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碩士論文

野生種白色念珠菌與 *cph1/cph1 efg1/efg1* 雙基因突變種於可誘發野生種形成菌絲環境下之基因表現比較

Comparison of Gene Expression between Wild-Type *Candida albicans* and *cph1/cph1 efg1/efg1* Double Mutant under the Condition of Inducing Filament Formation of Wild Type

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摘要

白色念珠菌 (*Candida albicans*) 是一種真菌類伺機性病源菌，在免疫系統不健全的人體中引起病害，嚴重時足以致命。在型態上，野生種白色念珠菌會形成菌絲，並且對寄主有致病力，一般也認爲形成菌絲與否跟有無致病能力有相當程度的關係；而在過去的研究中發現一株 *CPH1 EFG1* 雙基因突變種沒有形成菌絲的能力，亦無法致病。

本研究藉由抑制相減雜交技術 **(Suppression Subtractive hybridization, SSH)** 得到在能誘發野生種產生菌絲的條件下，野生種和突變種中表現不同之基因。其中包含影響形態或/及致病之基因，我們期望從中篩選出確實賦予白色念珠菌致病能力之基因，提供藥物設計者更精準的藥物標的 **(drug target)**。針對白色念珠菌特有之基因設計藥物，可以減少對寄主本身的危害，並且增加治療的效率，避免抗藥性的產生。

在本實驗之 SHH 結果中，逢機挑選 32 個基因片段進行分析，分離出種 **12** 種不同基因。其中包括與形成菌絲有關的 **EFG1** 基因，證實了此技術的可行性。在已分析的 **32** 個基因片段中，**EFG1** 共出現 18 次，可能表示該基因在野生種細胞內所被轉譯之套數。其他十二種基因，有些已有相關文獻或是可能牽涉形態改變或致病，例如：**serine/threonine kinase** 已知與真菌形成菌絲有關；白色念珠菌之 **squalene epoxidase (*CAERG1*)** 因爲與細胞膜生合成有關，所以可能涉及型態上的轉換；以及白色念珠菌之 *hxt6* **gene for galactose/glucose transporter** 與能量的代謝可能影響致病機制….等等。在未來的研究中，本實驗室將設計更多試驗以尋出與白色念珠菌致病直接相關之基因。

Comparison of Gene Expression between Wild-Type *Candida albicans* and *cph1/cph1 efg1/efg1* Double Mutant under the Condition of Inducing Filament Formation of Wild Type

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ABSTRACT

Candida albicans (*C. albicans*) is an opportunistic pathogenic fungus and one of the most commonly isolated human pathogens. Especially in immunocompromised patients, it can lead to lethal candidiasis. It is believed that filamentous growth is involved in virulence. Researchers have developed the avirulent *cph1/cph1 efg1/efg1 C. albicans*, which is unable to form filaments.

In recent years, the abuse of antibiotics and the emerging of resistance to existing antifungal drugs have made the treatment of fungal infection more and more difficult. To search for a new drug with higher efficiency and less toxicity, I have screened and isolated the virulence genes of *C. albicans* with the technique of suppression subtractive hybridization in the hope of unveiling suitable new targets for drug development.

Two sets of suppression substrate hybridization were performed. One subtracted mutant RNA from wild type RNA (WT-Mut). The other subtracted wild-type RNA from mutant RNA (Mut-WT). Genes in the WT-Mut group are expressed only in the wild type *C. albicans* or expressed at a higher level in wild-type than in mutant strain. Virulence genes could be in this group. 32 islated cDNA fragments were subjected for sequence analysis. Among them, 18 were *EFG1* DNA fragments. Since *EFG1* gene was included in the WT-Mut group as expected, this performance of this technique is reliable. It demonstrated that this suppression subtractive hybridization can indicate not only whether certain genes are expressed or not but also the difference in the expression level.

In the remaining 14 clones, grouped into 12 different genes, some have been reported to be associated in morphology or virulence: Serine/threonine kinase is involved in filamentous growth; squalene epoxidase (*CAERG1*) is in the pathway of synthesis of cell membrane; *hxt6* gene for galactose/glucose transporter is responsible for the metabolism of energy.

本論文は天国にいる父親に捧げる
この些細な成績と努力の結果を認めてくれるよう心から願っておる

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Introduction

A. *Candida albicans*

Candida albicans (*C. albicans*) is an opportunistic fungal pathogen and one of the most commonly isolated human pathogens (Tsarfaty et al, 2000). Especially in immunocompromised patients, it can lead to lethal candidiasis, which started in the digestive system by penetrating the mucous lining of gastrointestinal walls. In the previous study, a mouse can be killed by injection of adequate quantity of wild-type *C. albicans* (Lo et al, 1997).

The cells of *C. albicans* can have different morphology. For instance, it can switch from a unicellular budding yeast to a filamentous form. The fiamentous form may consist of hyphal or pseduohyphal cells. The ability of changing morphology is suspected to be involved in the virulence (Kobayashi and Cutler, 1998).

B. Filament Formation

Filamentous *C. albicans* have been observed to adhere to and invade host cells more readily than the yeast ones, suggesting that filamentous growth may contribute to the virulence of *C. albicans* (Leberer et al, 1997).

The genetic regulation of cell morphology in *C. albicans* has been discovered. The gene products of *CPH1*, *HST7*, and *CST 20* produced in *C albicans* are respectively homologous to those of *STE12*, *STE7*, and *STE20* in *S. cerevisiae*. *C. albicans* strains with either one of these genes mutated have demonstrated retarded filamentous growth but no impairment of serum-induced germ tube and hyphae formation (Liu et al, 1994; Kron et al, 1995). The study of *efg1/efg1*, *cph1/cph1 efg1/efg1*, and *tup1/tup1* mutants, whose capability to form hyphae were impaired

by the mutations, indicated that capabilities to invade and injure endothelial cells are significantly reduced (Phan et al, 2000).

C. Adherence

One critical step in the pathogenic process is their adherence to host tissues. When *C. albicans* cells disseminate in the bloodstream of hosts, the organisms are likely to adhere to and then penetrate the endothelial cell lining of blood vessels to invade the tissues (Filler et al, 1996).

Adherence of *C. albicans* to epithelial tissues is mediated primarily by specific adhesin-receptor interactions. Two major isolates among *Candida* species, *C. albicans* and *C. tropicalis*, (Asakura et al, 1991) adhere to host cells *in vitro* to a greater extent than the relatively nonpathogenic species. The correlation between adhesion and virulence has been confirmed in a comparative study of the adherence ability in different *Candida* species, including *C. albicans* serotype A, serotype B, *C. stellatoidea*, *C. tropicalis*, *C. krusei* and *C. glabrata*. The results showed that both serotypes of *Candida albicans* adhere to buccal epithelial cells to a significantly greater degree than the other species tested and there were no differences between *C. albicans* serotypes A and B (Ghezzi et al, 1986).

A study of strains of *C. albicans* with reduced ability to adhere *in vitro* has shown that they also have impaired abilities to cause infection in animal models (Calderone et al, 1991). Strains which had an increased ability to adhere to buccal epithelial cells and produced increased amounts of extracellular proteinase activity were shown to have increased lethality in a mouse model. The *C. albicans* isolates that adhered most strongly to buccal epithelial cells had the highest relative proteinase activities and were most pathogenic (Ghannoum et al, 1986).

Int1p is a *C. albicans* surface protein with limited similarity to vertebrate integrins. In *S. cerevisiae*, the expression of *INT1* was sufficient to direct the adhesion of this normally nonadherent yeast to human epithelial cells

(Gale et al, 1998). Furthermore, disruption of *INT1* in *C. albicans* suppressed hyphal growth, adhesion to epithelial cells, and virulence in mice (Gale et al, 1998).

Whether the hydrophobicity (CSH) of cell surface of the yeast cells may also contribute to virulence has not been definitively demonstrated. Nineteen isolates of *C. albicans* were grown in Sabouraud dextrose broth at either 23℃ or 37℃ and tested for CSH by a polystyrene microsphere assay and for the ability to adhere to HeLa cells, a human cervical epithelioid carcinoma cell line (Hazen, 1989). Growth temperature did not appear to determine adherence ability, as all isolates that did not differ in CSH after growth at either temperature also did not differ in the ability to adhere. No correlation (r = 0.44) was obtained between CSH and adherence when the isolates grown at 23℃ were evaluated as a group. However, higher correlation (r = 0.65) was obtained when the isolates were grown at 37℃. Interestingly, a significantly positive correlation between CSH and adherence was obtained when individual isolates were analyzed (Hazen, 1989).

It has been concluded that CSH had little effect on adherence once a moderately high level of CSH was attained. These results indicated that CSH is involved in adherence but is not the predominant mechanism and that the effect of CSH on adherence is isolate dependent (Hazen, 1989).

D. Proteinase Activity

The hydrophobicity on the cell surface is one of the physiological characteristics of *C. albicans*. Studies have revealed that hydrophobic *C. albicans* are more virulent than hydrophilic ones (Antley et al, 1988). The extracellular proteinase activity of *C. albicans* is thought to be associated with virulence. Secretory acid proteinase antigen and antibody can be demonstrated in patients infected with *C. albicans* (Macdonald et al, 1980). The enzyme may function in two ways affecting virulence. One is to

invade tissue by the proteolytic activity; the other is to colonize host tissues with production of enzymes (McCullough et al, 1996).

Previous studies showed that *C. albicans* could use serum as nitrogen source, and produce proteinase (Ganesan et al, 1991). Most of the publications focus on aspartyl acid proteinase. Scientists found that different strains of *C. albicans* can secrete proteinases with similar molecular weight, but with different pH optima, substrate specificity, and isoelectric point. This means the secreted proteinases are strain-specific (reviewed by Cutler, 1991).

