國立交通大學

生化工程研究所

碩 士 論 文

在啤酒酵母菌內
利用重組基因方式尋找 *CaENO1* 上的分泌訊號位置

Genetic screen to search for the secretion signal of *CaENO1* in *Saccharomyces cerevisiae*

研 究 生：許淑貞

指導教授：楊昀良　博士

中 華 民 國 九 十 八 年 八 月

在啤酒酵母菌內利用重組基因方式尋找 *CaENO1* 上的分泌訊號位置

Genetic screen to search for the secretion signal of *CaENO1* in *Saccharomyces cerevisiae*

研 究 生：許淑貞　　Student：Shu-Jen Shiu

指導教授：楊昀良　　Advisor：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 Biochemical Engineering

College of Biological Science and Technology

National Chiao Tung University

in partial Fulfillment of the Requirements

for the Degree of

Master

in

Biochemical Engineering

August 2009

Hsinchu, Taiwan, Republic of China

中華民國九十八年八月

在啤酒酵母菌內利用重組基因方式尋找 *CaENO1* 上的分泌訊號位置

學生：許淑貞　　指導教授：楊昀良教授

國立交通大學生化工程研究所碩士班

摘　　要

真核生物中，大部份的分泌性蛋白質含有一段約 10~30 個疏水性的胺基酸序列，且通常出現在蛋白質 N 端；透過辨識這類的訊號，細胞才能將正確的蛋白質分泌至胞外。然而，近年來有相關研究發現，許多真核模式生物的蛋白質不帶有典型 N 端的分泌訊號胜肽(N-terminal signal peptide)，卻可被運輸至細胞表面甚至胞外；而本實驗白色念珠菌(*Candida albicans*)的烯醇化酶(Enolase)即屬此類蛋白質。在白色念珠菌中，烯醇化酶主要是由 *CaENO1* 基因所表達，大部份存在於細胞質內，為胞內的醣解酵素之一；有趣的是，在細胞表面及胞外培養基中，也可偵測到此蛋白質的存在。本論文因此利用啤酒酵母菌(*Saccharomyces cerevisiae*)表達系統，研究烯醇化酶是否存在決定性的分泌訊號序列，藉以探討上述現象。首先，必須先確立啤酒酵母菌的系統可以表達外源的念珠菌 *CaENO1* 基因，於是將建構好的質體轉形至酵母菌胞內表達，再利用西方點墨(western blot)偵測蛋白質的表現；結果顯示念珠菌 *CaENO1* 基因不但成功的在酵母菌內表達，且可被分泌至胞外。確定表達系統建立後，利用建構不同的 *CaENO1* 片段並在 C 端接上螢光蛋白(EGFP)，建構的片段長度以 DNA 序列表示, *CaENO1* 的 1-150 bp, 1-279, 1-387, 1-450,1-510, 1-900, 280-1320, 388-1320, 451-1320, 901-1320, 451-901 共 11 個不同建構產物,並建構一對照組僅有 EGFP,比較各個的表現來探討此基因的序列與分泌表現的相關性。透過西方點墨法偵測結果，只有全長的 *CaENO1* 基因所表現之蛋白質可於胞外被偵測；另外，共軛焦顯微鏡下觀察，並未在細胞壁及細胞膜上觀察到 enolase-EGFP 蛋白質，而是主要位於胞質內。

Genetic Screen to search for the secretion signal of *CaENO1* in *Saccharomyces cerevisiae*

Student：Shu-Jen Shiu　　Advisors：Dr. Yun-Liang Yang

Institute of Biochemical Engineering
National Chiao Tung University

Abstract

The evidence of proteins at the yeast cell surface that lack N-terminal signal peptides was initially provided by morphological, biochemical and genetic studies. The existence of many such proteins has subsequently been demonstrated by proteomic approaches. In *Candida albicans*, the gene encoding enolase is named *CaENO1*. Enolase is an enzyme of glycolysis and gluconeogenesis as well as major cell-surface antigen, which binds host plasmin and plasminogen. It is immunoprotective, phagocytosis, biofilm-regulated, and farnesol-down regulated. Enolase is detected on the cell surface even in the culture medium, but the mechanism of secretion is still unknown. My study was focused on identifying the critical region of *CaENO1* for secretion. First step is to test whether *CaENO1* can be expressed in *Saccharomyces cerevisiae*, *CaENO1* was tagged with HA3HIS6 and EGFP, respectively for detecting the target protein. Second is to further analysis the protein secretion. For determining which region is critical, the *CaENO1* was truncated for obtaining the constructs with different fragments 1-150 bp, 1-279, 1-387, 1-450, 1-510, 1-900, 280-1320, 388-1320, 451-1320, 901-1320, 451-901 of *CaENO1* and negative control EGFP only construct, and then analyzed for the secretion of truncated CaENO1-EGFP protein with western blot. According to the result of western blot, only full length *CaENO1* can lead the tagged protein outside the cell. Using confocal laser scanning microscopy, the eno-EGFPp was localized in the cytoplasm but not in the cell membrane or cell wall.the. *S. cerevisiae* cell seems not recognize the eno-EGFPp as a cell wall protein in this study.

誌謝

燕去燕來，時間在無聲中流逝著，我的碩士生涯也隨之畫上句點。感謝我的指導教授-楊昀良老師，謝謝老師這段時間的不吝指導與教誨；在老師的身教言教之下，學習面對問題的態度及解決問題的方法，讓我在挫折之時不致彷徨失措，不論是科學實驗或待人處事，學生都獲益良多，能在老師的指導下完成學業，學生備感榮幸。感謝三位口試委員彭慧玲老師、羅秀容老師及藍忠昱老師，謝謝三位老師不辭辛勞，對我的論文鉅細靡遺的細心指正，讓我獲益匪淺。

再者，感謝一路上同行的朋友的陪伴。感謝惠菁學姐的熱心指引及實驗上的幫忙，志豪學長、欣彬學姐、金蓉學姐及育穎學長的指導與照顧。謝謝我的同學們，人脈超廣的有緣同鄉人淑萍、客家幽默一哥旻秀及神經構造特殊的敏書，彼此的加油打氣，在這忙碌的實驗生涯是一道強心針，使我能繼續前進不放棄。還有實驗室學弟妹們，皮膚很水的妹妹頭阿毛、眼睛超美的準人妻馨儀、作自己好自在的靚小倩、服裝儀容超有型的毓駿、老師兼美食家的佳真、實驗越做越晚的妍寧、笑點很多的阿大、總是 on line 的禎憶、愛貓一族的重延、豪邁大方的幸璇、實驗室助理阿鋘、萬年點名班長葉大哥及在這裡認識的所有朋友們，這裡的一切，因你們而交織成一幅多采多姿的回憶。另外，我的國高中及大學好友們，感謝你們對我默默消失的這段日子裡的關心及包容，特別感謝佑任的照顧及鼓勵，陪我度過各種的難題。最感謝我的家人，個性很急的爸爸、永遠煮我愛吃的菜的媽媽、互相取笑的姐姐們、一定要當好男人的姐夫們及我可愛的姪子們，你們的支持是我動力的來源。該感謝的人實在太多了，請容我無法一一道完，在此謝謝所有關心我的人。最後，僅以這本論文，獻給我的兩位偉大雙親，希望沒辜負你們對我的期望。

別了，交大生科，一個我永遠會懷念的地方。

Contents

Contents of Figures

I. Introduction

1.1 Protein secretion in yeast

Secretion is a universal cellular process occurring in simple organisms such as baker's yeast to complex multicellular organisms including humans. Cell secretion involved the transport of vesicular product from within cell to without. This is considered to be the canonical mechanism for protein secretion, and recognition of a signal peptide sequence in a gene is taken as a clear indication that the corresponding product is exported by the cell. The process involves the translocation apparatus of the ER and is operated by the Golgi and Golgi-derived secretory vesicles fusing with the plasma membrane to release their protein content into the extracellular space.

However, experimental evidence accumulated over the past few years clearly shows that a significant number of proteins, glycolytic enzymes and other cytosolic proteins that lack an N-terminal signal peptide reach the cell surface. Many of these studies have been carried out in two yeast systems, namely *Saccharomyces cerevisiae* and *Candida albican.* Evidence of proteins at the cell surface that lack N-terminal signal peptides was initially provided by morphological, biochemical and genetic studies. These findings were first documented in *C. albicans* because several of the aforementioned proteins were identified as adhesins and host ligands that mediate attachment and retention of the commensal yeast on host tissues [1, 2]. Furthermore, some of these proteins have also been found in the *S. cerevisiae* cell surface [3 – 7].

1.2 Signal-less proteins are grouped by their different functions

Although the proteomic characterization was not exhaustive, the presence of several members of at least three groups of proteins with different functions was

apparent [8]. Notably, in the first group, the levels of enzymes involved in glycolysis – including enolase and fructose-bisphosphate aldolase – are profoundly affected by the yeast-to-mycelium transition on *C. albicans*. For example, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was serologically identified on the cell surface of *C. albicans*, where it mediates binding to host fibronectin and laminutesin, and is expressed more abundantly in hyphal forms [9]. The second group consists of heat-shock proteins with the capacity to function as chaperones, a striking example being osmotically stabilized regenerating protoplasts of *S. cerevisiae*, which secrete many proteins including metabolic enzymes and heat-shock proteins [4]. The third group consists of translation elongation factors that are proteins that interact with complexes of other proteins and RNA. The level of enolase and phosphoglyceromutase associated with the cell wall is much reduced in *C. albicans* grown in the presence of fluconazole [10], providing further evidence that the localization of signal-less proteins is altered in response to the environment.

1.3 Hypothetical mechanisms for driving signal-less proteins

Several possibilities can be envisaged from observations made in yeasts and other organisms. Data from *S. cerevisiae* indicate that the ATP-binding cassette transporter is a potential driver for protein export. In addition, non-classical export (NCE) genes NCE101 and NCE102 are related to non-classical export of heterologous mammalian galectin. The existence of homologues for NCE101 and NCE102 in *C. albicans*, *Candida glabrata*, *Kluyveromyces lactis* and *Yarrowia lipolytica* is also relevant. Observations of alternative secretion in mammals and parasites [11] provide grounds for postulating membrane blebbing (bubble formation) and other options based on the specific recognition of alternative secretion proteins for export outside the plasma membrane.

When SignalP, an algorithm for the prediction of non-classically secreted human proteins [12], was applied to non-conventional exported yeast proteins, mixed results were obtained. One suggestion was that these proteins could leak into the secretory pathway and be exported in this way [13]. In the absence of potential glycosylation sites, leakage would be predicted to occur into Golgi or cargo vesicles. If this were the case, this mechanism would need to be sensitive to secretory-pathway inhibitors and would not operate in conditional mutants affected in classical secretion. Unfortunately, this possibility was examinutesed only briefly and a sec4 conditional mutant strain did not seem to be affected at the non-permissive temperature [14].

Secondary-structure elements might contribute to export because partial unfolding of the b-barrel structure of fibroblast growth factor (FGF)-1 and interleukin (IL)-1a [15] are part of an export mechanism. In addition, the unfolded state of the green fluorescent protein β-barrel structure leads to export of the unfolded fraction. None of the non-conventionally exported yeast proteins identified is of this structure, although some have a hybrid secondary structure.

1.4 The study of Enolase

Enolase (2-phospho-D-glycerate hydrolase) is an enzymatic component of the glycolytic pathway and is conserved through evolution. It catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the second step of he glycolytic pathway. The enzymatic activity of enolase requires certain divalent metal ions, and magnesium ion is its important natural cofactor [16,17]. Enolase can be detected in many prokaryotic and eukaryotic cells, and is localized in cytoplasm, cell surface and nucleus of various mammalian cells to possibly mediate distinct functions. Recent studies have revealed that enolase is involved in many different physiological and pathophysiological conditions [18].

The protein enolase is one of the most abundant enzymes in the cytosol, and is also a cell wall protein which may arrive at the cell wall via a currently unknown pathway in *S. cerevisiae* and *C. albicans.* The exact function of enolase in the cell wall is still unclear, but it is possible that enolase may have an alternative function, as do other glycolytic enzymes, most notably phosphoglycerate kinase, glyceraldehyde phosphate dehydrogenase, and alcohol dehydrogenase [3,5,10].

Different approaches had identified this protein among those isolated from the cell wall and immunogold electron microscopy demonstrated that this is associated to the surface of whole cells [3]. Preliminutesary evidence at the molecular level also confirmed that the protein sequence of enolase has the capacity to drive enolase to the external medium [4]. In addition, a global analysis using proteome chips also pointed to the capacity of enolase, and several other proteins involved in glucose metabolism, to bind to phospholipids, suggesting their interaction with membranes [19]. Enolase has also been described as a surface protein in the cells of many other species, ranging from prokaryotic microorganisms to human cells, and its putative role as binding to plasminutesogen, laminutesin or salivary mucin proteins [20] is coherent with an external location [21–15]. Plasminutes(ogen) binding of *C. albicans* enolase has been related to enhanced invasion of human brain microvascular endothelial cells [26] and in this organism enolase is also an immunodominutesant antigen.

1.5 Overview of the research project

In *C. albicans*, the gene encoding enolase is named *CaENO1*. It is a enzyme of glycolysis and gluconeogenesis. It is a major cell-surface antigen binding host plasminutes and plasminutesogen. It is involved in immunoprotection, phagocytosis, and is biofilm-regulated and farnesol-down regulated. The cDNA of *CaENO1* is 1320 bp. It is essential for cell growth in glucose-contained media. In previous study,

enolase of *C. albicans* was considered a secreted protein that lacks an N-terminal signal peptide through non-canonical mechanism. It was detected on the cell surface and even in the culture medium, but the mechanism of enolase secretion is still unknown.

There are two key issues in this project: how dose *C. albicans* secret enolase outside the cell and what is the sequence required for secretion. No matter what it uses for transport regardless channel, pump, or other approach, it must contain a region for interaction with receptor on the membrane, directly or indirectly.

My research was focused on searching the critical region of *CaENO1* for secretion. Because *C. albicans* is diploid without known plasmid; it is difficult to manipulate on molecular level. Therefore, in this study, the experiment is conduted with *CaENO1* in *S. cerevisiae* .

First step is to test whether *CaENO1* can be expressed in *S. cerevisiae*. For detecting the target protein, *CaENO1* was tagged with tag (either HA3HIS6 or EGFP) in this study. The second is further to determine which region of the protein is critical for secretion. Various trucated *CaENO1* genes were constructed. The flow chart is shown in Fgure1.

