國立交通大學

生化工程研究所

碩士論文

研究白色念珠菌 *REP3* 突變株的功能性補救

Study of *REP3* (Regulator of Efflux Pump) functional rescue in *rep3/rep3* homozygous mutants of *Candida albicans*

研究生：吳金蓉

指導教授：楊昀良博士

中華民國九十六年七月

研究白色念珠菌 *REP3* 突變株的功能性補救

Study of *REP3* (Regulator of Efflux Pump) functional rescue in *rep3/rep3* homozygous mutants of *Candida albicans*

研究生 ： 吳金蓉　　**Student : Chin-Jung Wu**

指導教授 ： 楊昀良 博士　　**Adviser : Dr. Yun-Liang Yang**

國立交通大學

生化工程研究所

碩士論文

A Thesis
Submitted to Institute of Biological Engineering
National Chiao Tung University
in Partial Fulfillment of the Requirements
for the Degree of
Master
in
Biological Engineering
July 2007
Hsinchu, Taiwan, Republic of China

中華民國九十六年七月

中文摘要

伺機性病原菌—白色念珠菌 (*Candida albicans*) 是造成人體黴菌感染最常見的病原。Azole 類藥物常用來治療白色念珠菌的感染。然而，廣泛使用 fluconazole 使得抗藥性白色念珠菌菌株之案例大增，進而導致臨床上治療失敗的機會增高。因此，瞭解白色念珠菌抗藥機轉對於克服黴菌感染是很重要的。排藥幫浦 *CDR1* 和 *CDR2* 的過量表現常見於抗藥性臨床菌株，然而其表現被如何調控卻所知甚少。實驗室先前經由在 *Saccharomyces cerevisiae* 所進行之 library screening 發現在白色念珠菌中，*REP3* (Regulator of Efflux Pump) 能增加 *CDR1* $_{Ym990348}$ 啟動子-*lacZ* 的 β-galactosidase 活性約四倍。再者根據藥物敏感性測試的結果顯示 *REP3* 突變株對 azole 類藥物的感受性有提高的現象，但 *REP3* 突變補救株對藥物感受性卻沒有恢復。本研究重新建構突變株，但結果仍同，經 Real-time PCR 檢測顯示 *REP3* mRNA 確實存在 *rep3/rep3::REP3* 突變補救株中且表現量與野生株 SC5314 相近。意外的是，在 *rep3/rep3* 突變株、*rep3/rep3::REP3* 突變補救株及野生株 SC5314 中 *CDR1* 的 mRNA 量相近。再者，在藥物誘導下，另一個距 *REP3* 基因下游處 266 個核酸的 orf19.3926 表現量在 *rep3/rep3::REP3* 突變補救株中比 *rep3/rep3* 突變株及野生株提高了 3.5 倍。即使利用 *SAT1* 的策略再次重新建構 *rep3/rep3::REP3* 突變補救株，以減少額外載體 DNA 序列介入 *rep3/rep3* 突變株中可能造成的干擾，仍然無法補救 *rep3/rep3* 突變株對藥物敏感的表現型。由於在 *rep3/rep3* 突變株中，*CDR1* 之 mRNA 並未下降，因此，對於 *REP3* 是否透過其他非調控 *CDR1* 的路徑而影響白色念珠菌的藥物抗藥性，以及 *rep3* 突變株的功能難以補救回復的原因，將來需做更進一步的研究。

Abstract

Candida albicans is an opportunistic fungal pathogen and is the most common cause of deep mycoses in humans. Azole therapy is commonly used to treat *C. albicans* infections. However the widespread use of azoles led to an increased frequency of treatment failure due to azole-resistant *C. albicans* in clinical setting. Therefore, understanding the molecular mechanisms of drug resistance in *C. albicans* is important to render the fungal infection. Over expression of drug efflux pumps— *CDR1* and *CDR2* (*C*andida Drug Resistance 1 and 2) in azole resistant *C. albicans* is commonly observed but the regulatory mechanism is poorly understood. Previously in the laboratory, *REP3* (Regulator of Efflux Pump) was isolated from *Candida* genomic library due to its ability to increase the β-galactosidase activity of $CDR1_{YM990348}$ promoter-*lacZ* about four folds in *Saccharomyces cerevisiae* in the presence of miconazole. According to the results of the drug susceptibility tests, *rep3/rep3* homozygous mutant seems to be more susceptible to miconazole, itraconazole, ketoconazole, fiuconazole and voriconazole than the SC5314 wild-type strain in spite of *rep3/rep3::REP3* rescued strains not showing a restoration of drug susceptibility phenotype. In this study, *REP3* rescued strains were re-constructed and the outcome remained the same. No *REP3* mRNA could be detected in *rep3/rep3* mutant and the real-time PCR results showed that the *REP3* mRNA could be detected in the *rep3/rep3::REP3* rescued strains in similar quantity as that of the SC5314 wild-type strain. Surprisingly, the expressions of *CDR1* were similar in the *rep3/rep3* mutants, *rep3/rep3::REP3* rescued strains, and SC5314 wild type strain. Furthermore, the expression level of the orf19.3926, which locates at the down stream 266 bps from *REP3*, increased about 3.5 folds in the *REP3* rescued strain than that of the wild-type strains and *rep3/rep3* mutants in the presence of miconazole. Even though the *REP3* rescued strains were reconstructed through *SAT1* flipping method to eliminate the potential interference from the integration of vector DNA fragment in *C. albicans*, the regenerated *rep3/rep3::REP3* rescued strains still cannot restore completely the drug susceptibility phenotype of *rep3/rep3* mutant. Since the mRNA level of *CDR1* in *rep3/rep3* mutant was the same as that in the wild type. *REP3* may be involved in drug resistance

through pathways other than *CDR1* in *C. albicans*. There is no clear reason as the why the *rep3/rep3::REP3* rescued strain could not restore the *REP3* phenotype. Both will require further studies.

誌謝

我常以為，學如逆水行舟，而今輕舟已過萬重山。一路行來，點滴在心，因此下筆之時，自己便預料這將是一篇冗長的謝詞，雖然這不是一本完美的論文，但這本論文的完成，要感謝的人真的很多，僅以此文表達我的誠摯謝意。

由衷的感謝楊昀良教授兩年來悉心指導與啟發，培養學生獨立思考與解決問題的能力，於論文研究及撰寫期間，細心斧正，使學業得以順利完成。論文研究期間，承蒙國家衛生研究院羅秀容教授之關愛，予以學生實驗上諸多協助與指導，銘感衷心。文稿初成，復蒙本校彭慧玲教授細心校稿與口試時的指導，使本論文得已益臻完善。

於大學專題期間，感謝中興大學林金和教授懇切的教導，大葉大學游志文與洪淑嫻教授的循循善誘與細心指導，帶領我進入科學世界，讓我對科學有更深一層的體認，於此一併敬表謝忱。

國家衛生研究院的一群夥伴們；學姊佳君不論生活或實驗上的幫助，訓練我對邏輯思考的重要性，伴我走過這沒有掌聲的舞台－沒有妳給我的引導，相信今天的研究之路不會如此順利；感謝學長彰勳(Scott)，不時的給予我幫助和鼓勵；學姊惠菁，謝謝你與我一同經歷交大與國衛院來往穿梭的旅程；俐君、國瓊、仁忠、誌偉、緯婷、安慧、志兆謝謝你們的相互砥礪與支持。另一群還在交大的熱血澎湃青年；學弟妹淑萍、淑貞、旻秀、敏書；小朋友佳叡、阿毛、小倩；你們的笑聲，是這緊湊、忙碌的研究生活中不可或缺的緩衝液，永遠在我耳邊迴響，請繼續加油喔！一起並肩作戰的同學新彬、育穎，好開心我們一起劃下碩班句點。已畢業的學長姐們，大小聲怡瑾、好人杏枚、好脾氣建孝、鑽石嘴砲志豪，謝謝你們讓我以歡笑而非淚水，度過了這研究所的歲月。

嘉佑，認識你讓我知道人生的相遇並非偶然，而是必然，距離對我們倆而言並非阻礙而是另一分幸運，何其有幸的是能透過遠距離擁有你默默在背後永不歇息的支持。

我最親愛的家人阿公，感謝有你在天上的庇祐；爸、媽，謝謝你們從小到大對我無怨無悔的栽培與付出，若不是你們的支持，我不會有今日小小的成就。

最後，將此論文獻給所有愛我及我愛的你們.....

Contents

Contents (Figures)

Contents (Tables)

Chapter 1. Introduction

1.1 Clinical significance of *Candida* infection

Candida albicans is a diploid asexual fungus, and a causal agent of opportunistic oral and genital infections in humans. There are a number of reasons for an increase in fungal infections. Systemic fungal infections have emerged as important causes of morbidity and mortality in immunocompromised patients (for example, AIDS, cancer chemotherapy, organ or bone marrow transplantation). *C. albicans* is a common commensal yeast that colonizes the gastrointestinal or genital tract of 15 – 30 % of healthy humans.

C. albicans is the fourth leading cause of bloodstream infections in the United States (Beck-Sague *et al*., 1993; Pfaller *et al*., 1998; Edmond *et al*., 1999). It can cause either benign and frequent infections such as oral and vaginal candidiasis or more serious infections such as life-threatening invasive infections in immunocompromised hosts. Invasive candidiasis mainly occur in hospitalized patients and *C. albicans*, which is currently a leading cause of nosocomial infections, is responsible for 60% of cases of candidemia. The annual Medicare coasts for treating candidemia estimated to exceed $1 billion in the USA (Miller *et al*., 2001). Through a simple calculation based on an incidence rate of 8 out of 100,000 every year (Kao *et al*., 1999), 40% mortality (Edmond *et al*., 1999), and 300 million population size, there are almost 10,000 deaths per year due to *Candida* infections in the USA.

In Taiwan the prevalence of nosocomial candidemia increased 27-fold from 1981 through 1993 at one medical center at National Taiwan University Hospital in Taipei and *C. albicans* is also the most common pathogen (50.4%) (Chen *et al*., 2003). In addition,

hospital-related infections in patients not previously considered at risk (for example, patients on an intensive care unit) have become a cause of major health concern.

1.2 *Candida albicans* is an opportunistic pathogen

C. albicans is among the gut flora, the many organisms that live in the human mouth and gastrointestinal tract. Under normal circumstances, *C. albicans* lives in 80% of the human population with no harmful effects, although overgrowth results in candidiasis. It is the causes of mucosal, cutaneous, and systemic infections, including oropharyngeal candidiasis (OPC), the most frequent opportunistic infection among AIDS patients (Klein et *al* ., 1984; Feigal *et al*., 1991).

Candidiasis is often observed in immunocompromised individuals such as HIV-positive patients. HIV-positive individuals and AIDS patients often develop oropharyngeal candidiasis and, in many countries, the triazole antifungals have been the mainstay of their treatment. The widespread use of prolonged fluconazole therapy in the 1990s led to an increased frequency of treatment failure due to fluconazole-resistant *C. albicans* (Ruhnke *et al*., 1994; Boschman *et al*., 1998). It also may occur in the blood and in the genital tract. Candidiasis, also known as "thrush", is a common condition that is usually easily cured in people who are not immunocompromised.

Among the different antifungal agents available, the class of azoles has been used extensively during the past 20 years. *C. albicans* cells repetitively exposed to these antifungals pressure can lead to phenomenon of azole resistance (Coste *et al*., 2006). Due to the repeated use of this agent especially in human immunodeficiency virus-positive patients with recurrent oropharyngeal candidiasis, treatment failures were observed to be associated with emergence of azole-resistant *C. albicans* strains (Coste *et al.*, 2004).

1.3 Resistance of *C. albicans* to antifungal agents

Antifungal agents exert their activity through a variety of mechanisms, some of which are poorly understood. Drug targets that are distinct between pathogens and hosts are more difficult to identify in fungi than in bacteria, at least in part because fungi and animals are relatively close as both are eukaryotes. The available drugs to therapy fungal infection suffer from many drawbacks in term of side effects, efficacy, spectrum, resistance and cost (Sundriyal *et al*., 2006). Currently, antifungal drugs that are available for treatment of *Candida* infections are those against ergosterol and its biosynthesis, nucleic acid synthesis, and cell wall synthesis (Ghannoum and Rice, 1999; Yang and Lo, 2001). Most of the antifungal drugs are directed against ergosterol, the major sterol of the fungal plasma membrane, which is analogous to cholesterol in mammalian cells. Ergosterol is the main component of the fungal plasma membrane and plays many important roles within the cell (Joseph-Horne and Hollomon, 1997; Morschhauser, 2003; Parks, 1995). It helps maintain membrane integrity and fluidity, as well as ensuring proper function of several membrane-bound enzymes (Lupetti, 2002). Accordingly, many antifungal drug classes target enzymes in the ergosterol biosynthetic pathway. In *Candida*, mutations or changes in levels of expression of *ERG* genes can lead to alterations in drug sensitivity (Jensen-Pergakes *et al*., 1998; Jia *et al.*, 2002; Sanglard *et al*.,2003; Young *et al*., 2004). Available antifungal drugs can be divided into four major classes including (1) polyenes (2) ergosterol biosynthesis inhibitors (3) nucleic acid synthesis inhibitor and (4) cell wall synthesis inhibitor.

1. polyenes

The polyenes, such as amphotericin B, are very potent agents acting by binding to the fungal cell membrane and causing the fungus to leak electrolytes. Amphotericin B is the

gold standard for severe, life-threatening systemic fungal infections.

The polyene compounds are so named because of the alternating conjugated double bonds that constitute a part of their macrolide ring structure. The polyene antibiotics are all products of *Streptomyces* species. These drugs interact with sterols in cell membranes (ergosterol in fungal cells; cholesterol in human cells) to form channels through the membrane, causing the cells to become leaky. The polyene antifungal agents include nystatin, amphotericin B, and pimaricin.

Amphotericin B binds to sterols, preferentially to the primary fungal cell membrane sterol, ergosterol. This binding disrupts osmotic integrity of the fungal membrane, resulting in leakage of intracellular potassium, magnesium, sugars, and metabolites and then cellular death. The mechanism of action is the same for all the preparations and is due to the intrinsic antifungal activity of amphotericin B (Vanden Bossche *et al*., 1994a).

2. ergosterol biosynthesis inhibitors

Polyenes including azoles are ergosterol biosynthesis inhibitors and specifically inhibit fungal ergosterol biosynthesis. Azoles are antifungal agents that inhibit the cytochrome P450-dependent 14-α-lanosterol demethylase enzyme. Inhibition of this enzyme results in the depletion of ergosterol, a major component needed to maintain the integrity of the fungal cell wall. The azoles are subdivided into two groups: the triazoles (fluconazole, itraconazole and voriconazole) and the imidazoles (clotrimazole, ketoconazole miconazole, econazole, and sulconazole). Fluconazole and voriconazole are the two triazoles that are most widely used to treat *C. albicans* infections. It inhibits the sterol 14-α-demethylase, which is encoded by *ERG11* (Hitchcock, 1991). Inhibition of lanosterol 14-α-demethylase by azoles not only results in ergosterol depletion but also in the accumulation of the methylated sterol, which inhibits cell growth (Kelly *et al*., 1997). The triazoles replaced earlier drugs like ketoconazole because they have better pharmacokinetics, are safer, and

have better efficacy when treating systemic fungal infections. Imidazoles are chiefly used to treat cutaneous fungal infections and vulvovaginal infections.

3. nucleic acid synthesis inhibitor

5-flucytosine (5-FC) is a synthetic fluorine analog of cytosine. It is deaminated to 5-fluorouracil (5-FU) and then converted to 5-fluorodeoxyuridylic acid monophosphate, a noncompetitive inhibitor of thymidylate synthetase. This interferes with DNA synthesis. It inhibits both DNA and RNA synthesis via intracytoplasmic conversion to 5-fluorouracil. The latter is converted to two active nucleotides: 5-fluorouridine triphosphate, which inhibits RNA processing, and 5-fluorodeoxyuridine monophosphate, which inhibits thymidylate synthetase and hence the formation of the deoxythymidine triphosphate needed for DNA synthesis (White *et al*., 1998). As with other antimetabolites, the emergence of drug resistance is a problem. Therefore, 5-fluorocytosine is seldom used alone. In combination with amphotericin B it remains the treatment of choice for cryptococcal meningitis and is effective against a number of other mycoses, including some caused by the dematiaceous fungi and perhaps even by *C. albicans*.

4. cell wall synthesis inhibitor

Although both fungi and humans are eukaryotics in nature, fungal cell wall is significantly unique structure feature. The major component of fungal cell wall is 1, 3-β-glucan. The glucan synthesis inhibitors are, collectively, agents that are presumed to block fungal cell wall synthesis by inhibiting the enzyme 1,3-β-glucan synthase. The lipopeptides papulocandins and echinocandins obtained from fungal kingdom through blocking the 1,3-β-glucan synthase to inhibit fungal cell wall synthesis (Leelaporn *et al*., 1994; Radding *et al*., 1998). Inhibition of this enzyme results in depletion of glucan polymers in the fungal cell, resulting in an abnormally weak cell wall unable to withstand

osmotic stress. There are three such agents at present, with all three belonging to the chemical family known as the echinocandins: caspofungin, micafungin, and anidulafungin. Caspofungin (MK-0991; L-743, 872) has antifungal activity against yeasts of the genus *Candida* (including isolates resistant to azoles and amphotericin B), several species of filamentous fungi, including *Aspergillus*, and certain dimorphic fungi, such as *Histoplasma*, *Blastomyces* and *Coccidioides* (Letscher-Bru and Herbrecht, 2003). It is fungicidal with minimal host toxicities and is the only compound in this class approved by US Federal Drug Agency for clinical use for therapy of aspergillosis and disseminated *Candida* infection (Georgopapadakou, 2001; Tkacz and DiDomenico, 2001; Odds *et al*., 2003).

1.4 Mechanism of resistance to azole antifungal agents

To date, four resistance mechanisms have been identified in the development of azole resistance in *C. albicans*: (1) the cellular content of the azole target encoded by the *ERG11* gene can be increased; (2) the affinity of Erg11p to azoles can be decreased by mutations in *ERG11*; (3) the ergosterol biosynthetic pathway can be altered; and (4) azoles can fail to accumulate inside the cells. (Sanglard *et al*., 1998b). In *C. albicans*, one of the well-documented mechanisms of resistance to azole antifungal agents is the upregulation of multidrug transporter genes (Perea *et al*., 2001; Sangland *et al*., 1997; Sangland *et al*., 1995; White *et al*., 1997). The upregulation of multidrug transporter genes leads to the enhanced efflux of azoles and therefore results in decreased drug accumulation and reduced inhibition of their target encoded by the *ERG11* gene. Among these mechanisms, the last is the most commonly observed in clinical strains developing azole resistance and involves the upregulation of multidrug transporter genes from at least two families, i.e. *CDR1* and *CDR2* (*Candida* drug resistance 1 and 2) of the ATP-binding cassette (ABC) transporter family (Prasad *et al*., 1995; Sanglard *et al*., 1995; 1997) and *CaMDR1* (*Candida albicans*

MultiDrug Resistance 1) of the major facilitator (MF) superfamily (Sanglard *et al*., 1995; Franz *et al*., 1998). The most important mechanism of resistance to azoles is the overexpression of multidrug transporters, encoded by either the major facilitator efflux pump *CaMDR1* (MultiDrug Resistance 1) or the ABC transporters *CDR1* (Candida Drug Resistance 1) and *CDR2*. *CaMDR1* expression is controlled by at least two regulatory cis-acting promoter regions as reported recently by Harry *et al* (2005).

1.5 *Candida* drug resistance gene: *CDR1*

The *Candida* drug resistance 1 (*CDR1*) gene, which encodes an ABC efflux pump, is identified by complementation of the pleiotropic drug resistance 5 (*pdr5*) mutant, which is hypersensitive to cycloheximide, chloramphenicol, and azole drugs, in *Saccharomyces cerevisiae* (*S. cerevisiae*) (Prasad *et al*., 1995).

Cdr1 protein (~170kDa) contains two highly hydrophobic transmembrane domains (TMD) and two cytoplasmically localized nucleotide binding domains (NBD). ABC transporter proteins are located in the plasma membrane, or in organelle membranes, of organisms as diverse as *Escherichia coli* and humans. They are ATP-dependent translocators of a wide variety of small molecules, including many xenobiotics, and typically comprise alternating pairs of cytoplasmic NBDs and membrane embedded TMDs that contain six transmembrane spans (Dean, 2005). All NBD regions contain conserved motifs: Walker A and Walker B also found in other nucleotide-binding proteins and the family defining C-loop or ABC signature motif（LSGGQ）. *Saccharomyces cerevisiae* Pdr5p is the archetype of the fungal pleiotropic drug resistance PDR family of drug transporters. *C. albicans* Cdr1p and Cdr2p show approximately 70% homology with Pdr5p.

Recent studies have made valuable progress in the determination of structure/function

relationships for the primary equences of the *Cdr* pump proteins of *C. albicans*. *C. albicans cdr1*mutant resulted in increasing susceptibilities to azole drugs (Coste *et al*., 1996), which is consistent with the observation that overexpression of *CDR1* contributes to the drug resistance of clinical isolates of *C. albicans* (Lopez-Ribot *et al*., 1999; Yang *et al*., 2001). However, the molecular mechanism and the gene network regulating the expression of *CDR1* and drug resistance are poorly understood.

Understanding the transcriptional control of *CDR* genes, by both cis- and trans-acting effectors, is therefore important for determining how azole resistance and transport mechanisms are regulated in *C. albicans*.

1.6 *Cis*- and *trans*-acting factors regulating of *CDR* genes

1.6.1 *Cis*-regulatory factors of efflux pumps involved in azole resistance

There are two major strategies to identify the important *cis*-regulatory elements of the efflux pumps involved in drug resistance. (1).Through comparing the promoter sequence of the *CDRs* and *MDR1* of fluconazole-resistant *C. albicans* strains with the published promoter sequences and that of fluconazole-susceptible strains. The difference among their promoters may cause the mRNA level of efflux pumps increasing in fluconazole-resistant strains. However, like previously mentioned in the *ERG11* sequence comparison, such sequence differences may simply reflect allelic variation and by themselves do not prove a causal relationship with resistance. Morschhäuser's group compared the promoter sequences of both *MDR1* alleles of two matched pairs of clinical *C. albicans* isolates in which fluconazole resistance correlated with constitutive *MDR1* expression and did not reveal promoter mutations that might be responsible for *MDR1* activation in the resistant isolates (Wirsching *et al*., 2000). (2). Using the method of

promoter mutation analysis, for example, deletion or site direct mutation. The expression of the *CDR1* gene has been shown to be induced by agents such as miconazole, fluconazole, nystatin, vinblastine or steroid hormones (Krishnamurthy *et al*., 1998). Using the promoter serial deletion analysis, four activating and four repressing domains were identified along the entire *CDR1* promoter. The AP-1 site and the drug-responsive element of the *CDR1* promoter have been reported to be the *cis*-regulatory elements (de Micheli *et al*., 2002; Puri *et al*., 1999). Several elements of *CDR* genes are important for the regulation of *CDR1* and *CDR2*. A basal response element (BRE) is located between nt -860 and -810 in the *CDR1* promoter, and a drug response element (DRE) is present in the promoters of both *CDR1* and *CDR2* (de Micheli *et al*., 2002). Further, the DNase I footprinting revealed four *cis*-acting regulatory elements (W1:-272/-265, W2:-243/-234, W3:-209/-198 and W4:-167/-160) in the proximal promoter (-289/-34). Their mutations led to modulation of basal promoter activity to varying degrees (W1: 4.5 folds, W2: 0.2 fold, W3: 0.6 fold, and W4: 1.9 folds). A considerable promoter activity enhancement caused by W1 mutation suggests that this region is involved in downregulating basal *CDR1* promoter activity by possibly interacting with a repressor protein, and thus this sequence was designated an NRE (negative regulatory element: -272~-265). Using the NRE sequence as the affinity matrix, a ~55-kDa nuclear protein specifically interacted with the NRE was purified (Gaur *et al*., 2004). In addition, another BRE (located between -243 and -234) and a negative regulatory element (NRE) located within the -289 region have been reported in *CDR1* (Puri *et al*.1999; Gaur *et al*. 2004). The BRE regulates basal expression of *CDR1* (de Micheli *et al*., 2002), while the DRE sequence (5'-CGGAA/TATCGGATA-3') is crucial for the upregulation of these genes in azole-resistant strains as well as for the transient upregulation of both genes in the presence of different drugs such as oestradiol, progesterone, or fluphenazine in azole-susceptible strains. Finally, in the same gene, Karnani *et al*. (2004) identified SRE1 and SRE2 (steroid response elements) between -696

and -521.

1.6.2 *Trans*-regulatory factors of efflux pumps involved in azole resistance

Trans-acting factors regulating *CDR1* and *CDR2* were reported recently.

1. This factor, *CaNDT80*, is a homolog to a meiosis specific transcription factor in *S. cerevisiae* (Chu *et al*., 1998). The overexpression of *CaNDT80* decreased the susceptibility of *S. cerevisiae* to both fluconazole and ketoconazole. According to the results of agar dilution assays, strains with mutations in either *CDR1* or *CaNDT80* were more susceptible to fluconazole (25μg/ml) and voriconazole (1μg/ml). According to the real-time PCR data, homozygous null mutations in *CaNDT80* abolished the induction of *CDR1* expression in the presence of miconazole (100μg/ml). Deletion of *CaNDT80* in *C. albicans* conferred hypersensitivity to azoles and decreased the inducible expression of *CDR1*. This result is consistent with the idea that CaNdt80 is an activator of *CDR1* (Chen *et al*., 2004).
2. *Tac1* (transcriptional activator of *CDR*), a transcription factor belonging to the family of zinc-finger proteins with a Zn2Cys6motif (Coste *et al*. 2004). Tac1p binds to the DRE, which contains two CGG triplets typical of the DNA-binding sites of Zn2Cys6 transcription factors. Tac1p is responsible for transient upregulation of both *CDR* genes in azole-susceptible strains in the presence of inducers. Interestingly, *TAC1* is located close to (within~14 kb) the mating-type-like (MTL) locus. *TAC1*, a *Candida albicans* transcription factor situated near the mating-type locus on chromosome 5, is necessary for the upregulation of the ABC-transporter genes *CDR1* and *CDR2*, which mediate azole resistance (Coste *et al*., 2006). Previous studies reported a strong correlation between homozygosity at the mating-type locus and azole resistance in a number of clinical isolates (Rustad *et al*. 2002).
3. *CKA2* : Catalytic subunit (alpha-subunit) of protein kinase CK2, is similar to *S.*

cerevisiae Cka2p. *CKA2* and its homologue *CKA1* specify catalytic subunits of protein kinase CK2. Although *cka1* mutations have little effect on fluconazole resistance, *CKA1* overexpression suppresses the fluconazole resistance of a *cka2* mutant. The protein phosphatase calcineurin is required for azole tolerance, and it is found that the calcineurin inhibitor cyclosporin reverses fluconazole resistance of *cka2* mutants. The *CDR1* and *CDR2* transcript was expressed at 1.9 fold higher levels in the *cka2/cka2* strain than in the *CKA2/CKA2* strain. Therefore, the *cka2* mutation affects both *CDR1* and *CDR2* expression. Because a loss of Cka2p causes elevated expression of *CDR1*, *CDR2*, *ERG11*, *MDR1* genes. It maybe because Cka2p functions as a negative regulator of *CDR1* and *CDR2* expression (Bruno and Mitchell, 2005).

The regulation of *CDR1* involves both the *cis* element on the promoter region and other genes acting in *trans*. The study here focuses on *trans*-acting regulatory factors of *CDR1*.

1.7 Previous work in the laboratory

Although *trans*-acting factors regulating *CDR1* were reported recently, the molecular mechanism and the gene network regulating the expression of *CDR1* and drug resistance are poorly understood. Other new *trans*-factors are still yet to be discovered. In this study, as in previous studies, *S. cerevisiae* was a successfully used model to study *C. albicans*, despite the differences between these two organisms. The *CDR1* promoter for the library screening is from the Ym990348 strain. The activity of the *CDR1* promoter from Ym990348 strain is relatively low compared to that of SC5314. In addition, the screening was performed in the presence of miconazole (100μg/ml). Under the screening system, four candidate open reading frames (ORFs) have been isolated. These five ORFs were

named *REP2, REP3*, *REP4*, *REP5*, and *REP6*.

The candidate genes, *REP3,* has the C_2H_2 type zinc finger domain, which is one of the major nucleic acid-binding structures, indicating their potential roles as transcription regulators. It was then analyzed for this functions by mutagenesis using the homologous recombination technique to understand more about the regulatory mechanism of drug resistance in *C. albicans* (Chin-Sheng Chi, 2004, Master thesis, Institute of Biological Science and Technology, National Chiao Tung University).

1.8 The purpose of the study

According to the results of the Etest and Agar dilution, *rep3/rep3* homozygous mutant seems to be more susceptible to azoles (Chin-Sheng Chi, 2004, Master thesis, Institute of Biological Science and Technology, National Chiao Tung University ; Wan-Jen Wu, 2005, Master thesis, Institute of Biological Science and Technology, National Chiao Tung University). However, this result has to be further confirmed by phenotype complementation. In addition, in their study, not all the strains have the same genetic background. They differ in whether *HIS* allele is present or not.

Hence, this study focuses on construction and characterization of *rep3/rep3::REP3* rescued strains. The homozygous knockout strains were rescued with single copy of wild-type *REP3* gene. If the change of phenotype is due to the disruption of *REP3*, the wild type phenotype may be restored when the wild type *REP3* gene is knock-in to the original location. Next, the *rep3/rep3::REP3* rescued strains were tested with other antifungal drugs to examine the susceptibility to drugs.

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Strains

1. *Escherichia coli* (DH5α) Bacteria

2. *Candida* strains : Table 1

2.1.2 plasmids and primers :

plasmids, and primers used in this study are listed in Table 2 and Table 3, respectively.

2.1.3 Chemicals and reagents

◎ **Difco laboratories**

Bacto agar (Cat.No.214040)

LB agar (Cat.No.24520)

yeast nitrogen base w/o amino acid (Cat.No.291940)

LB broth (Cat.No.244620)

YPD broth (Cat.No.242820)

◎ **Invitrogen**

Agarose (Cat.No.15510-027)

1 kb plus DNA ladder (Cat.No.12308-011)

◎ **BDH**

20XSSC (Cat.No.443527N)

◎ **Bio-Rad**

50×TAE(Cat.No.161-0773)

◎ **JRH BIOSCIENCES**

Fetal bovine serum (Cat.No.12003-500M)

◎ **Sigma Chemical Co.**

Dithiothreitol (DTT) (Cat.No.D9779)

Disodium ethylenediamine-tetraacetate (EDTA) (Cat.No.E-5134)

Isoamyl alcohol (Cat.No.81k1250)

Miconazole (Cat.No.M-3512)

Glassbeads (425~600 μm) (Cat.No.G9268-500G)

Lithium acetate (CH3COOLi) (Cat.No.L-6883)

PolyethyleneGlycol3350 (PEG3350)

L-leucine

Urdine (Cat.No.U-3003)

Histidine (Cat.No.H-8125) histidine (Sigma H-9511)

arginine (Sigma A-5131)

uridine (Sigma U-3003)

◎ **Merck KGaA, 64271 Darmsdct, Germany**

Ammonium acetate (CH3COONH4) (Cat.No.1.01116.0500)

Chloroform (Cat.No.1.0244511000)

Dodecyl sulfate sodium sat (SDS) (Cat.No.113760.0100)

Dimethyl sulfoxide (DMSO) (Cat.No.S26740)

Ethanol (Cat.No.K33534874)

Glucose (Cat.No.K33069537)

Isopropanol (Cat.No.K32632434)

Potassium chloride (KCl) (Cat.No.K24252236)

Sodium acetate (Cat.No.1.06268.0250)

Sodium carbonate (Na2CO3) (Cat.No.A375692)

Sodium hydroxide (NaOH) (Cat.No.B886298)

Tris (hydroxymethyl) aminomethane hydrogen chloride (Tris-HCl)

(Cat.No.8382T006) Sodium chloride (NaCl) (Cat.No.K29779304)

Maleic acid (Cat.No.S27857)

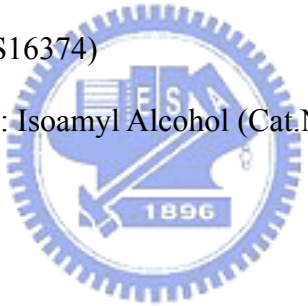
Tween 20 (Cat.No.P-1379)

Triton X-100 (Cat.No.K23841503)

◎ **USB**

Glycerol (Cat.No.US16374)

phenol : chloroform : Isoamyl Alcohol (Cat.No.US75831)



2.1.4 Buffers

◎ **TE buffer**

10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)

◎ **10X TAE buffer**

0.4 M Tris, 0.4 M glacial acetic acid, 10 mM EDTA

◎ **50％ PEG3350**

200 g polyethyleneglycol3350 added ddH_2O to 400 ml.

◎ **10X PCR buffer**

100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$ (pH 8.3)

◎ **Breaking buffer**

0.1 M Tris-HCl, 20% glycerol (v/v), 1 mM Dithiothreitol

◎ **Maleic acid buffer**

0.1 M maleic acid, 0.15 M NaCl, 0.3％ Tween 20 (pH 7.5)

◎ **Blocking soiution**

1％(W/V) blocking reagent dissolved in maleic acid buffer

◎ **Detection buffer**

0.1 M Tris-HCl, 0.1M NaCl (pH 9.5)

◎ **Denaturation Solution**

0.5 M NaCl, 1.5M NaCl

◎ **Neutralization Solution**

1.5 M NaCl, 0.5 M Tris-HCl (pH 7.5)

2.1.5 Medium

◎ **LB (Luria-Bertni) broth**

1% tryptone, 0.5% yeast extract, 1% NaCl

◎ **LB / ampicillin broth**

1% tryptone, 0.5% yeast extract, 1% NaCl, 50 g/ml ampicillin

◎ **LB / ampicillin agar**

1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar, 50 g/ml ampicillin

◎ **YPD/ Uridine broth**

2% Bacto-peptone, 1% yeast extract, 2% dextrose, 80 mg/l Uridine

◎ **YPD/Uridine agar**

2% Bacto-peptone, 1% yeast extract, 2% dextrose, 2% agar, 80 mg/l Uridine

◎ **SD (Synthetic Dextrose) / Histidine broth**

0.67% Bacto-yeast nitrogen base w/o amino acid, 2% dextrose, 20 mg/l Histidine-HCl

◎ **SD agar**

0.67% Bacto-yeast nitrogen base w/o amino acid, 2% dextrose, 2% agar, 20 mg/l Histidine-HCl

◎ **SD/ Histidine broth**

0.67% Bacto-yeast nitrogen base w/o amino acid, 2% dextrose, 20 mg/l Histidine-HCl

◎ **SD/ Histidine agar**

0.67% Bacto-yeast nitrogen base w/o amino acid, 2% dextrose, 2% agar, 20 mg/l Histidine-HCl

2.1.6 Enzymes

◎ **NEB**

*Afl*II (R0520S), *Bam*HI (R0136S), *Eco*RI (R0101S), *Eco*RV (R0195S), *Hind*III (R0104S), *Nru*I (R0192S), *Kpn*I (R0142S), *Not*I (R0189S), *Sac*II (R0157S), *Sac*I (R0156S), *Xho*I (R0146S)

CIP (M0290S)

◎ **Amersham Biosciences**

rTaq DNA polymerase (catalog no. 27-0799-06)

2.1.7 Equipments

Peltier Thermal Cycler PTC-200 (MJ Research)

Orbital shaking incubator OSI500 (TKS)

AlaImager 2000 (Molecular devices)

Vortex-2 genie (Scientific Industry)

Dry bath incubator (Violet Bioscience Inc.)

Brushless microcentrifuge Denville 260D (Scientific Industry)

Power pac 300 (Bio-Rad)

Elektroporator Gene pulser II (Bio-Rad)

Spectra max plus (Molecular devices)

Quick spin (Violet Bioscience Inc.)

Thelco laboratory incubator (Precision Scientific Inc.)

High speed refrigerated centrifuges J2-MC (Beckman)

High speed refrigerated centrifuges (Heraeus)

Centrifuge (SORVALL RT7)

Mettler AT261 DeltaRange (METTLER TOLEDO)

Mettler GG4002-S (METTLER TOLEDO)

TurboBlotter system (Schleicsher & Schuell)

Digitale camera COOLPIX 990 (Nikon)

Spectrophotometer

pH meter (HANHA instruments)

Hot plates / Stirrers (CORNING)

Water bath (CHERNG HUEI Co.)

UV crosslinker (Stratagene)

2.1.8 Strains and growth media

Escherichia coli DH5α was used as the host strain for all plasmid preparations, and was grown in Luria-Bertani (LB) medium. All constructed bacteria were grown in Luria-Bertani (LB) medium with or without antibiotics.

The *C. albicans* parental strains used in this study are SC5314 (wild type) (Gillum *et*

al., 1984) and its derivative BWP17 (*ura3*Δ::*imm434/ura3*Δ::*imm434 his1*::*hisG /his1*::*hisG arg4*::*hisG/arg4*::*hisG*) (Wilson *et al*., 1999). Yeast Peptone Dextrose, (pH6.7) was adjusting by 5 N NaOH, (YPD, 1% yeast extract, 2% peptone, and 2% dextrose and Synthetic Dextrose (SD, 0.67% yeast nitrogen base without amino acid and 2% dextrose) supplemented with necessary auxotrophic requirements [20 mg of each histidine, and arginine or 80 mg uridine per liter] were prepared as described in Sherman *et al* (Coste *et al*., 2004; Sherman, 2002). The pH of SD (pH5.4) was adjusted by 5 N NaOH.