Virulence of *C. albicans* strains with targeted disruption of secretory aspartyl proteinase genes (*SAP1* to *SAP6*) was assessed in an estrogen-dependent rat vaginitis model (De Bernardis et al, 1999). Null *sap1* to *sap3* mutants lost most of the virulence of their parental strain SC5314. In particular, the *sap2* mutant was almost avirulent in this model. Reinsertion of the *SAP2* into this latter mutant led to the recovery of the vaginopathic potential. The vaginal fluids of the animals infected by the wild-type strain or by the *sap1* or *sap3* mutants expressed a pepstatin-sensitive proteinase activity *in vitro*. No trace of this activity was found in the vaginal fluid of rats challenged by the *sap2* mutant. All strains were capable of developing true hyphae during infection. Thus, members of *SAP* family, in particular *SAP2*, play a clear pathogenic role in vaginitis and may constitute a novel target for chemoimmunotherapy of this infection. At least three proteinases have been found in the intracellular compartments of *C. albicans* (Portillo et al, 1986). They have distinctly different pH optima and other enzymatic characteristics. One of these proteinases has many characteristics in common with the putative virulence factor, aspartyl acid proteinase. Like the secreted enzyme, the intracellular one is a glycoprotein, an aspartyl acid proteinase that can act on a wide variety of proteins. It is not inhibited significantly by phenylmethylsulfonyl fluoride and is inhibited by pepstatin. But the molecular weight and pH optimum of the intracellular form is 60kd and

5.0; the secreted proteinase is 40-45kd and 2.2-4.5. The aspartyl acid proteinase can work at some particular tissues, which is acidic, because of the low pH optimum (Remold et al, 1968; Ruchel, 1981).

Factors involved in adherence, proteinase activity and filament formation were always considered important contributors to virulence. However, the study on molecular level has just begun. Recently, some scientists have begun to discover gene whose activities are related to the virulence of *Candida* (reviewed by Cutler, 1991).

E. *CPH1* Gene

Cph1p is the homologue of Ste12p. Ste12p in *S. cerevisiae* is an important transcription factor that regulates several signal transduction pathways (Burchett et al, 2001).

The genetic pathway of the yeast-hyphal dimorphism in *C. albicans* has not been established, but Cph1p has been shown to be involved in hyphal growth of *C albicans* (Liu et al, 1994).

F. *EFG1* Gene

EFG1p is the homologue of Phd1p. The overexpression of *phd1* in *S. cerevisiae* resulted in enhanced pseudohyphae growth and the *phd1 ste12* double mutants are more defective in pseudohyphal growth than the *ste12* mutant (reviewed by Ernst, 2000).

Homozygous *efg1* mutants form only pseudohyphae on solid media and do not germinate at all in liquid media, even in the presence of serum stimulation (Lo et al, 1997).

G. Subtractive Hybridization

a. Background

In 1994, a PCR-based technique called representational difference analysis has been announced. It does not require physical separation of single-stranded (ss) and double-stranded (ds) cDNAs. Representational difference analysis has been applied to enrich for genomic fragments that differ in size or representation (Lisitsyn et al, 1993) and to clone differentially expressed cDNAs (Hubank et al, 1994). However, representational difference analysis does not solve the problem of the wide differences in abundance of individual mRNA species. Consequently, multiple rounds of subtraction are still needed (Hubank et al, 1994). The mRNA differential display (Liang et al, 1992) and RNA fingerprinting by arbitrary primed PCR (Welsh et al, 1992) are potentially faster methods for identifying differentially expressed genes. However, both of these methods have a high level of false positives (Bauer et al, 1994; Sompayrac et al, 1995), biased for high-copy-number mRNA (Bertioli et al, 1995) and might be inappropriate in experiments in which only a few genes are expected to vary (Sompayrac et al, 1995).

Numerous cDNA subtraction methods have been reported. In general, they involve hybridization of cDNA from one population (tester) to excess of mRNA (cDNA) from other population (driver) and then separation of the unhybridized fraction (target) from hybridized common sequences. The latter step is usually accomplished by hydroxylapatite chromatography (Hedrick et al, 1984), avidin-biotin binding (Duguid et al, 1990; Sargent et al, 1983;Davis, 1984), or oligo(dT)30-latex beads (Hara et al, 1991). Despite the successful identification of numerous important genes such as the T-cell receptors (Hedrick et al, 1984) by these methods, they are usually inefficient for obtaining low abundance transcripts. These subtraction techniques often require greater then 20 μg of poly(A)$^+$ RNA, involve multiple or repeated subtraction steps, and are labor intensive.

b. Suppression Subtractive Hybridization, SSH

In 1996, Diatchenko et al. presented a new PCR-based cDNA subtraction

method, termed suppression subtractive hybridization (SSH), and demonstrated its effectiveness. SSH is used to selectively amplify target cDNA fragments (differentially expressed) and simultaneously suppress nontarget DNA amplification. The method is based on the suppression PCR effect previously described by Diatchenko's laboratories (Diatchenko et al, 1996).

This highly effective method, suppression subtractive hybridization (SSH), has been developed for the generation of subtracted cDNA libraries. It is based primarily on a recently described technique called suppression PCR and combines normalization and subtraction in a single procedure. The suppression PCR is mediated by long inverted terminal repeats. When attached to the ends of DNA fragments, these inverted repeats form stable panhandle-like loop structure after each denaturation and annealing cycle. In a PCR with primers derived from the sequences of the long inverted repeats, the panhandle-like structures cannot be amplified exponentially because intramolecular annealing of the long inverted terminal repeats is highly favored and is more stable than intermolecular annealing of the shorter PCR primer to the long inverted repeats (Lukyanov et al, 1995). By incooperating this suppression effect in a PCR amplification scheme, undesirable DNA fragments can be eliminated from a mixture of target sequences (Chenchik, 1996; Siebert, 1995).

In the process of SSH, the normalization step equalizes the abundance of cDNAs within the target population and the subtraction step excludes the common sequences between the target and driver populations. In a model system, the SSH technique enriched for rare sequences over 1,000-fold in one round of subtractive hybridization (Diatchenko et al, 1996).

c. Application

The suppression PCR effect has been applied in chromosome walking (Siebert et al, 1995) and rapid amplification of cDNA ends (Chenchik et al,

1996). The subtraction method described here overcomes the problem of differences in mRNA abundance by incorporating a hybridization step that normalizes (equalizes) sequence abundance during the course of subtraction by standard hybridization kinetics. It eliminates any intermediate step(s) for physical separation of single strand and double strand cDNAs, requires only one subtractive hybridization round, and can achieve greater than 1,000-fold enrichment for differentially expressed cDNAs. The effectiveness of the SSH method has been demonstrated by generating a testis-specific cDNA library and characterizing selected cDNA clones. Furthermore, the subtracted cDNA mixture can be used directly as a hybridization probe for screening recombinant DNA libraries, such as a human Y chromosome cosmid library, thereby identifying chromosome-specific and tissue-specific expressed sequences. Results of some studies have suggested that the SSH technique is applicable to many studies in molecular genetics and positional cloning for the identification of disease, developmental, tissue-specific, or other differentially expressed genes (Diatchenko et al, 1996).

H. Pharmaceutical Treatments for *C. albicans*

Recent years, the abuse of antibiotics and the increased number of immunocompromised cases had increased the prevalence of opportunistic fungal pathogens and made diagnosis and treatment important issues. Clinically applied antifungal drugs could bring side effects and develop resistance easily (Reviewed by Yang, 2003).

Three major categories of antifungal drugs are 5-flucytosine, polyenes and ergosterol biosynthesis inhibitors (Kerridge et al, 1986). The synthetic pyrimidine, 5-flucytosine inhibits the synthesis of DNA and protein in yeasts. The other two types of inhibitors target the main component of fungal plasma membranes, ergosterol. Since cholesterol of human cell membranes is very similar to cholesterol, polyenes are toxic.

The polyene macrolide, amphotericin B is one of the effectively and frequently applied antifungal drugs (reviewed by Gallis et al, 1990). Amphotericin B acts to slow down the growth and multiplication of susceptible fungi. If the concentrations of this medication are high enough, they can also destroy the fungi. It is approved by the FDA for the treatment of certain types of serious fungal infections, but there are many possible side effects, such as fever, chills, nausea, vomiting, headaches, electrolyte imbalance and renal failure.

I. The Aim of This Study

Molecular biology and genetics have been applied to the studies of *Saccharomyces cerevisiae* because of its genome stability and well-studied properties. Unlike *Saccharomyces*, life cycle of *C. albicans* is still unclear, and this makes the application of molecular biology and genetics difficult. Researchers have compared the same *Candida* strain with different morphologies resulted from different growth conditions or media and revealed the association between morphology and virulence. But these conditions, such as temperature, media or even interactions changed not only the virulence but also other phenotypes. To understand the virulence of *C. albicans* on the molecular level, it is necessary to identify and isolate the virulent genes. The technique of subtractive hybridization was used to isolate virulence factors that are involved by comparing the virulent (wild-type) strain and avirulent mutant strains under the same condition.

The aim of this study is to sieve out genes encoding virulence factors of *C. albicans* to pave the way for understanding the mechanisms of virulence and also provide information of more specified potential targets for drug design to develop higher efficiency and less toxicity antifungal drugs.

Materials and Methods

A. Culture of *Candida albicans*

The wild-type *C. albicans* (phenotype：CPH1/CPH1 EFG1/EFG1) strain SC5314 (Gillum et al, 1984) and *cph1/cph1 efg1/efg1* double mutant strain HLC54 (Lo et al, 1997) were used throughout this study. Yeast was precultured in 10 ml of YEPD medium (Clontech, pH 7) at 30°C overnight. Two milliliter of the overnight culture were transferred to 60 ml of YEPD containing 10% serum (Gibco BRL) and incubated at 37°C with shaking until the OD_{600} reaches 0.4-0.7.

B. 1. RNA Isolation

Cell was cultured overnight in YEPD containing 10% serum at 37°C. The cells were then spun down at 3200 rpm, 4°C for 5 minutes. The supernatant was then discarded. The cell pellets was then suspended in 0.3 ml RNA Isolation Buffer (50mM NaCl, 20mM Tris-HCl, pH7.6, 1% EDTA and 1% SDS) with vortex. One third volume of glass beads (Sigma G-9268, 425-600 microns unwashed glass beads, washed by acetic acid before use, Step B.2.) was then added to the cell suspension before being vortexed for 5 minutes to break the cell wall. A volume of 0.3 ml of phenol was then added and the mixture was vortexed for 5 minutes. A volume of 0.5 ml of RNA Isolation Buffer was added before being vortexed for 5 minutes. The whole mixture was then centrifuged at 14000 rpm, 4°C for 5 minutes. The supernatant was transferred to new tubes. In the process of transferring, the interface should not be taken. Equal volume of phenol was then added to the supernatant prior to vortex for 30 seconds followed by spinning at 14000 rpm for 5 minutes. The supernatant was transferred to new tubes with care to avoid the interface. The steps of 6 and 7 were repeated for one more time. The supernatant was transferred to new tubes. In the process of

transferring, one should avoid the interface. One eighth volume of 2.5 M NaOAc (pH 7) and 2.5 volume of 100% cold ethanol were added to the supernatant and kept on ice for 20 minutes prior to centrifugation at 3000 rpm for 1 min.

