragments were generated by the PCR process, subsequently the PCR products labeled with PCR DIG probe synthesis kit (Roche, catalog no. 11 636 090 910) and used as hybridization probe.

A total of 10 μg of genomic DNA were digested with restriction enzymes and size-fractionated by the suitable agarose gel electrophoresis on Sub-Cell GT system (BioRad, catalog no. 170-4404). To assess the quality, the DNA was electrophoresised and by stained in ethidium bromide and then destained with sterile water. The DNA transferred to a Nytran SuPerCharge membrane by capillarity (TurboBlotter Rapid Downward Transfer system, Schleicher & Schuell, Item no. 10416328) and fixed to the membrane by UV light in autocrosslinker (120 mj/pulse).

The DIG Easy Hyb (10 ml/100 cm^2 membrane) preheated to hybridization temperature (42°C). The blot was incubated for 30 minutes with gentle agitation. The DIG-labeled DNA probe (20 ng/ml hybridization buffer) was denatured by boiling for 10 minutes and rapidly chilled on ice. It was then added to preheat DIG Easy Hyb (3.5 ml/100 cm^2 membrane) and mixed properly before the addition to the membrane. The reaction was incubated with gentle agitation at 42°C for overnight.

The membrane was first washed under high-stringency conditions with 2X wash solution (2X SSC, 0.1% SDS) twice at room temperature for 5 minutes each, then washed with 0.5X wash solution (0.5X SSC, 0.1% SDS) twice under constant agitation at 65°C for 15 minutes each. The hybridized signals were detected with the DIG luminescent detection kit (Roche, catalog no. 11 363 514 910). Rinsing the membrane briefly for 1 to 5 minutes in washing buffer (0.1 M maleic acid, 0.15 M NaCl; pH 7.5; 0.3% (v/v) tween 20), and the membrane

was incubated for 30 minutes in 100 ml blocking solution and 20 ml anti-digoxigenin antibody solution sequentially. Subsequently, the membranes were washed in 100 ml washing buffer twice for 15 minutes each, followed by addition of 20 ml detection buffer twice for 2 to 5 minutes each. Next, the membrane was placed with DNA side up on a development folder (or hybridization bag) and applied 2 ml diluted CSPD solution in detection buffer (1:100). Afterward the reaction was incubated without air bubbles over the membrane for 5 minutes. Excess liquid was squeezed out and the edge of the development folder was sealed. The damp membrane was incubated at 37°C for 5 to 15 minutes to enhance the luminescent reaction, and then exposed to imaging instrument.

1.2.4 Morphology test of *CaENO1* mutants with germ tube analysis

The test strains were inoculated by aseptic toothpicks into YPG (1% yeast extract + 2% Bacto-peptone + 2% (v/v) glycerol) medium with or without 10 mM N-acetyl-glucosamine (GlcNAc) or 10 mM $NaNO_2$. The reaction was incubated at 37ºC for 2 to 4 hours and then the state of germ tube formation observed under microscope.

1.2.5 Growth assay of *CaENO1* mutants on different media

The different strains were spread on YPGE agar master plate. The plate was incubated for 1 to 2 days at 30°C and then replica-plated onto various plates. The following solid media were employed. YP agar (1% yeast extract (Bacto), 2% Bacto-peptone, 2% agar), YPD agar (1% yeast extract, 2% Bacto-peptone,

0.1% or 2% or 4% glucose, 2% agar), YPDE (1% yeast extract, 2% Bacto-peptone, 2% glucose, 2% (v/v) ethanol, 2% agar), YPDG (1% yeast extract, 2% Bacto-peptone, 2% glucose, 3% (v/v) glycerol, 2% agar), YPDGE (1% yeast extract, 2% Bacto-peptone, 2% glucose, 3% (v/v) glycerol, 2% (v/v) ethanol, 2% agar), YPDP (1% yeast extract, 2% Bacto-peptone, 2% glucose, 100 mM sodium pyruvate (Sigma, P2256), 2% agar), YPFru (1% yeast extract, 2% Bacto-peptone, 2% fructose), YPE (1% yeast extract, 2% Bacto-peptone, 2% (v/v) ethanol, 2% agar), YPG (1% yeast extract, 2% Bacto-peptone, 2% (v/v) glycerol, 2% agar), YPGala (1% yeast extract, 2% Bacto-peptone, 2% galactose, 2% agar), YPGE (1% yeast extract, 2% Bacto-peptone, 3% (v/v) glycerol, 2% (v/v) ethanol, 2% agar), YPP (1% yeast extract, 2% Bacto-peptone, 2% pyruvate, 2% agar), SA (0.67% Difco-yeast nitrogen base w/o amino acid, 2% agar), SE (0.67% Difco-yeast nitrogen base w/o amino acid, 2% (v/v) ethanol, 2% agar), SDE (0.67% Difco-yeast nitrogen base w/o amino acid, 2% glucose, 2% (v/v) ethanol, 2% agar), SP (0.67% Difco-yeast nitrogen base w/o amino acid, 2% Bacto-peptone, 2% agar), SPE (0.67% Difco-yeast nitrogen base w/o amino acid, 2% Bacto-peptone, 2% (v/v) ethanol, 2% agar), SG (0.67% Difco-yeast nitrogen base w/o amino acid, 2% (v/v) glycerol, 2% agar), SGE (0.67% Difco-yeast nitrogen base w/o amino acid, 3% (v/v) glycerol, 2% (v/v) ethanol, 2% agar), SG-CSM (0.67% Difco-yeast nitrogen base w/o amino acid, 2% (v/v) glycerol, 0.55 g/L CSM-HIS-LEU-LYS-TRP-URA, 2% agar), SG-Glu (0.67% Difco-yeast nitrogen base w/o amino acid, 2% (v/v) glycerol, 100 mg/L glutamic acid, 2% agar), SG-His (0.67% Difco-yeast nitrogen base w/o amino acid, 2% (v/v) glycerol, 20 mg/L histidine, 2% agar), SG-Leu (0.67% Difco-yeast nitrogen base w/o amino acid, 2% (v/v) glycerol, 100 mg/L leucine, 2% agar), SG-Lys (0.67% Difco-yeast nitrogen base w/o amino acid, 2% (v/v) glycerol, 50

mg/L lysine, 2% agar), SG-Trp (0.67% Difco-yeast nitrogen base w/o amino acid, 2% (v/v) glycerol, 50 mg/L tryptophan, 2% agar), SG-Ura (0.67% Difco-yeast nitrogen base w/o amino acid, 2% (v/v) glycerol, 20 mg/L uracil, 2% agar), SY (0.67% Difco-yeast nitrogen base w/o amino acid, 1% yeast extract, 2% agar), SYE (0.67% Difco-yeast nitrogen base w/o amino acid, 1% yeast extract, 2% (v/v) ethanol, 2% agar), SYP (0.67% Difco-yeast nitrogen base w/o amino acid, 1% yeast extract, 2% Bacto-peptone, 2% agar), SYPE (0.67% Difco-yeast nitrogen base w/o amino acid, 1% yeast extract, 2% Bacto-peptone, 2% (v/v) ethanol, 2% agar), SYPG (0.67% Difco-yeast nitrogen base w/o amino acid, 1% yeast extract, 2% Bacto-peptone, 2% (v/v) glycerol, 2% agar). After incubation for 1 to 2 days at 30°C, the plates were checked for growth.

1.2.6 Antifungal susceptibility assay of *CaENO1* mutants by Etest

Etest (AB Biodisk, Solna, Sweden) is an antimicrobial susceptibility testing method of *Candida* species. The Etest strips contained a gradient of 0.016 to 256 μg of fluconazole per ml, the others such as amphotericin B, caspofungin, fluorocytosine and voriconazole ranging in dilution from 0.002 to 32 μg of per ml and were stored at -20°C until use.

The Etest method was performed by modifying the manufacturer's instructions (AB Biodisk, 1998). The media used was 60 ml of YPGE (1% yeast extract, 2% Bacto-peptone, 3% (v/v) glycerol, 2% (v/v) ethanol) agar in 150-mm-diameter Petri plates. The inoculants prepared in 0.85% NaCl were adjusted VITEC colorimeter to the optical density equivalent of a 0.5-McFarland turbidity standard (Thermo scientific remel^{TM} colorimeter standard set, catalog no. R20343), then using an aseptic cotton swab dipped into

the inoculums suspension and smeared whole agar surface evenly in three directions. The drug strips tested amphotericin B, caspofungin, fluconazole, 5-fluorocytosine and voriconazole were determined, and the plates were incubated at 35°C until good growth was observed.

1.2.7 Chemicals and antifungal agents susceptibility test of *CaENO1* mutants with agar dilution assay

The agar dilution method was used to determine whether enolase involved in susceptibility to chemicals and antifungal agents for *C. albicans*. Individually, the agar plates prepared were contain 0.1 % H_2O, 0.1 % DMSO (dimethyl sulfoxide), 0.2 μg/ml amphotericin B, 25 μg/ml fluconazole, 2 μg/ml itraconazole, 2 μg/ml ketoconazole, 1 μg/ml miconazole, 2 μg/ml and 5 μg/ml voriconazole, 1 M and 2 M NaCl, 20 μg/ml calcofluor white, 100 μg/ml congo red, 20 mM, 30 mM and 40 mM $NaNO_2$, 0.25% and 0.5% cholate, 0.25% and 0.5% NaDOC, 0.25% SDS, 1% tween 20, 1% NP-40, 1% triton-X-100, 0.5 μg/ml and 1 μg/ml 4-nitroquinoline 1-oxide (abbreviated 4NQO).

First of all, test strains were inoculated on agar plate and allowed to grow at 30°C for one day. Next 0.85% NaCl solution was used to adjust the cells concentration to OD_{600} of 2 (approximately 2×10^7/ml) and the diluted cells used for 10-fold series dilution. 300 μl of cells of each concentration was placed in a separate well of the seed tray of replicator. The agar plates were incubated at 30°C or 35°C for 24 to 72 hours and before subjected for observation.

1.2.8 Broth microdilution susceptibility assay of *CaENO1* mutants

For broth microdilution testing, the antifungal agents stock solution were prepared in dimethyl sulfoxide and further diluted in YPGE (1% yeast extract, 2% Bacto-peptone, 3% (v/v) glycerol, 2% (v/v) ethanol) medium. The drugs and chemical used were kanamycin (concentration range, 0.39 to 200 μg/ml), amphotericin B (concentration range, 0.0078 to 4 μg/ml), fluconazole (concentration range, 0.125 to 64 μg/ml), miconazole (concentration range, 0.0039 to 2 μg/ml), voriconazole (concentration range, 0.0039 to 2 μg/ml), 4NQO (concentration range, 0.0039 to 2 μg/ml), triron-X-100 (concentration range, 0.039 to 20 mg/ml), sodium chloride (NaCl, concentration range, 0.0156 to 80 mg/ml), sodium deoxycholate (abbreviated NaDOC, concentration range, 0.156 to 80 mg/ml), $NaNO_2$ (concentration range, 0.078 to 40 mg/ml), calcofluor white (concentration range, 1.25 to 640 μg/ml), and congo red (concentration range, 0.625 to 320 mg/ml). Aliquots (100 μl) of drug containing serial twofold dilutions were dispensed into 96-well trays. The different strains were cultured on YPGE agar plates, and the inoculants prepared in 0.85% NaCl were adjusted spectrophotometrically to the optical density equivalent of a 2 McFarland standard, then added to drugs and incubated at 35°C for 24, 48 and 72 hours. The method was modified from National Committee for Clinical Laboratory Standards (NCCLS) guidelines M27-A2 (National Committee for Clinical Laboratory Standards, 2002).

1.2.9 Murine model virulence assay of *CaENO1* mutants

The *in vivo* virulence assay was modified from the previous reports (Chen

et al., 2006; Lo et al., 1997). The wild type strain (SC5314), YLO00367 and YLO00368 (*Caeno1*/*Caeno1*), YLO00369 and YLO00370 (*Caeno1*/*Caeno1*::*CaENO1*) and the HLC54 (*cph1*/*cph1 efg1*/*efg1*；Table 1) strains were grown on YPGE agar plates at 30°C for 2 days. Cells were cultured in YPGE medium and resuspend in 0.85% normal saline, then cell number counted by a hemocytometer.

Groups of 9-week-old male BALB/c mice (18 to 20 g) were obtained from the National Laboratory Animal Center (Taipei, Taiwan) and used for *in vivo* experiment. 70 mice per cage were housed and provided with nonallergic and nontoxic of sterile food, water, and bedding in the biohazardous isolate suite. All procedures were performed in accordance with the guidebook for the care and use of laboratory animals and standard operation procedure for humane handling, care at the Laboratory Animal Center of National Health Research Institutes in Taiwan.

Different strains of C. albicans were injected (1×10^6 / 0.5 ml) via lateral tail veins. Mice were observed once daily until day 20 after infection. The dying mice were euthanized and recorded as having died the following day; we gathered the kidney from 2, 3 and 10 mice on the third, fourth and 20^{th} day. Slide with histological specimen staining was performed to confirm that mice death associated with fungal infection. The left kidney was determined fungal burden by plating dilutions onto YPGE plates, whereas the right kidney was fixed in 10% formaldehyde and embedded in paraffin. Sections were taken and stained with Grocott Methenamine Silver (GMS) or Hematoxylin and Eosin (H&E) method to examine hyphal by light microscopy.

1.2.10 Growth curve analysis of *CaENO1* mutants

Bioscreen C analyzer (Oy Growth Curves AB Ltd, Helsinki, Finland) was used to monitor the growth kinetics of the different isolates, and approximately 1×10^4 *C. albicans* cells were cultured on the appropriate media in bio-screen plates. In this method, we formulated YPD (1% yeast extract (Bacto), 2% Bacto-peptone, 0.1% or 2% glucose), YPE (1% yeast extract, 2% Bacto-peptone, 2% (v/v) ethanol), YPGala (1% yeast extract, 2% Bacto-peptone, 2% galactose), YPG (1% yeast extract, 2% Bacto-peptone, 2% (v/v) glycerol), YPGE (1% yeast extract, 2% Bacto-peptone, 3% (v/v) glycerol, 2% (v/v) ethanol), YPP (1% yeast extract, 2% Bacto-peptone, 100 mM sodium pyruvate), YPDP (1% yeast extract, 2% Bacto-peptone, 2% glucose, 100 mM sodium pyruvate (Sigma, P2256), YPS (1% yeast extract, 2% Bacto-peptone, 10% or 50% serum); SD (0.67% Difco-yeast nitrogen base w/o amino acid, 0.1% or 2% glucose), SE (0.67% Difco-yeast nitrogen base w/o amino acid, 2% (v/v) ethanol), SGala (0.67% Difco-yeast nitrogen base w/o amino acid, 2% galactose), SG (0.67% Difco-yeast nitrogen base w/o amino acid, 2% (v/v) glycerol), SGE (0.67% Difco-yeast nitrogen base w/o amino acid, 3% (v/v) glycerol, 2% (v/v) ethanol), SP (0.67% Difco-yeast nitrogen base w/o amino acid, 100 mM sodium pyruvate), SDP (0.67% Difco-yeast nitrogen base w/o amino acid, 2% glucose, 100 mM sodium pyruvate (Sigma, P2256), SS (0.67% Difco-yeast nitrogen base w/o amino acid, 10% or 50% serum) as medium. After incubation at 30°C for 24, 48 and 72 hours, cells were observed under light microscope.

1.3 Results

1.3.1 Constructing the mutant and rescue strain of *CaENO1*

To verify the results of genomic DNA from the SC5314 strain, heterozygous, homozygous mutants and the rescued strain were analyzed using southern blotting (Fig. 9). Southern transfers of *C. albicans* SC5314 genomic DNA digested with the restriction endonuclease *Acc*I, which cut within the *CaENO1* open reading frame, showed a single DNA fragment hybridized to the molecular weight slightly higher than the 2.3-kb molecular weight standard (Fig. 9), which is consistent with the 2.4-kb *CaENO1* genomic fragment. The blot of the heterozygous knockout strain showed the 2.4-kb *CaENO1* band and the 1.7-kb fragment of the *Caeno1* knockout construct. The blot of the homozygous knockout strain *Caeno1*/*Caenol* showed a single 1.7-kb band. The blot of the rescued strain showed a band that corresponded to a molecular weight between 2.3 and 4 kb, which was consistent with the 3-kb *Caeno1*/*Caeno1*::*CaENO1* rescue allele.

1.3.2 Mutation of *CaENO1* affected germ tube formation of *Candida albicans*

Different strains were inoculated by aseptic toothpicks into YPG medium that contains 1% yeast extract, 2% Bacto-peptone and 2% glycerol, with either 10 mM N-acetyl-glucosamine (Sigma, A8625) or 10 mM $NaNO_2$ (Sigma, S2252) to YPG medium. Different strains of *C. albicans* may induce germ tube responsiveness by GlcNAc medium (Mattia et al., 1982). The hyphal form of

C. albicans is more susceptible than the yeast form in the presence of nitric oxide (Heilman et al., 2013). The chemisity natures of GlcNAc and $NaNO_2$ are as Table 4.

The germ tube formation was abnormal on the *Caeno1* mutant and caused defect on hyphal formation. The phenotype of *Caeno1*/*Caeno1* mutant exhibited reduced germ tube formation when compared with SC5314 strain in YPG media (Fig. 10), consistent with the previous report that glycerol induces hyphal growth (Argüelles et al., 1999).

The contribution of enolase to hyphal growth was also evident on examination of the colony morphology. Along the edge of the SC5314 colonies, hyphal were clearly visible, whereas none was observed for the null mutant colonies. The re-introduction of the *CaENO1* allele restored hyphal formation (Fig. 10).

1.3.3 Mutation on *CaENO1* inhibit *Candida albicans* growth on solid medium of yeast nitrogen base w/o amino acid in growth assay

Different tested strains including wild type strain (SC5314), *CaENO1*/*Caeno1*-1 (YLO00365), *Caeno1*/*Caeno1*-1 (YLO00367), *Caeno1*/*Caeno1*::*CaENO1*-1 (YLO00369), *CaENO1*/*Caeno1*-2 (YLO00366), *Caeno1*/*Caeno1*-2 (YLO00368), *Caeno1*/*Caeno1*::*CaENO1*-2 (YLO00370) were patched onto the YPGE media, then cells were replica-plated onto various media after incubation at 30°C for 1 to 2 days. The *CaENO1* null mutant cells were unable to grow on solid media of Difco-yeast nitrogen base w/o amino acid containing different carbon source excluded SYPG (Fig. 11).

1.3.4 Mutation on *CaENO1* inhibit *Candida albicans* growth on yeast extract and peptone with glucose or fructose carbon sources in growth assay

Seven strains including wild type strain (SC5314), *CaENO1*/*Caeno1*-1 (YLO00365), *Caeno1*/*Caeno1*-1 (YLO00367), *Caeno1*/*Caeno1*::*CaENO1*-1 (YLO00369), *CaENO1*/*Caeno1*-2 (YLO00366), *Caeno1*/*Caeno1*-2 (YLO00368), *Caeno1*/*Caeno1*::*CaENO1*-2 (YLO00370) were patched onto the YPGE media, then cells were replica-plated onto various media after incubation at 30°C for 1 to 2 days. The *CaENO1* null mutant cells were unable to grow on YPD and YPF solid media containing glucose or fructose, and also inhibited to a lesser extent on the YP media compared with the SC5314 strain, but not in the presence of glycerol, ethanol or pyruvate (Fig. 12). The results correlated with growth curve analysis in liquid medium (Fig. 14).

1.3.5 Mutation on *CaENO1* inhibit *Candida albicans* growth in liquid medium of yeast nitrogen base w/o amino acid by growth curve assay

Different tested strains including wild type strain (SC5314), *CaENO1*/*Caeno1*-1 (YLO00365), *Caeno1*/*Caeno1*-1 (YLO00367), *Caeno1*/*Caeno1*::*CaENO1*-1 (YLO00369) were cultured on the appropriate media in bio-screen plates at 30°C by time schedule of 24^{th}, 48^{th} and 72^{nd} for observation. The *CaENO1* null mutant cells inhibit growth in liquid media of Difco-yeast nitrogen base w/o amino acid containing different carbon source as glucose, ethanol, glycerol, galactose, pyruvate and serum (Fig. 13).

1.3.6 Mutation on *CaENO1* inhibit *Candida albicans* growth on yeast extract and peptone with glucose in growth curve assay

Several tested strains including wild type strain (SC5314), *CaENO1*/*Caeno1*-1 (YLO00365), *Caeno1*/*Caeno1*-1 (YLO00367), *Caeno1*/*Caeno1*::*CaENO1*-1 (YLO00369) were cultured on the appropriate media in bio-screen plates at 30°C by time schedule of 24^{th}, 48^{th} and 72^{nd} for observation. The *CaENO1* mutant cells were grown on media in the presence of ethanol, glycerol or pyruvate. The growth of *Caeno1*/*Caeno1* strain was inhibited in liquid media of yeast extract and peptone with glucose or serum, and mutation on *CaENO1* causes the growth rate in yeast extract and peptone medium with galactose less than SC5314 strain (Fig. 14).

1.3.7 *CaENO1* mutants increase the drug susceptibility in Etest

At first the susceptibilities to multiple antifungal agents incurred by *CaENO1* mutants were investigated by Etest performed on YPGE medium with modifications of the instruction from the Etest manufacturer. The plates were incubated at 35°C and examined after 24, 48 and 72 hours. The *CaENO1* mutant was characterized by notably raised Etest endpoints, and clear zones of inhibition were shown for amphotericin B, fluconazole and voriconazole sensitivity at 48 hours of incubation (Fig. 15).

1.3.8 Mutations on *CaENO1* increase the susceptibility to chemicals in *Candida albicans* by agar dilution analysis

To determine whether enolase involved in susceptibility to antifungals and chemicals for *C. albicans*, amphotericin B, fluconazole, itraconazole, ketaconazole, voriconazole, NaCl, calcofluor white, congo red, $NaNO_2$, cholate and NaDOC were used in this experiment. The natures of these compounds are as Table 4. The number of *Caeno1*/*Caeno1* null mutant cells that grew on media containing 0.2 μg/ml amphotericin B, 25 μg/ml miconazole or 1 M NaCl was significantly less than that of the SC5314 and rescued strains (Fig. 16).

1.3.9 Mutations on *CaENO1* increase the susceptibility to antifungal agents in *Candida albicans* by broth microdilution analysis

In vitro susceptibilities to antifungal agents were evaluated by broth microdilution method. The readings for *CaENO1* mutants were taken at 24, 48 and 72 hours for 35°C incubation, and the growth of each strain in the presence of the various drugs was normalized to that in the absence of drugs. The natures of these compounds are as Table 4. The wild type endpoints were higher than *CaENO1* mutants for amphotericin B, fluconazole, miconazole, voriconazole, 4NQO and NaCl in this study (Fig. 17).

The antifungal susceptibility results for the Etest, when compared with broth microdilution testing were in agreement in general susceptibility of *CaENO1* mutants to amphotericin B, fluconazole and voriconazole. Hence, the results obtained in a broth microdilution assay confirmed the findings of the Etest (Fig. 15).

CaENO1 mutants increased the susceptibility to NaCl stress, and the *CaENO1* may be involved in hyperosmotic signaling pathways or ion channels, in addition to influence for high-osmolarity glycerol response (HOG) pathway (Albertyn et al., 1994).

1.3.10 *CaENO1* mutants exhibit diminished virulent in the murine model

The *CaENO1* null mutants exhibited diminished virulence, compared to both the wild type SC5314 strain and the *Caeno1*/*Caeno1*::*CaENO1* strain when tested in a murine model for systemic infection. To determine fungal burden in the mice, the left mouse kidneys were harvested and ground with buffer, then plated onto YPGE plates for incubation. The colonies formed were quantified and the mouse kidneys were free from *CaENO1* mutant (Fig. 18 and 19). In accordance with previous report that *cph1*/*cph1 efg1*/*efg1* mutants strain was nonlethal (Chen et al., 2006). The histopathology of mouse kidney after *C. albicans* infections were displayed filamentous growth with the wild-type SC5314 strain and *Caeno1* rescued strain (YLO00369 and YLO00370), but *Caeno1* null mutants and *cph1*/*cph1 efg1*/*efg1* strains fail to form filaments (Fig. 20). The above-mentioned *CaENO1* null mutants affected germ tube formation and its growth was inhibited when glucose was present, consistent with the outcome of virulent assay. These findings may help to design new and more effective antifungal agents for preventing and treating bloodstream fungal infection since serum also contains 62-175 mg/dl of glucose (Lunsford et al., 2008).

1.4 Discussion

The construction of the mutants in *C. albicans* usually utilizes nutritional markers from auxotrophic strain, but this might interfere with the mutant phenotypes and affect virulence related traits (Bain et al., 2001; Cheng et al., 2003; Staab and Sundstrom, 2003). Traditionally, gene replacement in the autotrophic-defective strain was performed using nutrition-based selective markers (Lo et al., 2005; Yang et al., 2006; Chen et al., 2009). However, there are growing concerns regarding the effects of such markers on mutant phenotypes (Chibana et al., 2005; Sharkey et al., 2005). And in the study using tet-off strains, the regulation of gene expression required doxycycline promoter system, which may affect fungal growth, especially in the presence of fluconazole (Miceli et al., 2009). Hence, the *CaENO1* mutant strains were generated with the *SAT1* flipping method in SC5314 to avoid above possible pitfalls with the genetic analyses.

First question is the role of enolase on utilization of carbon source. Growth assay on solid medium and growth curve experiments in liquid medium were used to address the question. After glucose is converted to pyruvate by glycolysis pathway, the fermentation TCA cycle, aerobic repiration or amino acid biosynthesis may be implemented. Therefore, the effect of glucose for *Caeno1*/*Caeno1* must be considered. Different amino acid in medium may affect growing of *Caeno1*/*Caeno1* also needs to be considered. The result shows that glucose inhibits the growth of *Caeno1* mutant cells (Fig. 11 and 12) is consistent with the result of growth curve analysis (Fig. 13 and 14). Figures 11 and 13 show that medium with Difco-nitrogen base can not support the growing of *Caeno1*/*Caeno1* with or without amino acids (Fig. 11). And mutations on *CaENO1* inhibit growth on yeast nitrogen base media with different carbon sources except in SYPG (Fig. 11).

The *Caeno1* null mutant was capable of growth on YP, even though at a

slower rate than the parental strain (Fig. 12). Thus, the YP medium was enough to provide energy for glycolysis and gluconeogenesis for the null mutant. However, the growth of the null mutant on YP was completely inhibited when glucose was present (Mason et al., 1993).

Caeno1/*Caeno1* null mutant affect carbohydrate metabolism in *C. albicans*, it was also found that mutations on *CaENO1* inhibited cell growth within fructose (Fig. 12). That is not clear how *Candida albiccans* generated energy by using carbon source without glycolysis and gluconeogenesis, and it is also not clear any other pathway be affected.

However, glycolysis and gluconeogenesis pathway were interrelated with starch and sucrose metabolism, pentose phosphate pathway, propanoate metabolism, pyruvate metabolism, citrate cycle, carbon fixation and amino acid metabolism (Fig. 21).

The whole of pentose phosphate pathway participated in purine, pyrimidine and histidine metabolism (Appendix 2), and carbon fixation pathway partaken in phenylalanine, tyrosine and tryptophan biosynthesis (Appendix 3). The whole citrate cycle network identify with valine, leucine and isoleucine degradation, alanine, aspartate, glutamate, tyrosine, arginine, proline, glutamate, glutamine metabolism (Appendix 4). *CaENO1* may be involved in the complex metabolic network and affect other pathway.

This result is inconsistent with previous reports that the null mutant could utilize glycerol as a carbon source through sequential conversion to glycerol-3-phosphate, glycerone phosphate, and fructose-1,6-phosphate by aldolase (Porter et al., 2001), or conversion to acetyl-CoA as a substrate for the tricarboxylic acid cycle (Sorger and Daum, 2003). The results in this study are consistent with that of Postlethwait and Sundstrom who studied the effect of carbon source on *CaENO1* mRNA levels, and found that the growth of *C. albicans* was minimal on ethanol and no growth on glycerol (Postlethwait and Sundstrom, 1995).

In the series experiment, the *Caeno1* null mutant failed to grow in media contains 0.1% glucose (Fig. 12 and 14), suggesting that the *Caeno1* null mutants were avirulent in the mouse model because glucose exists in blood. The blood glucose concentrations are 0.075% to 0.1% in humans and 0.1% to 0.12% in mice (Keren et al., 2000; Tsuneki et al., 2004). Furthermore, in a mouse model of invasive candidiasis, *Candida* filamentation was only observed in the kidney but not in the spleen or liver, and the larger distribution of neutrophils accumulated in the kidney than brain, spleen and liver (Lionakis et al., 2010). According to above factors, the observation of fungal infection condition on kidney of murine had been selected. However, GSM stain method used to demonstrate fungus infection in tissue effect dark brown to black by deparaffinzation, stain and dehydration (McGregor et al., 1980). The *in vivo* experiments showed that no null mutant cell was detected in the kidneys of inoculated mice (Fig. 18 and 19), indicating that the null mutant were unable to proliferate in the mouse body, an observation that differs from *cph1*/*cph1 efg1*/*efg1* in *C. albicans* (Chen et al., 2006; Yang et al., 2009).

In the study of the relationship between *CaENO1* and drug susceptibility, the *Caeno1* mutant strain showed significant increased susceptibility to fluconazole and vorinazole by Etest. It was also been found that the *Caeno1* mutant strain was more sensitive than parental strain SC5314 to amphotericin B. Finally, affect of drug and chemicals for *CaENO1* mutants in liquid medium is evaluated. The *Caeno1*/*Caeno1* is involved in drug sensitivity to amphotericine B (0.2 ug/ml), miconazole (1 ug/ml), and sensitivity to high concentration of NaCl (1 M) stress (Fig. 16 and Fig. 17).

In addition, despite its ability to form colonies, the *CaENO1* null mutant is defective in germ tube formation in contrast to SC5314 in YPG medium. Thus, its inability to form germ tubes in liquid media (Fig. 10) was not due to cell death. However, morphogenesis in *C. albicans* can be induced by several mechanisms that are activated by various environmental triggers which might

have different effects on the fungal cell surface (Biswas, 2007).

1.5 Future Work

The results of this study provide the information that *CaENO1* is an essential gene for glycolysis and gluconeogenesis pathway in *C. albicans*. It is not clear whether *CaENO1* is involved in other metabolism pathways where it may also affect other molecules. It should be mentioned that the *CaENO1* null mutant was avirulent in mice. The result can be considered for the new antifungal drugs or vaccine development. It may help to break out the limitations of fungal resistance. The result that the *CaENO1* null mutant more susceptible to antifungal agent and NaCl showed that *CaENO1* maybe related with membrane permeability.

Glycolysis and gluconeogenesis pathways in *C. albicans* are correlated with different carbohydrate metabolism and amino acid metabolism pathway (Appendix 1, 2, 3 and 4). The following items can be the study subject. How does the *CaENO1* null mutant survive in YPD? What kind of energy and carbon sources can be use by the *CaENO1* null mutant in YPD? Does amino acid affect the growing of *CaENO1* null mutant cells? Is there interaction of enolase with other proteins necessary for posttranslational modification which helps the null mutant to survive in YPD?

As for the researches on the involvement of *CaENO1* in drug susceptibility, one can attempt to determine the expression of *CaCDR1*, *CaCDR2* of the ATP-binding cassette (ABC) transporter family (Prasad et al., 1995; Sanglard et al., 1995) or other genes known to involve in drug susceptibility by real-time PCR to determine whether there is a corelation. For example, one can study how *CaENO1* affect or involve the transportation and metabolism of antifungal agents.

The action of *Caeno1* mutants increased the susceptibility to NaCl may have relation with membrane permeability. Membrane permeability may also affect the drug uptake, so the following subjects may be studied. Does NaCl stress affect morphogenesis and drug absorption? How can the membrane permeability be affected by NaCl stress?

Chapter II

Study of *CaREP5* and *CaREP6* on Morphology and Drug Resistance in *Candida albicans*

2.1 Introduction

2.1.1 Antecedents of *CaREP5*

At first, the lab named the gene *CaREP5* (Regulator of Efflux Pump) (Chi, 2004), which is now also called *CaWOR1* (White-Opaque Regulator), *CaEAP2* (Enhanced Adhesion to Polystyrene) or *CaTOS9*, encoding by orf19.4884 (contig19-10215) and orf19.12348 on chromosome 1. In *Candida albicans*, the ORF of *CaWOR1* is 2358 nucleotides, encodeing 785 amino acids. And Wor1p is similar to *S. cerevisiae* transcriptional regulator Mit1p of pseudohyphal growth.

The multiple alignments of CaRep5 protein sequences in Saccharomycetales are shown in Fig. 22. The identified *CaREP5* protein sequence within fungal genomes show 98.7% similarity with CAWG_00418 *Candida albicans* WO-1, 61.3% similarity with Cd36-09540 *Candida dubliniensis CD36*, 49.3% similarity with CTRG_03345 *Candida tropicalis MYA-3404*, 50.5% similarity with CPAR2_805000 *Candida parapsilosis CDC317*, 51.0% similarity with CORT_0A05020 *Candida orthopsilosis Co 90-125*, 46.2% similarity with *Debaryomyces hansenii CBS767*, 70.1% similarity with *Lodderomyces elongisporus NRLL YB_4239*, 59.2% similarity with CLUG_00578 *Candida lusitaniae ATCC 42720*, 63.1% similarity with CAGL0L02453g *Candida glabrata CBS138*, 64.1% similarity with *Candida guilliermondii ATCC 6260* and 43.5% similarity with *PTH2* in *Candida albicans*

SC5314 (Fig. 23).

Li and Palecek suggested that this gene activated the expression of adhesins and was involved in regulating mating (Li and Palecee, 2005). Zordan et al. showed CaWor1p was required for establishing and maintaining the opaque state. It accumulates to high levels in opaque cells, but not in the white form (Zordan et al., 2006). Huang et al. speculated the feedback regulation of *CaWOR1* transcription that could be used commonly in phenotypic switching (Huang et al., 2006). Srikantha et al. indicated that Tos9p involved in white-opaque switch, and Tos9p misexpression blocked temperature-induced mass conversion from opaque to white (Srikantha et al., 2006).

2.1.2 Antecedents of *CaREP6*

CaREP6 (Chi, 2004) also known as *CaRME1*, is encoded by orf19.4438 (contig19-10205) and orf19.11918 at chromosome 1 (*Candida* Genome Database). The *CaREP6* mRNA was 1524 nucleotides that encodes 507 amino acids. *RME1* participate in meiosis and endocytosis pathway in *Candida albicans* (Appendix 5 and 6).

The multiple alignments of CaRep6 protein sequence in Saccharomycetales is shown in Fig. 24. The identified *CaREP6* protein sequence within fungal genomes show 98.0% similarity with CAWG_00686 *Candida albicans* WO-1, 86.8% similarity with Cd36_06830 *Candida dubliniensis CD36*, 43.8% similarity with CTRG_03993 *Candida tropicalis MYA_3404*, 44.4% similarity with LELG_01054 *Lodderomyces elongisporus NRLL YB_4239*, 41.0% similarity with CORT_0A11970 *Candida orthopsilosis Co 90-125*, 39.7% similarity with CPAR2_212670 *Candida parapsilosis CDC317*, 39.2% similarity with DEHA2F19778g *Debaryomyces hansenii CBS767*, 34.9% PGUG_00281 *Candida guilliermondii ATCC 6260*, 28.7% similarity with

CAGL0K04257g *Candida glabrata CBS138*, 27.9% similarity with CLUG_05819 *Candida lusitaniae ATCC 42720* and 28.3% similarity with *RME1*/YGR044C *Saccharomyces cerevisiae S288C* (Fig. 25).

In *Candida albicans*, *RME1* was expressed in a and α white cells (Tsong et al., 2003), and this gene encodes a zinc finger protein similar to *S. cerevisiae* meiosis regulator Rme1p (Kassir and Simchen, 1976; Tzung et al., 2001). Rme1 was a repressor of meiosis and sporulation in *S. cerevisiae* (Kassir and Simchen, 1976; Mitchell and Herskowitz, 1986). In *Kluyveromyces Lactis*, *RME1* was a regulator of mating-type switching, and upregulatted by starvation is cause upregulation of the heterotrimeric G proteins (Barsoum et al., 2010; Booth et al., 2010). Rme1 protein involved with the recycling of endocytic in mammals and *Caenorhabditis elegans* (Lin et al., 2001; Grant and Caplan, 2008).

2.1.3 Previous works

Although little is known about regulatory factors of *CDR1* in *C. albicans*, previous study in the lab has used *CDR1p-lacZ* fusion plasmid constructs to screen genomic DNA library of *C. albicans* for the regulatory factors in *S. cerevisiae* in the presence of miconazole (100 μg/ml). *CDR1* was initially isolated by complementation of the *pdr5* mutant, which was susceptible to cycloheximide, chloramphenicol and azole drugs in S. cerevisiae (Prasad et al., 1995). *CDR1* is an ATP-binding cassette transporter identified as an efflux pump contributing to drug resistance in *C. albicans* (Prasad et al., 1995; Lopez-Ribot et al., 1998; Yang and Lo, 2001), and *CDR1* has been connected to azole resistance and translocated lipid in *C. albicans* (Shukla et al., 2007).

The *CaREP5* and *CaREP6* have been identified for their ability to increase

the expression of *CDR1p-lacZ* in *S. cerevisiae* (Chi, 2004). Four other candidate open reading frames (ORFs) have also been isolated in the same study. They were *CaREP1*, *CaREP2*, *CaREP3*, and *CaREP4*, all of which increased the β-galactosidase activity of *CDR1p-lacZ* in *S. cerevisiae*. *CaREP1* turned out to be a negative regulator of *MDR1* (Chen et al., 2009). *CaREP2* was renamed *CaNDT80*, a positive regulator of *CDR1* and a major regulator of pathogenesis in *Candida albicans* (Chen et al., 2004).

2.1.4 The purposes of the study

Although previous study result shown that *CaREP5* and *CaREP6* may have the ability to regulate *CDR1*, the direct relation between *CaREP5* and *CaREP6* with drug resistance is not clear. The study of *CaREP5* on white-opaque switch had been done, but studies the function of *CaREP5* and *CaREP6* on *CDR1* are none.

This study focused on the functional study of *CaREP5* and *CaREP6*, especially their involvement in drug resistance in *C. albicans*. The study started by analyzing the effect of gene knockout on morphogenesis and drug susceptibility. *CaREP5* and *CaREP6* involved in drug resistance will be identified by agar dilution analysis and real-time PCR. The germ tube assay was used to analyze the effect of *CaREP5* and *CaREP6* mutants on morphology.

2.2 Methods and Materials

2.2.1 DNA method

QIAprep spin miniprep kit (Qiagen Inc., catalog no. 27106) was used to extract plasmid DNA within colibacillus and the QIAprep gel extraction kit (Qiagen Inc., catalog no. 28706) was used to perform isolation and purification from gel. The protocols were provided by the manufacturer. Restriction enzyme digestion was performed for one hour. DNA ligation reaction was at 4°C for overnight with ligase.

2.2.2 Preparing *Escherichia coli* competent cell using calcium chloride

Escherichia coli DH5α was inoculated in 3 ml LB medium at 37ºC with 150 rpm shaking overnight. The 100 ml (1:100) of LB medium were added into the overnight culture and then incubated at 37ºC with shaking to OD_{600} of 0.4 to 0.6. The subsequent culture was placed onto the ice prior to transferring to sterile tubes, for centrifugation at a speed of 4,000 rpm at 4°C for 10 minutes. The upper clear solution was then removed and 10 ml of ice-cold 0.1 M $CaCl_2$ added to suspend the pellet. The cells were centrifuged with a speed of 4,000 rpm at 4°C and then the upper clear solution was removed. Next, 2 ml of ice-cold $CaCl_2$ solution added to suspend the pellet. The suspended cells were divided into 200 μl fraction each in eppendorff to be stored at -70°C for future use.

2.2.3 *Escherichia coli* transformation using calcium chloride prepared competent cells

Plasmid DNA was added into 200 μl of competent cell and the mixture kept on the ice for 30 minutes before placed at 42°C for 2 minutes. Next, one ml of rich medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) was added to the mixture that maintained on ice to chill, then incubated on a shaker at 37°C for one hour. After that, appropriate amount of the mixture was transferred and spread on LB agar plate that contains ampicillin. The plates were incubated at 37°C for overnight.

2.2.4 Construct the plasmids and strains of *CaREP5* and *CaREP6*

Genetically engineered *C. albicans* strains used in this study are listed in Table 1. Primers used in this study are listed in Table 2, and plasmids used in this study are listed in Table 3.

2.2.4.1 pHC1: A region-pSFS2-*SAT1* (*CaREP5*)

In this study, plasmid pSFS2 carrying the dominant nourseothricin resistance marker (*SAT1*) along with several unique restriction sites was used for construction and recombination (Reuss et al., 2004).

The *C. albicans* SC5314 genomic DNA was used as a template for PCR amplification of a 300 bp DNA fragment of *CaREP5* using the primer HJL00840 (5'-ggtaccAGAAAGAGAGAGAGGGAACG-3') and HJL00841 (5'-ctcgagGTGCGGCGTAAATGGTCTTC-3'), containing *Kpn*I and *Xho*I

restriction sites respectively at the 5' end. The amplified fragment starts 529 bp upstream to the predicted start codon of the *CaREP5*. The PCR product was cloned into pGEM-T Easy vector. After *kpn*I and *Xho*I digestion, the fragment was ligated to a binary vector (pSFS2-*SAT*) that had been digested with *kpn*I and *Xho*I to create pHC1.