II. Material

2.1 Strains

- *E.coli* : DH5α
- *Saccharomyces cerevisae* :

Strain	Genotype	Reference
10560-2B	*MATa his3 : : hisG ura3–52 leu2 : : hisG*	Chen et al. (2004), Lab of H.-J. Lo
10560-2B-f0	10560-2B was transformed with plasmid YEP363	This study
10560-2B-f1	10560-2B was transformed with plasmid YEP363-CaENO1-TP	This study
10560-2B-f2	10560-2B was transformed with plasmid YEP363-CaENO1-EGFP	This study
10560-2B-f3	10560-2B was transformed with plasmid YEP363-CaENO1-EGFP-TP	This study
10560-2B-EGFP	10560-2B was transformed with plasmid YEP363-EGFP	This study
10560-2B-CaENO1[280-1320]	10560-2B was transformed with plasmid YEP363- CaENO1[280-1320]-EGFP	This study
10560-2B-CaENO1[388-1320]	10560-2B was transformed with plasmid YEP363- CaENO1[388-1320]-EGFP	This study
10560-2B-CaENO1[451-1320]	10560-2B was transformed with plasmid YEP363- CaENO1[451-1320]-EGFP	This study
10560-2B-CaENO1[901-1320]	10560-2B was transformed with plasmid YEP363- CaENO1[901-1320]-EGFP	This study
10560-2B-CaENO1[451-901]	10560-2B was transformed with plasmid YEP363- CaENO1[451-901]-EGFP	This study
10560-2B-CaENO1[1-150]	10560-2B was transformed with plasmid YEP363- CaENO1[1-150]-EGFP	This study
10560-2B-CaENO1[1-279]	10560-2B was transformed with plasmid YEP363- CaENO1[1-279]-EGFP	This study

Strain	Genotype	Reference
10560-2B-CaENO1[1-387]	10560-2B was transformed with plasmid YEP363- CaENO1[1-387]-EGFP	This study
10560-2B-CaENO1[1-1450]	10560-2B was transformed with plasmid YEP363- CaENO1[1-1450]-EGFP	This study
10560-2B-CaENO1[1-511]	10560-2B was transformed with plasmid YEP363- CaENO1[1-511]-EGFP	This study
10560-2B-CaENO1[1-900]	10560-2B was transformed with plasmid YEP363- CaENO1[1-900]-EGFP	This study

2.2 Plasmids

Plasmid	Description	Reference
pEGFP-N2	It contained EGFP.	BD Bioscience Clontech
YEP363	It contained selection marker *Leu2* gene, *lacZ* gene, ampR (ampicillin resistance), and 2μ (origin of yeast).	Valenzuela et.,1998
YEP363-CaENO1-TP	YEP363 plasmid contained *CaENO1* gene tagged with HA3HIS6 tag and pACT1 promoter	Made by Yao-Chen Chuang, Lab of Y.-L. Yang
YEP363-CaENO1-EGFP	YEP363 plasmid contained *CaENO1* gene tagged with EGFP and pACT1 promoter	This study
YEP363-CaENO1-EGFP-TP	YEP363 plasmid contained *CaENO1* gene tagged with EGFP and HA3HIS6 tag and pACT1 promoter	This study
YEP363-EGFP	YEP363 plasmid contained EGFP and pACT1 promoter	This study
YEP363-CaENO1[280-1320]-EGFP	YEP363 plasmid contained +280~+1320 bp of *CaENO1* gene tagged with EGFP and pACT1 promoter	This study
YEP363-CaENO1[388-1320]-EGFP	YEP363 plasmid contained +388~+1320 bp of *CaENO1* gene tagged with EGFP	This study

Plasmid	Description	Reference
	and pACT1 promoter	
YEP363-CaENO1[451-1320]-EGFP	YEP363 plasmid contained +451~+1320 bp of *CaENO1* gene tagged with EGFP and pACT1 promoter	This study
YEP363-CaENO1[901-1320]-EGFP	YEP363 plasmid contained +901~+1320 bp of *CaENO1* gene tagged with EGFP and pACT1 promoter	This study
YEP363-CaENO1[451-900]-EGFP	YEP363 plasmid contained +451~+901 bp of *CaENO1* gene tagged with EGFP and pACT1 promoter	This study
YEP363- CaENO1[1-150]-EGFP	YEP363 plasmid contained +1~+150 bp of *CaENO1* gene tagged with EGFP and pACT1 promoter	This study
YEP363- CaENO1[1-279]-EGFP	YEP363 plasmid contained +1~+279 bp of *CaENO1* gene tagged with EGFP and pACT1 promoter	This study
YEP363- CaENO1[1-387]-EGFP	YEP363 plasmid contained +1~+387 bp of *CaENO1* gene tagged with EGFP and pACT1 promoter	This study
YEP363- CaENO1[1-450]-EGFP	YEP363 plasmid contained +1~+450 bp of *CaENO1* gene tagged with EGFP and pACT1 promoter	This study
YEP363- CaENO1[1-510]-EGFP	YEP363 plasmid contained +1~+511 bp of *CaENO1* gene tagged with EGFP and pACT1 promoter	This study
YEP363- CaENO1[1-900]-EGFP	YEP363 plasmid contained +1~+901 bp of *CaENO1* gene tagged with EGFP and pACT1 promoter	This study

2.3 Primers

Name	Sequence (5'→3')	Location
EGFP-L-Xba I Spe I	ATTCTAGAACTAGTATGGTGAGCAAGGGC	EGFP gene: Nucleotide: +1~+15
EGFP-R-Xba I	ATTCTAGACTTGTACAGCTCGTCCATGC	EGFP gene: Nucleotide: +717~+698

Name	Sequence (5'→3')	Location
EGFP-R- Pst I	AACTGCAGTTACTTGTACAG CTCGTCC	EGFP gene: Nucleotide: +720~+702
pACT1 -F	CATTCATTCCCGGAATTCCC	YEP363-CaENO1-TP Nucleotide:+10509~+8
pACT1 -R	CCACTAGTTGTTAATTCAGT AAATTTTCG	YEP363-CaENO1-TP Nucleotide:+491~+471
eno/1-f	GGACTAGTTCTAGAATGTCT TACGCCACTAAA	CaENO1 gene: Nucleotide: +1~+18
eno/450-r	GGACTAGTAACTGGCAAAA CGAATTTACC	CaENO1 gene: Nucleotide: +450~+430
eno/451-f	GGACTAGTTCTAGAATGCCA TTCCAAAAC	CaENO1 gene: Nucleotide: +451~+462
eno/900-r	GGACTAGTGAATGGATCTTC AATAGAAACAA	CaENO1 gene: Nucleotide: +900~+878
eno/901-f	GGACTAGTTCTAGAATGGCT GAAGATGACT	CaENO1 gene: Nucleotide: +901~+913
eno/1320-r	GGACTAGTCAATTGAGAAG CCTTTTGG	CaENO1 gene: Nucleotide:+1320~+1302
eno1/280F	GGACTAGTCTAGATGTTCTT GTTGTCCTTGG	CaENO1 gene: Nucleotide:+280~+295
eno1/388F	GGACTAGTCTAGATGCCATT GTACAAACAC	CaENO1 gene: Nucleotide:+388~+402
eno1/150R	GGACTAGTCAATTCCAAAG CTTCGTG	CaENO1 gene: Nucleotide:+150~+133
eno1/279R	GGACTAGTTTCATCAATCTT AGCTTGGTCG	CaENO1 gene: Nucleotide:+279~+258
eno1/387R	GGACTAGTAATGCCTTGAGC AGCAGC	CaENO1 gene: Nucleotide:+387~+370
eno1/510R	GGACTAGTTTCTTGGAAAG CTAAAGCACC	CaENO1 gene: Nucleotide:+510~+490

2.4 Chemicals, enzymes, and regents

- **Alpha Biosciences :** LB agar (Cat. No.L12-111)
- **Amresco :** Agarose I (Cat. No. 0710-500G), EDTA (Cat. No.0105), Glycerol

(Cat.No.0854-1L-PTM), Tris base (Cat. No.0826), Tris-Hydrochloride (Cat. No.0234-500G), Acryl/Bis 37.5:1 solution (Cat. No.0524)

- **Amersham Biosciences :** Nitrocellulose Membrane (Cat. No.FM0053-1)
- **Anchor :** Non fat milk (Cat. No.EMB53084-D)
- **ApplinChem :** Ampicillin (Cat. No.A2839)
- **Bio-Rad :** Ammonium persulfate (APS) (Cat.No.161-0700)
- **Difco:** Bacto agar (Cat. No.143175)**,** Yeast nitrogen base w/o aminol acid (Cat. No.145368) ,YPD broth (Cat. No.135141XB)
- **Fermentas :** T4 DNA ligas (Cat. No.1812)
- **Fluka :** Acetic acid (Cat. No.33209), Calcofluor White Stain (Cat. No.18909), Phenyl methey sulfonyl fluoride (PMSF) (Cat.No.78830), Urea (Cat. No.SK-2644U)
- **Invitrogen :** Image-iT ™ LIVE Plasma Membrane and Nuclear Labeling Kit (Cat. No.I34406)
- **J.B.Baker :** Dextrose (Cat. No.1916-01)
- **Kodak :** GBX Developer and Replenisher (Cat. No.8875569) **,**GBX Fixer and Replenisher (Cat. No.1749837)**,** X-film (Cat. No.1651454)
- **Merck :** Dodecy Sulfate Sodium (SDS) (Cat. No.1.12012.0500) ,Ethanol (Cat. No.1.00983.2500), Tris –HCL (Cat. No.1.01547.1000), Sodium acetate (Cat. No.1.06267.0500), β-mercaptoethanol (Cat. No.1.1543.0100)
- **Mallinckrodt :** Methanol (Cat.No.3016-08)
- **MILLIPORE :** Amicon Ultra-15 (Cat. No.UFC9 010 08), Amicon Ultra-4 (Cat. No.UFC9 010 24), HRP substrate (Cat. No.WBKLS0500)
- **NEB :** Restriction Enzyme (in this study)
- **NOVUS :** pAb anti-EGFP[HRP] (Cat. No.NB600-313)
- **Protech :** ProTaq^{TM}DNA polymeras (Cat. No.PTM525)

- **Promega :** Shrimp Alkaline Phosphatase (SAP) (Cat. No.M8201)
- **Premier:** ExcelPure™ Plasmid Minutesi kit (Cat. No.PM250)**,** ExcelPure™ PCR Cleanup kit (Cat. No.CU250), PCR clean-up/Gel Extraction kit (Cat. No.N-DCE050)
- **Riedel-de Haen :** Sodium Chloride (Cat. No.13423), Sodium hydroxide (Cat. No.30620), $CaCl_2$(Cat. No.31307), Fomaldehyde (Cat. No.33220)
- **Santa Cruz :** HA-probe(F-7)HRP (Cat. No.SC-7392)
- **Scharlau:** LB broth (Cat. No.02-385)
- **Schleich&Schue II:** Nitrocellulose Transfer Membrane (Cat.No.10401396)
- **SibEneyme:** 1kb DNA leader (Cat. No.SEM11C001)
- **Sigma:** Crystal violet (Cat. No.C-3886), Ethidium bromide (Cat. No.E-7637) Glass Beads(Cat.No.G-9286), Lithium Acetate (Cat. No.L-6883), L-Histidine(Cat. No.H-8125), Polyethylene $Glycol_{3350}$(Cat. No.P-4338), 2-propanol(Cat.No.I9516), Uridine (Cat. No.U-0750), Lyticase (Cat. No. L4025-25KU), Tween 20 (Cat. No.P-1379), N,N,N', N',-tetramethylenediaminutese (TEMED) (Cat. No.T-9281)
- **TBB :** Prestain Protein marker (Cat. No.0901)
- **Thermo :** POLYSINE© SLIDES (Cat. No.2209-01)

2.5 Buffers

- **1% crystal violet solution**

  5 g crystal violet solution, 50 ml 37% formaldehyde, 450 ml H_2O

- **1M Lithium Acetate**

  40.8 g Lithium Acetate added ddH_2O to 400 ml (pH 7.5)

- **2X SDS-PAGE loading dye**

  0.5% bromphenol blue, 0.5M Tris-HCl (pH 8.0), 10% SDS, 100% glycerol

- **5% Blocking buffer**

  2.5 g nonfat powdered milk dissolved in 50 ml 1X TBS buffer

- **5M EDTA stock solution**

  186.1 g EDTA added ddH_2O to 800 ml (pH 8.0)

- **10% (v/v) Glycerol**

  12.6 g glycerol (density = 1.26 g/ml), make to 100 ml with dH2O

- **10X Running buffer (also called Laemmli buffer)**

  30.3 g Tris base, 144 g Glycine, 10 g SDS, make to 1L with dH_2O

- **10X TE buffer**

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

- **10X Transfer buffer**

  39 mM Glycine, 48mM Tri base, 10% SDS, 20% methanol

- **40% Dextrose**

  40g Dextrose added ddH2O to 100 ml

- **50% PEG_{3350}**

  75 g polyethylene glycol3350 added ddH2O to 150 ml

- **50X TAE buffer**

  48.4g Tris base, 0.5M EDTA (pH 8.0) 20ml, 11.42 ml acetic acid added ddH_2O to 200 ml

- **Breaking buffer**

  100 mM Tris-HCl (pH 8.0), 1% (w/s) SDS, 2% (v/v) Triton X-100, 100 mM NaCl, 10mM EDTA

- **LATE buffer**

  0.1 M Lithium acetate, 10 mM Tris-HCl (pH 7.5), 1mM EDTA

- **Lyticase digestion solution (in PBS)**

  100μg/ml Lyticase (Sigma L4025; 50kU $45; 500U/mg solid), 3μg/ml PMSF,

2μl/ml β-ME (13.8M stock at 4℃)

- **TBS buffer (Tris-buffered saline)**

  10 mM Tris-HCl (pH 8.0), 150 mM NaCl

- **TBST buffer**

  10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween

- **PBS buffer**

  8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, 0.24 g KH2PO4

  in 800 ml of distilled H2O. Adjust the pH to 7.4 with HCl. Add H2O to 1 liter.