The supernatant was discarded carefully and then the pellets were washed with 75% cold ethanol prior to centrifugation at 3000 rpm, 4°C for 1 minute. The supernatant was discarded. The RNA pellets were then dissolved in 1X TE before measurement of the OD260/280 to estimate the purity and concentration of RNA. To assess the integrity of RNA, 2 μg of isolated RNA were electrophoresed in 1% agarose gel.

2. Preparation of Glass Beads

Glass beads were poured into a 500ml-beaker, and acetic acid was added covering every glass beads. The beaker was covered with aluminum foil, and left in the laminar flow overnight. Liquid was poured out very carefully. The glass beads were washed with ddH_2O for 5-6 times and then dried in the oven. The glass beads were divided into 1.5ml eppendorf tubes and then autoclaved. The eppendorf tubes containing glass beads were dried in the oven.

C. Subtractive hybridization

a. cDNA Synthesis

Ten micromoles of both CDS (cDNA Synthesis) primer and SmartII SMART™ primer (Switch Mechanism At 5' end of the RNA Transcript) were used for every 3μg of RNA and H_2O was added to total volume of 5 μl.

The tubes were incubated in hot water at 70°C for 5 minutes and then cooled on ice before brief centrifugation. The following mixture was added to each tube and the tubes were vortexed and spun.

First-strand buffer, 5X, 2μl (250mM Tris-HCl, pH 8.5, 40 mM $MgCl_2$, 150 mM KCl, and 5 mM Dithiothreitol), DTT (100mM) 1μl, dNTP (10mM) 1μl, and Superscript II/MMLV RT (200u/μl) 1μl.

The tubes were incubated in a water bath at 42°C for 2 hours and then 40μl of TE was added into each tube. The reaction was inactivated by 7 minutes' hot water bath at 72°C.

b. cDNA Amplification by LD PCR

The master mix, containing 1050μl of H_2O, 126μl of 10X Advantage 2 PCR buffer (Clontech Laboratory, Inc.), 24μl of dNTP (10mM), 24μl of PCR primer (10μM), and 24μl of 50X Advantage 2 Polymerase Mix was prepared for 4 samples (12 tubes). Each tube contains 312μl of master mix.

Three microliter of cDNA template from the previous step, the synthesized cDNA, was added to each tube. The tubes were vortexed and spun. The mixture in tubes was divided into 3 tubes. The PCR condition was as following: 65°C's annealing, 68°C's elongation for 6 minutes, and totally 25 cycles. The program was interrupted at 15^{th} cycle. Two tubes of each sample were taken out and carefully kept. Eight microliters of mixture from the other tube was transferred into a new tube. PCR continued and 8 μl of sample was taken out every other cycle. Agarose gel (1%) at 250 Volt for 20 minutes was used to determine the optimal cycle for each sample. The 2 previous taken-away tubes were then continued with the rest of cycles. The 2 tubes of each sample were combined and then 4 μl of 0.5M EDTA was added to stop the reaction. Eight microliters from each sample was kept in a new tube for further analysis (Sample 1).

c. Column Chromatography

Samples from step b were mixed with equal volume of DNA phenol, vortexed for 30 seconds and then centrifuged at 12000g for 10 minutes. The water phase in the tube was transferred into the upper part of the Centricon 100 containing 1.8 ml of H_2O. The Centricon tube was centrifuged at 3000 rpm for 30 minutes. After centrifugation, the lower

part solution was discarded, 2 ml of H_2O was added and this step was repeated 3 times. The solution in the column was mixed by inverting and then centrifuged at 3000 rpm for 5 minutes. The solution in the cap was collected.

The column was inverted several times to re-suspend the gel matrix. The top and bottom plug of the column were removed and the inner buffer flowing through. TNE buffer (1.5ml, 10 mM Tris-HCl, pH 8, 10mM NaCl and 0.1mM EDTA) was added and after it flowing through, the buffer was collected and discarded. The sample was added in the center of gel in the column and followed 25 μl of TNE. After TNE buffer drained out, 150 μl of TNE was added and drained out. In the tube, 320 μl of TNE was added and the solution flowed through was collected. The sample 1 was run on 1.0% agarose gel to check the quantity of DNA. If the cDNA fragments on the gel were not able to be seen, another 75 μl of TNE should be added and the solution flowed through collected. The sample was confirmed on 1.0% agarose gel. Eight microliters of each sample was transferred into a clean tube for further analysis (Sample 2).

d. RsaI Digestion

The following procedure was performed to both wild type and mutant cDNA. The shorter, blunt-ended double-stranded cDNA and optimal fragments will be prepared in this step. The cDNA from Step c. was taken by 300 μl and mixed with 33.5 μl of 10X *Rsa*I restriction buffer attached to the restriction enzyme and 20 units of *Rsa*I. The mixture was incubated in 37°C of water bath overnight. The sample was run in 1.0% agarose gel and checked for the cDNA fragments. Each sample was added with 8 μl of 0.5M EDTA to stop the reaction and 8 μl of samples were transferred for further analysis (Sample 3).

DNA phenol was added and mixed by vortex and then centrifuged at 12000g for 10 minutes. The water phase was transferred into the

upper part of Centricon 100 containing 1.7 ml of H_2O. The centrifugation step was repeated at 3000 rpm for 30 minutes and the lower part of solution was discarded. Two milliliters of H_2O was added into the tube for 4 times. The column was inverted several times for mixing and centrifuged at 3000 rpm for 10 minutes. The solution was collected in the cap.

The samples was transferred into new tubes and concentrated by an evaporator (SpeedVac). The *Rsa*I digested cDNA fragments were dissolved in 10 μl of H_2O. From every sample, 1 μl was taken and mixed with 2 μl of dye and 7 μl of H_2O. Agarose gel (1%) was used to check Sample 3.

e. Adaptor Ligation

Every sample from Step d. was diluted with 5 times of H_2O; prepared for Tester cDNA T, and C. The Master Mix (4 μl of H_2O, 1 μl of 10X ligation buffer, and 1 μl of T4 DNA ligase for each reaction) was prepared for 2 samples (4 tubes). See the table below for recipes. The tubes were incubated at 16°C overnight and after incubation, 1 μl of EDTA/glycogen mix was added. The reaction was stopped in a thermal cycler at 72°C for 5 minutes. The fragments were stored at -20°C.

Sample	WT-A①	Mut-A②	WT-B③	Mut-B④
Diluted tester cDNA	WT, 2μl	Mut, 2μl	WT, 2μl	Mut, 2μl
Adaptor 1 (10 μM)	2	2	-	-
Adaptor 2R (10 μM)	-	-	2	2
Master Mix	6	6	6	6
Final Volume	10	10	10	10

①WT-A: wild type DNA ligased with A adaptor;

② Mut-A: mutant DNA ligased with A adaptor;

③WT-B: wild type DNA ligased with B adaptor;

④ Mut-B: mutant DNA ligased with B adaptor.

f. First Hybridization

Samples were prepared according the following recipe, and incubated in thermo cycler at 98°C for 2 minutes then 68°C for 6 hours. After incubation, step g was immediately performed.

Sample	WT-A-Mut①	Mut-A-WT②
Driver cDNA	Mut, 1.5 μl	WT, 1.5 μl
Tester cDNA	WT-A, 1.5μl	Mut-A, 1.5μl
4X hybridization buffer	1 μl	1 μl

Sample	WT-B-Mut③	Mut-B-WT④
Driver cDNA	Mut, 1.5 μl	WT, 1.5 μl
Tester cDNA	WT-B, 1.5μl	Mut-B, 1.5μl
4X hybridization buffer	1 μl	1 μl

①WT-A-Mut: mix WT-A DNA with mutant DNA;

②Mut-A-WT: mix Mut-A DNA with wild type DNA;

③ WT-B-Mut: mix WT-B DNA with mutant DNA;

④ Mut-B-WT: mix Mut-B DNA with wild type DNA.

g. Second Hybridization

Samples were prepared as follows:

	WT	Mut
Driver cDNA (μl)	1	1
4X Hybridization buffer (μl)	1	1
H_2O (μl)	2	1
Total volume (μl)	4	3

Tubes were spun and 1 μl of solution in each tube was transferred to new tubes. DNA fragments were denatured in thermal cycler at 98°C for 2 minutes. The denatured driver cDNA and T-DB were mixed and T-DA was then added. The mixture was thoroughly mixed and spun. Then the steps 4-6 were repeated and the mixture was incubated at 68°C overnight. One hundred of dilution buffer was added, then was mixed by vortex and spun down. The reaction was inactivated in thermal cycler at 68°C for 7 minutes. Samples were transferred to a new tube and stored at -20°C.

h. PCR Amplification

First PCR solution was prepared as follows and run the PCR program1※.

H_2O	19.5 μl
10X Advantage 2 PCR buffer	2.5 μl
dNTP mix, 10mM	0.5 μl
PCR primer 1, 10μM	1.0 μl
50X Advantage 2 polymerase mix	0.5 μl
Subtracted cDNA fragments from Step g-11	1.0 μl

Three microliter of PCR product was transferred and diluted with 27 μl of H_2O. The PCR product from first PCR was the template of second PCR

※PCR program1:

75°C	5 min	
94°C	25 sec	
94°C	15 sec	} 27 cycles
66°C	30 sec	
72°C	110 sec	

Second PCR solution was prepared as follows and run the PCR program2[€]. Agarose gel (1%) was used to check the PCR products.