2.2.4.2 pHC2: A fragment-pSFS2-*SAT1*-B fragment (*CaREP5*)

The *C. albicans* SC5314 genomic DNA was used as a template for PCR amplification of a 444 bp DNA fragment of *CaREP5* using the primer HJL00863 (5'-gcggccgcTTGAATTAATACGGTGATTC-3') and HJL00864 (5'-gagctcTTTATCTATTTGTTGCGGC-3'), containing *Not*I and *Sac*I restriction sites respectively at the 5' end. The amplified fragment started 2359 bp downstream to 2802 bp of the *CaREP5*. The PCR product was cloned into pGEM-T Easy vector. After *Not*I and *Sac*I digestion, the fragment was ligated with pHC1 to produce pHC2.

2.2.4.3 pHC3: A region-pSFS2-*SAT1* (*CaREP6*)

The *C. albicans* SC5314 genomic DNA was used as a template for PCR amplification of a 525 bp DNA fragment of *CaREP6* using the primer HJL00842 (5'-gggcccTATCATCACCACTACCTCC) and HJL00843 (5'-ctcgagAAGGAGAGGAAATGGAAGG), containing *Apa*I and *Xho*I restriction sites respectively at the 5' end. The amplified fragment started 528 bp upstream to the predicted start codon of the *CaREP6*. The PCR product was cloned into pGEM-T Easy vector. After *Apa*I and *Xho*I digestion, the

fragment was ligated to a binary vector (pSFS2-*SAT*) to create pHC3.

2.2.4.4 pHC4: A fragment-pSFS2-*SAT1*-B fragment (*CaREP6*)

The *C. albicans* SC5314 genomic DNA was used as a template for PCR amplification of a 556 bp DNA fragment of *CaREP6* using the primer HJL00865 (5'-gcggccgcTTCCTTTCGTCCTCCAAC) and HJL00866 (5'-gagctcTTCTTTGGTTCTTCTCTTC), containing *Not*I and *Sac*I restriction sites respectively at the 5' end. The amplified fragment started 1525 bp downstream to 2080 bp of the *CaREP6*. The PCR product was cloned into pGEM-T Easy vector. After *Not*I and *Sac*I digestion, the fragment was ligated with pHC3 to produce pHC4.

2.2.5 Transformation of *Candida albicans* by electroporation

The protocol for yeast transformation by electroporation was modified from the previous report (Köhler et al., 1997). *Candida albicans* cells were grown overnight in YPD medium on a shaker at 30°C. The culture was diluted into fresh YPD medium in 1/10,000 ratio (OD_{600} of 1.6 to 2.2) and incubated at 30°C with shaking. The cells were centrifuged at room temperature and re-suspended in 8 ml sterile water followed by adding 1 ml of 10X TE buffer and 1 ml of 1 M lithium acetate (pH=7.5). Then, 250 μl of 1 M DTT was added and the mixture shook at 30°C for 30 minutes followed by incubation at 30°C for 1 hour. The cells were then mixed with 40 ml of sterile water before centrifugation. The pellet was washed once with 25 ml sterile water, and 5 ml of 1 M ice-cold sorbitol, sequentially. Subsequently, the cell pellet was

re-suspended in 50 μl of 1 M sorbitol and kept on ice.

Transformation mixtures contained about 1 μg of the linear DNA and 40 μl of competent cells. The Elektroporator Gene pulser II (Bio-Rad) was set at 25 μF, 1.8 kV and 200 Ω, then electroporation was carried out in a chilled 0.2 cm electroporation cuvette.

The transformation mixture was added with 1 ml of 1 M sorbitol immediately before the re-suspension of the cells in 1 ml YPD medium. The suspension was transferred to a 15 ml centrifuge tube and incubated at 30°C for 1 hour with shaking. The cells were spread on YPD plates containing 100 μg/ml of nourseothricin and incubated at 30°C for 1 day to be selected for the nourseothricin-resistant cells. The resistant colonies were picked and inoculated in YP with 2% maltose liquid medium to pop-out the *SAT1*-cassette.

2.2.6 Replica-plating

The *SAT1* flipper excised transformants were plated on YPD plates at several dilutions in order to obtain about 100 colonies on each plate to be used as the master plate. Cells were replica-plated onto new YPD plates containing 100 μg/ml of nourseothricin. The colonies which were nourseothricin sensitive colonies were identified by their inability to grow on the YPD/nourseothricin plates and rescued from the primary plate for analyses.

2.2.7 Morphology test of *CaREP5* and *CaREP6* mutants with germ tube analysis

The different strains were inoculated by aseptic toothpicks into Brain Heart

Infusion medium (Difco) that contains 10% fetal bovine serum (JRH BIOSCIENCES, Australia). The cells were incubated at 37ºC for 2 to 4 hours and then the states of germ tube formations were observed under microscope.

2.2.8 White-opaque switching assay of *CaREP5*

The mating type-like a (MTLa) and α strains were isolated on YEPS (yeast extract-peptone plus 2% sorbose) plates. The strains were streaked on 2% Bacto-agar containing the nutrient components of the defined medium of Lee et al. (Lee et al., 1975), and colonies were resuspended into Lee's liquid medium. 5×10^8 cells were resuspended into 50 ml double-distilled water then transferred to Petri dish for UV treatment with 124 Jm^{-2}. Each Lee's media plate plus 5 μg/ml phloxine B with 34 μg/ml chloramphenicol, and contained a density between 500 and 1000 colonies roughly. Plates were incubated at room temperature for at least 7 days. The procedurd of integration refer to literature from Huang et al., Zordan et al., and srikantha et al (Huang et al., 2006; Zordan et al., 2006; and srikantha et al., 2006), and Morrow et al. demonstrated that UV irradiation induces white-opaque switching in *C. albicans* WO-1 (Morrow et al., 1989).

2.2.9 Quantitative analysis of the *CaREP5* and *CaREP6* mRNA level by real-time PCR (real-timepolymerase chain reaction)

The first day, strains were grown in centrifuge tube containing 3 ml SD (0.67% Difco-yeast nitrogen base w/o amino acid with 2% dextrose) medium at 30°C overnight. The second day, 20 μl culture were resuspended in 10 ml

fresh SD medium at 30°C overnight and the cells were diluted in 250 ml flask containing 50 ml fresh SD to OD_{600} of 0.2. After 5 hours at 30°C with shaking incubation, 22 ml culture were transferred into new flask containing final miconazole concentration of 100 ug/ml dissolved in DMSO or without miconazole but equal amount of DMSO added as controls. The cells were harvested 1 hour after incubated at 30°C with shaking.

Total RNAs were isolated by QIAGEN RNeasy Mini Kit (catalog no. 74106) and Baseline-ZERO^{TM} DNase (catalog no. DB0711K). The modified procedures is as follows: the cell pellet was loosened thoroughly by flicking the tube, then adding 600 μl buffer RLT (containing β-mercaptoethanol), and vortexing to resuspend the cell pellet. The acid-washed glass beads (~300 μl) were added to the sample. The cells were disrupted through FastPrep®-24 (4.5 M/s, 30 seconds 4 times, cooling intervals 30 seconds). The lysate (usually 350 μl) was then transferred to a new microcentrifuge tube and centrifuged for 2 minutes at full speed. The supernatant was then transferred to a new microcentrifuge tube. One volume of 70% ethanol was added to the homogenized lysate, and the mixture was mixed well by pipetting (do not centrifuge). The sample (usually 700 μl) was transferred to an RNeasy spin column placed in a 2 ml collection tube. After centrifugation for 15 seconds at ≧8000 g, the flow-through was discarded. 700 μl buffer RW1 was added to the RNeasy spin column. The column was centrifuged for 15 seconds at ≧8000 g to wash the spin column membrane. 500 μl buffer RPE was added to the RNeasy spin column. The column was centrifuged for 15 seconds at ≧8000 g to wash the spin column membrane. Another 500 μl buffer RPE was added to the RNeasy spin column. The column was centrifuged for 2 minutes

at ≧8000 g to wash the spin column membrane. The RNeasy spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 minute. The RNeasy spin column was placed in a new 1.5 ml collection tube and 30 μl RNase-free water was added directly to the spin column membrane. After incubation for 10 minutes, the column was centrifuged for 1 minute at ≧8000 g to elute the RNA. 30 μl RNase-free water was added to elute the RNA again. 6.5 μl of 10X Baseline-ZERO™ DNase reaction buffer and 2 μl (2 MBU) of Baseline-ZERO™ DNase were added to the sample and the mixture was incubated at 37°C for 60 minutes. 200 μl RNase-free water was added to the RNA sample. The mixture was kept on ice. One volume (200 μl) of 4 M LiCl-buffer (in DEPC water) and the mixture was set for at least 1 hour at -20°C before centrifugation at max speed for 30 minutes at 4°C. The pellet was washed twice with 70% ethanol and air dried. The pellet was dissolved in 30 to 35 μl of RNase-free water.

Reverse transcription was carried out by the ImProm-II™ reverse transcription system (Promega, catalog no. A3800) according to the technical manual of manufacturer. The primer pairs used were as follows: for *CaACT1*, HJL00693 (5'-AGTGCTGAAAGAGAAATTGT-3') and HJL00694 (5'-AGCAGCTTCCAAACCTA-3'); for *CaCDR1*, HJL00315 (5'-GTGCTGAACGTGAATATGT-3') and HJL00316 (5'-CTCTCTGTTACCCTTTGG-3'); for *CaCDR2*, HJL00395 (5'-GTTTACACATCAACTATGGGAC-3') and HJL00396 (5'-GCAGCTTCGGTATAAGG-3'); for *CaCPH1*, HJL00538 (5'-GCTACCACCTTGACCG-3') and HJL00539 (5'-GCATAACTTCCTGCCTGA-3'); for *CaEFG1*, HJL00540 (5'-GTGGTGCAGGAACTAGA-3') and HJL00541

(5'-TGGATTTGGGAGAAGATTATG-3'); for *CaNDT80*, HJL00319 (5'-AGAGTTGCCTGACCAC-3') and HJL00320 (5'-ATCTGCAAGTCCTCGT-3'); for *CaREP5*, HJL00586 (5'-gAACAACTgTCgggAAT) and HJL00587 (5'-CagTgTgAgTgATACTACCT-3'); *CaREP6*, HJL00588 (5'-gCAAcggTACTTACTgT-3') and HJL00589 (5'-gATgAgCAACCACTTgT-3'); *CaSNF3*, HJL00338 (5'-ACATTCAGCAACGTATCG-3') and HJL00339 (5'-TGTTCCACCACCACTT-3'). The mRNA levels of sample genes were measured by real-time PCR using Rotor-Gene RG-3000 amplification system (Corbett Research) with CAS-1200 robotic liquid handling system (Corbett Research), which allows amplification and measuring the binding of the FastStart Universal SYBR Green Master (ROX) (Roche, catalog no. 04 913 850 001) to double-stranded DNA. The condition for real-time PCR was: denaturation (120 seconds at 95°C), 35 cycles of repeated amplification (20 seconds at 95°C, 20 seconds at 65°C and 20 seconds at 72°C) and detected amplicon's fluorescence signal at 80°C. The expressions of *CaACT1* and *CaSNF3* were used to normalize the mRNA expression levels of target geness.

2.3 Results

2.3.1 Construction and confirmation of the heterozygous *CaREP5* null mutant

The 2358 bp genomic DNA sequence of *CaREP5* is obtained from The *Candida* Genome Database, which provides the sequence of assembly contig19-10215 of *C. albicans*. The *C. albicans* SC5314 genomic DNA was used as a template for PCR amplification using the primers HJL00840 and HJL00841, and the amplified fragment contained the sequence of *CaREP5* from -529 to -230. After *Kpn*I and *Xho*I digestion, the 300 bp fragment was ligated to pSFS2 to create pHC1. A fragment containing the sequence of *CaREP5* from +2360 to +2803 was PCR amplified by primers HJL00863 and HJL00864 from SC5314 genomic DNA. After *Not*I and *Sac*I digestion, the 444 bp fragment was ligated to pHC1 to create pHC2. The *Kpn*I-*Sac*I DNA fragment containing the constructed A and B fragments in pSFS2 was integrated into the wild type SC5314 strain to generate *CaREP5*/*Carep5* (Fig. 26) transformants YLO00324 and YLO00325. Using YLO00324 and YLO00325 as template, yeast colony PCR with primers HJL00814 (5'-CTCAACATGGAACGATCTAGC-3') and HJL00881 (5'-CCTATCTTTATCTTTCTATCT-3') generated a 1034 bp fragment due to the integration by *SAT1* cassette. The HJL00840 and HJL00881 primers generated an 861 bp fragments that pop-out the *SAT1* cassette already (Fig. 26).

2.3.2 Construction and confirmation of the homozygous *CaREP5* null mutant

Homozygous knock-out mutants of *C. albicans* SC5314 strains were obtained after two rounds of insertion and excision of the *SAT1* cassette. The *Kpn*I-*Sac*I DNA fragment containing the *SAT1* flipper cassette of pHC2 was integrated into the *CaREP5* heterozygous mutant strain to generate *Carep5*/*Carep5* (Fig. 27) transformants YLO00326 and YLO00327. In contrast, using the HJL00934 (5'-CATCAAGAATCCAAGGTCG) and HJL00935 (5'- GGTGTTGTTGTTGTTGTTG) primer set in PCR with the wild type chromosome as templates produced a 693 bp DNA fragment, whereas the mutated chromosomes did not generate any product. The strains of both heterozygous and homozygous null mutants were confirmed by southern blot analysis (Fig. 28) that the procedure was as same as above-mentioned in 1.2.3 (Page 16).

2.3.3 Construction and confirmation of the *Carep5*/*Carep5*::*CaREP5* rescued strains

The pHC2 was digested with *Kpn*I and *Xho*I to excise the 5' fragment of *CaREP5*, and then ligated with the fragment containing the sequence of *CaREP5* from -529 to +2804 which was amplified by primer HJL00840 (*Kpn*I) and HJL00959 (*Xho*I) (5'-CTCGAGCATTTATCTATTTGTTGCGGC) from wild type strain SC5314 genomic DNA to generate pHC5. The pHC5 digested with *Kpn*I-*Sac*I was integrated into the *Carep5*/*Carep5* mutants (YLO00326 and YLO00327) to create the *Carep5*/*Carep5*::*CaREP5* (Fig. 29) rescued strains

YLO00352 and YLO00353. Using the HJL00943 (5'-ACAACAAGAACAAAGAAGCC) and HJL00947 (5'-GCTGTTGATGATGATTCTGT) primer set, the rescued strains produced a 1794 bp DNA fragment, and the HJL01071 (5'- CTTCAACACCAACCACTTC) and HJL 00881 primer set generated a product of 1969 bp.

2.3.4 Germ tube test results of *CaREP5* mutants

To analyze the effect of mutations on morphogenesis, the germ tube analysis was performed on the *Carep5* mutant. It appeared that the germ tube formation was normal in the *Carep5* mutant with no defect on hyphal formation in the presence of serum. The phenotype of *Carep5*/*Carep5* mutant or the *Carep5*/*Carep5*::*CaREP5* rescued strain did not have significant difference compared with the wild type strain (Fig. 30).

2.3.5 Agar dilution assay results of *CaREP5* mutants

The agar dilution procedure was as same as above-mentioned in 1.2.7 (Page 21) and performed to examine antifungal susceptibility on SD or YPD plates. The natures of these compounds are as Table 4. All tested strains including wild type strain (SC5314), *CaREP5*/*Carep5*-1 (YLO00324), *Carep5*/*Carep5*-1 (YLO00326), *Carep5*/*Carep5*::*CaREP5*-1 (YLO00352), *CaREP5*/*Carep5*-2 (YLO00325), *Carep5*/*Carep5*-2 (YLO00327), *Carep5*/*Carep5*::*CaREP5*-2 (YLO00353) showed the same level of susceptibility to drugs indicated that the *CaREP5* is not involved in drug resistance (Fig. 31) in the tested conditions. The results also indicated that the

Carep5 mutant was not sensitive to bile salts and common detergents (Fig. 32).

2.3.6 White-opaque switching assay of *CaREP5*

The tested strains including the SC5314 α, SC5314 a, *CaREP5*/*Carep5* α, *CaREP5*/*Carep5* a, *Carep5*/*Carep5*::*CaREP5* α, *Carep5*/*Carep5*::*CaREP5* a have not significant switch. The *Carep5*/*Carep5* α and *Carep5*/*Carep5* a strains have not significant blocked opaque formation (Fig. 33).

2.3.7 Comparison of the genes expression level of *Carep5*/*Carep5* mutants by real-time PCR

The expression levels of *CaACT1* and *CaSNF3* used as loading control genes were not significantly induced by the treatment of 100 μg/ml miconazole. There is no *CaREP5* mRNA could be detected in *Carep5*/*Carep5* at real-time PCR result (Fig. 34). The results of real-time PCR showed that the expression levels of *CaCDR1*, *CaCDR2*, *CaNDT80*, *CaCPH1* and *CaEFG1* were not significantly by different in the cells treated with or without 100 μg/ml miconazole (Fig. 35, 36, 37, 38 and 39).

2.3.8 Construction and confirmation of the heterozygous *CaREP6* null mutant

The 1524 bp sequence of *CaREP6* is obtained from The *Candida* Genome Database, which provides the sequence of assembly contig19-10205 of *C. albicans*. The *C. albicans* SC5314 genomic DNA was used as a template for

PCR amplification using the primers HJL00842 (*Apa*I) and HJL00843 (*Xho*I), and the amplified fragment contained the sequence of *CaREP6* from -528 to -4. After *Apa*I and *Xho*I digestion, the 525 bp fragment was ligated to pSFS2 to create pHC3. A fragment contains the sequence of *CaREP6* from +1526 to +2081 and was amplified by primers HJL00865 (*Not*I) and HJL00866 (*Sac*I) from wild type strain SC5314 genomic DNA. After *Not*I and *Sac*I digestion, the 556 bp fragment was ligated to pHC3 to create pHC4. The *Apa*I-*Sac*I DNA fragment containing the *SAT1* flipper cassette of pHC4 was integrated into the wild type SC5314 strain to generate *CaREP6*/*Carep6* (Fig. 40) transformants YLO00348 and YLO00349. The HJL00814 and HJL00882 (5'-CGCAGAACAAAGAGAAGGA) primers generated a 1215 bp fragment in PCR due to the integration by *SAT1* cassette (Fig. 40). The HJL00866 and HJL00944 (5'-TGCTGAATCAACACAATATC) primers generated a 1308 bp fragments due to the pop-out of the *SAT1* cassette already (Fig. 40).

2.3.9 Construction and confirmation of the homozygous *CaREP6* null mutant

The *Apa*I-*Sac*I DNA fragment containing the *SAT1* flipper cassette of pHC4 was integrated into the *CaREP6* heterozygous mutant strain to generate *Carep6*/*Carep6* (Fig. 41) transformants YLO00350 and YLO00351. In contrast, using the HJL00936 (5'-TCATCATCATAGCCGTCAC) and HJL00937 (5'-CCGTTTGTGTGGAGATTC) primer set in PCR, the wild type chromosome produced an 814 bp DNA fragment, whereas the mutated chromosomes did not generate any product. Furthermore, the results of southern blot analysis verified the heterozygous and homozygous mutant constructs (Fig. 42).

2.3.10 Germ tube test results of *CaREP6* mutants

The germ tube formation was normal on the *Carep6* mutant and did not cause defect on hyphal formation in the presence of serum. The phenotype of *Carep6*/*Carep6* mutant did not have significant difference to that of the wild type SC5314 strain (Fig. 43).

2.3.11 Agar dilution assay results of *CaREP6* mutants

The agar dilution was performed to examine antifungal susceptibility on SD or YPD plates, and the natures of these compounds are as Table 4. Four tested strains including the wild type strain (SC5314), *CaREP6*/*Carep6*-1 (YLO00348), *Carep6*/*Carep6*-1 (YLO00350), *CaREP6*/*Carep6*-2 (YLO00349), *Carep6*/*Carep6*-2 (YLO00351) had the same result indicated that the *CaREP6* is not involved in drug resistance (Fig. 44) in the tested conditions. The results also indicated that the *Carep6* mutant was not sensitive to bile salts and common detergents (Fig. 45).

2.3.12 Comparison of the genes expression level of *Carep6*/*Carep6* mutants by real-time PCR

The expression levels of *CaACT1* and *CaSNF3* used as loading control genes were not significantly induced by the treatment of 100 μg/ml miconazole. There is no *CaREP6* mRNA could be detected in *Carep6*/*Carep6* at real-time PCR result (Fig. 46). The results of real-time PCR showed that the expression levels of *CaCDR1*, *CaCDR2*, *CaNDT80*, *CaCPH1* and *CaEFG1* were not

significantly by different in the cells treated with or without 100 μg/ml miconazole (Fig. 35, 36, 37, 38 and 39).

2.4 Discussion

Previous studies in the lab indicated that CaRep5p and CaRep6p can increase the β-galactosidase activity of $CDR1_{YM990348}$ promoter-*lacZ* in *S. cerevisiae* in the presence of miconazole. However, the results in this study showed the germ tube formation and filamentous growth of the *Carep5*/*Carep5* and *Carep6*/*Carep6* mutants were similar to the wild-type. A possible reason is that the experiment condition of germ tube analysis was not suitable to induce germ tube formation and/or filament growth.

Deletion of *CaREP5* and *CaREP6* have no effect on the antifungal and chemical susceptibility, and the mRNA expression profiles of *CaCDR1*, *CaCDR2*, *CaNDT80*, *CaCPH1* and *CaEFG1* were not change significantly following miconazole treatment. The results did not meet the anticipation. One reason is that previous experiments were conducted with the *CDR1p-lacZ* in *S. cerevisiae* but not in *C.albicans*. Or, there is no direct relation between *CaREP5*/*CaREP6* and drug susceptibility. The regulation needs the involvement of other genes.

Deletion of *CaREP5* failed to show defects in white-opaque switch. This is unexpected. In the literature, many studies have showed that *CaREP5* is a regulator of white-opaque switching and is opaque-specific (Huang et al., 2006; Sirkantha et al., 2006 and Zordan et al., 2006). According to these literatures about orf19.4884, WO-1 is the common strain used in white-opaque switching assays. WO-1 is *MTLα* genotype that high-frequency switching between white and opaque phenotypes (Slutsky et al., 1987; Lockhart et al., 2002). But in this study, SC5314 used as the strain to implement gene deletion and a/a and α/α strains construction. In addition, the condition of white-opaque switching

experiment may be inappropriate, and the reconfirmation or adjustment is necessary.

2.5 Future Work

In this study, the understanding of the relationship between *CaREP5*/*CaREP6* with *CaCDR1* and drug susceptibility is the key point. However, the result of agar dilution and real-time PCR showed *CaREP5* and *CaREP6* unaffected drug susceptibility, *CaREP5* and *CaREP6* mutants also not affect the morphogenesis. The function of *CaREP5* and *CaREP6* unable to understood by these results.

Later, the confirmation of the relationship between *CaREP5*/*CaREP6* with morphology is appropriate to investigate the experiment condition of germ tube growing. In the more recent studies suggest that *CaREP5* is required for establish and maintain the opaque cell，but *CaREP6* is expressed in the white cell (Tsong et al., 2003). The new experiment should be designed for the role of *CaREP5* and *CaRep6* in mechanisms of white-opaque switch, and the experiment condition may need to be reset. *CaREP5*/*CaREP6* of Parental strain SC5314 can compare with parental strain WO-1 in white-opaque switching assay. The biological significance and relationship to *CaREP5* and *CaREP6* is not clear, *CaREP5* and *CaREP6* interactive with other genes may investigated by microarray or quantitative PCR further. Furthermore, implementing the selection in *C. albicans* system for finding out the gene which can regulate *CDR1* in *C. albicans* anew.

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Table 1. Bacteria (*Escherichia coli*) and *Candida albicans* strains used in this study

Strain	Relevant genotype	Source
Bacteria		
E. coli DH5α	F⁻ψ80d*lacZ*ΔM15 Δ(*lac*ZYA-*arg*F) U169 *end*A1 *rec*A1 *hsd*R17 (r_k-m_k+)*deo*R thi-1 *pho*A *sup*E44λ⁻*gyr*A96 *rel*A1	Lo Lab. collection
Candida albicans		
SC5314	Wild type strain; pathogenic strain	Lo Lab. collection (Gillum et al., 1984)
YLO00133	*ura3*Δ::λ*imm434*/ *ura3*Δ::λ*imm434 his1*::*hisG*/ *his1*::*hisG arg4*::*hisG*/*arg4*::*hisG Candt80*::*GFP-Arg4*/*Candt80*::*URA3-dpl200 ENO1*/*eno1*::*ENO1-tetR-ScHAP4-3xHA-HIS1*	(Chen et al., 2004)
YLO00137	*ura3*Δ::λ*imm434*/ *ura3*Δ::λ*imm434 his1*::*hisG*/ *his1*::*hisG arg4*::*hisG*/*arg4*::*hisG Candt80*::*GFP-Arg4*/ *Candt80*::*URA3-dpl200*::*CaNDT80*::*HIS1*	(Chen et al., 2004)

(Continuous)

Strain	Relevant genotype	Source
YLO00324	*CaREP5*/*Carep5*△::*FRT*-1	This study
YLO00325	*CaREP5*/*Carep5*△::*FRT*-2	This study
YLO00326	*Carep5*△::*FRT*/*Carep5*△::*FRT*-1	This study
YLO00327	*Carep5*△::*FRT*/*Carep5*△::*FRT*-2	This study
YLO00348	*CaREP6*/*Carep6*△::*FRT*-1	This study
YLO00349	*CaREP6*/*Carep6*△::*FRT*-2	This study
YLO00350	*Carep6*△::*FRT*/*Carep6*△::*FRT*-1	This study
YLO00351	*Carep6*△::*FRT*/*Carep6*△::*FRT*-2	This study
YLO00352	*CaREP5*::*FRT*/*Carep5*△::*FRT*-1	This study
YLO00353	*CaREP5*::*FRT*/*Carep5*△::*FRT*-2	This study
YLO00365	*CaENO1*/*Caeno1*△::*FRT*-1	This study
YLO00366	*CaENO1*/*Caeno1*△::*FRT*-2	This study
YLO00367	*Caeno1*△::*FRT*/*Caeno1*△::*FRT*-1	This study
YLO00368	*Caeno1*△::*FRT*/*Caeno1*△::*FRT*-2	This study

(Continuous)

Strain	Relevant genotype	Source
YLO00369	*CaENO1*::*FRT*/*Caeno1*△::*FRT*-1	This study
YLO00370	*CaENO1*::*FRT*/*Caeno1*△::*FRT*-2	This study
HLC54	*Ura3*::*1 imm434*/*ura3*::*1 imm434 cph1*::*hisG*/*cph1*::*hisG* *efg1*::*hisG*/*efg1*::*hisG-URA3-hisG*	(Lo et al., 1997)

Table 2. Oligonucleotides used in the study

Primer	Sequence	Position
HJL00315	5'-GTGCTGAACGTGAATATGT	*CaCDR1*: +4205 ~ +4223
HJL00316	5'-CTCTCTGTTACCCTTTGG	*CaCDR1*: +4471 ~ +4488
HJL00319	5'-AGAGTTGCCTGACCAC	*CaNDT80*: +1080 ~ +1095
HJL00320	5'-ATCTGCAAGTCCTCGT	*CaNDT80*: +1382 ~ +1367
HJL00338	5'-ACATTCAGCAACGTATCG	*CaSNF3*: +1866 ~ +1883
HJL00339	5'-TGTTCCACCACCACTT	*CaSNF3*: +2207 ~ +2192
HJL00395	5'-GTTTACACATCAACTATGGGAC	*CaCDR2*: +3973 ~ +3994
HJL00396	5'-GCAGCTTCGGTATAAGG	*CaCDR2*: +4280 ~ +4264
HJL00538	5'-GCTACCACCTTGACCG	*CaCPH1*: +643 ~ +658
HJL00539	5'-GCATAACTTCCTGCCTGA	*CaCPH1*: +965 ~ +948
HJL00540	5'-GTGGTGCAGGAACTAGA	*CaEFG1*: +1520 ~ +1536
HJL00541	5'-TGGATTTGGGAGAAGATTATG	*CaEFG1*: +1815 ~ +1795
HJL00586	5'-gAACAACTgTCgggAAT	*CaREP5*: +172 ~ +188
HJL00587	5'-CagTgTgAgTgATACTACCT	*CaREP5*: +453 ~ +472

(Continuous)

Primer	Sequence	Position
HJL00588	5'-gCAAcggTACTTACTgT	*CaREP6*: +1185 ~ +1201
HJL00589	5'-gATgAgCAACCACTTgT	*CaREP6*: +1473 ~ +1489
HJL00693	5'-AGTGCTGAAAGAGAAATTGT	*CaACT1*: +1268 ~ +1287
HJL00694	5'-AGCAGCTTCCAAACCTA	*CaACT1*: +1477 ~ +1461
HJL00814	5'-CTCAACATGGAACGATCTAGC	*SAT1* flipper cassette: +3710 ~ +3730
HJL00840	5'-ggtaccAGAAAGAGAGAGAGGGAACG	*CaREP5*: -510 ~ -529
HJL00841	5'-ctcgagGTGCGGCGTAAATGGTCTTC	*CaREP5*: -230 ~ -249
HJL00842	5'-gggcccTATCATCACCACTACCTCC	*CaREP6*: -528 ~ -510
HJL00843	5'-ctcgagAAGGAGAGGAAATGGAAGG	*CaREP6*: -4 ~ -22
HJL00863	5'-gcggccgcTTGAATTAATACGGTGATTC	*CaREP5*: +2359 ~ +2378
HJL00864	5'-gagctcTTTATCTATTTGTTGCGGC	*CaREP5*: +2784 ~ +2802
HJL00865	5'-gcggccgcTTCCTTTCGTCCTCCAAC	*CaREP6*: +1515 ~ +1532
HJL00866	5'-gagctcTTCTTTGGTTCTTCTCTTC	*CaREP6*: +2062 ~ +2080
HJL00881	5'-CCTATCTTTATCTTTCTATCT	*CaREP5*: +2864 ~ +2884
HJL00882	5'-CGCAGAACAAAGAGAAGGA	*CaREP6*: +2213 ~ +2231

(Continuous)

Primer	Sequence	Position
HJL00934	5'-CATCAAGAATCCAAGGTCG	*CaREP5*: +221 ~ +239
HJL00935	5'-GGTGTTGTTGTTGTTGTTG	*CaREP5*: +895 ~ +913
HJL00936	5'-TCATCATCATAGCCGTCAC	*CaREP6*: +45 ~ +63
HJL00937	5'-CCGTTTGTGTGGAGATTC	*CaREP6*: +841 ~ +858
HJL00943	5'-ACAACAAGAACAAAGAAGCC	*CaREP5*: -673 ~ -654
HJL00944	5'-TGCTGAATCAACACAATATC	*CaREP6*: -721 ~ -702
HJL00947	5'-GCTGTTGATGATGATTCTGT	*CaREP5*: +1102 ~ +1121
HJL00959	5'-CTCGAGCATTTATCTATTTGTTGCGGC	*CaREP5*: +2784 ~ +2804
HJL00980	5'-ggtaccATTAAGCCGTGGGTTCTCAA	*CaENO1*: -578 ~ -559
HJL00981	5'-ctcgagAAAAAGGGAGAAAAGGAAAGAAA	*CaENO1*: -72 ~ -50
HJL00982	5'-GGCTTCTCAATTGTAAGTTTGC	*CaENO1*: +1308 ~ +1329
HJL00983	5'-CAGGATCTATTGACGAATTCCA	*CaENO1*: +1863 ~ +1884
HJL01071	5'-CTTCAACACCAACCACTTC	*CaREP5*: +916 ~ +934

Table 3. Plasmids used in this study

Plasmid	Description	Source
LOB317	The plasmid containing A region from -578 ~ -50 of *CaENO1* of *C. albicans*, and B region from +1308 ~ +1884 of *CaENO1* were cloned on both sides of the pSFS2A backbone. *CaSAT1*, Cap^R (A: *Kpn*I-*Xho*I; B: *Not*I-*Not*I)	This study
LOB318	The plasmid containing full length of *CaENO1* in the pSFS2A backbone. *CaSAT1*, Cap^R (orf19.395: -578 ~ +1884)	This study
pHC1	The plasmid containing A region from -529 ~ -230 of *CaREP5* of *C. albicans* was cloned into *Kpn*I-*Xho*I site of the pSFS2 backbone. *CaSAT1*, Amp^R	This study
pHC2	The plasmid containing B region from +2359 ~ +2802 of *CaREP5* of *C. albicans* was cloned into *Not*I-*Sac*I site of the pHC1 backbone. *CaSAT1*, Amp^R	This study
pHC3	The plasmid containing A region from -528 ~ -4 of *CaREP6* of *C. albicans* was cloned into *Apa*I-*Xho*I site of the pSFS2 backbone. *CaSAT1*, Amp^R	This study

(Continuous)

Plasmid	Description	Source
pHC4	The plasmid containing B region from +1525 ~ +2080 of *CaREP6* of *C. albicans* was cloned into *Not*I-*Sac*I site of the pHC3 backbone. *CaSAT1*, Amp^R	This study
pHC5	The plasmid containing A region and *CaREP5* from -529 ~ +2804 of *C. albicans* was cloned into *Kpn*I and *Xho*I site of the pHC2 backbone that excised A region. Used to rescue *CaREP5* function in *Carep5*/*Carep5* mutant strains. *CaSAT1*, Amp^R	This study
pSFS2	The plasmid contains a *C. albican*s-adapted nourseothricin resistance market, *CaSAT1*, Amp^R backbone.	(Wirsching et al., 2000)
pSFS2A	The plasmid contains a *C. albican*s-adapted nourseothricin resistance market, *CaSAT1*, Cap^R backbone.	(Wirsching et al., 2000)

Note. Amp^R, ampicillin resistant

Cap^R, chloramphenicol resistant

Table 4. Chemicals used in this study

Chemical	Catalog no.	Properties	Description
4NQO	Sigma, N8141	4-nitroquinoline 1-oxide $C_9H_6N_2O_3$	DNA damage-induced carcinogensis and induce intracellular production of oxidative stress (Nagao and Sugimura, 1976; Nunoshiba and Demple, 1993; Ramotar et al., 1998).
calcofluor white	Sigma, F3543	fluorescent brightener 28 $C_{40}H_{44}N_{12}O_{10}S_2$	Cell wall binding dye, used as a viability stain for cellulose in fungi (Elorza et al., 1983; Roncero and Durán, 1985).
cholate	Sigma, C1254	sodium cholate hydrate	Bile salts, non-denaturing detergent used for extraction of membrane proteins (Nicchitta and Blobel, 1990; Benson, 2006).
congo red	Sigma, C6767	$C_{32}H_{22}N_6Na_2O_6S_2$	Used as a stain for amyloid and polysaccharide (Teather and Wood, 1982; Roncero and Durán, 1985).

(Continuous)

Chemical	Catalog no.	Properties	Description
N-acetyl-glucosamine	Sigma, A8625	$C_8H_{15}NO_6$	Induction of hyphal morphogenesis, induce expression of virulence gene (Naseem et al., 2011), induce white-to –opaque switching in *C. tropicalis* (Xie et al., 2012).
NaCl	Merck, 106404	sodium chloride	Osmotic and salt stress (Park et al., 2011).
NaDOC	Sigma, D6750	sodium deoxycholate $C_{24}H_{39}NaO_4$	Bile salts, anionic detergent used for extraction of membrane proteins (Janssen et al., 1997).
$NaNO_2$	Sigma, S2252	sodium nitrite	Used as a precursor for nitrous acid (Misko et al., 1993).
NP-40	Sigma, I8896	octylphenyl-polyethylene glycol	Non-ionic detergents (Nakagawa et al., 2003).
SDS	Merck, 113760	dodecyl sulfate sodium salt	Anionic surfactant (Nakagawa et al., 2003; Nakao and Halldin, 2013).

(Continuous)

Chemical	Catalog no.	Properties	Description
triton-X-100	Merck, 108643	$(C_{16}H_{25}O)_{30}OH$	Non-ionic detergents (Nakagawa et al., 2003; Lyon et al., 2013).
tween 20	Merck, 822184	$C_{58}H_{114}O_{26}$	Non-ionic detergents (Sagong et al., 2013).

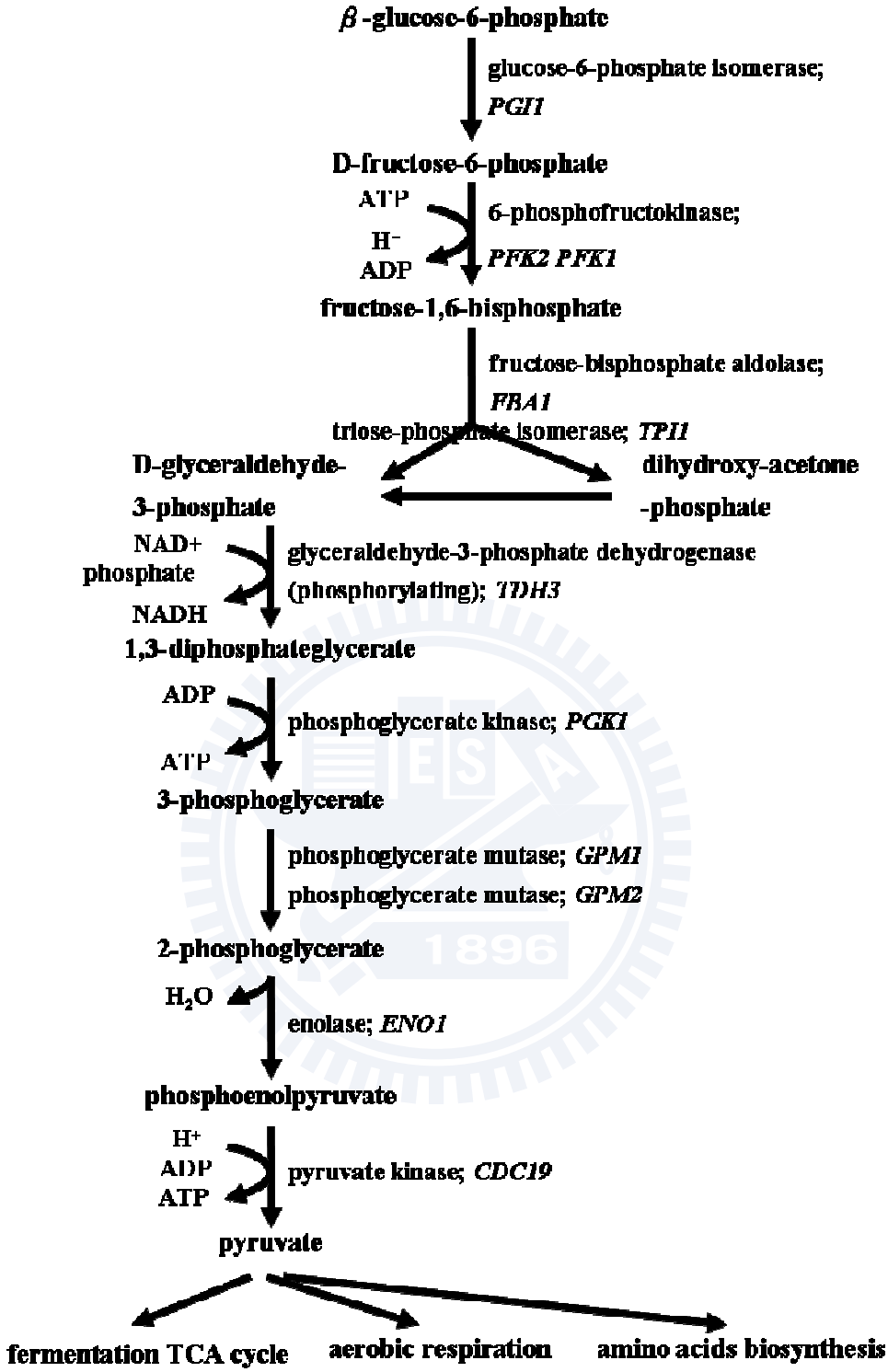


Fig. 1 Schematic diagram of the glycolysis pathway in *Candida albicans*. The pathway is modified from *Candida* genome database (http://pathway.candidagenome.org/CALBI/NEW-IMAGE?type=PATHWAY&object=GLYCOLYSIS&detail-level=2).