- **PLATE buffer**

  40% polyethylene $glycol_{3350}$ in LATE buffer

2.6 Medium

- **LB (Luri-Bertni) agar**

  1% tryptone, 0.5% yeast extract, 1%NaCl, 1.5% agar

- **LB agar /Ampicilline**

  1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar, 50μg/ml Ampicillin

- **LB broth**

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

- **LB broth/Ampicilline**

  1% tryptone, 0.5% yeast extract, 1% NaCl, 50μg/ml Ampicillin

- **SD agar**

  0.67% Bacto-yeast nitrogen base w/o aminol acid, 2% dextrose, 2% agar

- **SD broth**

  0.67% Bacto-yeast nitrogen base w/o aminol acid, 2% dextrose

- **SD agar/Uridine. Histidine**

  0.67% Bacto-yeast nitrogen base w/o aminol acid, 2% dextrose, 2% agar,

80 mg/l uridine, 100 mg/l histidine

- **SD broth/Uridine, Histidine**

  0.67% Bacto-yeast nitrogen base w/o aminol acid, 2% dextrose, 80 mg/l uridine, 100 mg/l histidine

- **YPD agar**

  2% Bacto-peptone, 1% yeast extract, 2% dextrose 2% agar

- **YPD broth**

  2% Bacto-peptone, 1% yeast extract, 2% dextrose

2.7 Equipments

- 4℃ refrigerator KS-101MS (MINUTESI KINGCON)
- -20℃ low temperature refrigerator (WHITE-WESTINGHOUSE)
- -80℃ low temperature refrigerator 925/926 (FRISTEK SCIENTIFIC)
- Bench top orbital shaker S101 (FIRSTEK)
- Certrifuge model 5100 (Kubota Corporation)
- Digital scale PB153-S (METTLER TOLEDO)
- Digital scale TB-214 (DENVER INSTRUMENT)
- Fluorescence Microscope ECLIPSE TE2000-U (NIKON)
- GeneQuant pro DNA/RNA calculator (AMERSHAM PHARMACIA BIOTECH)
- Gene CycleTM (BIO-RAD)
- Image system GEL DOC 2000 (BIO-RAD)
- Microcentrifuge MICRO 240A (DENVILLE SCIENTIFIC INC.)
- Olympus FluoView Confocal Laser Scanning Microscopes - FV500 (OLYMPUS)
- Orbital shaker IKA-VIBRAX-VXR
- pH meter Φ360 (BACKMAN)
- Spectrometer 20GENESYSRT (SPECTRONIC INSTRUMENT)

- Shaker VORTEX-GENIE2 G560 (SCIENTIFIC INDUATRIC)
- Thermal Cycler PTC-100RT (MJ RESEARCH INC.)
- Water bath B206-T1 (FIRSTEK SCIENTIFIC)
- TRANS-BLOT® SD CELL 221BR (BIO-RAD)

III. Method

3.1 *E. coli* Transformation

3.1.1 The preparation of competent cell

DH5α was inoculated into 5 ml LB broth and incubated at 37°C for 16~18 hours with vigorous shaking (~180 rpm/minutes). Then 2 ml of cultured cell solution was transferred to 50 ml LB broth and incubated at 37°C with vigorous shaking (~180 rpm/minutes). Until the OD_{600} arriving 0.6~0.8, the cultured cell solution was transferred to 50 ml centrifugation tube and then put on the ice for 20 minutes. The cells were collected by centrifugation of 1620×g (eppendorf 5804R) at 4°C for 10 minutes. Then the cells resuspended in cold 25 ml 0.1M $CaCl_2$ solution and put on the ice for 30 minutes. The cells were collected by centrifugation of 720×g (eppendorf 5804R) at 4°C for 10 minutes and then resuspended in cold 5 ml 0.1M $CaCl_2$ solution. The cell solution was stored at 4°C for 18 hours and then centrifuged at 4°C , 720×g (eppendorf 5804R) for 10 minutes. Then the cells were resuspended in cold 5 ml 0.05M $CaCl_2$ with 15% glycerol solution. Finally, the cell solution was separated into 100 μl for each eppendorf tube and then stored at -80°C.

3.1.2 Transformation of competent cell

The competent cell in the eppendorf tube from -80°C was put on the ice for unfreezing and then added 0.1~1μg plasmid DNA. After ice bath for 30 minutes, the competent cell was heat shock at 42°C for 1 minute and then added 300μl LB broth with 50μg/ml Ampicillin. The cell solution was incubated at 37°C for 1 hours with vigorous shaking (~180 rpm/minutes). Each 100μl of the cell solution was dispread on the LB agar medium with 50μg/ml Ampicillin and incubated at 37°C for 12~18 hours.

3.2 Isolation of *E.coli* plasmid DNA

The plasmid in *E.coli* was extracted with ExcelPure™ Plasmid Mini kit (Premier, Cat. No.PM250). The procedure is as following :

The DH5α with target plasmid was inoculated into 5 ml LB broth with 50μg/ml Ampicillin and incubated at 37°C for 16~18 hours with vigorous shaking (~180 rpm/minutes). 1.5 ml of cell solution was transferred to an eppendorf tube. The cells were recovered by centrifugation at 13000 rpm for 1 minute and then resupended in 200μl Solution I buffer (Premier, Cat. No.PM250). Then 200μl of Solution II buffer (Premier, Cat. No.PM250) was added to the mixture and mixed gently. Initially, 200μl of Solution III buffer (Premier, Cat. No.PM250) was added to the mixture and mixed gently again. The mixture was centrifuged at 13000 rpm for 5 minutes at room temperature. The supernatant was transferred to Mini-M™ Column (Premier, Cat. No.PM250) packed with collection tube and spun at 13000 rpm for 1 minute. The filtrate in the collection tube was discarded. 700 μl of Wash Solution (Premier, Cat. No.PM250) was added to Mini-M™ Column and spun at 13000 rpm for 1 minute. The filtrate was discarded and the washing step was repeated once again. After the filtrate was discarded, the column was centrifuged at 13000 rpm for 3 minutes to remove residual ethanol. The Mini-M™ Column was transferred to a new eppendorf tube and incubated at 60°C for 5 minutes to evaporate the ethanol. Finally, the DNA in the column was eluted in 50μl ddH_2O by spinning at 13000 rpm for 1 minute. The plasmid DNA was stored at -20°C.

3.3 Restriction enzyme digestion

The plasmid DNA was digested into DNA fragment by restriction enzyme. The

reaction mixture was added by 0.5~10μg plasmid DNA, 0.1~1μl restriction enzyme and reaction buffer to appropriate volume (10~50μl). The reaction for each kind of restriction enzyme was according to the recommend condition in operation manual. After the reaction was finished, the DNA was analyzed by agarose gel electrophoresis.

3.4 Agarose Gel Electrophoresis

Preparation of sample for running gel: 2 ul 6 X loading dye was added to 1 0 ul reaction mix. Electrophoresis : 0.8% agarose gel (0.6-1.2 % depends on the size of DNA). Every 0.8 g agarose was add 100 ml 1 X TBE (or TAE) buffer. The mixture was microwaved till the agarose melt complete and then cooled to 50^{o}C. While agarose solution was cooling, the gel box and comb was assembled and then the gel was poured into it. After the gel was solidified, the sample was loaded and run with 50-150V. After the DNA dye was on appropriate site, the gel was stained in EtBr 10 ug/ml for 5 minutes and then destained in running water for 10-15 minutes. The gel was take picture under UV box.

3.5 PCR reaction

The primers used in this study were designed from NCBI database for amplifying target genes. The total volume of reaction mixture was 50μl, contained DNA template(10~20 ng), 5μl of 10× reaction buffer, 1μl of each 5μM primer of pairs, 4μl of 2.5μM dNTP, 0.5μl of Tag (5U/μl) and fulfilled with ddH_2O. The PCR reaction was as following :

Step 1 : 95^{o}C for 10 minutes

Step 2 : 95^{o}C for 1 minutes

Step 3 : 50~60^{o}C for 1 minutes

Step 4 : 72^{o}C for 1 minutes

Step 5 : back to step 2 and repeat for 29 cycles

Step 6 : 72°C for 5 minutes

After the PCR reaction was finished, the PCR products were analyzed by agarose gel electrophoresis.

3.6 PCR clean up

For removing the extra material from PCR reaction mixtures, the PCR products were clean up with ExcelPure™ PCR Cleanup kit (Cat. No.CU250).

3.7 Gel extraction

For isolating DNA from agarose gel, the band was excised out of the agarose gel with PCR clean-up/Gel Extraction kit (Premier, N-DCE050).

3.8 Dephosphorylation

Restriction-digested vector (restriction enzyme and buffer removed, DNA in water or TE buffer) was incubated with Shrimp Alkaline Phosphatase (1 unit/μg DNA) (Promega, Cat. No.M8201) at 37°C for 15 minutes in 1X SAP reaction buffer in a final volume of 30–50μl. This is a sufficient amount of SAP to completely dephosphorylate the vector regardless of overhang type (5′, 3′ or blunt). Inactivate SAP by heating to 65°C for 15 minutes.

3.9 DNA ligation

For ligation of vector DNA and insert DNA, the total reaction volume was 10~30μl. The reaction solution contained 1 unit T4 DNA ligase, 1~3μl of 10× ligase buffer, vector DNA and insert DNA (the ratio of vector DNA to insert DNA was 1:3) and fulfilled with ddH_2O. The mixture was stored at 4°C for 14~18 hours.

3.10 Yeast transformation

S. cerevisiae cells were grown in 10 ml of YPD or selective medium overnight at 30℃ for 18 hours with 300 rpm (OD_{600} > 1.0). 1 ml of culture was transfered into eppendorf tubes (If more cells needed, use 2 ml of overnight culture, especially, when selective medium is used). Cells was spun for 30 seconds - 1 minutesute at high speed , and poured off supernatant. Cells was resuspended with 1 ml of 0.1 M of LioAC/TE, then spun for 30 secconds - 1 minutesute at low speed and poured off supernatant, repeated twice. Then cells was resuspended gently with 0.1 ml of 0.1 M of LioAC/TE. 0.1~1 μg plasmid (to be transformed) and 3 ~10 μl of boiled 10 mg/ml salmon sperm DNA as carrier (DNA was boiled for 1 minutesute and kept on ice right away) were added. The reaction mixture were mixed gently, then 0.4 ml of 50% PEG_{3350} /0.1 M of LioAC/TE was added. The reaction was then incubated at 30℃ on wheel for 30 minutes or sit in 30℃ incubator for 30-60 minutes. The reaction mixture was then subjected to heat shock at 42℃ for 20 minutes in water bath. Cells were spun very gently at low speed for 30 seconds, and the supernatant was discarded. The reaction was further spun for 5 seconds, then all supernatant was removed with 200 μl pipette tip. 150 μl of SD or TE was added to resuspend cells. Then the cells were plated on selective media (SD agar/Uridine,Histidine) and incubated at 30℃ incubator for 3-5 days.

3.11 Western blot analysis

3.11.1 Preparation of protein sample

Protein sample from cell extract : *S. cerevisiae* cells were grown in 5 ml of SD broth/Uridine,Histidine at 30℃ for 48 hours with 300 rpm (OD_{600} ~ 2.0). The cell solution was centrifuged at 2500 rpm at 4℃ for 5 minutes. After the supernatant was

discarded, the cells was resuspended in 10 ml Z buffer and then centriguged at 2500 rpm at 4℃for 5 minutes once again. The cells was resuspended in 200μl breaking buffer in the eppendorf, and added 12.5μl PMSF and glass beads (near the fluid surface). The mixture was vortex at 4℃for 15 seconds, repeated four times. After the cells was broken completely, 200μl breaking buffer was added and vortex for 10 seconds. The cell mixture was centrifuged at 4℃, 13000 rpm for 15 minutes. The supernatant was transferred to cooled eppendorf and added the same volume of 2× SDS-PAGE loading dye. The protein sample was stored at -20℃.

Protein samples from supernatant of 5 ml cultured media was obtained as following : *S. cerevisiae* cells were grown in 5 ml of SD broth/Uridine,Histidine at 30℃ for 48 hours with 300 rpm (OD_{600} ~ 2.0) . The cell solution was centrifuged at 2500 rpm at 4℃for 5 minutes. Then the supernatant was transferred to 15ml tube or concentrated by Amicon Ultra-15 (Cat. No.UFC9 010 08). Equal amount of the protein sample was added the same volume of 2× SDS-PAGE loading dye and stored at -20℃.

Protein samples from supernatant of 50 ml cultured media was obtained as following: *S. cerevisiae* cells were grown in 5 ml of SD broth/Uridine,Histidine at 30℃ for 48 hours with 300 rpm (OD_{600} ~ 2.0). Then 2ml of cell solution was transferred to 50 ml of SD broth/Uridine,Histidine and incubated at 30℃ for 48 hours (OD_{600} ~ 2.0). The cell solution was centrifuged at 2500 rpm at 4℃ for 5 minutes. The supernatant was concentrated by Amicon Ultra-15 (Cat. No.UFC9 010 08). The protein sample was added the same volume of 2× SDS-PAGE loading dye and stored at -20℃.

3.11.2 SDS-PAGE Electrophoresis

First is to set up gel plates before mixing the gel mixture (14% SDS-PAGE). The

thin spacer was used and a comb was chosen--number of wells varies. Then APS and TEMED was added to resolving gel and mixed. The resolving gel mixture was poured into the spacer about 3-3.5 ml per gel and kept in room temperature for polymerizing. After resolving gel polymerizes, APS and TEMED were added to stacking gel mixture and poured gently on top of the resolving gel. Then the comb was set up to solidify. After the gel was solidified, the comb was removed and the gel was placed in holder/electrode and then was transferred to running tank. The tank was filled with 1X Running Buffer (the inside and outside buffer chambers was kept separated). 10~50µl of protein samples were loaded in each well. The electrophoresis was conducted at 100V through the stacking gel, and then at 100V-200V until the dye front reached the bottom of the gel.