H_2O	18.5 μl
10X Advantage 2 PCR buffer	2.5 μl
dNTP mix, 10mM	0.5 μl
Nested PCR primer 1, 10μM	1.0 μl
Nested PCR primer 2R, 10μM	1.0 μl
50X Advantage 2 polymerase mix	0.5 μl

[€]PCR program2:

94°C	10 sec	10/12/14 cycles
68°C	30 sec	
72°C	110 sec	

i. Final PCR cycles

First step PCR solution was prepared as follows and 1 μl of subtracted cDNA fragments from step g-11 was added to run the PCR program[‡]. The PCR product was transferred and diluted with 4 times of H_2O.

H_2O	19.5 μl
10X Advantage 2 PCR buffer	2.5 μl
dNTP mix, 10mM	0.5 μl
PCR primer 1, 10μM	1.0 μl
50X Advantage 2 polymerase mix	0.5 μl

PCR program[‡]:

75°C	5 min	
94°C	25 sec	
94°C	15 sec	20 cycles
66°C	30 sec	
72°C	110 sec	

Second step PCR solution was prepared as follows and 1 μl of diluted first PCR product was added as templates (2 for each sample) The PCR program†.

H_2O	41 μl
10X PCR buffer	5 μl
dNTP mix, 10mM	1 μl
Nested PCR primer 1, 10μM	1 μl
Nested PCR primer 2R, 10μM	1 μl
50X Advantage cDNA polymerase	1 μl

PCR program†

94°C	10 sec	optimized cycles
68°C	30 sec	
72°C	110 sec	

Two of each sample was combined and taken out 8 μl for 1.0% agarose gel.

The PCR products were purified and ligated (TA cloning). The water phase of PCR products from previous step were transferred into the upper part of Centricon 100 containing 1.8 ml of H_2O. Tubes were centrifuged at 3000 rpm for 30 minutes. The lower part of the solution was removed and 2 ml of H_2O was added for 4 times.

The column was inverted for mixing and centrifuged at 3000 rpm for 5 minutes. The solution in the cap was collected. Taq DNA polymerase, 10X PCR buffer and 25 mM dNTP were added. The mixture was incubated at 72°C in a thermal cycler for 10 minutes.

Two hundred microliters of DNA phenol and 150 μl of H_2O were added and mixed by vortex, and then centrifuged at 12000g for 10 minutes. The water phase was transferred into the upper part of Centricon 100 containing 1.8 ml of H_2O. Tubes were centrifuged at 3000 rpm for 30 minutes. The lower part of the solution was removed and 2 ml of H_2O

was added for 4 times. The column was inverted for mixing and centrifuged at 3000 rpm for 5 minutes. The solution in the cap was collected. Samples were concentrated by evaporator (SpeedVac) for 3 hours. The cDNA fragments were dissolved in 12 μl H_2O. Small amount of the cDNA sample was taken for 1.0% agarose gel.

The cDNA fragments were taken by 6 μl to be ligated with master mix (vector 2 μl, buffer 1 μl and ligase 1 μl) and spun down. Samples were incubated at 14.5°C in thermal cycler overnight.

j. Transformation (Invitrogen TA Cloning One Shot competent cell)

A tube containing frozen competent cell DH5α (50μl) was placed on ice and 2 μl of DNA was added slowly and mixed gently with the pipette tip. The tube was tapped gently and then kept on ice for 30 minutes. The tube was incubated at 37°C for 20 seconds; shaking should be avoided. After incubation, it was kept on ice for 2 minutes and then 0.45 ml of room temperature SOC medium was added. The mixture was transferred into a new centrifugation tube and then centrifuged at 225 rpm at 37°C for 50 minutes. The solution was dispersed averagely 70μl on medium plate containing ampicillin.

D. Plasmid DNA Isolation

Cells were cultured in 24-well-plates with LB medium (Scharlau) containing ampicillin (10mg/ml) and incubated at 37°C overnight with shaking. Plates containing cells were spun at 3000 rpm for 15 minutes and the supernatant was poured out. Cell pellets were re-suspended with 200 μl of P1 buffer (re-suspension buffer, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 μg/ml RNase A, QIAGEN) and rocked for 5 minutes. The solution was incubated at room temperature for 8 minutes with 300 μl of P2 buffer (lysis buffer, 200mM NaOH/ 1% SDS, QIAGEN) added, and then on ice for 2 minutes. Cold P3 buffer (neutralizing buffer, 3M potassium acetate, pH 5.5) was added and placed on ice for

at least 15 minutes (Sheibani, 1997). The mixture was spun at 3000 rpm for 15 minutes and the supernatant was carefully poured to new 24-well plates. Equal volume of iso-propanol was added and spun at 3000 rpm for 15 minutes. The supernatant was poured out. Plates were washed with 1 ml of 70% ethanol and spun at 3000 rpm for 10 minutes. The supernatant was poured out and the plate was placed on the bench until it dries. The pellets were resuspended in ddH_2O and stored at –20°C.

E. Restriction Mapping

DNA samples and pCRII-TOPO vector (Invitrogen, Figure 4) were digested with *Hae*III and *Sau*3AI endonucleases (Gibco), respectively. Plasmids were isolated in 24-well microplate.

The enzyme solution was prepared as follows and incubated at 37°C for 2 hours (Sambrook et al, 1989).

Hae III (10 unit/μl)	0.2μl / *Sau* 3AI (10 unit/μl)	0.2μl
REACT® 2 Buffer	1.5μl / **RE**ACT® 4 Buffer	1.5μl
H_2O	3.3μl	
Plasmid DNA	10.0μl	
Total	15.0 μl/ Reaction	

F. Acrylamide gel for endonuclease digested DNA fragments

The gel electrophoresis cassette, Mini-PROTEAN 3 (Bio-rad), was set up to cast a polyacrylamide gel. The gel size was 8.3 cm wide, 7.3 cm height and 0.075 cm thick. The solution was prepared by mixing 1.2 ml of 30% acrylamide/bis acrylamide (29:1), 0.6 ml of 10X TBE, 30 μl of 10% ammonium persulfate and 4.2 ml of H_2O. The polymerization

stated when 3 μl of TEMED added. The mixture was immediately poured in between the glass plates and then the 10-well comb was inserted. The gel was completely polymerized at room temperature for 25-30 minutes. The comb was removed and the cassettes were transferred to the electrode assembly. The electrode assembly was placed in the clamping frame and the tank was filled with running buffer (0.5X TBE Buffer, Gibco BRL).

The first lane was always loaded with size marker, 1kb Plus (Gibco BRL) and the tenth was vector sequence digested by restriction enzyme, *Hae*III or *Sau*3AI. The gel was run at 100 volts for 50 minutes.

G. Visualization of the Gel

The gels were stained with Ethidium bromide (0.5 μg/ml, Sambrook et al, 1989) for 30 seconds and destained in H_2O for another 30 seconds. The bands were visualized and stored with Gel Doc 2000 (Bio-rad).

H. Classification Restriction Maps

The molecular weight of each band of samples can be determined according to the 1kb size marker and the enzyme digested vector fragments on the same gel. First, samples were classified by the number of bands and then similar band patterns. Finally, samples within the same group carrying the same molecular weights of bands are regarded as the same fragment of DNA.

Results and Discussion

A. Cell Culture

C. albicans were inoculated into fresh media and incubated with shaking at 37°C overnight. The cell density was estimated by spectrophotometer at 600nm. The optical density measured at 600nm of mutant cells reached 0.41 (Table 1) at the 4th hour and then cells were harvested. Wild-type *C. albicans* cells tended to aggregate at the bottom of tubes and the absorbance was varying and difficult to determine, so they were harvested at the same time as mutant cells. The double mutant strain cells were suspending in the media (Figure 1). The morphological expression of wild type and that of mutant strain were corresponding to the referred publication (Lo et al, 1997).

B. RNA Isolation

The RNA isolation steps followed the protocol in Chapter 7 of Molecular Cloning (Sambrook et al, 1989). The quantity of RNA was estimated by spectrophotometer at 260nm. The OD_{260} of isolated RNA of the wild type was 0.296 and that of the double mutant was 0.598. From 100 ml of cell culture media, 118.4 μg of total RNA were isolated from wild-type cells, while 239.2 μg of total RNA from double mutant strain. The electrophoresis results were shown in Figure 2.

Theoretically, the whole process of RNA isolation should be kept under the circumstance of 4°C to ensure the stability of RNA. In this study, RNA isolation procedure was performed five times following the same protocol. Among them, three times were at room temperature and two in cold room at 4°C. There were different degrees of degradation in some samples, the bottom bands are the degradation products. Lane 3 has the strongest degradation band, Land 2 and 6 also have these bands (Figure 2).

C. Subtractive Hybridization

There were 991 transformants resulted from subtractive hybridization

using wild type as testers and mutant strain as drivers (WT-Mut group). On the other hand, 340 clones were isolated when mutant RNA was used as testers and wild-type RNA as drivers (Mut-WT group).

C. albicans cph1/cph1 efg1/efg1 double mutant is unable to form filaments, and it is avirulent in a mouse model (Lo et al, 1997). Genes regulated by *CPH1* and *EFG1* genes are related to morphology and the pathogenic ability, and each gene represents a separate pathway of control (Braun et al, 2000). By comparing the gene expression in wild type and mutant cells, genes from both pathways were found. Further studies will be necessary to determine which pathway, morphology,or virulence, these genes are involved in. Of course, some genes may be involved in both.

Among the 32 randomly picked and sequenced clones, 18 of them were different restriction DNA fragment of the *EFG1* gene and 3 of them *CAERG1* gene (Table3).

Genes in the WT-Mut group are expressed only in wild type *C. albicans* or expressed at higher level in wild-type than in the mutant strain. Virulence genes could be in this group. The discovery of *EFG1* gene in this group demonstrated that subtractive hybridization can actually single out the specifically expressed genes in WT-Mut group. Since *EFG1* gene was included in the WT-Mut group as expected, this technique has been proven reliable in this case. According to the results of the restriction mapping patterns of *Hae* III digested DNA fragments in WT-Mut group, there were 130 clones carrying no insert and only the vector. The efficiency was 86.9%.