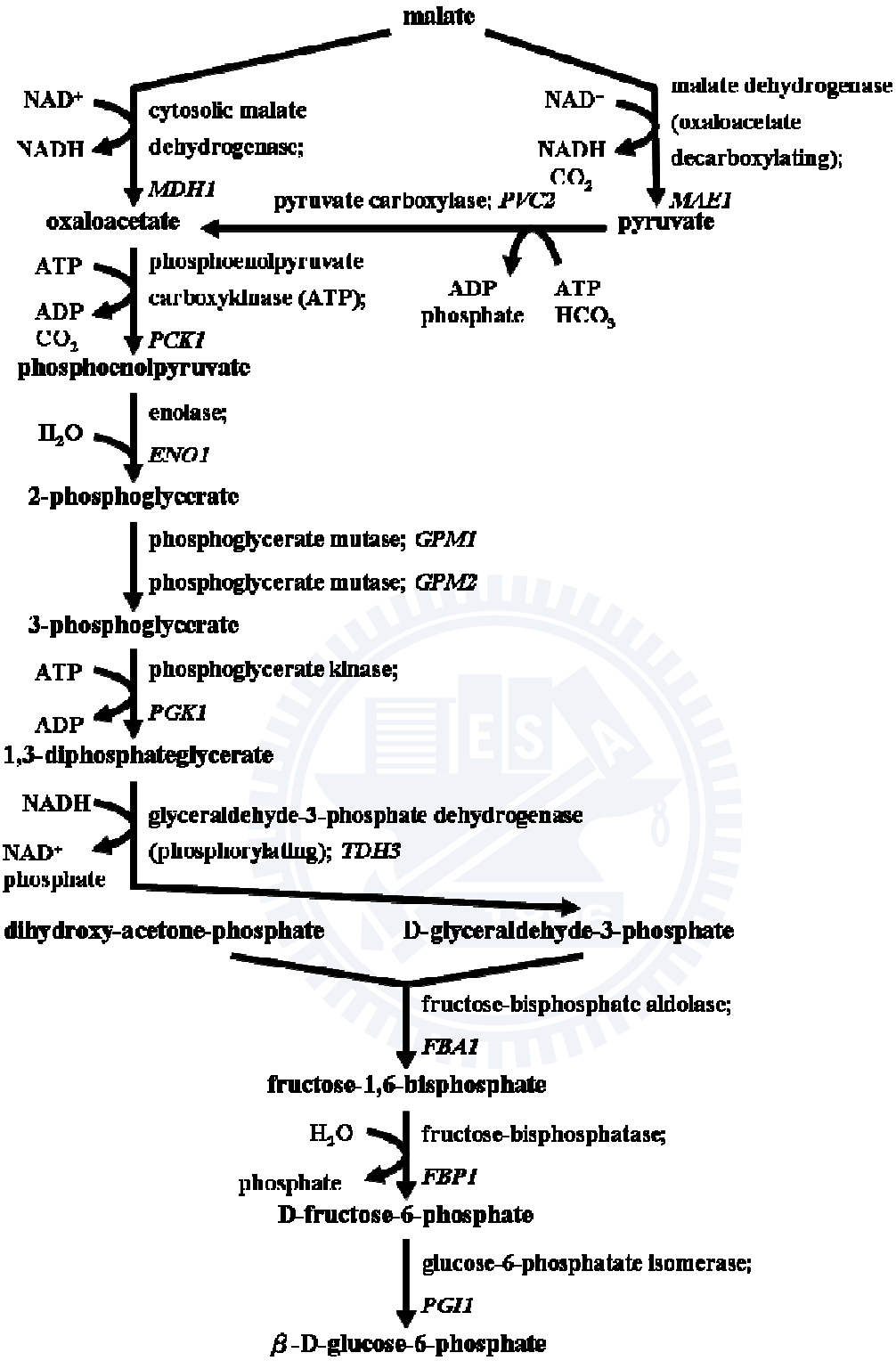


Fig. 2 Schematic diagram of the gluconeogenesis pathway in *Candida albicans*. The pathway is modified from *Candida* genome database (http://pathway.candidagenome.org/CALBI/NEW-IMAGE?type=PATHWAY&object=GLUCONEO-PWY&detail-level=2).

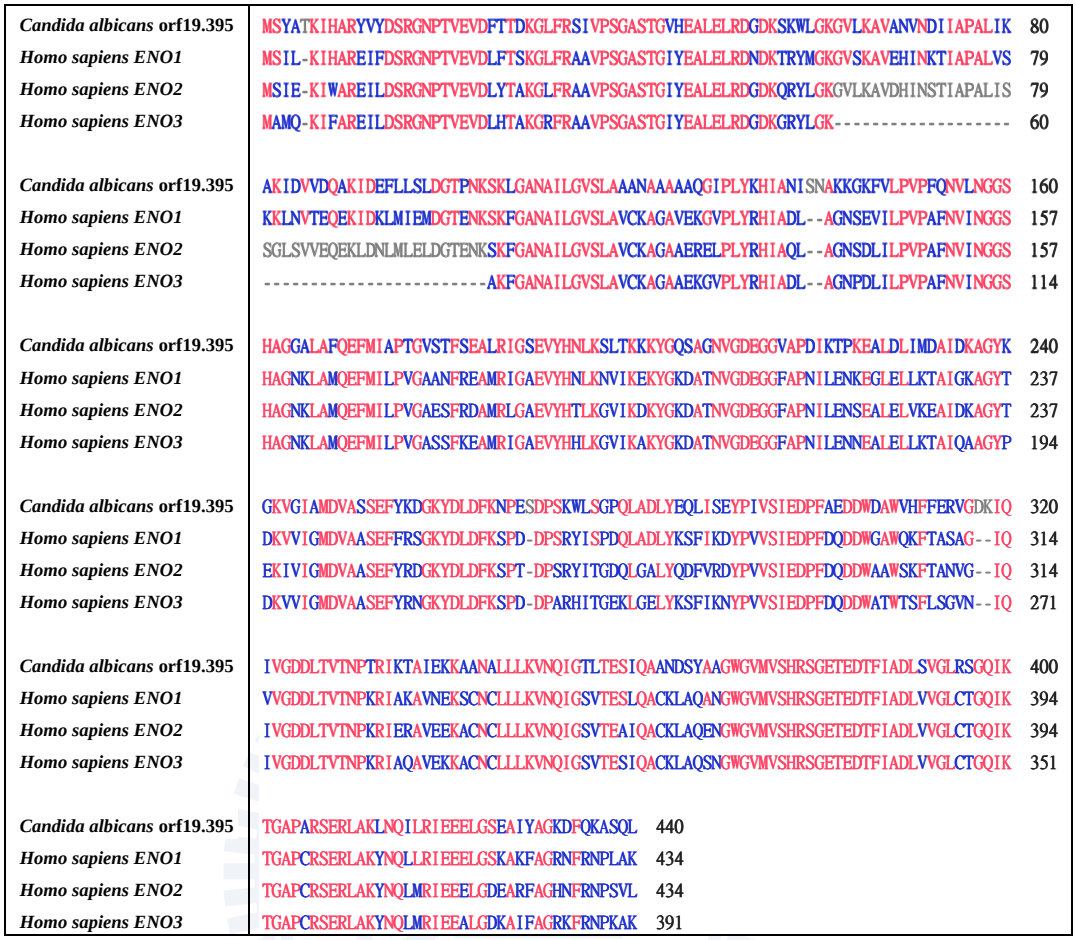


Fig. 3 Sequence alignment of CaEno1 and *Homo sapiens* enolase. The protein sequences of *CaENO1* compared with *Homo sapiens* alpha-enolase isoform 1 (*ENO1*), gamma-enolase isoform 2 (*ENO2*), and beta-enolase isoform 3 (*ENO3*) by multiple alignment tool (http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi?link_loc=BlastHomeLink).

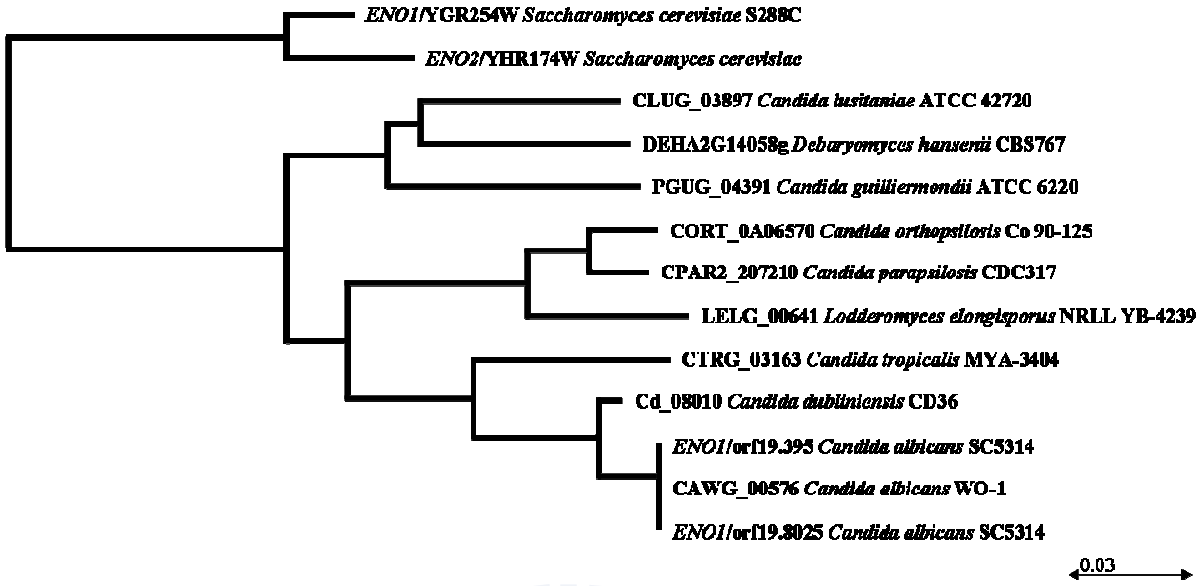


Fig. 4 The phylogenetic tree view approach for Saccharomycetales species of which the genome alike to *CaENO1*. The phylogenetic tree view align protein sequences by fast minimum evolution of COBALT multiple aligment.

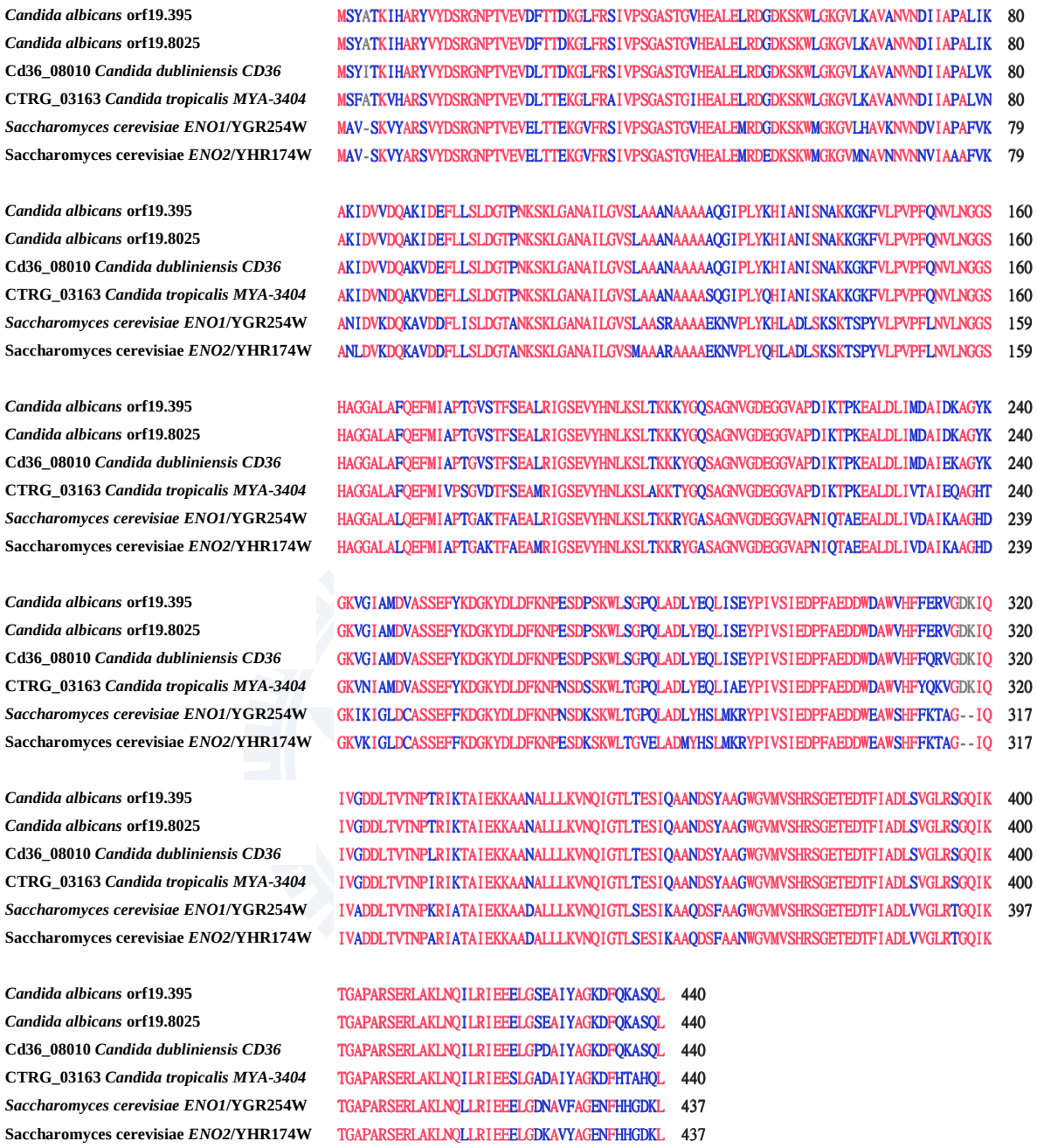


Fig. 5 The multiple alignment result for *ENO1*. The protein sequences of *CaENO1* compared with Cd36_08010 *C. dubliniensis*, CTRG_03163 *C. tropicalis*, *S. cerevisiae ENO1* and *ENO2* by multiple alignment tool (http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi?link_loc=BlastHomeLink).

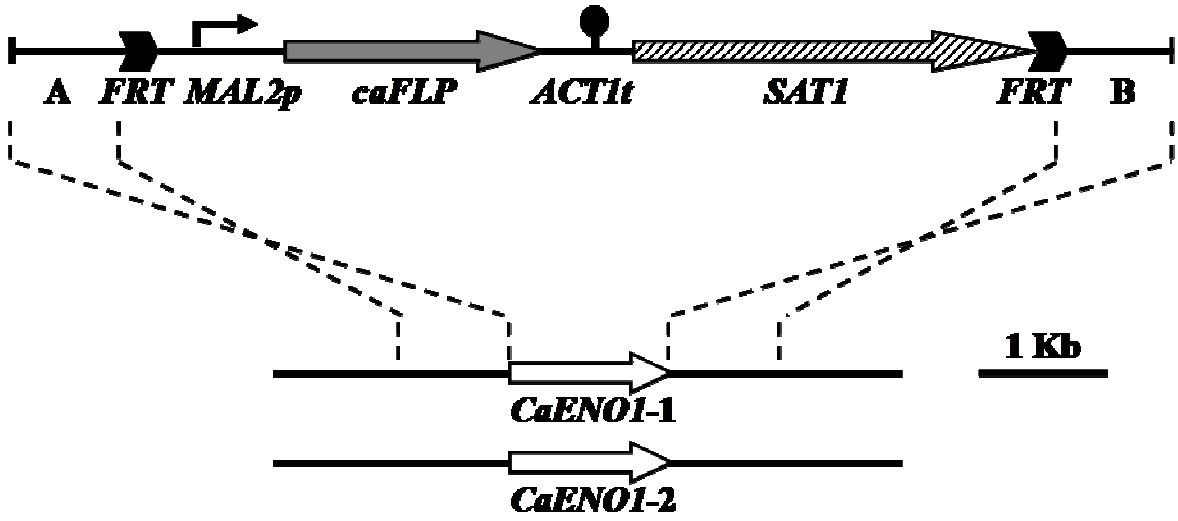


Fig. 6 Schematic diagram of construction *CaENO1* heterozygous mutant plasmid by *SAT1* flipper method. Structure of the deletion cassette from plasmid LOB317 (top), in which the *CaENO1* coding region is replaced by *SAT1* flipper cassette, and genomic structure of the *CaENO1* alleles in the parent strain SC5314 (bottom). *CaENO1-1* and *CaENO1-2* represent the two alleles of *CaENO1* in the genome, in which *CaENO1*-1 was replaced with the *SAT1* flipper cassette to obtain heterozygous mutant strains YLO00365 and YLO00366.

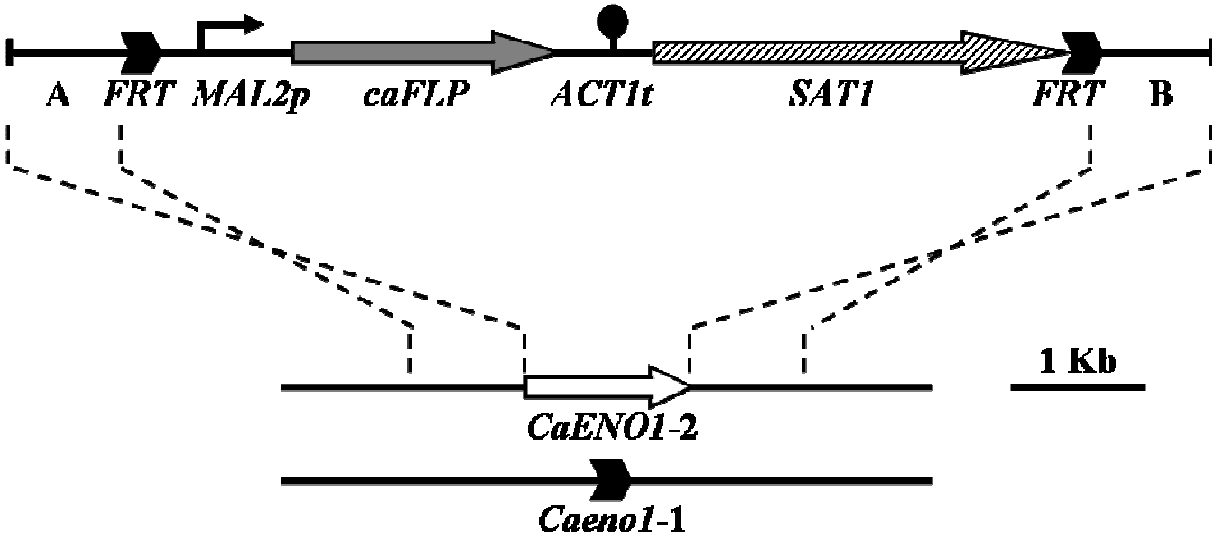


Fig. 7 Schematic diagram of construction *CaENO1* homozygous mutant by *SAT1* flipper method. Schematic maps show the deletion cassette from plasmid LOB317 (top), and one disrupted alleles of *CaENO1* (bottom). The second allele of *CaENO1* was replaced with the *SAT1* flipper cassette to obtain homozygous mutant strains YLO00367 and YLO00368.

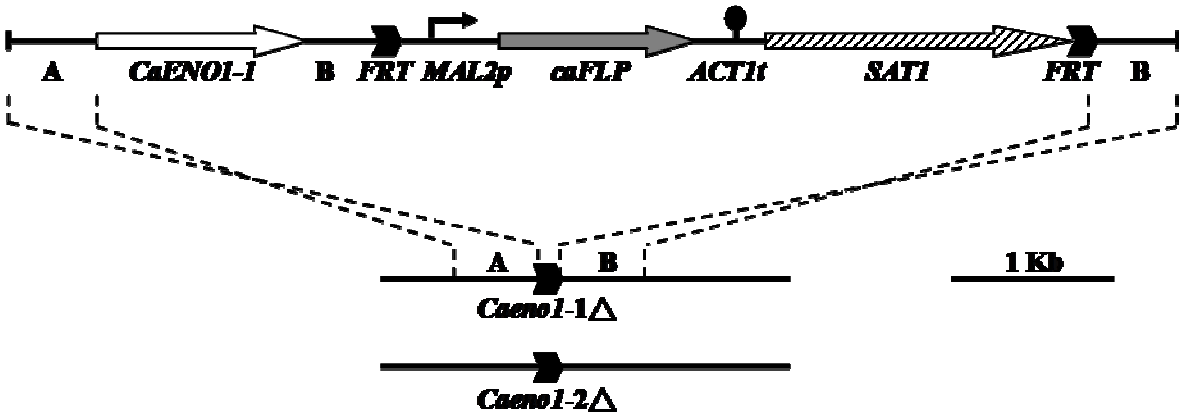


Fig. 8 Schematic diagram of construction *CaENO1* rescued strain by *SAT1* flipper method. Structure of the *SAT1* cassette from LOB318 used for reintegration of an intact *CaENO1* allele into the inactivated *Caeno1* locus to produce the *CaENO1* rescued strains YLO00369 and YLO00370.

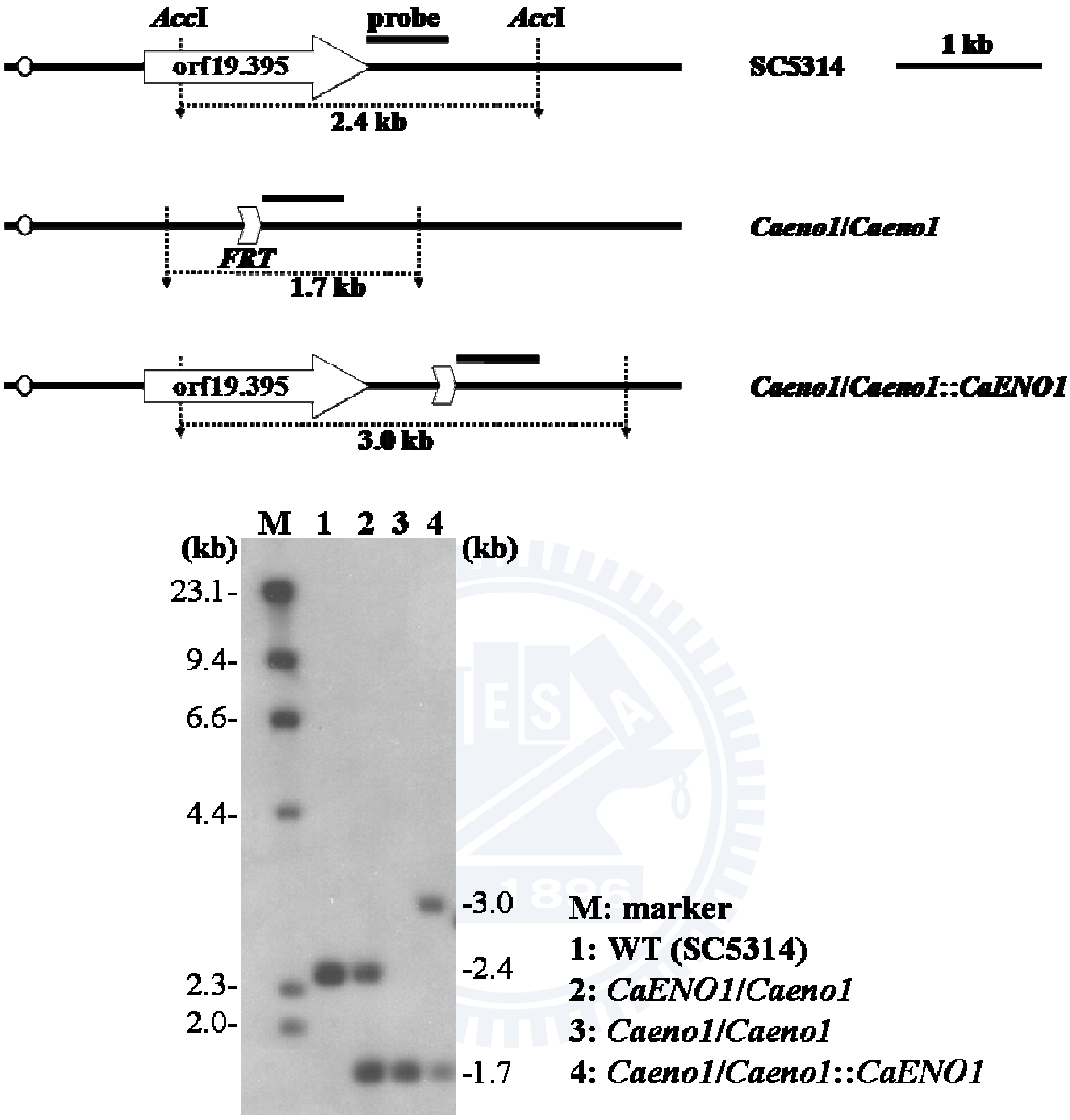


Fig. 9 Southern blot analysis to verify the *CaENO1* mutant derivatives in *Candida albicans*. Genomic DNA from heterozygous mutant strain and homozygous mutant strain were digested with *Acc*I and probed with the 577 bp PCR fragment of the *CaENO1* downstream. The sizes of the hybridizing fragments were given on the right side of the blot.

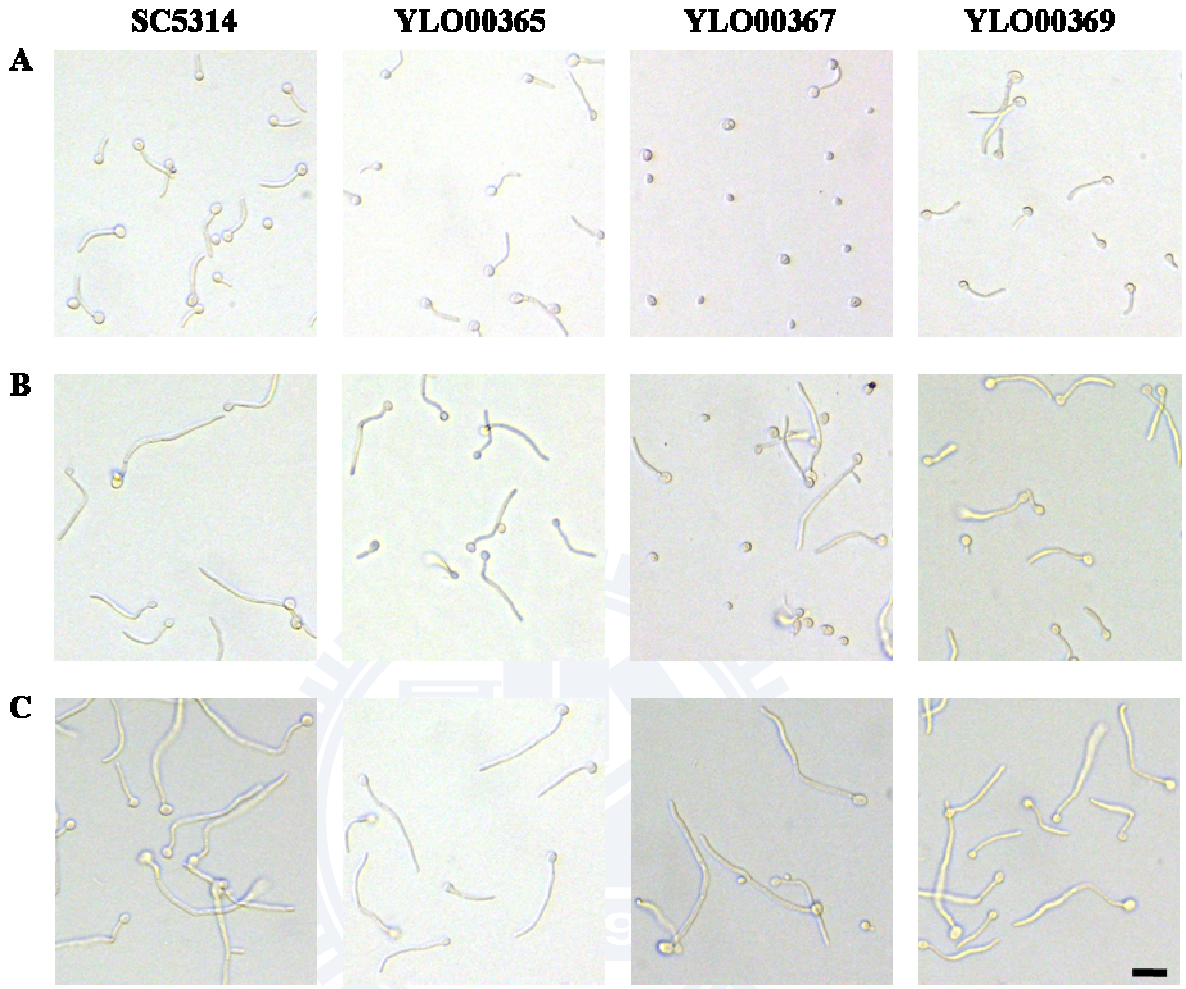


Fig. 10 Mutations on *CaENO1* affected germ tube formation of *Candida albicans*. The morphology of four strains, included *CaENO1*/*CaENO1* (SC5314), *CaENO1*/*Caeno1* (YLO00365), *Caeno1*/*Caeno1* (YLO00367) and *Caeno1*/*Caeno1*::*CaENO1* (YLO00369), were incubated in (A) YPG medium (1% yeast extract, 2% Bacto-peptone and 2% glycerol), (B) YPG medium plus 10 mM N-acetyl-glucosamine or (C) YPG medium plus 10 mM $NaNO_2$ for 3 hours at 37°C. Scale bar, 20 μm.

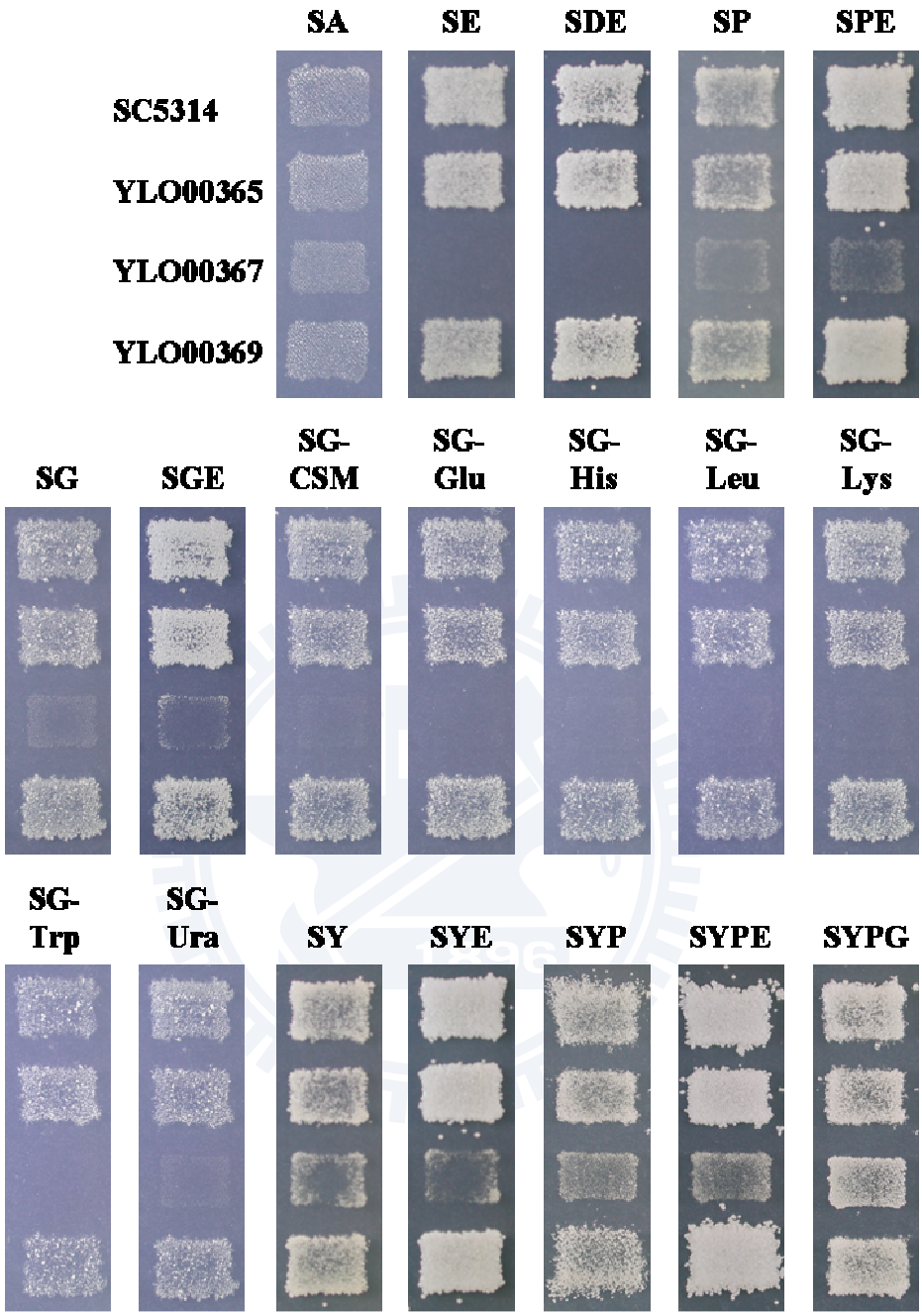


Fig. 11 Growth assay in yeast nitrogen base media of *Caenol* mutants. SA, Difco-yeast nitrogen base w/o amino acid + agar; SE, Difco-yeast nitrogen

base w/o amino acid + ethanol + agar; SDE, Difco-yeast nitrogen base w/o amino acid + dextrose + ethanol + agar; SP, Difco-yeast nitrogen base w/o amino acid + Bacto-peptone + agar; SPE, Difco-yeast nitrogen base w/o amino acid + Bacto-peptone + ethanol + agar; SG, Difco-yeast nitrogen base w/o amino acid + glycerol + agar; SGE, Difco-yeast nitrogen base w/o amino acid + glycerol + ethanol + agar; SG-CSM, Difco-yeast nitrogen base w/o amino acid + glycerol + CSM-HIS-LEU-LYS-TRP-URA + agar; SG-Glu, Difco-yeast nitrogen base w/o amino acid + glycerol + glutamic acid + agar; SG-His, Difco-yeast nitrogen base w/o amino acid + glycerol + histidine + agar; SG-Leu, Difco-yeast nitrogen base w/o amino acid + glycerol + leucine + agar; SG-Lys, Difco-yeast nitrogen base w/o amino acid + glycerol + lysine + agar; SG-Trp, Difco-yeast nitrogen base w/o amino acid + glycerol + tryptophan + agar; SG-Ura, Difco-yeast nitrogen base w/o amino acid + glycerol + uracil + agar; SY, Difco-yeast nitrogen base w/o amino acid + yeast extract + agar; SYE, Difco-yeast nitrogen base w/o amino acid + yeast extract + ethanol + agar; SYP, Difco-yeast nitrogen base w/o amino acid + yeast extract + Bacto-peptone + agar; SYPE, Difco-yeast nitrogen base w/o amino acid + yeast extract + Bacto-peptone + ethanol + agar; SYPG, Difco-yeast nitrogen base w/o amino acid + yeast extract + Bacto-peptone + glycerol + agar. The strains were YLO00365: *CaENO1*/*Caeno1*, YLO00367: *Caeno1*/*Caeno1*, YLO00369: *Caeno1*/*Caeno1*::*CaENO1* and parental strain SC5314.

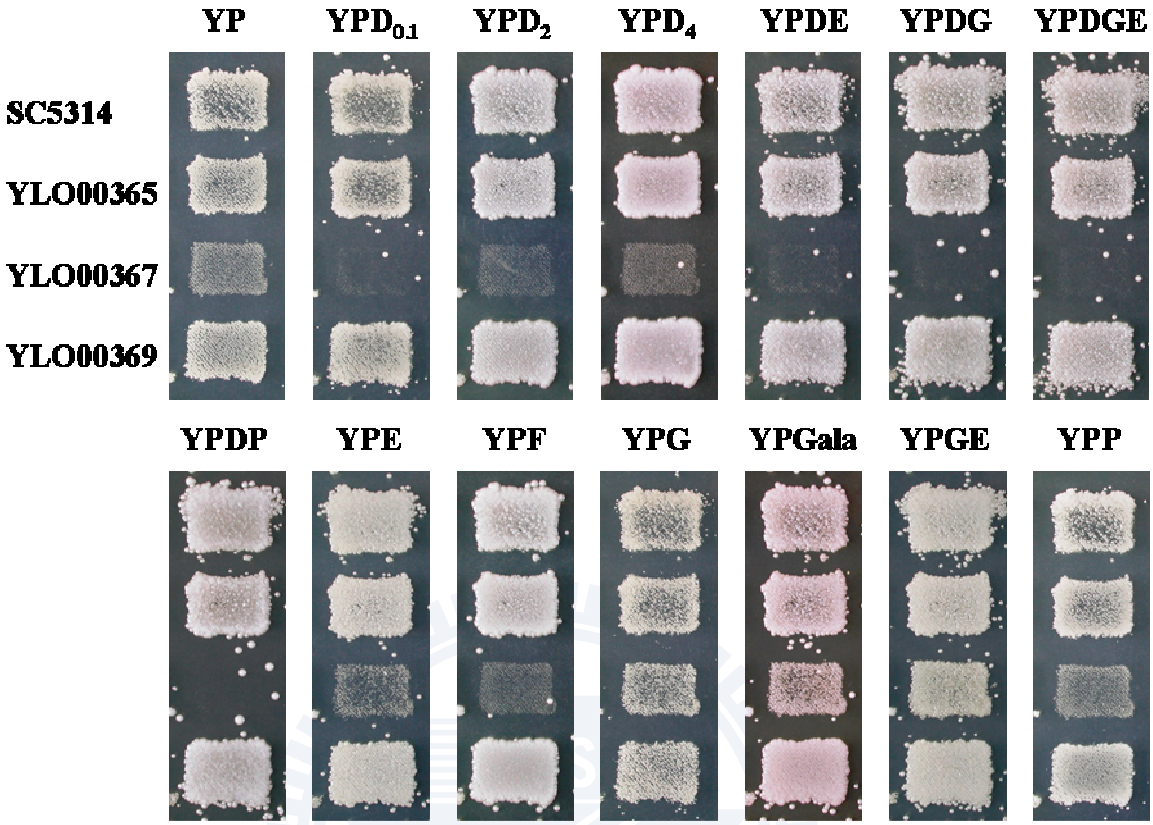
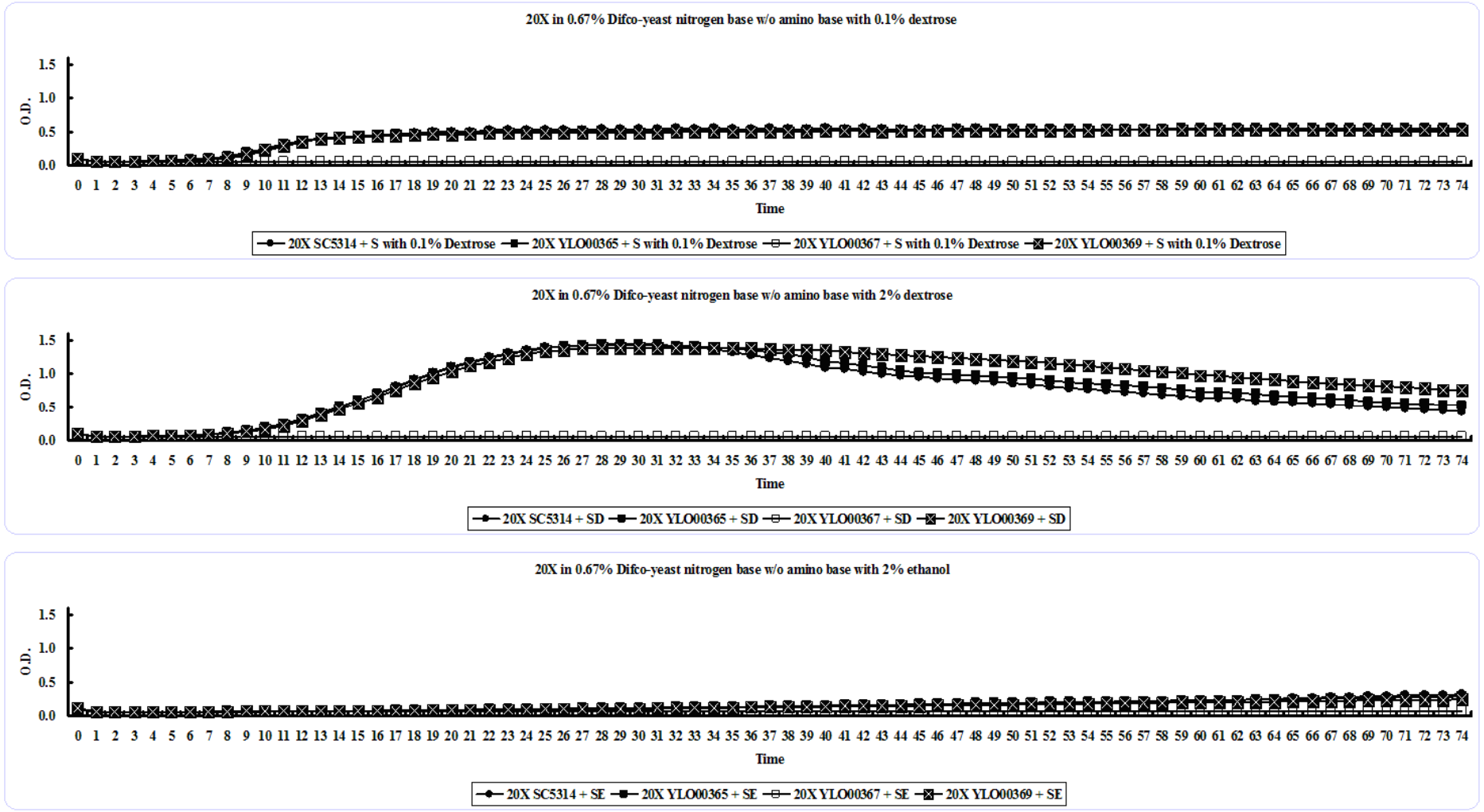


Fig. 12 Growth assay in yeast extract-peptone based media of *Caenol* mutants. YP, yeast extract and Bacto-peptone plus agar; YPD, Yeast extract + Bacto-peptone + dextrose (0.1% or 2% or 4%) + agar; YPDE, YPD + ethanol + agar; YPDG, YPD + glycerol + agar; YPDGE, YPD + glycerol + ethanol + agar; YPDP, YPD + pyruvate + agar; YPE, YP + ethanol + agar; YPF, YP + fructose + agar; YPG, YP + glycerol + agar; YPGala, YP + galactose + agar; YPGE, YP + glycerol + ethanol + agar; YPP, YP + pyruvate + agar. The strains were YLO00365: *CaENO1*/*Caeno1*, YLO00367: *Caeno1*/*Caeno1*, YLO00369: *Caeno1*/*Caeno1*::*CaENO1* and parental strain SC5314. The *CaENO1* null mutant inhibits growth to YP media in the presence of glucose or fructose but not other carbon sources in the assay.