2.2 Methods

2.2.1 DNA methods

All DNA manipulations were performed by standard procedures (Joseph Sambrook and David W.Russell, 1989). Kits from Qiagen, Inc. were used to isolate plasmids (catalog no. 12143, 12125, 27104, 20021) and to purify DNA fragments (catalog no. 28106, 28706) according to the manufacturer's protocols. Enzymes for digestion, ligation, and dephosphorylation of DNA were from Gibco BRL, *BioLabs* and Roche Molecular Biochemicals. Taq DNA polymerase (AmershamBiosciences, catalog no. 27-0799-06) and Vent DNA polymerase (*BioLabs*, catalog no. M0254S) for PCR were purchased from Amersham Pharmacia Biotech and *BioLabs*, respectively.

2.2.2 Preparing electrocompetent cells

Escherichia coli DH5α was precultured in a tube containing 5 ml of LB broth at 37ºC overnight. 5 ml overnight culture of *E.coli*. DH5α was inoculated into 1 liter LB. Cells were grown at 37ºC with shaking to an OD_{600} of 0.4 ~ 0.6 (about 3 ~ 4 h). The flask along

with the cells were chilled on ice for 15 ~ 30 min. It is essential to keep the cells cold from this point onwards. The cells were transfered to 4 chilled 500 ml centrifuge bottles on ice and spun at 5 K rpm (Beckman J2-MC) for 5 min at 4℃. The supernatant was decanted gently and pellets were resuspended in 200 ml of chilled water per centrifuge bottle (800 ml in total). Then the cells were spun at 5 K rpm (Beckman J2-MC) for 5 min at 4℃. The supernatant was decanted gently. The cells were resuspend in 50 ml of chilled water per centrifuge bottle (200 ml in total). Then the cells were spun at 5 K rpm (Beckman J2-MC) for 5 min at 4℃. The supernatant was decanted gently. The cells were resuspend in 10 ml of chilled 10% of glycerol per centrifuge bottle. Then the cells were transferred cells into 50 ml centrifuge tube and spun at 5 K rpm (Beckman J2-MC) for 5 min at 4℃. The supernatant was decanted gently. The cells were resuspend in 2 ml of chilled 10% of glycerol. 80 μl of cells were used per transformation. The cells can store at –70ºC freezer.

2.2.3 *E. coli.* transformation with electroporation

Frozen cells were thawed on ice. 80 μl of cells were mixed with 2 μl of transforming DNA in a chilled 1.5 ml microcentrifuge tube. The Bio-Rad Gene Pulse was setted at 25 μF and 2.45 kV. Pulse controller was set to 200 Ω.The cell/DNA mixture were transferred to a chilled 0.2 cm electroporation cuvette and the suspension were shacked to the bottom of the cuvette. The moisture on the outside of the cuvette was wiped off with a tissue and the cuvette was put in the slide, which was pushed into the chamber until the cuvette was seated between the contacts in the base of the chamber. Two red buttons on the machine were pushed on the left at the same time (pulse once and heard the beep). The cuvette was removed from the chamber and 1 ml of SOC broth was added immediately before the resuspension of the cells. The cells were transferred to a 1.5 ml centrifuge tube and

incubated at 37℃ for 1 hr with shaking. Cells were spun for 5 min at 3 K rpm (SORVALL RT7) and the supernatant was poured off (remained medium approximately 100 μl) before the cells were plated on LB contained ampicillin (100 μg/ml) plates after suspension.

2.2.4 Competent cell prepared using Calcium chloride

A single colony from a plate freshly grown was picked and inoculated in 5 ml LB broth for 16 ~ 20 hr. The cells transferred into 100ml LB (1:100) broth and incubated the culture for about 3 hr at 37℃ with 180 rpm till the cells reached OD_{600} = 0.4 ~ 0.6. The cells transferred to 50 ml sterile tubes. The culture store on ice for 10 min. The cells were spun 4 K rpm (SORVALL RT7) for 10 min at 4℃. Then the media were decanted and the tubes was inverted for 1 min to allow the last traces of media to drain away. Each pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and stored on ice for 15 min. The cells were spun at 4 K rpm (SORVALL RT7) for 10 min at 4℃. The media was decanted. And pellets were resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$ for each 50 ml of original culture. The cells were stored as 200 μl aliquots at -70 ℃.

2.2.5 *E. coli.* transformation using Calcium chloride prepared competent cells

One 200 μl aliquots of competent cells was taken from -70℃ and thawed on ice. Then the DNA (no more than 50 ng in a volume of 10 μl or less) was added into each tube. The tube was mixed and swirling gently. The cells with DNA were stored on ice for 30 min. The tubes (containing mixture of cells and DNA) were left at 42℃ for 2 min. The tubes were transferred rapidly to ice for 2min. Then 800 μl of SOC broth was added into each tube. The cultures were incubated for 90 min at 37℃ to allow the bacteria to recover and express the antibiotic resistance marker encoded by the plasmid. The cultures were

spun 5 K rpm (SORVALL RT7) for 5 min at room temperature. The supernatant was removed about 800 μl. The cells were resuspended and used up to 200 μl of transformed competent cells per 90-mm plate. Appropriate antibiotics were added to the plate; if blue/white selection is used, plates were coated with X-Gal. Then the plates were left at room temperature, until the liquid has been absorbed. The plates were inverted and incubated at 37℃. The transformants should appear in 12 ~ 16 h.

2.2.6 Constructions of plasmids and strains

The plasmids and primers used in this study are listed in Table II and Table III.

(1). pWJB24: (constructed by Wan-Jan Wu, 2005, Master thesis, Institute of Biological Science and Technology, National Chiao Tung University) pGEM-*REP3*-*CaHIS1*—The *C. albicans* SC5314 genomic DNA was used as a template for PCR amplification using the primers HJL504 and HJL505, both containing a *Bam*HI restriction site at the 5' end. The amplified 2.849 kb fragment started upstream to the predicted 906 bps of the orf19.3929 start codon and 91 bps downstream to the predicted orf19.3926 stop codon (TAA). After *Bam*HI digestion, the 2.849 kb fragment was introduced in a sense orientation into a binary vector (pGEM-*CaHIS1*) to create pWJB24. To rescue *REP3* function in the *rep3/rep3 his1::hisG/his1::hisG* mutant strains (CSC80 and CSC81), pWJB24 digested with *Afl*II at 310 bps upstream of the translation initiation site of the orf19.3929 was transformed into CSC80 and CSC81 to generate CJC7 ~ CJC9 and CJC10 ~ CJC12, respectively..

(2). pCJB1: A region-pSFS2-*CaSAT1*—A 201 bps fragment amplified by primers HJL930 (*kpn*I) and HJL931 (*Xho*I) from *rep3*::*ARG4*/*rep3*::*URA3*-dpl200 *C. albicans* genomic DNA. The PCR amplified program was 94℃ for 3 min followed by 30 cycles of 94 ℃ for 1 min, 55℃ for 1 min and 72℃ for 1 min 20 sec. After a final extension at

72℃ for 1 min then stop reaction at 4℃. The amplified fragment contains the sequences of dpl200:*URA3*:dpl200 from -558 ~ -721 and the sequence of *REP3* from +130 ~ +218 (Fig.18-B). After *kpn*I and *Xho*I digestion, the 201 bps fragment was ligated to a binary vector (pSFS2-*CaSAT*) to create pCJB1 ~ pCJB5.

(3). pCJB6 ~ pCJB10: pGEM-T- B fragment (*REP3*)—The *C. albicans* SC5314 genomic DNA was used as a template for PCR amplification using the primers HJL928 and HJL929, both containing *Not*I and *Sac*II restriction sites at the 5' end. The amplified fragment started +1188 of orf19.3926 to +58 of orf19.3929 (Fig.18-B). The PCR product was amplified by template of SC5314 genomic DNA, 1 μl of a 50 μM of each primer, 5 μl of 10 X PCR buffer (Amersham Biosciences), 0.6 μl of 25 mM deoxynucleside triphosphates (Amersham Biosciences), 2 μl of 25 mM $MgCl_2$, 39.2 μl of water and 0.2 μl (~ 0.9 U) of DNA polymerase mixture (*Taq*/*Vent*:9/1). The PCR amplified program was 94℃ for 3 min followed by 30 cycles of 94℃ for 1 min, 55 ℃ for 1 min and 72℃ for 3 min 20 sec. After a final extension at 72℃ for 5 min then stop reaction at 4℃. The PCR product was cloned into pGEM-T to grate pCJB6 ~ pCJB10.

(4). pCJB11 and pCJB15: A fragment-pSFS2-*CaSAT1*-B fragment (*REP3*)—The DNA fragment containing full length *REP3* purified from pCJB6 and pCJB8 was ligated with the pCJB1 plasmid (A region-pSFS2-*CaSAT1*) at *Not*I site to produce pCJB11 and pCJB15.

2.2.7 Complementation of the *REP3* gene in *rep3/rep3* mutant strains (CSC80 and CSC81)

To verify that the mutant phenotypes were indeed caused by deletion of the target gene, a functional *REP3* copy was reinserted into the CSC80 and CSC81 (*rep3/rep3 his1::*

hisG/his1:: hisG).

Figure 6 showed the genome map of reintegrating the *REP3* gene into *REP3* knockout strains (CSC80 and CSC81). pWJB24 was digested by *Afl*II which restricted at the +310 of orf19.3929. Then it was transformed into *rep3/rep3* strains and the reaction mixture was transformed on SD medium.

The *rep3/rep3* rescued strains (CJC7 ~ CJC12) were assessed by PCR with primers HJL458 and HJL505 (Fig.7).

2.2.8 Complementation of the *REP3* gene in *rep3/rep3 his1::hisG/his1::hisG::HIS1* mutants (CJC2 and CJC4) by *SAT1* method (Morschha *et al*.,2004)

The *kpn*I-*Sac*II fragments from pCJB11 (pCJB15) containing A and B fragment. B fragment is the complete open reading frame as well as 509 bps of upstream and 279 bps of downstream flanking sequences of *REP3* alleles. A fragment includes from -558 ~ -721 of dpl200:*URA3*:dpl200 to the sequence +130 ~ +218 of *REP3*. The B fragment from pCJB6 ~ pCJB8 was constructed into pCJB1 in respectively to generate plasmids pCJB11 ~ pCJB13 and pCJB14 ~ pCJB16.

Before transformation, the *REP3* rescue plasmids, pCJB11 and pCJB15, (pSFS2 plasmids with both A and B fragments) were restricted by *kpn*I-*Sac*II in respectively to generate the 6 kb fragment (Fig.18-C) were purified using QIAprep gel extraction Kit (QIAGEN). Generally, 2 ~ 5 μg of gel purified product was suspended in 10 μl of sterile distilled water and was used for one transformation. *C. albicans* strains were transformed by electroporation as described previously (Kohler *et al*., 1997), with slight modifications. The *kpn*I-*Sac*II fragment from pCJB11 and pCJB15 was transformed into strain *rep3/rep3 his1::hisG/his1::hisG::HIS1* (CJC2) in respectively to generate CJC13 ~ CJC15. pCJB11 is generated CJC13; pCJB15 is generated CJC14 and CJC15. Another *rep3/rep3*

his1::hisG/his1::hisG::HIS1 (CJC4) was integrated the *kpn*I-*Sac*II fragment from pCJB11 and pCJB15 in respectively to generate CJC16 ~ CJC18. pCJB11 is generated CJC16; pCJB15 is generated CJC17 and CJC18. For the selection of nourseothricin-resistant (NouR) transformants, 200 μg/ml of nourseothricin was added to YPD agar plates. Cells were then spread on YPD plates containing 200 μg/ml of nourseothricin and grown for 2 days at 30 ℃. The integration of the *SAT1* cassette into the *rep3/rep3 his1::hisG/his1::hisG::HIS1* genome is selected in the presence of nourseothricin.

First, using the HJL855 and HJL856 primer set, the *REP3* integration of *rep3/rep3* chromosome will give a 923 bps DNA fragment, when the chromosome carrying *rep3:: dpl200-URA3-dpl200-SAT1-REP3*. Then were given these strains celled CJC13 ~ CJC15, of which the parent is CJC2. And the CJC14 ~ CJC15 were obtained by the integration of pCJB15 and the CJC13 was of pCJB11. The strains of CJC16 ~ CJC18 (parent is CJC4)； CJC17 ~ CJC18 were obtained by integration of pCJB15 and the CJC16 was of pCJB11.

2.2.9 The *REP3* rescued strains (CJC13 ~ CJC18) excise the *SAT1* flipping (Fig 28)

In this work, FLP-mediated marker used a new dominant selection marker, *caSAT1*, conferring resistance to nourseothricin. The *REP3* rescued strains (CJC13 ~ CJC18) containing the *SAT1* cassette integrated into the *rep3/rep3 his1::hisG/his1::hisG::HIS1* genome are selected in the presence of nourseothricin. Originally, I intended to use the *MAL2* promoter to induce *caFLP* expression by growing the transformants in medium containing maltose instead of glucose (Backen *et al*., 2000; Sanchez-Martinez and Perez-Martin, 2002).

FLP-mediated excision of the *SAT1* flipper cassette is achieved by simply growing the transformants for a few days in medium with 2% maltose without selective pressure, and nourseothricin-sensitive (Nou^S) derivatives can easily be identified. After about seven

days, screening for Nou^S derivatives in which the *SAT1* flipper cassette were excised by FLP-mediated recombination. To pick up the single colony from (CJC13 ~ CJC18) after treated with 2% maltose can not grow on YPD plates containing 200 μg/ml nourseothricin to generate CJC19 ~ CJC24.

The CJC19 ~ CJC24 strains were generated and analyzed by PCR with HJL458-HJL133, HJL963-HJL964 and HJL855-HJL856 primers. To confirm the results of excision the *SAT1* flipper in the *REP3* rescued strains (CJC19 ~ CJC24) by PCR with primers HJL963-HJL964. If the *SAT1* flipper was excised, the results would be generated a 935 bps fragment (Fig.28-C). Primers of HJL458-HJL133 is positive control the *rep3::ARG4 /rep3::URA3* allele would be generated a 1.2 kb fragment (Fig. 28-B). The reintegration of *REP3* in CJC19 ~ CJC24 was confirmed by PCR with HJL855 and HJL856 that generated a 923 bps fragment (Fig.28-D). As result shown in Fig.28, I have been got the *REP3* rescued strains without *SAT1* cassette (CJC19 ~ CJC24).

2.2.10 Isolation of genomic DNA

An isolated single colony was picked and inoculated at 6 ml YPD or YPD + uridine (80 mg/l) broth in 15 ml centrifuge tube. The cells were shaken at about 180 rpm and incubated at 30℃ for 24 ~ 48 h. Cells were spun at 3 K rpm (SORVALL RT7) for 10 minutes, the supernatant was poured off and the cell pellets were washed with 5 ml ddH_2O. Cells were spun at 3,000 rpm (SORVALL RT7) for 10 min, the supernatant was poured off and the cell pellets were resuspended with 500 μl DNA breaking buffer by vortexing and then acid-washed glass beads (~ 400 μl) and 500 μl of phenol/choroform/isoamyl alcohol (25:24:1) were added. The mixtures were vortexed at highest speed for 10 min at room temperature. 500 μl TE was added and the mixtures were vortexes for a few seconds.

Cells were spun at 3 K rpm (SORVALL RT7) for 15 min at room temperature (RT) and aqueous layer were transferred to a new 15 ml centrifuge tube.

Then about 850 μl of (equal volume) phenol/choroform/isoamyl alcohol (25:24:1) were added and the mixtures were vortexed for 60 sec. Phenol extract steps were repeated 3 times then aqueous layer was transferred to 2 new 1.5 ml eppendorf tube (~ 400 μl /tube). 1 ml (2.5 X volume) of cold 100% ETOH was added and the solution was mixed by inversion. The solution were spun at 13 K rpm (Brushless microcentrifuge Denville 260D, Scientific Industry) for 10 min at 4℃ , the supernatant was discarded carefully. The DNA pellet was dried in the air about 10 ~ 15 min. The pellets were resuspended in 0.4 ml of TE buffer with 3 μl of 10 mg/ml RNase A. They were mixed and incubated for ~ 15 min at 37℃. Then, 40 μl of 3 M ammonium acetate (pH 5.2) and 1 ml of cold 100% ETOH were added and mixed by inversion. They were spun at 13 K rpm (Brushless microcentrifuge Denville 260D, Scientific Industry) for 10 min at 4℃ and the supernatant was discarded carefully followed by adding 1ml of cold 70% ETOH to wash DNA pellet. They were spun at 13 K rpm (Brushless microcentrifuge Denville 260D, Scientific Industry) for 5 min at 4℃. The supernatant was removed carefully. The DNA pellet was dried in the air about 15 min and then resuspended in 100 ~ 200 μl of ddH_2O.

2.2.11 Transformation of *Candida albicans* by lithium acetate method

The protocol for yeast transformation was modified from the previous report (Gietz *et al*., 1995). *C. albicans* cells were grown overnight in YPD/Uri medium on a shaker at 30 ℃ to stationary phase. The culture was diluted into fresh YPD/Uri medium in 1/100 ratio (OD_{600} of about 0.1) and was incubated at 30℃ with shaking (about 150 rpm) for an additional 6 h (OD_{600} of about 1.0 ~ 0.7). Cells were centrifuged at 3000 rpm in SORVALL RT7 for 10 min at room temperature, washed once with sterile ddH_2O, TE buffer

(pH 7.5), and lithium acetate/TE buffer 〔100 mM lithium acetate (Sigma L6883), 10 mM Tris-HCl, 1 mM EDTA〕, sequentially. The washed cell pellet from 10 ml of original culture was resuspended in 0.1 ml lithium acetate/TE buffer for one transformation reaction. Transformation mixtures contained 5–10 μg DNA, 10 μl of 10 mg/ml of boiled Salmon Sperm DNA (sperm DNA was boiled in a PCR machine for 2 min and then it was kept on ice right away), and 100 μl of *C. albicans* cells. The transformation mixture was incubated at room temperature for 30 min. To this mixture, 700 μl of PEG/lithium acetate/TE buffer 〔40% polyethyleneglycol 3350PEG (Sigma P4338), 100 mM lithium acetate, 10 mM Tris-HCl, 1 mM EDTA〕 was added and briefly vortexed. Subsequently, the transformation mixture was incubated for overnight at 30°C, followed by a heat shock of 60 min at 42°C. Cells were pelleted, washed once with TE buffer (pH 7.5) and plated onto selective media and incubated at 30°C for at least 2 ~ 3 days.

2.2.12 Transformation of *Candida albicans* by electroporation method

C. albicans strains were transformed by electroporation as described previously (Kohler *et al*., 1997), with slight modifications. Cells from a YPD preculture were diluted 10^{-4} in 50 ml fresh YPD medium and grown overnight at 30℃ to an optical density at 600 nm (OD_{600}) of 1.6 ~ 2.2. The cells were collected by centrifugation and resuspended in 8 ml of water. After addition of 1 ml of 10 ×TE (100 mM Tris–HCl, 10 mM EDTA, pH 7.5) and 1 ml of 1 M lithium acetate (Sigma-Aldrich Chemie, Steinheim, Germany), pH 7.5, the suspension was incubated in a rotary shaker at 150 rpm for 60 min at 30℃. A 250 μl volume of 1 M dithiothreitol was then added, and the cells were incubated for a further 30 min at 30℃ with shaking. After addition of 40 ml of water the cells were centrifuged, washed sequentially in 25 ml of ice-cold water and 5 ml of ice-cold 1 M sorbitol, resuspended in 50 μl of 1M sorbitol, and kept on ice.

The inserts from plasmids were purified by agarose gel electrophoresis and elution with the QIAprep gel extraction kit (Cat. NO 28706). Five microliters (approximately 1 μg) of the linear DNA fragments was mixed with 40 μl of electrocompetent cells, and electroporation was carried out in a 0.2 cm electroporation cuvette, 1.8 kV.

After electroporation, the cells were washed in 1 ml of 1 M sorbitol, resuspended in 1 ml YPD medium, and incubated for 4 h with shaking at 30℃. The cells were spread on YPD plates containing 100 μg/ml of 4 h nourseothricin and grown at 30℃. Resistant colonies were picked after 1 day of growth and inoculated in YPD liquid medium containing 100 μg/ml of nourseothricin for DNA isolation. In parallel, the transformants were streaked on YPD plates with 100 μg/ml of nourseothricin for further use.

2.2.13 Southern blot analysis

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. Briefly, probe1 is the 634 bps of PCR fragment of *ARG4* from plasmid pRSARG4△Spe1 (LOB16) which was amplified by primer HJL311 and HJL312. And probe2 is the 613 bps of PCR fragment of *URA3* from plasmid pGEM-*URA3* (LOB15) which was amplified by primer HJL632 and HJL633. The probes are DIG labeled by random priming (PCR DIG Probe Synthesis Kit. Cat. No. 11 636 090 910), using a 1:3 ratio of DIG-dUTP:dTTP according to the length of the probe 1 (634 bps)、probe 2 (613 bps). The PCR amplified program was 95℃ for 2 min followed by 10 cycles of 95℃ for 30 sec, 58℃ for 30 sec and 72℃ for 40 sec followed by 20 cycles of 95℃ for 30 sec, 58℃ for 30 sec and 72℃ for 1 min in a Peltier Thermal Cycler-200 (MJ Research, Watertown, MA). After a final extension at 72 ℃ for 4 min then the reaction was stopped at 4℃.

A total of 10 μg of genomic DNAs from BWP17、SC5314、*rep3::ARG4/REP3* (CSC21

and CSC22)、*rep3::ARG4/rep3::URA3* (CSC80 and CSC81) and *rep3/ rep3::REP3* (CJC7 and CJC10) were digested with *Hind*III and size-fractionated by 0.8% agarose gel electrophoresis on Sub-Cell GT system (BioRad, catalog no. 170-4404). The 634 bps the *ARG4* fragment amplified by primers HJL311 and HJL312 was used as a positive control. Another positive control *URA3* was amplified by HJL631 and HJL633.

The DNAs were transferred by capillarity (TurboBlotter Rapid Downward Transfer system, Schleicher & Schuell, Item no. 10416328) onto Nytran SuPerCharge membrane (Schleicher & Schuell, Item no. 10416296). On UV crosslinker (Stratagene, catalog no. 400075), DNA was immobilized on membrane by pressing two times of autocrosslink mode (120mJ/ pulse).

Appropriate volume of DIG Easy Hyb (approx.20 ml/100 cm^2) was pre-heated to hybridization temperature 42℃. The blot was incubated for 30 min with gentle agitation. The DIG-labeled DNA probe (20 ng/ml for DNA-probe) was boiled for 10 min for denaturation and was rapidly cooled on ice. It was then added to pre-heated DIG Easy Hyb (at least 3.5 ml/100 cm^2 membrane) and mixed well. Prehybridization solution was then discarded and immediately, the probe/DIG Easy Hyb mixture was added to membrane. The hybridization mixture was incublated with gentle agitation at 42℃ overnight.

The membrane was washed as recommended by the manufacturer under high-stringency conditions with twice of 2 X wash solution (2 X SSC, 0.1% SDS) at room temperature for 5 min, and twice of 0.5 X wash solution (0.5 X SSC, 0.1% SDS) at 65℃ for 15 min. After hybridization and stringency washes, the membrane was rinsed briefly 5 min in Washing buffer. Then it was incubated for 30 min in 100 ml Blocking solution. And then it was incubated for 30 min in 20 ml Antibody solution. Next, it was washed 2*15 min in 100 ml Wahing buffer. After equilibrated for 2 ~ 5 min in 20 ml Dectection buffer, the membrane was placed with DNA side facing up on a development folder (or hybridization bag) and 2 ml diluted CSPD solution was applied. The membrance was

covered immediately with the second sheet of the folder to spread the substrate evenly and without airbubbles over the membrane. It was then incubated for 5 min. Excess liquid was squeezed at the edges of the development folder was sealed. The damp membrane was incubated for 15 min at 37℃ to enhance the luminescent reaction.

2.2.14 Antifungal susceptibility test with agar dilution assay

The agar dilution method was used to determine the susceptibility to antifungal agents for *C. albicans*. First of all, cells were diluted to an OD_{600} of 2 (approximately 2 X 10^7/ml). And the original cells were treated with 10-fold serial dilution with 0.85% NaCl (dilute it to 10, 100 and 1000 times).

The seeding tray was placed on the Replicator device (0.5 μl/spot). The lid was removed from each repliplate media in turn and then the plate was placed on the Replicator device. Each plate was inoculated by smoothly swinging the inoculating head assembly from the wells to the plate surface. Cells were grown on pH5.4 SD plates, DMSO or water as control, respectively for 2 days at 35ºC.

2.2.15 Antifungal susceptibility test with broth microdilution method

Antifungal susceptibility assay was performed by broth microdilution method according to the M-27A standard protocol approved by the National Committee of Clinical Laboratory Standards (NCCLS). Briefly, *C. albicans* strains were grown on SD plates containing drugs for 2 days at 30℃. Single colonies were resuspended in SD medium 30 ℃ overnight. The antifungal agents were freshly prepared as a stock at the concentration in DMSO. A drug-free culture and a sterile control were included in each microtiter plate. Cultures were further serially diluted 20-fold with SD medium and 100 ml of the final

dilution was added to each of a 96-well microtitre plate containing 100 ml of SD medium with the appropriate concentration of drugs.

Cells were incubated in 96-well U-bottom microtiter plates at 35°C, and the OD600 was measured by a microplate spectrophotometer (Benchmark Plus; Bio-Rad Laboratories) at 24 and 48 hr. The MIC50 was defined as the drug concentration required for 50% growth inhibition compared to that in the drug-free culture.

2.2.16 Isolation of RNA by the hot acid phenol method

C. albicans was scratched from the plate into a 15 ml disposable tube containing 3 ml sterilized SD or other desired medium and then was incubated at 30℃, 180 rpm, overnight.

The cells were grown in SD medium at 30℃ overnight and next day the cells started with adjusting to MaFarland 2.0, and then diluted 1/10 fold into SD medium for 6 hour growing at 35℃ with shaking at 200 rpm. The miconazole (Sigma M3512) dissolved in dimethyl sulfoxide (DMSO) was added to the cell cultures to a final concentration of 40 μg/ml after cells were incubated at 35℃ with shaking until OD_{600} = 0.7 ~ 0.9 about 6 hrs. The cells were harvested 1.5 hr after the addition of miconazole. Equal amount of DMSO was added to cell cultures as controls.

The cells were then centrifuged at 3,200 rpm, 4°C in SORVALL™ RT7. The supernatants discarded and re-suspend pellet was kept in 1 ml ice-cold DEPC-H_2O. The culture was transferred to 2 ml centrifuge tube and was centrifuged at 13,000 rpm for 1 min in a table centrifuge (Brushless microcentrifuge Denville 260D, Scientific Industry) at 4℃. The supernatant was discarded. Pellet was re-suspended in 1 ml ice-cold water and cells were centrifuged at 13,000 rpm in a table centrifuge (Brushless microcentrifuge Denville 260D, Scientific Industry) for 1 min at 4℃ then the supernatant was discarded. Next, the supernatant was removed after the cells were microcentrifuged for 10 sec at 4℃. If

desired immediately freeze pellet by placing tube in dry ice then stored in -80℃.

The cells were then to add 400 μl RNA isolation buffer (50mM NaOAc+10mM EDTA) and mix well by vortex. And then 40 μl 10% SDS and a lot of glass beads were added. The mixtures mixed well by vortex. Then 500 μl pre-heat (65℃) acid phenol (pH 4.0) were added to incubate 10 min at room temperature by vortexing each minute at 65℃. The upper layer was transferred into a new 2 ml tube and spun at 13,000 rpm for 5 min at 4℃. Then 500 μl pre-heat (65℃) acid phenol (pH 4.0) were added by vortex for 1 min at 65℃ and incubated 10 min. Equal volume phenol/chloroform/isoamyl alcohol (25:24:1) were added. The mixtures were vortexed for1 min at 4℃ and then were centrifuged for 5 min. Upper layer was transferred into a new 2 ml tube. Equal volume chloroform/isoamyl alcohol (24:1) were added and mixtures were vortexes for 1 min. The mixtures were spun at 13,000 rpm (Brushless microcentrifuge Denville 260D, Scientific Industry) for 5 min at 4℃. RNA was precipitated from the resulting aqueous layer by transferring that protein to new 2 ml tube containing 1/10 volume 3M, pH 5.2, NaOAc and 2.5 volume ice-cold 100% EtOH, mixing well. The mixtures were mix well and were placed -80℃ for 30 min.

The supernatant was removed and was spun at 13,000 rpm (Brushless microcentrifuge Denville 260D, Scientific Industry) for 20 min at 4℃. Then RNA pellet was washed with 700 μl ice-cold 70% EtOH and was spun at 13,000 rpm (Brushless microcentrifuge Denville 260D, Scientific Industry) for 10 min at 4℃. The supernatant was removed. RNA pellet was air-dried (White pellet become invisible). Re-suspend in 51.5 μl of DEPC-H_2O.

(DNase treatment)

DEPC-H_2O 140 μl and 200 μl 65℃ acid phenol (pH 4.0) were added. The mixtures were vortexes for 1 min and spun at 13,000 rpm (Brushless microcentrifuge Denville 260D, Scientific Industry) for 5 min at 4℃. The upper layer was transfered into a new 2

ml tube. Equal volume phenol/chloroform/isoamyl alcohol (25:24:1) was added. To vortex the mixtures for 1 min and centrifuge at 13,000 rpm (Brushless microcentrifuge Denville 260D, Scientific Industry) for 5 min at 4℃. The upper layer was transfered into a new 2 ml tube. Equal volume chloroform/isoamyl alcohol (24:1) was added. Then the mixtures was vortexes for 1 min and was spun at 13,000 rpm (Brushless microcentrifuge Denville 260D, Scientific Industry) for 5 min at 4℃. To adjust final volume to 125 μl with DEPC-H_2O. The culture mix well with 12.5 μl 3M, pH 5.2 NaOAc and 315 μl ice-cold 100% EtOH to place at -80℃, overnight. Then the sample were spun at 13,000 rpm (Brushless microcentrifuge Denville 260D, Scientific Industry) for 20 min at 4°C. The supernatant was removed. RNA pellet was washed with 700 μl ice-cold 70% EtOH and was spun at 13,000 rpm (Brushless microcentrifuge Denville 260D, Scientific Industry)for 5 min at 4°C. Then the supernatant was removed. RNA pellet was air-dried. RNA pellet was re-suspended in 20 μl DEPC-H_2O. To measure RNA concentration: aliquot 1 μl RNA into 59 μl DEPC-H_2O.

2.2.17 Isolation of RNA by the Masterpure Yeast Purification Kit

C. albicans cells was scratched from a saturated 1.5 ml culture (approximately 4 X 10^7 cells) by centrifugation in a microcentrifuge at 13,000 rpm for 2 ~ 5 minutes. All growth medium was removed. Extraction Reagent for RNA containing the Proteinase K was added 300 μl to each microcentrifuge tube of tissue collected in cells. The cells was suspended by either vortex mixing or pipetting the cells repeatedly using a 1 ml capacity pipet tip. The suspended cells were incubated at 70℃ for 15 min. And the cells were vortex to mix every 5 min. The samples were placed on ice for 5 min. 175 μl of MPC Protein Precipitation Reagent was added and the mixtures were vortex to mix for 10 sec. The debris of pellet was spun at 13,000 rpm in a microcentrifuge (Brushless

microcentrifuge Denville 260D, Scientific Industry) for 10 min at 4℃. The supernatant was transferred into a clean microcentrifuge tube and 500 μl of isopropanol was added. The mixtures were mix thoroughly by inversion 30 ~ 40 times. The RNA was pelleted by being spun at 13,000 rpm in a microcentrifuge (Brushless microcentrifuge Denville 260D, Scientific Industry) for 10 min at 4℃. All of the residual isopropanol were removed with a pipet. 200 μl of DNase I solution was prepared for each sample. 20 μl of 10 X DNase buffer was added to 175 μl of deionized water, then was mixed with 5 μl of RNase-Free DNase I. The nucleic acid pellet was resuspended completely in 200 μl of DNase I solution. The pellet was incubated at 37℃ for 30 min. 200 μl of 2 X T and C Lysis Solution was added then the mixtures was vortexes for 5 sec. Then 200 μl MPC Protein Precipitation Reagent (solution may become cloudly) was added. Next, the mixtures were vortexed for 10 sec, and were placed on ice for 3 ~ 5 min. The debris of pellet was spun at 13000 rpm (Brushless microcentrifuge Denville 260D, Scientific Industry) for 10 min at 4 ℃ in a microcentrifuge. The supernatant containing the RNA was transferred into a clean microcentrifuge tube and the pellet was discarded. 500 μl of isopropanol was added to the supernatant. The tube was inverted 30 ~ 40 times. Then the mixtures were spun at 13000 rpm (Brushless microcentrifuge Denville 260D, Scientific Industry) for 10 min at 4℃ in a microcentrifuge. The isopropanol was poured off or aspirated without dislodging the RNA pellet carefully. The pellet was rinsed twice with 70% ethanol. If the pellet is dislodged, spin the tube briefly. All of the residual ethanol was removed with a pipet. The RNA was resuspended in 50 μl of DEPC-H_2O.

2.2.18 Quantitative analysis of the mRNA level by real-time polymerase chain reaction (real-time PCR)

The real-time PCR was performed in a Rotor-Gene™ 3000 instrument (Corbett

Research, Australia) with a TITANIUM ™ Taq PCR kit (BD Clontech 639210) and SYBR®Green I Nucleic Acid Stain (Cambrex 50513) to determine the level of mRNA.

The real-time PCR was performed according to the instructions from the manufacturer and the expression of *CaSNF3* in each strain was used as the loading control. The relative quantitation was based on two standard curves for comparisons and the results were given as a ratio (Kofron *et al*., 1999). The primer pairs HJL360-HJL361 for *ERG3,* HJL590-HJL591 for *ERG11*, HJL393-HJL394 for *MDR1,* HJL272-HJL273 for *CPH1*, HJL315-HJL316 for *CDR1*, HJL712-HJL713 for *REP3*, HJL896-HJL897 for orf19.3926, HJL898-HJL899 for orf19.3929 were used to amplify PCR products by using the LightCycler Probe Design program provided by the manufacturer. The Light Cycler DNA Master SYBR Green I kit (Roche, Germany, catalog no. 2 015 099) was used as described by the manufacturer. The condition for Real-Time PCR was: denaturation (2 min at 95℃), 45 cycles of repeated amplification (20 sec at 95℃, 20 sec at 62℃ and 20 sec at 72℃) and detected amplicon's fluorescence signal at 80 ~ 82°C. A serial dilution of cDNA obtained from the SC5314 strain were analyzed in each Light Cycler experiment and the results were used to create standard curves. Using these standard curves, I could compare the levels of mRNA among different strains. After cycling, relative quantitation used two standard (the reference gene and the target gene) curves for comparisons and the results were given as a ratio (Kofron *et al*., 1999; Filion *et al*., 2003). The expression of *CaSNF3* (HJL178-HJL179) in each strain was used to normalize the expression levels of target genes. For miconazole induction assay, the level of RNA isolated from different strains without miconazole treatment was defined as 100%. The relative level of mRNA isolated from a certain strain with miconazole treatment was normalized with the level of mRNA isolated from the same strain without miconazole treatment.

The primer pair, HJL293 and HJL294, of which the sequences were deduced from the intron and exon of the *C. albicans CaEFB1* gene was used to distinguish the PCR products

resulting from either the genomic DNA (928 bps DNA fragment) or the spliced RNA (564 bps DNA fragment) (Schaller *et al*., 2003).

Chapter 3. Results

3.1 Generation of histidine prototrophy of *rep3/rep3* mutant (Fig.2)

The *REP3* (orf19.3928 ; contig19-10194) was previously identified for increasing the β-galactosidase activity of *CDR1p-lacZ* about four-fold in *S. cerevisiae* in the presence of miconazole (Chin-Sheng Chi, 2004, Master thesis, Institute of Biological Science and Technology, National Chiao Tung University). In a previous study, *rep3/rep3* homozygous mutants (CSC80 and CSC81; *rep3/rep3*; *his1:: hisG/his1:: hisG*) have been generated in BWP17 (Chin-Sheng Chi, 2004, Master thesis, Institute of Biological Science and Technology, National Chiao Tung University). For comparative analysis of the phenotype of *rep3/rep3* mutant and the nutrition prototrophy *C. albicans*, SC5314, I started to rescue the *rep3/rep3* histidine auxotrophy in CSC80 and CSC81. The *CaHIS1*-containing plasmid pDDB78 (Elisabetta *et al.*, 2003) was digested with *Nru*I and transformed into *rep3/rep3*; *his1/his1* strains (CSC80 and CSC81) to obtain histidine prototrophy of *rep3/rep3* strains by being selected on SD without histidine. The CJC1 ~ CJC6 strains were picked and analyzed by PCR with HJL606-HJL607 primers. If the *HIS1* integrated into *his1* locci, an 890 bps fragment would be generated (Fig.2). There were *HIS1* rescued strains (CJC1 ~ CJC6) generated (Fig.2). In addition, I confirmed the *HIS1* phenotype of these strains (CJC1 ~ CJC6) on his$^-$ medium.

3.2 The antifungal drugs susceptibility of histidine prototrophy *rep3/rep3* is identical with histidine auxototrophy *rep3/rep3* (Fig.3).

In a previous study, the *rep3/rep3* homozygous mutants (CSC80 and CSC81) appeared

to be more susceptible to fuconazole, itraconazole, and voriconazole according to the E-test assay (Chin-Sheng Chi, 2004, Master thesis, Institute of Biological Science and Technology, National Chiao Tung University) and more sensitive to miconazole in agar dilution assay (Wan-Jen Wu, 2005, Master thesis, Institute of Biological Science and Technology, National Chiao Tung University). As shown in Fig.3, the *cdr1/cdr1* cells required at least 1000 cells to grow, *REP3/REP3* (SC5314) cells required 10 cell and *rep3/rep3* cells required at least 100 cells. The *rep3/rep3* (CSC80 and CSC81) is more sensitive to fluconazole (6 μg/ml), miconazole (0.3 μg/ml), ketoconazole (0.6 μg/ml), itraconazole (0.4 μg/ml) and voriconazole (1 μg/ml). When compared, the phenotype of *rep3/rep3; his1/his1::HIS1* (lane 4 ~ lane 6 and lane 10 ~ lane 12 ; CJC1 ~ CJC6) is the same as the *rep3/rep3* (lane 3 & lane 9 ; CSC80 & CSC81).