Avirulence genes are supposed to be found in the Mut-WT group, which means the genes were expressed at a higher level in a mutant cell than in a wild-type cell or expressed only in the mutant strain. So far, *TUP1* gene has been found in the Mut-WT group. *TUP1* was reported to repress genes responsible for the filamentous growth in *C. albicans* (Braun et al, 2000). Many genes with unknown function were found in this group,

which could be potential therapeutic targets. There were 66 clones carrying vector only in the Mut-WT group digested by *Sau*3AI and 29 in the group digested by *Hae*III. The efficiency was 80.6% and 91.5%.

D. Restriction Mapping

There are four groups of results, cDNA clones from WT-Mut digested by *Hae*III, WT-Mut digested by *Sau*3AI, Mut-WT digested by *Hae*III and Mut-WT digested by *Sau*3AI.

Many lanes on the polyacrylamide gel showed no DNA at all. There are some reasons of the absence of the DNA fragments. First, the contaminant was mistakenly identified as a clone. Second, the plasmid was lost in the process of isolation.

Some lanes on the gel were too weak to be identified. Quantifying the plasmid before incubation with the restriction enzyme helps to determine how much plasmid to be added and would have a better result for classification. The quantity of the restriction enzyme added to each well should also depend on the quantity of the plasmid extracted. If the quantification has been done in this research, the results of restriction mapping and classification may have been improved.

The clones with vector only (no insert) should have been eliminated by using agarose gel to identify whether the clone carries an insert or not. After elimination of the empty clones, inadequate plasmid ones, vector only ones and unclassified ones, the remaining 363 clones in the WT-Mut digested by *Hae*III group were classified into 61 different patterns (Table 2). In the Mut-WT group, the restriction mapping results were classified into 47 different patterns (Table 5).

The pCRII-TOPO vector used for cloning the cDNA fragments was digested into 17 fragments by *Hae*III and 25 fragments by *Sau*3AI. The cutting positions and lengths of fragments were shown in Table 4.

E. Sequence Analysis

Figure 3 shows different *EFG1* fragments discovered in this study and how they matched to the *EFG1* sequence. The repeated presence of *EFG1* DNA fragments may imply the abundance of its mRNA in wild type *C. albicans*. On the other hand, there was not *CPH1* gene found which may stand for the expression level of *CPH1* was much lower than that of *EFG1* in wild type.

In addition to *EFG1* and *CAERG1*, there were *C. albicans* partial hxt6 gene for galactose/glucose transporter, *C. albicans* estrogen-binding protein gene (*EBP1*), and pyruvate kinase homologue. Other included homologues of *Homo sapiens* regulator of G protein signaling 10 mRNA, *Homo sapiens* serine/threonine kinase 24 (*Ste 20*, yeast homologue), *Gadus morhua* microsatellite *Gmo36* sequence, *Homo sapiens* sodium channel beta 2 sequence, *Arabidopsis thaliana* DNA chromosomes, *Homo sapiens* hypothetical protein FLJ13149, *Homo sapiens* clone hRPK.36_A_1, and *Homo sapiens* MAX dimerization protien (MAD) mRNA (Table 3). These may contribute to the regulation of the virulence.

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Table 1. OD_{600} of Cell Culture

Strain / OD_{600}	Wild type (*CPH1/CPH1 EFG1/EFG1*)	HLC54 (*cph1/cph1 efg1/efg1*)
Initiation	0.086	0.052
4 hour-Incubation	0.038-0.068	0.410

Table 2. Groups of Restriction Mapping Pattern In WT-Mut Group Digested by *Hae* III

Number of Bands	Group Description	Number of Clones
0	Clones Without Plasmid	159
	Inadequate Plasmid Isolated	228
	Unclassified Clones	111
11	Pattern 1	7
12	Pattern 2 (Vector Only)	130
13	Pattern 3	11
13	Pattern 4	6
13	Pattern 5	12
13	Pattern 6	17
13	Pattern 7	18
13	Pattern 8	46
13	Pattern 9	4
13	Pattern 10	22
13	Pattern 11	5
13	Pattern 12	4
13	Pattern 13	2
13	Pattern 14	1
13	Pattern 15	13
13	Pattern 16	25
13	Pattern 17	5
13	Pattern 18	23
13	Pattern 19	2
14	Pattern 20	1
14	Pattern 21	1
14	Pattern 22	5
14	Pattern 23	1
14	Pattern 24	2
14	Pattern 25	2
14	Pattern 26	3
14	Pattern 27	1
14	Pattern 28	11

14	Pattern 29	12
14	Pattern 30	9
14	Pattern 31	1
14	Pattern 32	5
14	Pattern 33	2
14	Pattern 34	2
14	Pattern 35	5
14	Pattern 36	6
14	Pattern 37	1
14	Pattern 38	1
14	Pattern 39	12
15	Pattern 40	25
15	Pattern 41	1
15	Pattern 42	1
15	Pattern 43	3
15	Pattern 44	2
15	Pattern 45	1
15	Pattern 46	1
15	Pattern 47	1
15	Pattern 48	1
15	Pattern 49	1
15	Pattern 50	2
15	Pattern 51	3
15	Pattern 52	1
15	Pattern 53	2
15	Pattern 54	1
15	Pattern 55	3
16	Pattern 56	2
16	Pattern 57	1
16	Pattern 58	2
17	Pattern 59	1
17	Pattern 60	1
17	Pattern 61	1
	Total	991

Table 3. DNA Sequencing Results of Randomly Chosen Clones from WT-Mut Group

Result of Sequence Analysis	Number of Clones
C. albicans EFG1 gene	18
C. albicans squalene epoxidase (*CAERG1*)	3
C. albicans partial *hxt6* gene for galactose/glucose transporter	1
C. albicans estrogen-binding protein gene (*EBP1*)	1
C. albicans pyruvate kinase homologue	1
Homologue of Homo sapiens regulator of G protein signaling 10 mRNA	1
Homologue of Homo sapiens serine/threonine kinase 24 (Ste 20, yeast homologue)	1
Homologue of Homo sapiens sodium channel beta 2 sequence	1
Homologue of Homo sapiens hypothetical protein FLJ13149	1
Homologue of Homo sapiens clone hRPK.36_A_1	1
Homologue of Homo sapiens MAX dimerization protien (MAD) mRNA	1
Homologue of Gadus morhua microsatellite Gmo36 sequence	1
Homologue of Arabidopsis thaliana DNA chromosomes	1
Total	32

Table 4. pCRII-TOPO Vector Fragments Digested by *Hae*III and Sau3AI

***Hae*III Cutting at (base)**	**Fragments (base)**	**Sau3AI Cutting at (base)**	**Fragments (base)**
38	272	294	250
310	60	544	733
370	24	1277	17
394	40	1294	6
434	102	1300	5
536	290	1305	19
826	142	1324	311
968	404	1635	78
1372	174	1713	81
1546	391	1794	9
1937	27	1803	78
1964	580	1881	11
2544	267	1892	360
2811	80	2252	36
2891	458	2288	17
3349	434	2305	258
3783	29	2563	46
3812		2609	18
		2627	341
		2968	105
		3073	12
		3085	78
		3163	8
		3171	11
		3182	75
		3257	

Table 5. Groups of Restriction Mapping Pattern In Mut-WT Group Digested by *Hae* III

Group Description	Number of Clones
Clones Without Plasmid	33
Inadequate Plasmid Isolated	90
Vector Only	29
Pattern 1	5
Pattern 2	19
Pattern 3	9
Pattern 4	1
Pattern 5	1
Pattern 6	1
Pattern 7	1
Pattern 8	2
Pattern 9	1
Pattern 10	3
Pattern 11	2
Pattern 12	8
Pattern 13	1
Pattern 14	2
Pattern 15	1
Pattern 16	1
Pattern 17	1
Pattern 18	1
Pattern 19	1
Pattern 20	1
Pattern 21	1
Pattern 22	1
Pattern 23	7
Pattern 24	4
Pattern 25	5
Pattern 26	5
Pattern 27	7
Pattern 28	9
Pattern 29	2

Pattern 30	**13**
Pattern 31	**6**
Pattern 32	**8**
Pattern 33	**3**
Pattern 34	**34**
Pattern 35	**4**
Pattern 36	**4**
Pattern 37	**2**
Pattern 38	**1**
Pattern 39	**1**
Pattern 40	**2**
Pattern 41	**1**
Pattern 42	**1**
Pattern 43	**1**
Pattern 44	**1**
Pattern 45	**1**
Pattern 46	**1**
Pattern 47	**1**
Total	**340**