Fig. 13



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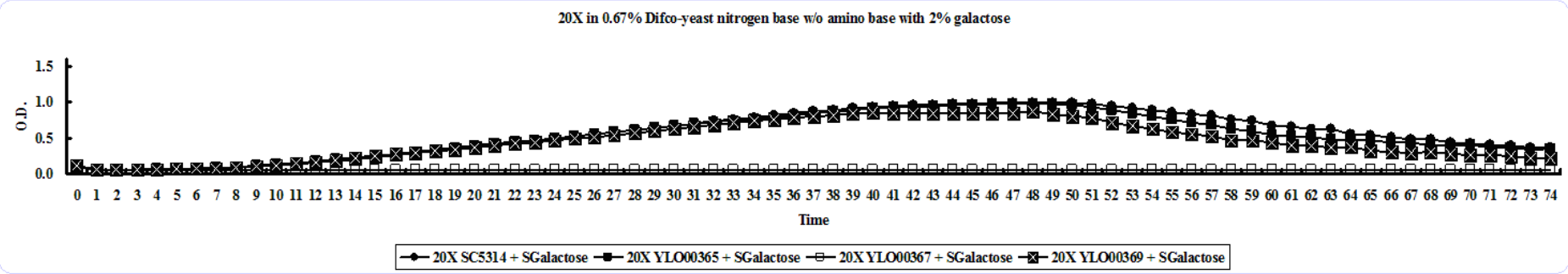
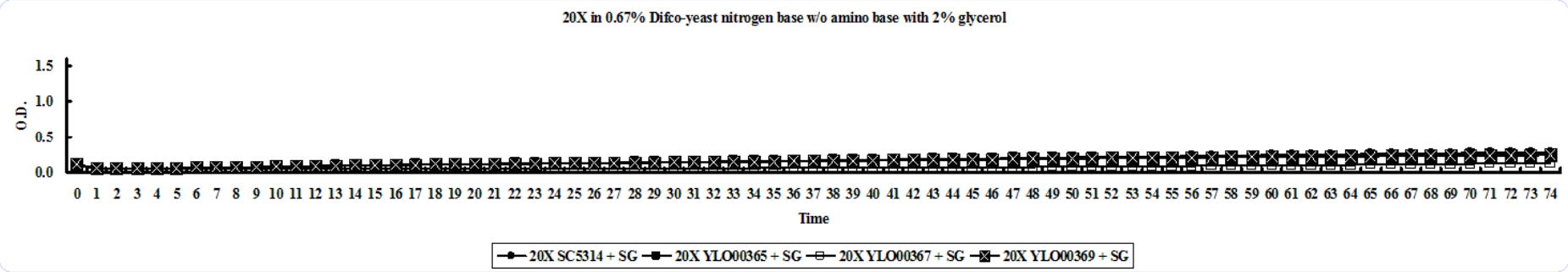
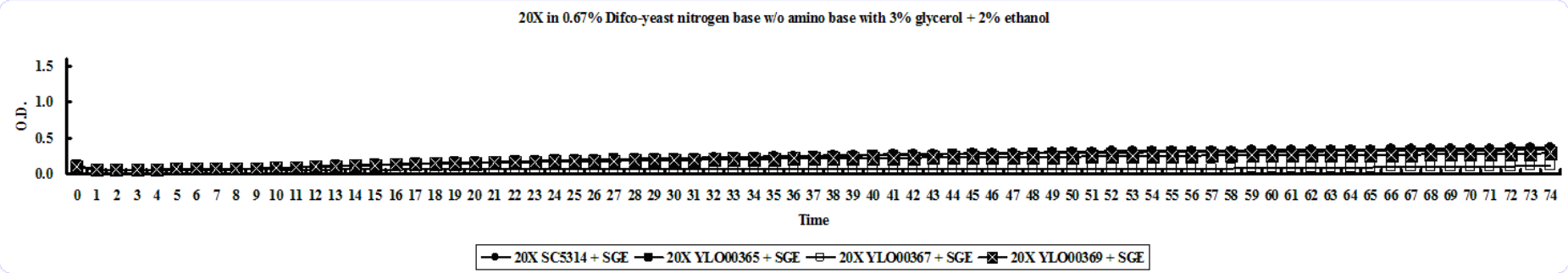
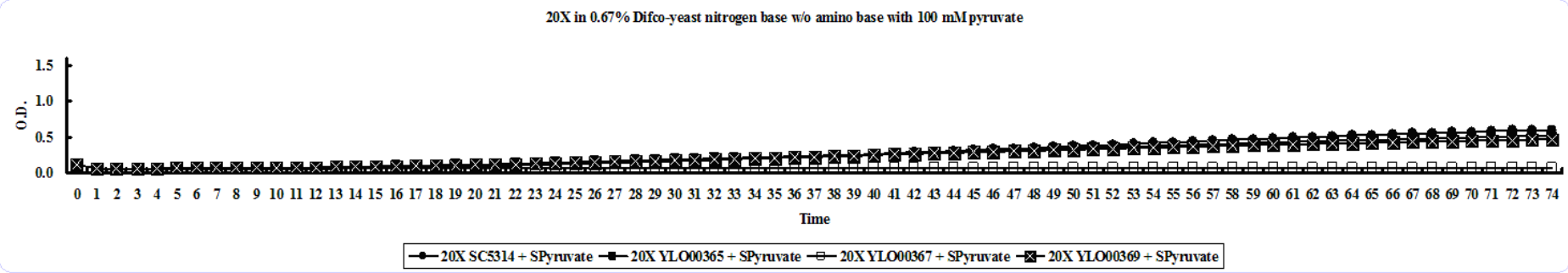
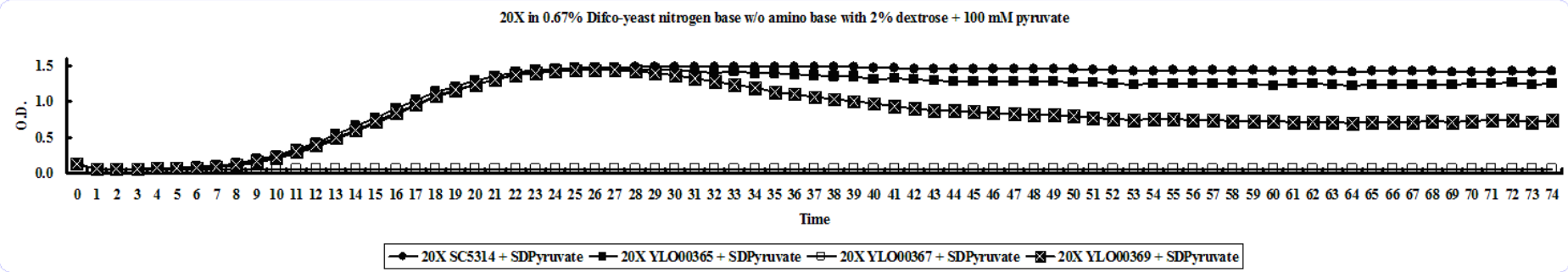

20X in 0.67% Difco-yeast nitrogen base w/o amino base with 2% galactose
O.D.
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74
Time
20X SC5314 + SGalactose
20X YLO00365 + SGalactose
20X YLO00367 + SGalactose
20X YLO00369 + SGalactose


20X in 0.67% Difco-yeast nitrogen base w/o amino base with 2% glycerol
O.D.
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74
Time
20X SC5314 + SG
20X YLO00365 + SG
20X YLO00367 + SG
20X YLO00369 + SG


20X in 0.67% Difco-yeast nitrogen base w/o amino base with 3% glycerol + 2% ethanol
O.D.
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74
Time
20X SC5314 + SGE
20X YLO00365 + SGE
20X YLO00367 + SGE
20X YLO00369 + SGE

（Continuous）


20X in 0.67% Difco-yeast nitrogen base w/o amino base with 100 mM pyruvate
O.D.
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74
Time
20X SC5314 + SPyruvate
20X YLO00365 + SPyruvate
20X YLO00367 + SPyruvate
20X YLO00369 + SPyruvate


20X in 0.67% Difco-yeast nitrogen base w/o amino base with 2% dextrose + 100 mM pyruvate
O.D.
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74
Time
20X SC5314 + SDPyruvate
20X YLO00365 + SDPyruvate
20X YLO00367 + SDPyruvate
20X YLO00369 + SDPyruvate

（Continuous）

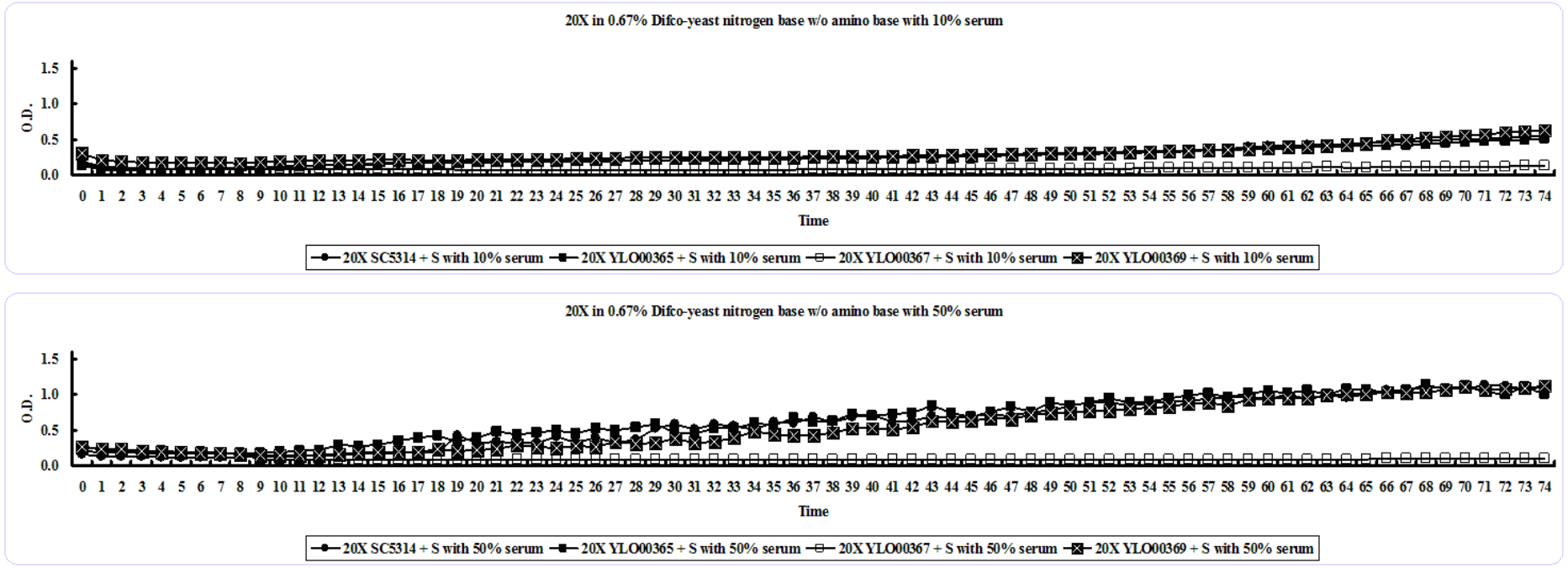
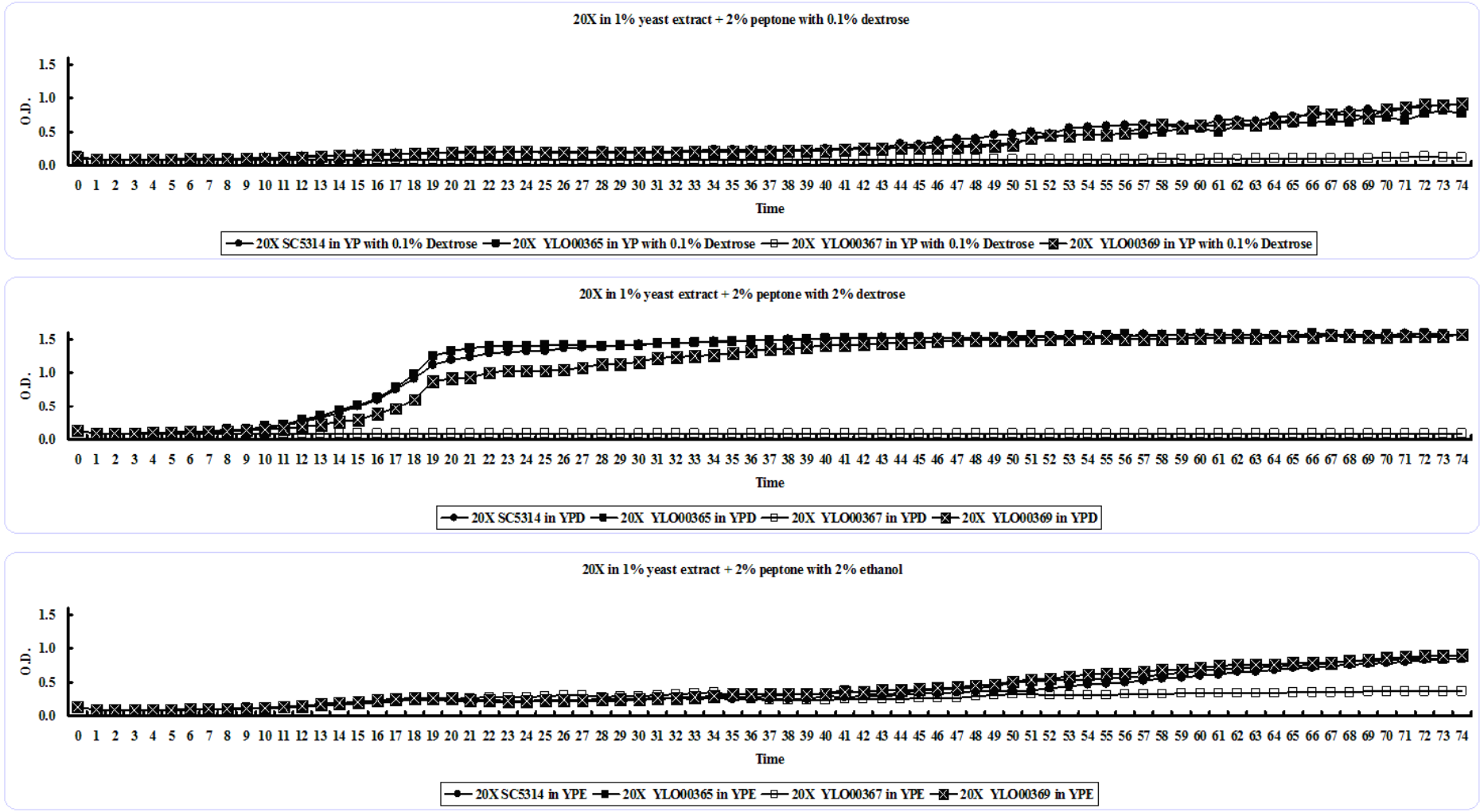
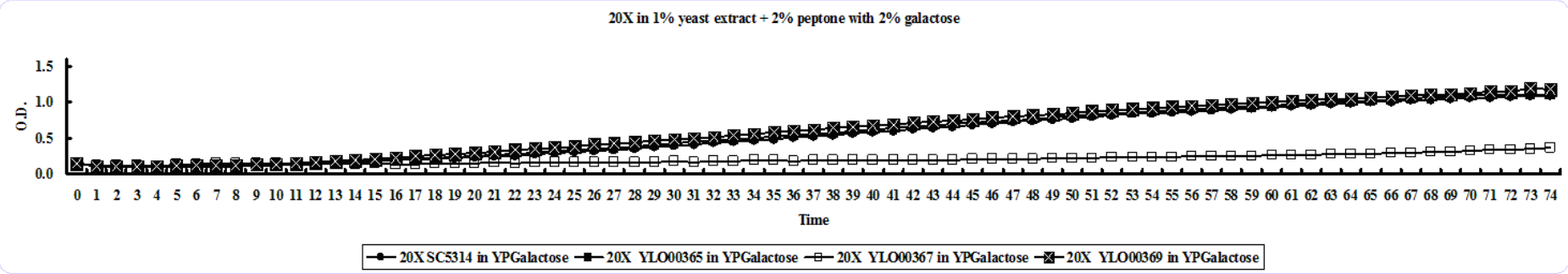


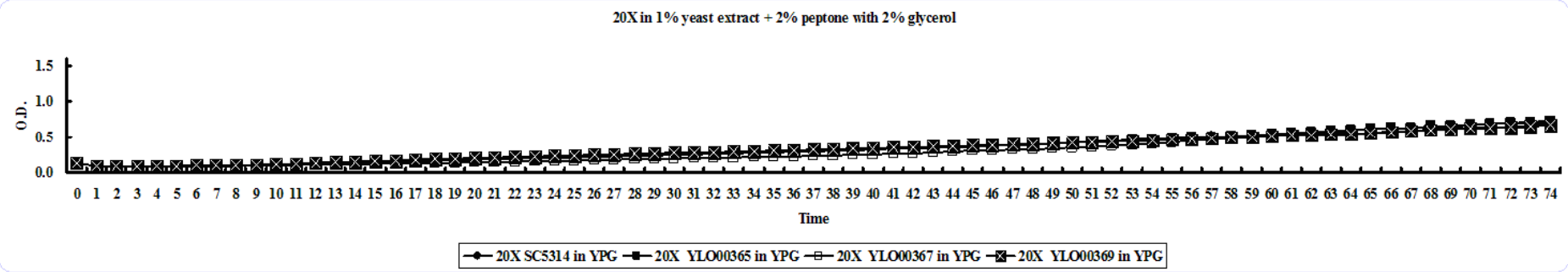
Fig. 13 Growth curve analyses of *Caenol* mutants in yeast nitrogen based liquid media. The strains were YLO00365: *CaENO1*/*Caeno1*, YLO00367: *Caeno1*/*Caeno1*, YLO00369: *Caeno1*/*Caeno1*::*CaENO1* and parental strain SC5314.

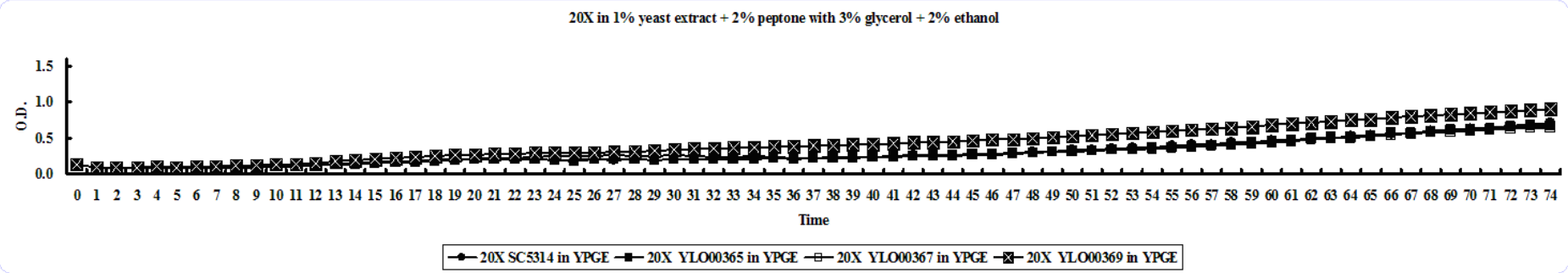
Fig. 14



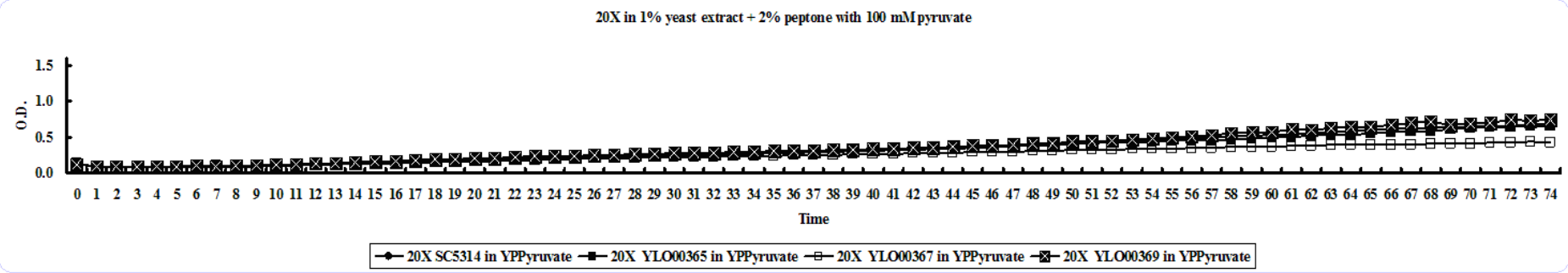
（Continuous）

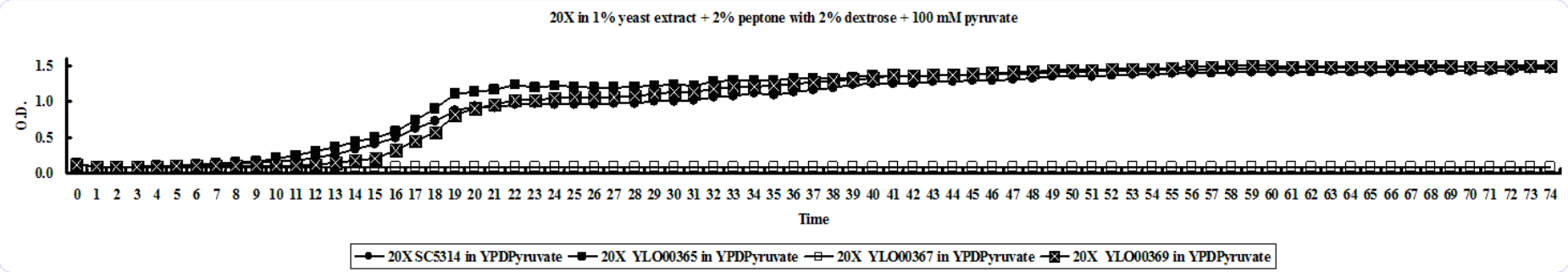

20X in 1% yeast extract + 2% peptone with 2% galactose
O.D.
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74
Time
20X SC5314 in YPGalactose
20X YLO00365 in YPGalactose
20X YLO00367 in YPGalactose
20X YLO00369 in YPGalactose


20X in 1% yeast extract + 2% peptone with 2% glycerol
O.D.
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74
Time
20X SC5314 in YPG
20X YLO00365 in YPG
20X YLO00367 in YPG
20X YLO00369 in YPG


20X in 1% yeast extract + 2% peptone with 3% glycerol + 2% ethanol
O.D.
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74
Time
20X SC5314 in YPGE
20X YLO00365 in YPGE
20X YLO00367 in YPGE
20X YLO00369 in YPGE

（Continuous）


20X in 1% yeast extract + 2% peptone with 100 mM pyruvate
O.D.
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74
Time
20X SC5314 in YPPyruvate
20X YLO00365 in YPPyruvate
20X YLO00367 in YPPyruvate
20X YLO00369 in YPPyruvate


20X in 1% yeast extract + 2% peptone with 2% dextrose + 100 mM pyruvate
O.D.
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74
Time
20X SC5314 in YPDPyruvate
20X YLO00365 in YPDPyruvate
20X YLO00367 in YPDPyruvate
20X YLO00369 in YPDPyruvate

（Continuous）

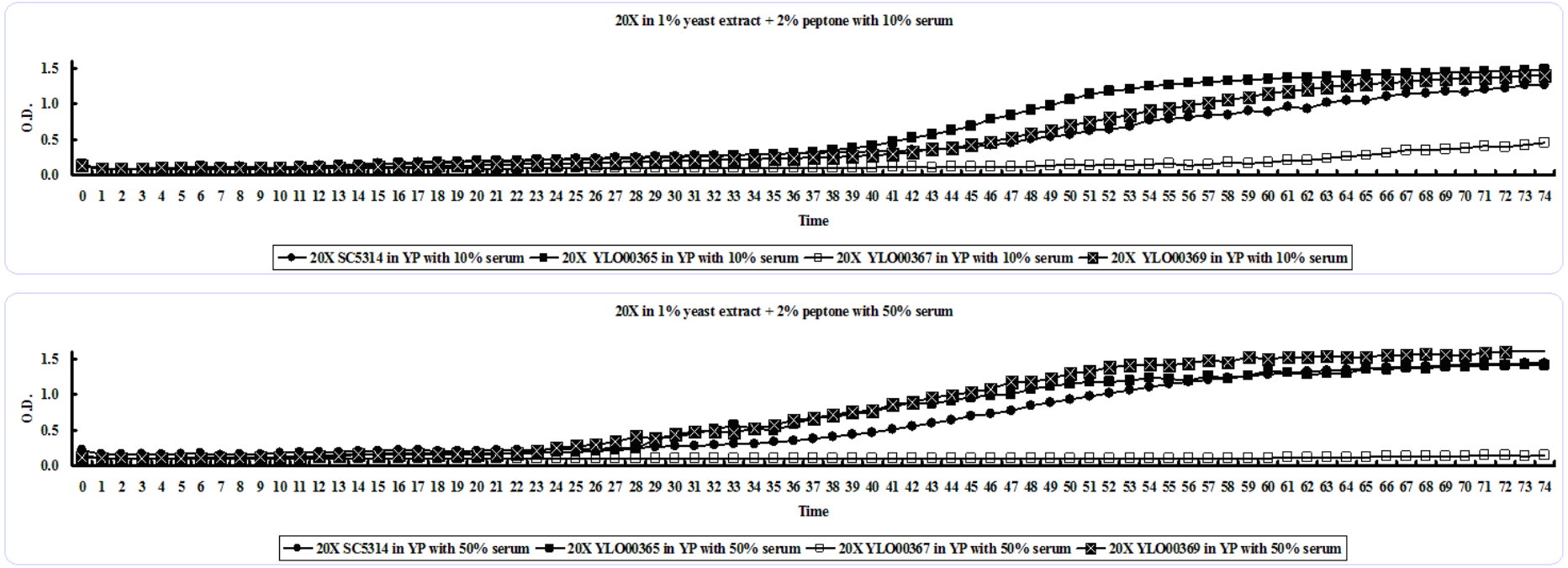


Fig. 14 Growth curve analyses of *Caenol* mutants in yeast extract-peptone based liquid media. The strains were YLO00365: *CaENO1*/*Caeno1*, YLO00367: *Caeno1*/*Caeno1*, YLO00369: *Caeno1*/*Caeno1*::*CaENO1* and parental strain SC5314.

Fig. 15

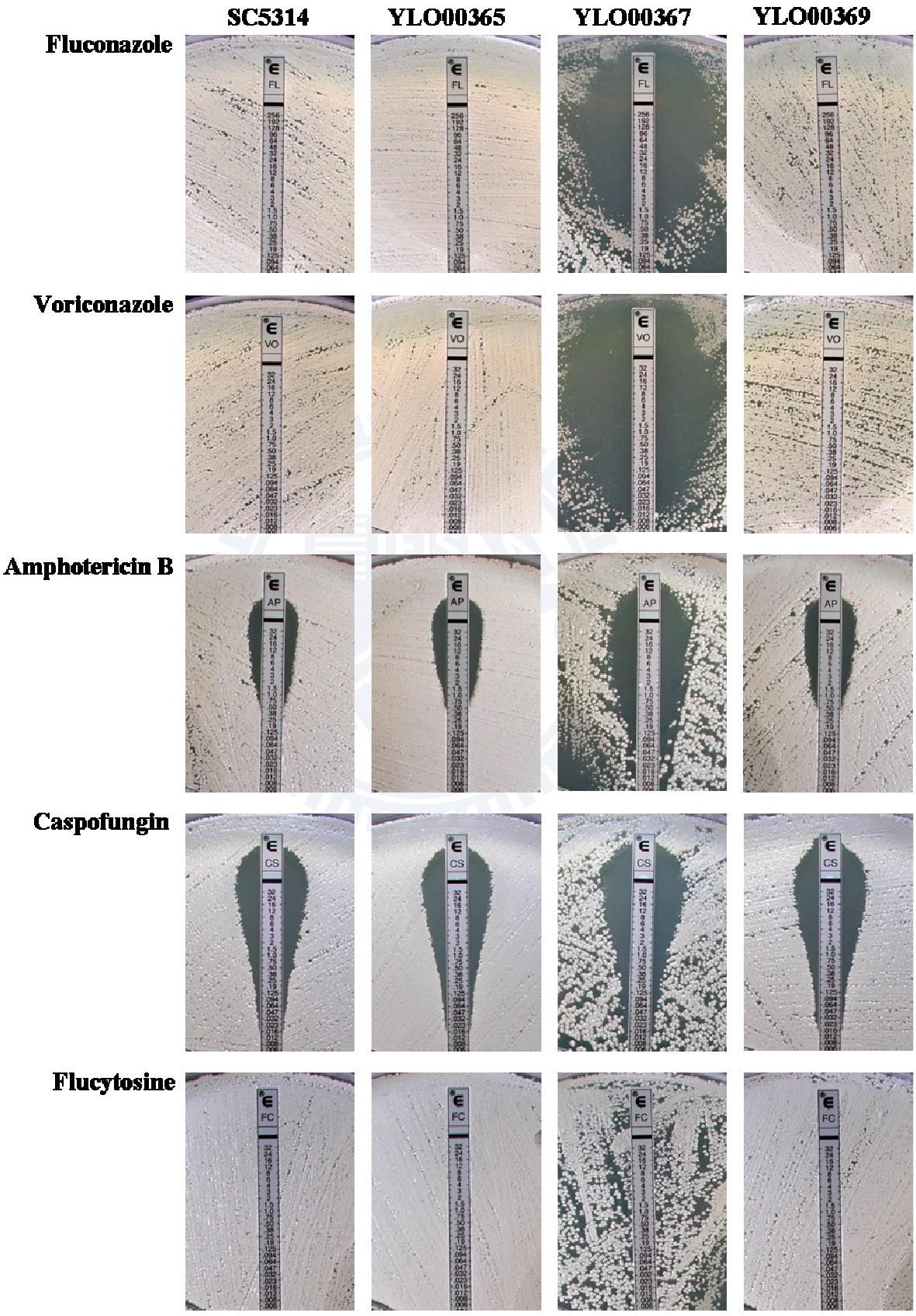

SC5314
YLO00365
YLO00367
YLO00369
Fluconazole
Voriconazole
Amphotericin B
Caspofungin
Flucytosine
FL
VO
AP
CS
FC

Fig. 15 Etest for *CaENO1* mutants on drug susceptibility. Antifungal susceptibility test was performed inYPGE agar plates (1% yeast extract, 2% Bacto-peptone, 3% glycerol and 2% ethanol) with Etest strips (AB BIODISK, Solna, Sweden). The strains were YLO00365: *CaENO1*/*Caeno1*, YLO00367: *Caeno1*/*Caeno1*, YLO00369: *Caeno1*/*Caeno1*::*CaENO1* and parental strain SC5314. The plates were incubated at 35°C for 48 hours.

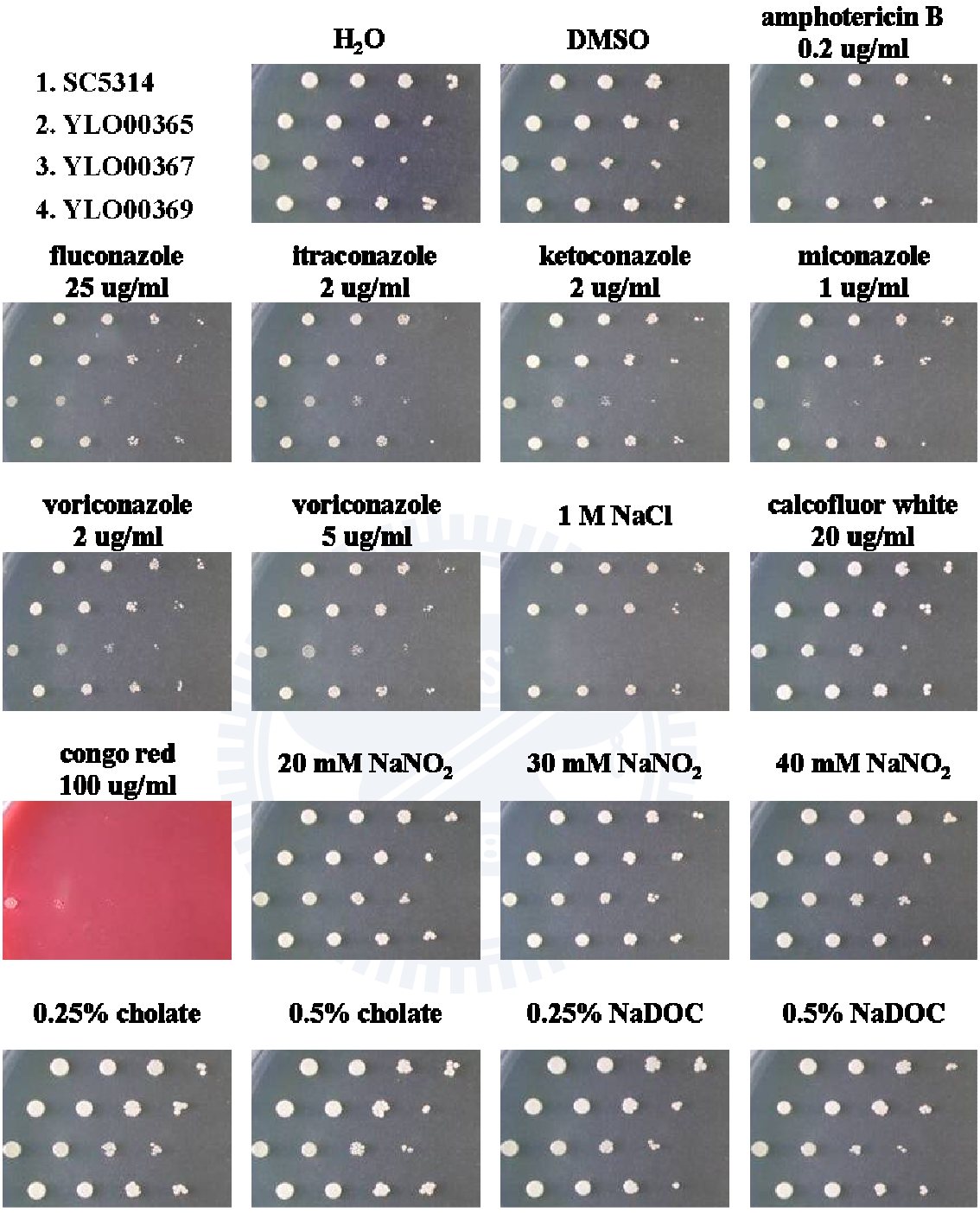
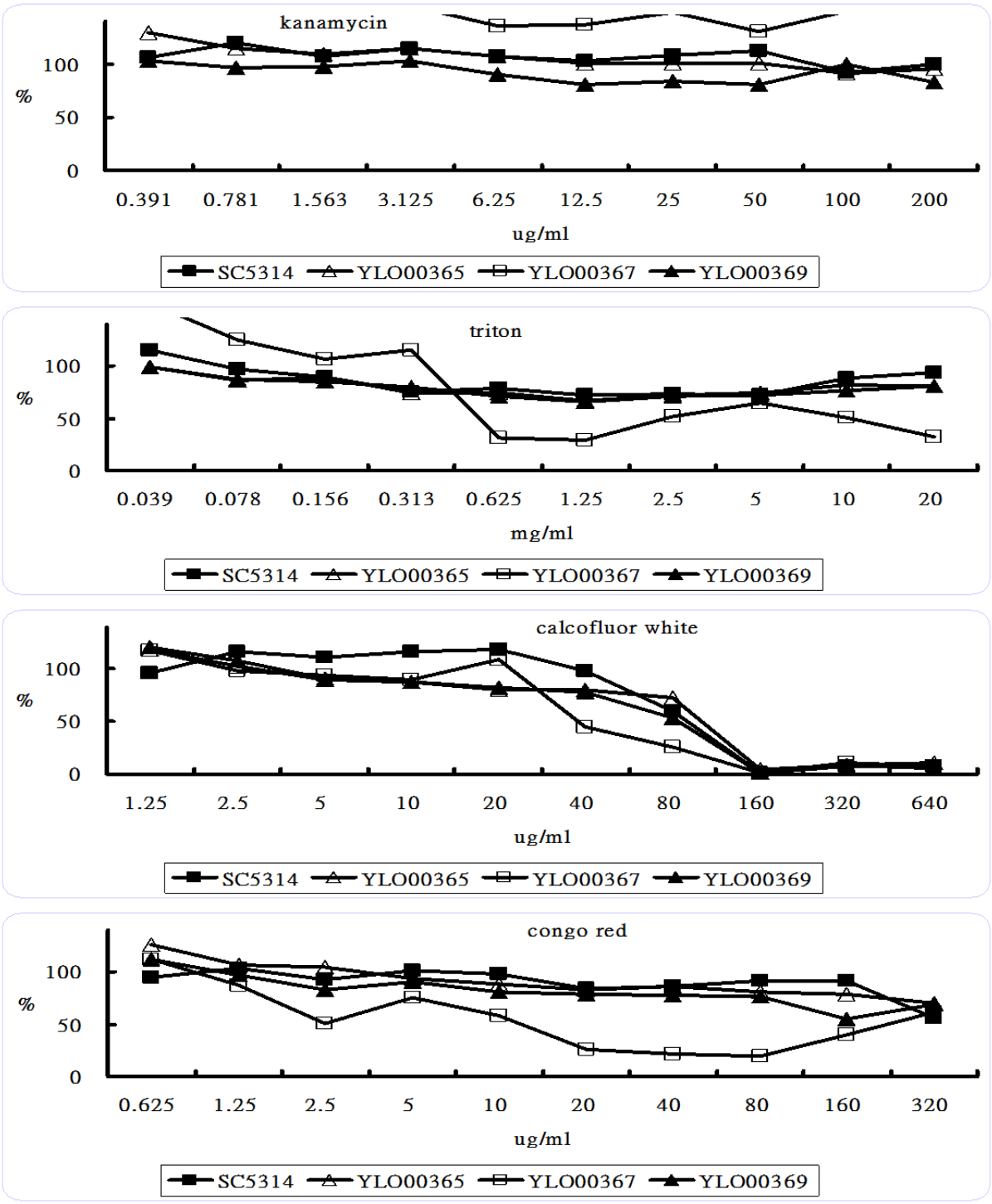
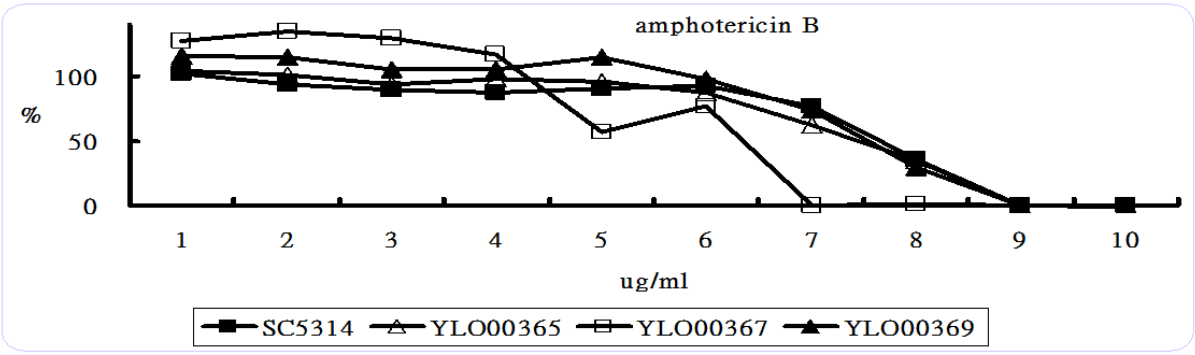


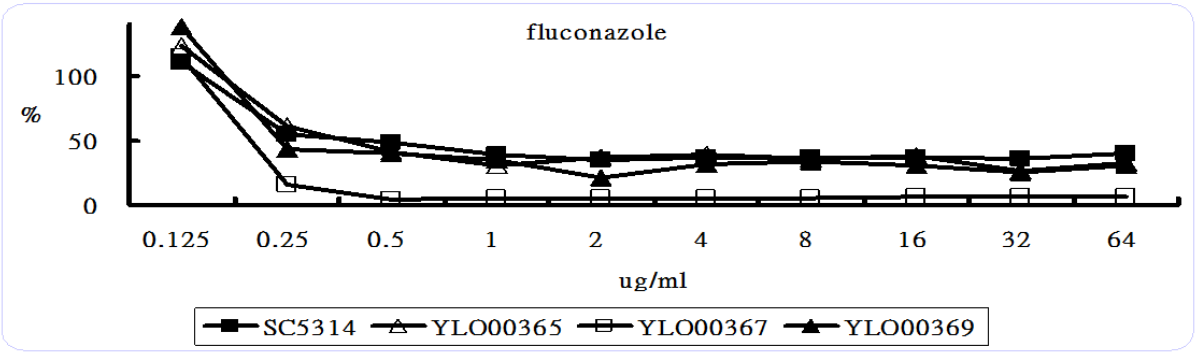
Fig. 16 Agar dilution assay for *CaENO1* mutants on drug susceptibility. The strains were YLO00365: *CaENO1*/*Caeno1*, YLO00367: *Caeno1*/*Caeno1*, YLO00369: *Caeno1*/*Caeno1*::*CaENO1* and parental strain SC5314.