3.11.3 Western blot

After preparation of samples and SDS-PAGE electrophoresis, the gel was incubated in about 20 ml of transfer buffer for 15 minutes with shaking. In the mean time, one piece of Nitrocellulose membrane for each gel was incubated in methanol for 1 minute. After that, methanol was replaced with transfer buffer. The Nitrocellulose membrane was placed on top of the gel, and one filter paper placed on top of that and two filter papers below. of that. The proteins were transferred to Nitrocellulose membrane at 0.09 A for 37 minutes by TRANS-BLOT® SD CELL 221BR (BIO-RAD) for a gel. Non-specific binding sites were blocked by incubation of Nitrocellulose membrane in 20 ml of blocking buffer for 60 minutes at room temperature with continuous, gentle rotary agitation. Nitrocellulose membrane was then incubated with a dilution 1:1000 primary antibody-HRP conjugated (in blocking buffer) for 3 hours at room temperature, with continuous gentle agitation, or for 16 hours overnight at 4 °C. Nitrocellulose membrane was rinsed in 10 ml of TBST twice

and was washed in 20 ml of TBST for 1 x 20 minutes followed by 4 x 5 minutes, with continuous, rotary agitation on each occasion. Again, agitation should be vigorous but not too much so as to damage the membrane. The peroxidase detection method was applied. The membrane was incubated with HRP substrate and covered with saran wrap after removing the excessive solution from the surface. Then it was exposed to X-ray film in dark room for proper exposure time and treated with developer and fixer solution.

3.12 Fluorescence staining

3.12.1 Fix cells/Permerbilize

S. cerevisiae cells were grown in 5 ml of SD broth/Uridine,Histidine at 30℃ for 48 hours with 300 rpm (OD_{600} ~ 2.0). For fixing, 50µl of 36.5% formaldehyde was added to 316 µl of cell solution and the mixture was incubated at room temperature for 15 minutes. Then the cells were centrifuged at 13000 rpm for 5 minutes. The supernatant was removed with 200µl pipette, and the cells were washed with 500µl of PBS solution. Then the cells were centrifuged at 13000 rpm for 5 minutes and washed once again. The cells were resuspended in 100 µl of lyticase digestion solution at 30℃ for 30 minutes and then added 900 µl PBS. Then the cells were centrifuged at 4℃, 2000 rpm for 5 minutes. The supernatant was removed with 200µl pipette, and the cells were washed with 500µl of PBS solution. Then the cells were centrifuged a 4℃, 2000 rpm for 5 minutes and wash once again. The cells were resuspended in 200 µl PBS. 10 µl of cell solution was placed on the polylysine coated slide at room temperature for 5~10 minutes. Then the slide was washed with 4℃ PBS and dry at room temperature.

3.12.2. Cell staining

For membrane labeling, the slide coated cells was added 10 μl of 1 μM Alexa Fluor 594 WGA for 5 minutes. Then the slide was washed with PBS to remove excessive dye and dry the slide. After the slide was dry, 75% glycerol was added on the slide and the cover glass was covered on it. The slide was sealed with nail polish. Then the samples were viewed with a fluorescence microscope or confocalmicroscope with a standard fluorescent filter. For cell wall labeling, the procedure was the same as above, but the cells were stained with 10 μl of Calcofluor white without litycase treating.

IV. Result

In this study, the *CaENO1* gene of *C. albicans* was cloned into YEP363 plasmid (named YEP363-CaENO1-TP) and expressed in *S. cerevisiae* (10560-2B). In order to examine whether the protein was secreted, I modified the *CaENO1*gene with HAHIS and EGFP tags fused to 3' end.

4.1 The *CaENO1* gene was expressed in *S. cerevisiae*

4.1.1 The restriction enzyme map of the YEP363-CaENO1-TP plasmid

The YEP363-CaENO1-TP plasmid was made previously in the laboratory. It was diagramed into a circular map about 10 kb (shown in the Figure 2A) and composed of the *CaENO1* gene tagged with HA3HIS6 tag, pACT1 promoter, *Leu2* gene, *lacZ* gene, ampR (ampicillin resistance), and 2μ (origin of *S. cerevisiae*). For selecting the correct plasmid, the possible samples were subjected to *Nde* I restriction digestion. Accordingly, three DNA fragments of 1868 bp, 3022 bp and 5632 bp will be produced, as those in the band A, band B and band C in lane1-6 of Figure 2B. Lane 7 was undigested plasmid. The plasmid samples from lane 1-6 were in accord with the prediction and used for following experiments.

4.1.2 The expression of *CaENO*1 gene

The YEP363-CaENO1-TP plasmid was transformed into strain 10560-2B to express *CaENO1* gene, and this transformed strain was named 10560-2B-f1 in this study. The protein product of the *CaENO1* gene tagged HA3HIS6 was about 53 kDa, named eno-tp (tp, tag protein HA3HIS6) in this study. To ensure the strain 10560-2B-f1 can express target protein eno-tp, I incubated the cells at 30℃ for 48

hours and broke them to extract the proteins. Then the protein sample was checked with western blot. If the protein was expressed in the cell, a signal in the protein sample will be detected at about 53 kDa by anti-HA-HRP antibody with western blot. In the western blot result shown in Figure 3, a signal correspond to the size of the protein sample was detected (band A near 55kDa in lane 1-4) and the positive control (E-tag, E-protein tagged with HAHIS tag was about 40 kDa) and negative control (the strain transformed YEP363 was named 10560-2B-f0 in this study) were in lane 5 and lane 6, respectively. According to this result, the protein eno-tp was in accord with the prediction. The positive control (E-tag) was extracted from *E. coli,* and it was detected at about 55 kDa (band A in lane 5) lager than the prediction in this experiment. It might be result from incompletely denaturing.

4.1.3 The secretion of the eno-tp

In order to detect the secreted eno-tp, the centrifugation of cultured medium was collected and checked with western blot. If the protein was secreted outside the cell, a signal about 53 kDa would be detected in the medium by anti-His-HRP antibody with western analysis. In the western blot result shown in Figure 4, a signal was detected in the medium sample by anti-HA-HRP antibody (as shown in the band B' in lane 3-6) at about 55 kDa. According to this result, the eno-tp can be secreted outside the *S. cerevisiae* cell. The positive control (E-tag, E-protein tagged with HAHIS tag was about 40 kDa) and the negative control was shown in lane 2 and lane 1, respectively. The positive control (E-tag) was detected at about 40 kDa (the band A in lane 2) in accord with prediction, but also detected at about 55 kDa and 80 kDa (the band B and C in lane 2) larger than the prediction in this experiment. It might be result from the incompletely denaturing.

4.2 The analysis of *CaENO1* -EGFP fusion protein

Because the eno-tp protein cannot be observed for its localization inside the cell, *CaENO1* gene was further tagged with Enhanced Green Fluorescent Proteins (EGFP) for visualization in this study. The EGFP is a 29 kDaa recombinant protein with Ex/Em = 488/507 nm, and it is a powerful tool for the visualization of tagged proteins.

4.2.1 The construction of the expressed plasmid

For the construction, the EGFP gene was amplified by PCR from pEGFP-N2 plasmid. Two kinds of PCR products (*XbaI*-EGFP-*XbaI* and *XbaI*-EGFP-PstI) were obtained through two pairs of primers (EGFP-L-*Xba I Spe I*, EGFP-R-*Xba I* and EGFP-L-*Xba I Spe I*, EGFP-R- *Pst I*) by PCR. The PCR products of *XbaI*-EGFP-*XbaI* was predicted to be 739 bp (the band A in lane 1of Figure 5B) and *Xba1*-EGFP-*Pst1* was predicted to be 742 bp, (the band B in lane 2 of Figure 5B). And then the PCR products as insert were ligated into vector (YEP363-CaENO1-TP plasmid) in frame to form recombinant plasmids. The diagram of construct was as shown in Figure 5A, and one of the construct was tagged with HA3HIS6 and the other not, named YEP363-CaENO1-EGFP-TP and YEP363-CaENO1-EGFP respectively in this study. The recombinant plasmids were checked by restriction enzyme digestion. The plasmid YEP363-CaENO1-EGFP was about 11115 bp and would be digested into four DNA fragments 721 bp, 929 bp , 5694 bp and 3771 bp (the band a, b, c and d in lane1 of Figure 6B **)** by *XbaI* and *BsrGI.* The plasmid YEP363-CaENO1-EGFP-TP was about 11251 bp and would be digested into four DNA fragments 721 bp, 929 bp, 3771 bp and 5830 bp DNA fragments (the band a, b, c and d in lane1 of Figure 6C **)** by *XbaI* and *BsrGI*. Both of them were in accord with the prediction. The recombinant plasmids would be transformed into 10560-2B to express the fusion protein

eno-EGFP-tp and eno-EGFPp (named in this study).

The *S. cerevisiae* strain transformed with the YEP363-CaENO1-EGFP-TP plasmid and YEP363-CaENO1-EGFP plasmid was named 10560-2B-f2 and 10560-2B-f3 in this study.

4.2.2 The expression of CaENO1-EGFP fusion protein

To ensure the strain 10560-2B-f2 and 10560-2B-f3 can express target fusion protein, eno-EGFP-tp and eno-EGFPp, the cells were incubated at 30℃ for 48 hours and broken to extract the protein sample. Then the protein sample was analyzed with western blot. The molecular weight of eno-EGFP-tp and eno-EGFPp was predicted to be about 79 kDa and 75kDa. If the fusion protein was expressed in the cell, the protein sample would be detected by antibody with western blot. In this experiment, two kinds of antibody (anti-HA-HRP antibody and anti-EGFP antibody) was used.

The result with the anti-HA-HRP antibody was shown in Figure 7A. The positive control E-tag (the band A in lane 1) and the negative control was shown in lane 1 and lane 2, respectively. Tow signal in the sample of the eno-EGFP-tp was detected at about 79kDa and 72 kDa (band C and D in lane 4) and there was no signal for eno-EGFPp (lane 3). In addition, the result used anti-EGFP antibody was shown in Figure 7B, the eno-EGFPp was detected at about 75kDa (band E) in lane 2 and eno-EGFP-tp about 79kDa (band F) in lane 3. It follows that eno-EGFP-tp and eno-EGFPp were expressed in the cells.

4.2.3 The secretion of the CaENO1-EGFP fusion protein

In order to examine whether the EGFP fusion protein could be secreted outside the *S. cerevisiae* cell, the centrifugate of cultured medium was collected and checked with western blot. If the protein was secreted outside the cell, the medium sample would be

detected by antibody with western blot. In this experiment, two kinds of antibodies (anti-HA-HRP antibody and anti-EGFP antibody) were used.

In Figure 8A (anti-HA-HRP antibody used), the eno-tp was detected at about 55kDa (band B in lane 2) and the eno-EGFP-tp was detected at about 79kDa (band C in lane 3); the positive control E-tag was about 40 kDa (band A in lane 1) and the negative control was in lane 2. In Figure 8B (anti-EGFP-HRP antibody used), the eno-EGFPp was detected at about 75kDa (band D in lane 2) and the eno-EGFP-tp can not be detected. Negative control was in lane 1.

According to the result, both eno-EGFP-tp and eno-EGFPp can be detected in the culture medium.

4.2.4 The fluorescence observation of CaENO1-EGFP fusion protein

To further examine whether the EGFP fusion protein could be expressed, I incubated the transformed cells at 30℃ for 48 hours and observed under fluorescence microscope with 400X magnification.

As shown in Figure 9, 10560-2B-f2 (shown in B) and 10560-2B-f3(shown in C) were fluorescent under fluorescence microscope. 10560-2B-f0 (shown in A) was negative control in this data. It follows that the EGFP fusion protein was fluorescent.

4.3 The analysis of truncated *CaENO1*

In this experiment, in order to determine which region of *CaENO1* gene was critical to secretion, I designed different constructs of truncated-*CaENO1* fused to EGFP and compared their activities to the control construct. The diagram of this experiment was shown in Figure 10.

4.3.1 The construction of the expressed plasmid

The fragments of truncated *CaENO1* were amplified by PCR. I designed several pairs of primers for PCR amplification of different length of truncated *CaENO1*. The PCR products were then treated with restriction enzymes for ligation with vector. The diagrams of constructs are shown in Figure 11.

4.3.1.1 The negative control YEP363-EGFP plasmid

For this construct, the vector and insert were ligated together to form the negative control plasmid named YEP363-EGFP in this study (shown in Figure 12A). The PCR products containing *BamHI* and *SpeI* site on 3' and 5'end (band A in lane 1-2 of Figure 12B) as insert about 500 bp were obtained with primers ACT1-F and ACT1-R and then treated with *BamHI* and *SpeI*. The plasmid YEP363-CaENO1-EGFP, as vector, was also digested with restriction enzymes and then ligated with insert. The recombinant plasmid was checked by restriction enzyme digestion. The plasmid YEP363-EGFP was about 9783 bp and would be digested into two DNA fragments 3643 and 6140 bp (the band a and d in lane1-2 of Figure 13B **)** by *BsrGI*. The plasmid was in accord with the prediction.

4.3.1.2 The YEP363- CaENO1[1-150]-EGFP plasmid

For this construct, the YEP363-EGFP plasmid, as vector, was digested with restriction enzyme *SpeI*. The PCR product containing *SpeI* site on both 3' and 5'end as insert was obtained with primers (eno/1-f and eno1/150R) and then treated by *SpeI*. It is about 150 bp (the band A in lane 1 of Figure 14B). The vector and insert were ligated together to form the recombinant plasmid named YEP363-CaENO1/[1-150]-EGFP in this study. The recombinant plasmid was checked by restriction enzyme digestion. The plasmid was about 9945 bp and would be digested into 871 bp, 3643 bp and 5431 bp DNA fragments (b, f and g in lane 1 of Figure 15B) by *XbaI* and *BsrGI*. The plasmid was in accord with the prediction.

4.3.1.3 The YEP363- CaENO1[1-279]-EGFP plasmid

For this construct, the YEP363-EGFP plasmid, as vector, was digested with restriction enzyme *SpeI*. The PCR product containing *SpeI* site on both 3' and 5'end as insert was obtained with primers (eno/1-f and eno1/279R) by PCR and then treated by *SpeI*. It is about 279 bp (the band B in lane 2 of Figure 14B). The vector and insert were ligated together to form the recombinant plasmid named YEP363-CaENO1[1-279]-EGFP in this study. The recombinant plasmid was checked by restriction enzyme digestion. The plasmid was about 10074 bp and would be digested into 1000 bp, 3643 bp and 5431 bp DNA fragments (b, f and g in lane 2 of Figure 15B) by *XbaI* and *BsrGI*. The plasmid was in accord with the prediction.

4.3.1.4 The YEP363- CaENO1[1-387]-EGFP plasmid

For this construct, the YEP363-EGFP plasmid, as vector, was digested with restriction enzyme *SpeI*. The PCR product containing *SpeI* site on both 3' and 5'end as insert was obtained with primers (eno/1-f and eno1/387R) by PCR and then treated by *SpeI*. It is about 279 bp (the band C in lane 3 of Figure 14B). The vector and insert were ligated together to form the recombinant plasmid named YEP363-CaENO1[1-387]-EGFP in this study. The recombinant plasmid was checked by restriction enzyme digestion. The plasmid was about 10182 bp and would be digested into 1108 bp, 3643 bp and 5431 bp DNA fragments (d, f and g in lane 3 of Figure 15B) by *XbaI* and *BsrGI*. The plasmid was in accord with the prediction.