3.3 The *REP3* rescued strains YLO221 and YLO222 cannot restore the drug susceptibility phenotype of *rep3/rep3* (Fig.4).

The *REP3* homozygous null mutant strain is more sensitive to some of the azoles. This result has to be further confirmed by phenotype rescuing (complementation). A previous student in the laboratory, (Wan-Jen Wu, 2005, Master thesis, Institute of Biological Science and Technology, National Chiao Tung University) has constructed the *rep3/rep3::REP3* strains YLO221 and YLO222. According to the agar dilution assay, when compared *rep3/rep3; his1/his1::HIS1* (CJC2 and CJC4) with the *rep3/rep3::REP3* strains (YLO221 and YLO222), both required at least 100 cells in the fluconazole (6 μg/ml), miconazole (0.3 μg/ml), ketoconazole (0.6 μg/ml), itraconazole (0.4 μg/ml) and voriconazole (1 μg/ml). The *rep3/rep3::REP3* strains (YLO221 and YLO222) do not restore the drug susceptibility phenotype of *rep3/rep3* (Fig.4).

3.4 The *REP3* sequence of the rescue plasmid (pWJB24) is identical with the *REP3* sequence of *Candida* Genome Database (CGD) (Fig.5).

To understand the problem why *rep3/rep3::REP3* strains (YLO221 and YLO222) can not restore the drug susceptibility phenotype of *rep3/rep3*, the *REP3* sequence of the rescue plasmid (pWJB24) was analysis against *REP3* sequence in the *Candida* Genome Database (CGD). For this purpose, the *REP3* rescue plasmid (pWJB24), which has been constructed previously in the laboratory (Wan-Jan Wu, 2005, Master thesis, Institute of Biological Science and Technology, National Chiao Tung University) was subjected to sequencing analysis. The *Candida* Genome Database (CGD) (http://www.candida genome.org/) provides the sequences of assembly contig19-10194 of *C. albicans*. The pWJB24 integrative plasmid, which derived from pGEM-*CaHIS1*, contains a 2.849 kb fragment which was amplified with primers HJL504 and HJL505 including full-length ORF of *REP3* (orf19.3928). The fragment starts from 91 bps downstream of the stop codon of orf19.3926 to 906 bps upstream of start codon of orf19.3929. The sequences comparison between the *REP3* sequence of the rescue plasmid (pWJB24) with orf19.3928 of contig19-10194 is shown in Fig.5. Besides *REP3* sequences, there are some other ORFs in the 2.849 kb fragment, including 906 bps of the orf19.3929 and 91 bps of the orf19.3926.

3.5 Re-construction the *REP3* rescued strains (CJC7~CJC12) with pWJB24 (Fig.7)

What I want to know is whether the rescue transformation causes mutations in *rep3/rep3::REP3* to affect the rescue strain not able to restore the phenotype of *rep3/rep3*. The integration strategy for re-rescuing *REP3* in *rep3/rep3* mutant strains is shown in Fig.6 (Wan-Jan Wu, 2005, Master thesis, Institute of Biological Science and Technology,

National Chiao Tung University). To rescue *REP3* function in the *rep3/rep3* mutant strain, the linear pWJB24 (digested with *Afl*II) was transformed into the *rep3/rep3; his1 /his1* strains (CSC80 and CSC81) to generate CJC7 ~ CJC9 and CJC10 ~ CJC12, respectively. The transformants were selected for His^+ phenotype. As depicting in Fig.7, the HJL505 and HJL458 primer set will generate DNA fragments in 3 different sizes : 3.7 kb, 3.4 kb, and 2.7 kb DNA fragments. The integration of *REP3* can generate a 2.7 kb fragment. An approximately 3.4 kb PCR product would be generated due to the integrated *URA3* allele, and finally a 3.7 kb was due to the integration by *ARG4* allele (Fig. 7 and 8).

3.6 The *REP3* was disrupted at the proper chromosomal location and the *ARG4* or *URA3* marker did not integrated into unpredicted chromosome locations (Fig. 8 and 9).

The *REP3* homozygous knockout and rescued strains have to be confirmed by Southern-blot analysis. Previously, (Chin-Sheng Chi, 2004, Master thesis, Institute of Biological Science and Technology, National Chiao Tung University) the vicinity around the *ARG4* or *URA3* integrated sites have been determined by PCR. But it can not determine the genomic structure. Therefore, in this study, Southern blot was used to confirm whether the marker gene is integrated at the proper chromosomal locations and whether the copy number of the marker is correct.

In this study, I performed Southern blot analysis on *rep3/rep3*; *his1/his1*::*HIS1* (CJC2 and CJC4) and *REP3/rep3*::*ARG4* (CSC21 and CSC22) (Chin-Sheng Chi, 2004, Master thesis, Institute of Biological Science and Technology, National Chiao Tung University) and *rep3/rep3*::*REP3* (CJC7 and CJC10) to determine whether the *ARG4* or *URA3* markers were integrated correctly with probes from *ARG4* and *URA3* sequences, Probe 1 and Probe 2 (represented by the gray arrows in the Fig.8 and Fig.9, respectively). Southern analyse

were performed on *Hind* III-digested genomic DNA of the wild type strain SC5314, parental strain BWP17, heterozygous mutants, homozygous knockout strains and rescued derivatives with the *ARG4*-specific Probe 1 (Fig.8). If the *ARG4* fragment locates at the correct position in the *REP3/rep3* heterozygous mutant and *rep3/rep3* homozygous mutant strains, the sizes of the hybridizing fragments (in kb) would be 4.1 kb. And in *rep3/rep3*::*REP3* strains (CJC7), it would produce a 12.6 kb band. According to the result of Southern blot, the *REP3* was integrated into the predicted site. In Fig.8 lane 2 ~ lane 8, a 6.1 kb signal was detected. The parental strain of lanes 2 ~ lane 8 is BWP17 (*arg4/arg4 ura3/ura3 his1/his1*), of which part of the indigenous *ARG4* sequence is still in the genome, therefore the 6.1 kb signal.

Fig.9 shows the result of the Southern hybridization using probes against *URA3*. *REP3/rep3* heterozygous mutant strains can not produce signals because there is no *URA3* sequence that can be detected by the probe. The 4.2 kb fragments were as expected from these *rep3/rep3* heterozygous mutants, indicating that the *URA3* was located at the correct position. A 12.5kb fragment was observed in the products from these *rep3/rep3*::*REP3* rescued strains (CJC10), indicating that the *REP3* was integrated into the predicted site.

According to the Southern blot (Fig.8 and Fig.9), *REP3* was disrupted at proper chromosomal location in the *REP3/rep3* heterozygous (CSC21 and CSC22) and *rep3/rep3* homozygous (CJC2 and CJC4) mutants. The process of *REP3* deletion did not cause the *ARG4* or *URA3* the insert to gene locations other than *REP3*. The *REP3* was integrated into the *rep3::ARG4* and the *rep3::URA3* allele in *REP3* rescued strain (CJC7) and CJC10, respectively.

3.7 The *REP3* rescued strains (CJC7 and CJC10) did not show a restored drug susceptibility phenotype of *rep3/rep3* (Fig. 10, 11, 12 and 13).

3.7.1 The agar dilution assay (Fig.10)

As previously discussed, the *rep3/rep3::REP3* strains (YLO221 and YLO222) can not restore the drug susceptibility phenotype of *rep3/rep3* (Fig.4). The *REP3* sequences of the *REP3* rescue plasmid (pWJB24) were identical with that in *Candida* Genome Database (CGD) according to sequencing analysis. Southern blots confirmed the constructions of *REP3/rep3* heterozygous (CSC21 and CSC22), *rep3/rep3* homozygous mutants (CJC2 and CJC4) and *REP3* re-rescued strains (CJC7 and CJC10).

To understand the *REP3* involvement in drug resistance, I tested the drug susceptibility of the re-constructed *rep3/rep3* homozygous mutants (CJC2 and CJC4) and the *rep3/rep3::REP3* strains (CJC7 and CJC10) by agar dilution assay. The concentrations of drugs used were fluconazole (6 μg/ml), miconazole (0.3 μg/ml), ketoconazole (0.6 μg/ml), itraconazole (0.4 μg/ml) and voriconazole (1 μg/ml).

On the plates, the *rep3/rep3* cells (lane 3 and lane 4) required at least 100 cells to grow and SC5314 (lane 1) cells only required 10 cell. But the level of drug susceptibility of *rep3/rep3::REP3* rescued strains (CJC7 and CJC10 ; lane 5 and lane 6) were the same as *rep3/rep3* mutants strains (lane 3 and lane 4). In the presence of serum, the *rep3/rep3* mutant cells were as resistant to drugs as wild type. The *rep3/rep3* mutant cells were more susceptible to five azoles than the wild type. Although the *rep3/rep3::REP3* rescued strains contains one copy of the wild-type *REP3*, it did not behave like a wild type.

3.7.2 Broth microdilution method (according to NCCLS standard) (Fig.11, 12, and 13)

To confirm that *REP3* is involved in the pathway of antifungal drugs resistance in *C. albicans*, the MICs of antifungal drugs for various *C. albicans* strains with mutations on *REP3* were measured.

The drug susceptibilities of *rep3/rep3* mutant and *rep3/rep3::REP*3 rescued strains were determined by broth microdilution method according to the CLSI (formerly NCCLS) M-27A. The wild type strain (*REP3/REP3*) was 50% inhibited by 0.25 mg/ml miconazole, but *rep3/rep3* homozygous mutants (CJC2 and CJC4) and the *rep3/rep3::REP3* strains (CJC7 and CJC10) were inhibited completely. The *rep3/rep3* mutant cells were more susceptible to miconazole (MIC, 0.125 ~ 0.25 μg/ml) (Fig.11). As the result shown in Fig.12, wild-type strain (*REP3/REP3*) was 50% inhibited by 0.0625 mg/ml itraconazole, but *rep3/rep3* homozygous mutants (CJC2 and CJC4) and the *rep3/rep3::REP3* strains (CJC7 and CJC10) were completely inhibited. The *rep3/rep3* mutant cells were more susceptible to introconazole (MIC, 0.0312 ~ 0.0625 μg/ml). According to the result of the real-time PCR (Fig.15), the expression level of *MDR1* in *rep3/rep3* homozygous mutants (CJC2 and CJC4) was about 3.5 folds higher than that in *REP3/REP3*. However, in Fig.13 the *rep3/rep3* mutant cells were not more resistant to 4-nitroquinoline-N-oxide (4-NQO) (MIC, 0.03125 ~ 0.125 μg/ml) than the wild-type cells.

This is consistent with the result of agar dilution assay. The *rep3/rep3::REP3* rescued strains were the same as the homozygous *rep3/rep3* mutant strains.

3.8 Comparison of the genes expression level of *rep3/rep3* mutants and the rescued strains (CJC7 and CJC10)

A real-time PCR was performed with an LC FastStartDNA Master SYBR Green I kit in a LightCycler instrument (catalog no. 2239264; Roche, Mannheim, Germany) to determine the level of mRNA of *REP3* and several other genes. The real-time PCR was performed according to the instructions of the manufacturer. The expression of *SNF3* in each strain was used as a control.

3.8.1 *REP3* expressed in wild type and rescued strains (CJC7 and CJC10) but not in the knockout strains (CJC2 and CJC4) (Fig.14).

There was no *REP3* mRNA detected in the *rep3/rep3* mutant (CJC2 and CJC4), consistent with the null mutation on *REP3*. As expected, the expression of *REP3* can be detected in the *REP3* rescued strains (CJC7 and CJC10) (Fig.14) with the same quantity of wild-type (*REP3/REP3*).

3.8.2 Mutations in *REP3* do not decrease the *CDR1* expression (Fig.14).

The expression of *CDR1* was increased by treating the cells with miconazole (Fig.14). In the presence of miconazole, the level of *CDR1* mRNA in the *REP3/REP3* strain was increased 3.5 folds. (Fig.14). However, homozygous null mutations in *REP3* do not reduce the expression of *CDR1* in the presence of miconazole. The *CDR1* expression level is not significantly different in the *rep3/rep3* null mutations (CJC2 and CJC4) and the rescued strains (CJC7 and CJC10) in the present of miconazole (Fig.14).

The expression of *ERG3*, *ERG11* and *CPH1* in the *rep3/rep3* null mutations (CJC2 and

CJC4) showed no difference in the present of miconazole (Fig.15 and Fig.16). Surprisingly the expression of *MDR1* is about 3.5 folds higher in the *rep3/rep3* than that in *REP3/REP3*. And in the presence of miconazole, the expression level of *MDR1* increased about 3 ~ 3.5 folds in *rep3/rep3* and *rep3/rep3::REP3* (Fig.15).

3.8.3 The expression level of orf19.3926 increased about 3.5 folds in the *REP3* rescued strains than that of wild-type and *rep3/rep3* in the presence of miconazole. (Fig.17).

The primers HJL896-HJL897 and HJL898-HJL899 design for real-time PCR to detect the expression level orf19.3926 and orf19.3929, respectively. The genome locations of these primers in *REP3/REP3* and *rep3/rep3::REP3* are indicated respectively in Fig.17-A and Fig.17-B.

Interestingly, the expression level of the orf19.3926, which locates at the down stream 266 bps from *REP3*, increased about 3.5 folds in the *REP3* rescued strain than that of the wild-type strains and *rep3/rep3* mutants in the presence of miconazole (Fig.17-C).

3.9 Construction of the *REP3* rescue plasmid (*SAT1* flipping method).

Since the *REP3* rescued strains (CJC7 and CJC10) can not restore the *REP3* phenotype, I tried to reconstruct a new rescued strain through *SAT1* flipping method.

3.9.1 Construction of A fragment into pSFS2 to create CJB1 ~ CJB5:

First I constructed plasmids pCJB1 ~ pCJB5 based on the plasmid pSFS2 (Fig.18-A) with A fragment (pCJB1 ~ pCJB5) (Fig.18-B). The pSFS2 plasmid carries the *SAT1*

cassette and with several unique restriction sites on both sides of the *SAT1* flipper target gene for homologous recombination. A fragment containing the sequences of dpl200: *URA3*:dpl200 from -558 ~ -721 and the sequence of *REP3* from +130 ~ +218 was amplified by primers HJL930 (*kpn*I) and HJL931 (*Xho*I) from *rep3*::*ARG4* /*rep3*::*URA3*-dpl200 genomic DNA (Fig.18-B). After *kpn*I and *Xho*I digestion, the purified 201 bps fragment (A fragment) was ligated to pSFS2 to create pCJB1 (Fig.19-A). The transformants (pCJB1 ~ pCJB5) were confirmed by *kpn*I-*Xho*I with 7 kb and 201 bps (Fig.20) and *Sac*I-*Sac*II with 4 kb and 2.8 kb (Fig.21).

3.9.2 Construction of CJB6 ~ CJB10: pGEM-T easy vector with B fragment.

The 1.931 kb B fragment of *REP3* (from orf19.3926 start codon +1188 to +58 of orf19.3929) was amplified by PCR from Can14 genomic DNA with primers HJL928 (*Not*I) and HJL929 (*Sac*II) (Fig.18-B). The purified B fragment was digested with *Not*I and *Sac*II and then was cloned into pGEM-T easy vector to generate pCJB6 and pCJB8 (Fig.19-B). The transformants (pCJB6 ~ pCJB10) were confirmed by *Eco*RI to generate fragments of 3 kb, 1.5 kb and 400 bps (Fig.22).

3.9.3 Comparison of the *REP3* rescue plasmid sequence between pWJB24 and pCJB6 ~ pCJB8.

The transformants (pCJB6 ~ pCJB8) were subjected to sequence analysis. Previous result has showed that *REP3* on the rescue plasmid WJB24 has the same sequence as the *C. albicans* genome database (CGD) (http://www.candidagenome.org/). On the other hand, I found that the *REP3* sequences from pCJB6 ~ pCJB8 plasmids have some differences with that of pWJB24 and CGD (Fig.23). The comparison of the amino acid sequences of the

Rep3p from pWJB24 and pCJB6 ~ pCJB8 is shown in Fig.24.

3.9.4 Construction of the plasmid CJB11 and CJB15 with A and B fragments on both sides of *SAT1* from pSFS2 (Fig.24).

The B fragment contains the full length of *REP3* from pCJB6~pCJB10. According to the sequencing result (Fig.23), I decided to choose pCJB6 and pCJB8 plasmids for digestion with *Not*I and cloning the B fragment into CJB1 (pSFS2 with the A fragment) (Fig.20), respectively, to create pCJB11 ~ pCJB13 and pCJB14 ~ pCJB16. Then, *Sac*II digestion was used to check candidates for either sense or anti-sense directions (Fig.25). If the insert is in the sense direction, *Sac*II digestion can produce 9.131 fragments ; otherwise it will produce 7.2 & 1.93 kb fragment. Therefore, pCJB14 is an anti-sense direction. The plasmids pCJB11, pCJB12, pCJB13 and pCJB15 are pSFS2 plasmids with both A and B fragments (Fig.26).

3.10 Construction and confirming the *rep3/rep3*::*REP3* (with *SAT1* method) rescued strains (Fig.28)

Plasmids pCJB11 and pCJB15 contain A and B fragments. A fragment was used to amplify the *rep3*::*URA3-dpl200*-based cassette with primers HJL930 and HJL931, which replaced the region from the +130 ~ +218 bps of *REP3*. The B fragment contains sequence from the 279 bps down stream of the stop codon to 509 bps up stream of the translation initiation site of the *REP3* gene. The wild-type genomic DNA was used as the DNA template to amplify this 1.9 kb fragment. To obtain the *REP3* rescued strains, pCJB11 and pCJB15 digested with *kpn*I-SacII respectively were integrated into the *rep3/rep3* mutants (CJC2 and CJC4) to create the strains CJC13~CJC18. The parental

strain of CJC13-pCJB11, CJC14-pCJB15 and CJC15-pCJB15 is CJC2. The parental strain of CJC16-pCJB11, CJC17-pCJB15 and CJC18-pCJB15 is CJC4. During the 2% maltose treatment, the *rep3/rep3::REP3* rescued strains (CJC13 ~ CJC18) adapted *FLP* gene that allows the subsequent excision of the *SAT1* cassette, which is flanked by *FLP* target sequences, from the genome (Joachim Morschha *et al*.,2004). It was assessed by PCR with HJL963 and HJL964; HJL458 and HJL133 and HJL855 and HJL856. As depicted in figure 28-B,C,D, the HJL458 and HJL133 primer set; the HJL963 and HJL964 primer set and the HJL855 and HJL856 primer set will generate 1,200 bps, 935 bps and 923 bps DNA fragments, respectively. The strains with chromosomes carrying *rep3::URA3-dpl200 -REP3* were called CJC19 ~ CJC24.

3.11 The *REP3* rescued strains (*SAT1* cassette method) (CJC19 ~ CJC24) can not show a restored phenotype of drug susceptibility (Fig.29, 30 and 31).

3.11.1 The agar dilution (Fig.29)

The susceptibility of these mutants and rescued strains to different classes of antifungal were determined by the agar dilution assay on SD plates (Fig.29) with different drug concentrations, of which the ranges were determined by a broth microdilution method. Cells grew in all spots in the absence of drugs. The concentrations of drugs used were fluconazole (6 μg/ml), miconazole (0.3 μg/ml), ketoconazole (0.6 μg/ml), itraconazole (0.4 μg/ml) and voriconazole (1 μg/ml) in *C. albicans*. The results presented in Fig.29 indicate that, in general, the mutants with *rep3/rep3* mutations (CJC2 and CJC4) (lane 3 and lane 10) were more susceptible than the wild type to miconazole, itraconazole, ketoconazole, fluconazole and voriconazole (Fig.29). On the plates, the *rep3/rep3* cells required at least

100 cells to grow and SC5314 (wild type) cells only required 10 cell. Few *cdr1*/*cdr1* cells grew on medium with drugs inoculated with 1000 cells. Above all, the phenotypes of the rescued strains (CJC19 ~ CJC21 or CJC7) (CJC22 ~ CJC24 or CJC10) were not restored significantly (Fig.29). The rescued strains CJC19 ~ CJC21 (CJC22 ~ CJC24) showed slightly lower susceptibility than CJC7 (CJC10) (Fig.29).

3.11.2 Broth microdilution method (according to NCCLS standard) (Fig.30 and 31)

The antifungal drugs susceptibilities of the *rep3/rep3* mutant (CJC2) and *rep3/rep3::REP*3 rescued strains (CJC19 ~ CJC21 and CJC7) were determined by broth microdilution tests following the NCCLS M-27A protocol. The *rep3/rep3* mutant cells were found to be more sensitive to miconazole (MIC, 0.125 ~ 0.25 μg/ml) (Fig.30) and introconazole (MIC, 0.0312 ~ 0.0625 μg/ml) (Fig.31) than the wild-type cells. Comparison of the *rep3* mutant strain (CJC2) with the *rep3/rep3::REP3* rescued strains with *SAT1* flipper method (in gray color) (CJC19 ~ CJC21) shows there was no restoration of the drug susceptibility phenotype to *rep3/rep3*. Although the *rep3/rep3::REP3* (CJC7) rescued strain is more sensitive than the *rep3/rep3::REP3* (CJC19 ~ CJC21) but their MICs are the same.

As the results of the agar dilution and broth microdilution, the *rep3/rep3::REP3* reconstructed through *SAT1* flipping method still can not restore completely the drug susceptibility phenotype to *rep3/rep3*.

Chapter 4. Discussion

4.1 Genomic organization of the *REP3* locus

REP3 is a putative transcription factor with zinc finger DNA-binding motif known as orf19.3928 in the Stanford *Candida albicans* genome sequence database assembly 19. It is also known as: orf19.11410*, IPF23263.1, IPF10936.2, Contig4-2817_0008,* orf*6.4048, CA2230*. *REP3* has two sequence identical alleles, one is orf19.3928 and the other is orf19.11410. Orf19.3929 and orf19.3926 are located upstream 451 bps and downstream 266 bps to *REP3* (orf19.3928), respectively. It is possible that the expression of *REP3* is controlled not only by the up stream 451 nts sequences from *REP3* start codon but also the sequence in orf19.3929. The *REP3* special genome organization also of *REP3* cause the difficulty for the construction of *rep3/rep3::REP3* rescued strains. Since *REP3* have been almost completely deleted in *rep3/rep3* mutant strains (CSC80 and CSC81), it is very difficult to have a suitable restriction enzyme site on *REP3* upstream of the *REP3* rescue plasmid that can create the homologous sequence ends for integrating *REP3* in *rep3/rep3* mutant strains and at the same time, these *REP3* rescue events would not affect the expression of the neighboding orf19.3929 and orf19.3926.

4.2 The phenotype of *REP3* rescued strains

According to the agar dilution data, the *rep3* homozygous null mutant strains (CJC2 and CJC4) were more susceptible to drugs than the wild-type strain. When the wild type *REP3* gene was integrated back to homozygous knockout mutants (CSC80 and CSC81), there is no complementation. This result was confirmed by agar dilution and the broth

microdiluction method according to the CLSI M-27A guideline. Firstly, the sequence data showed that the *REP3* sequence in the rescue plasmid pWJB24 does not have any point mutation. Secondly, Southern blot showed that *URA3* and *ARG4* genes were integrated at proper chromosomal locations and the copy numbers of the integrated marker were correct in the *REP3* heterozygous (CSC21 and CSC22) and *rep3* homozygous mutant strains (CJC2 and CJC4). For these *REP3* rescued strains (CJC7 and CJC10), the *REP3* gene was integrated at *ARG4* allele in the CJC7 and at *URA3* allele in CJC10. According to the sequence data and southern blot assay results, the construction should be correct. These results indicate that *REP3* rescued strains (CJC7 and CJC10) may have other issues.

The possible conjectures are: (1). Since *rep3/rep3* homozygous mutant strains are from "the same" *REP3/rep3* heterozygous strain, the drug susceptibility phenotype of those *rep3/rep3* may be due to factors in the *REP3/rep3* heterozygous strain. Thus, the role, if any, of the drug susceptibility phenotype in *REP3/rep3* heterozygous requires to be further determined. (2). *REP3* (orf19.3928) is very close to neighboring genes; orf19.3929 and orf19.3926. On this situation, it is difficult to design integration of *REP3* in *rep3* homozygous strains. This may cause alteration in genome structure of the rescued strains (CJC7 and CJC10). Such difference will then affect these two ORFs. (3). According to the real-time PCR data, the *REP3* rescued strains (CJC7 and CJC10) can express *REP3*. This demonstrated that the transcription level does not have any problems in the *REP3* rescued strains. I conjecture that it means the process of translation is not normal. The process of translation may have problems and affects the phenotype of drug susceptibility in *REP3* rescue strains. (4). Interestingly, real-time PCR result indicated that orf19.3926 expression level differed in the wild-type, *rep3* mutants, and the *REP3* rescued strains in the present of miconazole (Fig.16).

Although proper *REP3* rescued strains design can avoid possible pitfalls mentioned above, construction of rescued strains directly by *SAT1* flipping method would eliminated

all potential problems associated with the use of original strategy. After the *SAT1* flipping cassette was excised, the construct is confirmed by PCR in the rescued strains CJC19 ~ CJC24. The results of broth microdilution assay indicates that the phenotype of drug susceptibility in the *REP3* rescued strains (CJC19 ~ CJC24) using *SAT1* flipping method was not restored in the presence of miconazole and itraconazole. These results indicate that *REP3* rescued strains (CJC7 and CJC10) may still have other issues. The *SATI* flipping method was used to construct the *REP3* rescued plasmid and sequencing of three independent clones (pCJB11, pCJB13 and pCJB15) showed that the two *REP3* alleles differed from one another, which was not noted in CGD (Candida Genome Database). These sequence differences might have functional consequences, but it have to be demonstrated in the future.

4.3 The relationship with drug resistance between *trans*-regulator factor, *REP3* and *CDR1*.

Although Rep3p can increase the β-galactosidase activity of $CDR1_{YM990348}$ promoter-*lacZ* about four folds in *S. cerevisiae* in the presence of miconazole, it is not known whether it also involved in drug resistance through regulation of the expression of *CDR1* in *C. albicans*. *CDR1* is known to be involved in efflux of fluconazole, ketoconazole, itraconazole, and miconazole (Nakamura *et al.*, 2001) and cells lacking Cdr1p becomes hypersensitive to fluconazole, itraconazole, and ketoconazole (Sanglard *et al.*, 1996). However, homozygous null mutations in *REP3* do not abolish the induction of *CDR1* expression in the presence of the miconazole (Fig.14). This maybe because (A). The experiment conditions are not suitable for real-time PCR. In this condition, I can not detect the *CDR1* expression level decrease in the *REP3* null mutant strains. (B). In this study, as in previous studies, the labratory has successfully used *S. cerevisiae* as a model to

study *C. albicans*. However, there are differences between these two organisms. Therefore, overexpression of *REP3* induces the expression of $CDR1_{YM990348}$ promoter-*lacZ* in *S. cerevisiae*, otherwise, mutations on *REP3* does not affect the expression of *CDR1* in *C. albicans*. Alternatively, the observation of overexpression of *REP3* induces the expression of $CDR1_{YM990348}$ promoter-*lacZ* in *S. cerevisiae* may be due to an artifact of overexpression. (C). *REP3* may be involved in another unknown gene in drug resistance. The genes known to involve in drug susceptibility include *MDR1*, *ERG3*, *ERG11*, and *CPH1*. However neither in the presence of miconazole nor not, gene expressions of *ERG3*, *ERG11*, and *CPH1* in the *rep3/rep3* null mutations showed no difference with *REP3/REP3*. It suggests that *REP3* affects the drug susceptibility in *C. albicans* through another mechanism to regulate the expression of another gene. The expression of *MDR1* increased about 3.5 folds in the *rep3/rep3* mutants in the absence or presence of miconazole beyond expectation. Mdr1p is classified in the major facilitator superfamily of efflux transporters and exports a variety of structurally unrelated compounds, including fluconazole, benomyl, methotrexate, cycloheximide, 4-NQO, and sulfometuron methyl (Ben-Yaacov *et al*., 1994). *C. albicans* cells lacking the *MDR1* gene are hypersensitive to most of the drugs listed above (Goldway *et al*., 1995). However, according to the agar dilution *rep3/rep3* mutants are more susceptible to fluconazole (Fig. 3). Similarly, mutations on *REP3* is more sensitive to 4-NQO than wild-type according to the broth microdilution. These drug susceptibility results raise the question why the mutant phenotype can not correspond with the real-time PCR. Maybe the expression of *MDR1* in *rep3/rep3* can be further determined with Northern blot. However, one possible interpretation of these different results is that there are multiple mechanisms control-ing the expression of *MDR1*.

4.4 Other uncharacterized protein factors probably bind to elements within the *MDR1* promoter and *trans*-activate the *MDR1* gene.

Despite the frequency with which *MDR1*-overexpressing, fluconazole-resistant strains being isolated, the molecular mechanisms leading to high-level expression of *MDR1* are not well understood (Lyons *et al*., 2000). Wirsching *et al*. demonstrated that in two independent fluconazole-resistant clinical strains of *C. albicans*, *MDR1* overexpression was due to mutations in an unidentified *trans*-regulatory factor(s) (Wirsching *et al*., 2000). Recent work by Hiller *et al*. indicates that three independent regions of the *MDR1* promoter are capable of contributing to *MDR1* expression in a fluconazole-resistant strain overexpressing *MDR1*. Three regions of the *MDR1* promoter are important for *trans*-activation in the *MDR1*-overexpressing strain from a fluconazole-resistant clinical isolate: region 1 (−397 to −300), region 2 (−588 to −500), and region 3 (−287 to 209) (Hiller *et al*., 2006). The portion of the *MDR1* promoter they term region 2 (−588 to −500) mediates benomyl induction of *MDR1* transcription in the fluconazole-sensitive laboratory strain CAI4 (Hiller *et al*., 2006). The more-proximal *cis*-acting element (−399 to −299) was responsible for benomyl-induced transcription of *MDR1* while the more-distal *cis*-acting element (−601 to −500) was implicated in *MDR1* induction by oxidizing agents. The most-distal region identified by both groups (−601/−588 to −500) contains a sequence that resembles and functions like the YAP1-responsive element of *S. cerevisiae* (Harry *et al*., 2005). Yap1p is a member of the bZIP family of transcription factors and regulates the *S. cerevisiae FLR1* gene, encoding a multidrug efflux protein that is similar to *C. albicans* Mdr1p (Alarco *et al*., 1997). The homologous *C. albicans* gene is termed *CAP1* (for *C. albicans* AP-1) (Alarco *et al*., 1999). Surprisingly, deletion of *CAP1* in *C. albicans* did not lead to FLC hypersensitivity (Alarco *et al*., 1999). When the *CAP1* gene was disrupted in a laboratory-derived FLC^R strain that overexpresses *MDR1*, increased levels of *MDR1*

mRNA were observed, indicating that Cap1p acts as a negative regulator of *MDR1* in this strain and is not responsible for the preexisting *MDR1* overexpression.

The MDRE (*MDR1* drug resistance element) is a small region of the *MDR1* promoter. The fluconazole-resistant strains contain a factor that binds the MDRE which was sufficient to mediate high-level *trans*-activation of *MDR1* (Perry *et al*., 2006). The MDRE (−295 to −261) shares a 26-bp overlap with the 78-bp region 3 (−287 to −209) described by Hiller *et al*. and may identify the same *cis*-acting element. These results raise the possibility that the MDRE contributes to *MDR1* expression in both laboratory-isolated and clinically isolated fluconazole-resistant strains.

It has also been demonstrated that independently isolated fluconazole-resistant laboratory strains, *MDR1* overexpression was also achieved through *trans*-activation of *MDR1*, suggesting that this may be the most common mechanism for *MDR1* overexpression (Perry *et al*., 2006). Therefore, other uncharacterized protein factors probably bind to elements within the *MDR1* promoter and *trans*-activate the *MDR1* gene in fluconazole-resistant strains.

4.5 *REP3* is a homolog of *CRZ1*, a target of the calcineurin pathway, in *Candida albicans.*

REP3 is known as orf19.3928. Rep3p containing six zinc finger C_2H_2 type repeats, which bind nucleic acids, has weak similarity to human *ZNF655*, which is a putative transcription factor that interacts with human *VAV1* and human *CDK4* and downregualtes cell cycle progression. *REP3* is homologous to *CRZ1* (calcineurin-responsive zinc finger 1 gene) at about 35%. Previous in vitro studies reveal that the antifungal properties of fluconazole, terbinafine, and fenpropimorph against *C. albicans* can be synergistically enhanced by the addition of FK506 or cyclosporine (Cruz *et al*., 2002; Marchetti *et al*., 2000;

Onyewu *et al*., 2003). These agents inhibit calcineurin, a serine/threonine phosphatase that plays a central role in calcium signaling. *C. albicans* mutants lacking calcineurin are hypersensitive to serum and antifungal agents that target ergosterol biosynthesis in vitro (Blankenship *et al*., 2003; Cruz *et al*., 2002; Onyewu *et al*., 2003; Sanglard *et al*., 2003), and they are attenuated for virulence in a murine model of disseminated candidiasis (Bader *et al*., 2003; Blankenship *et al*., 2003; Sanglard *et al*., 2003). However, the components of the calcineurin pathway are still poorly investigated in this yeast species. Using *S. cerevisiae* as a model to reconstitute this pathway, two *CRZ1*-like genes, *CRZ1* and *CRZ2* have been tested. Only *CRZ1* was able to restore the calcium hypersusceptibility of a *S. cerevisiae* *crz1*/*crz1* mutant and to mediate calcium-dependent gene expression in this yeast species (Mahir *et al*., 2006).

Calcineurin is a major player in calcium-dependent signal transduction pathways of eukaryotes. In the human pathogen *C. albicans*, calcineurin function is required for cell morphogenesis, azole tolerance, membrane stress responses, survival in serum and virulence in mice (Santos *et al*., 2005). In *C. albicans*, the calcineurin pathway is involved in tolerance to antifungal agents, cation homeostasis and virulence. In *S. cerevisiae*, Crz1 has been extensively characterized as a primary target of calcineurin signaling in response to a variety of environmental stresses (Matheos et al., 1997; Stathopoulos et al., 1997; Stathopoulos-Gerontides et al., 1997). The *S. cerevisiae* Crz1 protein contains an N-terminal glutamine-rich region, a PxIxIQ motif (where x is any amino acid) that serves as a docking site for calcineurin, two C_2H_2-type zinc finger domains, and a serine-rich region that contains a nuclear export signal (Boustany *et al*., 2002; Matheos *et al*., 1997; Stathopoulous *et al*., 1997; Stathopoulous *et al*., 1999). These C_2H_2-type zinc fingers are conserved among *S. cerevisiae*, *C. albicans*, and the putative *Ashbya gossypii* Crz1 homologs, and sequences resembling the other motifs are present in *C. albicans* but have not yet been characterized. And a motif [5'-G(C/T)GGT-3'] with similarity to the target

sequence of Crz1p (GNGGCG/TCA) from *S.cerevisiae* was identified as a putative regulatory sequence in the upstream regions of these calcineurin/Crz1p-dependent genes. Opposite to calcineurin, *CRZ1* was not involved in tolerance to antifungal agents (fluconazole, terbinafine). It was only moderately influencing virulence in a mice model of infection which is in sharp contrast to the strong avirulence of *can*/*can* mutant in the same animal model (Chiatogu *et al*., 2004). The calcineurin-dependent pathways for virulence and for responses to antifungal agents appear to be functionally distinct and may even be unrelated. It showed that calcineurin may have other targets in addition to *CRZ1*. In rescent studies, other calcineurin targets, such as Hph1 and Hph2, have been identified in *S. cerevisiae* since the discovery of Crz1 (Heath *et al*., 2004). Even though it establishes *CRZ1* as a calcineurin target, the functions of *C. albicans* homologs will need to be explored in *C. albicans* itself and not extrapolated from the model yeast system.

4.6 Orf19.3926 has high similarity to uncharacterized *Candida albicans* Rny1p.

Since according to the real-time PCR, the expression level of orf19.3926 which locates at down stream 266 bps from *REP3* increased 3.5 folds in the *REP3* rescued strain. However, role of orf19.3926 *in C. albicans* is unknown. Orf19.3926 is also known as: IPF10934; orf6.4050; orf19.11408. Its protein has six C2H2 type zinc finger domains containing a ribounclease T2 family domain, and it has high similarity to *C. albicans RNY1* at about 59%. The T_2 RNases (EC 3.1.27.1), a family of endoribonucleases with no absolute base specificity, are found in all organisms so far examined (Irie *et al*., 1997). Most T_2 enzymes are secretory RNases and therefore are found extracellularly or in compartments of the endomembrane system that would minimize their contact with cellular RNA. Since the biological functions of various T_2 RNases have been postulated on the

basis of enzyme location or gene expression patterns, the cellular roles of these enzymes are generally unknown. Although ubiquitous in nature, most are located where RNA is not thought to be readily available (e.g., outside the cell or in the vacuole). This idea has led researchers to propose biological functions for these enzymes other than the processing of cellular RNA (Irie *et al*., 1997).

Previously report advances understanding of the biology of T_2 RNases by characterizing the only member of this RNase family in *S. cerevisiae*, *RNY1*. *RNY1* encodes an active RNase with a signal sequence that, when overexpressed, was found primarily outside the cell. The enzyme is glycosylated and may be processed proteolytically. Rny1 was found to be an active, secreted RNase whose gene expression is controlled by heat shock and osmotic stress. Inactivation of *RNY1* leads to unusually large cells that are temperature-sensitive for growth. The cell wall is an important osmotic barrier and also plays a key role in morphogenesis and cell–cell recognition (Smits *et al*., 1999). Possibly an effect of the RNase, such as modifying or loosening the cell wall through an as-yet-unknown mechanism, could explain some of the observed phenotypes. However, a more appealing connection between *RNY1* and its mutant phenotype is suggested by the recent finding that specific RNAs can bind to biological membranes and affect their permeability to ions (Khvorova *et al*., 1999). If some RNAs still carry out this ancient function, then secreted RNases could have an important role in controlling membrane permeability or stability. Accordingly, *RNY1* expression would be expected to increase in response to changing environmental conditions, such as osmotic stress and heat shock, that have important membrane-related implications. Yeast may regulate membrane permeability or stability during stress conditions by secreting an RNase to degrade membrane-bound RNAs.