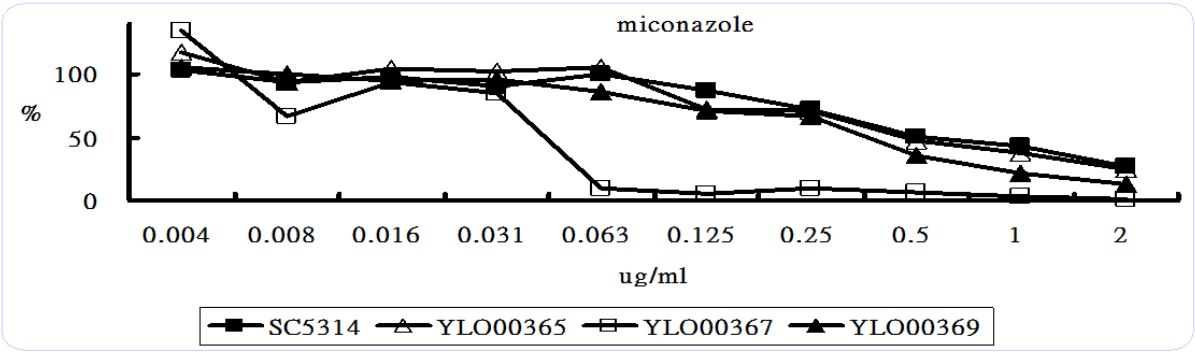
Fig. 17

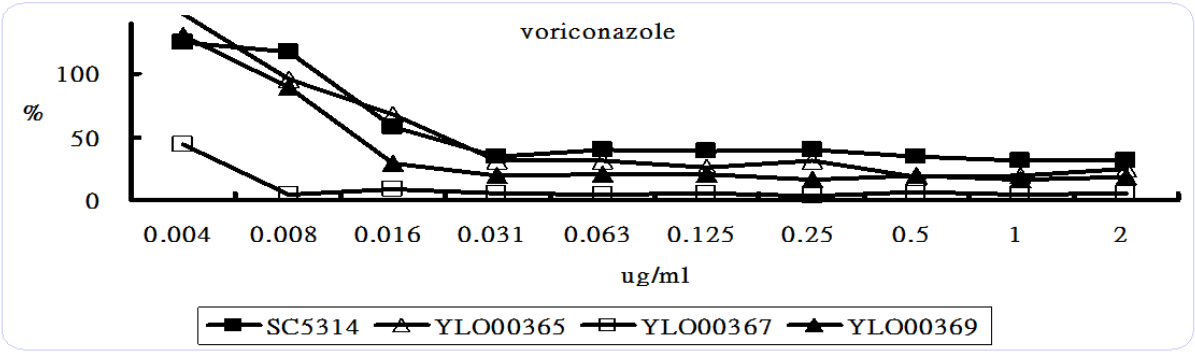


（Continuous）

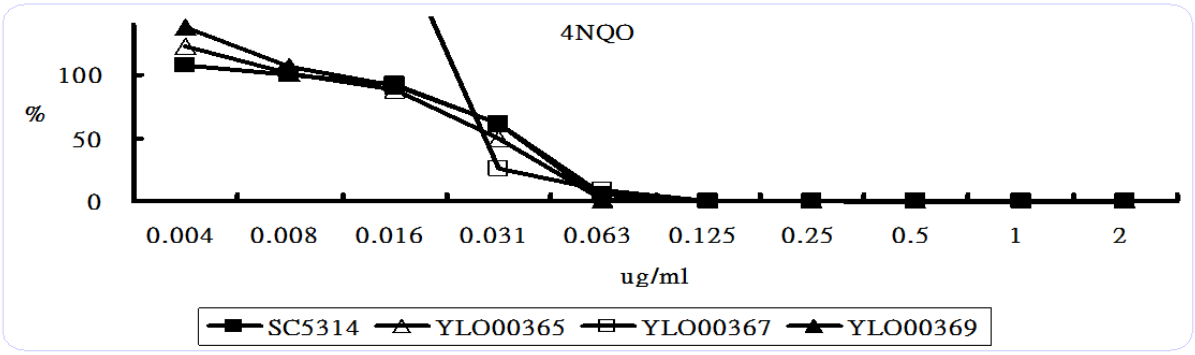

amphotericin B
100
%
50
0
1
2
3
4
5
6
7
8
9
10
ug/ml
SC5314
YLO00365
YLO00367
YLO00369

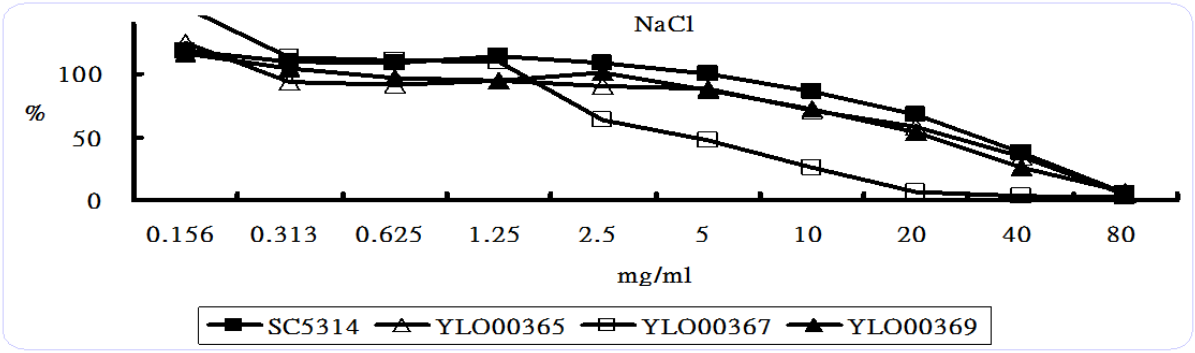

fluconazole
100
%
50
0
0.125
0.25
0.5
1
2
4
8
16
32
64
ug/ml
SC5314
YLO00365
YLO00367
YLO00369

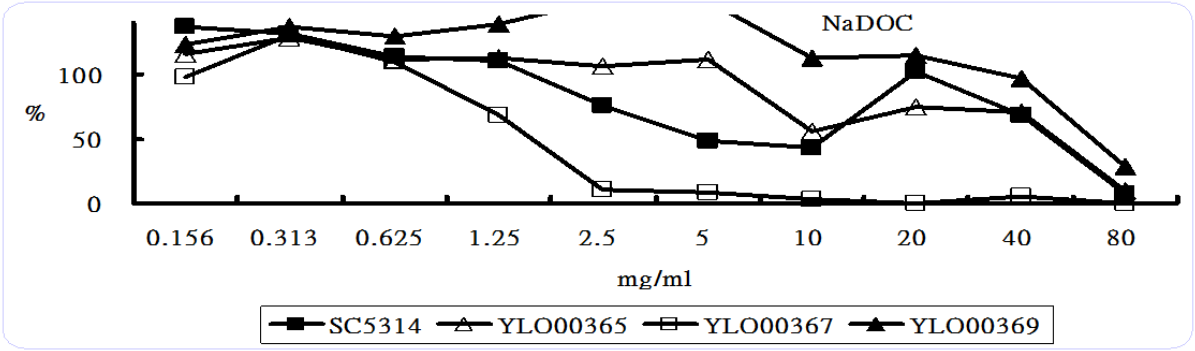

miconazole
100
%
50
0
0.004
0.008
0.016
0.031
0.063
0.125
0.25
0.5
1
2
ug/ml
SC5314
YLO00365
YLO00367
YLO00369


voriconazole
100
%
50
0
0.004
0.008
0.016
0.031
0.063
0.125
0.25
0.5
1
2
ug/ml
SC5314
YLO00365
YLO00367
YLO00369

（Continuous）


4NQO
100
%
50
0
0.004
0.008
0.016
0.031
0.063
0.125
0.25
0.5
1
2
ug/ml
SC5314
YLO00365
YLO00367
YLO00369


NaCl
100
%
50
0
0.156
0.313
0.625
1.25
2.5
5
10
20
40
80
mg/ml
SC5314
YLO00365
YLO00367
YLO00369


NaDOC
100
%
50
0
0.156
0.313
0.625
1.25
2.5
5
10
20
40
80
mg/ml
SC5314
YLO00365
YLO00367
YLO00369

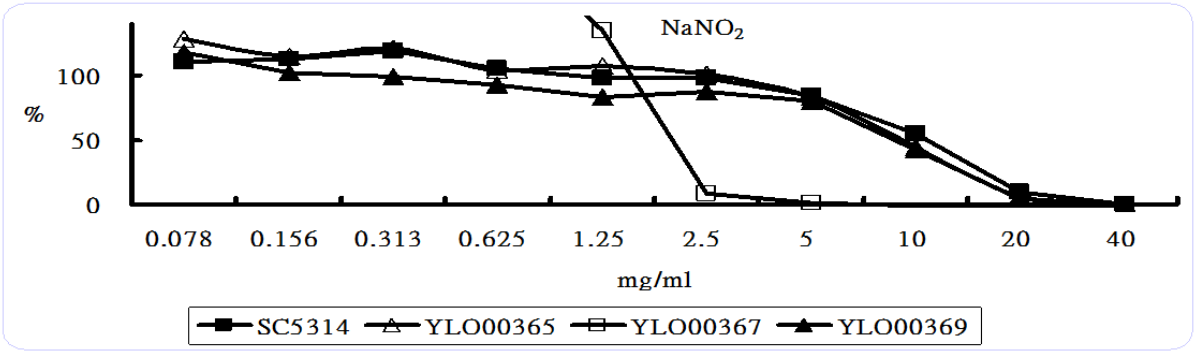

NaNO2
100
%
50
0
0.078
0.156
0.313
0.625
1.25
2.5
5
10
20
40
mg/ml
SC5314
YLO00365
YLO00367
YLO00369

Fig. 17 Broth microdilution assay for *CaENO1* mutants on compound susceptibility. The compounds tested are kanamycin, triton-X-100, calcofluor white, congo red, amphotericine B, fluconazole, miconazole, voriconazole, 4NQO, NaCl, NaDOC and $NaNO_2$. The strains were YLO00365: *CaENO1*/*Caeno1*, YLO00367: *Caeno1*/*Caeno1*, YLO00369: *Caeno1*/*Caeno1*::*CaENO1* and parental strain SC5314.

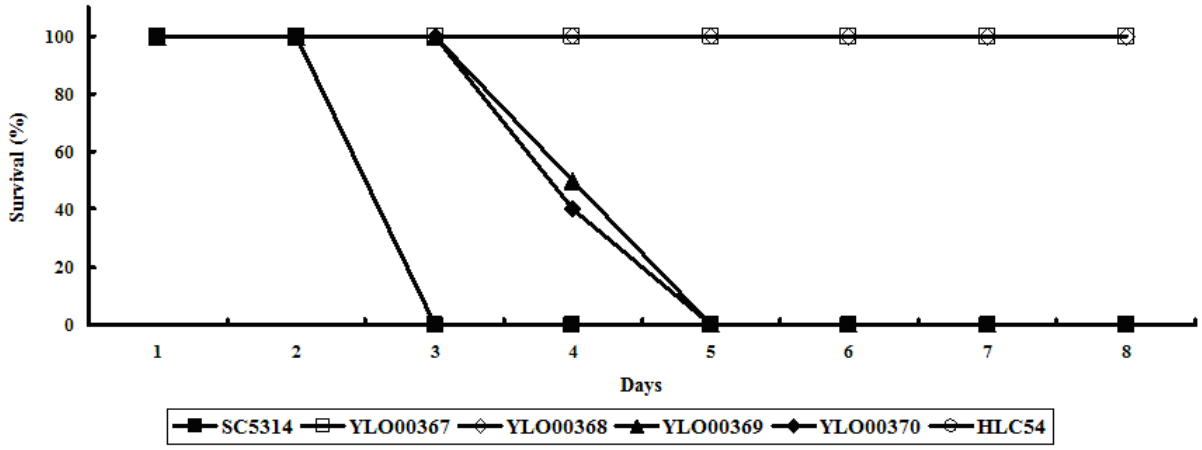


Fig. 18 *Caeno1* mutants of *Candida albicans* are avirulent. The BALB/c mice were injected with the 2×10^6 cells of SC5314 (■), YLO00367 (□; *Caeno1*/*Caeno1*-1), YLO00368(◇ ; *Caeno1*/*Caeno1*-2), YLO00369 (▲; *Caeno1*/*Caeno1*::*CaENO1*-1), YLO00370 (◆; *Caeno1*/*Caeno1*::*CaENO1*-2) and HLC54 (○; *cph1*/*cph1 efg1*/*efg1*).

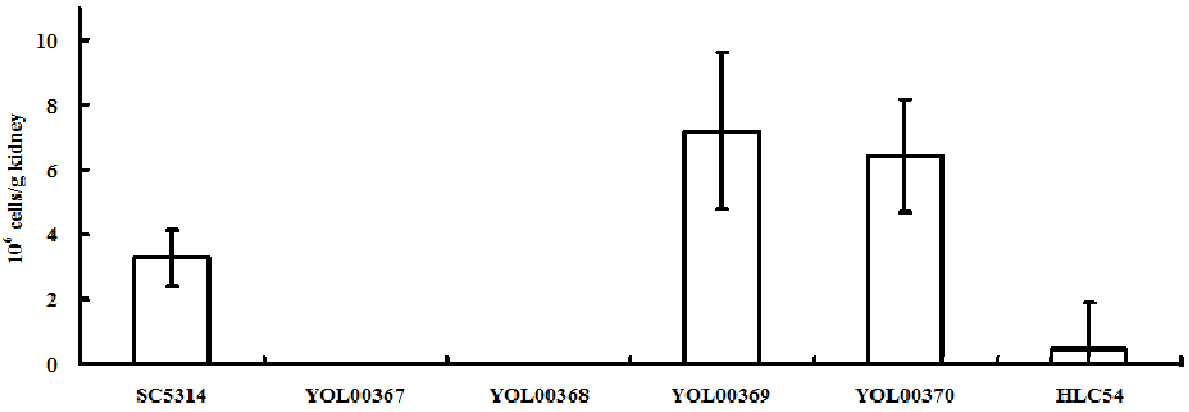


Fig. 19 Fungal loads of the infected kidneys. *C. albicans* cells in the left-side kidneys from the infected mice were scored. The strains were parental strain SC5314, YLO00367: *Caeno1*/*Caeno1*-1, YLO00368: *Caeno1*/*Caeno1*-2, YLO00369: *Caeno1*/*Caeno1*::*CaENO1*-1, YLO00370: *Caeno1*/*Caeno1*::*CaENO1*-2 and HLC54: *cph1*/*cph1 efg1*/*efg1*. The *CaENO1* null mutants were avirulent when tested in a mouse model of systemic infection.

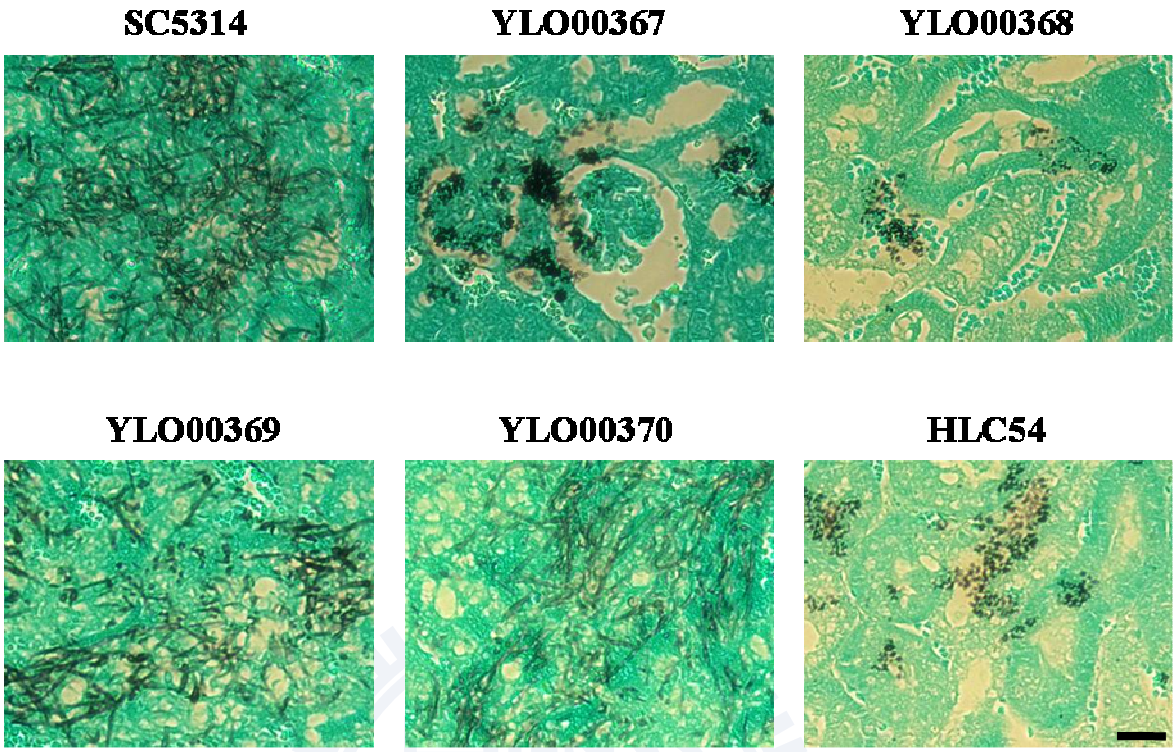


Fig. 20 The histopathology of mouse kidney after *Candida albicans* infections by Gomori Methenamine Silver (GMS) stain. The strains were wild-type parental strain SC5314, YLO00367: *Caeno1*/*Caeno1*-1, YLO00368: *Caeno1*/*Caeno1*-2, YLO00369: *Caeno1*/*Caeno1*::*CaENO1*-1, YLO00370: *Caeno1*/*Caeno1*::*CaENO1*-2 and HLC54: *cph1*/*cph1 efg1*/*efg1*. The histopathology of mouse kidney after *C. albicans* infections were displayed filamentous growth with the wild-type SC5314 strain and *Caeno1* rescued strain (YLO00369 and YLO00370), but fail to form filaments with *Caeno1* null mutants and *cph1*/*cph1 efg1*/*efg1* mutant strains. Scale bar, 20 μm.

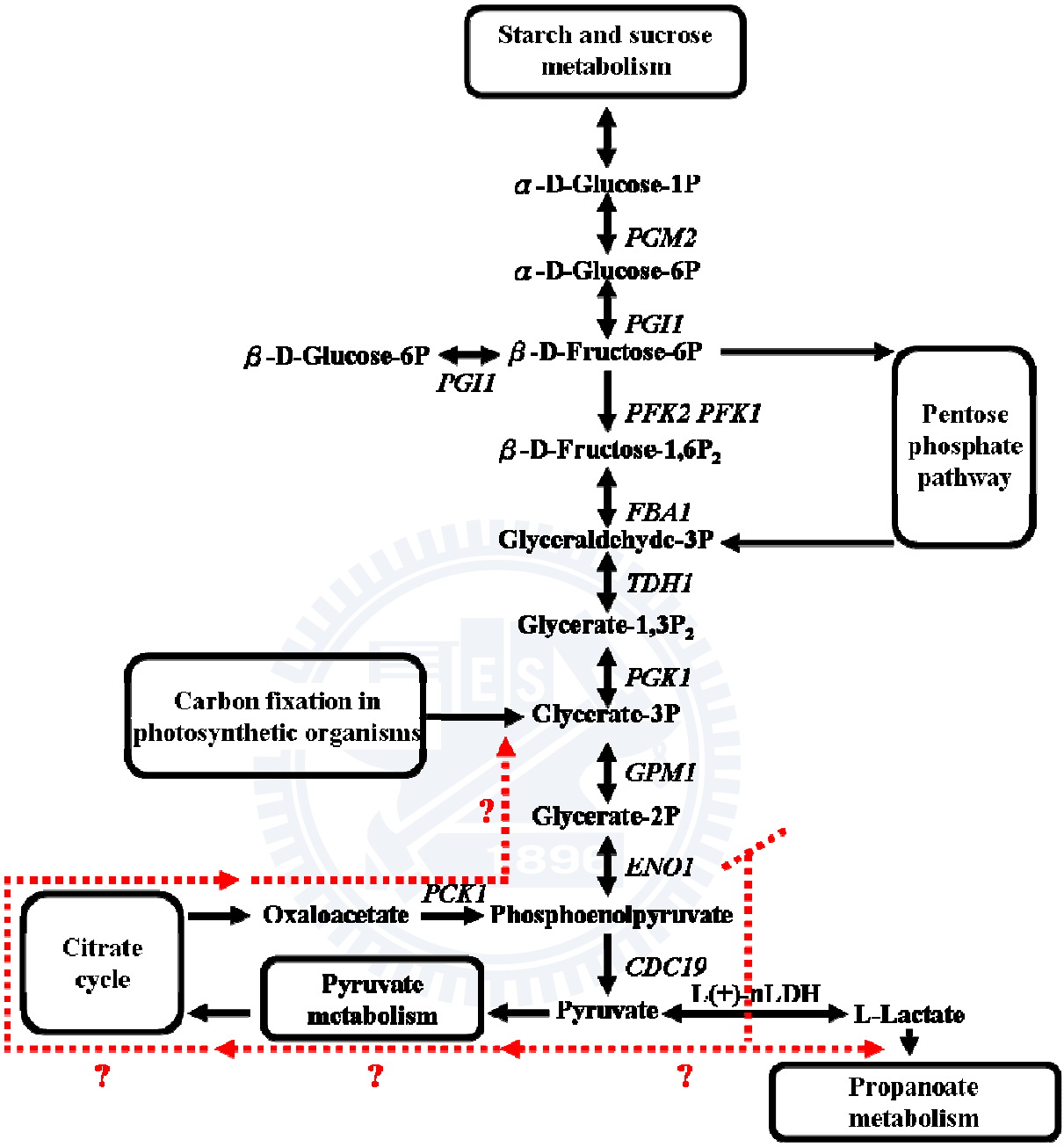


Fig. 21 Schematic diagram of the carbohydrate metabolism in *Candida albicans*. The glycolysis and gluconeogenesis pathway in *C. albicans* is modified from KEGG cal00010 (http://www.genome.jp/kegg-bin/show_pathway?cal00010).

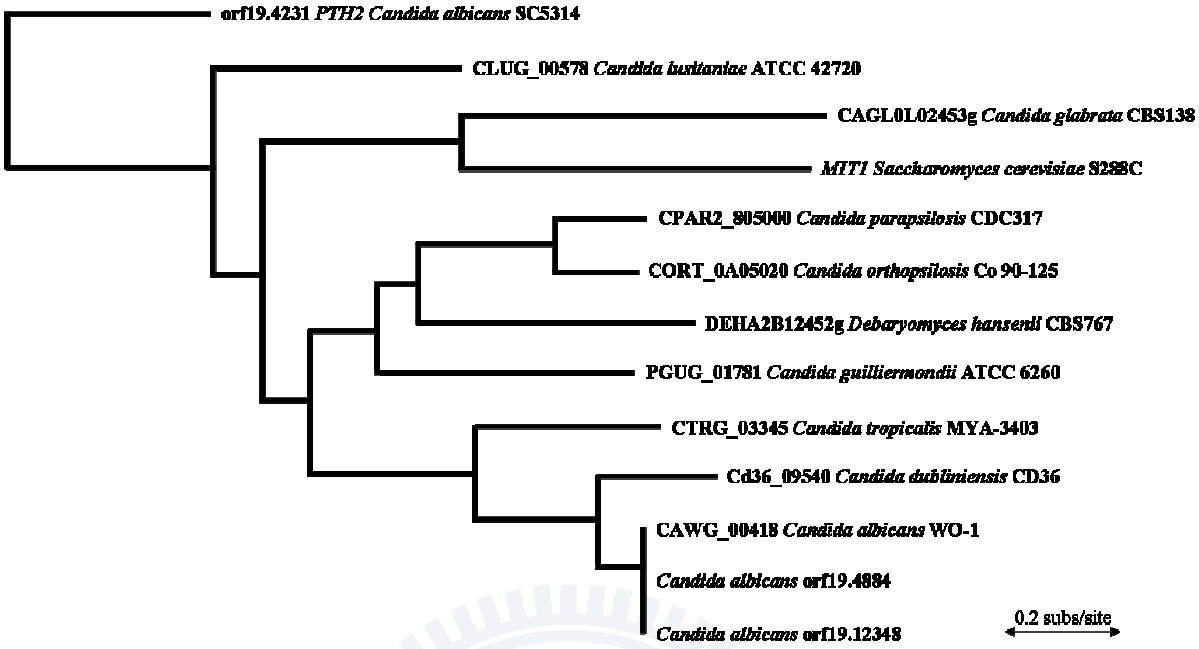
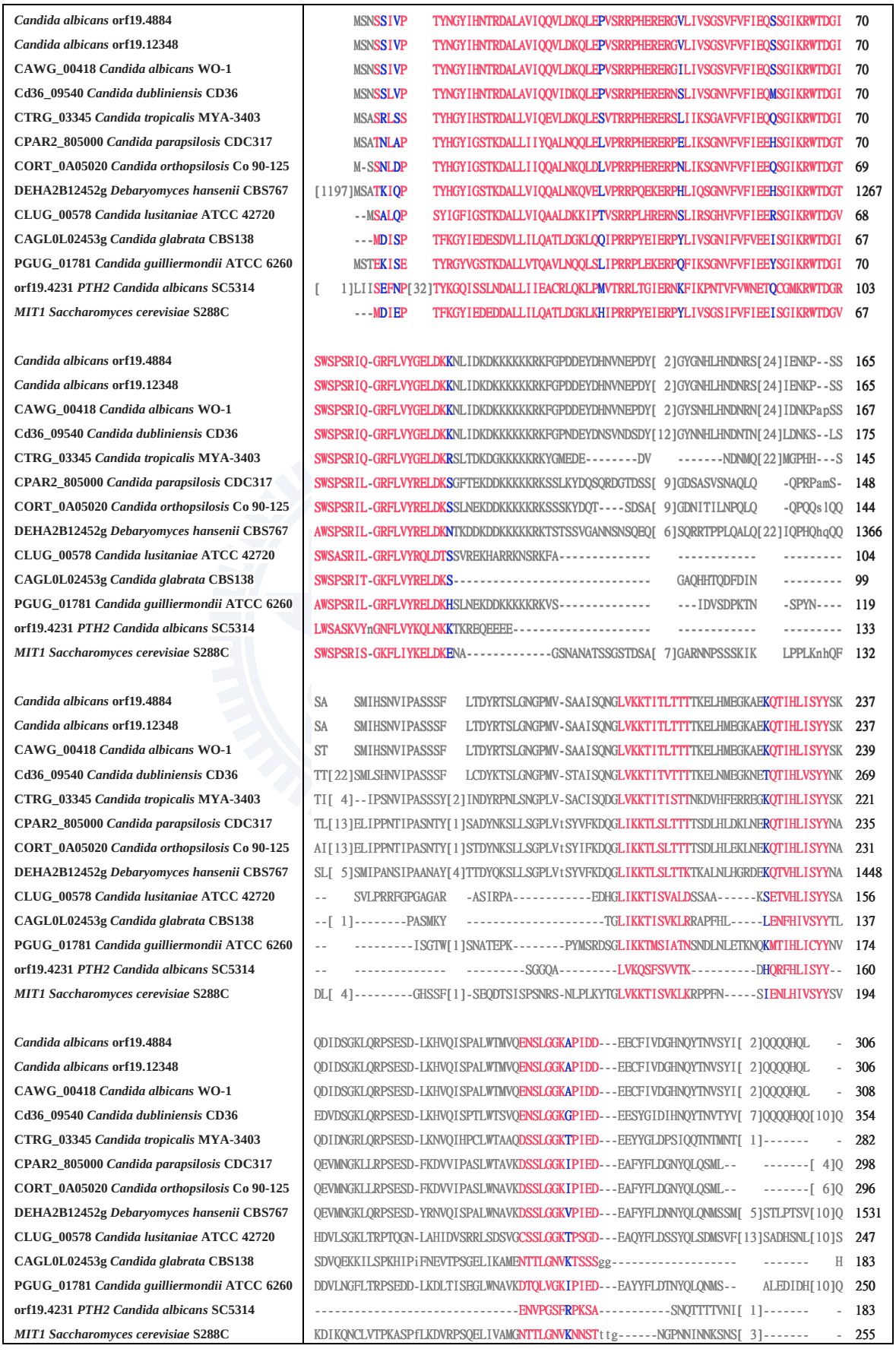
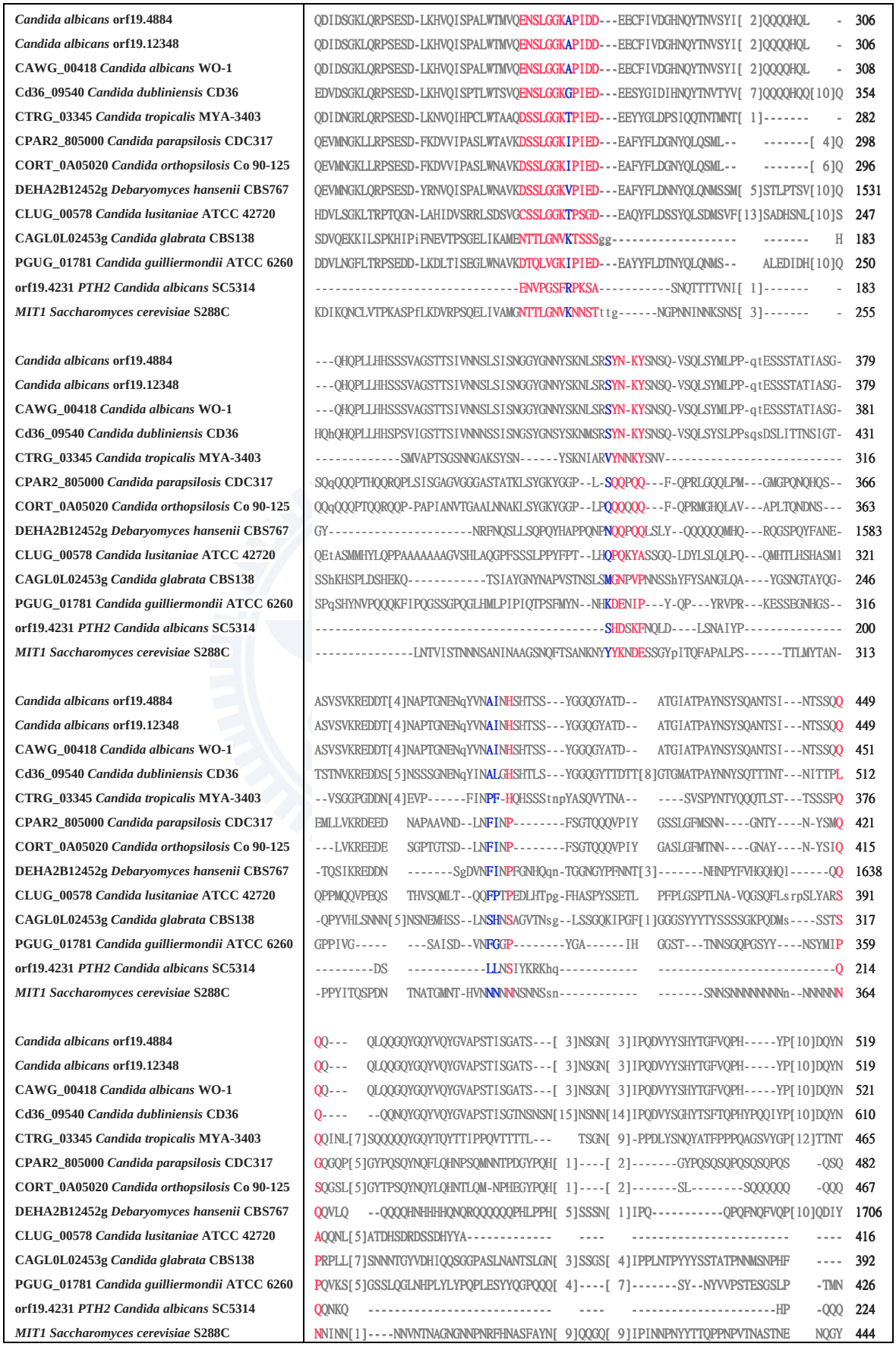


Fig. 22 The phylogenetic tree view approach for Saccharomycetales species of which the genome alike to *CaREP5* (orf19.4884/orf19.12348). The phylogenetic tree view align protein sequences by fast minimum evolution of COBALT multiple aligment.

Fig. 23


Candida albicans orf19.4884
Candida albicans orf19.12348
CAWG_00418 Candida albicans WO-1
Cd36_09540 Candida dubliniensis CD36
CTRG_03345 Candida tropicalis MYA-3403
CPAR2_805000 Candida parapsilosis CDC317
CORT_0A05020 Candida orthopsilosis Co 90-125
DEHA2B12452g Debaryomyces hansenii CBS767
CLUG_00578 Candida lusitaniae ATCC 42720
CAGL0L02453g Candida glabrata CBS138
PGUG_01781 Candida guilliermondii ATCC 6260
orf19.4231 PTH2 Candida albicans SC5314
MIT1 Saccharomyces cerevisiae S288C


Candida albicans orf19.4884 QDIDSGKLQRPSESD-LKHVQISPALWTMVQENSLGGKAPIDD---EECFIVDGHNQYTNVSYI[2]QQQQHQL - 306
Candida albicans orf19.12348 QDIDSGKLQRPSESD-LKHVQISPALWTMVQENSLGGKAPIDD---EECFIVDGHNQYTNVSYI[2]QQQQHQL - 306
CAWG_00418 Candida albicans WO-1 QDIDSGKLQRPSESD-LKHVQISPALWTMVQENSLGGKAPIDD---EECFIVDGHNQYTNVSYI[2]QQQQHQL - 308
Cd36_09540 Candida dubliniensis CD36 EDVDSGKLQRPSESD-LKHVQISPTLWTSVQENSLGGKGPIED---EESYGIDIHNQYTNVTYV[7]QQQQHQQ[10]Q 354
CTRG_03345 Candida tropicalis MYA-3403 QDIDNGRLQRPSESD-LKNVQIHPCLWTAAQDSSLGGKTPIED---EEYYGLDPSIQQTNTMNT[1]------- - 282
CPAR2_805000 Candida parapsilosis CDC317 QEVMNGKLLRPSESD-FKDVVIPASLWTAVKDSSLGGKIPIED---EAFYFLDGNYQLQSML-- -------[4]Q 298
CORT_0A05020 Candida orthopsilosis Co 90-125 QEVMNGKLLRPSESD-FKDVVIPASLWNAVKDSSLGGKIPIED---EAFYFLDGNYQLQSML-- -------[6]Q 296
DEHA2B12452g Debaryomyces hansenii CBS767 QEVMNGKLQRPSESD-YRNVQISPALWNAVKDSSLGGKVPIED---EAFYFLDNNYQLQNMSSM[5]STLPTSV[10]Q 1531
CLUG_00578 Candida lusitaniae ATCC 42720 HDVLSGKLTRPTQGN-LAHIDVSRRLSDSVGCSSLGGKTPSGD---EAQYFLDSSYQLSDMSVF[13]SADHSNL[10]S 247
CAGL0L02453g Candida glabrata CBS138 SDVQEKKILSPKHIPiFNEVTPSGELIKAMENTTLGNVKTSSSgg------------------ ------- H 183
PGUG_01781 Candida guilliermondii ATCC 6260 DDVLNGFLTRPSEDD-LKDLTISEGLWNAVKDTQLVGKIPIED---EAYYFLDTNYQLQNMS-- ALEDIDH[10]Q 250
orf19.4231 PTH2 Candida albicans SC5314 ----------ENVPGSFRPKSA----------SNQTTTTVNI[1]------- - 183
MIT1 Saccharomyces cerevisiae S288C KDIKQNCLVTPKASPfLKDVRPSQELIVAMGNTTLGNVKNNSTttg-----NGPNNINNKSNS[3]------- - 255
Candida albicans orf19.4884 ---QHQPLLHHSSSVAGSTTSIVNNSLSISNGGYGNNYSKNLSRSYN-KYSNSQ-VSQLSYMLPP-qtESSSTATIASG- 379
Candida albicans orf19.12348 ---QHQPLLHHSSSVAGSTTSIVNNSLSISNGGYGNNYSKNLSRSYN-KYSNSQ-VSQLSYMLPP-qtESSSTATIASG- 379
CAWG_00418 Candida albicans WO-1 ---QHQPLLHHSSSVAGSTTSIVNNSLSISNGGYGNNYSKNLSRSYN-KYSNSQ-VSQLSYMLPP-qtESSSTATIASG- 381
Cd36_09540 Candida dubliniensis CD36 HQhQHQPLLHHSPSVIGSTTSIVNNNSSISNGSYGNSYSKNMSRSYN-KYSNSQ-VSQLSYSLPPsqsDSLITTNSIGT- 431
CTRG_03345 Candida tropicalis MYA-3403 ------------SMVAPTSGSNNGAKSYSN------YSKNIARVYNNKYSNV---------- 316
CPAR2_805000 Candida parapsilosis CDC317 SQqQQQPTHQQRQPLSISGAGVGGGASTATKLSYGKYGGP--L-SQQPQQ---F-QPRLGQQLPM---GMGPQNQHQS-- 366
CORT_0A05020 Candida orthopsilosis Co 90-125 QQqQQQPTQQRQQP-PAPIANVTGAALNNAKLSYGKYGGP--LPQQQQQQ---F-QPRMGHQLAV---APLTQNDNS--- 363
DEHA2B12452g Debaryomyces hansenii CBS767 GY----------NRFNQSLLSQPQYHAPPQNFNQQPQQLSLY--QQQQQQMHQ---RQGSPQYFANE- 1583
CLUG_00578 Candida lusitaniae ATCC 42720 QEtASMMHYLQPPAAAAAAAGVSHLAQGPFSSSLPPYFPT--LHQPQKYASSGQ-LDYLSLQLPQ---QMHTLHSHASM1 321
CAGL0L02453g Candida glabrata CBS138 SShKHSPLDSHEKQ----------TSIAYGNYNAPVSTNSLSMGNPVPNNSShYFYSANGLQA----YGSNGTAYQG- 246
PGUG_01781 Candida guilliermondii ATCC 6260 SPqSHYNVPQQQKFIPQGSSGPQGLHMLPIPIQTPSFMYN--NHKDENIP---Y-QP---YRVPR---KESSEGNHGS-- 316
orf19.4231 PTH2 Candida albicans SC5314 ----------SHDSKFNQLD----LSNAIYP---------- 200
MIT1 Saccharomyces cerevisiae S288C ----------LNTVISTNNNSANINAAGSNQFTSANKNYYYKNDESSGYpITQFAPALPS------TTLMYTAN- 313
Candida albicans orf19.4884 ASVSVKREDDT[4]NAPTGNENqYVNAINHSHTSS---YGGQGYATD-- ATGIATPAYNSYSQANTSI---NTSSQQ 449
Candida albicans orf19.12348 ASVSVKREDDT[4]NAPTGNENqYVNAINHSHTSS---YGGQGYATD-- ATGIATPAYNSYSQANTSI---NTSSQQ 449
CAWG_00418 Candida albicans WO-1 ASVSVKREDDT[4]NAPTGNENqYVNAINHSHTSS---YGGQGYATD-- ATGIATPAYNSYSQANTSI---NTSSQQ 451
Cd36_09540 Candida dubliniensis CD36 TSTNVKREDDS[5]NSSSGNENqYINALGHSHTLS---YGGQGYTTDTT[8]GTGMATPAYNNYSQTTTNT---NITTPL 512
CTRG_03345 Candida tropicalis MYA-3403 --VSGGPGDDN[4]EVP------FINPPF-HQHSSStnpYASQVYTNA-- ----SVSPYNTYQQQTLST---TSSSPQ 376
CPAR2_805000 Candida parapsilosis CDC317 EMLLVKRDEED NAPAAVND--LNFINP--------FSGTQQQVPIY GSSLGFMSNN----GNTY----N-YSMQ 421
CORT_0A05020 Candida orthopsilosis Co 90-125 ---LVKREEDE SGPTGTSD--LNFINP--------FSGTQQQVPIY GASLGFMTNN----GNAY----N-YSIQ 415
DEHA2B12452g Debaryomyces hansenii CBS767 -TQSIKREDDN -------SgDVNFINPFGNHQqn-TGGNGYPFNNT[3]-------NHNPYFVHGQHQl------QQ 1638
CLUG_00578 Candida lusitaniae ATCC 42720 QPPMQQVPEQS THVSQMLT--QQFPTPEDLHTpg-FHASPYSSETL PFPLGSPTLNA-VQGSQFLsrpSLYARS 391
CAGL0L02453g Candida glabrata CBS138 -QPYVHLSNNN[5]NSNEMHSS--LNSHNSAGVTNsg--LSSGQKIPGF[1]GGGSYYYTYSSSSGKPQDMs----SSTS 317
PGUG_01781 Candida guilliermondii ATCC 6260 GPPIVG----- ---SAISD--VNFGGP--------YGA------IH GGST---TNNSGQPGSYY----NSYMIP 359
orf19.4231 PTH2 Candida albicans SC5314 ---------DS ------------LLNSIYKRKhq------------ --------------------------Q 214
MIT1 Saccharomyces cerevisiae S288C -PPYITQSPDN TNATGMNT-HVNNNNNNSNNSsn------------ -------SNNSNNNNNNNNn--NNNNNN 364
Candida albicans orf19.4884 QQ--- QLQQGQYGQYVQYGVAPSTISGATS---[3]NSGN[3]IPQDVYYSHYTGFVQPH-----YP[10]DQYN 519
Candida albicans orf19.12348 QQ--- QLQQGQYGQYVQYGVAPSTISGATS---[3]NSGN[3]IPQDVYYSHYTGFVQPH-----YP[10]DQYN 519
CAWG_00418 Candida albicans WO-1 QQ--- QLQQGQYGQYVQYGVAPSTISGATS---[3]NSGN[3]IPQDVYYSHYTGFVQPH-----YP[10]DQYN 521
Cd36_09540 Candida dubliniensis CD36 Q---- --QQNQYGQYVQYGVAPSTISGTNSNSN[15]NSNN[14]IPQDVYSGHYTSFTQPHYPQQIYP[10]DQYN 610
CTRG_03345 Candida tropicalis MYA-3403 QQINL[7]SQQQQQYGQYTQYTTIPPQVTTTTL--- TSGN[9]-PPDLYSNQYATFPPPQAGSVYGP[12]TTNT 465
CPAR2_805000 Candida parapsilosis CDC317 GQGQP[5]GYPQSQYNQFLQHNPSQMNNTPDGYPQH[1]----[2]-------GYPQSQSQPQSQSQPQS -QSQ 482
CORT_0A05020 Candida orthopsilosis Co 90-125 SQGSL[5]GYTPSQYNQYLQHNTLQM-NPHEGYPQH[1]----[2]-------SL--------SQQQQQQ -QQQ 467
DEHA2B12452g Debaryomyces hansenii CBS767 QQVLQ --QQQQHNHHHQNQRQQQQQQPHLPPH[5]SSSN[1]IPQ----------QPQFNQFVQP[10]QDIY 1706
CLUG_00578 Candida lusitaniae ATCC 42720 AQQNL[5]ATDHSDRDSSDHYYA---------- ---- ---------- ---- 416
CAGL0L02453g Candida glabrata CBS138 PRPLL[7]SNNNTGYVDHIQQSGGPASLNANTSLGN[3]SSGS[4]IPPLNTPYYYSSTATPNNMSNPHF ---- 392
PGUG_01781 Candida guilliermondii ATCC 6260 PQVKS[5]GSSLQGLNHPLYLYPQPLESYYQGPQQQ[4]----[7]-------SY--NYVVPSTESGSLP -TMN 426
orf19.4231 PTH2 Candida albicans SC5314 QQNKQ ---------- ---- ----------HP -QQQ 224
MIT1 Saccharomyces cerevisiae S288C NNINN[1]----NNVNTNAGNGNNPNRFHNASFAYN[9]QQGQ[9]IPINNPNYYTTQPPNPVTNASTNE NQGY 444