4.3.1.5 The YEP363- CaENO1[1-510]-EGFP plasmid

For this construct, the YEP363-EGFP plasmid, as vector, was digested with restriction enzyme *SpeI*. The PCR product containing *SpeI* site on both 3' and 5'end as insert was obtained with primers (eno/1-f and eno1/510R) by PCR and then treated by *SpeI*. It is about 279 bp (the band D in lane 4 of Figure 14B). The vector and insert were ligated together to form the recombinant plasmid named YEP363-CaENO1[1-510]-EGFP in this study. The recombinant plasmid was checked by

restriction enzyme digestion. The plasmid was about 10305 bp and would be digested into 397 bp, 843 bp, 3643 bp and 5431 bp DNA fragments (a, b, f and g in lane 4 of Figure 15B) by *XbaI* and *BsrGI*. The plasmid was in accord with the prediction.

4.3.1.6 The YEP363- CaENO1[280-1320]-EGFP plasmid

For this construct, the YEP363-EGFP plasmid, as vector, was digested with restriction enzyme *SpeI*. The PCR product containing *SpeI* site on both 3' and 5'end as insert was obtained with primers (eno1/280F and eno/1320-r) by PCR and then treated by *SpeI*. It is about 1041 bp (the band E in lane 6 of Figure 14C). The vector and insert were ligated together to form the recombinant plasmid named YEP363-CaENO1[280-1320]-EGFP in this study. The recombinant plasmid was checked by restriction enzyme digestion. The plasmid was about 10837 bp and would be digested into 1644 bp, 3643 bp and 5550 bp DNA fragments (h, i and j in lane 5 of Figure 15B) by *BsrGI*. The plasmid was in accord with the prediction.

4.3.1.7 The YEP363- CaENO1[388-1320]-EGFP plasmid

For this construct, the YEP363-EGFP plasmid, as vector, was digested with restriction enzyme *SpeI*. The PCR product containing *SpeI* site on both 3' and 5'end as insert was obtained with primers (eno1/388F and eno/1320-r) by PCR and then treated by *SpeI*. It is about 933 bp (the band G in lane 7 of Figure 14C). The vector and insert were ligated together to form the recombinant plasmid named YEP363-CaENO1[388-1320]-EGFP in this study. The recombinant plasmid was checked by restriction enzyme digestion. The plasmid was about 10729 bp and would be digested into 1644 bp, 3643 bp and 5442 bp DNA fragments (h, i and j in lane 6 of Figure 15B) by *BsrGI*. The plasmid was in accord with the prediction.

4.3.1.8 The YEP363- CaENO1[1-450]-EGFP plasmid

For this construct, the YEP363-EGFP plasmid, as vector, was digested with restriction enzyme *SpeI*. The PCR product containing *SpeI* site on both 3' and 5'end

as insert was obtained with primers (eno/1-f and eno/450-r) by PCR and then treated by *SpeI*. It is about 450 bp (the band H in lane 8 of Figure 14D). The vector and insert were ligated together to form the recombinant plasmid named YEP363-CaENO1[1-450]-EGFP in this study. The recombinant plasmid was checked by restriction enzyme digestion. The plasmid was about 10245 bp and would be digested into 447 bp, 774 bp, 3643 bp and 5431 bp DNA fragments (l, m, r and s in lane 7 of Figure 15B) by *XbaI* and *BsrGI*. The plasmid was in accord with the prediction.

4.3.1.9 The YEP363- CaENO1[451-900]-EGFP plasmid

For this construct, the YEP363-EGFP plasmid, as vector, was digested with restriction enzyme *SpeI*. The PCR product containing *SpeI* site on both 3' and 5'end as insert was obtained with primers (eno/451-f and eno/900-r) by PCR and then treated by *SpeI*. It is about 450 bp (the band H in lane 9 of Figure 14D). The vector and insert were ligated together to form the recombinant plasmid named YEP363-CaENO1[451-900]-EGFP in this study. The plasmid was about 10248 bp and would be digested into 1174 bp, 3643 bp and 5431 bp DNA fragments (n, r and s in lane 8 of Figure 15B) by *XbaI* and *BsrGI*. The plasmid was in accord with the prediction.

4.3.1.10 The YEP363- CaENO1[901-1320]-EGFP plasmid

For this construct, the YEP363-EGFP plasmid, as vector, was digested with restriction enzyme *SpeI*. The PCR product containing *SpeI* site on both 3' and 5'end as insert was obtained with primers (eno/901-f and eno/1320-r) by PCR and then treated by *SpeI*. It is about 420 bp (the band I in lane 10 of Figure 14D). The vector and insert were ligated together to form the recombinant plasmid named YEP363-CaENO1[901-1320]-EGFP in this study. The plasmid was about 10218 bp and would be digested into 1144 bp, 3643 bp and 5431 bp DNA fragments (n, r and s in lane 9 of Figure 15B) by *XbaI* and *BsrGI*. The plasmid was in accord with the prediction.

4.3.1.11 The YEP363- CaENO1[1-900]-EGFP plasmid

For this construct, the YEP363-EGFP plasmid, as vector, was digested with restriction enzyme *SpeI*. The PCR product containing *SpeI* site on both 3' and 5'end as insert was obtained with primers (eno/1-f and eno/900-r) by PCR and then treated by *SpeI*. It is about 900 bp (the band J in lane 11 of Figure 14D). The vector and insert were ligated together to form the recombinant plasmid named YEP363-CaENO1[901-1320]-EGFP in this study. The plasmid was about 10695 bp and would be digested into 397 bp, 1224 bp, 3643 bp and 5431 bp DNA fragments (k, n, r and s in lane 10 of Figure 15B) by *XbaI* and *BsrGI*. The plasmid was in accord with the prediction.

4.3.1.12 The YEP363- CaENO1[451-1320]-EGFP plasmid

For this construct, the YEP363-EGFP plasmid, as vector, was digested with restriction enzyme *SpeI*. The PCR product containing *SpeI* site on both 3' and 5'end as insert was obtained with primers (eno/451-f and eno/1320-r) by PCR and then treated by *SpeI*. It is about 869 bp (the band K in lane 12 of Figure 14D). The vector and insert were ligated together to form the recombinant plasmid named YEP363-CaENO1[451-1320]-EGFP in this study. The plasmid was about 10668 bp and would be digested into 1594 bp, 3643 bp and 5431 bp DNA fragments (q, r, sin lane 11 of Figure 15B) by *XbaI* and *BsrGI*. The plasmid was in accord with the prediction.

All of above plasmids were transformed into 10560-2B strain to expressed truncated CaENO1-EGFP fusion protein. The transformed strain was named according to its target plasmid in this study; for example, the 10560-2B-CaENO1[1-150] strain was transformed by the YEP363- CaENO1[1-150]-EGFP plasmid.

4.3.2 The analysis of the truncated CaENO1-EGFP fusion protein

The expressed protein was named according to the remaining region of *CaENO1* in this study; for example, the YEP363-CaENO1[1-150]-EGFP plasmid would

express eno[1-150]-EGFPp. To ensure the target protein was successfully expressed in *S. cerevisiae*, I incubated the transformed cells at 30℃ for 48 hours and broke them to extract the protein sample for western blot analysis.

4.3.2.1 The expression of recombinant protein

The protein of eno[1-150]-EGFPp was predicted to be about 31 kDa and there was a signal at the corresponding location (the band A in lane 1 of Figure 16). In addition, there was a lower weight signal near 26 kDa in lane 1. From this, it seems that the protein might be degraded. The sample of eno[1-279]-EGFPp was predicted to be about 36 kDa. The result of western blot showed that it was about 43kDa (the band B in lane 2 of Figure 16), which is larger than prediction. It seems that the protein might be modified. The protein of eno[1-387]-EGFPp was predicted to be about 40 kDa (the band C in lane 3 of Figure 16) and there was a signal at the corresponding location. In addition, there was a lower weight signal near 34 kDa in lane 3. From this, it seems that the protein was expressed but also degraded. The protein of eno[1-450]-EGFPp was predicted to be about 43 kDa (the band D in lane 4 of Figure 16) and there was a signal at the correct location. In addition, there were lower signals near 34 kDa and 26 kDa in lane 4. From this, it seems that the protein was expressed but also degraded. The sample of eno[1-510]-EGFPp was predicted to be about 45 kDa (the band E in lane 5 of Figure 16) and there was a signal at the correct location. In addition, there were lower signals near 34 kDa and 26 kDa in lane 5. From this, it seems that the protein was expressed but also degraded. The sample of eno[1-900]-EGFPp was predicted to be about 59 kDa (the band F in lane 6 of Figure 16) and there was a signal at the correct location. The protein was expressed in *S. cerevisiae*. The sample of EGFPp was predicted to be about 27 kDa (the band G in lane 7 of Figure 16) and there is a signal at the correct location. The protein was expressed in *S. cerevisiae*. The sample of eno[280-1320]-EGFPp was predicted to be about 65 kDa (the band H

in lane 8 of Figure 16) and there was a signal at the correct location. In addition, there was a lower signal near 55 kDa in lane 8. From this, it seems that the protein was expressed but also degraded. The sample of eno[388-1320]-EGFPp was predicted to be about 62 kDa (the band I in lane9 of Figure 16) and there was a signal at the correct location. In addition, there were lower signal near 55 kDa in lane 9. From this, it seems that the protein was expressed but also degraded. The sample of eno[451-1320]-EGFPp was predicted to be about 58 kDa (the band J in lane10 of Figure 16) and there was a signal at the correct location. In addition, there was a lower signal near 55 kDa in lane 10. From this, it seems that the protein was expressed but also degraded. The sample of eno[451-900]-EGFPp was predicted to be about 43 kDa. The result of western blot showed a signal at about 34 kDa (the band K in lane11 of Figure 16), which was lower than prediction. It seems that the protein was expressed but also degraded in the *S. cerevisiae*. The sample of eno[901-1320]-EGFPp was predicted to be about 43 kDa. The result of western blot showed a signal at about 34 kDa (the band L in lane12 of Figure 16), which was lower than prediction. It seems that the protein was expressed but also degraded in the *S. cerevisiae*.

4.3.2.2 Fluorescence observation of the truncated CaENO1-EGFP fusion protein

To examine whether the truncated CaENO1-EGFP fusion protein could express fluorescence, the live cells were incubated at 30℃ for 48 hours and then observed under fluorescence microscope with 400X magnification.

As shown in Figure 17, all of the constructs were fluorescent under fluorescence. 10560-2B-f0 was negative control in this experiment. It follows that truncated CaENO1-EGFP fusion protein was fluorescent.

4.3.3 The secretion analysis of the truncated CaENO1-EGFP fusion protein

4.3.3.1 The detection of culture medium sample with western blot

For detecting the secretion, the cells were incubated at 30℃ for 48 hours and centrifuged, and then the centrifugate of cultured medium was collected and checked with western blot. If the protein was secreted outside the cell, a signal would be detected in the medium by anti-EGFP-HRP antibody with western blot.

According to the result of western blot (shown in the Figure 18), no signal was detected from the supernatant sample of cultured medium. The positive control of western blot was eno-EGFPp extracted from *S. cerevisiae* cell.

4.3.3.2 The detection of the concentrated sample with western bolt

In order to obtain more secreted proteins, the samples of truncated CaENO1-EGFP fusion protein in this study were concentrated. The cells were incubated at 30℃ for 48 hours and centrifuged, and then the supernatant of cultured medium was concentrated by centrifugal filter devices (MILLIPORE) and checked with western blot. If the protein was secreted outside the cell, a signal would be detected by anti-EGFP-HRP antibody with western blot.

The sample of a 5 ml was concentrated to 250μl for the western blot analysis. According to the result of western blot (Figure 19), only eno-EGFPp can be detected at about 75 kDa (shown in the band A in lane 1), and no truncated CaENO1-EGFP fusion protein was detected. The positive control of western blot was eno-EGFPp extracted from *S. cerevisiae* cell.

Next, a 50 ml sample was concentrated for the western analysis to 250μl. No signal was detected from the concentrated supernatant sample of cultured medium even though the full-length eno-EGFPp (Figure 20). The positive control of western blot was eno-EGFPp extracted from *S. cerevisiae* cell.

It follows that the full-length eno-EGFPp was detected from the concentrate of 5 ml supernatant but not from 50 ml, and all the truncated CaENO1-EGFP fusion protein cannot be detected in this experiment.

4.4 The localization of CaENO1p in cell

To examine the localization of CaENO1p, I tagged the *CaENO1* with EGFP, a fluorescent protein for visualization in the *S. cerevisiae* cell. For observation, the transformed cell was stained with Alexa Fluor 594 WGA for cell membrane labeling and Calcofluor white for cell wall labeling. The treated cell was fixed on slide to observe under confocal microscope with 1000X magnification. Confocal microscope is capable of observing selected thin layers. In a confocal microscope only the features located within a thin plane of focus are seen.

4.4.1 The localization of eno-EGFPp

The observation of eno-EGFPp under confocal microscope was shown in Figure 21. In Figure 21A, the cell membrane labeled Alexa Fluor 594 WGA was showed in red (image A) and the eno-EGFPp showed in green (image B). The red and green images were merged to determine whether the eno-EGFPp localized in cell membrane (image C) , and DIC (Differential Interference Contrast) was shown in image D. As image C showed, the red and green signals didn't merged in the same localization.

In addition, In Figure 21B cell wall labeled with Calcofluor white was blue (image A) and the eno-EGFPp was green (image B). The red and green images were merged to confirm whether the eno-EGFPp was localized in cell wall (image C) and DIC was shown in image D. As image C showed, the red and green signal did not merged at the same localization,

From this data, it seems that the eno-EGFPp was localized in the cytoplasm but not in the cell membrane or cell wall.

4.4.2 The localization of EGFPp

In this experiment, the EGFPp as control was observed in the same way.

The observation of EGFPp under confocal microscope was shown in Figure 22. In the Figure 22A, cell membrane was showed in red (image A) and the EGFPp in green (image B). And the red and green images were merged to determine whether the EGFPp was localized in cell membrane (image C). As image C showed, the red and green signals didn't merged at the same localization, and DIC was shown in image D.