As a consequence, yeast cells lacking *RNY1* would be osmosensitive, because they cannot secrete such RNases (Gustavo *et al*., 2001). Because other systems (such as ion

channels and transporters) also contribute to maintaining cell homeostasis at the level of membrane permeability, one explanation for the alternative effects of genetic background on the *rny1Δ* phenotype is that many strains have systems that compensate for the loss of Rny1 function (Gustavo *et al*., 2001). RNases are essential to a variety of biological processes for reasons that are not yet understood fully. If other members of the T_2 RNase family, and possibly members of other families of secreted RNases as well, can affect membrane permeability and/or stability, then a number of RNase-requiring phenomena, such as cytotoxicity, can be considered from a new perspective. The hypothesis put forward here that RNases can act as modifiers of biological membranes could therefore have implications for understanding the mechanism through which other extracellular RNases act.

Chapter 5. Future work

In previously study, *REP3* has been identified as a potential *trans*-acting regulatory factor in the regulation of *CDR1* expression. The result of this study provides the information that *rep3/rep3* homozygous knockout is more sensitive to azoles than wild type. However, complementation of *REP3* in *rep3/rep3* homozygous strains can not restore the phenotype of drug susceptibility. However, the molecular mechanism and the *REP3* network regulating the expression of *CDR1* and drug resistance are poorly understood. There are still more for future analyses.

1. In order to investigate which molecular mechanism that *REP3* is involved in drug resistance, it may be necessary to test the experiment conditions determining the expression of *CDR1* and other genes of drug susceptibility in *C. albicans* by real-time PCR or Northern blot. The conditions for the test may include the temperations of growth, time of drug treatment. This is to determine whether the *REP3* is involved in the drug resistance through the regulation of *CDR1* or other genes in *C. albicans*.

2. The *rep3/rep3::REP3* rescued strains with *SAT1* flipping method shall be confirmed by Southern blot. Southern blot analysis can demonstrate that *REP3* is correctly integrated at the proper chromosome location and does not disrupt other genes. It can also determine that all Nou^s clones had correctly excised the *SAT1* flipper by FLP-mediated recombination.

3. Sequencing results of three independent plasmids which integrated into homozygous mutants at *REP3* locus showed differences with the sequence of contig19-10194 from

Assembly 19 (http://www.candidagenome.org/). If the point mutations on the *REP3* rescue plasmids cause the failure of rescued strains, it is necessary to construct the specific *REP3* sequence in rescue plasmids in the further.

4. ORF19.3926, which locates at the down stream 266 bps from *REP3*, increased the expression level in the presence of miconazole in the strains rescued by pWJB24. It is necessary to determine the espression of orf19.3926 in the *rep3/rep3::REP3* rescued strains reconstructed with *SAT1* flipping method.

5. Construction of the *REP3* over-expression strains for investigating the drug susceptibility. Consistently, cells overexpressing *REP3* were hyper-resistant to specific kind of azoles than the wild-type cells. The phenotype may be opposite in the homozygous knockout strains.

6. Since *rep3/rep3* homozygous mutants are from the same *REP3/rep3* heterozygous mutant strain. Consequently, it is important to determine the drug susceptibility of this *REP3/rep3* heterozygous mutant strain in further study. The phenotype of *rep3/rep3* homozygous mutants has probably existed in *REP3/rep3* heterozygous mutant strain.

References

1. Alarco, A. M. and Raymond, M. 1999. The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in *Candida albicans*. *J. Bacteriol.* 181:700-708.
2. Alarco, A. M., Balan, I., Talibi, D., Mainville, N. and Raymond, M. 1997. AP1-mediated multidrug resistance in *Saccharomyces cerevisiae* requires *FLR1* encoding a transporter of the major facilitator superfamily. *J. Biol. Chem.* 272:19304-19313.
3. Bader, T., Bodendorfer, B., Schröppel, K. and Morschhäuser, J. 2003. Calcineurin is essential for virulence in *Candida albicans*. *Infect. Immun.* 71:5344-5354.
4. Beck-Sague, C. M. and Jarvis, W. R. 1993. Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980–1990. National Nosocomial Infections Surveillance. Syst. *J. Infect. Dis*. 167:1247-1251.
5. Ben-Yaacov, R., Knoller, S., Caldwell, G. A., Becker, J. M. and Koltin, Y. 1994. *Candida albicans* gene encoding resistance to benomyl and methotrexate is a multidrug resistance gene. *Antimicrob. Agents Chemother.* 38:648-652.
6. Blankenship, J. R., Wormley, F. L., Boyce, M. K., Schell, W. A., Filler, S. G., Perfect, J. R. and Heitman, J. 2003. Calcineurin is essential for *Candida albicans* survival in serum and virulence. *Eukaryot. Cell* 2:422-430.
7. Boustany, L. M. and Cyert, M. S. 2002. Calcineurin-dependent regulation of Crz1p nuclear export requires Msn5p and a conserved calcineurin docking site. *Genes Dev*. 16:608-619.
8. Boschman, C. R., Bodnar, U. R., Tornatore, M. A., Obias, A. A., Noskin, G. A., Englund, K., Postelnick, M. A., Suriano, T. and Peterson, L. R. 1998. Thirteen-year evolution of azole resistance in yeast isolates and prevalence of resistant strains carried by cancer

patients at a large medical center. *Antimicrobial Agents and Chemotherapy*. 42:734-8.

9. Bruno, V. M. and Mitchell, A. P. 2005. Regulation of azole drug susceptibility by *Candida albicans* protein kinase CK2. *Mol. Microbiol*. 56:559-573.
10. Chen, Y. C., Chang, S. C., Luh, K. T. and Hsieh, W. C. 2003. Stable susceptibility of *Candida* blood isolates to fluconazole despite increasing use during the past 10 years. *J. Antimicrob. Chemother*. 52:71-77.
11. Chen, C. G., Yang, Y. L., Shih, H. I., Su, C. L. and Lo, H. J. 2004. CaNdt80 is involved in drug resistance in *Candida albicans* by regulating *CDR1*. Antimicrob. *Agents Chemother*. 48:4505-4512.
12. Chu, S. and Herskowitz, I. 1998. Gametogenesis in yeast is regulated by a transcriptional cascade dependent on Ndt80. *Mol. Cell*. 1:685-696.
13. Cruz, M. C., Goldstein, A. L., Blankenship, J. R., Del Poeta, M., Davis, D., Cardenas, M. E., Perfect, J. R., McCusker, J. H. and Heitman, J. 2002. Calcineurin is essential for survival during membrane stress in *Candida albicans*. *EMBO J*. 21:546-559.
14. Coste, A. T., Karababa, M., Ischer, F., Bille, J. and Sanglard, D. 2004. *TAC1*, transcriptional activator of *CDR* genes, is a new transcription factor involved in the regulation of *Candida albicans* ABC transporters *CDR1* and *CDR2*. *Eukaryot. Cell*. 3:1639-1652.
15. Coste, A., Turner, V., Ischer, F., Morschhauser, J., Forche, A., Selmecki, A., Berman, J., Bille, J. and Sanglard, D. 2006. A mutation in Tac1p, a transcription factor regulating *CDR1* and *CDR2*, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in Candida albicans. *Genetics*. 172(4):2139–2156.
16. D'Alessio, G. and Riordan, J. S. 1997. *Ribonucleases: Structures and Functions*. New York: *Academic*; pp. 101–130.
17. Dean, M. 2005 The genetics of ATP-binding cassettetransporters. *Methods Enzymol*. 400: 409-429.
18. de Micheli, M., Bille, J., Schueller, C. and Sanglard, D. 2002. A common

drug-responsive element mediates the upregulation of the *Candida albicans* ABC transporters *CDR1* and *CDR2*, two genes involved in antifungal drug resistance. *Mol. Microbiol*. 43:1197-1214.

19. Edmond, M. B., Wallace, S. E., McClish, D. K., Pfaller, M. A., Jones, R. N. and Wenzel, R. P. 1999. Nosocomial bloodstream infections in United States hospitals: a three-year analysis. *Clin. Infect. Dis*. 29:239-244.
20. Feigal, D. W., Katz, M. H., Greenspan, D., Westenhouse, J., Winkelstein, W. J., Lang, W., Samuel, M., Buchbinder, S. P., Hessol, N, A. and Lifson, A. R. 1991. The prevalence of oral lesions in HIV-infected homosexual and bisexual men: three San Francisco epidemiological cohorts. *AIDS*. 5:519-525.
21. Franz, R., Kelly, S. L., Lamb, D. C., Kelly, D. E., Ruhnke, M. and Morschhauser, J. 1998. Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. *Antimicrob Agents Chemother* 42:3065-3072.
22. Georgopapadakou, N. H. 2001. Update on antifungals targeted to the cell wall: focus on beta-1,3-glucan synthase inhibitors. *Expert. Opin. Investig. Drugs.* 10:269-280.
23. Ghannoum, M. A. and Rice, L. B. 1999. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin. Microbiol. Rev.* 12:501-517.
24. Goldway, M., Teff, D., Schmidt, R., Oppenheim, A. B. and Koltin, Y. 1995. Multidrug resistance in *Candida albicans*: disruption of the *BEN*^{r} gene. *Antimicrob. Agents Chemother.* 39:422-426.
25. Harry, J. B., Oliver, B. G., Song, J. L., Silver, P. M. and Little, J. T. 2005 Drug-induced regulation of the *MDR1* promoter in *Candida albicans. Antimicrob. Agents Chemother.* 49:2785-2792.

26. Heath, V. L., Shaw, S. L., Roy, S. and Cyert, M. S. 2004. Hph1p and Hph2p, novel components of calcineurin-mediated stress responses in *Saccharomyces cerevisiae. Eukaryot. Cell*. 3:695-704.

27. Hiller, D., Stahl, S. and Morschhäuser, J. 2006. Multiple *cis*-acting sequences mediate upregulation of the MDR1 efflux pump in a fluconazole-resistant clinical *Candida albicans* isolate. *Antimicrob. Agents Chemother.* 50:2300-2308.

28. Hitchcock, C. A. 1991. Cytochrome P-450-dependent 14 alpha-sterol demethylase of *Candida albicans* and its interaction with azole antifungals. *Biochem. Soc. Trans.* 19: 782-787.

29. Jensen-Pergakes, K. L., Kennedy, M. A., Lees, N. D., Barbuch, R., Koegel, C. and Bard, M. 1998. Sequencing, disruption, and characterization of the *Candida albicans* sterol methyltransferase (*ERG6*) gene: drug susceptibility studies in *erg6* mutants. *Antimicrob. Agents Chemother*. 42:1160-1167.

30. Jia, N., Arthington-Skaggs, B. A., Lee, W., Pierson, C. A., Lees, N. D., Eckstein, J., Barbuch, R. and Bard., M. 2002. *Candida albicans* sterol C-14 reductase, encoded by the *ERG24* gene, as a potential antifungal target site. *Antimicrob. Agents Chemother*. 46:947-957.

31. Karnani, N., Gaur, N. A., Jha, S., Puri, N., Krishnamurthy, S., Goswami, S. K., Mukhopadh-yay, G. and Prasad, R. 2004. SRE1 and SRE2 are two specific steroid-responsive modules of *Candida* drug resistance gene 1 (*CDR1*) promoter. *Yeast*. 21:219-239.

32. Kelly, S. L., Lamb, D. C., Kelly, D. E., Manning, N. J., Loeffler, J., Hebart, H., Schumacher, U. and Einsele, H. 1997. Resistance to fluconazole and cross-resistance to amphotericin B in *Candida albicans* from AIDS patients caused by defective sterol delta5,6-desaturation. *FEBS Lett.* 400:80-82.

33. Khvorova, A., Kwak, Y. G., Tamkun, M., Majerfeld, I. and Yarus, M. RNAs that bind and change the permeability of phospholipid membranes. 1999. *Proc Natl Acad Sci.* 96:10649–10654.

34. Klein, R. S., Harris, C. A., Small, C. B., Moll, B., Lesser, M. and Friedland, G. H. 1984. Oral candidiasis in high-risk patients as the initial manifestation of the acquired immunodeficien- cy syndrome. *N. Engl. J. Med*. 311:354-358.

35. Kofron, M., Demel, T., Xanthos, J., Lohr, J., Sun, B., Sive, H., Osada, S., Wright, C., Wylie, C. and Heasman, J. 1999. Mesoderm induction in Xenopus is a zygotic event regulated by maternal VegT via TGFbeta growth factors. *Development.* 126:5759-5770.

36. Krishnamurthy, S., Gupta, V., Prasad, R., Panwar, S. L. and Prasad, R. 1998. Expression of *CDR1*, a multidrug resistance gene of *Candida albicans*: transcriptional activation by heat shock, drugs and human steroid hormones. *FEMS Microbiol. Lett.* 160:191-197.

37. Leelaporn, A., Firth, N., Byrne, M. E., Roper, E. and Skurray, R. A. 1994. Possible role of insertion sequence IS257 in dissemination and expression of high- and low-level trimethop-rim resistance in *staphylococci*. *Antimicrob. Agents Chemother.* 38:2238-2244.

38. Letscher-Bru, V. and Herbrecht, R. 2003. Caspofungin: the first representative of a new antifungal class. *J. Antimicrob. Chemother*. 51:513-521.

39. Lyons, C. N. and White, T. C. 2000. Transcriptional analyses of antifungal drug resistance in *Candida albicans*. *Antimicrob. Agents Chemother.* 44:2296-2303.

40. Lopez-Ribot, J. L., McAtee, R. K., Perea, S., Kirkpatrick, W. R., Rinaldi, M. G. and Patterson, T. F. 1999. Multiple Resistant Phenotypes of *Candida albicans* Coexist during Episodes of Oropharyngeal Candidiasis in Human Immunodeficiency Virus-Infected Patients. *Antimicrob. Agents Chemother.* 43:1621-1630.

41. Lupetti, A., Danesi, R., Campa, M., Tacca, M. D. and Kelly, S. 2002. Molecular basis of resistance to azole antifungals. *Trends Mol. Med*. 8:76–81.

42. MacIntosh, G. C., Bariola, P. A., Newbigin, E. and Green, P. J. 2001. Characterization of Rny1, the *Saccharomyces cerevisiae* member of the T_2 RNase family of RNases: Unexpected functions for ancient enzymes? *Proc Natl Acad Sci U S A*. January 30; 98(3): 1018–1023.

43. Marchetti, O., Entenza, J. M., Sanglard, D., Bille, J., Glauser, M. P. and Moreillon, P. 2000. Fluconazole plus cyclosporine: a fungicidal combination effective against experimental endocarditis due to *Candida albicans. Antimicrob. Agents Chemother.* 44:2932-2938.

44. Marchetti, O., Moreillon, P., Glauser, M. P., Bille, J. and Sanglard, D. 2000. Potent synergism of the combination of fluconazole and cyclosporine in *Candida albicans. Antimicrob. Agents Chemother.* 44:2373-2381.

45. Matheos, D. P., Kingsbury, T. J., Ahsan, U. S. and Cunningham, K. W. 1997. Tcn1p/Crz1p, a calcineurin-dependent transcription factor that differentially regulates gene expression in *Saccharomyces cerevisiae. Genes Dev*. 11:3445-3458.

46. Miller, L. G., Hajjeh, R. A. and Edwards, J. E., Jr. 2001. Estimating the cost of nosocomial candidemia in the united states. *Clin. Infect. Dis.* 32:1110.

47. Morschhauser, J**.** 2002. The genetic basis of fluconazole resistance development in Candida albicans. Biochim. Biophys. *Acta—Mol. Basis Dis.* 1587:240-248.

48. Odds, F. C., Brown, A. J. and Gow, N. A. 2003. Antifungal agents: mechanisms of action. *Trends Microbiol*. 11:272-279.

49. Onyewu, C., Blankenship, J. R., Del Poeta, M. and Heitman, J. 2003. Ergosterol biosynthesis inhibitors become fungicidal when combined with calcineurin inhibitors in *Candida albicans*, *Candida glabrata*, and *Candida krusei. Antimicrob. Agents Chemother.* 47:956-964.

50. Parks, L. W. and Casey, W. M. 1995. Physiological implications of sterol biosynthesis in yeast. *Annu. Rev. Microbiol.* 49:95-116.

51. Perea, S., Lopez-Ribot, J. L., Kirkpatrick, W. R., McAtee, R. K., Santillan, R. A., Martinez, M., Calabrese, D., Sanglard, D. and Patterson, T. F. 2001. Prevalence of molecular mechan-isms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. *Antimicrob. Agents Chemother*. 45:2676-2684.

52. Pfaller, M. A., Jones, R. N., Messer, S. A., Edmond, M. B. and Wenzel, R. P. 1998. National surveillance of nosocomial blood stream infection due to *Candida albicans*: frequency of occurrence and antifungal susceptibility in the SCOPE Program. *Diagn. Microbiol. Infect. Dis.* 31:327-332.

53. Prasad, R., De Wergifosse, P., Goffeau, A. and Balzi, E. 1995. Molecular cloning and characterization of a novel gene of *Candida albicans*, *CDR1*, conferring multiple resistance to drugs and antifungals. *Curr Genet*. 27:320-329.

54. Puri, N., Krishnamurthy, S., Habib, S., Hasnain, S. E., Goswami, S. K. and Prasad, R. 1999. *CDR1*, a multidrug resistance gene from *Candida albicans*, contains multiple regulatory domains in its promoter and the distal AP-1 element mediates its induction by miconazole. *FEMS Microbiol. Lett.* 180:213-219.

55. Radding, J. A., Heidler, S. A. and Turner, W. W. 1998. Photoaffinity analog of the semisynthetic echinocandin LY303366: identification of echinocandin targets in *Candida albicans*. *Antimicrob. Agents Chemother*. *42*:1187-1194.

56. Reuß, O., Vik, Å., Kolter, R. and Morschhäuser, J. 2004. The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*. *Gene*. 341:119-127.

57. Ruhnke, M., Eigler, A. and Tennagen, I. 1994. Emergence of fluconazole-resistant strains of *Candida albicans* in patients with recurrent oropharyngeal candidosis and human immunodeficiency virus infection. *Journal of Clinical Microbiology*. 32:2092-8.

58. Rustad, T. R., Stevens, D. A., Pfaller, M. A. and White, T. C. 2002 Homozygosity at the *Candida albicans* MTL locus associated with azole resistance. *Microbiology*. 148:1061-

1072.

59. Sanglard, D., Ischer, F., Parkinson, T., Falconer, D. and Bille, J. 2003. *Candida albicans* mutations in the ergosterol biosynthetic pathway and resistance to several antifungal agents. Antimicrob. *Agents Chemother*. 47:2404-412.

60. Sanglard, D., Isher, I., Calabrese, D., de Micheli, M. and Bille, J., (1998b) Multiple mechanisms to azole antifungals in yeast. *Drug Resistance Updates*. 1:255-265.

61. Sanglard, D., Ischer, F., Marchetti, O., Entenza, J. and Bille, J. 2003. Calcineurin A of *Candida albicans*: involvement in antifungal tolerance, cell morphogenesis and virulence. *Mol. Microbiol.* 489:59-76.

62. Sanglard, D., Kuchler, K., Ischer, F., Pagani, J. L., Monod, M. and Bille, J. 1995. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. *Antimicrob Agents Chemother*. 39: 2378-2386.

63. Smits, G. J., Kapteyn, J. C., van den Ende, H, and Klis, F. M. 1999. Cell wall dynamics in yeast. *Curr Opin Microbiol*. 2:348–352.

64. Sundriyal, S., Sharma, R. K. and Jain, R. 2006. Current advances in antifungal targets and drug development. *Curr. Med. Chem*. 13, 1321-1335.

65. Spreghini, E., Davis, D. A., Subaran, R., Kim, M. and Mitchell, A. P. 2003. Roles of *Candida albicans* Dfg5p and Dcw1p cell surface proteins in growth and hypha formation. *Eukaryot Cell. Aug*. 2(4):746-55.

66. Stathopoulos, A. M. and Cyert, M. S. 1997. Calcineurin acts through the *CRZ1*/*TCN1*-encoded transcription factor to regulate gene expression in yeast. *Genes Dev*. 11:3432-3444.

67. Stathopoulos-Gerontides, A., Guo, J. J. and Cyert, M. S. 1999. Yeast calcineurin regulates nuclear localization of the Crz1p transcription factor through dephosphorylation. *Genes Dev*. 13:798-803.

68. Tkacz, J. S. and DiDomenico, B. 2001. Antifungals: what's in the pipeline. *Curr. Opin. Microbiol.* 4:540-545.

69. Vanden Bossche, H., Marichal, P. and Odds, F. C. 1994a. Molecular mechanisms of drug resistance in fungi. *Trends Microbiol*. 2:393-400.

70. White, T. 1997. Increased mRNA levels of *ERG16*, *CDR*, and *MDR1* correlates with increased resistance in azole resistance in *Candida albicans* isolates from patients infected with human immunodeficiency virus. *Antimicrob Agents Chemother*. 41:1482-1487.

71. White, T. C., Marr, K. A. and Bowden, R. A. 1998. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. *Clin. Microbiol. Rev.* 11:382-402.

72. Wirsching, S., Michel, S., Kohler, G. and Morschhauser, J. 2000. Activation of the multiple drug resistance gene *MDR1* in fluconazole-resistant, clinical *Candida albicans* strains is caused by mutations in a trans-regulatory factor. *J. Bacteriol*. 182:400-404.

73. Yang, Y. L. and Lo, H. J. 2001. Mechanisms of antifungal agent resistance. *J Microbiol Immunol Infect*. 34:79-86.

74. Young, L. Y., Hull, C. M. and Heitman, J. 2003. Disruption of ergosterol biosynthesis confers resistance to amphotericin B in *Candida lusitaniae*. Antimicrob. *Agents Chemother*. 47:2717-2724.

75. 紀錦昇 Chin-Sheng Chi 2004

在藥物誘導下分離鑑定白色念珠菌之調控因子

Isolation and identification of *trans*-regulatory factors of *CDR1* in *Candida albicans*.

Master thesis, Institute of Biological Science and Technology, National Chiao Tung University.

76. 吳宛真 Wan-Jen Wu 2005

白色念珠菌抗藥基因 *CDR1* 之異位調控因子 *REP3*、*REP4* 與 *REP5* 之遺傳學研究

Genetic study of *trans*-regulatory factors *REP3*, *REP4* and *REP5* of the drug resistant

gene *CDR1* in *Candida albicans*.

Master thesis, Institute of Biological Science and Technology, National Chiao Tung University.

Table 1. The recombinant plasmid.

Plasmid	Lab No.	Description	Source
pGEM-*HIS1*	LOB14	A vector contained *CaHIS1* , Amp^R	(Wilson *et al* ., 1999)
pDDB78	LOB83	A vector constructed from in vivo recombination of pRS314 and *CaHIS1* fragment from pGEM-*HIS1* , AmpR	(Spreghini *et al* ., 2003)
pGEM-*URA3*	LOB15	A vector contained *CaURA3* , Amp^R	(Wilson *et al* ., 1999)
pRS*ARG4* △Spe1	LOB16	yeast 2μ *ori* , *E. coli* ColE1 *ori* , *ARG4* , Amp^R	(Wilson *et al* ., 1999)
pSFS2	LOB223	The plasmid contains a *C. albicans* -adapted nourseothricin resistance marker, *CaSAT1* , Amp^R	(Wirsching *et al* ., 2000b)
pWJB24	WJB24	backbone. Used to rescue *REP3* function in *rep3/rep3* mutant strains. *CaHIS1* , Amp^R	(Wan-Jan Wu, 2005)
pCJB1	CJB1	The plasmid containing A region(dpl200:*URA3* :dpl200 from -558 ~ -721 and *REP3* from +130 ~ +218) in the pSFS2 backbone. *CaSAT1* , Amp^R	This study
pCJB6	CJB6	The plasmid containing full length of *REP3* in the pGEM-T easy backbone. (orf19.3926 : +1188 ~ orf19.3929 : +58), Amp^R	This study

pCJB7	CJB7	The plasmid containing full length of *REP3* in the pGEM-T easy backbone. (orf19.3926 : +1188 ~ orf19.3929 : +58), Amp^R	This study
pCJB8	CJB8	The plasmid containing full length of *REP3* in the pGEM-T easy backbone. (orf19.3926 : +1188 ~ orf19.3929 : +58), Amp^R	This study
pCJB11	CJB11	The plasmid containing B region full length of *REP3* in the pCJB6 backbone. Used to rescue *REP3* function in *rep3/rep3* mutant strains by *SAT1* method. *CaSAT1*, Amp^R	This study
pCJB15	CJB15	The plasmid containing B region full length of *REP3* in the pCJB8 backbone. Used to rescue *REP3* function in *rep3/rep3* mutant strains by *SAT1* method. *CaSAT1*, Amp^R	This study

Note. Amp^R, ampicillin resistant

Table 2. *Candida albicans* used in this study

Strain	Genotype or relevant features	parental strain	Source
SC5314	Wild type strain.		(Sanglard *et al* .,1996)
BWP17	SC5314 derivative; *ura3△::λimm434/ ura3△::λimm434 his1::hisG/ his1::hisG arg4::hisG/arg4::hisG*		(Wilson *et al* ., 1999)
YLO159	*ura3△::λimm434/ ura3△::λimm434 △cdr1::hisG-URA3-hisG/△cdr1::hisG*	CAF4-2 (SC5314)	(Sanglard *et al* .,1996)
CSC21	*ura3△::λimm434/ ura3△::λimm434 his1::hisG/ his1::hisG arg4::hisG/arg4::hisG rep3::ARG4/REP3*	BWP17	(Chin-Sheng Chi,2004)
CSC22	*ura3△::λimm434/ ura3△::λimm434 his1::hisG/ his1::hisG arg4::hisG/arg4::hisG rep3::ARG4/REP3*	BWP17	(Chin-Sheng Chi,2004)
CSC80	*ura3△::λimm434/ ura3△::λimm434 his1::hisG/ his1::hisG arg4::hisG/arg4::hisG rep3::ARG4/rep3::URA3-dpl200*	CSC21	(Chin-Sheng Chi,2004)
CSC81	*ura3△::λimm434/ ura3△::λimm434 his1::hisG/ his1::hisG arg4::hisG/arg4::hisG rep3::ARG4/rep3::URA3-dpl200*	CSC21	(Chin-Sheng Chi,2004)
CJC2	*ura3△::λimm434/ ura3△::λimm434 his1::hisG/ his1::hisG::HIS1 arg4::hisG/arg4::hisG rep3::ARG4/rep3::URA3-dpl200*	CSC80	This study
CJC4	*ura3△::λimm434/ ura3△::λimm434 his1::hisG/ his1::hisG::HIS1 arg4::hisG/arg4::hisG rep3::ARG4/rep3::URA3-dpl200*	CSC81	This study
CJC7	*ura3△::λimm434/ ura3△::λimm434 his1::hisG/ his1::hisG arg4::hisG/arg4::hisG rep3::ARG4/rep3::URA3-dpl200, rep3::REP3-HIS1* (pWJB24)	CSC80	This study
CJC10	*ura3△::λimm434/ ura3△::λimm434 his1::hisG/ his1::hisG arg4::hisG/arg4::hisG rep3::ARG4/rep3::URA3-dpl200, rep3::REP3-HIS1* (pWJB24)	CSC81	This study
CJC13	*ura3△::λimm434/ ura3△::λimm434 his1::hisG/ his1::hisG::HIS1 arg4::hisG/arg4::hisG rep3::ARG4/rep3::URA3-dpl200, rep3::SAT1-REP3* (pCJB11)	CJC2	This study

CJC14	*ura3△::λimm434/ura3△::λimm434 his1::hisG/his1::hisG::HIS1 arg4::hisG/arg4 rep3::ARG4/rep3::URA3-dpl200, rep3::SAT1-REP3* (pCJB15)	CJC2	This study
CJC15	*ura3△::λimm434/ura3△::λimm434 his1::hisG/his1::hisG::HIS1 arg4::hisG/arg4::hisG rep3::ARG4/rep3::URA3-dpl200, rep3::SAT1-REP3* (pCJB15)	CJC2	This study
CJC16	*ura3△::λimm434/ura3△::λimm434 his1::hisG/his1::hisG::HIS1 arg4::hisG/arg4::hisG rep3::ARG4/rep3::URA3-dpl200, rep3::SAT1-REP3* (pCJB11)	CJC4	This study
CJC17	*ura3△::λimm434/ura3△::λimm434 his1::hisG/his1::hisG::HIS1 arg4::hisG/arg4::hisG rep3::ARG4/rep3::URA3-dpl200, rep3::SAT1-REP3* (pCJB15)	CJC4	This study
CJC18	*ura3△::λimm434/ura3△::λimm434 his1::hisG/his1::hisG::HIS1 arg4::hisG/arg4::hisG rep3::ARG4/rep3::URA3-dpl200, rep3::SAT1-REP3* (pCJB15)	CJC4	This study
CJC19	*ura3△::λimm434/ura3△::λimm434 his1::hisG/his1::hisG::HIS1 arg4::hisG/arg4::hisG rep3::ARG4/rep3::URA3-dpl200, rep3::REP3* (pCJB11)	CJC13	This study
CJC20	*ura3△::λimm434/ura3△::λimm434 his1::hisG/his1::hisG::HIS1 arg4::hisG/arg4::hisG rep3::ARG4/rep3::URA3-dpl200, rep3::REP3* (pCJB15)	CJC14	This study
CJC21	*ura3△::λimm434/ura3△::λimm434 his1::hisG/his1::hisG::HIS1 arg4::hisG/arg4::hisG rep3::ARG4/rep3::URA3-dpl200, rep3::REP3* (pCJB15)	CJC15	This study
CJC22	*ura3△::λimm434/ura3△::λimm434 his1::hisG/his1::hisG::HIS1 arg4::hisG/arg4::hisG rep3::ARG4/rep3::URA3-dpl200, rep3::REP3* (pCJB11)	CJC16	This study
CJC23	*ura3△::λimm434/ura3△::λimm434 his1::hisG/his1::hisG::HIS1 arg4::hisG/arg4::hisG rep3::ARG4/rep3::URA3-dpl200, rep3::REP3* (pCJB15)	CJC17	This study
CJC24	*ura3△::λimm434/ura3△::λimm434 his1::hisG/his1::hisG::HIS1 arg4::hisG/arg4::hisG rep3::ARG4/rep3::URA3-dpl200, rep3::REP3* (pCJB15)	CJC18	This study

Table 3. Primer used in this study

Name	Sequence	Position
HJL133	ACCAGTAGCACAGCGATT	*CaURA3:* (pDDB57) +51~+68
HJL178	GATACAGGTCAACATACTGC	*CaSNF3* :+1348~+1367
HJL179	GATGTGATTGTGACGATGAC	*CaSNF3* :+1834~+1815
HJL272	GAAGCCACACCAAGTCAATA	*CaCPH1* : +834~+851
HJL273	CCAGTTTGCGGTATTGCCT	*CaCPH1* : +1361~+1343
HJL293	AGTCATTGAACGAATTCTTGGCTG	*CaEFB1* :+38~+61
HJL294	TTCTTCAACAGCAGCTTGTAAGTC	*CaEFB1* :+591~+568
HJL311	AGTAGAAATCACACCAGTAGCAA	*CaARG4* :+1077~+1055
HJL312	CGGCAATTCTTGAACGAGCAC	*CaARG4* :+443~+463
HJL315	GTGCTGAACGTGAATATGT	*CaCDR1* :+4205~+4223
HJL316	CTCTCTGTTACCCTTTGG	*CaCDR1* :+4471~+4488
HJL360	TACCGCTTGTCACACTG	*CaERG3* :+930~+946
HJL361	ACCTTCAACTTCTCCTCTAAT	*CaERG3* :+1128~+1108
HJL393	GCTACTACTGCTTCTGGT	*CaMDR1* :+1396~+1413
HJL394	CTCAACTTTGGTCCGTTC	*CaMDR1* :+1670~+1653
HJL458	CCACCCGGGCTCGGAGCTTTATCATCG	orf19.3926:+1267~+1294
HJL504	CGGGATCCGTCACTGGATCTGGATCAAC	orf19.3926:+1114~+1133
HJL505	CGGGATCCATAGTTCCATAGGCAGCCTC	orf19.3929:+902~+883
HJL590	TTTCTCCAGGTTATGCTCA	*CaERG11* :+1211~+1229
HJL591	TCAGGGTCAGGCACTTTA	*CaERG11* :+1494~+1511
HJL606	CAGAAGAGGCTATTGACAGG	*CaHIS1* : -605~-586
HJL607	CCCAGTTATACCCAAGTCAC	*CaHIS1* : +285~+266

HJL632	GCCTCACCAGTAGCACAG	*CaURA3* : + 46~+ 63
HJL633	GTTGTCCTAATCCATCACC	*CaURA3* :+ 643~+661
HJL712	TTGCTACAGAGCACCCT	*CaREP3* :+791~+807
HJL713	TGAGACTGCACAGCAT	*CaREP3* :+1103~+1088
HJL855	GCTGAACATAGCGGAAG	orf19.3928*(REP3)* :-444~-460
HJL856	CAAGTCCACAGTGCTC	orf19.3928*(REP3)* :+469~+454
HJL896	CCCTCCAAAAGGCAATTC	orf19.3926:+768~+785
HJL897	CAGTGACAGCACCCAA	orf19.3926:+1105~+1120
HJL898	GGTGCTGTTGATGCTC	orf19.3929:+718~+733
HJL899	GTCCTCCACTGTGTCGA	orf19.3929:+981~+997
HJL928	GCGGCCGCCTTAGATATGAGGCGT	orf19.3926:+1118~+1142
HJL929	CCCCGCGGCTCACTAATGCTTTCGA	orf19.3929:+6~+31
HJL930	GTGGTACCCAAATAAGCATTCCAAC	*CaURA3* :(pGEM-*URA3*) -558~-721
HJL931	GCCACTCGAGAGAACACGAGGCA	orf19.3928:-1230~-1253
HJL963	CCTTCAATATCTGGGAG	*CaURA3* :(pGEM-*URA3*) -601~-618
HJL964	CGACAAGTTGATGGACC	orf19.3928:+787~+770

*The A of the ATG translation initiation site is +1

*restriction enzyme sites are underlined

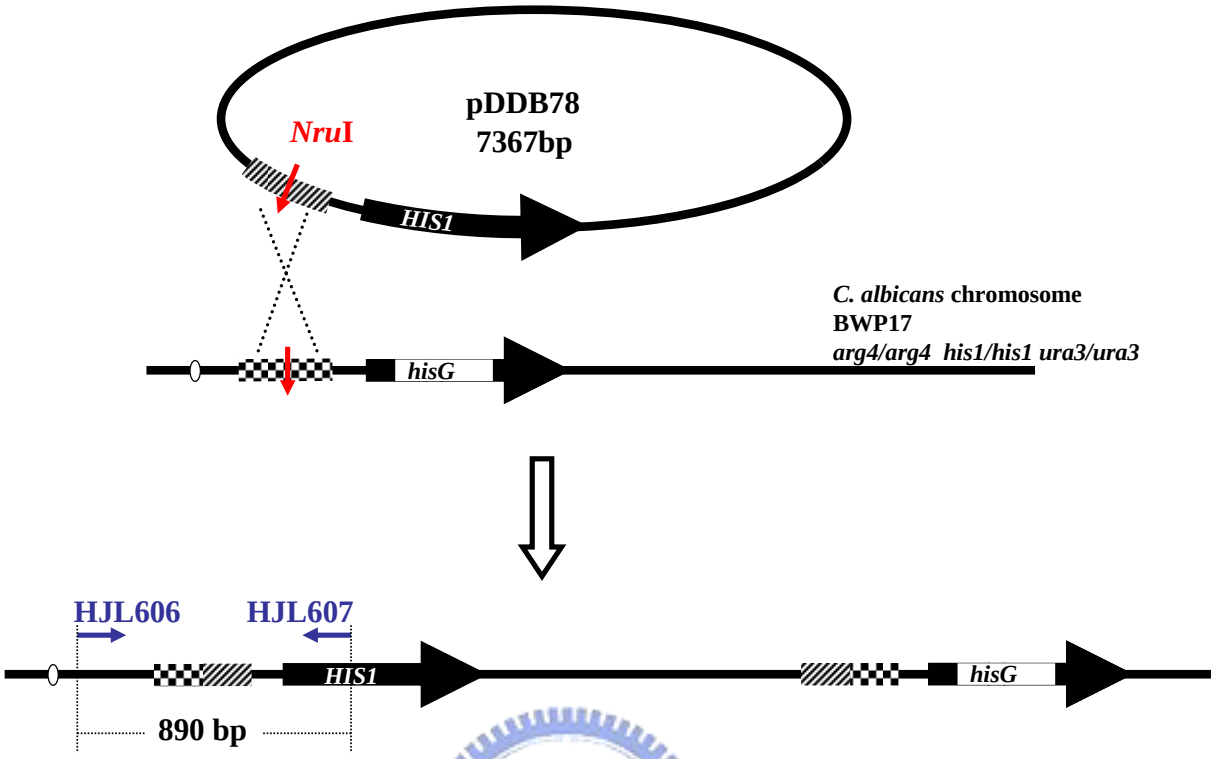


Fig. 1. Schematic diagram of PCR analysis of the integration strategy of *HIS1*.

Plasmid pDDB78 is a *HIS1* vector and the *HIS1* is represented by the black arrow. A unique *Nru*I sites in *HIS1* promoter sequences (hatched arrow) that is used to target integration to the *C. albicans HIS1* locus. Plasmid integration was targeted to the *his1::hisG* locus by digestion with *Nru*I. The integrated genomes were assessed by PCR with primers HJL606 and HJL607.