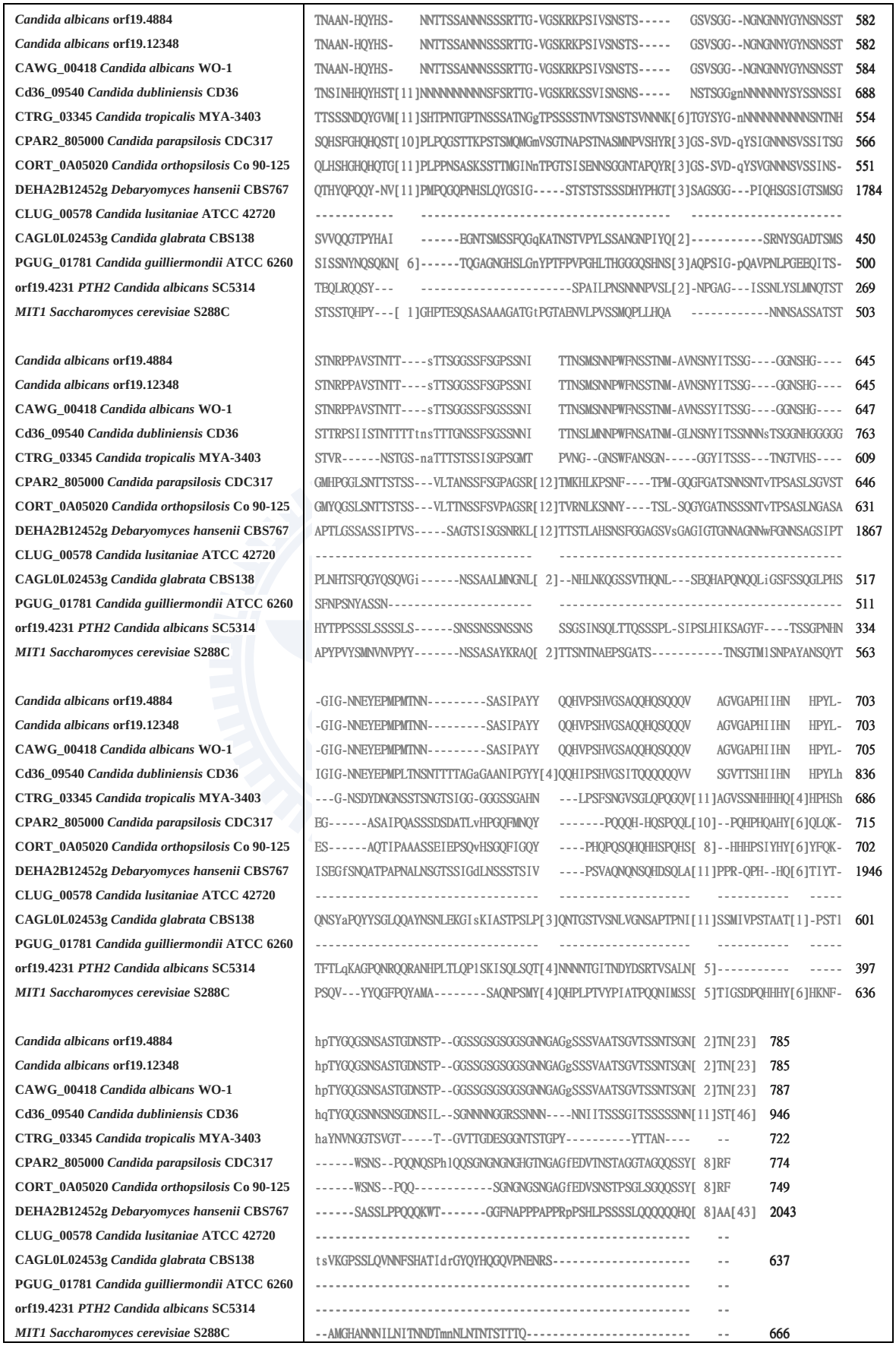

Candida albicans orf19.4884 TNAAN-HQYHS- NNTTSSANNNSSSRTTG-VGSKRKPSIVSNSTS----- GSVSGG--NGNGNNYGYNSNSST 582
Candida albicans orf19.12348 TNAAN-HQYHS- NNTTSSANNNSSSRTTG-VGSKRKPSIVSNSTS----- GSVSGG--NGNGNNYGYNSNSST 582
CAWG_00418 *Candida albicans* WO-1 TNAAN-HQYHS- NNTTSSANNNSSSRTTG-VGSKRKPSIVSNSTS----- GSVSGG--NGNGNNYGYNSNSST 584
Cd36_09540 *Candida dubliniensis* CD36 TNSINHHQYHST[11]NNNNNNNNNSFSRTTG-VGSKRKSSVISNSNS----- NSTSGGgnNNNNNNYSYSSNSSI 688
CTRG_03345 *Candida tropicalis* MYA-3403 TTSSSNDQYGVM[11]SHTPNTGPTNSSSATNGgTPSSSSTNVTSNSTSVNNNK[6]TGYSYG-nNNNNNNNNNSNTNH 554
CPAR2_805000 *Candida parapsilosis* CDC317 SQHSFGHQHQST[10]PLPQGSTTKPSTSMQMGmVSGTNAPSTNASMNPVSHYR[3]GS-SVD-qYSIGNNNSVSSITSG 566
CORT_0A05020 *Candida orthopsilosis* Co 90-125 QLHSHGHQHQTG[11]PLPPNSASKSSTTMGINnTPGTSISENNSGGNTAPQYR[3]GS-SVD-qYSVGNNNSVSSINS- 551
DEHA2B12452g *Debaryomyces hansenii* CBS767 QTHYQPQQY-NV[11]PMPQGQPNHSLQYGSIG-----STSTSTSSSDHYPHGT[3]SAGSGG---PIQHSGSIGTSMSG 1784
CLUG_00578 *Candida lusitaniae* ATCC 42720 ------------ ------------------------------------ ----------
CAGL0L02453g *Candida glabrata* CBS138 SVVQQGTPYHAI ------EGNTSMSSFQGqKATNSTVPYLSSANGNPIYQ[2]----------SRNYSGADTSMS 450
PGUG_01781 *Candida guilliermondii* ATCC 6260 SISSNYNQSQKN[6]------TQGAGNGHSLGnYPTFPVPGHLTHGGGQSHNS[3]AQPSIG-pQAVPNLPGEEQITS- 500
orf19.4231 *PTH2 Candida albicans* SC5314 TEQLRQQSY--- ----------SPAILPNSNNNPVSL[2]-NPGAG---ISSNLYSLMNQTST 269
MIT1 Saccharomyces cerevisiae S288C STSSTQHPY---[1]GHPTESQSASAAAGATGtPGTAENVLPVSSMQPLLHQA ----------NNNSASSATST 503
Candida albicans orf19.4884 STNRPPAVSTNTT----sTTSGGSSFSGPSSNI TTNSMSNNPWFNSSTNM-AVNSNYITSSG----GGNSHG---- 645
Candida albicans orf19.12348 STNRPPAVSTNTT----sTTSGGSSFSGPSSNI TTNSMSNNPWFNSSTNM-AVNSNYITSSG----GGNSHG---- 645
CAWG_00418 *Candida albicans* WO-1 STNRPPAVSTNTT----sTTSGGSSFSGSSSNI TTNSMSNNPWFNSSTNM-AVNSSYITSSG----GGNSHG---- 647
Cd36_09540 *Candida dubliniensis* CD36 STTRPSIISTNTTTTtnsTTTGNSSFSGSSNNI TTNSLMNNPWFNSATNM-GLNSNYITSSNNNsTSGGNHGGGGG 763
CTRG_03345 *Candida tropicalis* MYA-3403 STVR------NSTGS-naTTTSTSSISGPSGMT PVNG--GNSWFANSGN-----GGYITSSS---TNGTVHS---- 609
CPAR2_805000 *Candida parapsilosis* CDC317 GMHPGGLSNTTSTSS---VLTANSSFSGPAGSR[12]TMKHLKPSNF----TPM-GQGFGATSNNSNTvTPSASLSGVST 646
CORT_0A05020 *Candida orthopsilosis* Co 90-125 GMYQGSLSNTTSTSS---VLTTNSSFSVPAGSR[12]TVRNLKSNNY----TSL-SQGYGATNSSSNTvTPSASLNGASA 631
DEHA2B12452g *Debaryomyces hansenii* CBS767 APTLGSSASSIPTVS-----SAGTSISGSNRKL[12]TTSTLAHSNSFGGAGSVsGAGIGTGNNAGNNwFGNNSAGSIPT 1867
CLUG_00578 *Candida lusitaniae* ATCC 42720 ---------- ----------
CAGL0L02453g *Candida glabrata* CBS138 PLNHTSFQGYQSQVGi------NSSAALMNGNL[2]--NHLNKQGSSVTHQNL---SEQHAPQNQQLiGSFSSQGLPHS 517
PGUG_01781 *Candida guilliermondii* ATCC 6260 SFNPSNYASSN---------- ---------- 511
orf19.4231 *PTH2 Candida albicans* SC5314 HYTPPSSSLSSSSLS------SNSSNSSNSSNS SSGSINSQLTTQSSSPL-SIPSLHIKSAGYF----TSSGPNHN 334
MIT1 Saccharomyces cerevisiae S288C APYPVYSMNVNVPYY-------NSSASAYKRAQ[2]TTSNTNAEPSGATS----------TNSGTM1SNPAYANSQYT 563
Candida albicans orf19.4884 -GIG-NNEYEPMPMTNN---------SASIPAYY QQHVPSHVGSAQQHQSQQQV AGVGAPHIIHN HPYL- 703
Candida albicans orf19.12348 -GIG-NNEYEPMPMTNN---------SASIPAYY QQHVPSHVGSAQQHQSQQQV AGVGAPHIIHN HPYL- 703
CAWG_00418 *Candida albicans* WO-1 -GIG-NNEYEPMPMTNN---------SASIPAYY QQHVPSHVGSAQQHQSQQQV AGVGAPHIIHN HPYL- 705
Cd36_09540 *Candida dubliniensis* CD36 IGIG-NNEYEPMPLTNSNTTTTAGaGAANIPGYY[4]QQHIPSHVGSITQQQQQQVV SGVTTSHIIHN HPYLh 836
CTRG_03345 *Candida tropicalis* MYA-3403 ---G-NSDYDNGNSSTSNGTSIGG-GGGSSGAHN ---LPSFSNGVSGLQPQGQV[11]AGVSSNHHHHQ[4]HPHSh 686
CPAR2_805000 *Candida parapsilosis* CDC317 EG------ASAIPQASSSDSDATLvHPGQFMNQY -------PQQQH-HQSPQQL[10]--PQHPHQAHY[6]QLQK- 715
CORT_0A05020 *Candida orthopsilosis* Co 90-125 ES------AQTIPAAASSEIEPSQvHSGQFIGQY ----PHQPQSQHQHHSPQHS[8]--HHHPSIYHY[6]YFQK- 702
DEHA2B12452g *Debaryomyces hansenii* CBS767 ISEGfSNQATPAPNALNSGTSSIGdLNSSSTSIV ----PSVAQNQNSQHDSQLA[11]PPR-QPH--HQ[6]TIYT- 1946
CLUG_00578 *Candida lusitaniae* ATCC 42720 ---------- ---------- ---------- -----
CAGL0L02453g *Candida glabrata* CBS138 QNSYaPQYYSGLQQAYNSNLEKGIsKIASTPSLP[3]QNTGSTVSNLVGNSAPTPNI[11]SSMIVPSTAAT[1]-PST1 601
PGUG_01781 *Candida guilliermondii* ATCC 6260 ---------- ---------- ---------- -----
orf19.4231 *PTH2 Candida albicans* SC5314 TFTLqKAGPQNRQQRANHPLTLQP1SKISQLSQT[4]NNNNTGITNDYDSRTVSALN[5]---------- ----- 397
MIT1 Saccharomyces cerevisiae S288C PSQV---YYQGFPQYAMA--------SAQNPSMY[4]QHPLPTVYPIATPQQNIMSS[5]TIGSDPQHHHY[6]HKNF- 636
Candida albicans orf19.4884 hpTYGQGSNSASTGDNSTP--GGSSGSGSGGSGNNGAGgSSSVAATSGVTSSNTSGN[2]TN[23] 785
Candida albicans orf19.12348 hpTYGQGSNSASTGDNSTP--GGSSGSGSGGSGNNGAGgSSSVAATSGVTSSNTSGN[2]TN[23] 785
CAWG_00418 *Candida albicans* WO-1 hpTYGQGSNSASTGDNSTP--GGSSGSGSGGSGNNGAGgSSSVAATSGVTSSNTSGN[2]TN[23] 787
Cd36_09540 *Candida dubliniensis* CD36 hqTYGQGSNNSNSGDNSIL--SGNNNNGGRSSNNN----NNIITSSSGITSSSSSNN[11]ST[46] 946
CTRG_03345 *Candida tropicalis* MYA-3403 haYNVNGGTSVGT-----T--GVTTGDESGGNTSTGPY----------YTTAN---- -- 722
CPAR2_805000 *Candida parapsilosis* CDC317 ------WSNS--PQQNQSPh1QQSGNGNGNGHGTNGAGfEDVTNSTAGGTAGQQSSY[8]RF 774
CORT_0A05020 *Candida orthopsilosis* Co 90-125 ------WSNS--PQQ----------SGNGNGSNGAGfEDVSNSTPSGLSGQQSSY[8]RF 749
DEHA2B12452g *Debaryomyces hansenii* CBS767 ------SASSLPPQQQKWT-------GGFNAPPPAPPRpPSHLPSSSSLQQQQQQHQ[8]AA[43] 2043
CLUG_00578 *Candida lusitaniae* ATCC 42720 ---------- --
CAGL0L02453g *Candida glabrata* CBS138 tsVKGPSSLQVNNFSHATIdrGYQYHQGQVPNENRS---------- -- 637
PGUG_01781 *Candida guilliermondii* ATCC 6260 ---------- --
orf19.4231 *PTH2 Candida albicans* SC5314 ---------- --
MIT1 Saccharomyces cerevisiae S288C --AMGHANNNILNITNNDTmnNLNTNTSTTTQ---------- -- 666

Fig. 23 The multiple alignment of CaRep5 protein sequences. The protein sequences of *CaREP5* compared with CAWG_00418 *C. albicans*, Cd36_09540 *C. dubliniensis*, CTRG_03345 *C. tropicalis*, CPAR2_805000 *C. parapsilosis*, CORT_0A05020 *C. orthopsilosis*, DEHA2B12452g *Debaryomyces hansenii*, CLUG_00578 *C. lusitaniae*, CAGL0L02453g *C. glabrata*, PGUG_01781 *C. guilliermondii*, *C. albicans PTH2* and *Saccharomyces cerevisiae MIT1* by multiple alignment tool (http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi?link_loc=BlastHomeLink).

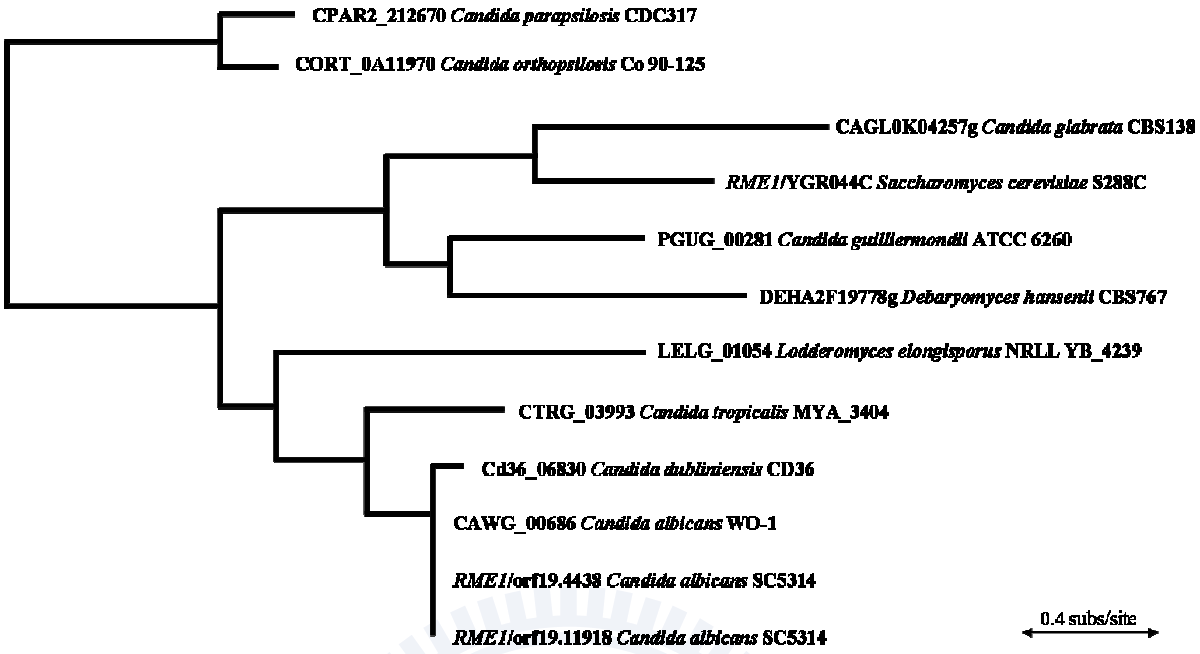
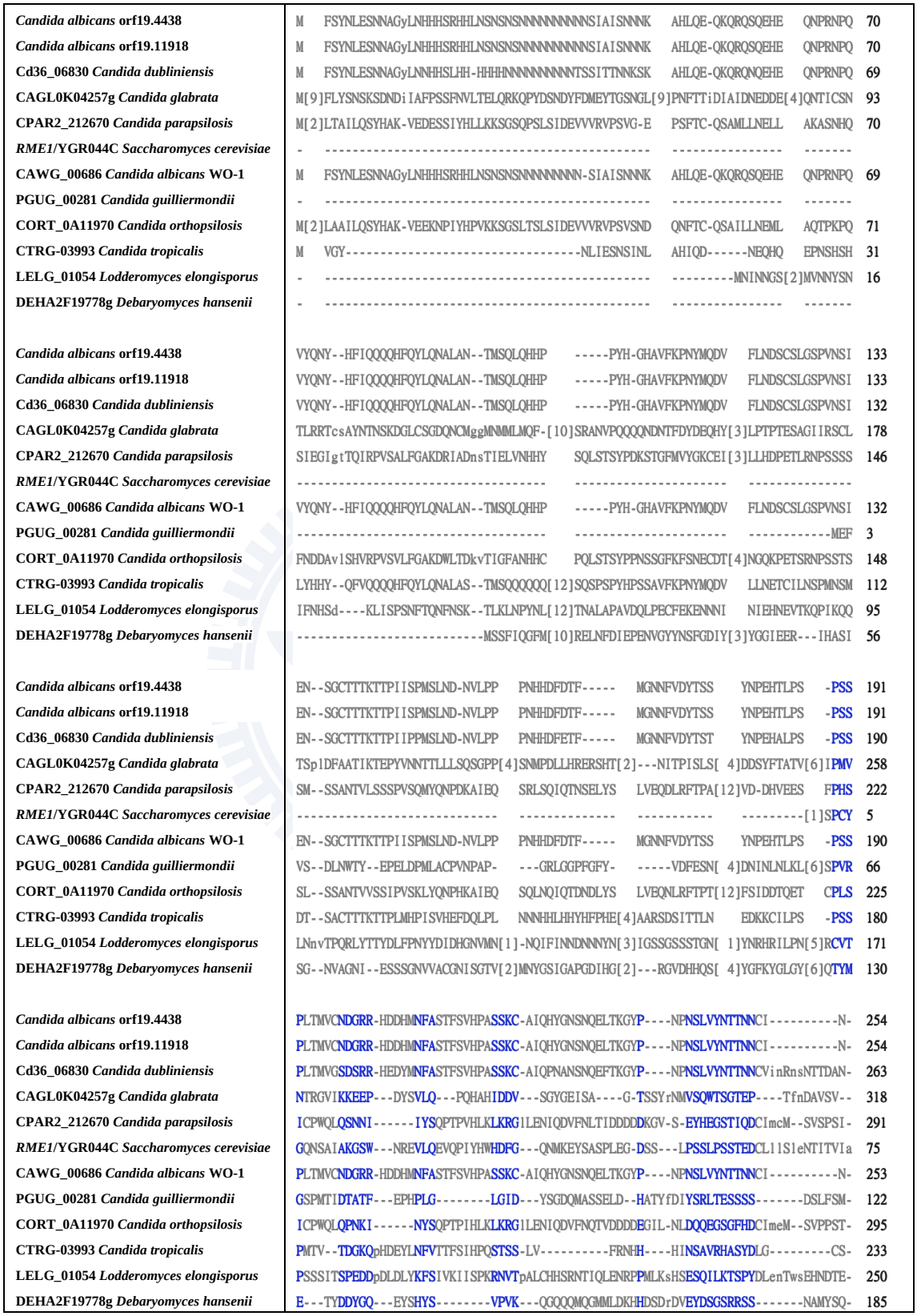


Fig. 24 The phylogenetic tree view approach for Saccharomycetales species of which the genome alike to *CaREP6* (orf19.4438/orf19.11918). The phylogenetic tree view align protein sequences by fast minimum evolution of COBALT multiple aligment.

Fig. 25



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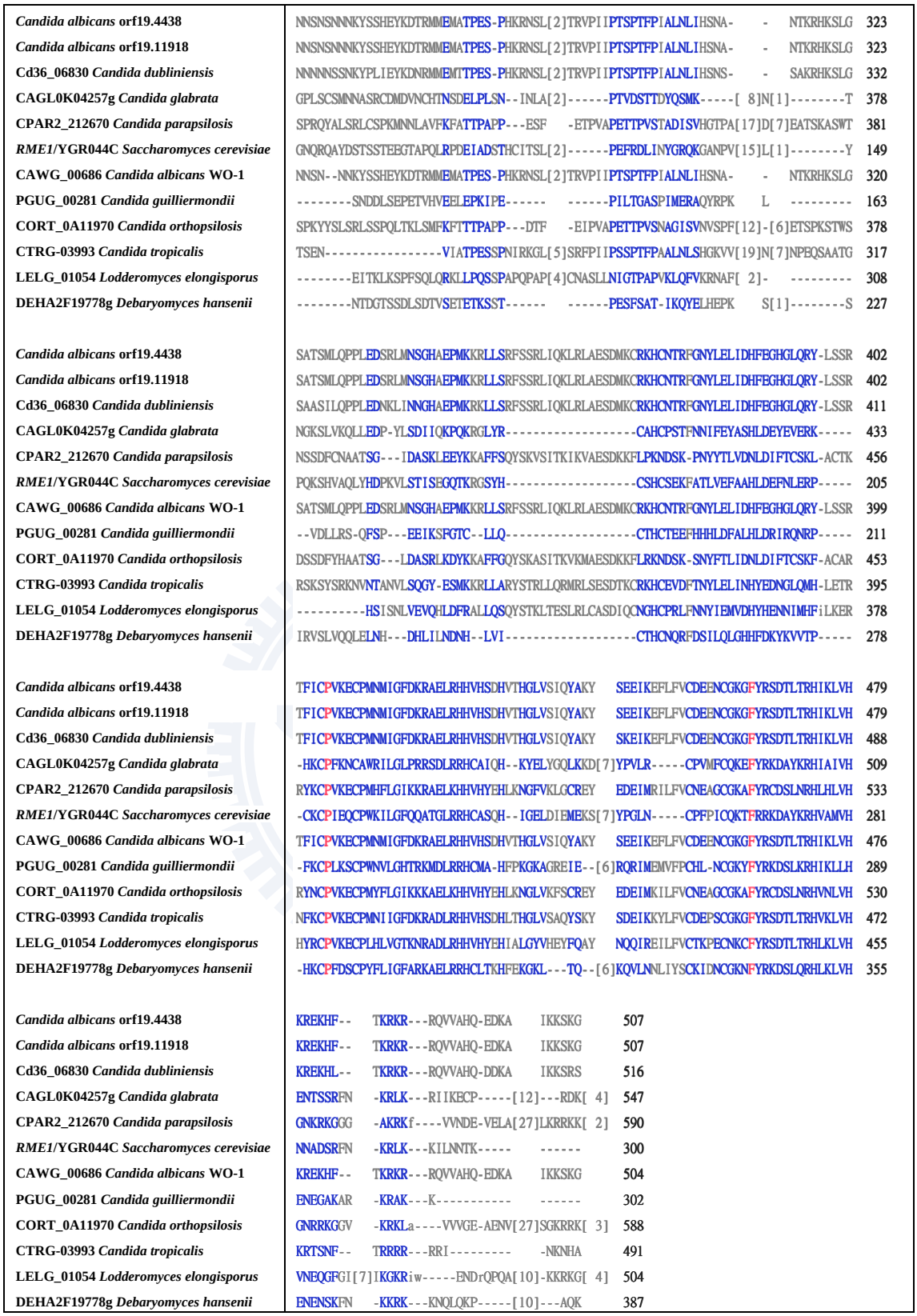

Candida albicans orf19.4438
Candida albicans orf19.11918
Cd36_06830 Candida dubliniensis
CAGL0K04257g Candida glabrata
CPAR2_212670 Candida parapsilosis
RME1/YGR044C Saccharomyces cerevisiae
CAWG_00686 Candida albicans WO-1
PGUG_00281 Candida guilliermondii
CORT_0A11970 Candida orthopsilosis
CTRG-03993 Candida tropicalis
LELG_01054 Lodderomyces elongisporus
DEHA2F19778g Debaryomyces hansenii

Fig. 25 The multiple alignment of CaRep6 protein sequences. The protein sequences of *CaREP6* compared with Cd36_06830 *C. dubliniensis*, CAGL0K04257g *C. glabrata*, CPAR2_212670 *C. parapsilosis*, *RME1*/YGR044C *Saccharomyces cerevisiae*, CAWG_00686 *C. albicans*, PGUG_00281 *C. guilliermondii*, CORT_0A11970 *C. orthopsilosis*, CTRG-03993 *C. tropicalis*, LELG_01054 *Lodderomyces elongisporus* and DEHA2F19778g *Debaryomyces hansenii* by multiple alignment tool (http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi?link_loc=BlastHomeLink).

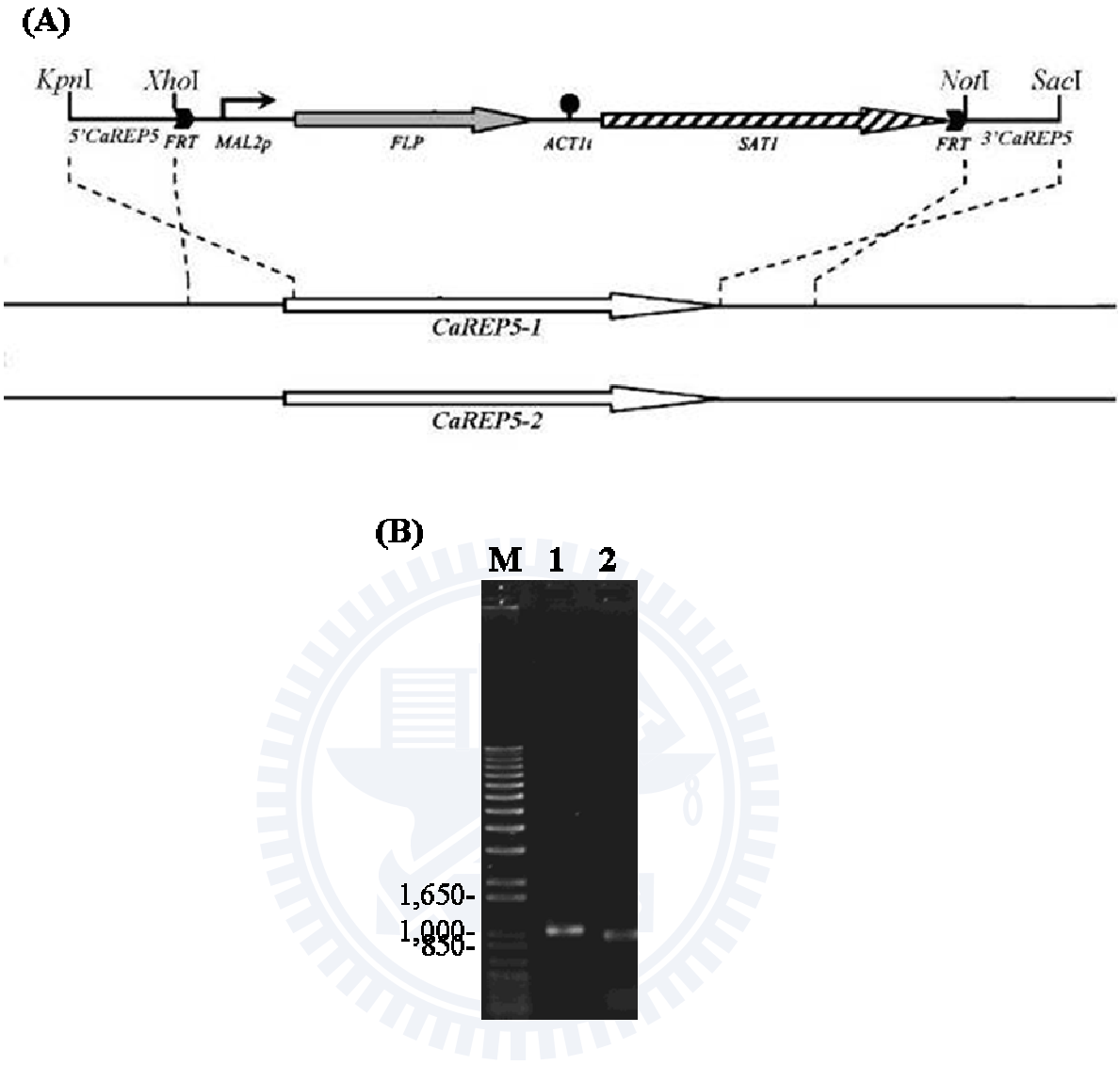


Fig. 26 Schematic diagram of construction *CaRE5* heterozygous mutant by *SAT1* flipper method. (A) Structure of the deletion cassette from plasmid pHC2 (top), in which the *CaREP5* coding region is replaced by *SAT1* flipper cassette, and genomic structure of the *CaREP5* alleles in the parent strain SC5314 (bottom). *CaREP5-1* and *CaREP5-2* represent the two alleles of

CaREP5 in the genome, in which *CaREP5*-1 was replaced with the *SAT1* flipper cassette to obtain YLO00324 and YLO00325. (B) Lane M: 1 kb Plus DNA ladder maker. Lane 1: Assessing the *SAT1* cassette integrated into *CaREP5* by PCR, and the fragment approximate sizes were 1034 bp. Lane 2: The excision of the *SAT1* cassette in *CaREP5* heterozygous strain, and the fragment approximate sizes were 861 bp.

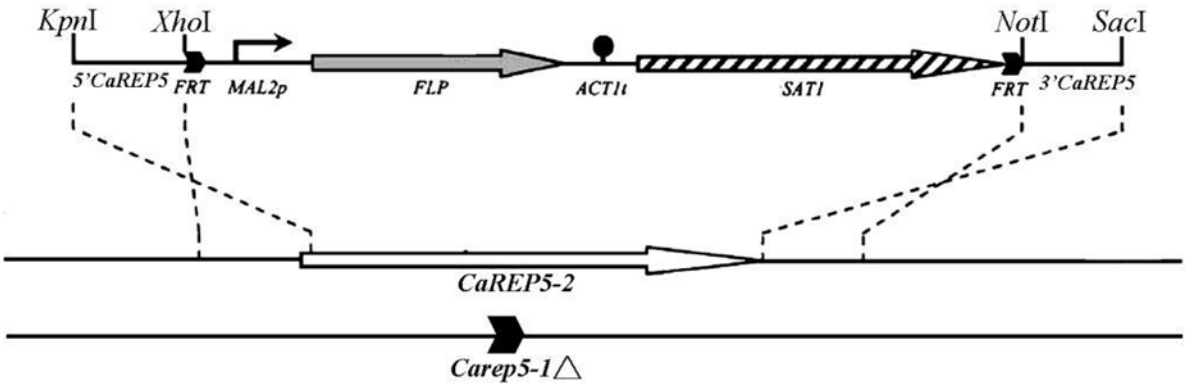


Fig. 27 Schematic diagram of construction *CaREP5* homozygous mutant by *SAT1* flipper method. Schematic maps show the deletion cassette from plasmid pHC2 (top), and either one disrupted alleles of *CaREP5* (bottom). The second allele of *CaREP5* was replaced with the *SAT1* flipper cassette to obtain homozygous mutant strains YLO00326 and YLO00327.

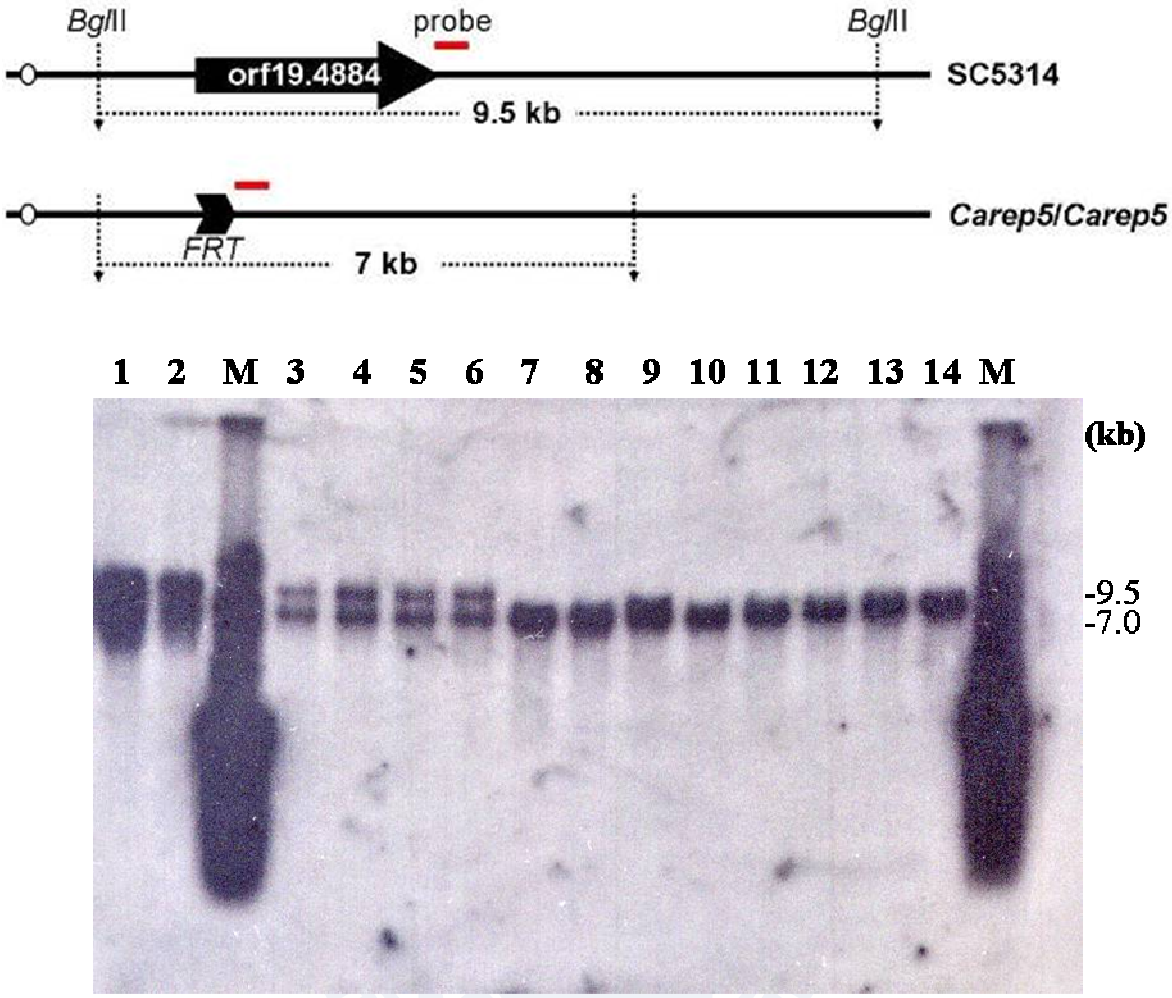
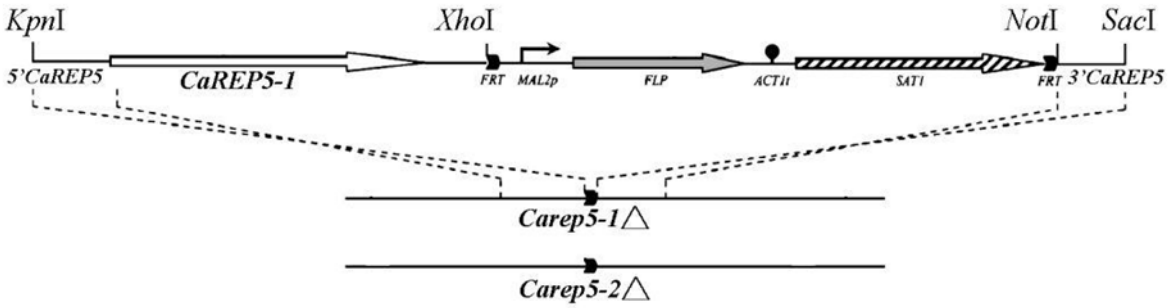


Fig. 28 Southern blot analysis to verify the *Carep5* mutant derivatives in *Candida albicans*. Genomic DNA from four heterozygous mutant strains and eight homozygous mutant strains were digested with *Bgl*II and probed with the 525 bp PCR fragment of the *CaREP5* downstream. The sizes of the hybridizing fragments were given on the right side of the blot. Lane 1 to 2 were wild-type SC5314, predicted almost 9.5 kb; lane 3 to 6 were *CaREP5*/*Carep5* heterozygous mutants, predicted almost 7.0 kb and 9.5 kb; lane 7 to 14 were *Carep5*/*Carep5* homozygous mutants, predicted almost 7.0 kb; land M was about 0.4 ug of 1 kb plus DNA ladder.



Figu. 29 Schematic diagram of construction *CaREP5* rescued strain by *SAT1* flipper method. Structure of the *SAT1* cassette from pHC5 used for reintegration of an intact *CaREP5* allele into the inactivated *Carep5* locus to produce the *CaREP5* rescued strains YLO00352 and YLO00353.

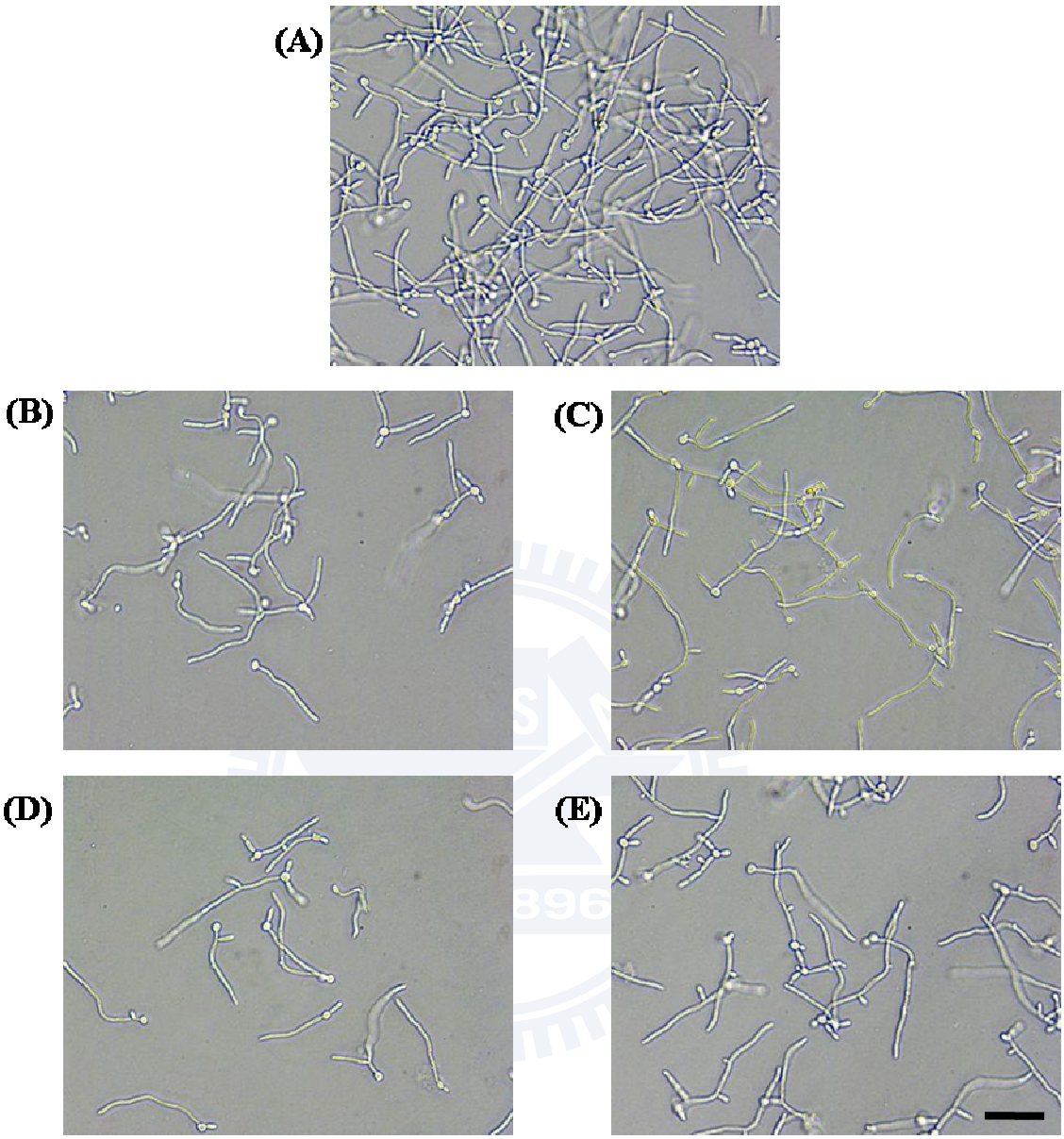


Fig. 30 Mutations on *CaREP5* do not affecting germ tube formation of *Candida albicans*. The morphology of five strains, (A) *CaREP5*/*CaREP5* (SC5314), (B) *CaREP5*/*Carep5*-1 (YLO00324), (C) *CaREP5*/*Carep5*-2 (YLO00325), (D) *Carep5*/*Carep5*-1 (YLO00326), and (E) *Carep5*/*Carep5*-2 (YLO00327), were incubated in YPD medium containing 10% fetal bovine serum for 4 hours at 37°C. Scale bar, 20 μm.