In addition, In Figure 21B, cell wall labeled with Calcofluor white was blue (image A) and the EGFPp was green (image B). The red and green images were merged to determine whether the eno-EGFPp was localized at cell wall (image C). As image C showed, the red and green signal did not merged at the same localization, and DIC was shown in image D.

From this data, it seems that the EGFPp was localized in the cytoplasm but not in the cell membrane or cell wall. To compare both of EGFPp and eno- EGFPp, the same result was observed from confocal microscope.

V. Discussion

Enolase is a ubiquitous enzyme involved in glycolysis and gluconeogenesis. It catalyzes the reversible conversion of 2-phosphoglycerate to phosphoenolpyruvate. However, besides its metabolic role, an involvement of enolase in various intra- or extracellular functions has also been reported for different organisms [18]. Proteins lacking the canonical N-terminal secretion signal that gives access to the surface of live cells must be able to cross the lipophilic plasma membrane. Direct genetic proof of the capacity of these proteins to be driven through the plasma membrane while lacking an N-terminal signal peptide has been provided by fusion with reporter proteins. Hybrid truncates carrying the N-terminal moiety of GAPDH, enolase or fructose 1,6-bisphosphate aldolase can drive reporter proteins to the cell surface [2,4].

In this study, I developed a system for screening the signal region of *CaENO1* gene in *S. cerevisiae.* The gene was truncated and tgged with EGFP on C-terminal for expressing different fragments of *CaENO1* gene. Through this modification, the localization of the CaENO1p can be detected in the cell.

The vector plasmid used in this research was YEP363,which contained selection marker *Leu2* gene, *lacZ* gene, ampR (ampicillin resistance), and 2μ (origin of *S. cerevisiae*). For expressing, the *CaENO1* gene tagged with HA3HIS6 tag and pACT1 promoter was cloned into YEP363 plasmid (made by Yao-Chen Chuang, Lab of Y.-L. Yang) , and the construct was named YEP363-CaENO1-TP.

5.1 The *CaENO1* gene was expressed in *Saccharomyces cerevisiae*

5.1.1 The expression of eno-tp

In order to construct a system for expressing heterologous protein in *S. cerevisiae* 10560-2B *(Leu⊿, Uri⊿, His⊿)* used in this study, the pACT1 *S. cerevisiae* promoter was cloned into the plasmid YEP363-CaENO1-TP. Due to the plasmid contained

selection marker *Leu2* gene, the transformed *S. cerevisiae* cell can be selected through nutrition selection. To ensure the protein eno-tp can be expressed in *S. cerevisiae*, I have analyzed the protein sample extract from cells with western blot analysis. Because protein eno-tp contained HAHIS tag, the protein sample can be detected by anti-HA antibody in western bolt. If the protein was expressed successfully, there would be a signal at about 53 kDa (band A of Figure 3) with western blot. According to the result of western analysis, the protein eno-tp can be expressed in *S. cerevisiae.*

5.1.2 The secretion of eno-tp

To examine whether the protein eon-tp also can be secreted in *S. cerevisiae,* the protein sample from culture medium were analyzed with western blot analysis. If the protein was secreted into cultured medium, the sample from the supernatant of cultured media would show a signal at about 53 kDa (Band D of Figure 4) with western blot. According to the result of western analysis, the protein eno-tp can be secreted outside the cell of *S. cerevisiae.* The result of comparing the aminol acid sequences of ScENO2p and CaENO1p shows that the identity between ScENO2p and CaENO1p was 76% (Figure 24**)**. If eno-tp was secreted outside cell through a mechanism share with *S. cerevisiae,* the secretion signal might be the conserve region of enolase.

5.2 The analysis of *CaENO1*-EGFP fusion protein

5.2.1 The expression of CaENO1-EGFP fusion protein

According to the constructs with EGFP–HA3HIS6 tag or EGFP only, the proteins would be detected by anti-HA antibody or anti-EGFP antibody.

The western blot shows that the sample of eno-EGFP-tp was detected at about 79 kDa (Band C of Figure 7A and Band F of in Figure 7B) by anti-HA and anti-EGFP antibodies but also detected the lower weight. The same result was happened on

eno-EGFP. It seems that protein might be degraded in the cell or in the period of treating samples with detergent.

To further determine if the proteins were expressed in the cells, the transformed cells were observed under phase contrast microscope for fluorescence. All the constructs were fluorescent under microscope. This result shows the protein was expressed in frame and the folding of EGFP was correct and the resulting fluorescence. According to the result, both eno-EGFP-tp and eno-EGFPp were successfully expressed in *S. cerevisiae*.

5.2.2 The secretion of CaENO1-EGFP fusion protein

To confirm whether the CaENO1-EGFP fusion protein can be secreted outside the cells, the protein samples from cultured media were analyzed with western blot analysis. According to the result of western blot, a signal at about 79kDa was detected in the sample of eno-EGFP-tp (Band C of Figure 8A) by anti-HA antibody. But there was no signal detected by anti-EGFP antibody. In addition, a signal at about 75kDa was detected in the sample of eno-EGFP (Band E of Figure 8B) from cultured media by anti-EGFP antibody. It follows that both eno-EGFP-tp and eno-EGFPp were secreted in *S. cerevisiae*. However, the protein eno-EGFP-tp from cultured media can be detected by anti-HA antibody but not by anti-EGFP antibody. Therefore, it is like that HAHIS tag would affect the anti-EGFP antibody and decrease its activity.

5.3 The analysis of truncated CaENO1-EGFP protein

5.3.1 The truncation and expression of *CaENO1*-EGFP

A recent study has demonstrated that an N-terminal fragment of enolase consisting of 169 aminol acids can target cytoplasmic invertase to the external medium [21]. It showed the evidence on *ScENO2* gene for genetic screening secretion critical region of enolase. Following this result, CaENO1p was truncated into 11 fragments tagged

with EGFP in this study (Figure 11) and then analyzed for the secretion of truncated CaENO1-EGFP protein with western blot.

For the truncation, the *CaENO1* was truncated by PCR into parts of DNA fragment 1-150bp, 1-279bp, 1-387bp, 1-450bp, 1-510bp, 1-900bp, 151-1320bp, 280-1320bp, 388-1320bp. 451-1320bp, 511-1320bp, 901-1320bp and 451-900bp. The treated PCR product was ligated into treated vector to form truncated CaENO1-EGFP fusion constructs.

To determine whether the protein can be expressed in *S. cerevisiae*, I analyzed the protein samples extracted from transformed cells by anti-EGFP antibody with western blot.

The western blot of truncated CaENO1-EGFP fusion protein were shown in Figure 16. It revealed that all but three of the proteins were detected in accord to prediction but most of them also degraded into smaller size. The protein eno[1-279]-EGFPp (Band B) was detected larger than predict and it might be result of modification from other molecules. The eno[451-900]-EGFPp and eno[901-1320]-EGFPp was detected the lower weight by western analysis, and this might be result from the degradation in the cell.

To further determine if the protein were expressed in the cells, the transformed cells were observed under phase contrast microscope for fluorescence (as shown in Figure 17). According to the result, all the constructs were fluorescent under microscope.

5.3.2 The secretion analysis of truncated CaENO1-EGFP protein

This experiment was for determine which region of *CaENO1* was critical to secretion. If the truncated CaENO1-EGFP fusion protein was secreted, it would be detected by anti-EGFP antibody with western analysis.

The western blot of supernatant from cultured medium showed that there was no signal detected (as shown in Figure 18). However in this experiment, the positive control eno-EGFP cannot be detected either. It seems that the protein secretion was not stable or there were artificial mistakes.

In order to increase the density of the protein sample from cultured media, the 5 ml cultured media was concentrated to about 250μl by centrifugal filter device (Amicon Ultra-4, which was cut off 10K NMWL). The result in Figure 19 showed that only eno-EGFPp was detected at about 75kDa (Band A).

To further concentrate more protein sample, I collected 50 ml of cultured media and concentrated to about 250μl by centrifugal filter device (Amicon Ultra-15, which was cut off 10K NMWL). The result in Figure 20 showed that there was no signal detected.

Through increasing the volume of cultured medium for concentration, the protein eno-EGFPp seems to be lost. There were two possibilities, one is the secretion of protein might be too weak to detect, and the other is recovery was lost through operational mistake such as the residual protein on the membrane. Comparing the results of truncated CaENO1-EGFP protein with eno-EGFPp, I think that full length CaENO1 was required for secretion, but the truncated fusion proteins were unknown.

5.4 The localization of CaENO1p in cell

Several reports have showed that enolase is located in the cytoplasm, cell membrane and cell wall. It is considered that the enolase was covalently bound on the cell surface and also secreted into medium in some condition. Therefore if the enolase bound on the cell surface seems a significant sight for secretion.

To examine the localization of CaENO1p, the *CaENO1* was tagged with EGFP for visualization in *S. cerevisiae* cells. For observation, The *S. cerevisiae* cells were

stained with Alexa Fluor 594 WGA (which binds to sialic acid and N-acetylglucosaminyl residues) for cell membrane labeling and Calcofluor white (which binds to beta-1,3 and beta-1,4 polysaccharides) for cell wall labeling. The treated cell was fixed on the slide to observe under confocal microscope with 1000X magnification.

Using confocal laser scanning microscopy, the fusion protein was clearly localized at the cell periphery around nucleus (Image B of Figure 21)**.** In the image C of Figure 21 A and Figure 21B, the localization of eno-EGFPp (green) was not co-localized with cell wall stained with Calcofluor white(blue) or cell membrane stained with Alexa Fluor 594 WGA (red). The same result was observed with EGFP (Image C of Figure 22 A and Figure 22B). According to the result, the *S. cerevisiae* cell seems not recognize the eno-EGFPp as a cell wall protein in this study.

5.5 Conclusion

I would like to conclude that the *CaENO1* gene can be expressed and secreted in *S. cerevisiae.* Furthermore, the CaENO1p tagged with EGFP in frame also can be expressed and displayed fluorescence in the cells, and secreted into the medium. For the truncated proteins, it revealed that all but three of the proteins were detected but most of them also degraded into several smaller size. According to the degradation sizes of the truncated proteins, it seems that the cells would digest the proteins on a specific site in truncated CaENO1-EGFP protein. Although they can be expressed in *S. cerevisiae*, none of the truncated *CaENO1* fusion protein was detected in medium samples even after concentration. According to the result, only full length *CaENO1* can lead the tagged proteins outside the cells. According to the microscope observation, *S. cerevisiae* cell seems not to recognize the eno-EGFPp as a cell surface binding protein in this study. The enolase encoded by *CaENO1* gene was recognized

as secreted protein in *S.cerevisiae* and exported outside the cell. It suggested the heterologous enolase was undergo the secretion mechanism in *S.cerevisiae*. The previous report (Elena, L.V., 2006) showed that enolase encoded by ScEno2 can reach the cell surface and described the protein regions involved in its cell surface targeting in *S.cerevisiae.* Since the protein between *C. albicans* with *S. cerevisiae* is similar (ScEno2p and CaEno1p are 76% identities and ScEno1p and CaEno1p are 77% identities), the conserved region of both enolases maybe are responsible for the targeting. However the result of eno1[1-510]-EGFPp (N-terminal 170 amino acids of CaEno1p) compared to the N-terminal 169 amino acids residue of ScEno2p did not confirm the the idea that this similar region was also critical to secretion in the *CaENO1* gene. Further experiments are needed to determine the mechanism of enolase export in *Candida albicans*.

VI. Future Work

In this study, only full-length *CaENO1* can be detected the secretion in *S.cerevisiae*. Therefore, there are three issues for the research in the future.

First is the detection system seems not sensitive for the recombinant proteins in this study. Since the CaENO1p was heterologus to *S. cerevisiae,* when *CaENO1* was truncated, it might be not recognized by *S. cerevisiae.* Therefore, it prefers to identify the secretion signal of *CaENO1* in *C. albicans*. However, the EGFP reporter system contained several CUG codons, and *C. albican*s translates the standard leucine-CUG codon as serine partially. Therefore the EGFP reporter system cannot be used to the full efficiency in *C. albican*s, it is need to be changed or modified.

Second is the secretion was not detected within truncated constructs, it might be result from the protein abnormal folding, and therefore the cell would not recognize them as a normal protein. Because the truncated recombinant protein was not stable in the cell, it might indirectly affect the protein secretion. For this problem, one could replace the approach of truncation with random mutagenesis. The random mutagenesis can be used for construction of large and diverse clones to find essential residues of the protein.

Third is that the growth condition might be a factor for the secretion when study in *C. albicans*. A research by Roger et al. [28] showed that enolase was indentified from cell wall-enriched fraction only in hyphae form *C. albicans*. It seems that the growth condition (YPD+10 % serum at 37℃ , hyphae indution) might induce the morphic transition and influence the enolase export directly or indirectly, and therefore it is necessary to be considered as a factor for expressing secreted proteins.

VII. Reference

[1] Martinez, J. P., Gil, M. L., Lopez-Ribot, J. L. and Chaffin, W. L. (1998) Serologic response to cell wall mannoproteins and proteins of *Candida albicans*. *Clin. Microbio.l Rev.* 11, 121 – 141.

[2] Delgado, M. L., Gil, M. L. and Gozalbo, D. (2003) Candida albicans TDH3 gene promotes secretion of internal invertase when expressed in Saccharomyces cerevisiae as a glyceraldehyde-3-phosphate dehydrogenase-invertase fusion protein. *S. cerevisiae* 20, 713 – 722.

[3] Edwards, S. R., Braley, R., Chaffin, W. L. (1999) Enolase is present in the cell wall of *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* 177, 211–216.

[4] Pardo, M., Monteoliva, L., Pla, J., Sánchez, M., Gil, M. and Nombela, C. (1999) Two-Dimensional analysis of proteins secreted by *Saccharomyces cerevisiae* regenerating protoplasts: a novel approach to study the cell wall. *S. cerevisiae* 15, 459 – 472.

[5] Pardo, M., Ward, M., Bains, S., Molina, M., Blackstock, W., Gil, C. and Nombela, C. (2000) A proteomic approach for the study of *Saccharomyces cerevisiae* cell wall biogenesis. *Electrophoresis* 21, 3396 – 3410.

[6] Motshwene, P., Brandt,W. and Lindsey, G. (2003) Significant quantities of the glycolytic enzyme phosphoglycerate mutase are present in the cell wall of *S. cerevisiae Saccharomyces cerevisiae. Biochem. J.* 369, 357 – 362.