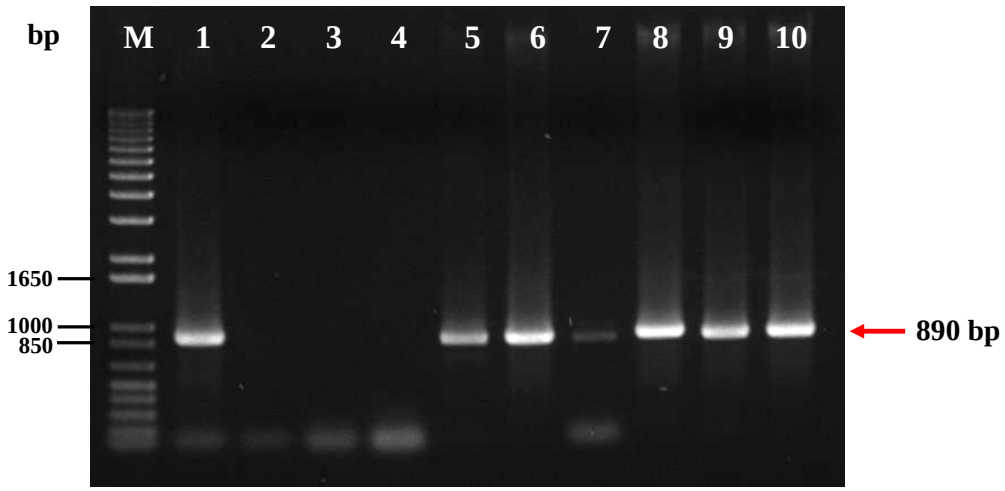


Fig. 2. Assessment of *HIS1* integration into *rep3/rep3* by PCR.

Lane M: 1 kb Plus DNA ladder marker (0.4 μg/ml). Lane 1: CS90 is the positive control. Lane 2: BWP17. Lane 3 and 4: *rep3/rep3* strains (CSC80 and CSC81). Lane5 ~ 10: Analysis of transformant strains (CJC1 ~ CJC6). Arrows indicate the position of the estimated 890 bp fragment.

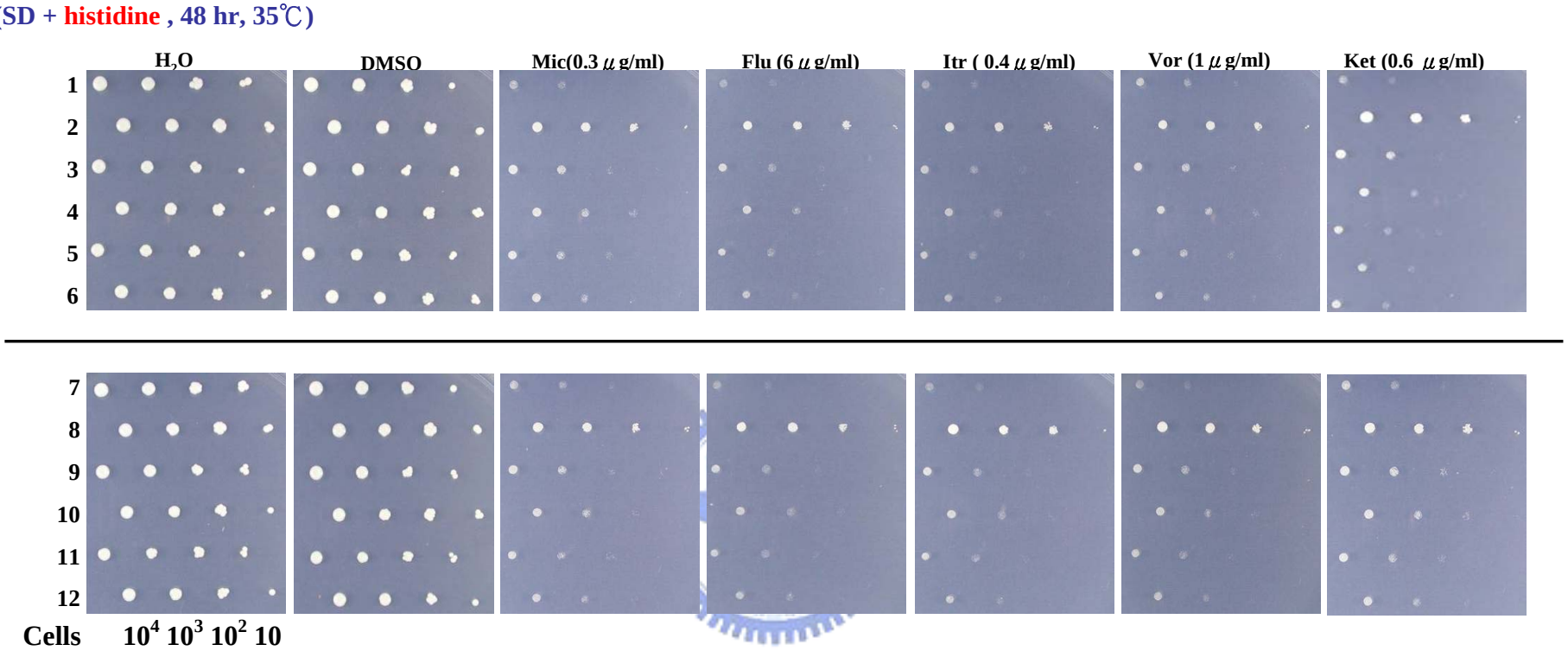


Fig. 3. The antifungal drugs susceptibility of histidine prototrophy *rep3/rep3* is identical with histidine auxotrophy *rep3/rep3*.

The agar dilution was performed to analyze antifungal susceptibility on SD + Histidine (20 mg/l) agar plates. The cells were diluted to an OD_{600} of 2 and spotted onto drug plates along with 10-fold serial dilution. Azoles were prepared to the final concentrations in SD agar, and the same volume (1/1000 in SD agar) of drug solvent-DMSO was used to prepare the control plate. Cells were grown on media in the absence of drug, or in the presence of 6 μg Fluconazole ml^{-1} (Flu), 0.3 μg Miconazole ml^{-1} (Mic), 0.6 μg Ketoconazole ml^{-1} (Ket), 0.4 μg Itraconazole ml^{-1} (Itr) and 1 μg Voriconazole ml^{-1} (Vor). Cells including; (1),(7) *cdr1/cdr1*(YLO159); (2),(8) *REP3/REP3*(SC5314); (3) *rep3/rep3*; *his1:: hisG/his1:: hisG* (CSC80); (9) *rep3/rep3*; *his1:: hisG/his1:: hisG* (CSC81); (4) ~ (6) *rep3/rep3*; *his1:: hisG/his1:: hisG*-CSC80*::HIS1*(CJC1 ~ CJC3); (10 ~ (12) *rep3/rep3*; *his1:: hisG/his1:: hisG*-CSC81*::HIS1* (CJC4 ~ CJC6). The results were photographed after 2 days of growth at 35 ℃.

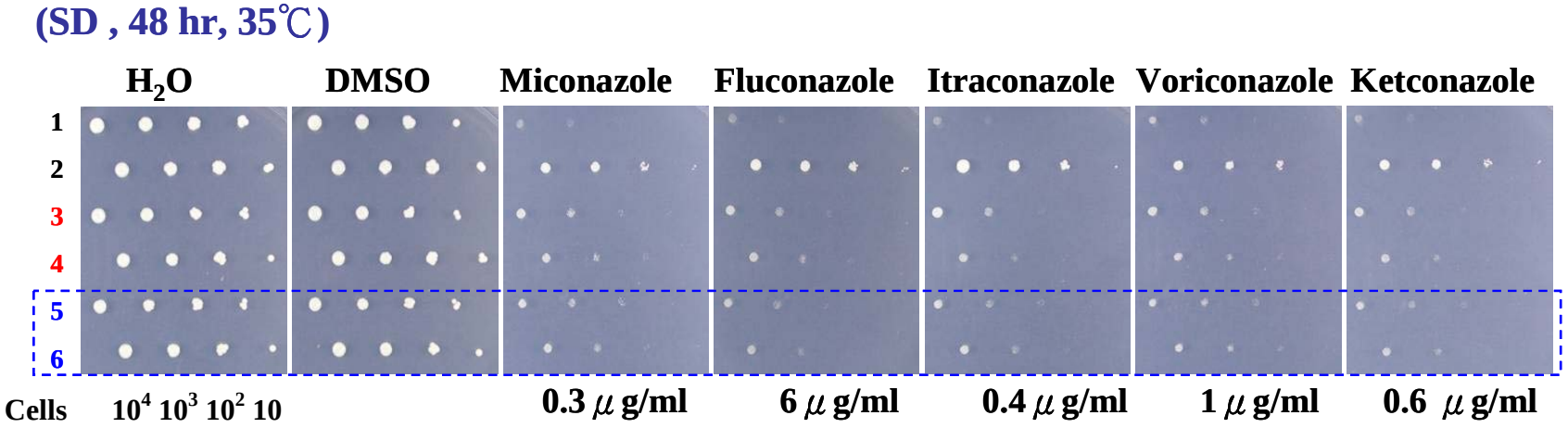
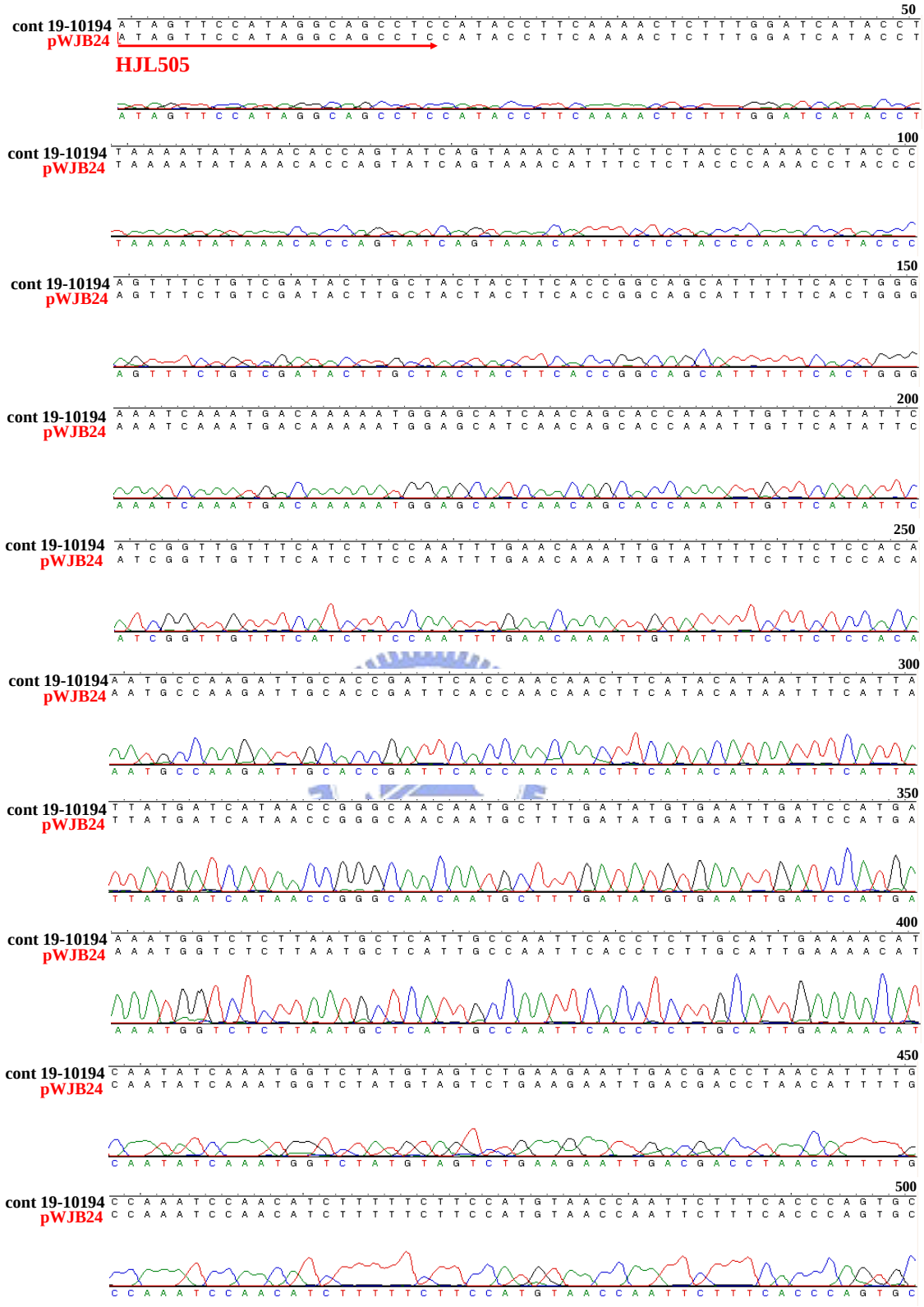
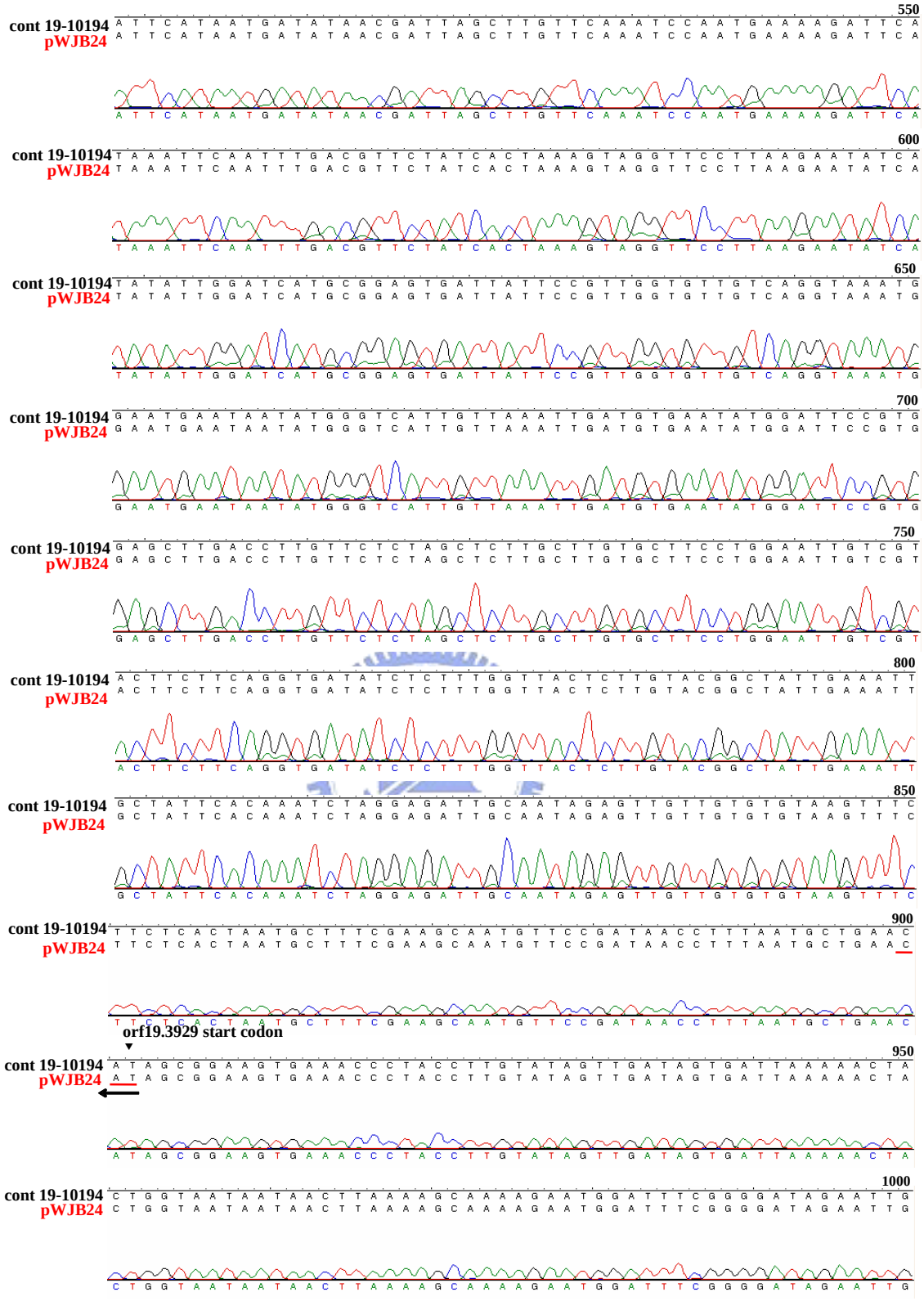
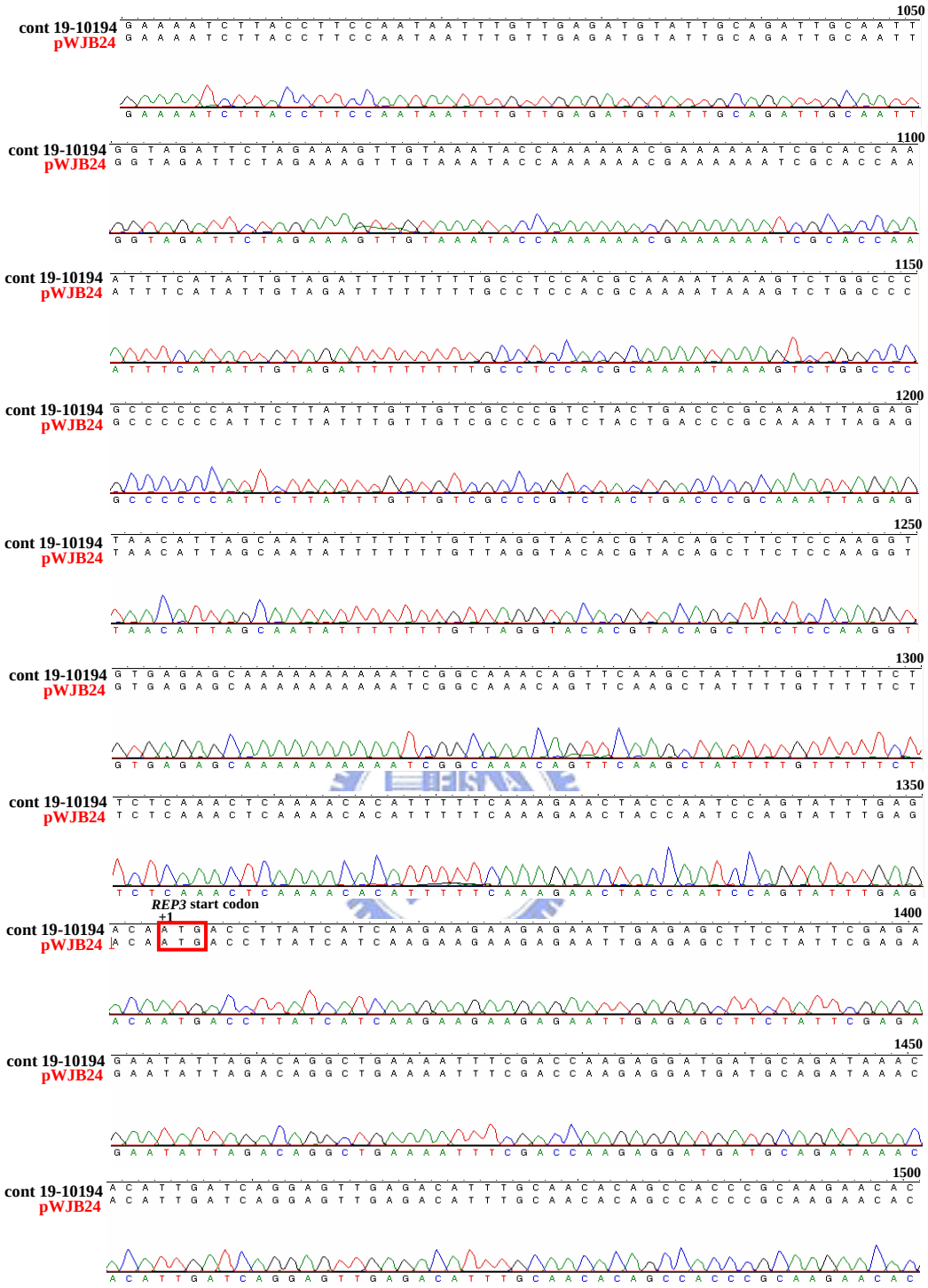


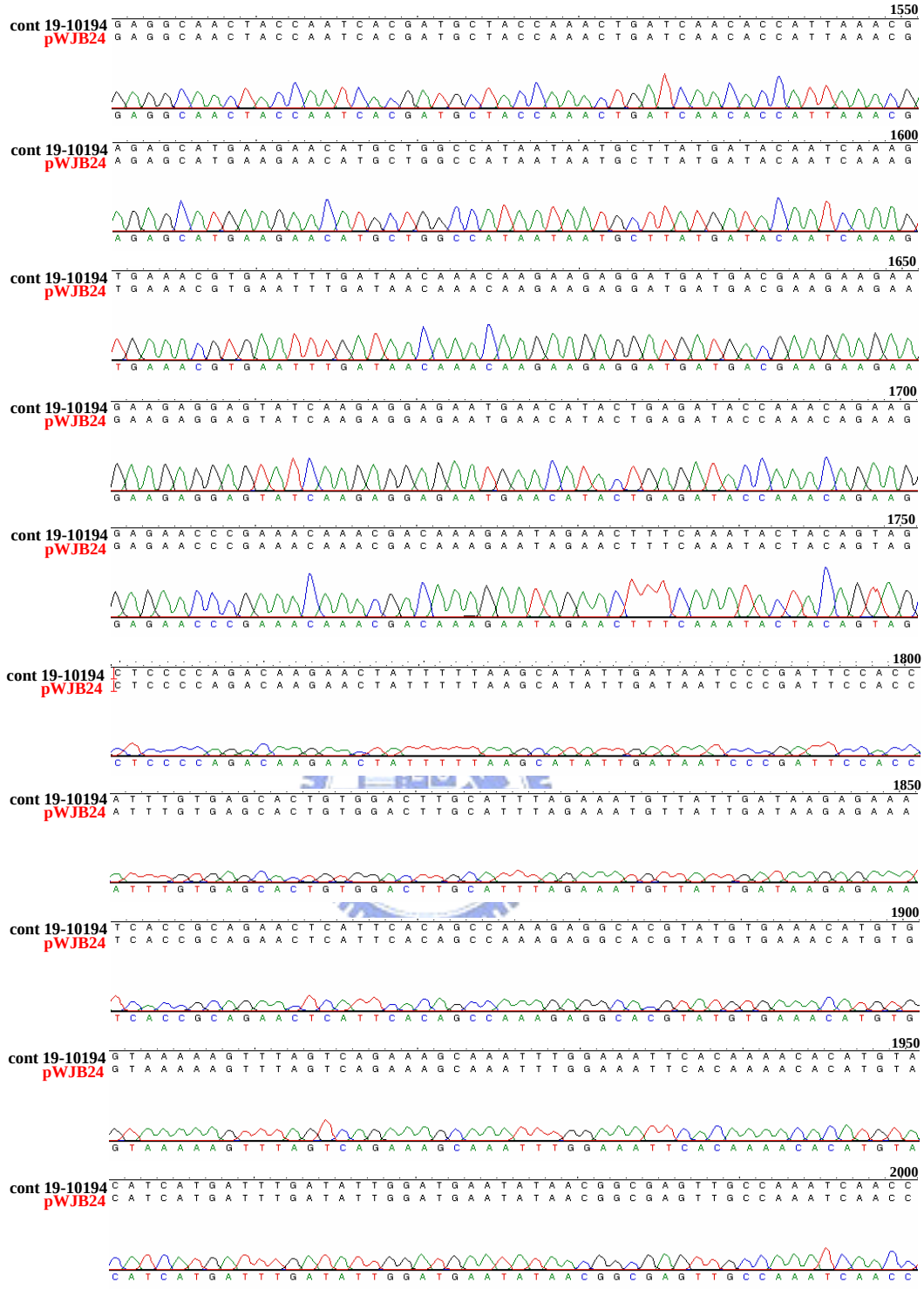
Fig. 4. The *REP3* rescued strains YLO221 and YLO222 can not restore the drug susceptibility phenotype of *rep3/rep3*.

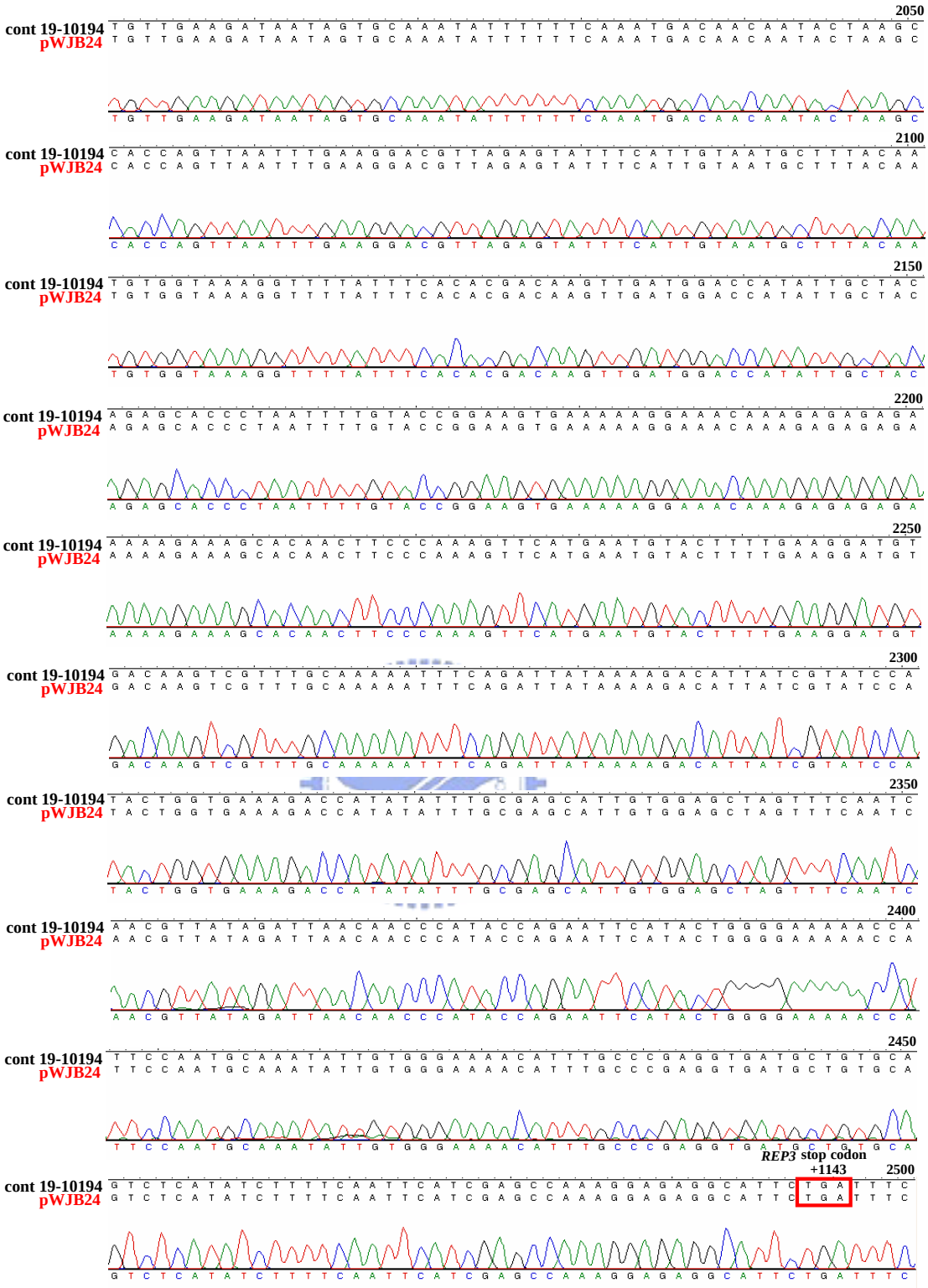
The agar dilution was performed to analyze antifungal susceptibility on SD agar plates. The cells were diluted to an OD_{600} of 2 and spotted onto drug plates along with 10-fold serial dilution. Azoles were prepared to the final concentration in SD agar, and the same volume (1/1000 in SD agar) of drug solvent-DMSO was used to prepare the control plate. Cell were grown on media in the absence of drug, or in the presence of 6 μg Fluconazole ml^{-1} (Flu), 0.3 μg Miconazole ml^{-1} (Mic), 0.6 μg Ketoconazole ml^{-1} (Ket), 0.4 μg Itraconazole ml^{-1} (Itr) and 1 μg Voriconazole ml^{-1} (Vor). Cells including; (1) *cdr1/cdr1*(YLO159); (2) *REP3/REP3*(SC5314); (3) *rep3/rep3*; *his1:: hisG/his1:: hisG*-CSC80*::HIS1*(CJC2); (4) *rep3/rep3*; *his1:: hisG/his1:: hisG*-CSC81*::HIS1*(CJC4); (5) *rep3/rep3::REP3*(YLO221); (6) *rep3/rep3::REP3*(YLO222). The results were photographed after 2 days of growth at 35℃.


cont 19-10194
pWJB24
HJL505
50
100
150
200
250
300
350
400
450
500


550
cont 19-10194
pWJB24
600
cont 19-10194
pWJB24
650
cont 19-10194
pWJB24
700
cont 19-10194
pWJB24
750
cont 19-10194
pWJB24
800
cont 19-10194
pWJB24
850
cont 19-10194
pWJB24
900
cont 19-10194
pWJB24
orf19.3929 start codon
950
cont 19-10194
pWJB24
1000
cont 19-10194
pWJB24


cont 19-10194
pWJB24
1050
1100
1150
1200
1250
1300
1350
REP3 start codon
+1
1400
1450
1500


1550
cont 19-10194
pWJB24
1600
cont 19-10194
pWJB24
1650
cont 19-10194
pWJB24
1700
cont 19-10194
pWJB24
1750
cont 19-10194
pWJB24
1800
cont 19-10194
pWJB24
1850
cont 19-10194
pWJB24
1900
cont 19-10194
pWJB24
1950
cont 19-10194
pWJB24
2000
cont 19-10194
pWJB24


2050
cont 19-10194
pWJB24
2100
cont 19-10194
pWJB24
2150
cont 19-10194
pWJB24
2200
cont 19-10194
pWJB24
2250
cont 19-10194
pWJB24
2300
cont 19-10194
pWJB24
2350
cont 19-10194
pWJB24
2400
cont 19-10194
pWJB24
2450
cont 19-10194
pWJB24
REP3 stop codon
+1143
2500
cont 19-10194
pWJB24

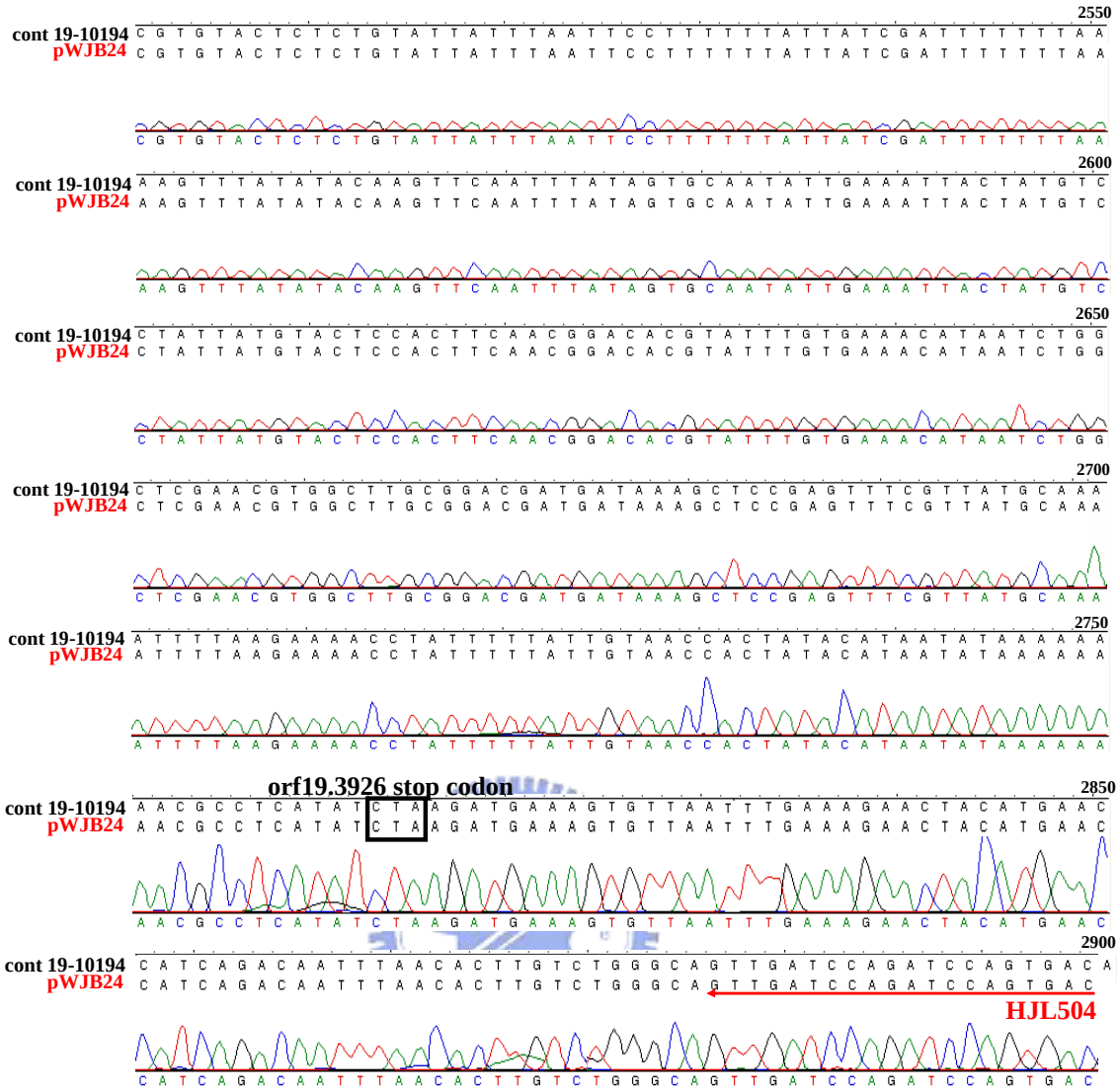


Fig. 5. The *REP3* sequence of the rescue plasmid pWJB24 is identical to the *REP3* sequence of *Candida* Genome Database (CGD).

The pWJB24 integrative plasmid, which derived from pGEM-*CaHIS1* contains a 2.849 kb fragment which was amplified with primers HJL504-HJL505 including full-length ORF of *REP3* and staring from 91 bps downstream of the stop codon of orf19.3926 to 906 bps upstream of start codon of orf19.3929. Alignment of the orf19.3928 in assembly 19 contigs of *C. albicans* and *REP3* rescue plasmid (pWJB24). Translational start and stop codons of *REP3* are indicated by +1 and +1143. The orf19.3929 start codon is indicated by ▼. The arrow is indicated the direction of orf19.3929 expression. The orf19.3926 stop codon is indicated.

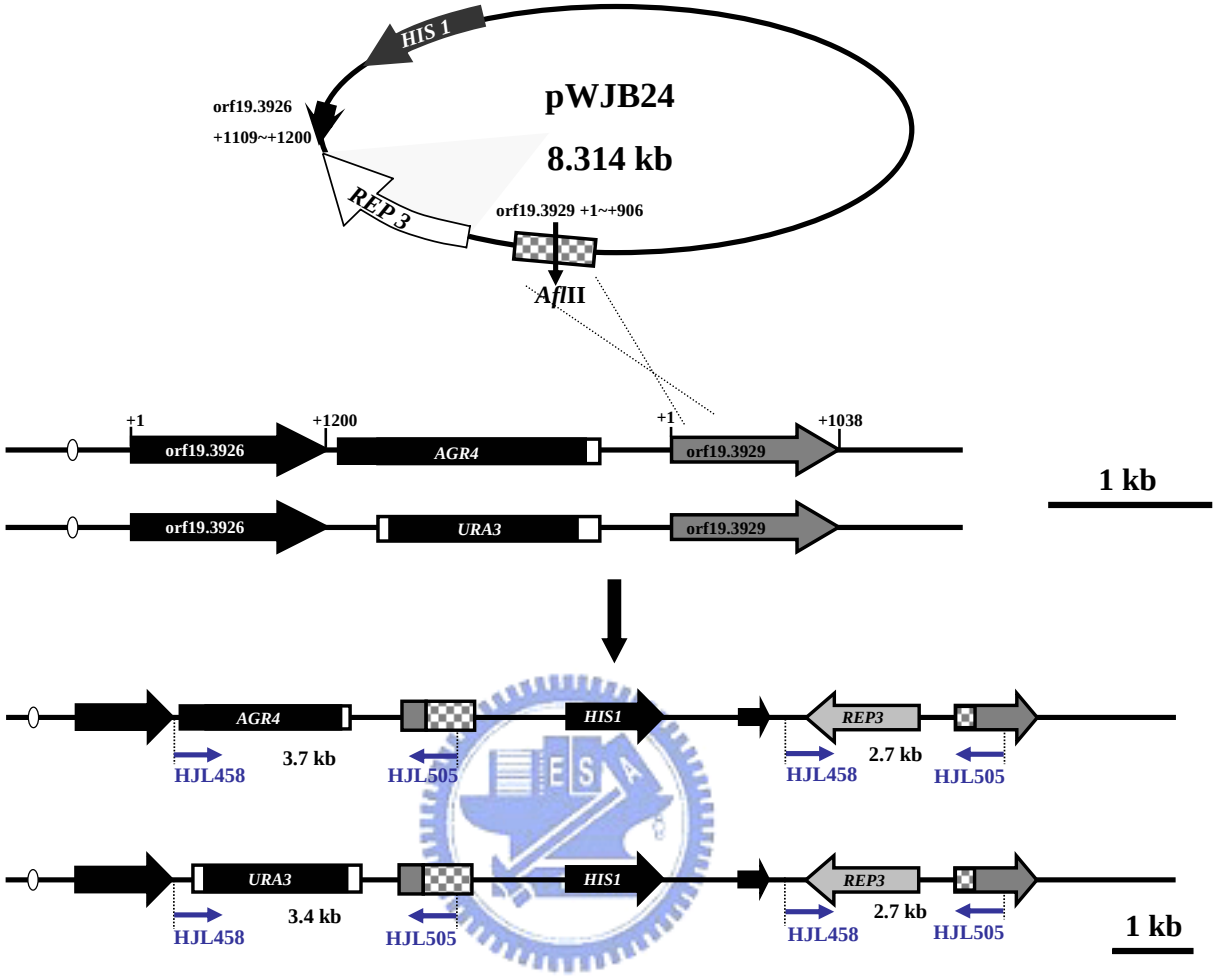


Fig. 6. Schematic diagram of analysis of integration of *REP3* into rescued strains with pWJB24 by PCR.