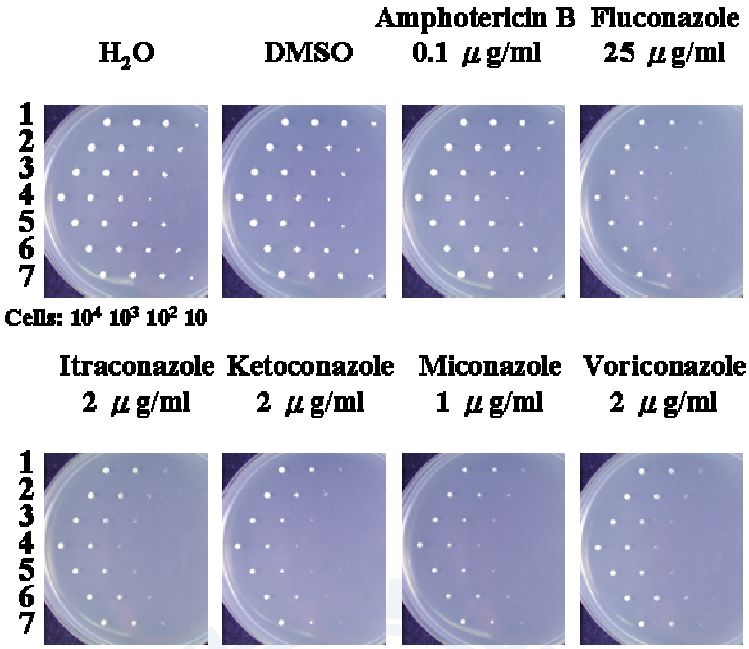


Fig. 31 Mutations on *CaREP5* do not affect the antifungal susceptibility of *Candida albicans*. The agar dilution was performed to analyze antifungal susceptibility on SD agar plates. DMSO and H_2O were used to prepare the control plates. Cells including, (1) *CaREP5*/*CaREP5* (SC5314), (2) *CaREP5*/*Carep5*-1 (YLO00324), (3) *Carep5*/*Carep5*-1 (YLO00326), (4) *Carep5*/*Carep5*::*CaREP5*-1 (YLO00352), (5) *CaREP5*/*Carep5*-2 (YLO00325), (6) *Carep5*/*Carep5*-2 (YLO00327), (7) *Carep5*/*Carep5*::*CaREP5*-2 (YLO00353). The results were photographed after 48 hours of growth at 30°C.

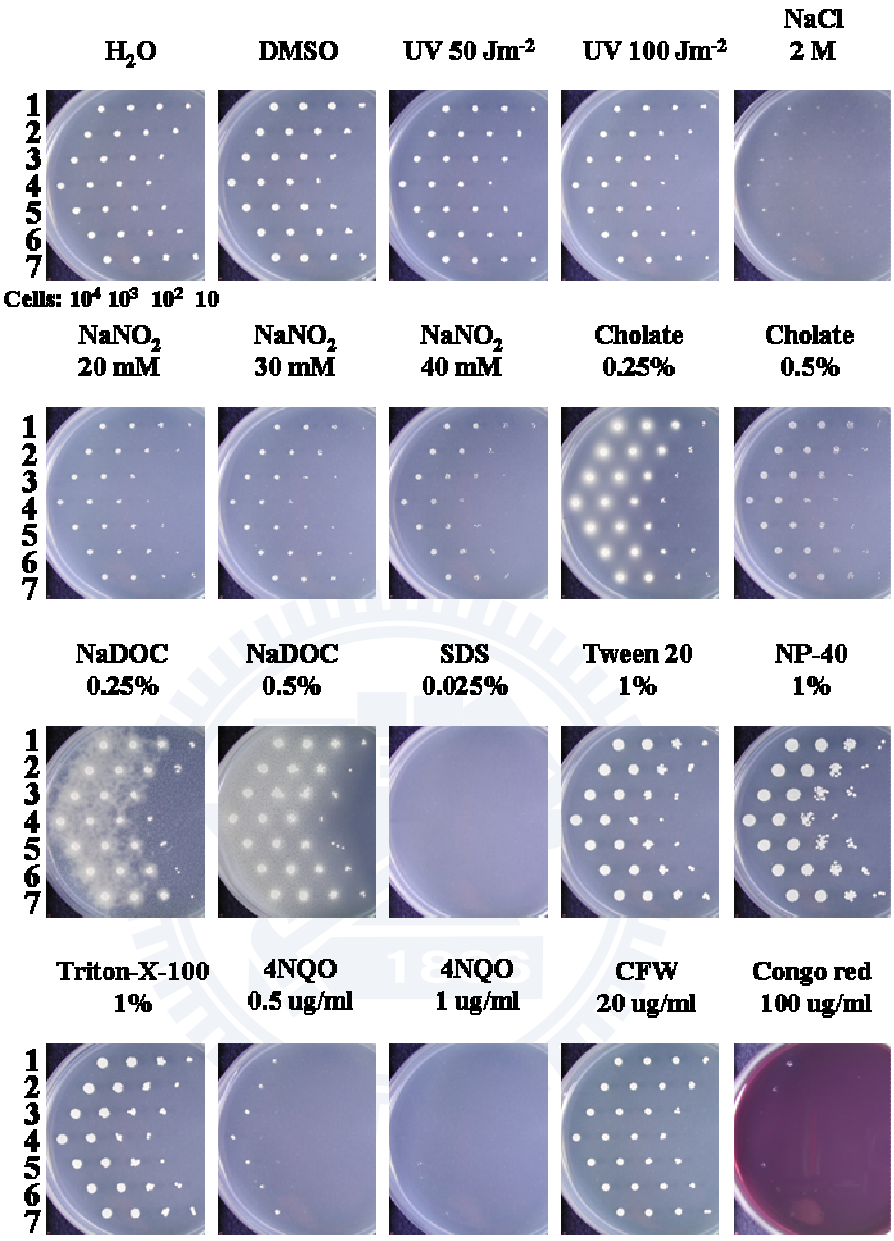


Fig. 32 Mutations on *CaREP5* are not sensitive to bile salts and common detergents. The agar dilution was performed to analyze antifungal susceptibility on SD agar plates. DMSO and H_2O were used to prepare the

control plates. Cells included, (1) *CaREP5*/*CaREP5* (SC5314), (2) *CaREP5*/*Carep5*-1 (YLO00324), (3) *Carep5*/*Carep5*-1 (YLO00326), (4) *Carep5*/*Carep5*::*CaREP5*-1 (YLO00352), (5) *CaREP5*/*Carep5*-2 (YLO00325), (6) *Carep5*/*Carep5*-2 (YLO00327), (7) *Carep5*/*Carep5*::*CaREP5*-2 (YLO00353). The results were photographed after 48 hours of growth at 30°C.

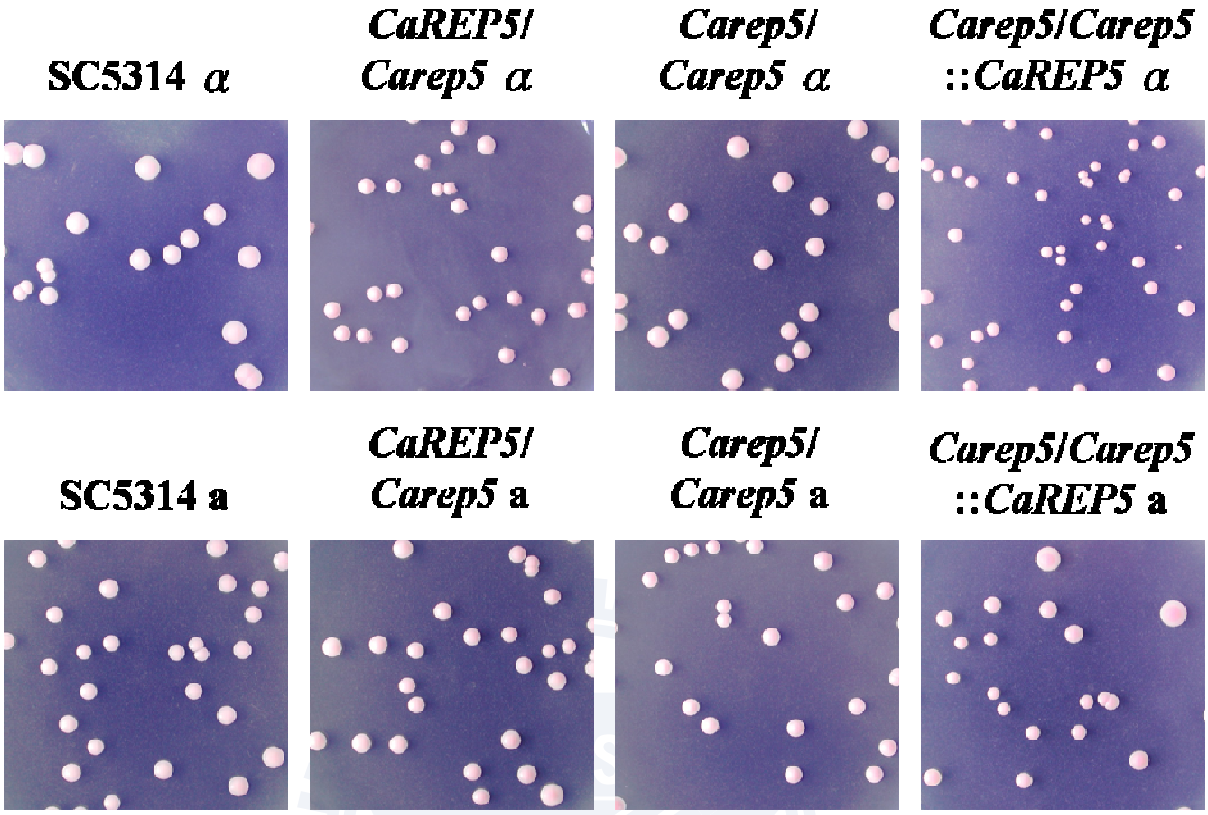


Fig. 33 White-opaque switching assay of *CaREP5* strains. The plates were incubated at room temperature for 14 days.

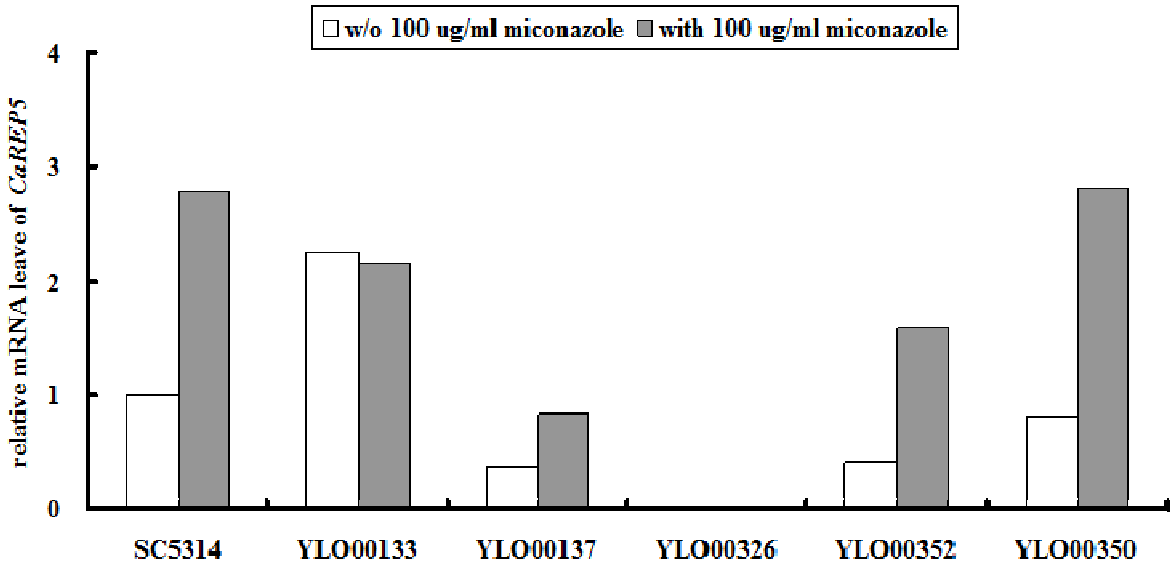


Fig. 34 *Carep5*/*Carep5* is confirmed by real-time PCR analysis. Total RNAs were isolated from cells with or without 100 μg/ml miconazole treatment at 30°C for 1 hour. The transcript levels of different samples as follow: parental strain SC5314, *Candt80*/*Candt80* (YLO00133), *Candt80*/*Candt80*::*CaNDT80* (YLO00137), *Carep5*/*Carep5*-1 (YLO00326), *Carep5*/*Carep5*::*CaREP5*-1 (YLO00352), *Carep6*/*Carep6*-1 (YLO00350) were normalized with the level of *CaSNF3* mRNA isolated from the same conditions. The primer pairs used were HJL00586 and HJL00587.

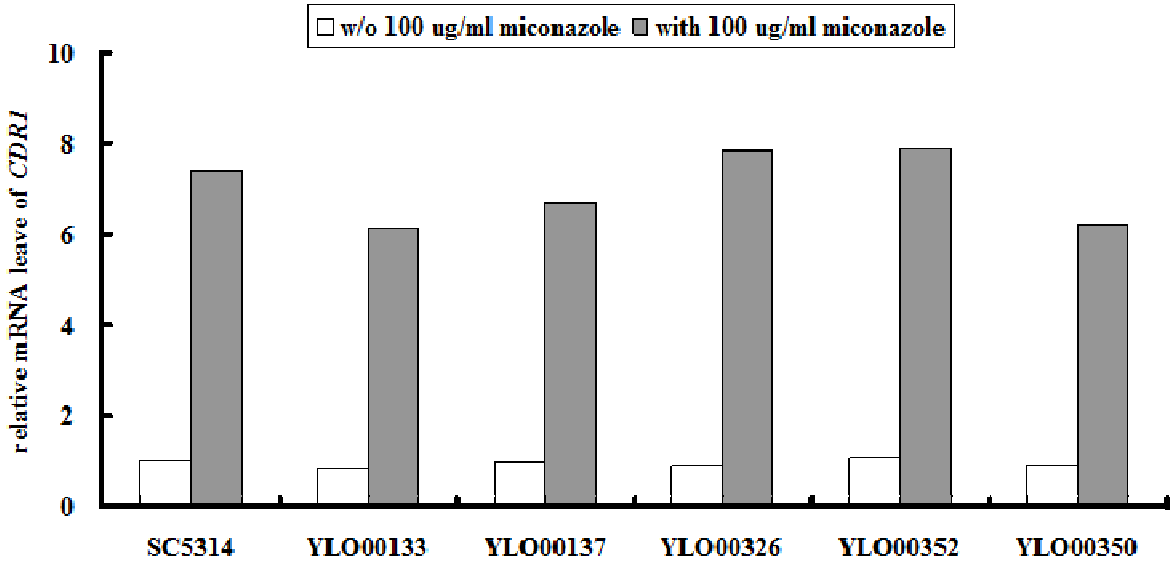


Fig. 35 Confirmation of the miconazole-induced change in *CDR1* expression. *Carep5*/*Carep5* messenger RNA expression observed by real-time PCR. Total RNAs were isolated from cells after treated with or without 100 μg/ml miconazole at 30°C for 1 hour. The transcript levels of different samples as follow: parental strain SC5314, *Candt80*/*Candt80* (YLO00133), *Candt80*/*Candt80*::*CaNDT80* (YLO00137), *Carep5*/*Carep5*-1 (YLO00326), *Carep5*/*Carep5*::*CaREP5*-1 (YLO00352), *Carep6*/*Carep6*-1 (YLO00350). Signals from experimental samples were normalized to the *CaACT1* gene expression level. The primer pairs used were HJL00315 and HJL00316.

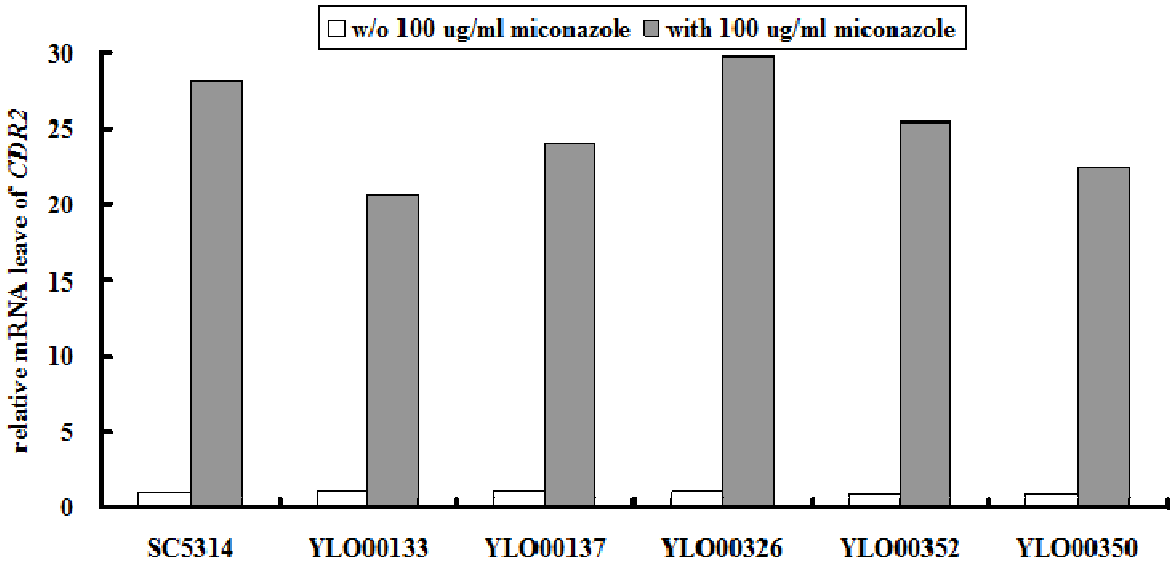


Fig. 36 Confirmation of the miconazole-induced change in *CDR2* expression. *Carep5*/*Carep5* messenger RNA expression observed by real-time PCR. Total RNAs were isolated from cells after treated with or without 100 μg/ml miconazole at 30°C for 1 hour. The transcript levels of different samples as follow: parental strain SC5314, *Candt80*/*Candt80* (YLO00133), *Candt80*/*Candt80*::*CaNDT80* (YLO00137), *Carep5*/*Carep5*-1 (YLO00326), *Carep5*/*Carep5*::*CaREP5*-1 (YLO00352), *Carep6*/*Carep6*-1 (YLO00350). Signals from experimental samples were normalized to the *CaACT1* gene expression level. The primer pairs used were HJL00395 and HJL00396.

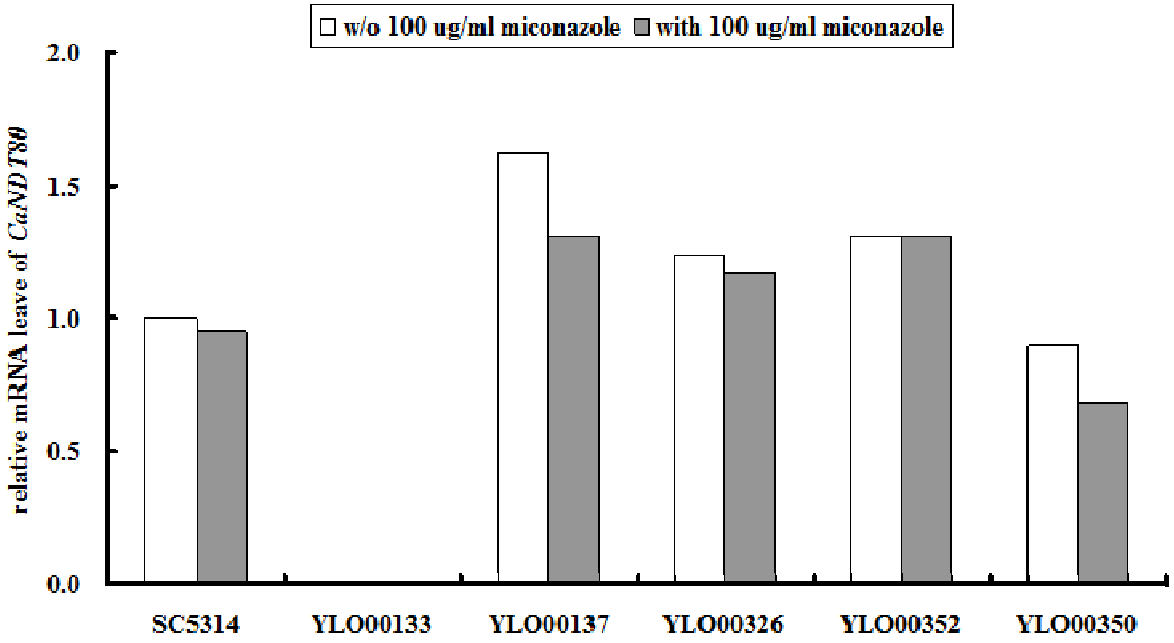


Fig. 37 Confirmation of the miconazole-induced change in *CaNDT80* expression. *Carep5*/*Carep5* messenger RNA expression observed by real-time PCR. Total RNAs were isolated from cells after treated with or without 100 μg/ml miconazole at 30°C for 1 hour. The transcript levels of different samples as follow: parental strain SC5314, *Candt80*/*Candt80* (YLO00133), *Candt80*/*Candt80*::*CaNDT80* (YLO00137), *Carep5*/*Carep5*-1 (YLO00326), *Carep5*/*Carep5*::*CaREP5*-1 (YLO00352), *Carep6*/*Carep6*-1 (YLO00350). Signals from experimental samples were normalized to the *CaACT1* gene expression level. The primer pairs used were HJL00319 and HJL00320.

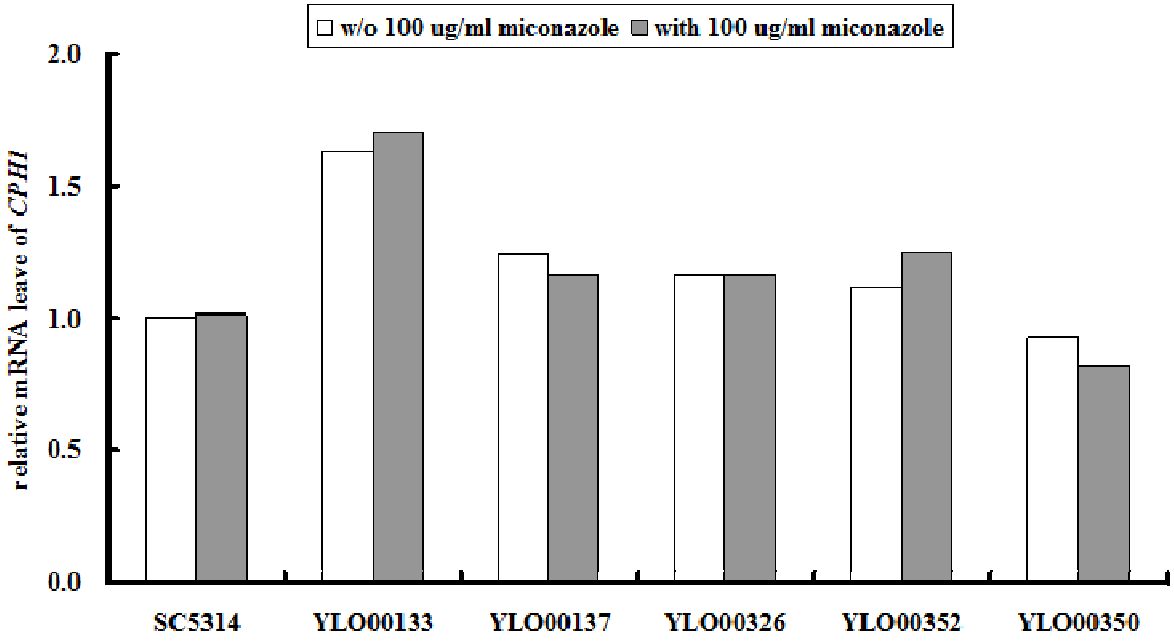


Fig. 38 Confirmation of the miconazole-induced change in *CPH1* expression. *Carep5*/*Carep5* messenger RNA expression observed by real-time PCR. Total RNAs were isolated from cells after treated with or without 100 μg/ml miconazole at 30°C for 1 hour. The transcript levels of different samples as follow: parental strain SC5314, *Candt80*/*Candt80* (YLO00133), *Candt80*/*Candt80*::*CaNDT80* (YLO00137), *Carep5*/*Carep5*-1 (YLO00326), *Carep5*/*Carep5*::*CaREP5*-1 (YLO00352), *Carep6*/*Carep6*-1 (YLO00350). Signals from experimental samples were normalized to the *CaACT1* gene expression level. The primer pairs used were HJL00538 and HJL00539.

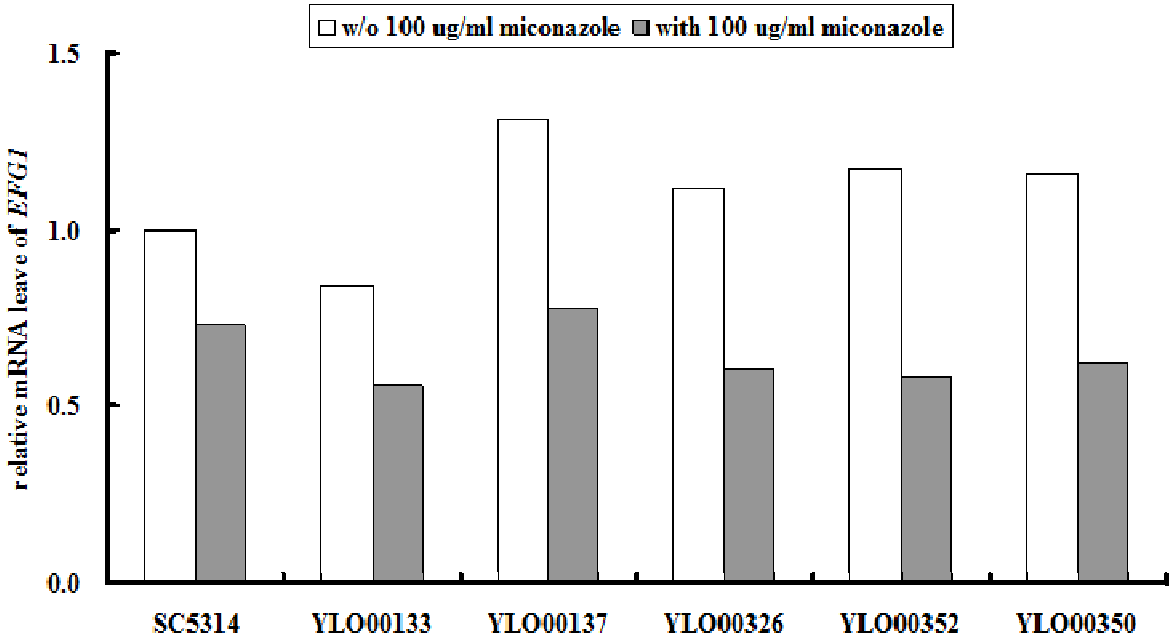


Fig. 39 Confirmation of the miconazole-induced change in *EFG1* expression. *Carep5*/*Carep5* messenger RNA expression observed by real-time PCR. Total RNAs were isolated from cells after treated with or without 100 μg/ml miconazole at 30°C for 1 hour. The transcript levels of different samples as follow: parental strain SC5314, *Candt80*/*Candt80* (YLO00133), *Candt80*/*Candt80*::*CaNDT80* (YLO00137), *Carep5*/*Carep5*-1 (YLO00326), *Carep5*/*Carep5*::*CaREP5*-1 (YLO00352), *Carep6*/*Carep6*-1 (YLO00350). Signals from experimental samples were normalized to the *CaACT1* gene expression level. The primer pairs used were HJL00540 and HJL00541.

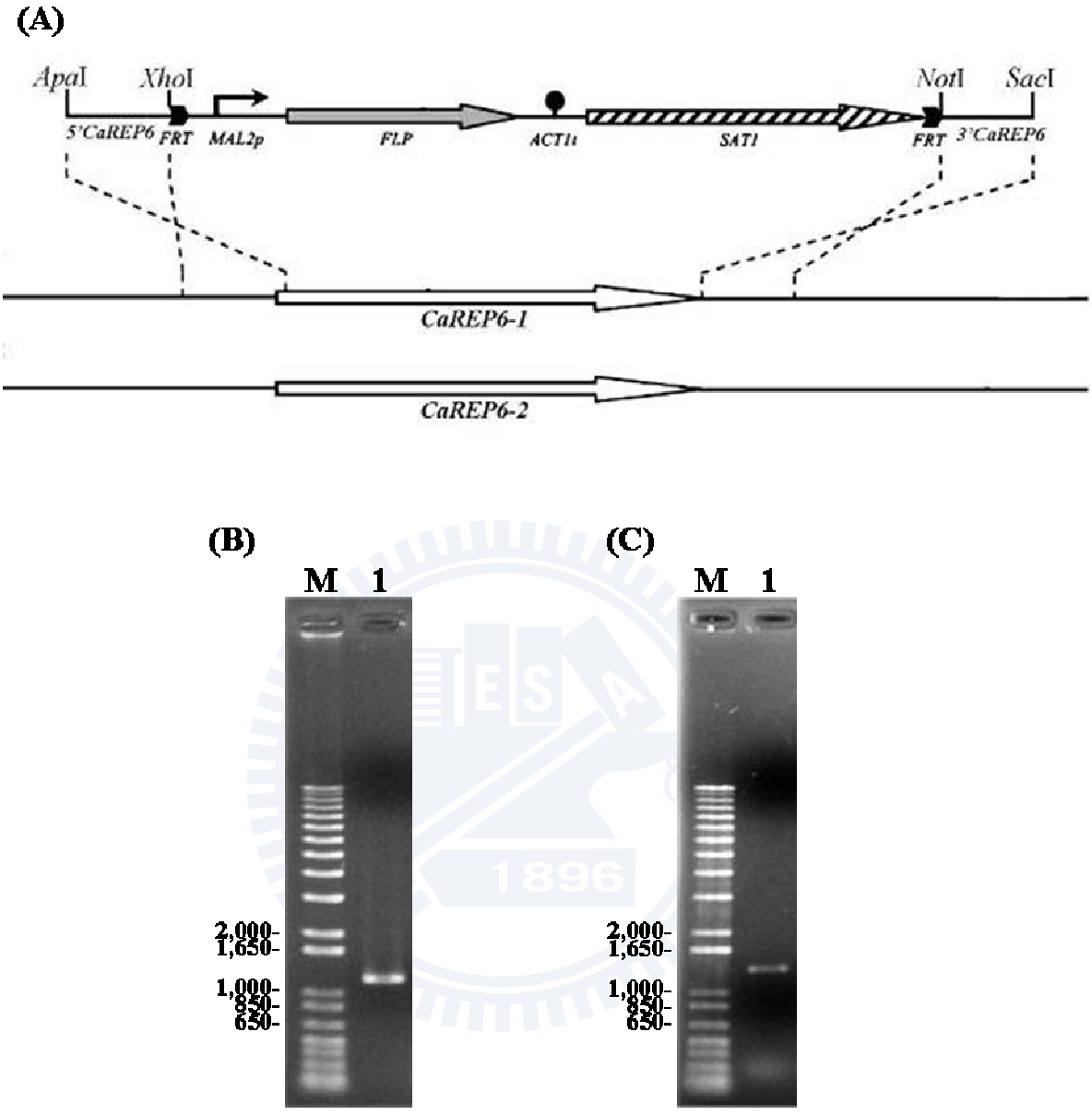


Fig. 40 Schematic diagram of construction *CaREP6* heterozygous mutant by *SAT1* flipper method. Structure of the deletion cassette from plasmid pHC4 (top), in which the *CaREP6* coding region is replaced by *SAT1* flipper

cassette, and genomic structure of the *CaREP6* alleles in the parent strain SC5314 (bottom). *CaREP6-1* and *CaREP6-2* represent the two alleles of *CaREP6* in the genome, in which *CaREP6*-1 was replaced with the *SAT1* flipper cassette to obtain YLO00348 and YLO00349. (B) Lane M: 1 kb Plus DNA ladder maker. Lane 1: Assessing the *SAT1* cassette integrated into *CaREP6* by PCR, and the fragment approximate sizes were 1215 bp. (C) Lane M: 1 kb Plus DNA ladder maker. Lane 1: Assessing the *SAT1* cassette integrated into *CaREP6* by PCR, and the fragment approximate sizes were 1308 bp.

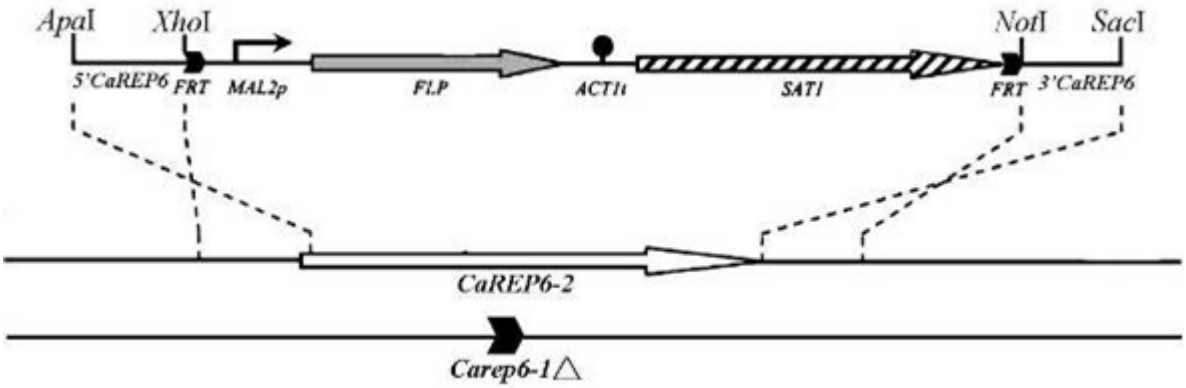


Fig. 41 Schematic diagram of construction *CaREP6* homozygous mutant by *SAT1* flipper method. Schematic maps show the deletion cassette from plasmid pHC4 (top), and either one disrupted alleles of *CaREP6* (bottom). The second allele of *CaREP6* was replaced with the *SAT1* flipper cassette to obtain homozygous mutant strains YLO00350 and YLO00351.

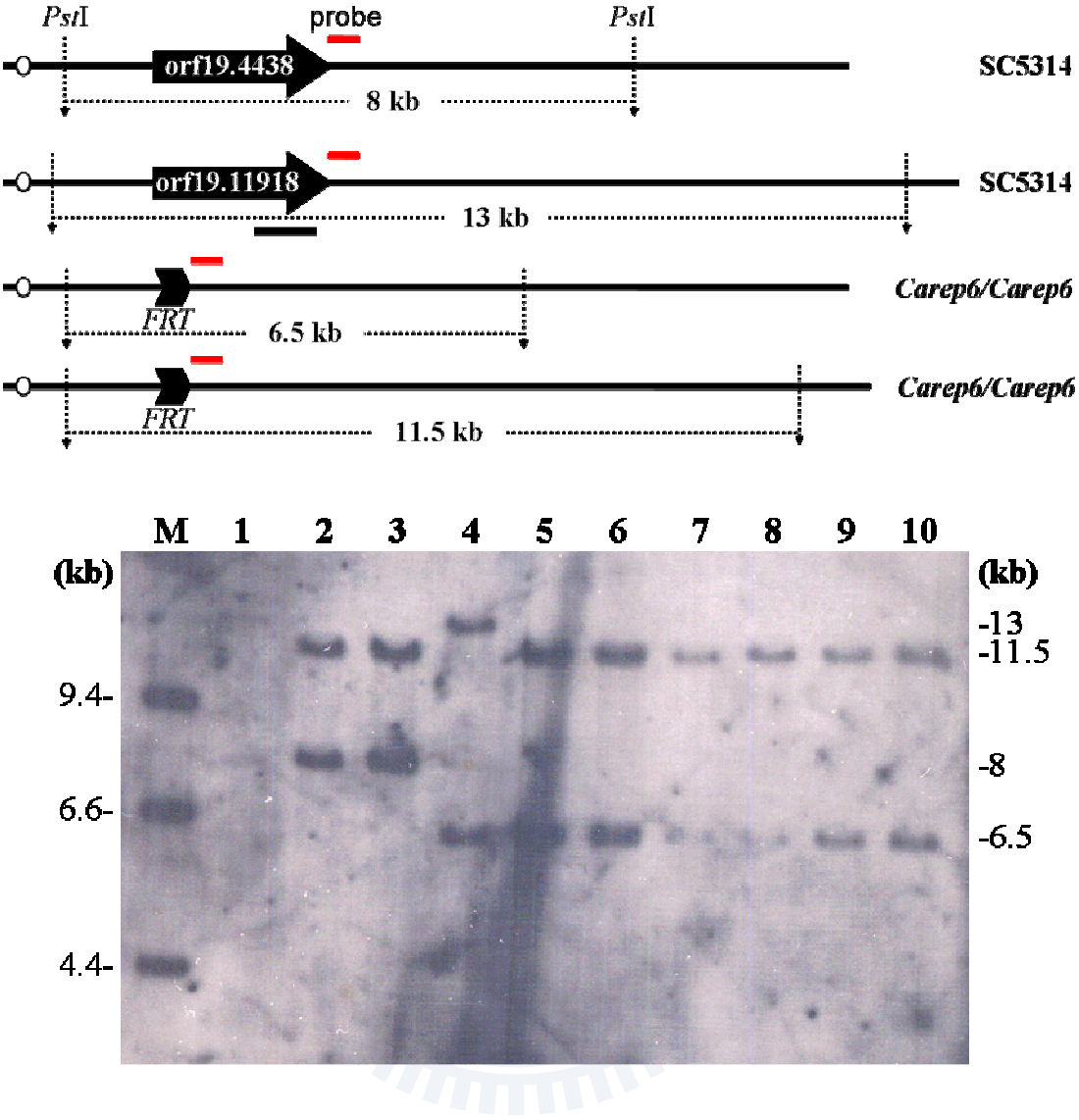


Fig. 42 Southern blot analysis to verify the *CaREP6* mutant derivatives in *Candida albicans*. Genomic DNA from three heterozygous mutant strains and six homozygous mutant strains were digested with *Pst*I and probed with the 556 bp PCR fragment of the *CaREP6* downstream. The sizes of the hybridizing fragments were given on the right side of the blot. Lane 1: wild-type allele (SC5314), predicted almost 8 or 13 kb; lane 2 to 4: *CaREP6*/*Carep6*, predicted almost 8/11.5 kb or 13/6.5 kb; lane 5 to 10: *Carep6*/*Carep6*, predicted almost 6.5/11.5 kb.