[7] Nombela, C., Gil, C. and Chaffin, W. L. (2006) Non-conventional protein secretion in *S. cerevisiae*. *TRENDS Microbiol*. 14, 1, 15-21.

[8] Pitarch, A., Sánchez, M., Nombela, C. and Gil C. (2002) Sequential fractionation and two-dimensional gel analysis unravels the complexity of the dimorphic

fungus *Candida albicans* cell wall proteome. *Mol. Cell Proteomics* 1, 967–982.

[9] Gozalbo, D., Gil-Navarro, I., Azorín, I., Renau-Piqueras, Martínez, J. P. and Gil, M. L. (1998) The cell wall-associated glyceraldehyde-3-phosphate dehydrogenase of *Candida albicans* is also a fibronectin and laminutesin binding protein. *Infect. Immun*. 66, 2052–2059.

[10] Angiolella, L., Micocci, M. M., D'Alessio, S., Girolamo, A., Maras, B. and Cassone, A. (2002) Identification of major glucan-associated cell wall proteins of Candida albicans and their role in fluconazole resistance. *Antimicrob. Agents Chemother.* 46, 1688–1694.

[11] Nickel, W. (2003) The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. *Eur.J. Biochem*. 270, 2109–2119.

[12] Bendtsen, J. D. Jensen, L. J., Blom, N., von Heijne, G. and Brunaket, S. (2004) Feature-based prediction of non-classical and leaderless protein secretion. *Protein Eng. Des. Sel.* 17, 349–356.

[13] De Groot, P.W. J., Ramb, A. F. and Klis, F. M. (2005) Features and functions of covalently linked proteins in fungal cell walls. *Fungal Genet. Biol.* 42, 657–675.

[14] Cleves, A.E., Cooper, D. N. W., Barondes, S. H. and Kelly, R. B. (1996) A new pathway for protein export in *Saccharomyces cerevisiae*. *J. Cell Biol.* 133, 1017–1026.

[15] Prudovsky, I., Mandinova, A., Soldi, R., Bagala C., Graziani, I., Landriscina, M., Tarantini, F., Duarte, M., Bellum, S., Doherty, H. and Maciag, T. (2003) The non-classical export routes: FGF1 and IL-1α point the way. *J. Cell Sci.* 116, 871–4881.

[16] Brewer, J. M. (1981) *S. cerevisiae* enolase: mechanism of activation by metal ions. *CRC Crit. Rev. Biochem.* 11, 209-254.

[17] Brewer, J. M. and Ellis P. D. (1983) 31P-NMR studies of the effect of various

metals on substrate binding to *S. cerevisiae* enolase. *J. Inorg. Biochem.* 18, 71-82.

[18] Pancholi V. (2001) Multifunctional α-enolase: its role in diseases. *Cell. Mol. Life Sci.* 58, 902-920.

[19] Zhu, H., Bilgin, M., Bangham, R., Casamayor, A.,1 Bertone, P., Lan, N., Jansen, R.,2 Bidlingmaier, S., Houfek, T., Mitchell, T., Miller, P., Dean, R. A., Gerstein, M. and Snyder M. (2001) Global Analysis of Protein Activities Using Proteome Chips. *Science* 293, 2101–2105.

[20] Pancholi, V. and Fischetti, V. A., (1998) α-Enolase, a Novel Strong Plasminutes(ogen) Binding Protein on the Surface of Pathogenic Streptococci. *J. Biol. Chem*. 273, 14503–14515.

[21] Bernal, D., de la Rubia, J. E., Carrasco-Abad, A. M., Toledo, R., Mas-Coma, S. and Marcilla, A. (2001) Identification of enolase as a plasminutesogen-binding protein in excretory-secretory products of *Fasciola hepatica*. *FEBS Lett.* 2004, 563, 203–206.

[22] Fox, D. and Smulian, A. G. (2004) Plasminutesogen-binding activity of enolase in the opportunistic pathogen *Pneumocystis carinii*. *Med. Mycol.* 39, 495–507.

[23] Ge, J., Catt, D. M. and Gregory, R. L. (2004) *Streptococcus mutans* surface α-enolase binds salivary mucin MG2 and human plasminutesogen. *Infect. Immun.* 72, 6748–6752.

[24] Carneiro, C. R. W., Postol, E., Nomizo, R., Reis, L. F. L. and Brentani, R. R. (2004) Identification of enolase as a laminutesin-binding protein on the surface of *Staphylococcus aureus*. *Microbes Infect.* 6, 604–608.

[25] Schaumburg, J., Diekmann, O., Hagendorff, P., Bergmann, S., Rohde, M., Hammerschmidt, S., Jänsch, L., Wehland, J. and Kärst, U. (2004) The cell wall subproteome of *Listeria monocytogenes*. *Proteomics* 4, 2991–3006.

[26] Jong, A.Y., Chen, S. H., Stins, M. F., Kim, K. S., Tuan, T. L. and Huang, S. H. (2003) Binding of Candida albicans enolase to plasminutes(ogen) results in enhanced invasion of human brain microvascular endothelial cells. *J Med Microbiol* 52, 615–622.

[27] Elena, L. V., Lucia, M., Martin, R. L., Emmanuelle, S., Mohammed, S., Mercedes, P., Jesus, P., Concha, G., Peter, R. and Cesar, N.(2006) Genetic and proteomic evidences support the localization of *S. cerevisiae* enolase in the cell surface. *Proteomics* 6, S107–S118.

[28] Roger, O.E., Kenneth, C., Stewart, M., Malcolm, W., Devanand , M. P. (2006) Proteomic analysis of Candida albicans *S. cerevisiae* and hyphal cell wall and associated proteins. *Proteomics* 6, 2147-2156.

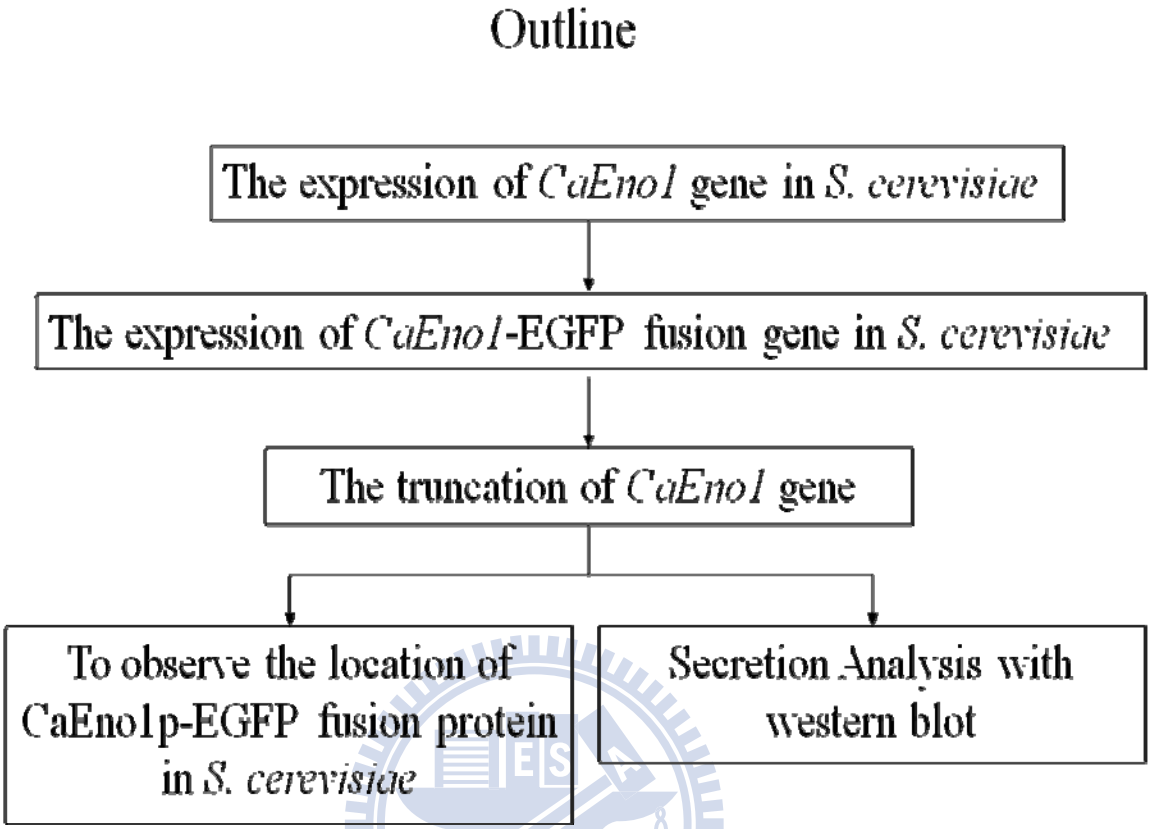
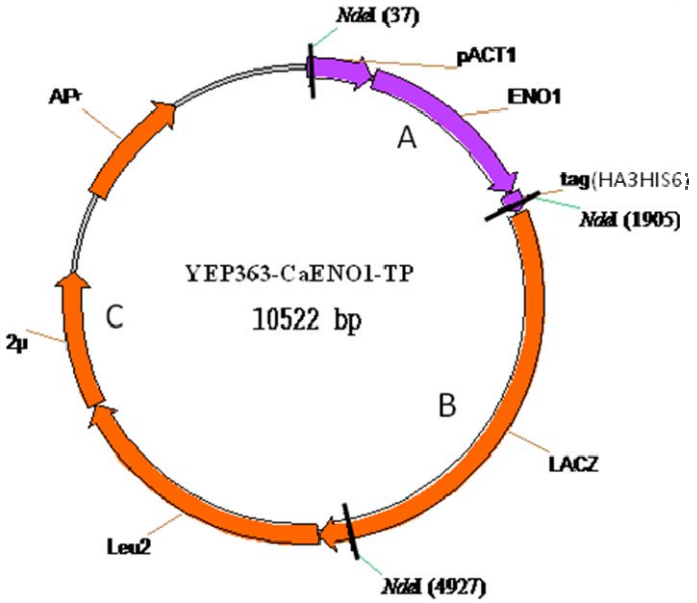


Figure 1. Strategy and flow chart of the experiments in this study.

(A)



(B)

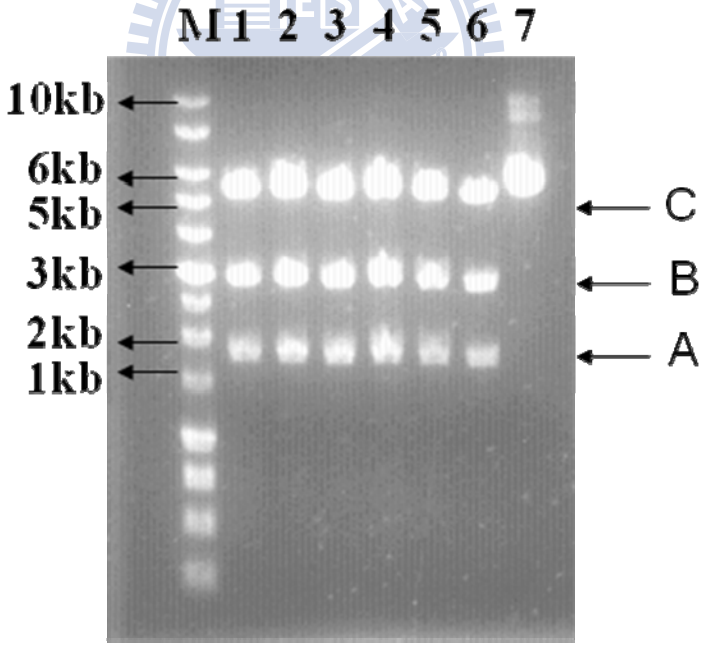


Figure 2. Restriction enzyme digestion of YEP363-CaENO1-TP.

(A) The map of YEP363-CaENO1-TP. (B) The result of *Nde I* digestion.

Lane 1-6 :YEP363–CaENO1-TP plasmid digested by *Nde I*. Lane 7 : untreated plasmid.

A: 1868 bp DNA fragment. B: 3022bp DNA fragment. C: 5632 bp DNA fragment.

M : 1kb DNA leader.

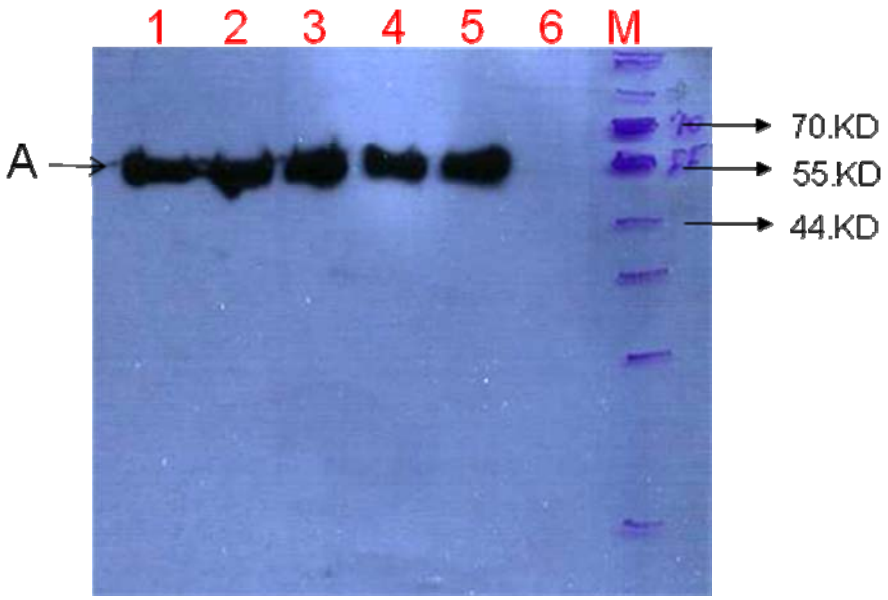


Figure 3. Western blot of the eno-tp expressed in cell.

Protein sample extracted from *S.cerivisiae* cell were detected by anti-HA antibody.

Each lane was loaded 10 μl of protein sample mixture.

Lane 1-4: protein sample (eno-tp) extracted from 10560-2B-f1

Lane 5 : E-tag protein sample as positive control of western blot.

Lane 6 : protein sample extracted from 10560-2B-f0 contained plasmid YEP363.