Plasmid pWJB24 (a vector carrying *HIS1*) was integrated to orf19.3929 in *rep3/rep3*; *his1:: hisG/his1:: hisG* (CSC80 and CSC81) by digestion with *Afl*II. The integration of *REP3* was assessed using primers HJL458 and HJL505.

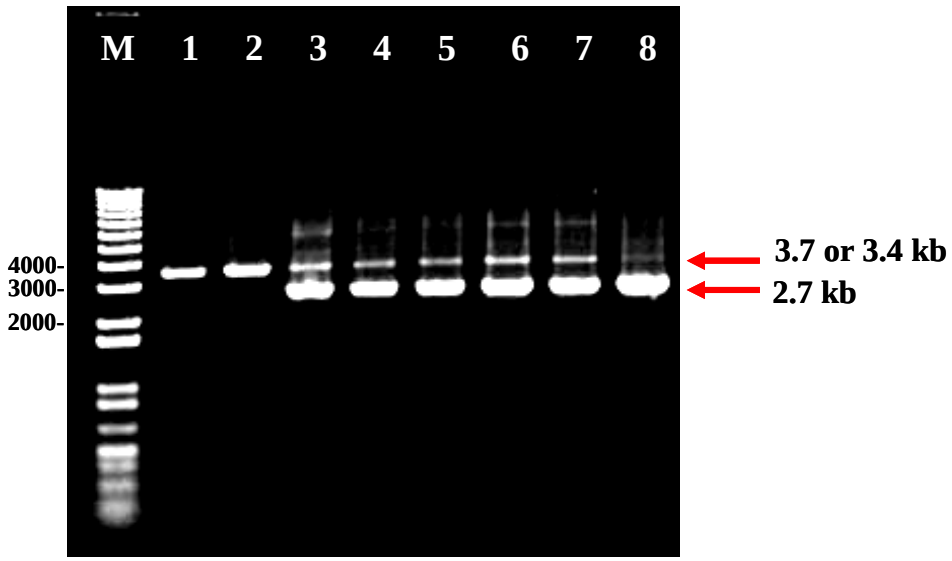
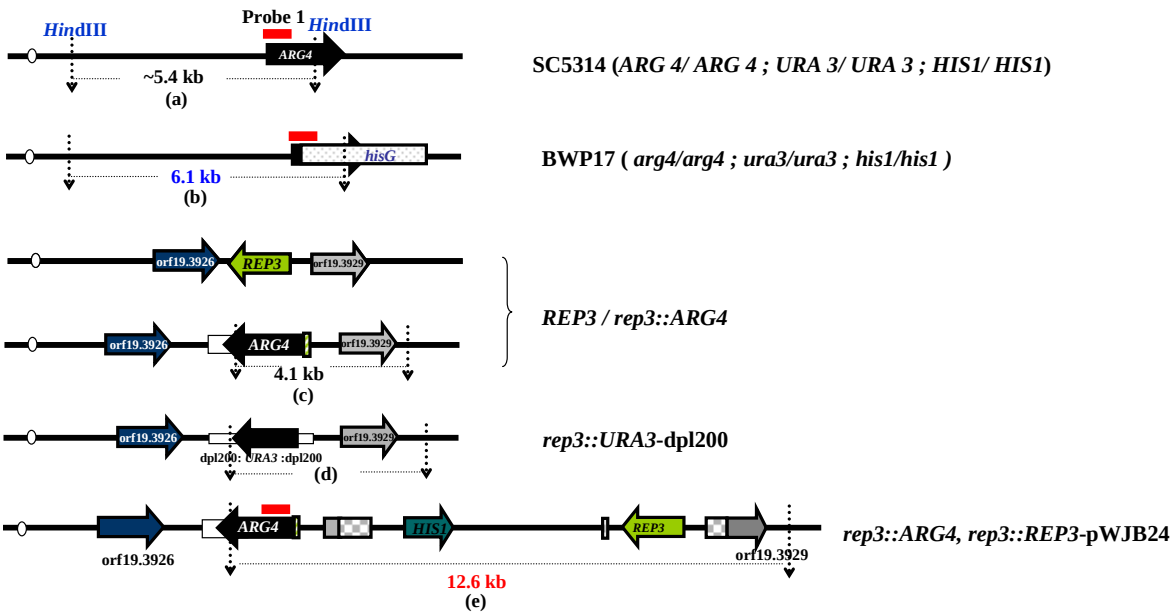


Fig. 7. Assessment of the *REP3* gene transformed to *rep3/rep3* null mutant strains by PCR with primers HJL458, HJL505.

Lane M : 1 kb Plus DNA ladder marker (0.5 μg/ml). Lane 1, 2: *rep3/rep3*; *his1:: hisG/his1:: hisG* (CSC80 and CSC81). Lane3 ~ 8: Analysis of transformant strains (CJC7 ~ CJC12). Arrows indicated 3.7 or 3.4 kb is the positions of the primers on the *ARG4* or *URA3* genome. Arrows indicated 2.7 kb is integrated of *REP3*.



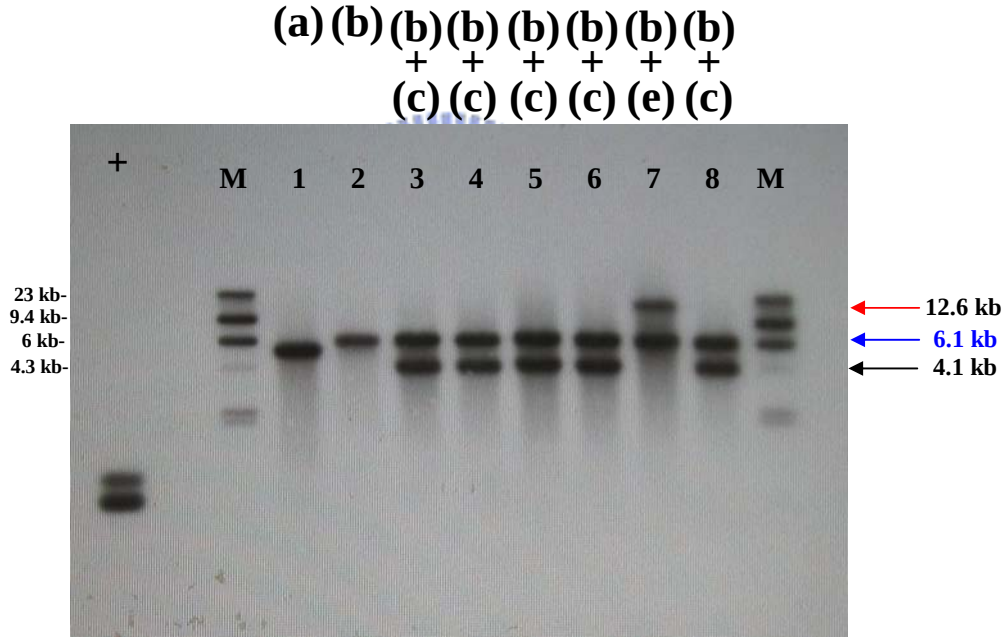
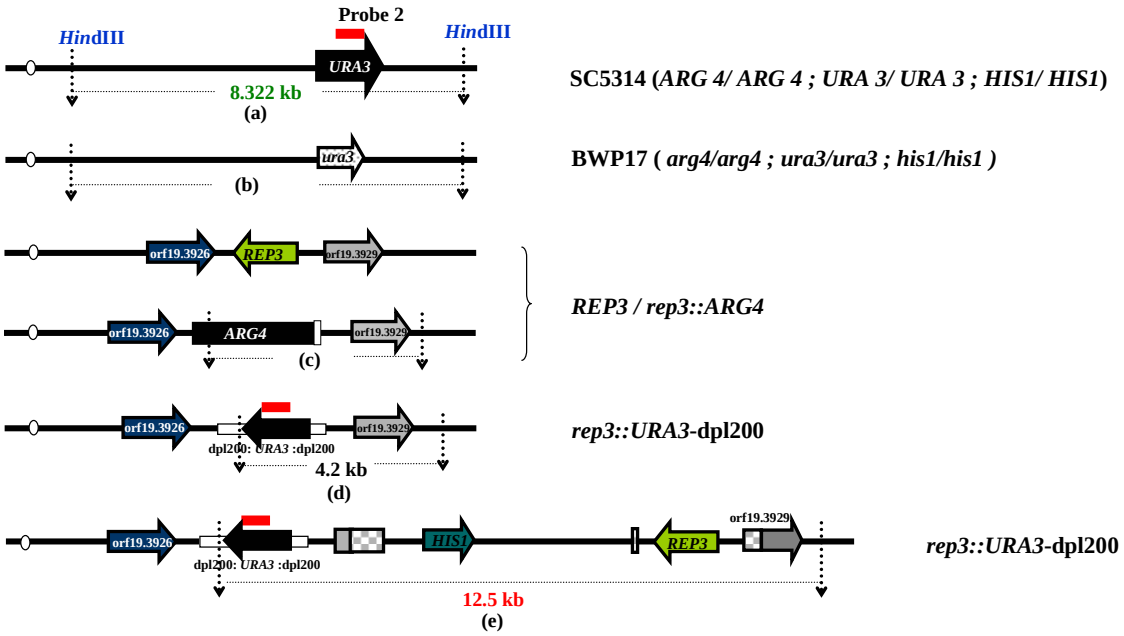


Fig. 8. Southern blot analysis of *Hin*dIII-digested genomic DNA of the parent strain BWP17, mutant and rescued derivatives with *ARG4*-specific probe 1.

The *ARG4* probe detects the indicated fragments derived from pRS*ARG4*△*Spe*I. The expected sizes are shown at right side of the Southern blot and with arrows. (+) : positive control. M : λ*Hin*dIII marker. The sizes of the molecular mass standards (bacteriophage λ restricted by *Hin*dIII). Lane 1 : wild-type allele (SC5314), 5.387 kb; lane 2 : parental strain (BWP17), 6.1 kb; lane 3 and lane 4 : *REP3/rep3::ARG4* (CSC21 and CSC22), 4.1 kb; lane 5 and lane 6 : *rep3::URA3*-dpl200*/rep3::ARG4* (CJC2 and CJC4), 4.1 kb; lane 7 : *rep3::URA3*-dpl200*/rep3::ARG4*, *rep3::REP3*-pWJB24 (CJC7), 12.6kb; lane 8 : *rep3::URA3*-dpl200 */rep3::ARG4*, *rep3::REP3*-pWJB24 (CJC10), 4.1 kb. The bolts were hybridized at 42℃ and wash at 65℃ in 0.5×SSC/0.1％SDS for 15 mins.



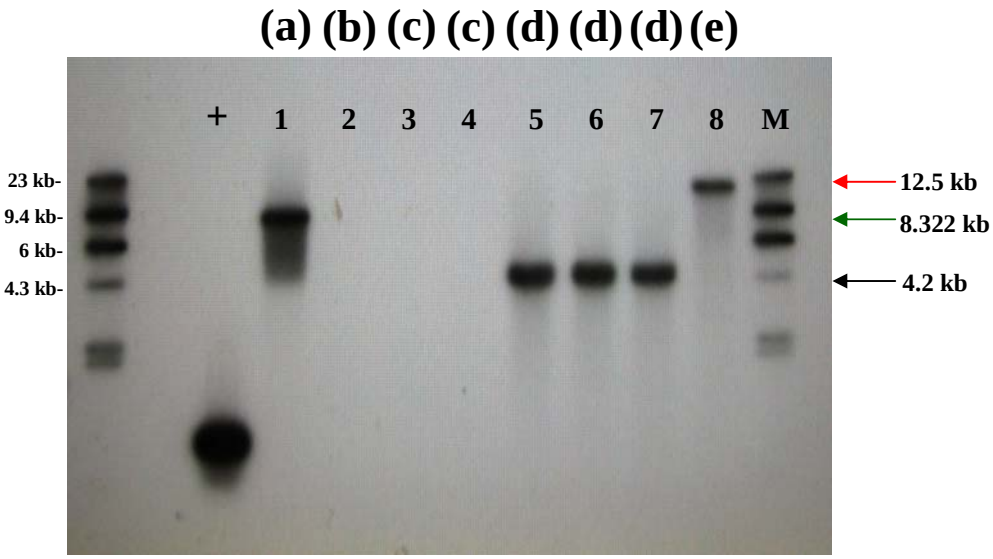


Fig. 9. Southern blot analysis of *Hin*dIII-digested genomic DNA of the parent strain BWP17, mutant and rescued derivatives with *URA3*-specific probe 2.

The *URA3* probe detects the indicated fragments derived from pGEM-*URA3*. The expected size is shown at right side of the Southern blot and with arrows. (+) : positive control. M: λ *Hin*dIII marker. The sizes of the molecular mass standards (bacteriophage λ restricted by *Hin*dIII). Lane 1 : wild-type allele (SC5314), 8.322 kb; lane 2 : parental strain (BWP17); lane 3 and lane 4 : *REP3/rep3::ARG4* (CSC21 and CSC22); lane 5 and lane 6 : *rep3::URA3*-dpl200/*rep3::ARG4* (CJC2 and CJC4), 4.2 kb; lane 7 : *rep3::URA3*-dpl200/*rep3::ARG4, rep3::REP3*-pWJB24 (CJC7), 4.2 kb; lane 8 : *rep3::URA3*-dpl200 /*rep3::ARG4, rep3::REP3*-pWJB24 (CJC10), 12.5 kb. The bolts were hybridized at 42℃ and wash at 65℃ in 0.5×SSC/0.1％SDS for 15 mins.

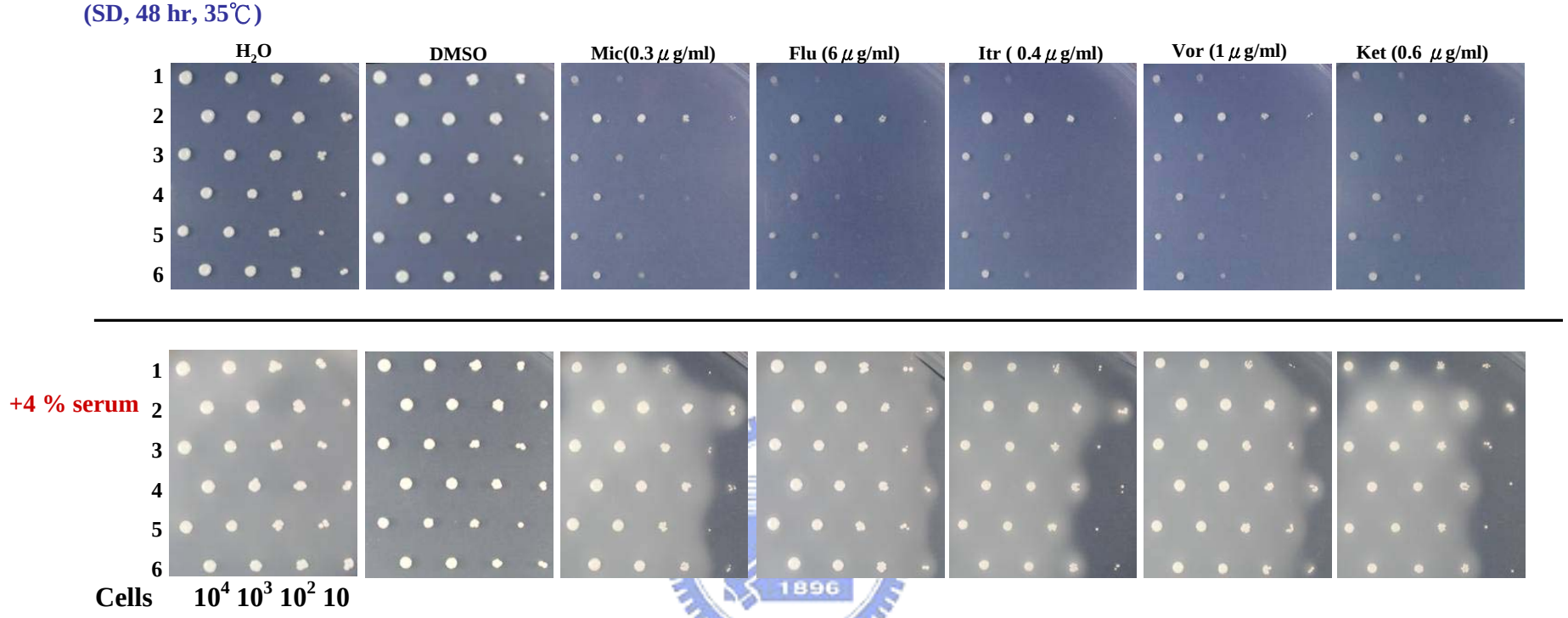


Fig. 10. Mutations on *REP3* increase the susceptibility to antifungal agents but the rescued strains (CJC7 and CJC10) did not show a restored drug susceptibility phenotype in *C. albicans*.

The agar dilution was performed to analyze antifungal susceptibility on SD agar plates and added the 4% FBS (fetal bovine serum) respectively. The cells were diluted to an OD_{600} of 2 and spotted onto drug plates along with 10-fold serial dilution. Azoles were prepared to a final concentration in SD agar, and the same volume (1/1000 in SD agar) of drug solvent-DMSO was used to prepare the control plate. Cells were grown on media in the absence of drug, or in the presence of 6 μg Fluconazole ml^{-1} (Flu), 0.3 μg Miconazole ml^{-1} (Mic), 0.6 μg Ketconazole ml^{-1} (Ket), 0.4 μg Itraconazole ml^{-1} (Itr) and 1 μg Voriconazole ml^{-1} (Vor). The results were photographed after 2 days of growth at 35 ºC. Cells including; (1) *cdr1/cdr1* (YLO159); (2) *REP3/REP3* (SC5314); (3),(4) *rep3/rep3*; *his1:: hisG/his1:: hisG::HIS1* (CJC2 and CJC4); (5),(6) *rep3/rep3::REP3* (CJC7 and CJC10).

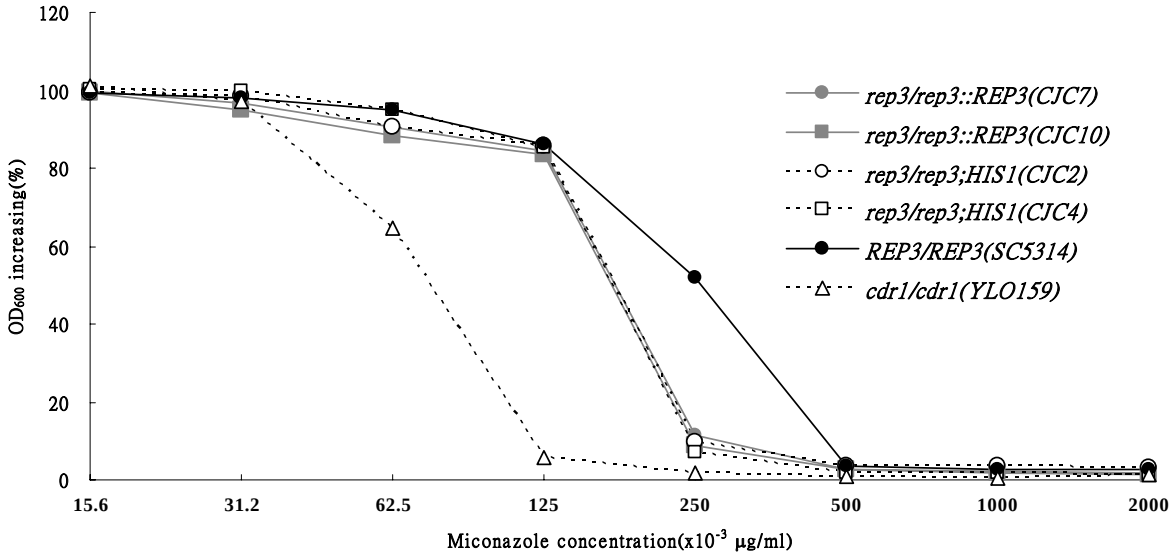


Fig. 11. Mutations on *rep3* gene of *C. albicans* increases the susceptibility to miconazole.

The antifungal drugs susceptibility of six strains; (—●—) wild-type SC5314 (*REP3/REP3*), (···○···) (···□···) *rep3* null mutant (*rep3/rep3)*, (—●—) (—■—) *REP3* rescue (*rep3/rep3*::*REP3*) and (···△···) *cdr1/cdr1* (YLO159) were determined by the microdilution method with some modifications. The antifungal agent miconazole was freshly prepared as a stock at the concentration of 4 mg/ml in DMSO. To prepare working concentration (2 ~ 0.156 μg/ml), the drug was processed by stepwise twofold dilutions in SD medium. A drug-free culture and a sterile control were included in each microtiter plate. The optical density (OD) in each well of the microtiter plate was read with a microplate reader at 600 nm after incubated at 35°C for 48 h. The drug inhibitory curve was presented by the OD of each well with different concentrations of miconazole relative to the OD of the drug-free control.

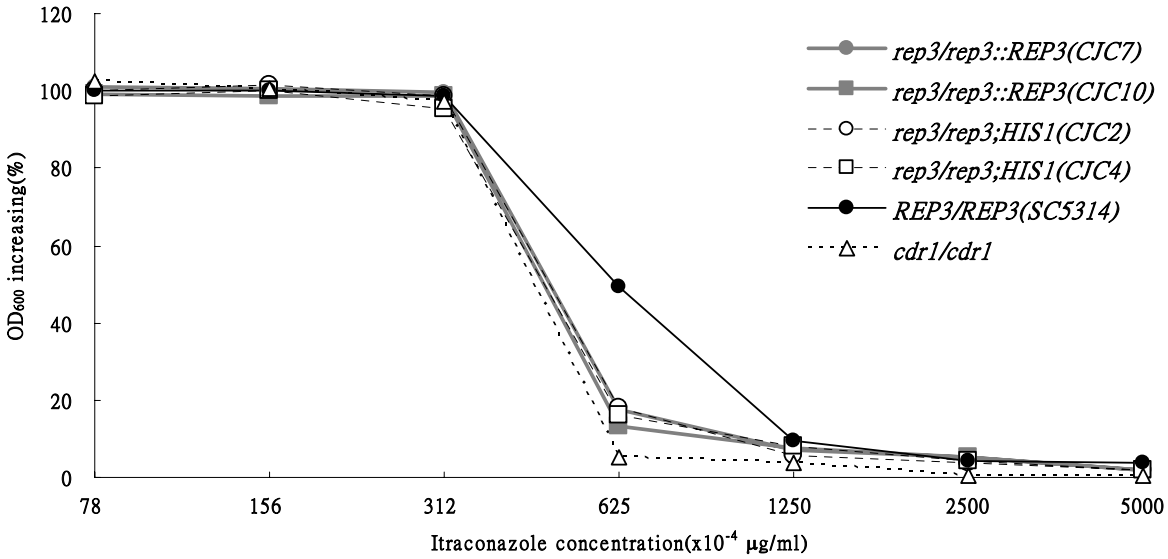


Fig. 12. Mutations on *rep3* gene of *C. albicans* increases the susceptibility to itraconazole.

The antifungal drugs susceptibility of six strains; (—●—) wild-type SC5314 (*REP3/REP3*), (···○···) (···□···) *rep3* null mutant (*rep3/rep3)*, (—●—) (—■—) *REP3* rescue (*rep3/rep3*::*REP3*) and (··△··) *cdr1/cdr1* (YLO159) were determined by the microdilution method with some modifications. The antifungal agent itraconazole was freshly prepared as a stock at the concentration of 1 mg/ml in DMSO. To prepare working concentration (0.5 ~ 0.0078 μg/ml), the drug was processed by stepwise twofold dilutions in SD medium. A drug-free culture and a sterile control were included in each microtiter plate. The optical density (OD) in each well of the microtiter plate was read with a microplate reader at 600nm after incubated at 35°C for 48 h. The drug inhibitory curve was presented by the OD of each well with different concentrations of itraconazole relative to the OD of the drug-free control.

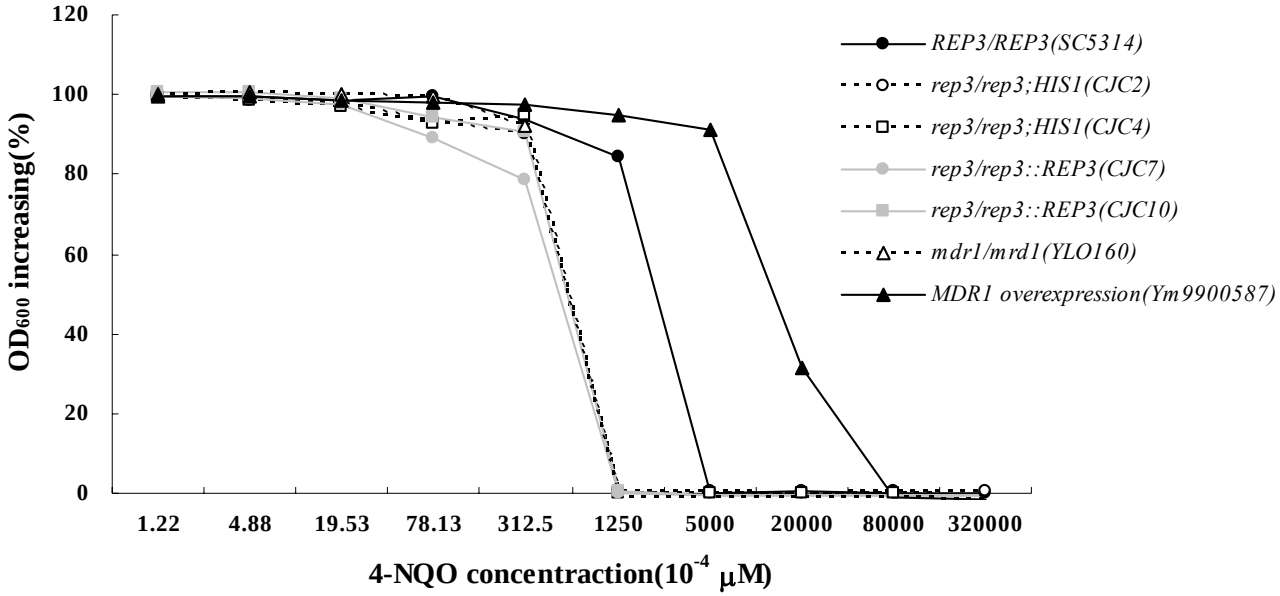


Fig. 13. Mutations on *rep3* gene of *C. albicans* increases the susceptibility to 4-NQO.

The antifungal drugs susceptibility of six strains; (—●—) wild-type SC5314 (*REP3/REP3*), (···○··) (···□··) *rep3* null mutant (*rep3/rep3*), (—●—) (—■—) *REP3* rescue (*rep3/rep3*::*REP3*) and (··△··) *cdr1/cdr1* (YLO159) were determined by the microdilution method with some modifications. The antifungal agent 4-NQO was freshly prepared as a stock at the concentration of 64 mg/ml in DMSO. To prepare working concentration (32 ~ 0.000122 μg/ml), the drug was processed by stepwise twofold dilutions in SD medium. A drug-free culture and a sterile control were included in each microtiter plate. The optical density (OD) in each well of the microtiter plate was read with a microplate reader at 600nm after incubated at 35°C for 48 h. The drug inhibitory curve was presented by the OD of each well with different concentrations of 4-NQO relative to the OD of the drug-free control.

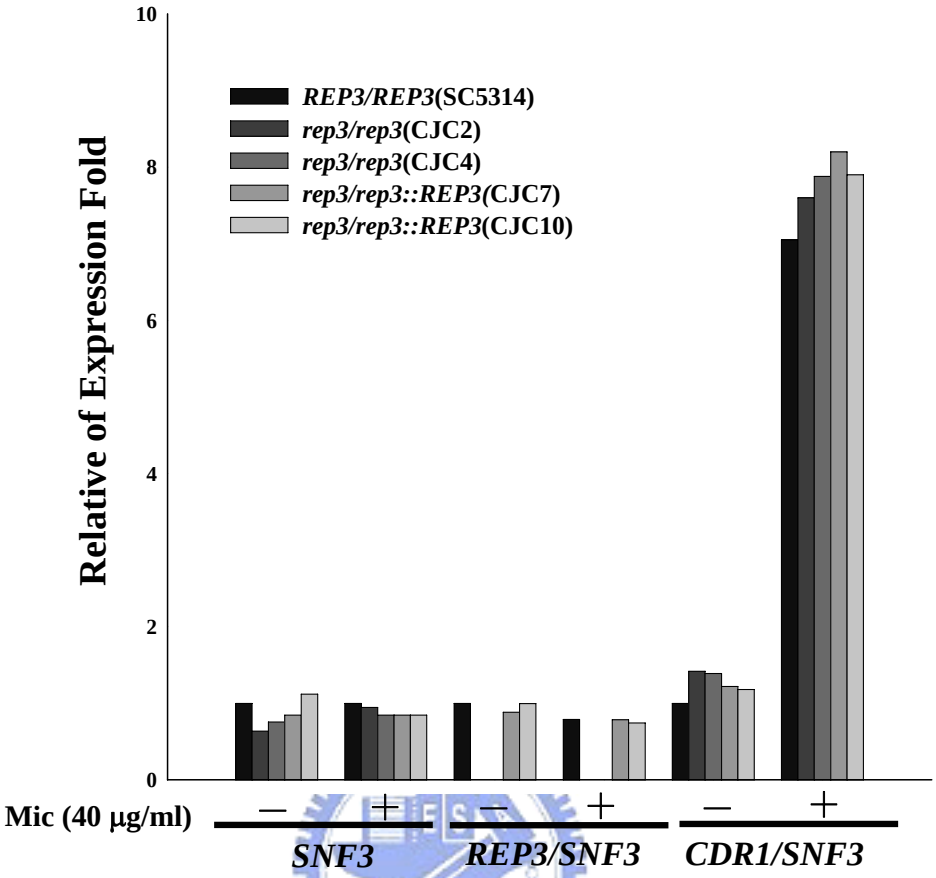


Fig. 14. Mutations in *REP3* do not decrease the *CDR1* expression in *C. albicans*.

Total RNAs were isolated from cells without or with 40 μg/ml of miconazole (Mic) treatment for 1.5 h at 35ºC. The transcript levels of different samples; wild type SC5314 (*REP3/REP3*), *rep3* null mutant (*rep3/rep3*) (CJC2 and CJC4), and *REP3* rescue (CJC7 and CJC10) were normalized with the level of *SNF3* mRNA isolated from the same conditions. The relative transcript levels of target genes from cells without drug treatment were defined as 1. The locations of primers in (*REP3/REP3*) HJL712-HJL713 for *REP3* are indicated in Fig.17-A.

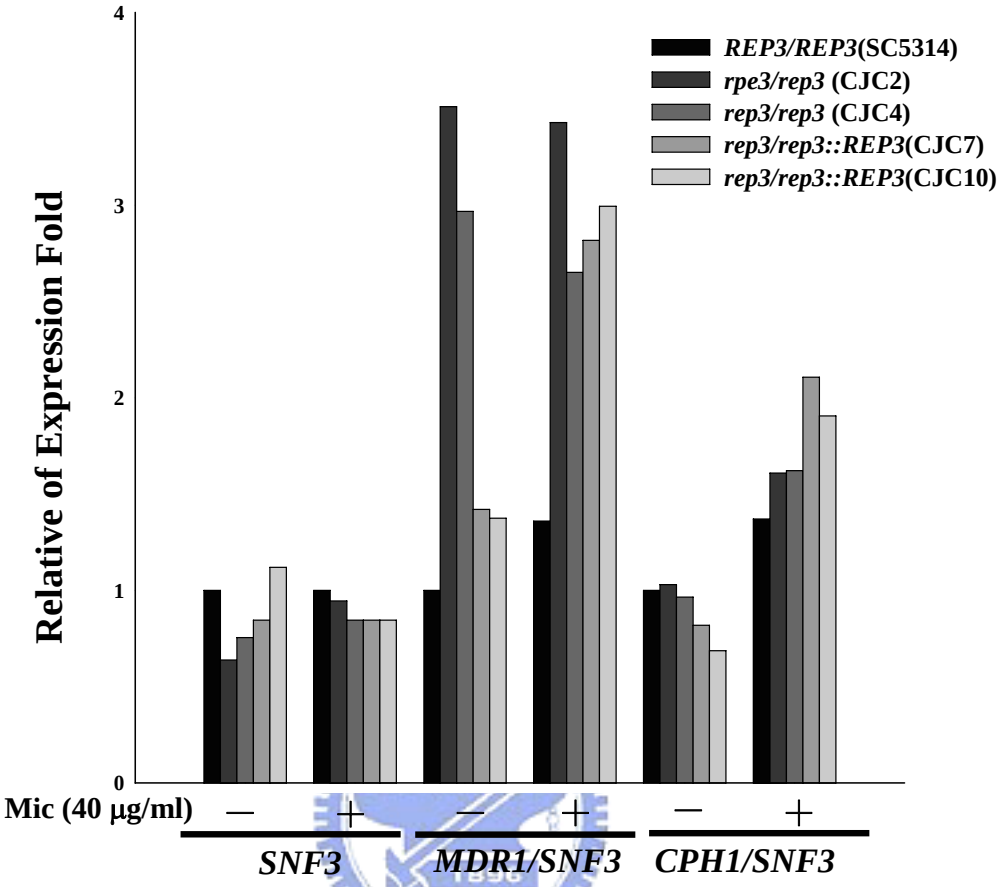


Fig. 15. The expression of *CPH1* and *MDR1* in the *rep3/rep3* null mutations (CJC2 and CJC4) and rescued strains (CJC7 and CJC10) in the present of miconazole.

Total RNAs were isolated from cells without or with 40 μg/ml of miconazole (Mic) treatment for 1.5 h at 35ºC. The mRNA abundances of azoles resistance related structure genes *MDR1* and *CPH1* were measured by real-time PCR. The transcript levels of different samples; wild type SC5314 (*REP3/REP3*), *rep3* null mutant (*rep3/rep3*) (CJC2 and CJC4), and *REP3* rescue (CJC7 and CJC10) were normalized with the level of *SNF3* mRNA isolated from the same conditions. The relative transcript levels of target genes from cells without drug treatment were defined as 1.

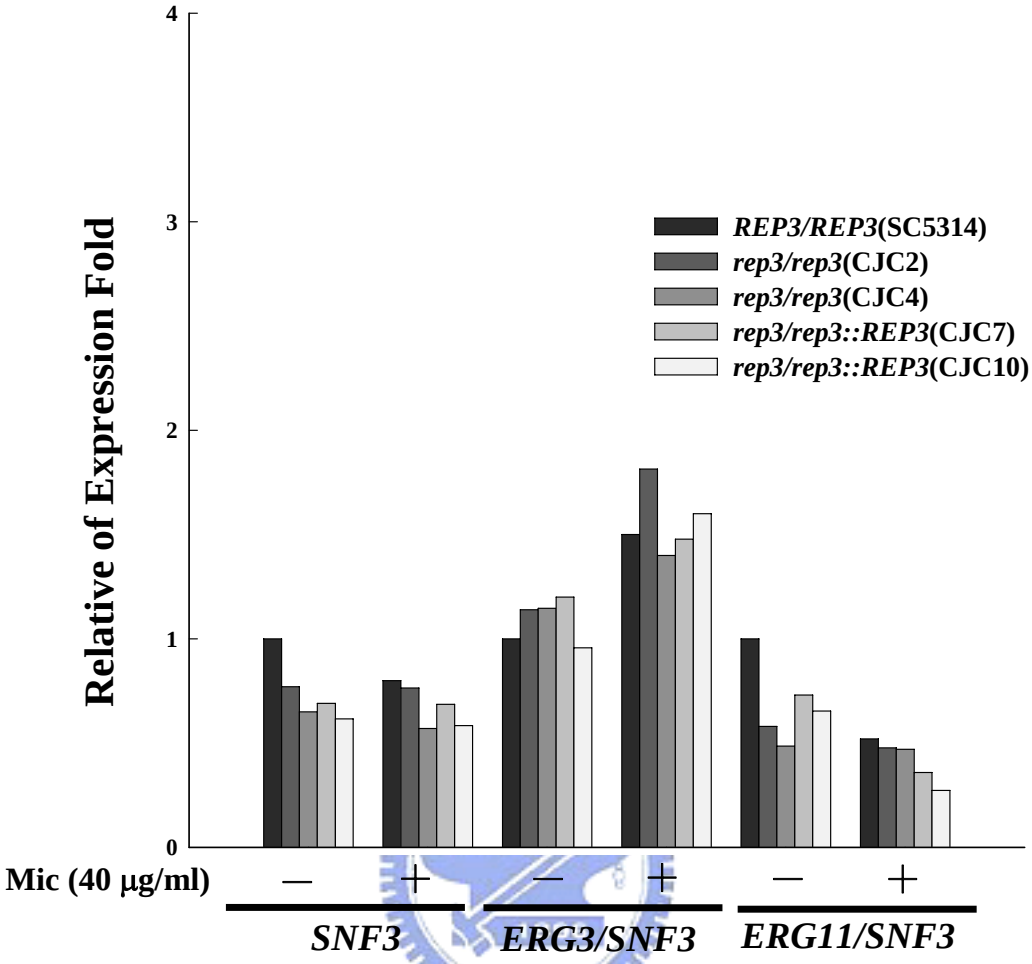
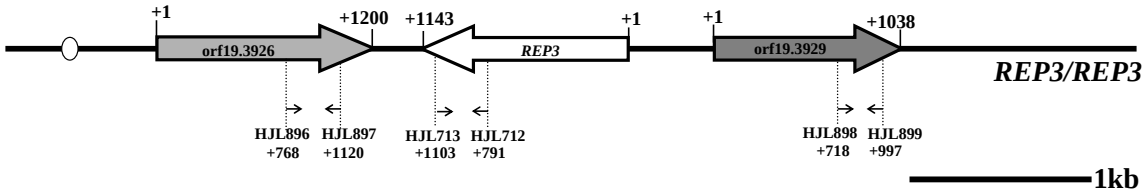


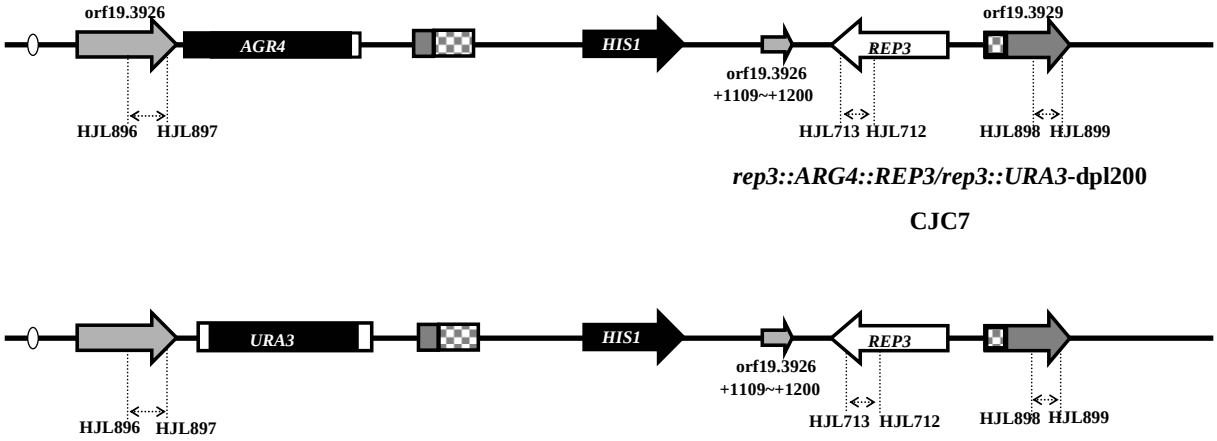
Fig. 16. The expression of *ERG3* and *ERG11* in the *rep3/rep3* null mutations (CJC2 and CJC4) in the present of miconazole.