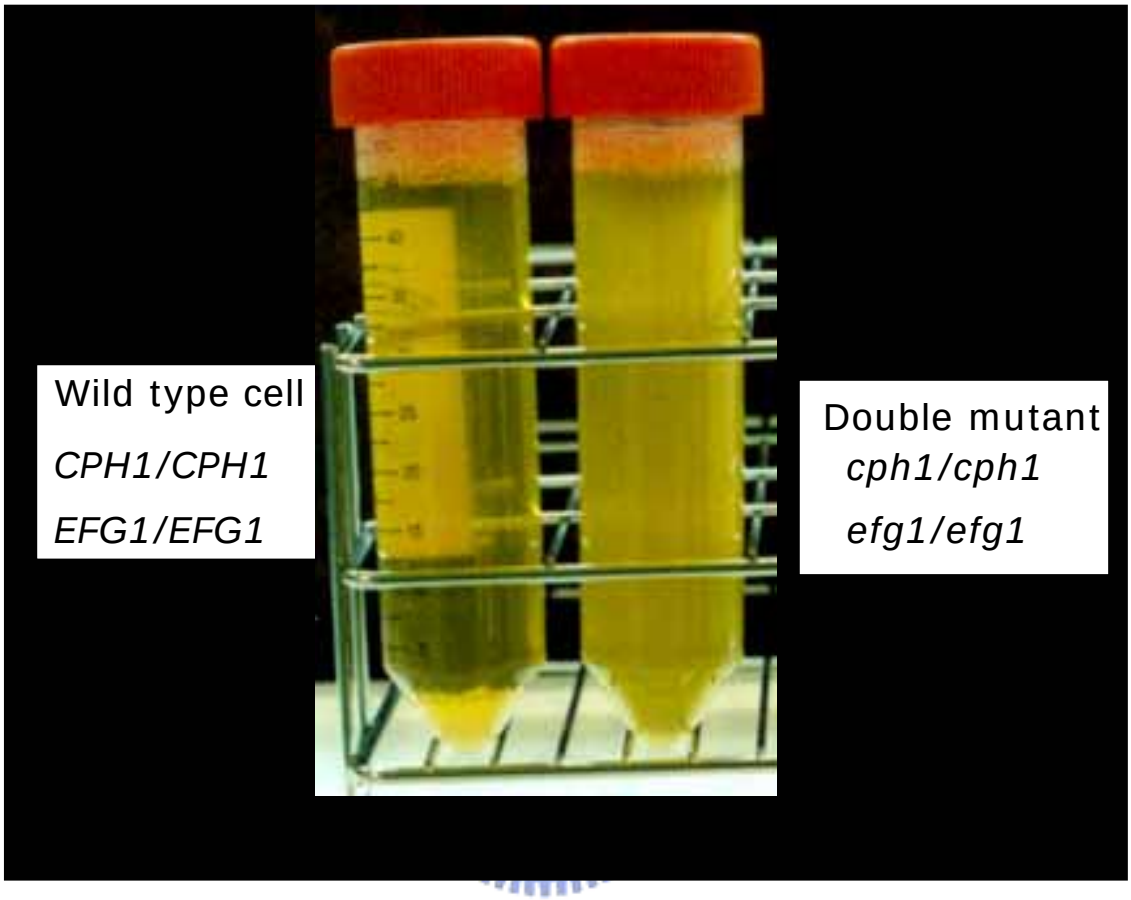


Figure 1. Wild Type and *cph1/cph1 efg1/efg1 Candida albicans* grown in the media

1 2 3 4 5 6

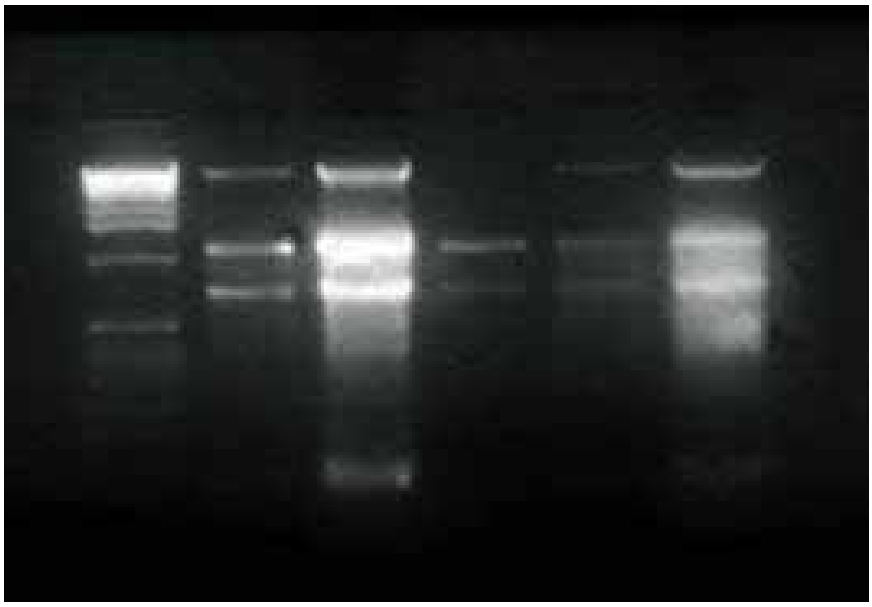


Figure 2. 1% Agarose Gel of RNA Isolated from Wild Type and Double Mutant Strains

Lane 1: 1 kb PLUS DNA Ladder (Gibco BRL), 1μg;

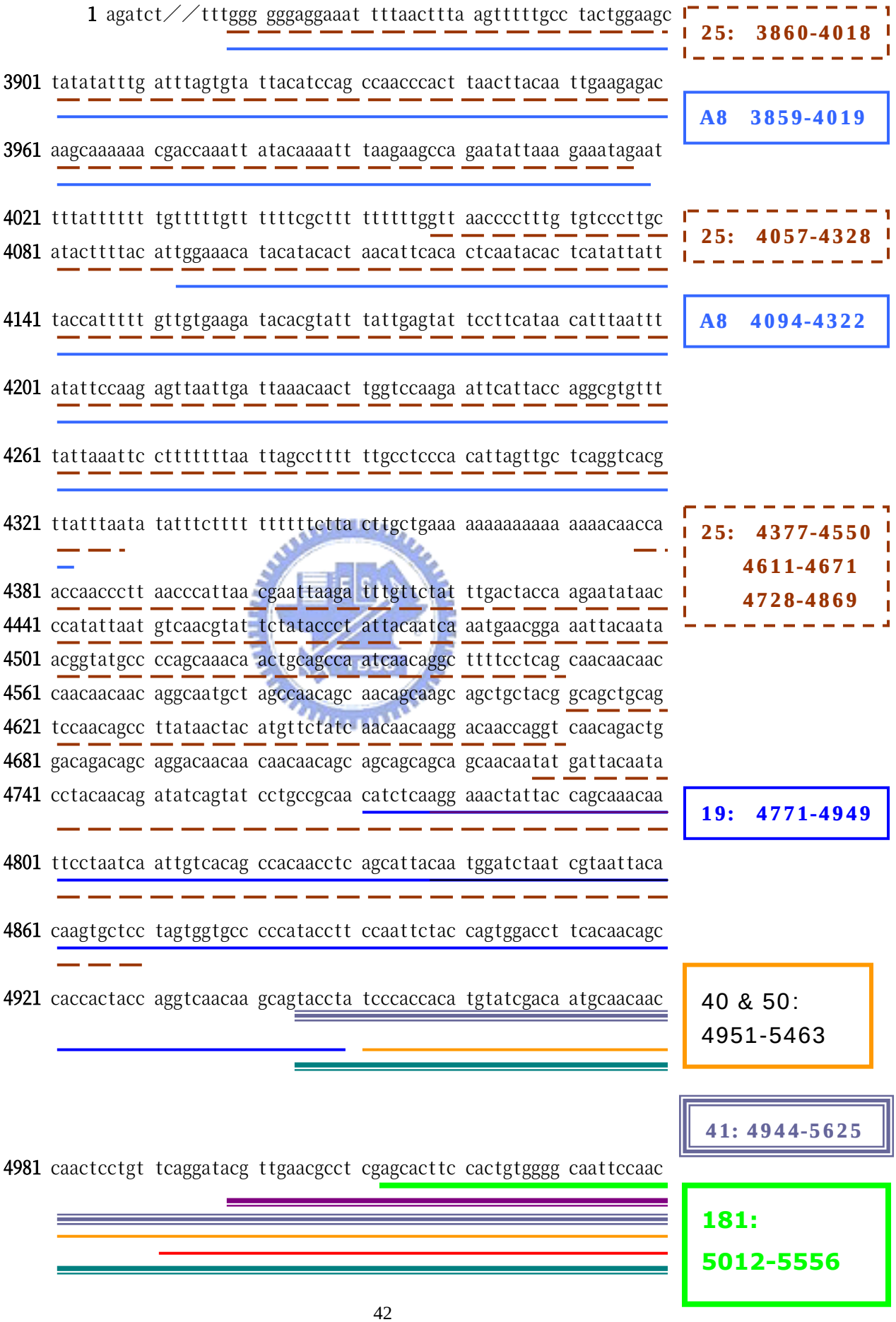
Lane 2: Wild Type Strain, Dec. 2 1999,
RNA concentration undetermined;

Lane 3: Double Mutant Strain, Dec. 2 1999,
RNA concentration undetermined;

Lane 4: The Same as Lane 2

Lane 5: Wild Type Strain, Nov. 19 1999,
RNA 2 μg (sample used in this study);