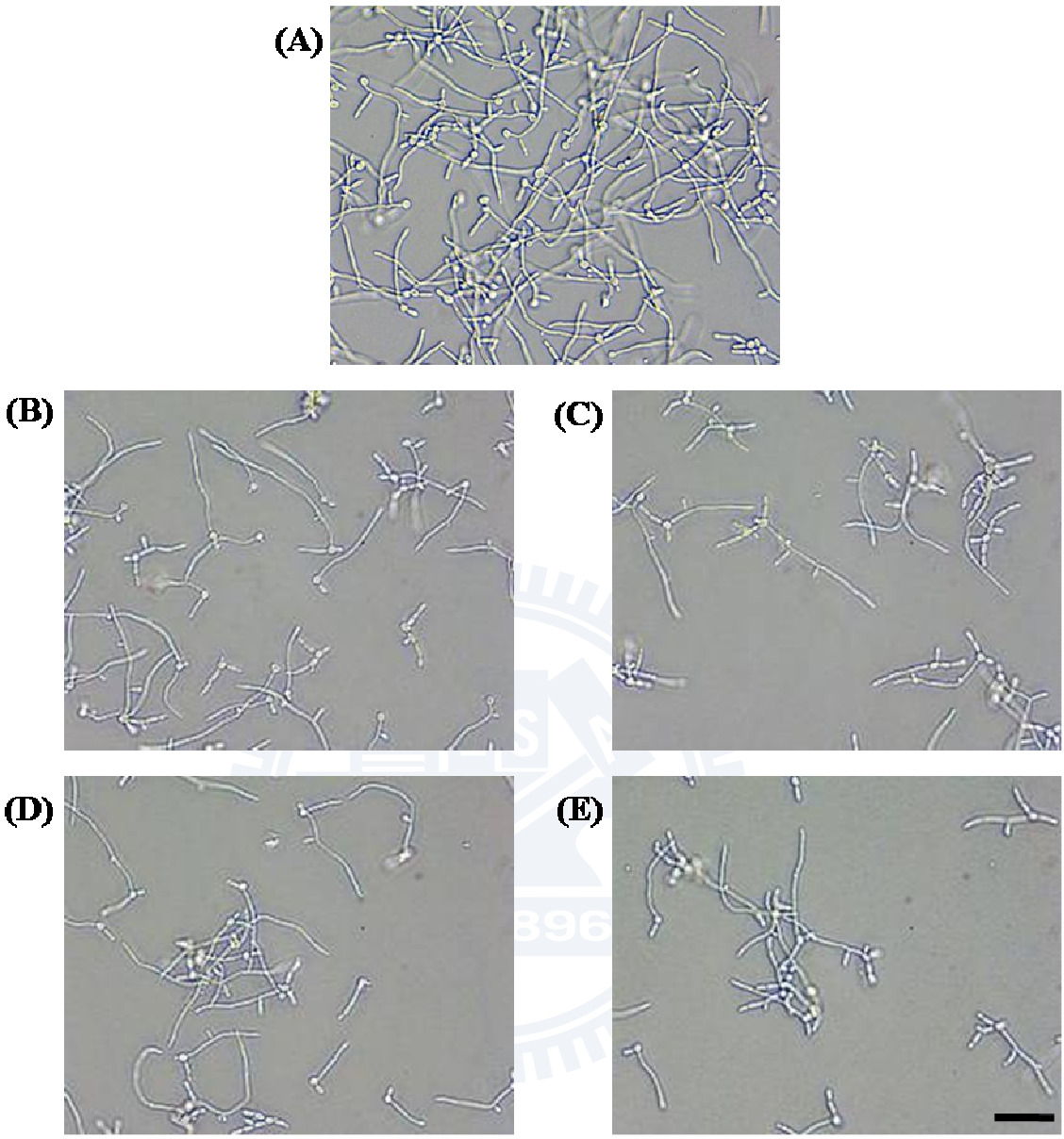


Fig. 43 Mutations on *CaREP6* do not affect germ tube formation of *Candida albicans*. The morphology of five strains, (A) *CaREP6*/*CaREP6* (SC5314), (B) *CaREP6*/*Carep6*-1 (YLO00348), (C) *CaREP6*/*Carep6*-2 (YLO00349), (D) *Carep6*/*Carep6*-1 (YLO00350), and (E) *Carep6*/*Carep6*-2 (YLO00351), were incubated in YPD medium containing 10% fetal bovine serum for 4 hours at 37°C. Scale bar, 20 μm.

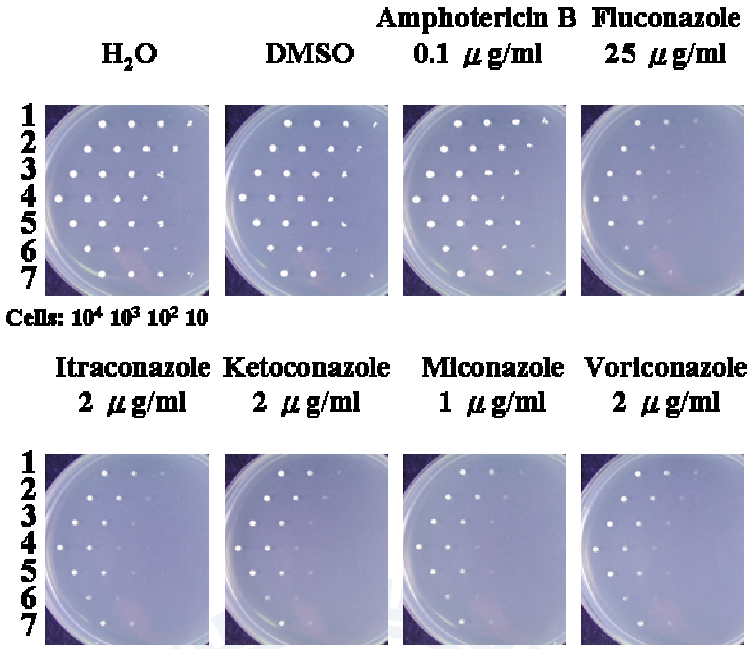


Fig. 44 Mutations on *CaREP6* do not affect the antifungal susceptibility of *Candida albicans*. The agar dilution was performed to analyze antifungal susceptibility on SD agar plates. DMSO and H_2O were used to prepare the control plates. Cells tested included, (1) *CaREP6*/*CaREP6* (SC5314), (2) *CaREP6*/*Carep6*-1 (YLO00348), (3) *Carep6*/*Carep6*-1 (YLO00350), (4) *CaREP6*/*Carep6*-2 (YLO00349), (5) *Carep6*/*Carep6*-2 (YLO00351), (6) *Candt80*/*Candt80* (YLO00133), (7) *Candt80*/*Candt80*::*CaNDT80* (YLO00137). The results were photographed after 48 hours of growth at 30°C.

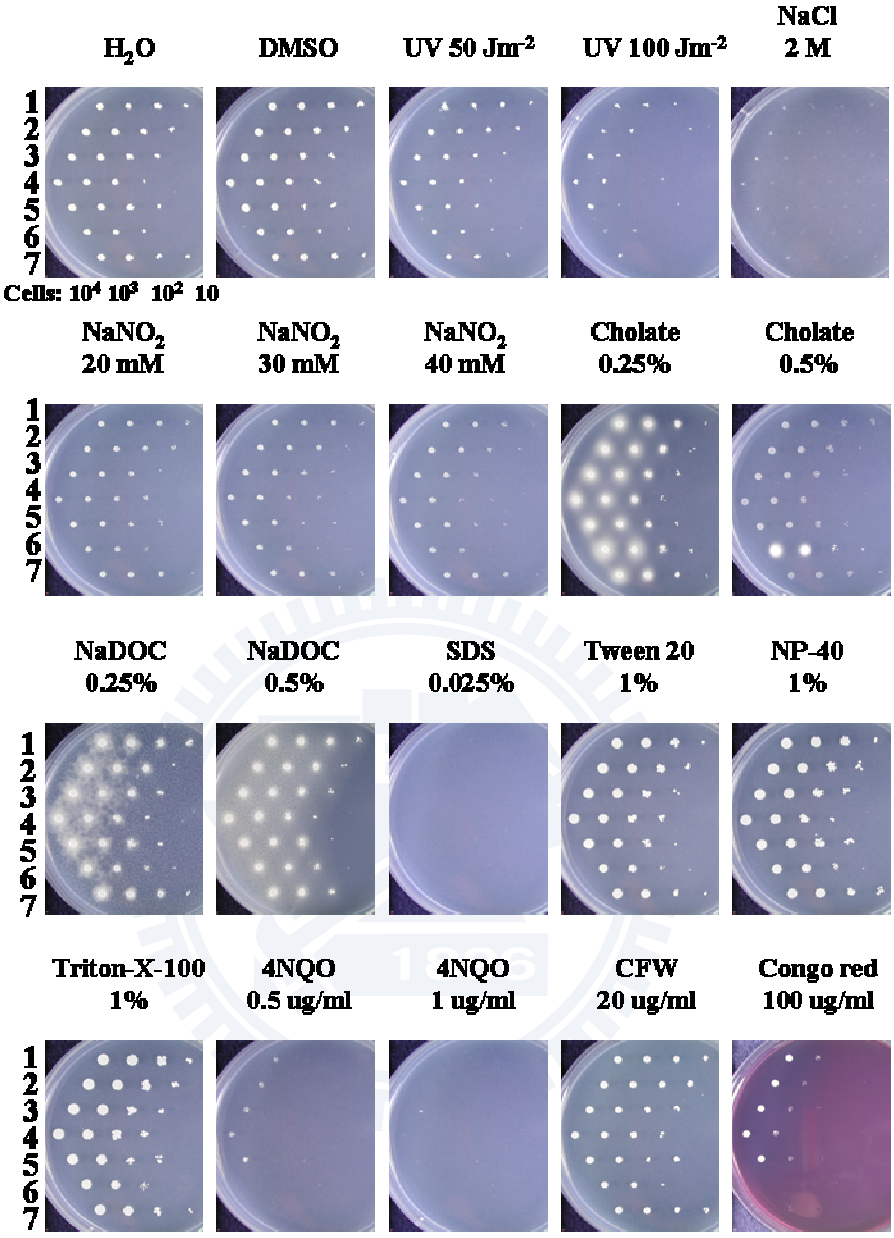


Fig. 45 Mutations on *CaREP6* are not sensitive to bile salts and common detergents. The agar dilution was performed to analyze antifungal susceptibility on SD agar plates. DMSO and H_2O were used to prepare the

control plates. Cells tested included, (1) *CaREP6*/*CaREP6* (SC5314), (2) *CaREP6*/*Carep6*-1 (YLO00348), (3) *Carep6*/*Carep6*-1 (YLO00350), (4) *CaREP6*/*Carep6*-2 (YLO00349), (5) *Carep6*/*Carep6*-2 (YLO00351), (6) *Candt80*/*Candt80* (YLO00133), (7) *Candt80*/*Candt80*::*CaNDT80* (YLO00137). The results were photographed after 48 hours of growth at 30°C. CFW indicates the cell wall binding dye calcofluor white. 4NQO: 4-nitroquinoline 1-oxide; NaDOC: sodium deoxycholate; NP-40: octylphenyl-polyethylene glycol; SDS: sodium dedocyl sulfate.

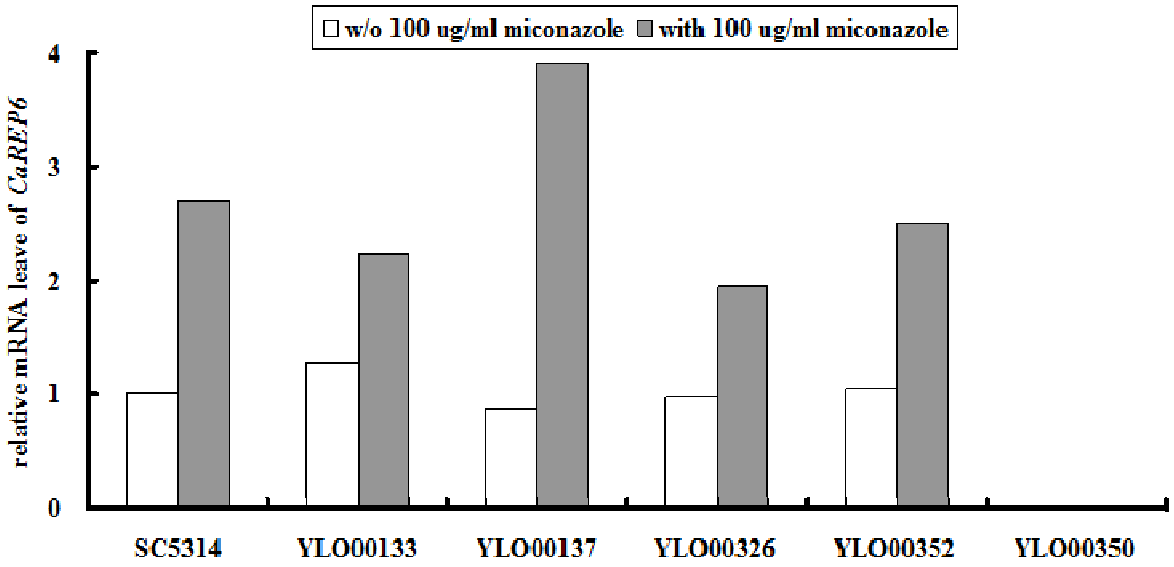
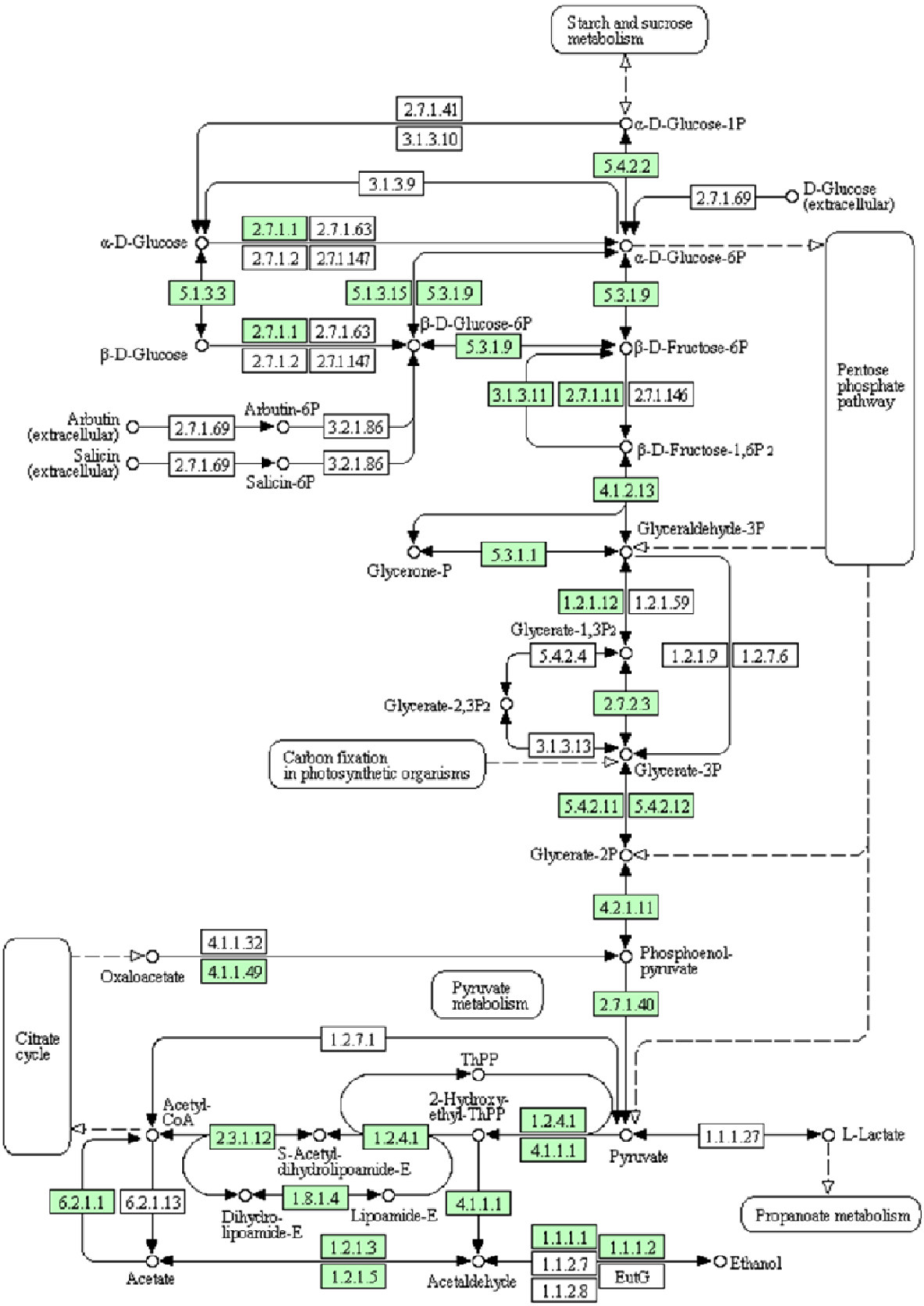
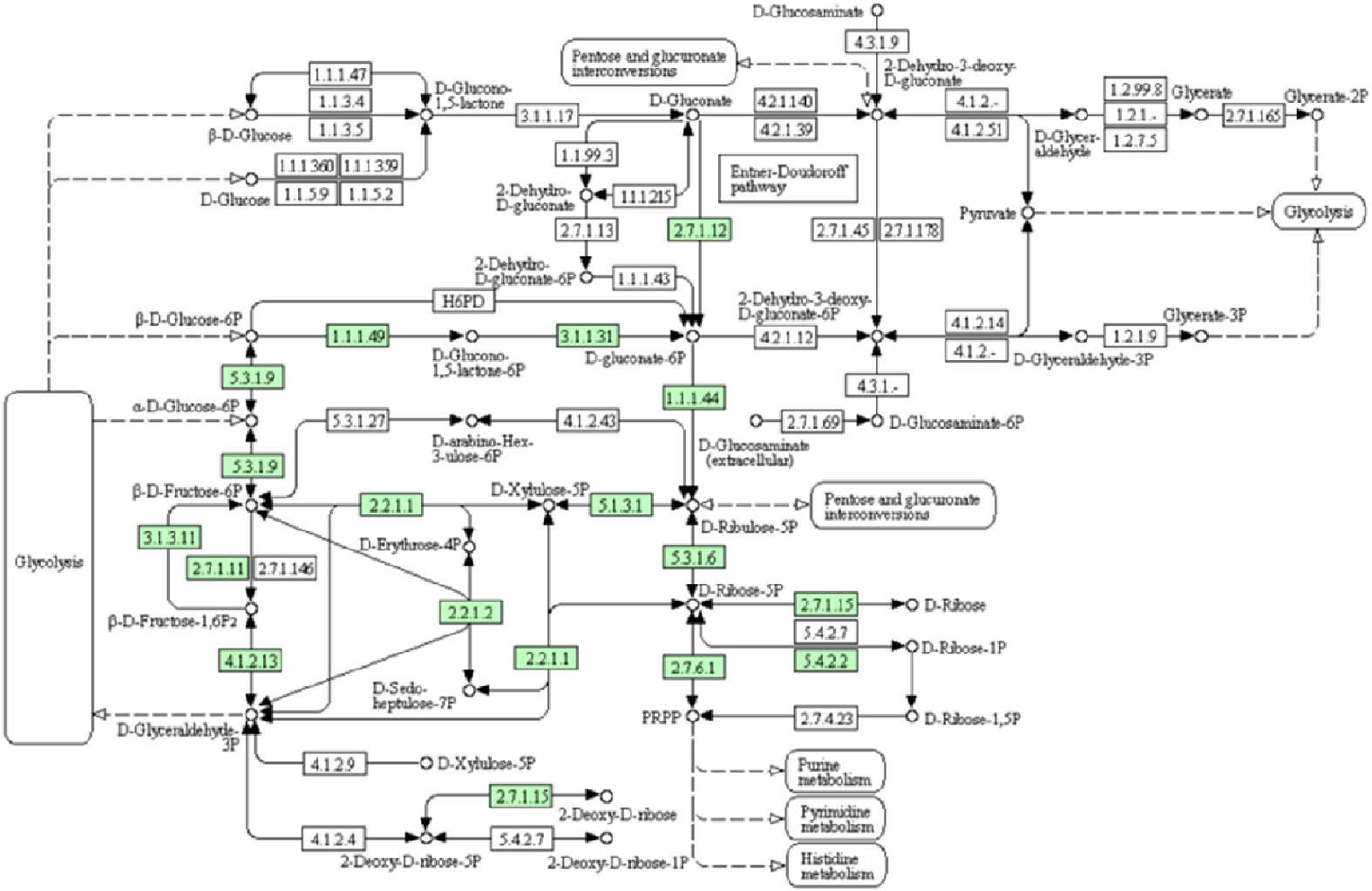


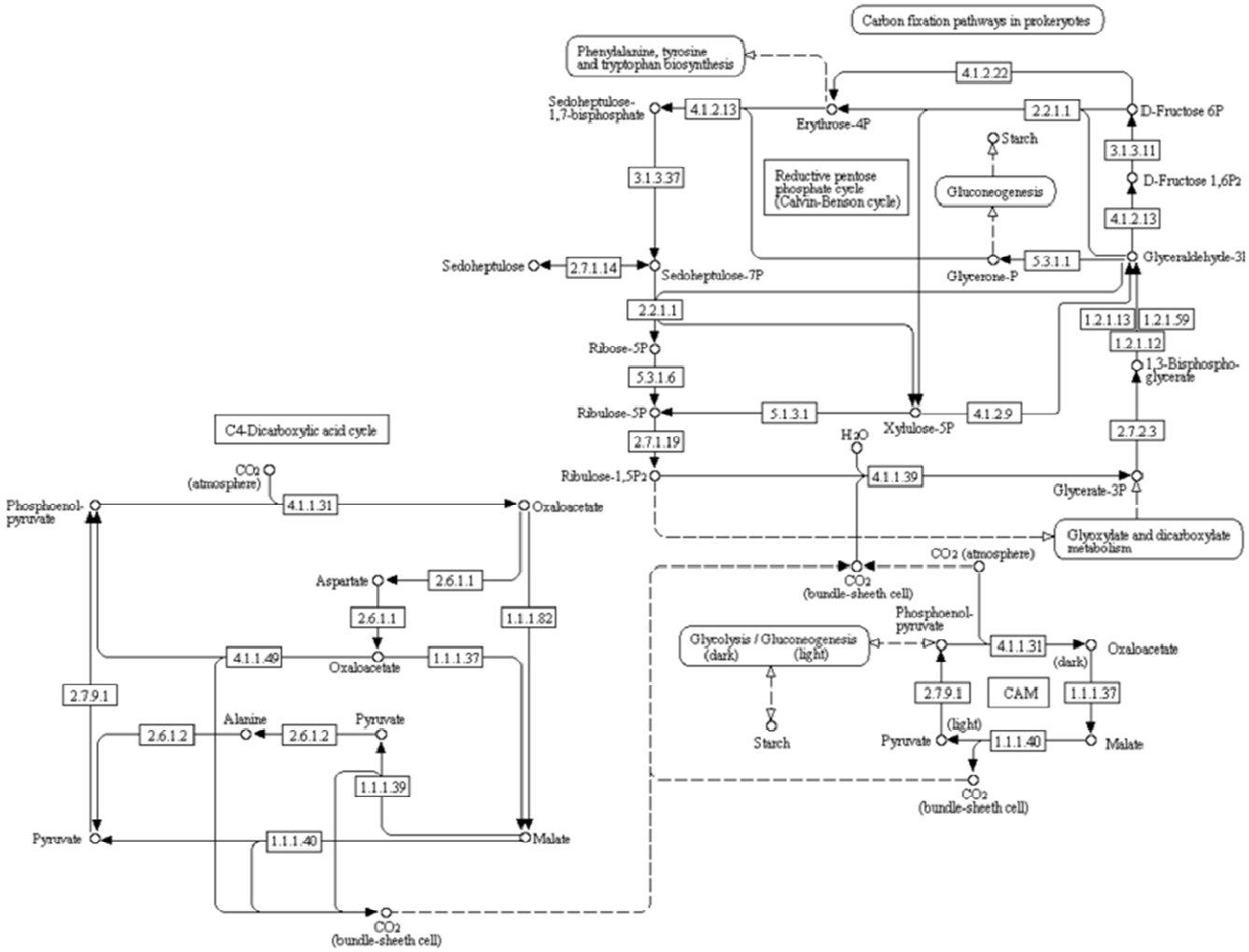
Fig. 46 *Carep6*/*Carep6* is confirmed by real-time PCR analysis. Total RNAs were isolated from cells with or without 100 μg/ml miconazole treatment at 30°C for 1 hour. The transcript levels of different samples as follow: parental strain SC5314, *Candt80*/*Candt80* (YLO00133), *Candt80*/*Candt80*::*CaNDT80* (YLO00137), *Carep5*/*Carep5*-1 (YLO00326), *Carep5*/*Carep5*::*CaREP5*-1 (YLO00352), *Carep6*/*Carep6*-1 (YLO00350) were normalized with the level of *CaSNF3* mRNA isolated from the same conditions. The primer pairs used were HJL00588 and HJL00589.



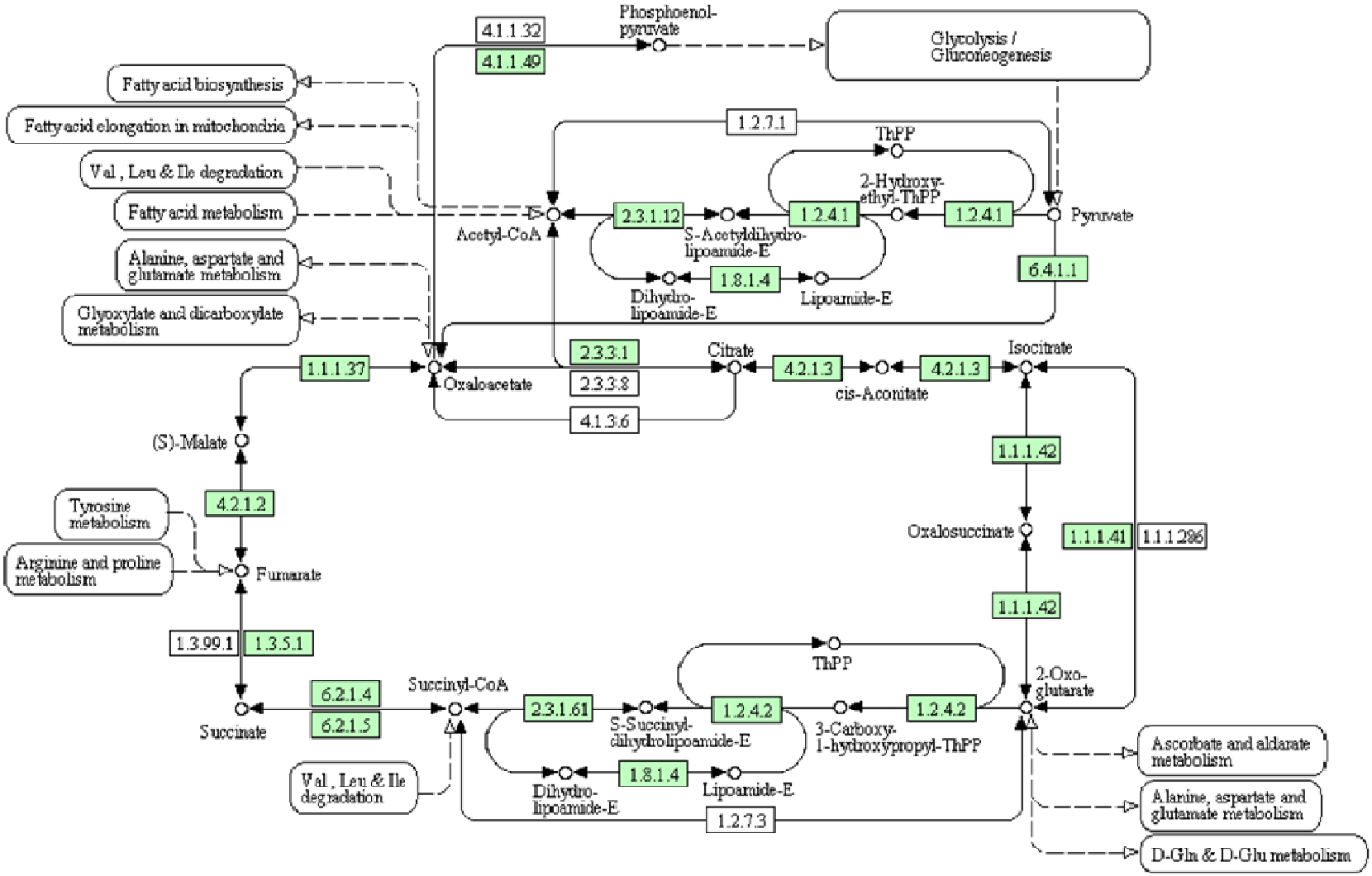
Appendix 1. Glycolysis and gluconeogenesis in *Candida albicans* from KEGG cal00010 pathway.



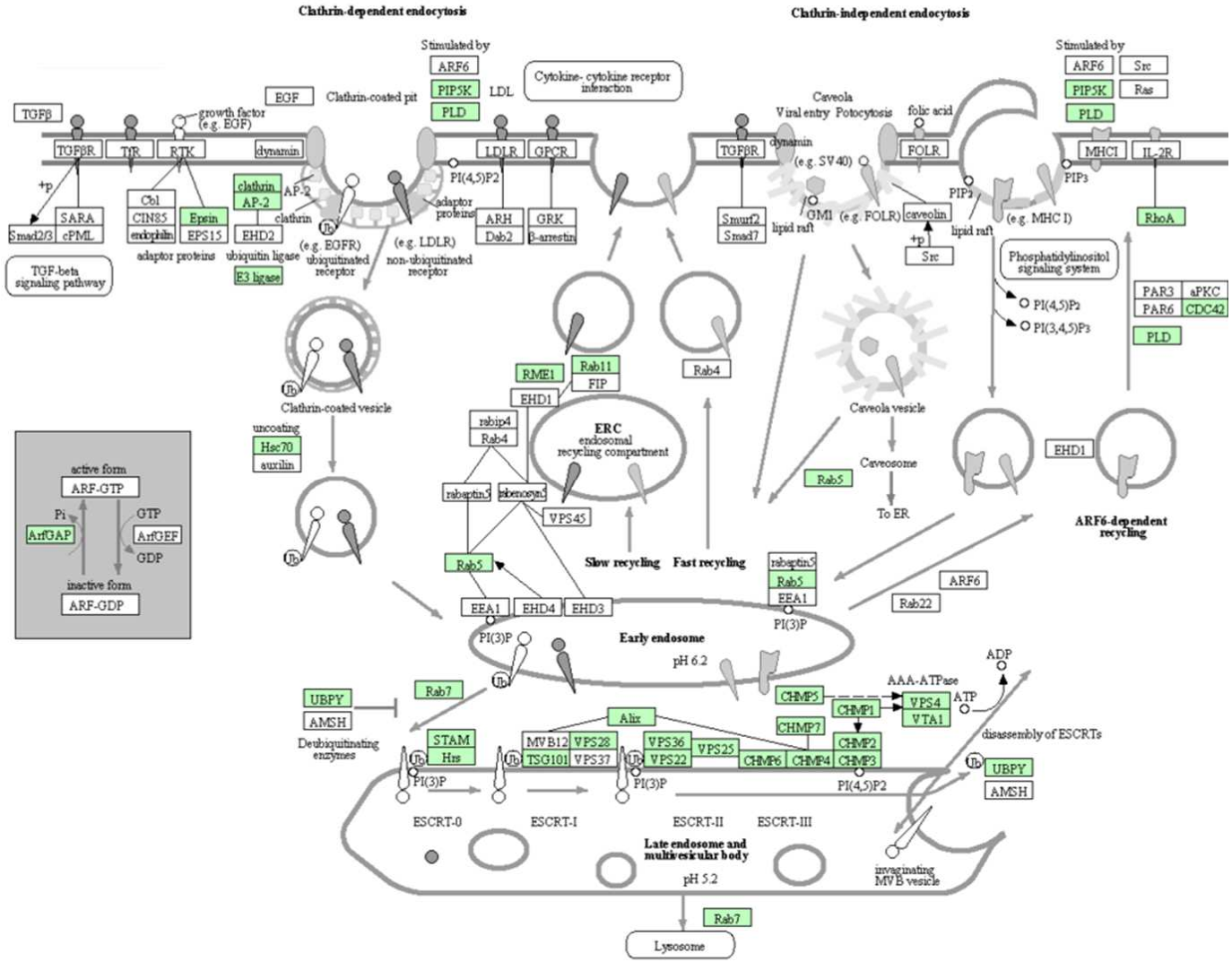
Appendix 2. Pentose phosphate pathway in *Candida albicans* from KEGG cal00030 pathway.



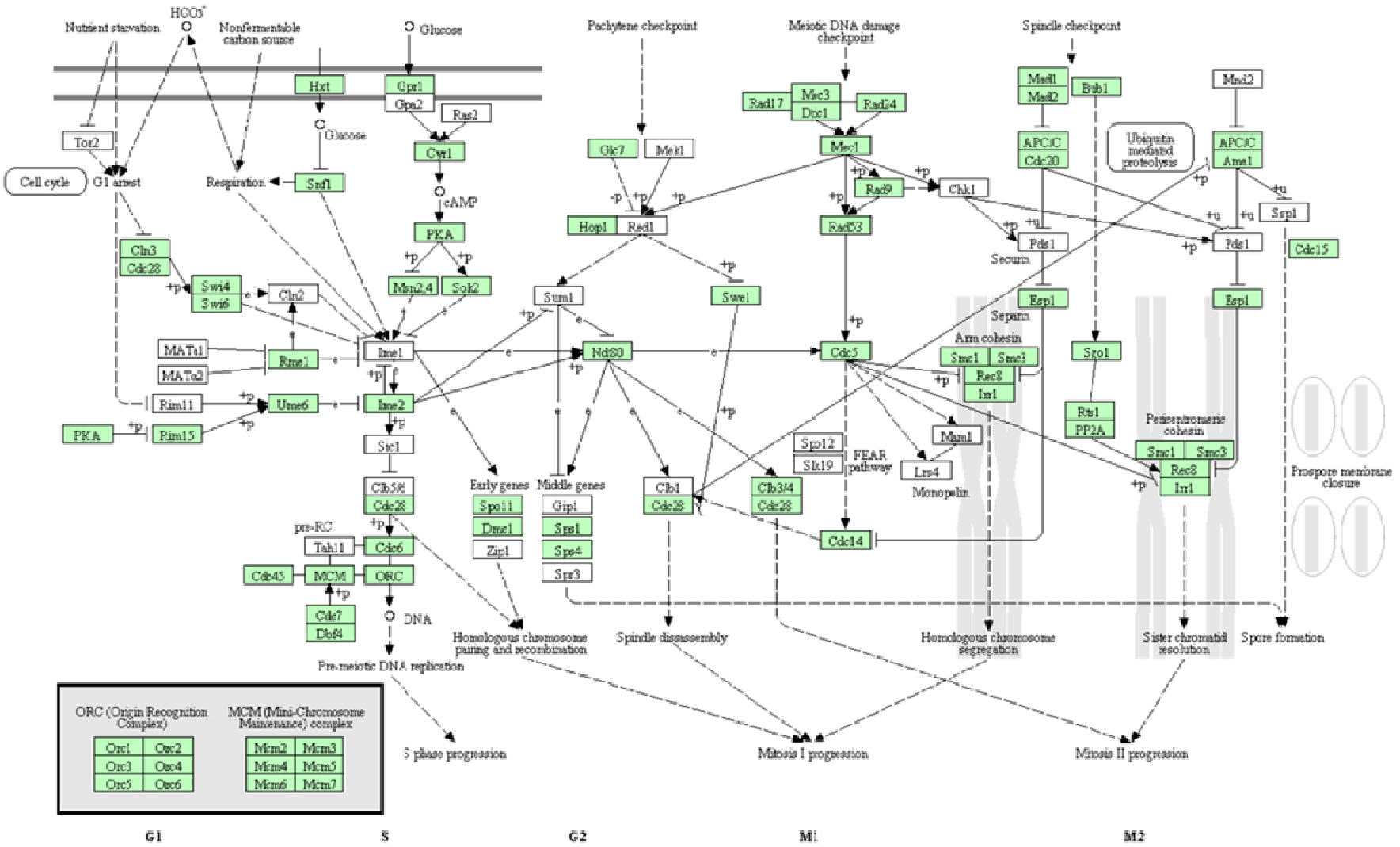
Appendix 3. Carbon fixation in photosynthetic organisms from KEGG map00710 pathway.



Appendix 4. Citrate cycle in *Candida albicans* from KEGG cal00020 pathway.



Appendix 5. Endocytosis in *Candida albicans* from KEGG cal04144 pathway.



Appendix 6. Meiosis in *Candida albicans* from KEGG cal04113 pathway.

Publication List

Journals:

4. **Ko, Hui-Ching**, Hsiao, Ting-Yin, Chen, Chiung-Tong, and Yang, Yun-Liang*. (2013). *Candida albicans ENO1* Null Mutants Exhibit Altered Drug Susceptibility, Hyphal Formation, and Virulence. Journal of Microbiology 51: 345-351. (June). SCI.

3. Chen, Yen-Ning, Lo, Hsiu-Jung, Wu, Chia-Chen, **Ko, Hui-Ching**, Chang, Te-Pin, and Yang, Yun-Liang*. (2011). Loss of Heterozygosity of *FCY2* Leading to the Development of Flucytosine Resistance in *Candida tropicalis.* Antimicrobial Agents and Chemotherapy 55: 2506-2514. (June). SCI.

2. Li, Shu-Ying, Yang, Yun-Liang, Lin, Yu-Hsin, **Ko, Hui-Ching**, Wang, An-Huei, Chen, Kuo-Wei, Wang, Chih-Wei, Chi, Hsin, Lo, Hsiu-Jung, and TSARY Hospitals. (2009). Two Closely Related Fluconazole-resistant *Candida tropicalis* Clone Circulating in Taiwan from 1999 to 2006. Microbial Drug Resistance 15: 205-210. (Sept). SCI.

1. Lin, En-Shyh. and **Ko, Hui-Ching**. (2005). Glucose Stimulates Production of the Alkaline-Thermostable Lipase of the Edible Basidiomycete *Antrodia cinnamomea*. Enzyme and Microbial Technology 37:261-265. (July). SCI.

Abstracts for Conferences:

10. Y. Chen, H. Lo, C. Wu, **H. Ko**, T. Chang, Y. Yang. (2012). Loss-of-heterozygosity of *FCY2* leading to the development of flucytosine resistance in *Candida tropicalis*. 22nd European Congress of Clinical Microbiology and Infectious Diseases. P169, Poster 822. March 31-April 3, 2012. London, UK.

9. Yang, Y.-L., **Ko, H.-C.**, Lan, C.-Y., Chen, P.-L., Lo, H.-J. (2010). Elucidating the functions of pathogenesis factors by functional genomic approaches in *Candida albicans*. Symposium of the National Research Program for Genomic Medicine. P220. March 14-15, 2010. Taipei, Taiwan.

8. **Ko, H.-C.**, Yang, Y.-L., Lo, H.-J., Chen, C.-T. (2009). *CaENO1* in *Candida albicans* is involved in the drug sensitivity and virulence in a mouse model. The Biotechnology Taiwan 2009 Conference. P83. November 6-8, 2009. Taipei, Taiwan.

7. **Ko, H.-C.**, Chen, P.-L., Tsai, H.-Y., Yang, Y.-L. (2009). *CaENO1* is involved in the drug susceptibility and virulence in *Candida albicans*. 21st IUBMB and 12th FAOBMB International Congress of Biochemistry and Molecular Biology. P68. August 2-7, 2009. Shanghai, China.

6. Yang, Y.-L., **Ko, H.-C.**, Wang, C.-W., Liou, C.-H., Lin, C.-C., Chen, H.-F., Hsiao, T.-Y., Chen, C.-T., Lo, H.-J. (2009). Mutations of *CaENO1* affect cell growth, virulence, susceptibilities to drug, and the resistance to sodium chloride in *Candida albicans*. The 17th Congress of the International Society for Human and Animal Mycology. P281, PP-01-18. May 25-29, 2009. Tokyo, Japan.

5. **Ko, H.-C.**, Lo, H.-L., Wang, C.-W., Lin, C.-C., Chen, C.-T., Yang, Y.-L. (2009). Mutations on *CaENO1* in *Candida albicans* affect the sensitivity to drugs and sodium chloride stress. Genetics and Genomics of Infectious Diseases. P14. March 21-24, 2009. Singapore.

4. **Ko, H.**, Lo, H., Chen, H., Wang, C., Liou, C., Lin, C., Chen, C., Yang, Y. (2009). *CaENO1* is involved in carbon-utilization, cell growth, and the resistances to antifungal drugs and sodium chloride stress in *Candida albicans*. The 7th International Symposium on Antimicrobial Agents and Resistance. P167, MF007. March 18-20, 2009. Bangkok, Thailand.

3. Yang, Y.-L., **Ko, H.-C.**, Lan, C.-Y., Shiu, S.-J., Lo, H.-J. (2009). Elucidating the functions of pathogenesis factors by functional genomic approaches in *Candida albicans*. 2009 Symposium of the National Research Program for Genomic Medicine. P259-260. March 8-9, 2009. Taipei, Taiwan.

2. Lo, H., Li, S., Yang, Y., Lin, Y., **Ko, H.**, Wang, A., Chen, K., Wang, C. (2008). Two dominant diploid sequence types of *Candida tropicalis* isolates with less susceptibility to fluconazole in Taiwan from 1999 to 2006. 48th Annual ICAAC/IDSA 46th Annual Meeting. P642, M-1536. October 25-28, 2008. Washington DC, USA.

1. **Ko, H.-C.**, Chi, C.-S., Lo, H.-J., Yang, Y.-L. (2008). *CaREP5* and *CaREP6* affecting drug efflux pump function in *Candida albicans*. 7th Taiwan Yeast Biology Annual Meeting (酵母菌新知研討會). September 1, 2008. Taipei, Taiwan. (Oral Presentation).

簡歷

柯惠菁
Hui-Ching Ko

教育程度

國立交通大學	生物科技學系博士	民國 94 年 ~ 民國 103 年
國立高雄大學	生物科技研究所理學碩士	民國 92 年 ~ 民國 94 年
嘉南藥理專科學校	藥學科	民國 76 年 ~ 民國 82 年

專業證照

中華民國技術士證 美容乙級	100-024104	民國 102 年
中華民國技術士證 美容丙級	100-306434	民國 102 年
教育部部定講師證書	講字第 084274 號	民國 94 年
行政院衛生署藥師證書	藥字第 025042 號	民國 89 年
考試院檢覈及格證書		民國 89 年

專業書籍著作

柯惠菁（2008）生技化粧品學，合記圖書出版社。