Total RNAs were isolated from cells without or with 40 μg/ml of miconazole (Mic) treatment for 1.5 h at 35ºC. The mRNA abundances of azoles resistance related structure genes *ERG3* and *ERG11* were measured by real-time PCR. The transcript levels of different samples; wild type SC5314 (*REP3/REP3*), *rep3* null mutant (*rep3/rep3*) (CJC2 and CJC4), and *REP3* rescue (CJC7 and CJC10) were normalized with the level of *SNF3* mRNA isolated from the same conditions. The relative transcript levels of target genes from cells without drug treatment were defined as 1.

(A).


+1
+1200
+1143
+1
+1
+1038
orf19.3926
REP3
orf19.3929
REP3/REP3
HJL896 +768
HJL897 +1120
HJL713 +1103
HJL712 +791
HJL898 +718
HJL899 +997
1kb

(B).


orf19.3926
AGR4
HIS1
orf19.3926 +1109~+1200
REP3
orf19.3929
HJL896
HJL897
HJL713
HJL712
HJL898
HJL899
rep3::ARG4::REP3/rep3::URA3-dpl200
CJC7
URA3
HIS1
orf19.3926 +1109~+1200
REP3
HJL896
HJL897
HJL713
HJL712
HJL898
HJL899
rep3::ARG4/rep3::URA3-dpl200::REP3
CJC10

(C).

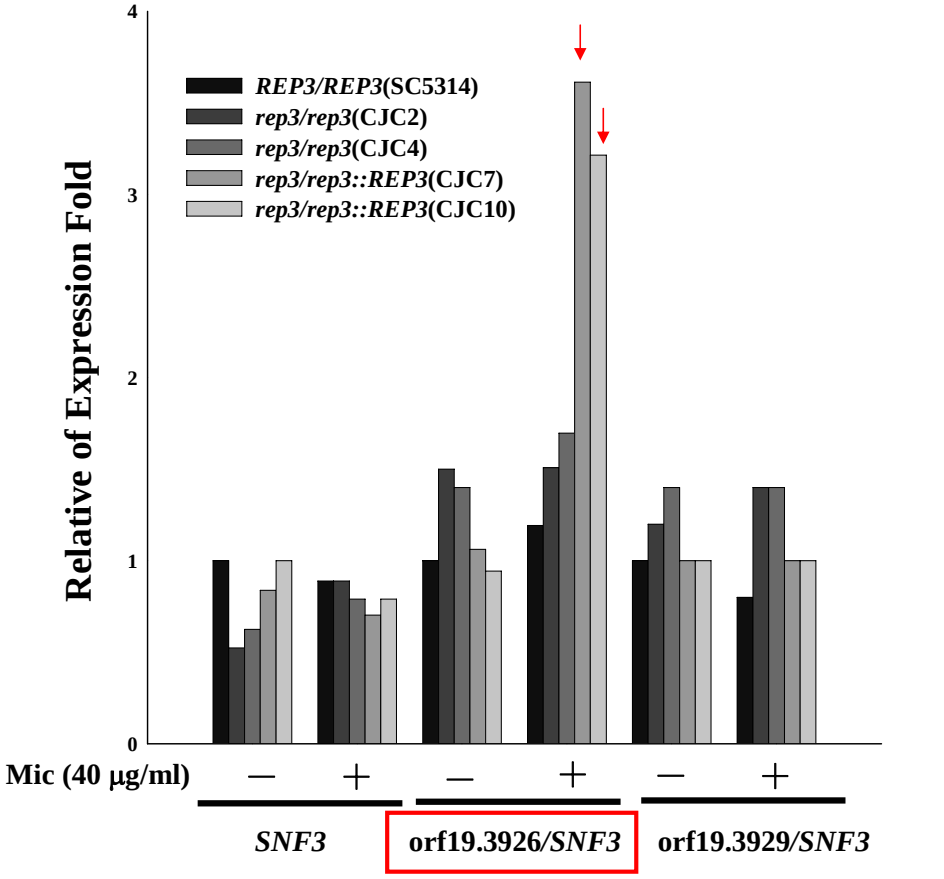
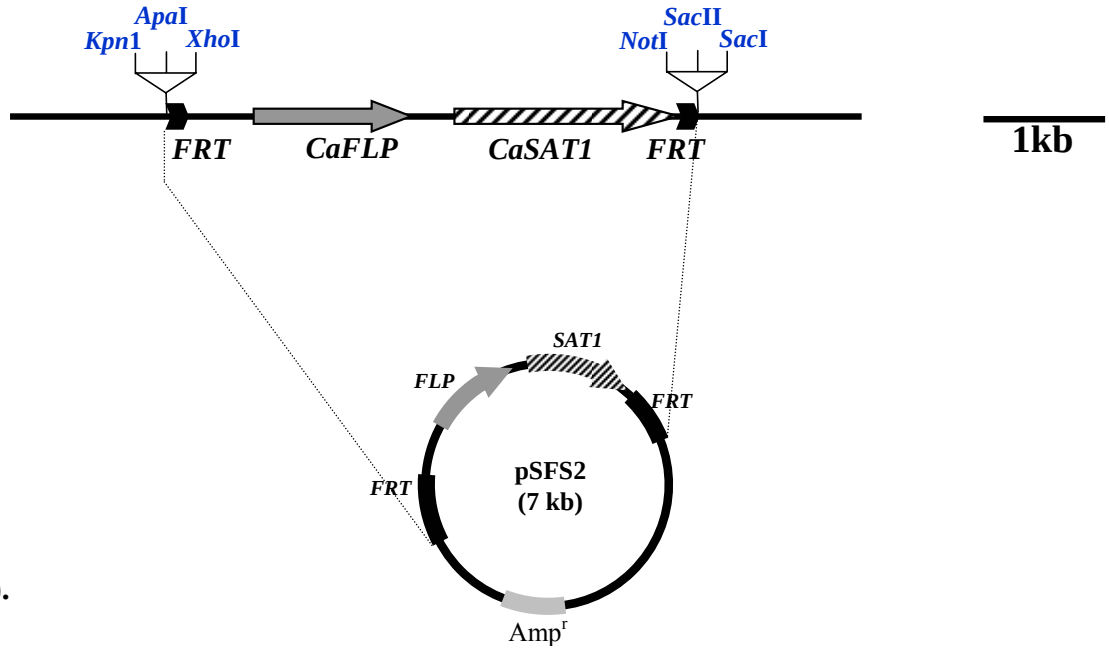

1 kb
Relative of Expression Fold
0
1
2
3
4
REP3/REP3(SC5314)
rep3/rep3(CJC2)
rep3/rep3(CJC4)
rep3/rep3::REP3(CJC7)
rep3/rep3::REP3(CJC10)
Mic (40 μg/ml)
—
+
—
+
—
+
SNF3
orf19.3926/SNF3
orf19.3929/SNF3

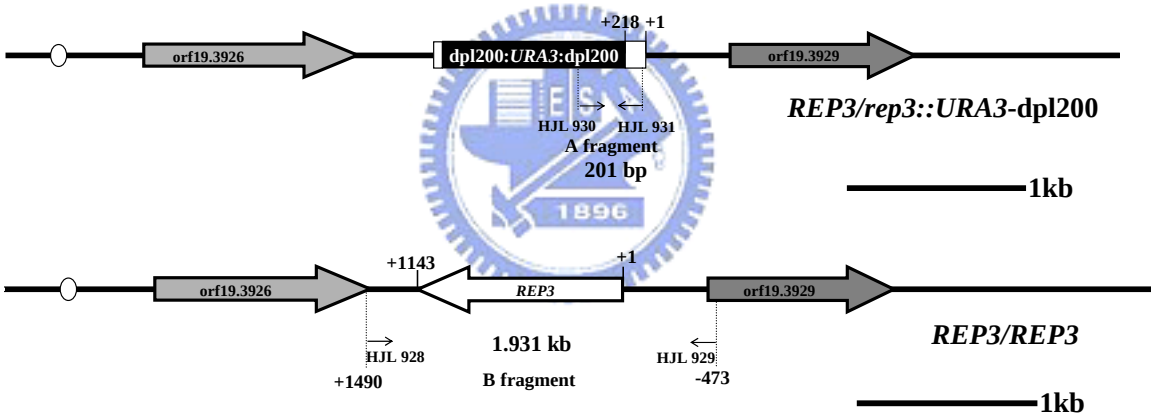
Fig. 17. The orf19.3926 in the *REP3* rescued strains showed higher mRNA level after the cells were treated with miconazole.

(A). The locations of primers in (*REP3/REP3*) ; HJL712-HJL713 for *REP3,* HJL896-HJL897 for orf19.3926 and HJL898-HJL899 for orf19.3929 were used to amplify PCR products using the LightCycler Probe Design program provided by the manufacturer. The arrows indicate the primers. (B). The arrows indicate the location of real-time primers on *rep3/rep3::REP3* (CJC7 and CJC10). (C). Total RNAs were isolated from cells without or with 40 μg/ml of miconazole (Mic) treatment for 1.5 h at 35ºC. The mRNA abundances of azoles resistance related structure genes orf19.3926 and orf19.3929 were measured by real-time PCR. The transcript levels of different samples; wild type SC5314 (*REP3/REP3*), *rep3* null mutant (*rep3/rep3*) (CJC2 and CJC4), and *REP3* rescue (CJC7 and CJC10) were normalized with the level of *SNF3* mRNA isolated from the same conditions. The relative transcript levels of target genes from cells without drug treatment were defined as 1.

(A).


ApaI
Kpn1
XhoI
SacII
NotI
SacI
FRT
CaFLP
CaSAT1
FRT
1kb
SAT1
FLP
FRT
FRT
pSFS2
(7 kb)
Amp^r

(B).


orf19.3926
dpl200:URA3:dpl200
+218 +1
orf19.3929
REP3/rep3::URA3-dpl200
HJL 930
HJL 931
A fragment
201 bp
1kb
+1143
+1
orf19.3926
REP3
orf19.3929
HJL 928
+1490
1.931 kb
B fragment
HJL 929
-473
REP3/REP3
1kb

(C).

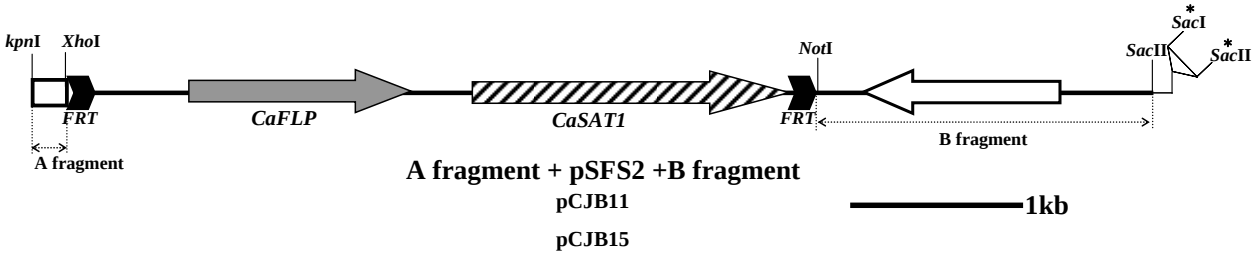

kpnI
XhoI
FRT
A fragment
CaFLP
CaSAT1
NotI
FRT
SacII
SacI
SacII
B fragment
A fragment + pSFS2 +B fragment
pCJB11
pCJB15
1kb

Fig. 18. Schematic diagram of construction *REP3* rescue plasmid (pCJB11 and pCJB15) by *SAT1* flipper method to transform *rep3/rep3* (CJC2 and CJC4) homozygous mutants.

(A). Structure of the *SAT1* flipper cassette contained in plasmid pSFS2. Partial restriction map of plasmid pSFS2 are showed blow. The *C. albicans*-adapted *FLP* gene (*caFLP*) is represented by the gray arrow, the nourseothricin resistance marker (*caSAT1*) by the hatched arrow, the *MAL2* promoter (*MAL2*) by the bent arrow, and the transcription termination sequence of the *C. albicans ACT1* gene by the filled circle. The 34bp *FLP* recombination target sequences (*FRT*, black arrows) (5'-GAAGTTCCTATACTTTCTAGAGAATAGGAA CTTC-3'). (B). The rescue plasmid constructed A and B fragments into pSFS2. A fragment contains the sequences of dpl200:*URA3*:dpl200 from -558 ~ -721 and the sequence of *REP3* from +130 ~ +218 and was amplified by primers HJL930 and HJL931 from *rep3::ARG4/rep3::URA3* genomic DNA. B fragment from *REP3* after stop codon 289 bp to +22 of orf19.3929 and was amplified by primers HJL928 and HJL929 from *REP3/REP3* (SC5314) genomic DNA. (C). Partial structure of the pCJB11 and pCJB15 which were ligated A and B fragments on pSFS2 both sides. The plasmid pCJB11 (pCJB15) was restricted by *kpn*I-*Sac*II to generated into *rep3/rep3* homozygous mutants (CJC2 and CJC4). The restriction enzyme on the plasmid pSFS2 is indicated by "*".

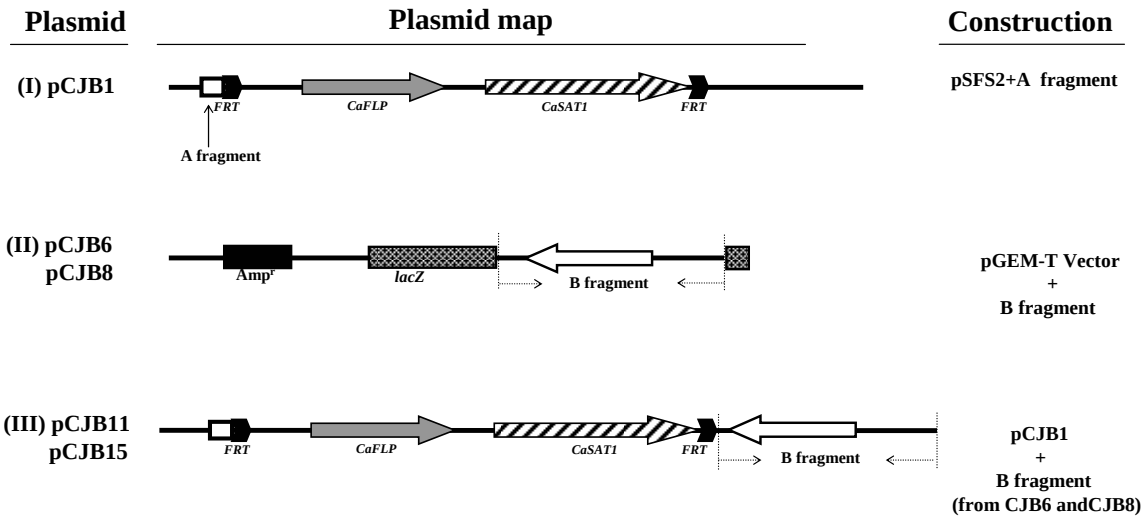
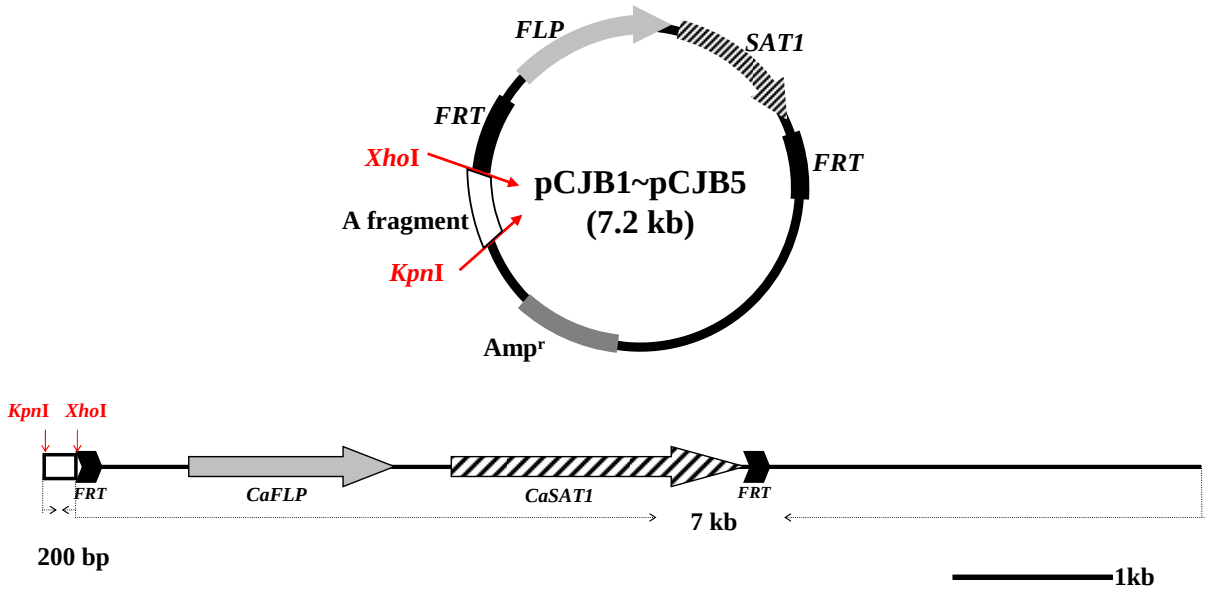


Fig. 19. Processes of construction *REP3* rescue plasmid (pCJB11 and pCJB15) by *SAT1* flipper method.

The *FLP* gene (*caFLP*) is represented by the gray arrow, the nourseothricin resistance marker (*caSAT1*) by the hatched arrow, the *MAL2* promoter (*MAL2*) by the bent arrow, and the transcription termination sequence of the *C. albicans ACT1* gene by the filled circle. The 34bp *FLP* recombination target sequences (*FRT*, black arrows). First, the plasmid pCJB1 (I) based on the plasmid pSFS2 with A fragment. Then B fragment was amplified by PCR which was cloned into pGEM-T easy vector to grate pCJB6 & pCJB8 (II). The plasmid pCJB6 (or pCJB8) was digested with *Not*I which ligated to pCJB1 to produce pCJB11 (or pCJB15) (III).

(A).



(B).

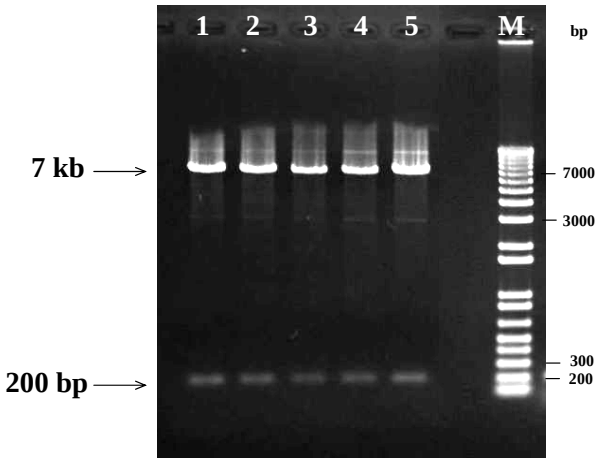
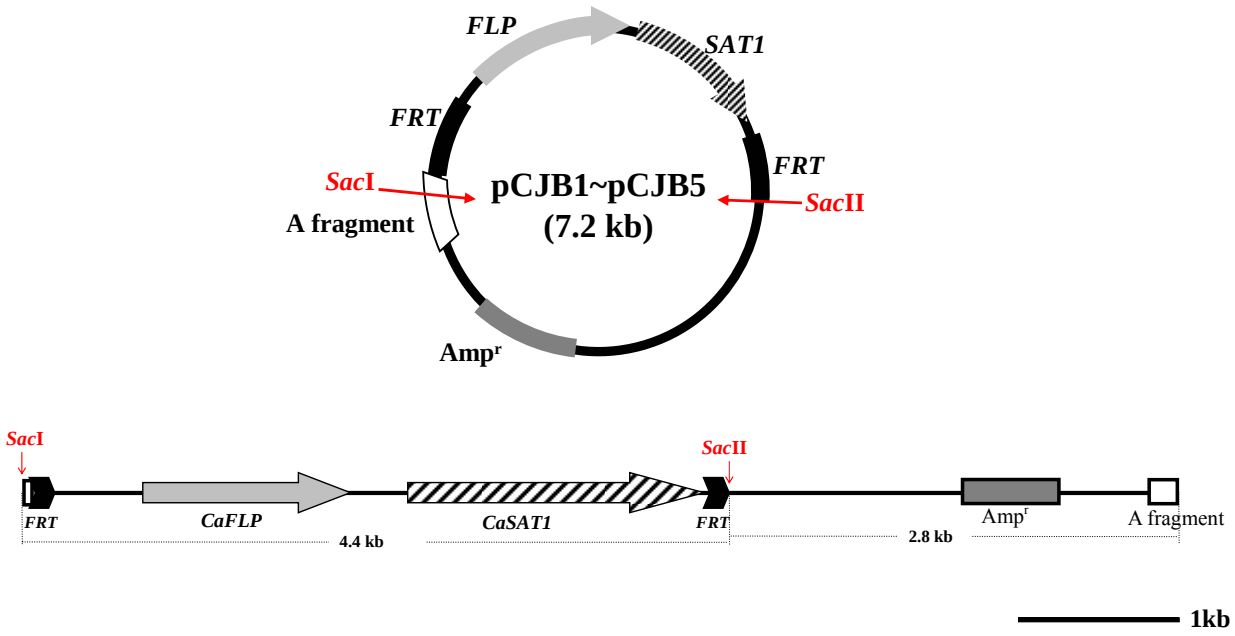


Fig. 20. Constructing the plasmid pCJB1 ~ pCJB5 and confirming the transformants by *kpn*I and *Xho*I digestion.

(A). Schematic drawing of assessing plasmid pCJB1 ~ pCJB5 digested with *kpn*I and *Xho*I. (B). Result of the enzyme digestion. M: 1kb plus DNA ladder 0.4 μ g. Lane 1 ~ 5 : *kpn*I and *Xho*I digested of plasmid DNA isolated from candidates (pCJB1 ~ pCJB5). The fragments sizes that were obtained are indicated to the left of the figure.

(A).



(B).

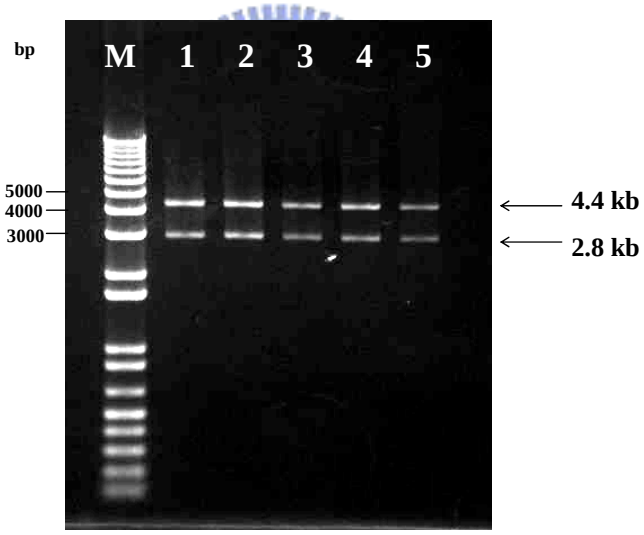
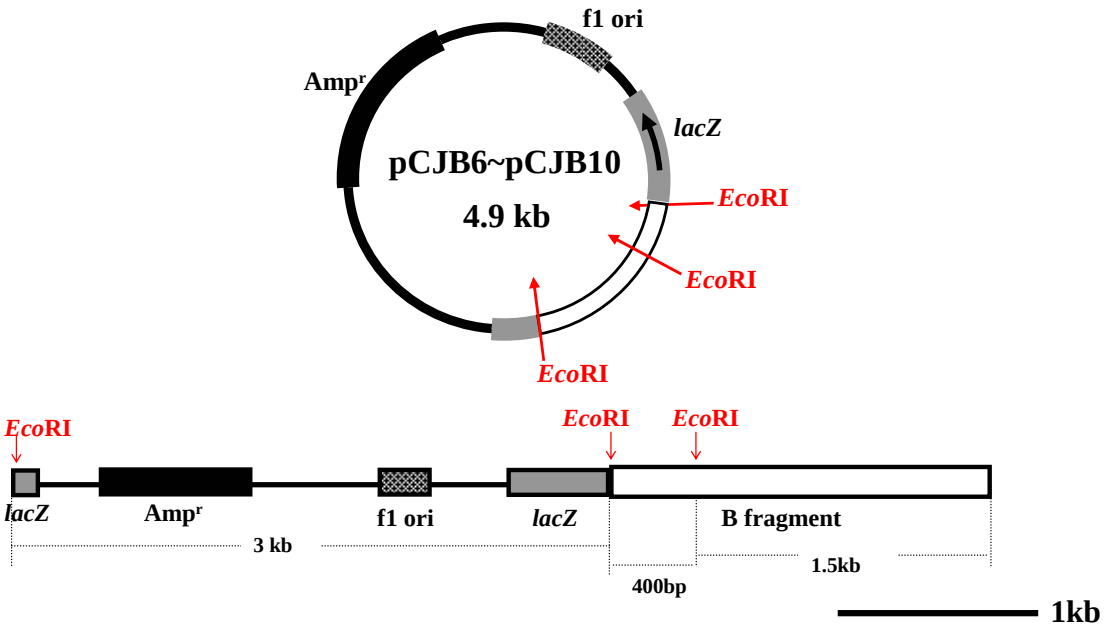


Fig. 21. Constructing the plasmid pCJB1 ~ pCJB5 and confirming the transformants by *Sca*I and *Sac*II digestion.

(A). Schematic drawing of assessing plasmid pCJB1 ~ pCJB5 digested with *Sca*I and *Sac*II. (B). Result of the enzyme digestion. M: 1kb plus DNA ladder 0.4 μ g. Lane 1 ~ 5 : *Sca*I and *Sac*II digested of plasmid DNA isolated from candidates (pCJB1 ~ pCJB5). The fragments sizes that were obtained are indicated to the right of the figure.

(A).



(B).

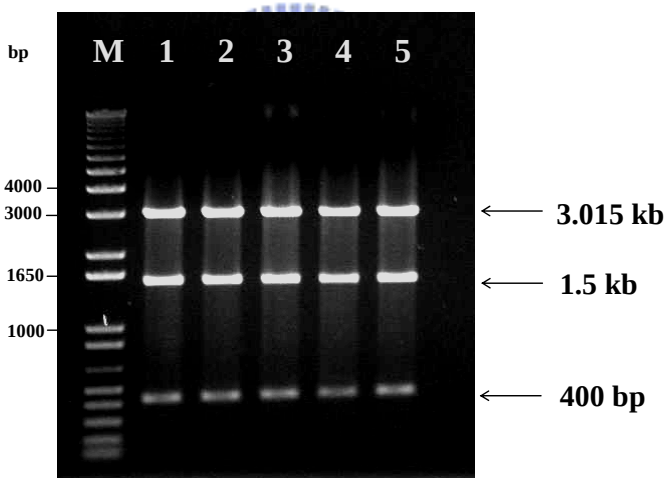
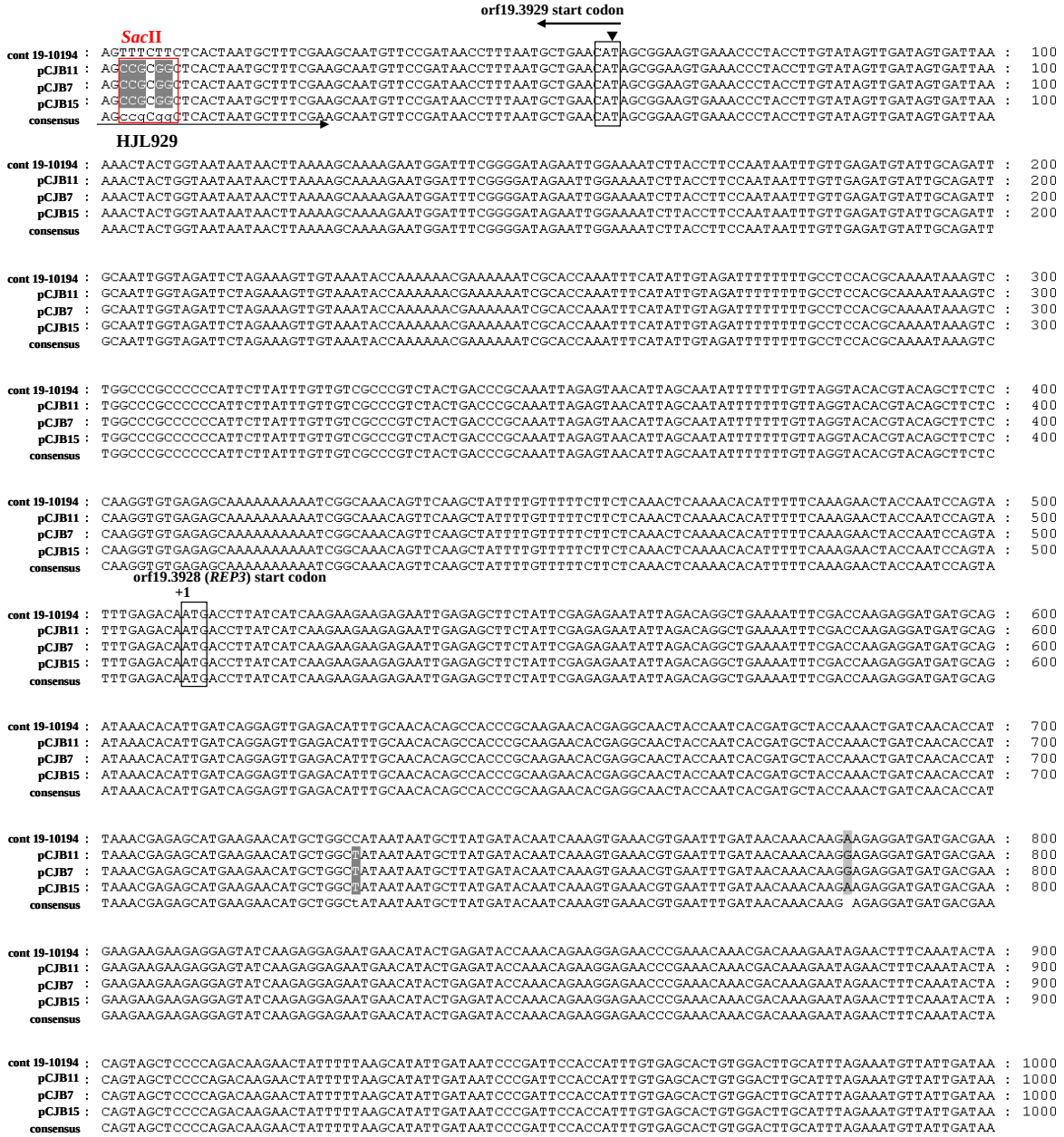


Fig. 22. Constructing the plasmid pCJB6 ~ pCJB10 and confirming the transformants by enzyme digested.

(A). Schematic drawing of assessing plasmids pCJB6 ~ pCJB10 digested with *Eco*RI . A 1.931 kb B fragment (*REP3*) was amplified by PCR from Can14 genomic DNA with primers HJL 928 (*Not*I) and HJL 929 (*Sac*II) which is cloned into pGEM-T easy vector. (B). *Eco*RI digested of plasmid DNA isolated from candidates (pCJB6 ~ pCJB10). Result of the enzyme digestion. M: 1kb plus DNA ladder 0.4 μg. Lane 1 ~ 5 : *Eco*RI digested of plasmid DNA isolated from candidates. The fragments sizes that were obtained are indicated to the right of the figure.


orf19.3929 start codon
SacII
HJL929
orf19.3928 (REP3) start codon
+1

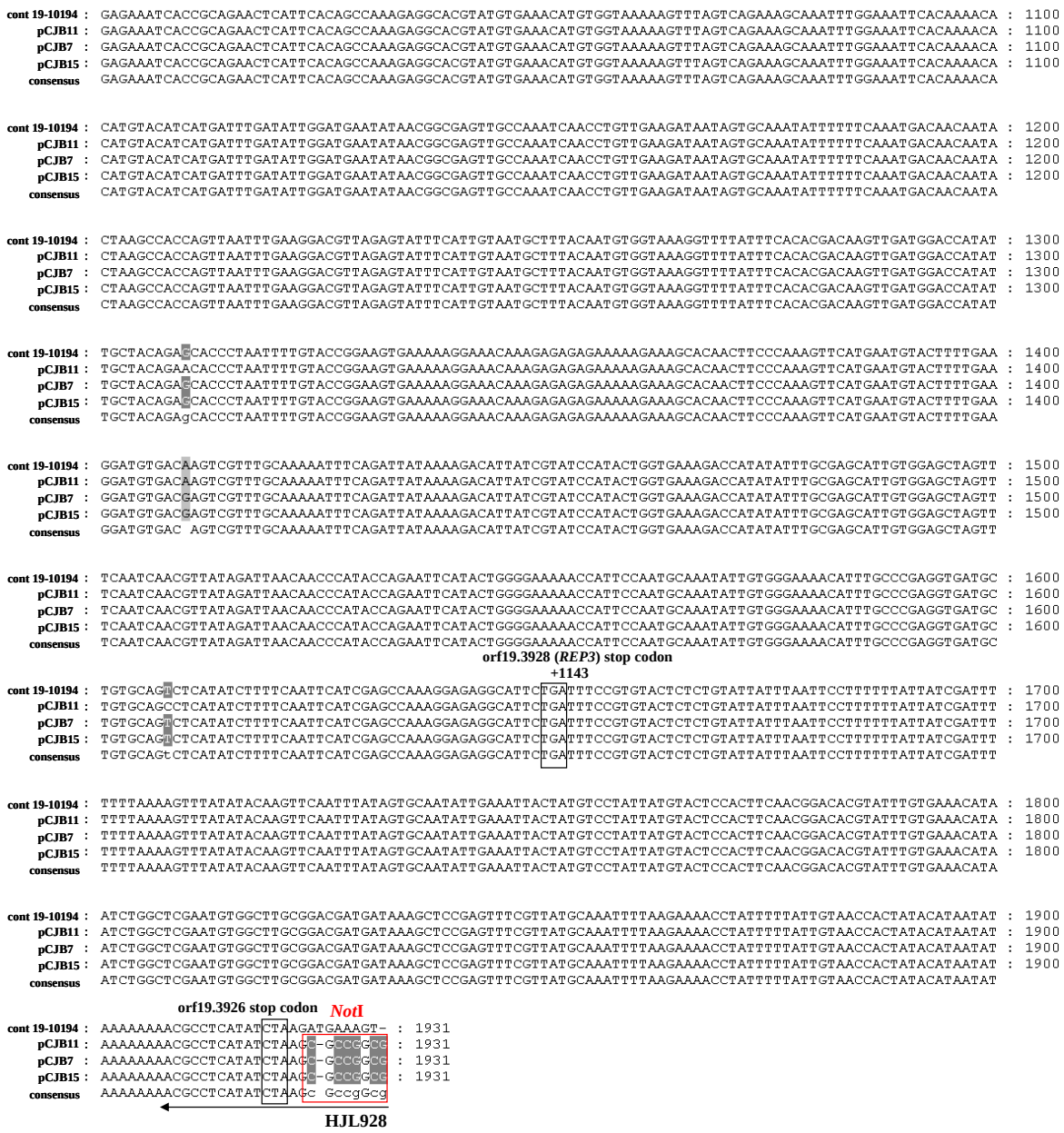


Fig. 23. Comparison *REP3* sequences of cont19-10194 nucleotide sequences with pCJB11, pCJB7 and pCJB15.