Lane 6: Double Mutant Strain, Nov. 19 1999,
RNA 2 μg (sample used in this study).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25: 3860-4018
3901 tatatatttg atttagtgta ttacatccag ccaacccact taacttacaa ttgaagagac
A8 3859-4019
3961 aagcaaaaaa cgaccaaatt atacaaaatt taagaagcca gaatattaaa gaaatagaat
4021 tttatttttt tgtttttgtt ttttcgcttt ttttttggtt aacccctttg tgtcccttgc
25: 4057-4328
4081 atacttttac attggaaaca tacatacact aacattcaca ctcaatacac tcatattatt
4141 taccattttt gttgtgaaga tacacgtatt tattgagtat tccttcataa catttaattt
A8 4094-4322
4201 atattccaag agttaattga ttaaacaact tggtccaaga attcattacc aggcgtgttt
4261 tattaaattc ctttttttaa ttagcctttt ttgcctccca cattagttgc tcaggtcacg
4321 ttatttaata tatttctttt ttttttctta cttgctgaaa aaaaaaaaaa aaaacaacca
25: 4377-4550
4611-4671
4728-4869
4381 accaaccctt aacccattaa cgaattaaga tttgttctat ttgactacca agaatataac
4441 ccatattaat gtcaacgtat tctataccct attacaatca aatgaacgga aattacaata
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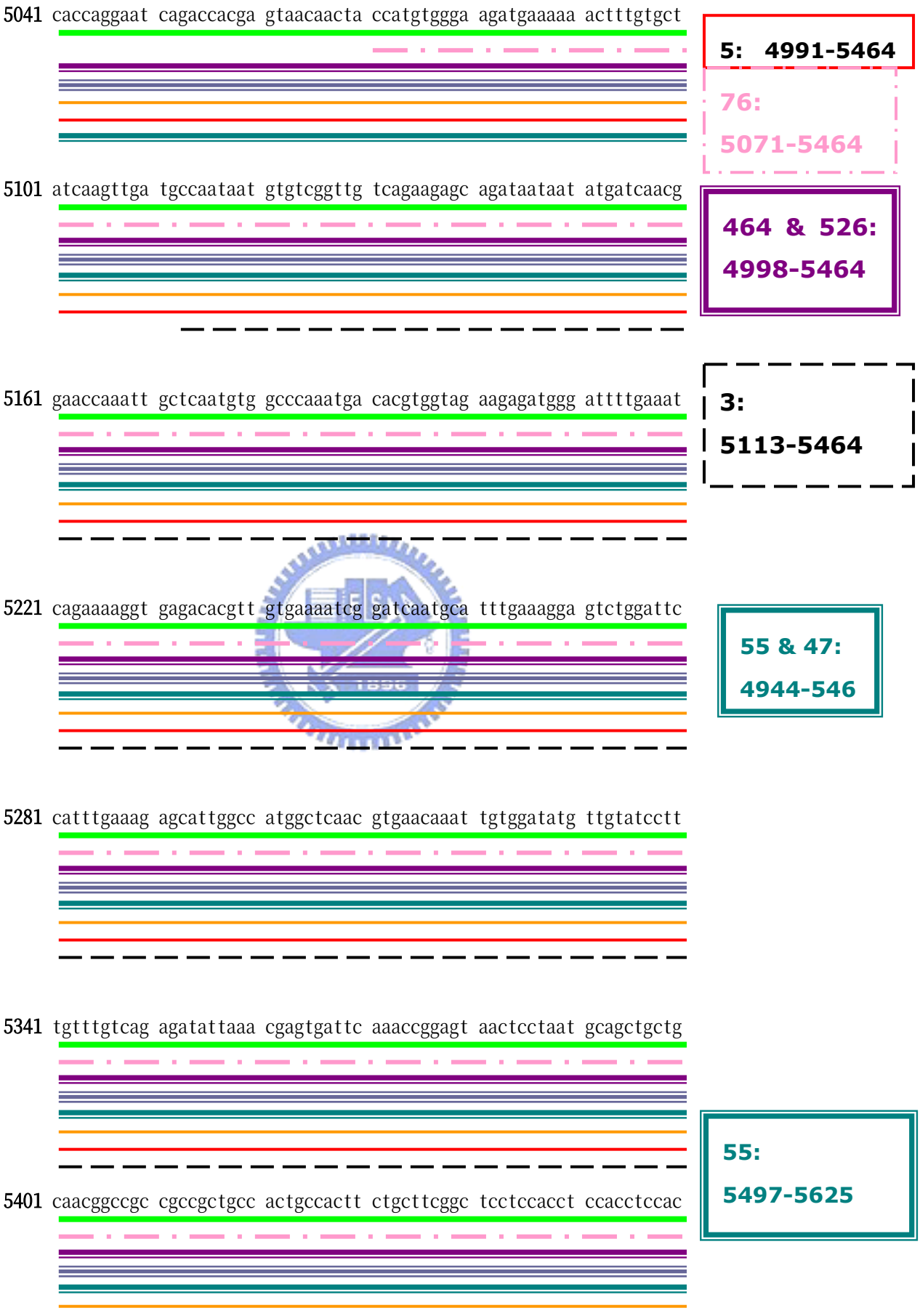
4561 caacaacaac aggcaatgct agccaacagc aacagcaagc agctgctacg gcagctgcag
4621 tccaacagcc ttataactac atgttctatc aacaacaagg acaaccaggt caacagactg
4681 gacagacagc aggacaacaa caacaacagc agcagcagca gcaacaatat gattacaata
4741 cctacaacag atatcagtat cctgccgcaa catctcaagg aaactattac cagcaaacaa
19: 4771-4949
4801 ttcctaatca attgtcacag ccacaacctc agcattacaa tggatctaat cgtaattaca
4861 caagtgctcc tagtggtgcc cccatacctt ccaattctac cagtggacct tcacaacagc
4921 caccactacc aggtcaacaa gcagtaccta tcccaccaca tgtatcgaca atgcaacaac
40 & 50:
4951-5463
41: 4944-5625
4981 caactcctgt tcaggatacg ttgaacgcct cgagcacttc cactgtgggg caattccaac
181:
5012-5556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76: 5071-5464
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464 & 526: 4998-5464
5161 gaaccaaatt gctcaatgtg gcccaaatga cacgtggtag aagagatggg attttgaaat
3: 5113-5464
5221 cagaaaaggt gagacacgtt gtgaaaatcg gatcaatgca tttgaaagga gtctggattc
55 & 47: 4944-546
5281 catttgaaag agcattggcc atggctcaac gtgaacaaat tgtggatatg ttgtatcctt
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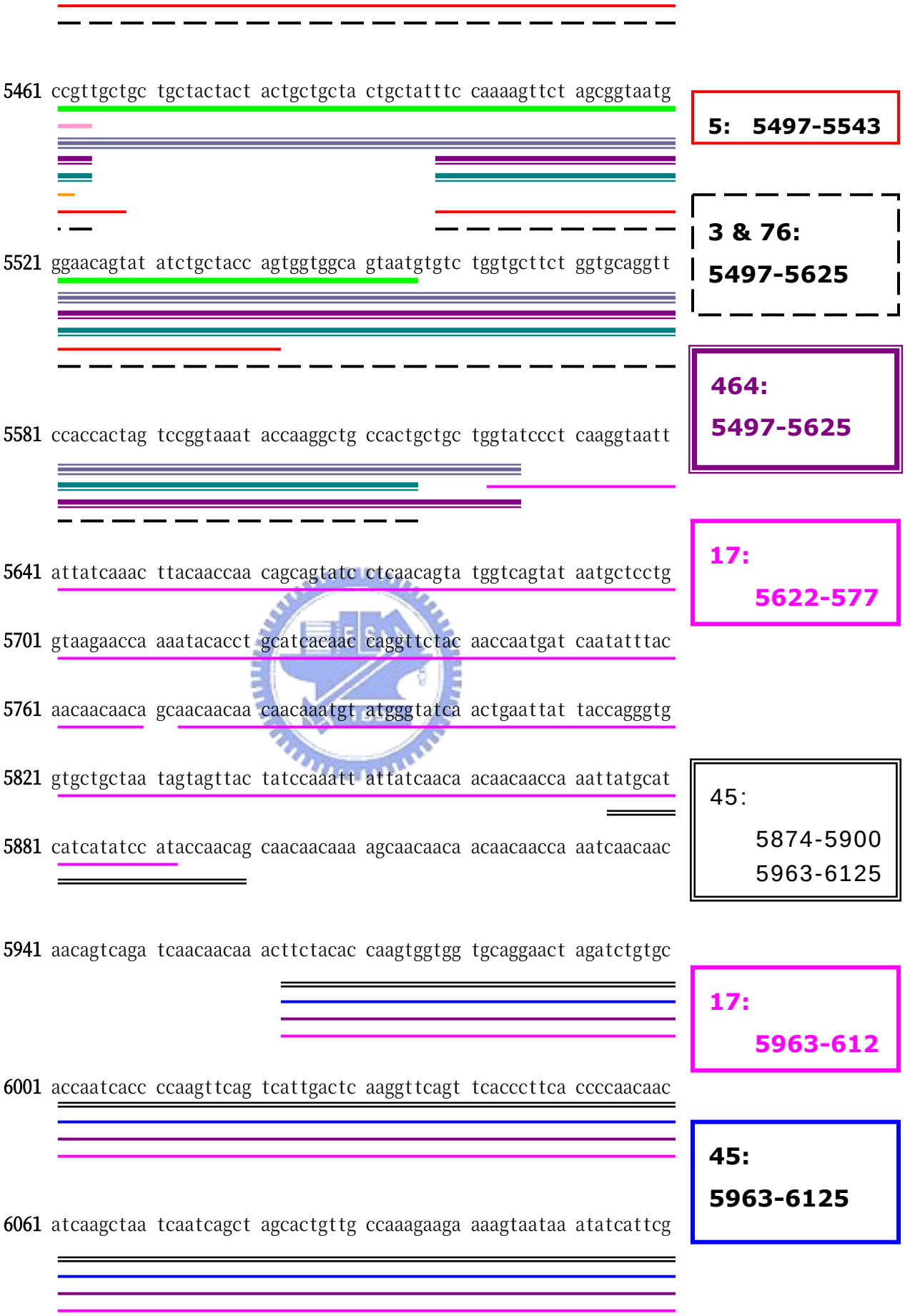
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5: 5497-5543
3 & 76:
5497-5625
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464:
5497-5625
5581 ccaccactag tccggtaaat accaaggctg ccactgctgc tggtatccct caaggtaatt
17:
5622-577
5641 attatcaaac ttacaaccaa cagcagtatc ctcaacagta tggtcagtat aatgctcctg
5701 gtaagaacca aaatacacct gcatcacaac caggttctac aaccaatgat caatatttac
5761 aacaacaaca gcaacaacaa caacaaatgt atgggtatca actgaattat taccagggtg
5821 gtgctgctaa tagtagttac tatccaaatt attatcaaca acaacaacca aattatgcat
45:
5874-5900
5963-6125
5881 catcatatcc ataccaacag caacaacaaa agcaacaaca acaacaacca aatcaacaac
5941 aacagtcaga tcaacaacaa acttctacac caagtggtgg tgcaggaact agatctgtgc
17:
5963-612
6001 accaatcacc ccaagttcag tcattgactc aaggttcagt tcacccttca ccccaacaac
45:
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6061 atcaagctaa tcaatcagct agcactgttg ccaaagaaga aaagtaataa atatcattcg