Concensus in sequences are shown with a white background and black lettering. Residues that are not identical among those five sequences are highlighted with a grey background. The *REP3* start and stop codon are indicated by +1 and +1143 below the sequence. The arrow indicates the direction of orf19.3929 expression.

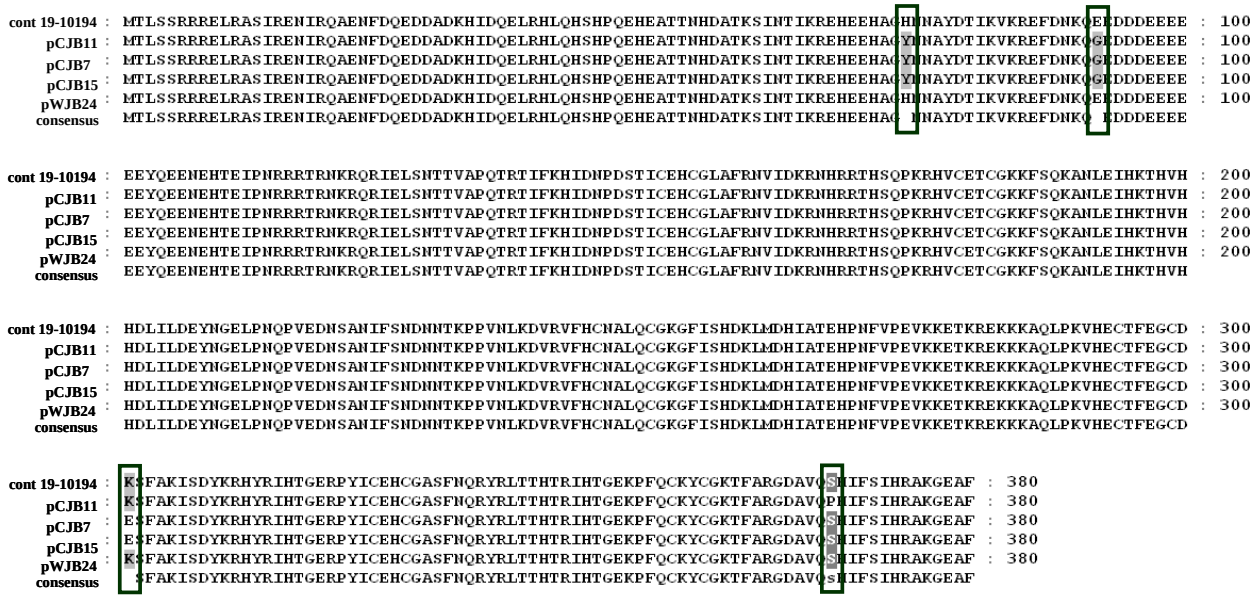


Fig. 24. Comparison of the *REP3* amino acid sequences among pCJB11, pCJB7 and pCJB15.

Residues identical in all the proteins are shown with a white background and black lettering. Grey background shades the similarity amino acid among at least three proteins.

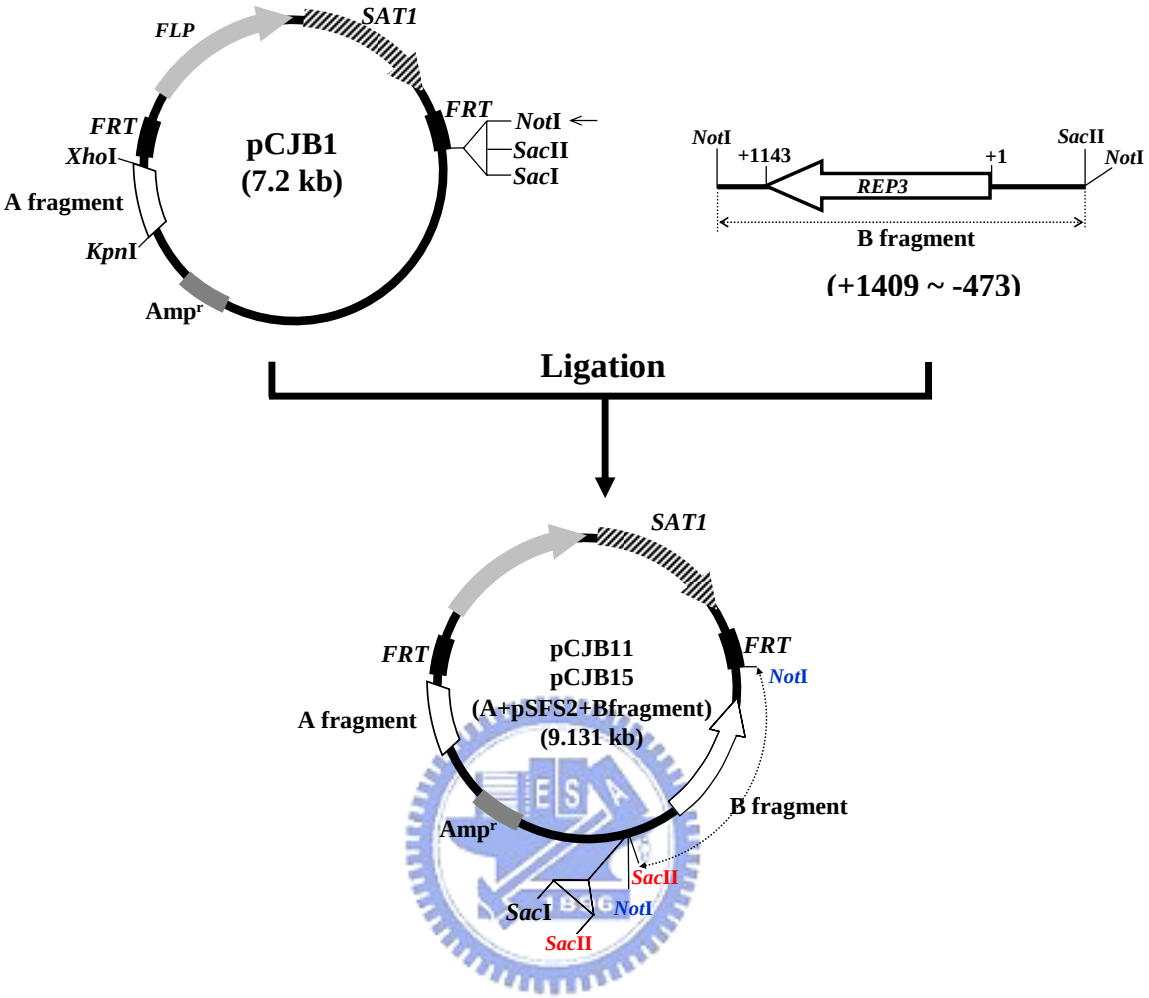
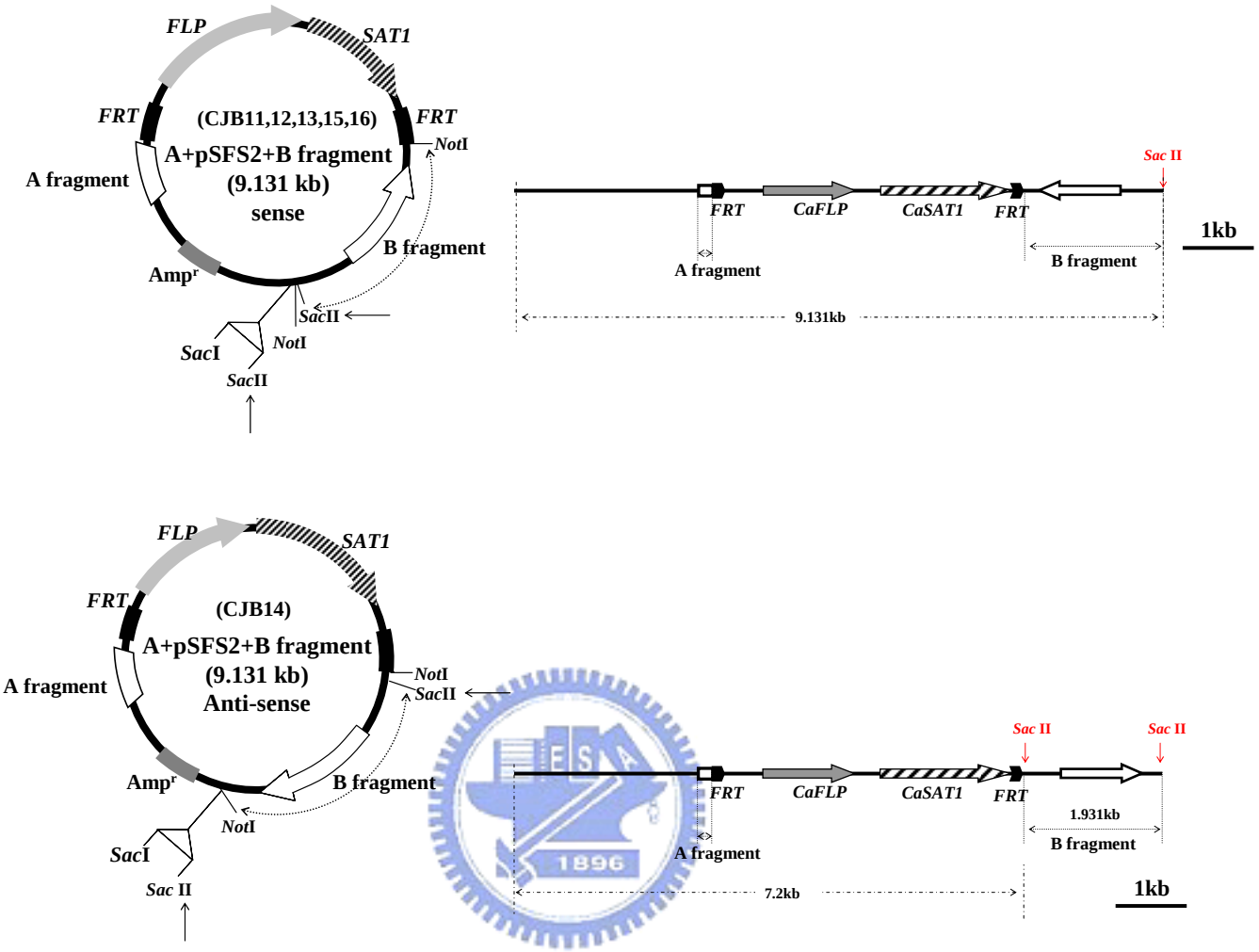


Fig. 25. Schematic diagram of construction strategy of pCJB11 and pCJB15.

The B fragment containing full length *REP3* (+1409 ~ -473) from pCJB6 (pCJB8) was ligated with pCJB1 at *Not*I site to produce pCJB11 (pCJB15). The A fragment contains the sequences of URA3 (-558 ~ -721) and the sequence of *REP3* (+130 ~ +218).

(A).


FLP
SAT1
FRT
FRT
(CJB11,12,13,15,16)
A+pSFS2+B fragment
(9.131 kb)
sense
NotI
A fragment
B fragment
Amp^r
SacII
SacI
NotI
SacII
Sac II
FRT
CaFLP
CaSAT1
FRT
A fragment
B fragment
1kb
9.131kb
FLP
SAT1
FRT
(CJB14)
A+pSFS2+B fragment
(9.131 kb)
Anti-sense
NotI
SacII
A fragment
Amp^r
B fragment
NotI
SacI
Sac II
Sac II
Sac II
FRT
CaFLP
CaSAT1
FRT
1.931kb
A fragment
B fragment
7.2kb
1kb

(B).

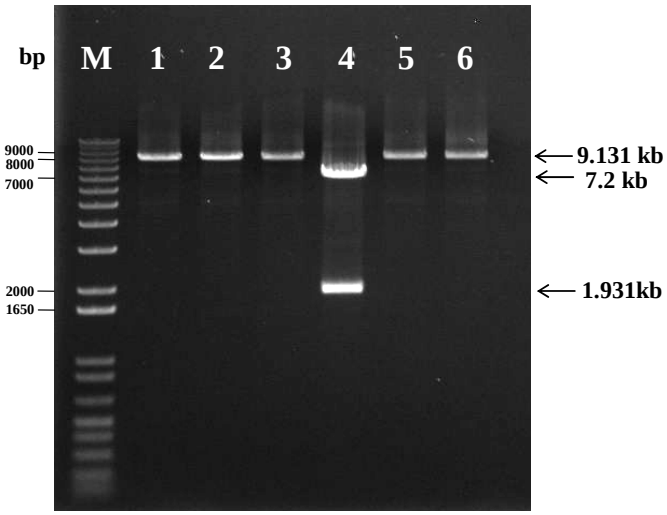

bp
M
1
2
3
4
5
6
9000
8000
7000
2000
1650
9.131 kb
7.2 kb
1.931kb

Fig. 26. Constructing the plasmid pCJB11 ~ pCJB16 and confirming the transformants by enzyme digestions.

(A). Schematic diagram of the plasmid map of pCJB11 ~ pCJB16. *Sac*II digestion was used to check candidates for either sense or anti-sense directions. Arrows indicate the position of the *Sac*II on plasmids pCJB11 ~ pCJB16. (B). Electrophoresis of the enzyme digestion reaction mixtures. Lane M : 1kb plus DNA ladder 0.4 μ g. Digestion of transformants with *Sac*II were obtained are indicated to the right of the figure. Lane 1 ~ 6: *Sac*II digested of plasmid DNA isolated from candidates (pCJB11 ~ pCJB16).

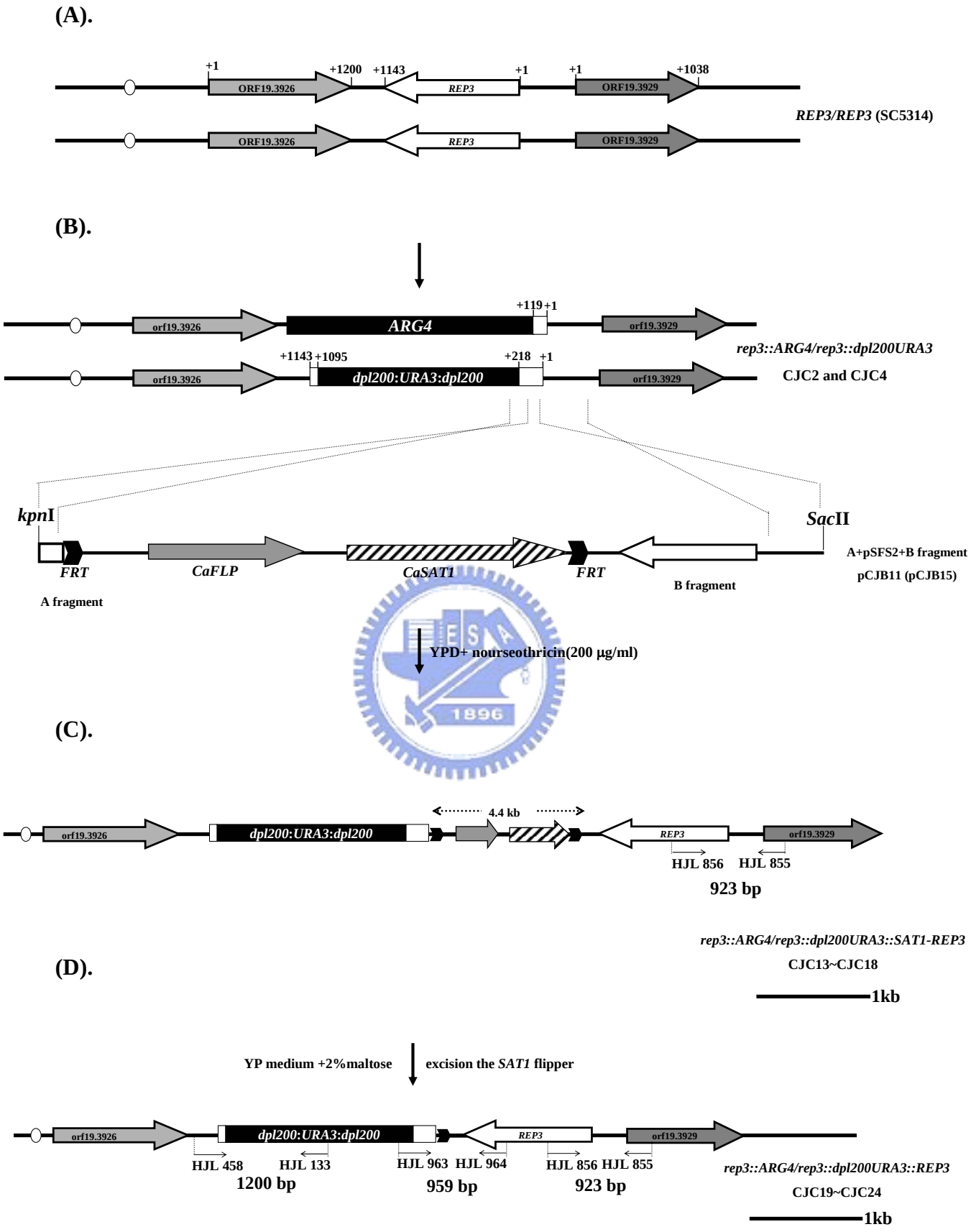
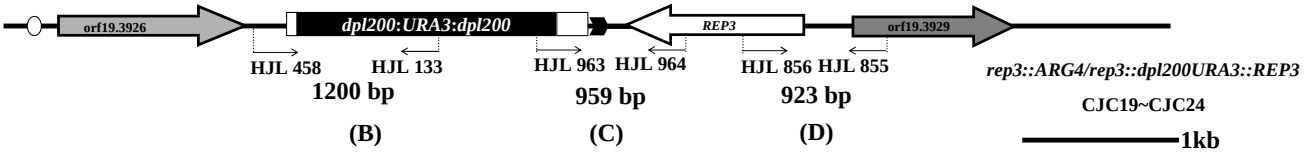

(A).
+1
+1200
+1143
+1
+1
+1038
ORF19.3926
REP3
ORF19.3929
REP3/REP3 (SC5314)
ORF19.3926
REP3
ORF19.3929
(B).
+119 +1
orf19.3926
ARG4
orf19.3929
rep3::ARG4/rep3::dpl200URA3
+1143 +1095
+218 +1
orf19.3926
dpl200:URA3:dpl200
orf19.3929
CJC2 and CJC4
kpnI
SacII
FRT
CaFLP
CaSAT1
FRT
A+pSFS2+B fragment
pCJB11 (pCJB15)
A fragment
B fragment
YPD+ nourseothricin(200 μg/ml)
(C).
4.4 kb
orf19.3926
dpl200:URA3:dpl200
REP3
orf19.3929
HJL 856
HJL 855
923 bp
rep3::ARG4/rep3::dpl200URA3::SAT1-REP3
CJC13~CJC18
1kb
(D).
YP medium +2%maltose
excision the SAT1 flipper
orf19.3926
dpl200:URA3:dpl200
REP3
orf19.3929
HJL 458
HJL 133
HJL 963
HJL 964
HJL 856
HJL 855
1200 bp
959 bp
923 bp
rep3::ARG4/rep3::dpl200URA3::REP3
CJC19~CJC24
1kb

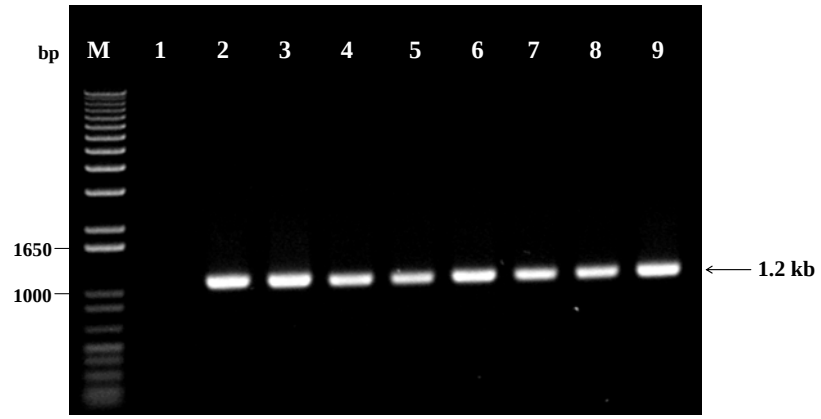
Fig. 27. Schematic diagram of integration strategy of *REP3* by *SAT1* flipping method.

(A). *REP3/REP3* (SC5314). The white arrow indicates the ORF of *REP3* (+1~ +1143). Up stream 451 bp from *REP3* is orf19.3929 (+1 ~ +1038). Another ORF; orf19.3926 (+1 ~ +1200) is down stream of *REP3*. (B). *rep3*::*ARG4*/*rep3*::*URA3*-dpl200 (CJC2 and CJC4) structure map was shown below. Black box indicates the selection construction of markers *ARG4 and* selection cassette *URA3*-dpl200. The open box represents the ORF of the *REP3* gene. The *kpn*I-*Sac*II fragments from pCJB11 (pCJB15) were transformed into CJC2 and CJC4. Dotted lines indicate the homology region for recombination. Integration of the cassette into genome is selected in the presence of nourseothricin. (C). Assessing the *rep3*::*ARG4*/*rep3*::*URA3*-dpl200::*SAT1*-*REP3* strains (CJC13 ~ CJC18) by PCR. The reintegration of *REP3* was confirmed by PCR with HJL855 and HJL856 that generated 923 kb fragments. Arrows indicates the positions of the primers on the genome. (D). Confirming the *REP3* rescued strains constructed by *SAT1* flipping method (CJC19 ~ CJC24) excising the *SAT1* cassette. The reintegration of *REP3* was confirmed by PCR with primers HJL855 and HJL856 that generated 923 kb fragments from orf19.3928 (*REP3*) -460 ~ +469. To confirm the result of excision in *REP3* rescued strains (CJC19 ~ CJC24) by PCR primers with HJL963 and HJL964 from -618 of *CaURA3* (pGEM-URA3) to +787 of orf19.3928. HJL458 and HJL133 is the positive control from +1333 ~ +1316 of orf19.3928 (*REP3*) and *CaURA3* (pDDB57):+51 ~ +68. Arrows indicated the positions of the primers on the genome.

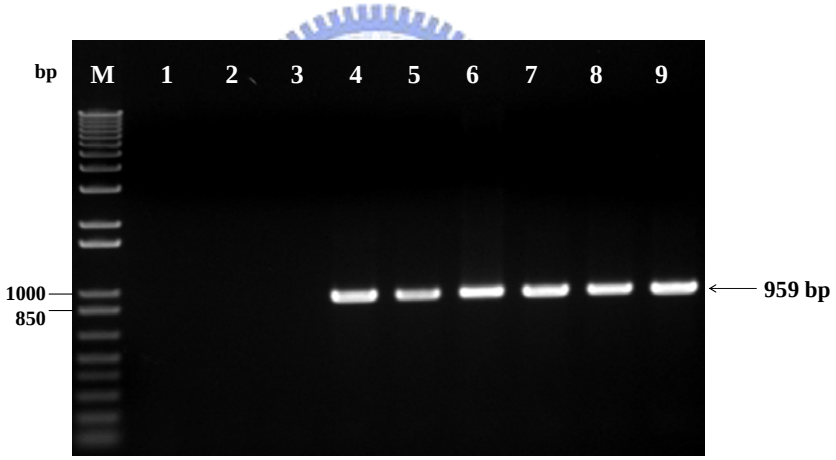
(A).


orf19.3926
dpl200:URA3:dpl200
REP3
orf19.3929
HJL 458
HJL 133
HJL 963
HJL 964
HJL 856
HJL 855
1200 bp
959 bp
923 bp
(B)
(C)
(D)
rep3::ARG4/rep3::dpl200URA3::REP3
CJC19~CJC24
1kb

(B).


bp
M 1 2 3 4 5 6 7 8 9
1650
1000
1.2 kb

(C).


bp
M 1 2 3 4 5 6 7 8 9
1000
850
959 bp

(D).

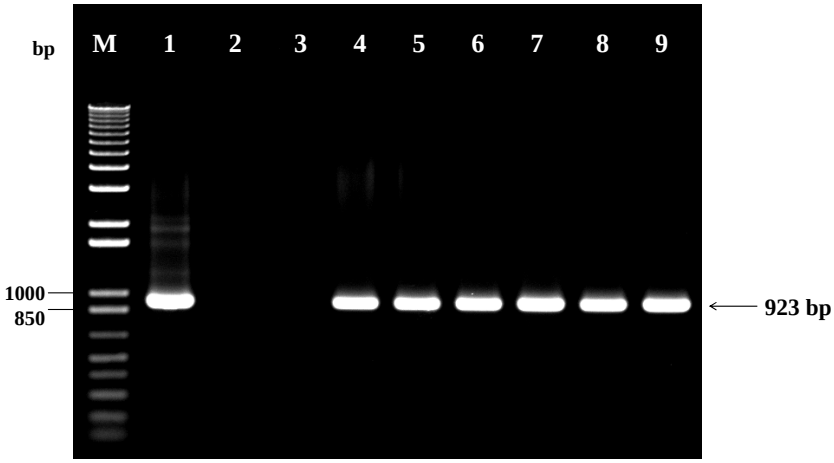

bp
M 1 2 3 4 5 6 7 8 9
1000
850
923 bp

Fig. 28. Assessing the *REP3* integration to *rep3* null mutant strains and excision of the *SAT1* cassette by PCR.

(A). Schematic drawing of assessing *REP3* rescued strains (CJC19 ~ CJC24) using primers HJL458 and HJL133; HJL963 and HJL964; HJL855 and HJL856. (B) ~ (D). Result of the PCR. M: 1kb plus DNA ladder. Lane 1: wild type (BWP17); lane 2 and lane 3: *rep3* homozygous mutant strains (CJC2 and CJC4); lane 4 ~ 9: The excision of the *SAT1* cassette in *REP3* rescued strains (CJC19 ~ CJC24). Arrows indicate the positions of the primers on the genome.

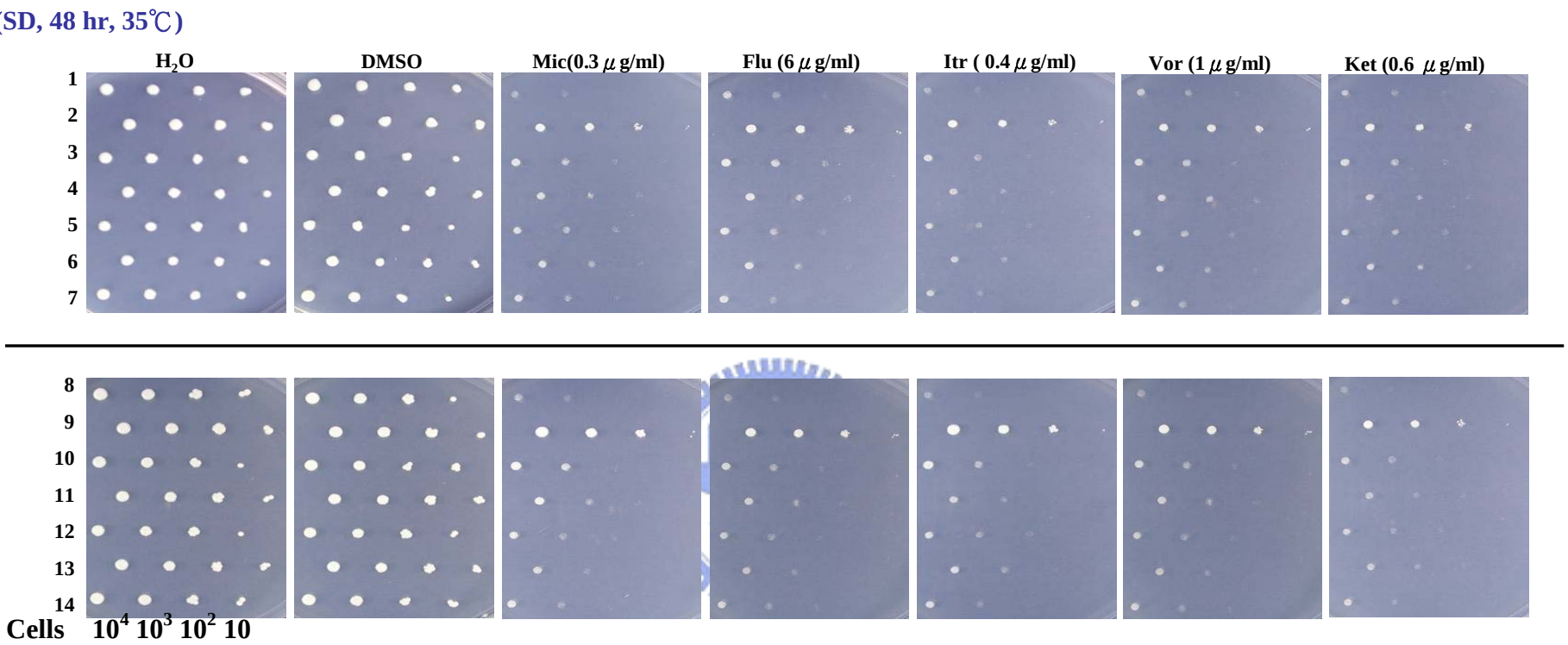


Fig. 29. Mutations on *REP3* increase the susceptibility to antifungal agents but the rescued strains (CJC19 ~ CJC24) did not show a restored drug susceptibility phenotype in *C. albicans*.

The cells were diluted to an OD_{600} of 2 and spotted onto drug plates along with 10-fold serial dilution. Azoles were prepared to a final concentration in SD agar, and the same volume (1/1000 in SD agar) of drug solvent-DMSO was used to prepare the control plate. Cells were grown on media in the absence of drug, or in the presence of 6 μg Fluconazole ml^{-1} (Flu), 0.3 μg Miconazole ml^{-1} (Mic), 0.6 μg Ketconazole ml^{-1} (Ket), 0.4 μg Itraconazole ml^{-1} (Itr) and 1 μg Voriconazole ml^{-1} (Vor). The results were photographed after 2 days of growth at 35 ºC. (1),(8) *cdr1/cdr1*(YLO159); (2),(9) *REP3/REP3*(SC5314); (3) *rep3/rep3*; *his1:: hisG/his1:: hisG::HIS1* (CJC2); (4) *rep3/rep3*::*REP3*-CJC2-pCJB11 (CJC19); (5),(6) *rep3/rep3*::*REP3*-CJC2-pCJB15 (CJC20 and CJC21); (7) *rep3/rep3*::*REP3*-CSC80-pWJB24 (CJC7); (10) *rep3/rep3*; *his1:: hisG/his1:: hisG::HIS1* (CJC4); (11) *rep3/rep3*::*REP3*-CJC4-pCJB11 (CJC22); (12),(13) *rep3/rep3*::*REP3*-CJC4-pCJB15 (CJC23 and CJC24); (14) *rep3/rep3*::*REP3*-CSC81-pWJB24 (CJC10). The results were photographed after 2 days of growth at 35℃

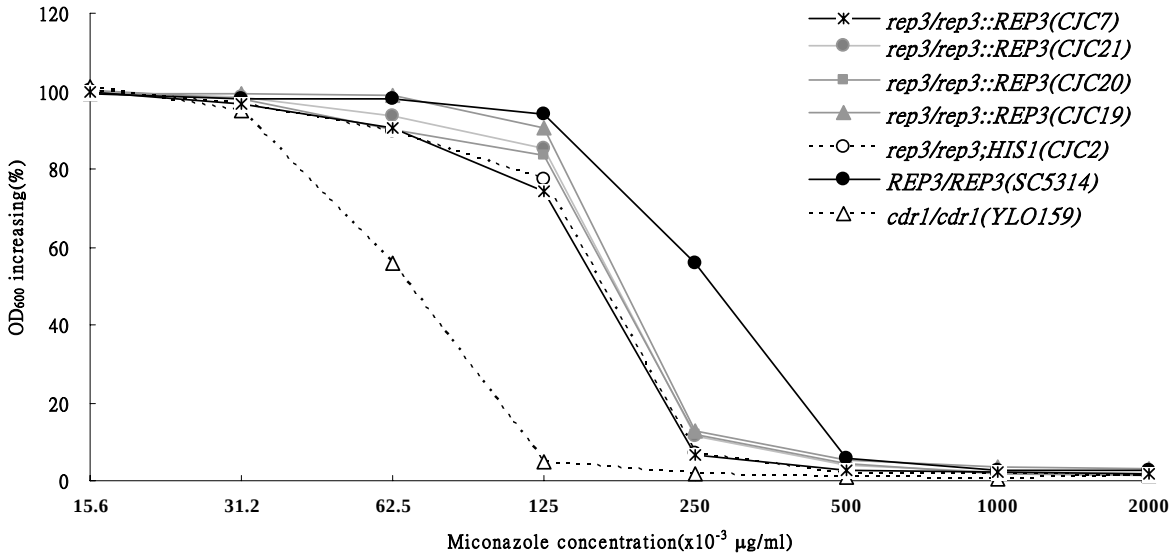


Fig. 30. Comparing the *rep3* homozygous mutants and the *REP3* rescued strains (CJC19 ~ CJC21) by *SAT1* flipper method for the susceptibility to miconazole.

The antifungal drugs susceptibility of seven strains; (—●—) wild-type SC5314 (*REP3/REP3*), (···○···) *rep3* null mutant (*rep3/rep3*) (CJC2), (—●—) (—▲—) (—■—) *REP3* rescue with *SAT1* flipper method (*rep3/rep3::REP3*) (CJC19 ~ CJC21), (—✳—) *REP3* rescue (CJC7) and (···△···) *cdr1/cdr1* (YLO159) were determined by the microdilution method with some modifications. The antifungal agent miconazole was freshly prepared as a stock at the concentration of 4 mg/ml in DMSO. To prepare working concentration (2 ~ 0.156 μg/ml), the drug was processed by stepwise twofold dilutions in SD medium. A drug-free culture and a sterile control were included in each microtiter plate. The optical density (OD) in each well of the microtiter plate was read with a microplate reader at 600 nm after incubated at 35° C for 48 h. The drug inhibitory curve was presented by the OD of each well with different concentrations of miconazole relative to the OD of the drug-free control.

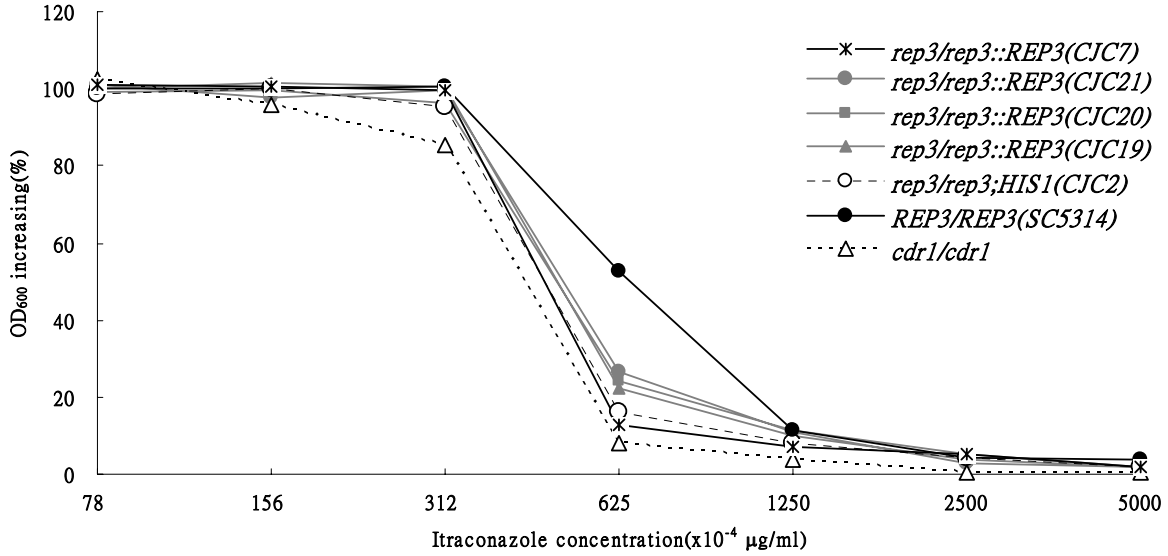


Fig. 31. Comparing the *rep3* homozygous mutants and the *REP3* rescued strains (CJC19 ~ CJC21) by *SAT1* flipper method for the susceptibility to itraconazole.

The antifungal drugs susceptibility of seven strains; (—●—) wild-type SC5314 (*REP3/REP3*), (···○···) *rep3* null mutant (*rep3/rep3*) (CJC2), (—●—) (—▲—) (—■—) *REP3* rescue with *SAT1* flipper method (*rep3/rep3*::*REP3*) (CJC19 ~ CJC21), (—✳—) *REP3* rescue (CJC7) and (···△···) *cdr1/cdr1* (YLO159) were determined by the microdilution method with some modifications. The antifungal agent itraconazole was freshly prepared as a stock at the concentration of 1 mg/ml in DMSO. To prepare working concentration (0.5 ~ 0.0078 μg/ml), the drug was processed by stepwise twofold dilutions in SD medium. A drug-free culture and a sterile control were included in each microtiter plate. The optical density (OD) in each well of the microtiter plate was read with a microplate reader at 600 nm after incubated at 35° C for 48 h. The drug inhibitory curve was presented by the OD of each well with different concentrations of itraconazole relative to the OD of the drug-free control.

簡歷

吳金蓉

Chin Jung Wu

主要求學歷程:

✧ 2005-2007 國立交通大學

生物科技學系　生化工程所

✧ 2001-2005 私立大葉大學

生物產業科技學系

✧ 1994-2000 私立金陵女中

主要得獎經歷:

✧ 2005 年　斐陶斐學會榮譽會員

✧ 2003 年　國科會大專生暑期研究計畫

育成含降血脂機能性多胜肽轉基因水稻

期刊論文:

Wang, J. S., Yang, Y. L., **Wu, C. J.**, Ouyang, K. J., Tseng, K. Y., Chen, C. G., Wang, H. and Lo, H. J.* (2006) The DNA-binding domain of CaNdt80p is required to activate *CDR1* involved in drug resistance in *Candida albicans*. *Journal of Medical Microbiology* 55, 1403–1411