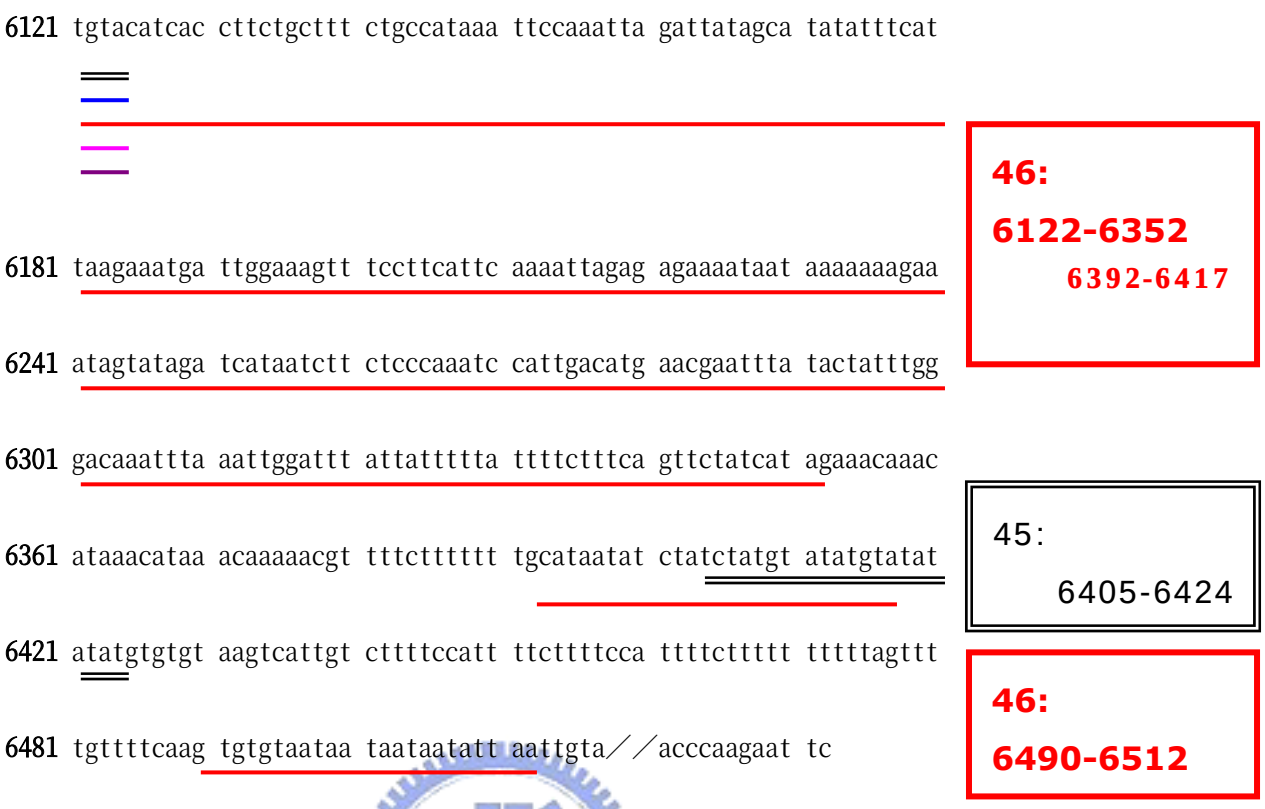


Figure 3. Different DNA Fragments of *Candida albicans EFG1* Gene

Example:

46: 6490-6512

46：the number of the clone.

6490 and 6512 mean the base position in the sequence of ***C. albicans EFG1***.

After sequencing and alignment, the sequences of colne 46 match those of *EFG1* base 6490-6512 and the color of the frame corresponds to that of the undelines.

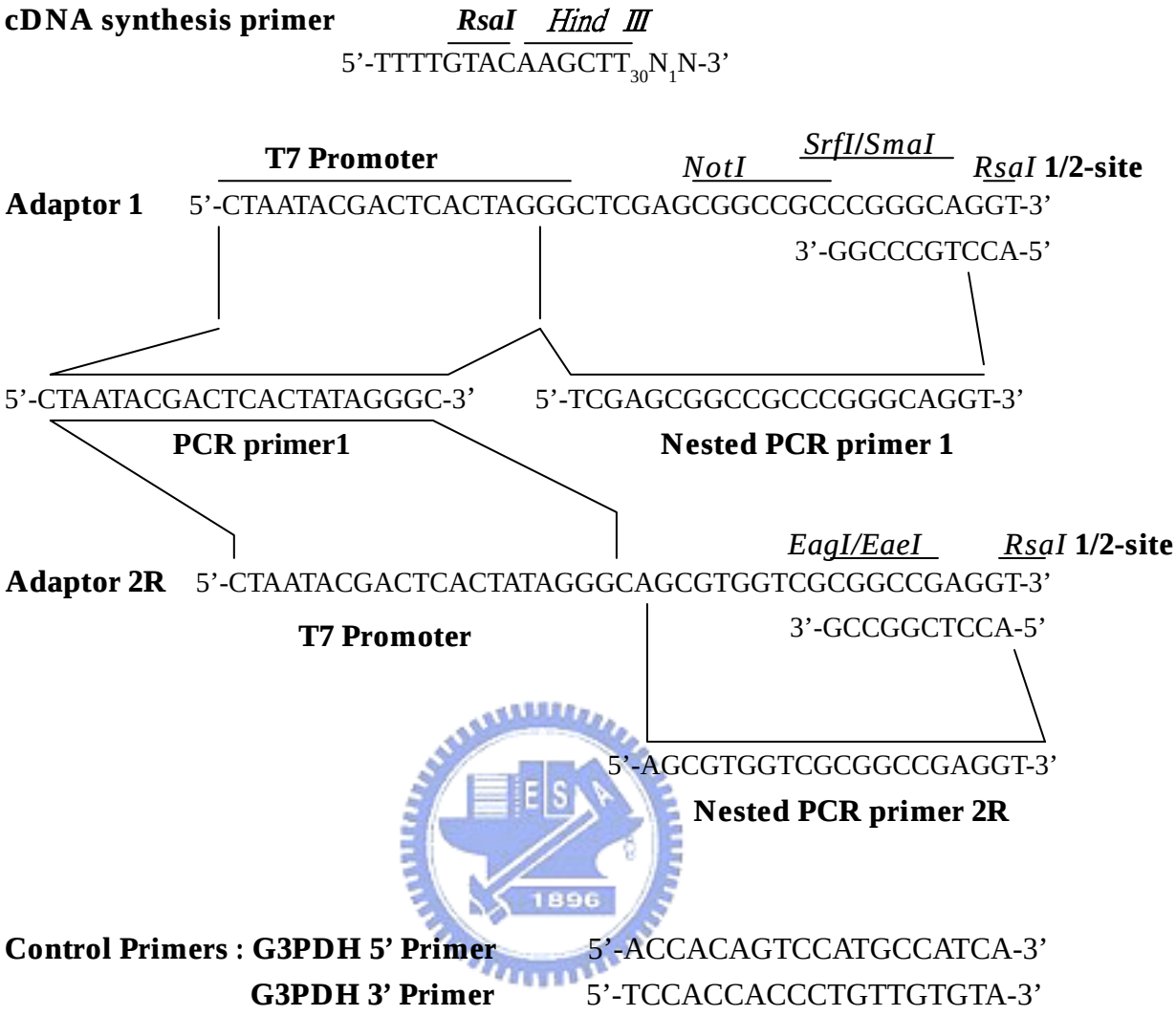
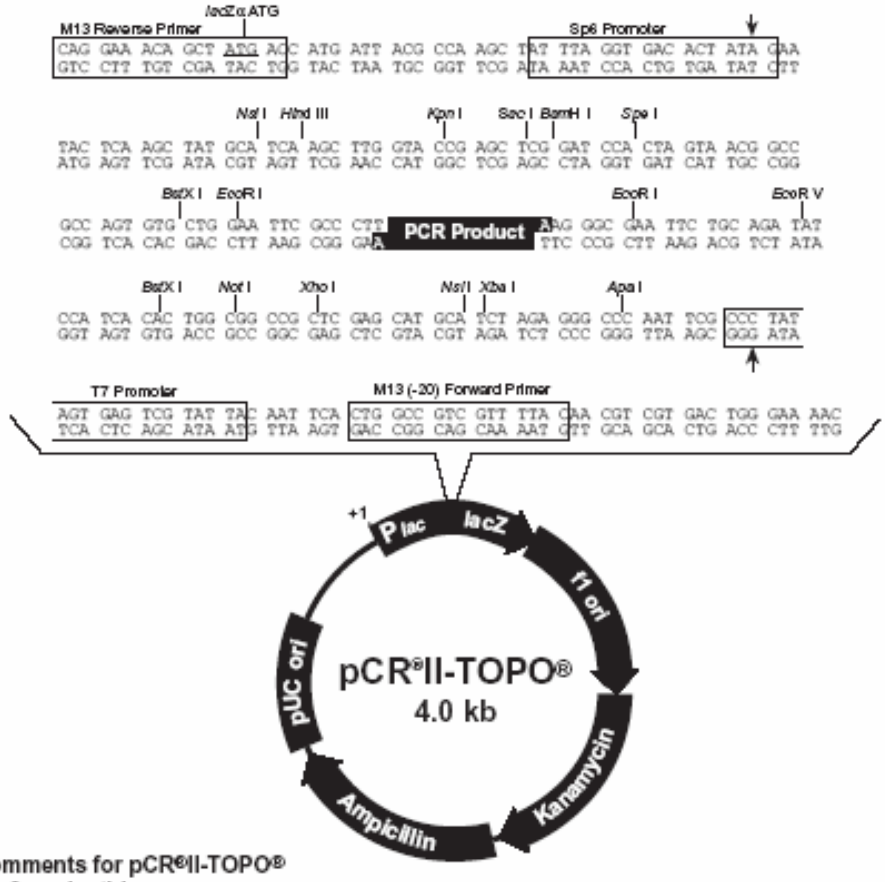


Figure 4. Sequences of the PCR-Select cDNA Synthesis Primer, Adaptors, and PCR Primers

When the adaptors are ligaed to Rsa-digested cDNA, the site is restored. Adaptor 2R was reformulated in December 1996. Before reformulation, this adaptor was called Adaptor 2; the sequence of the old Adaptor 2 was different. However, please note that in some lots of the PCR-Select Kit, the reformulated Adaptor 2R was still called Adaptor 2. If you received version PR6Y996 of the Use Manual with your PCR-Select Kit, your Adaptor 2 has the same sequence as Adaptor 2R shown here. (From : CLONTECH PCR-Select™ cDNA Subtraction Kit Use Manual)



Comments for pCR®II-TOPO®
3973 nucleotides

*Lac*Zα gene: bases 1-589
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
Multiple Cloning Site: bases 269-383
T7 promoter: bases 406-425
M13 (-20) Forward priming site: bases 433-448
f1 origin: bases 590-1027
Kanamycin resistance ORF: bases 1361-2155
Ampicillin resistance ORF: bases 2173-3033
pUC origin: bases 3178-3851

Figure 5. Map of pCR® II-TOPO®