A: 55 kDa protein of eno-tp

M : Prestain Protein marker (Cat. No.0901)

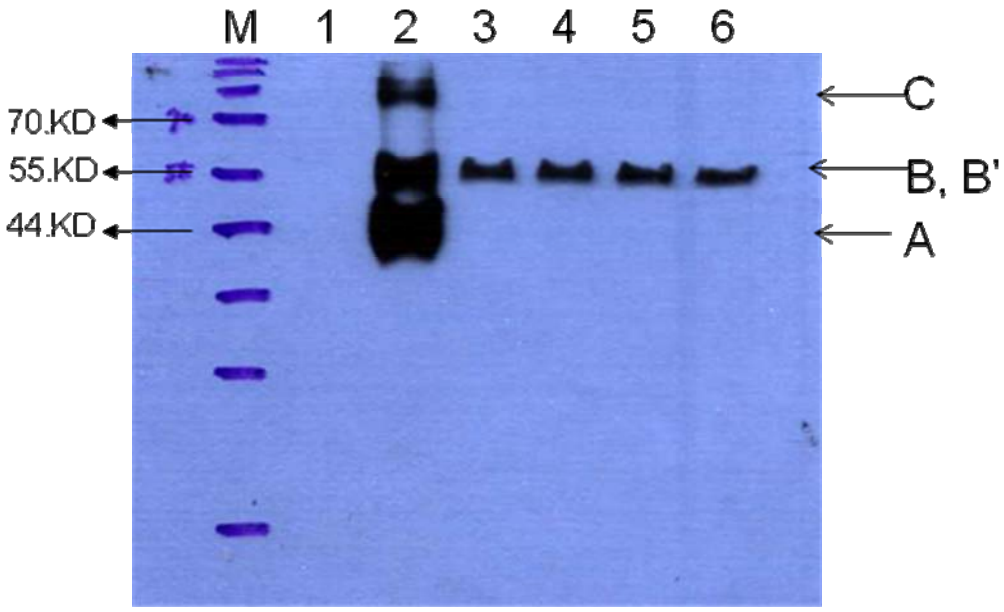


Figure 4. Wstern Blot of the eno-tp secreted outside the cell.

Protein sample extracted from cultured medium were detected by anti-HA antibody.

Each lane was loaded 10 μl of protein sample mixture.

Lane 1: protein sample as negative control from 10560-2B-f0

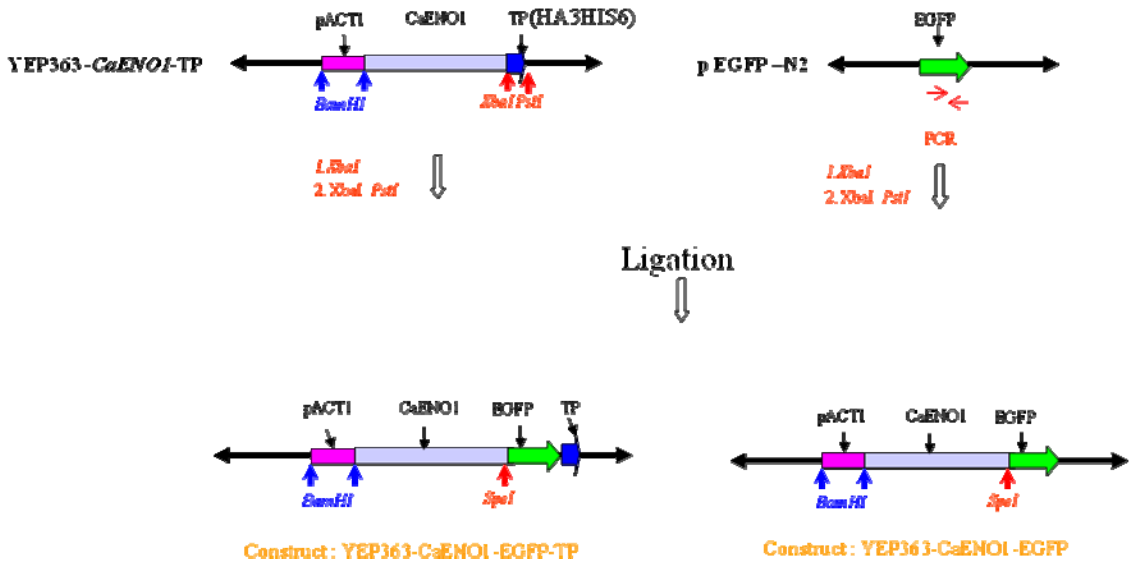
Lane 2: E-tag sample as positive control (bands A, B, C).

Lane 3-6 : protein sample from 10560-2B-f1 (band B')

A: 40 kDa of E-tag. B: 55 kDa of E-tag. B': 55 kDa of eno-tp. C: 80kDa of E-tag.

M : Protein marker : Prestain Protein marker (Cat. No.0901)

(A)



(B)

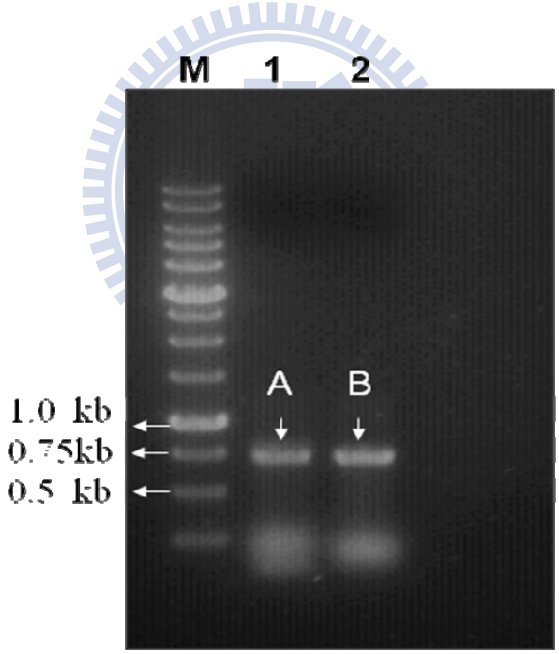


Figure 5. The construction of the plasmid expressed CaENO1-EGFP fusion protein in the *S. cerivisiae* cell.

(A) The strategy of the construction. (B) The PCR product of EGFP on agarose gel. Lane 1 : the PCR product of *XbaI*-EGFP -*XbaI* . Lane 2 : the PCR product of *XbaI*-EGFP-PstI. A: 739 bp DNA fragment of *XbaI*-EGFP–*XbaI*. B: 742 bp DNA fragment of *XbaI*-EGFP-PstI.

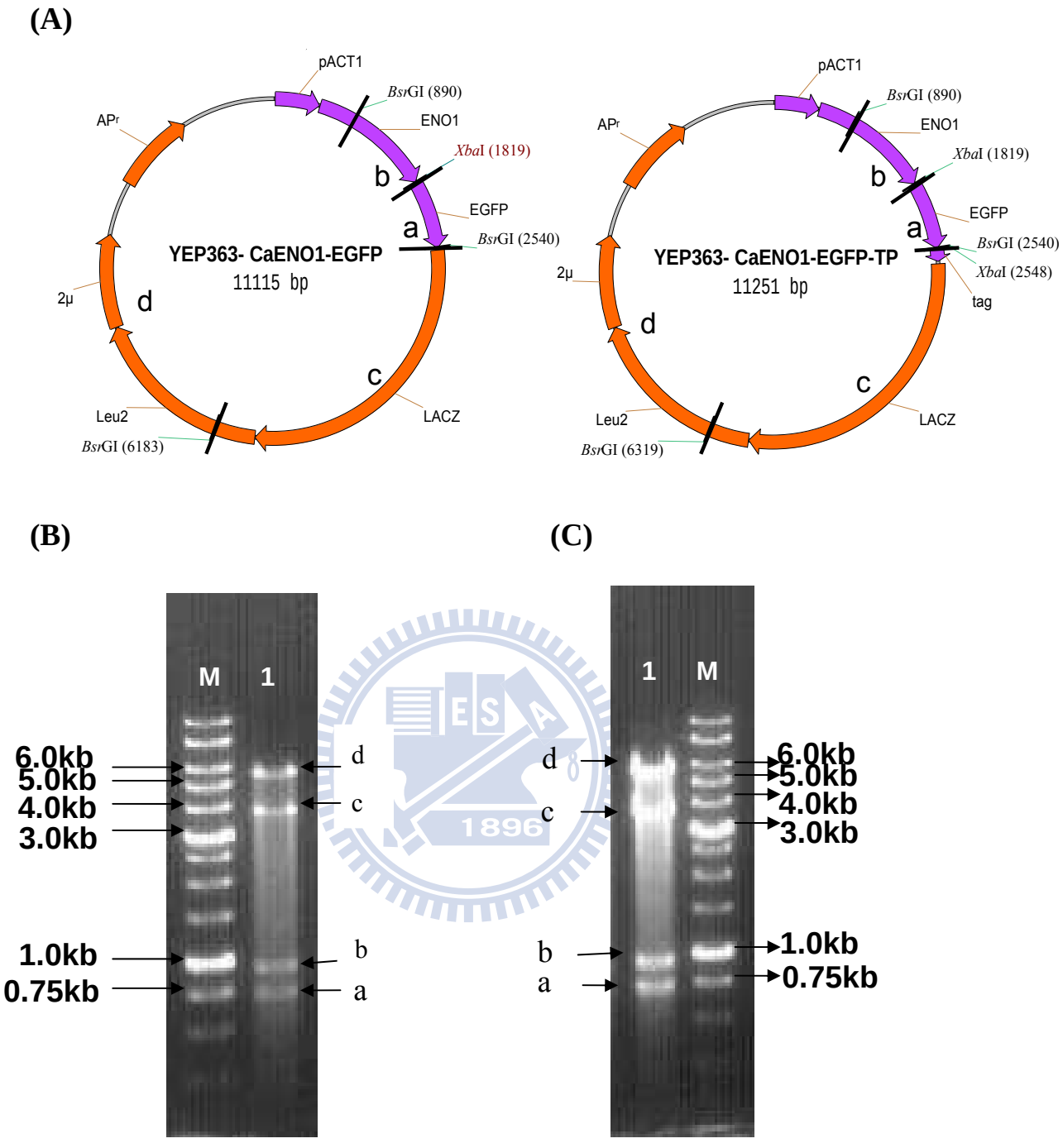


Figure 6. Restriction digestion of the plasmids expressed CaENO1-EGFP fusion genes. (A) The maps of the plasmids YEP363-CaENO1-EGFP and YEP363-CaENO1-EGFP-TP. (B) The result of *XbaI* and *BsrGI* digestion on YEP363-CaENO1-EGFP. Lane 1: the plasmid was digested into 721 bp, 929 bp, 3771 bp and 5694 bp DNA fragments (the arrows showed bands a, b, c, d). (C) The result of *XbaI* and *BsrGI* digestion on YEP363-CaENO1-EGFP-TP. Lane 1: the plasmid was digested into 721 bp, 929 bp, 3771 bp and 5830 bp DNA fragments (the arrows showed bands a, b, c, d). M: 1kb DNA leader.

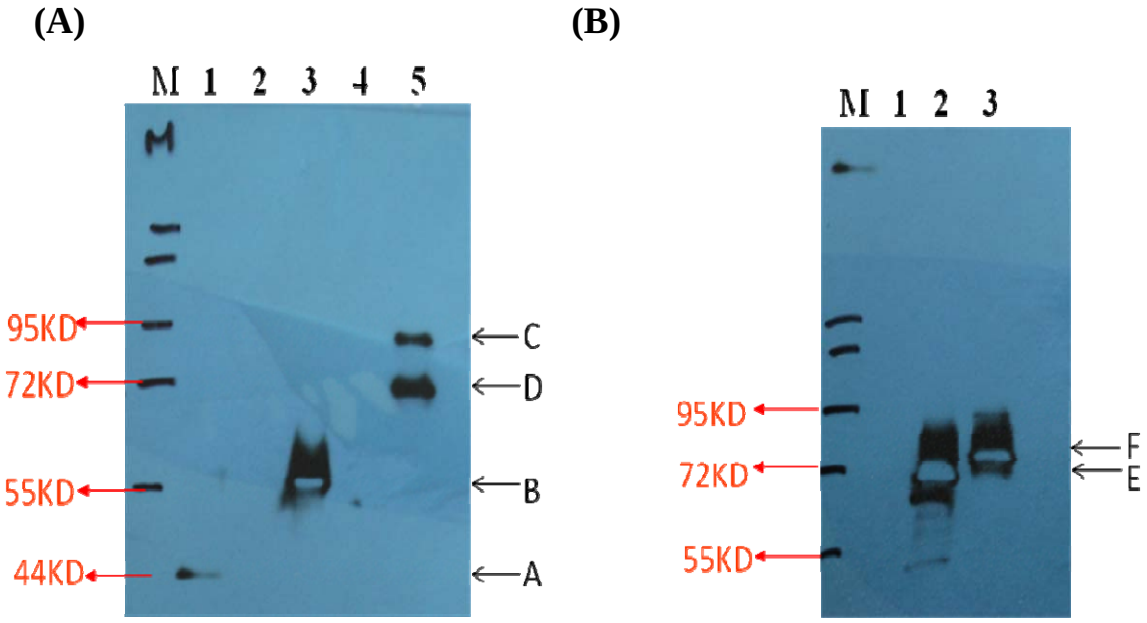


Figure 7. The western blot of CaENO1-EGFP fusion protein.

The protein samples of cell extract were detected by antibody. Each lane was loaded 10 μl of protein sample mixture. (A) the result of western bolt with anti-HA antibody. Lane 1: E-tag sample.(E-tag construct tagged with HAHIS tag was about 40 kDa, but it also can be detected to be about 55 kDa and 80 kDa in this study). Lane 2: the protein sample from 10560-2B-f0. Lane 3: the protein sample (eno-tp) from 10560-2B-f1. Lane 4: the protein sample (eno-EGFPp) from 10560-2B-f3. Lane 5: the protein sample (eno-EGFP-tp) from 10560-2B-f2. (B) the result of western bolt with anti-EGFP antibody. Lane 1: the protein sample from 10560-2B-f0. Lane 2: the protein sample(eno-EGFPp) from 10560-2B-f3. Lane 3: the protein sample(eno-EGFP-tp)from 10560-2B-f2.

A: 40 kDa protein of E-tag. B: 53 kDa of eno-tp .C and D: 79kDa and 72 kDa of eno-EGFP-tp. E : 75 kDa of eno-EGFPp. F: 79 kDa of eno-EGFP-tp. M : Prestain Protein marker (Cat. No.0901)

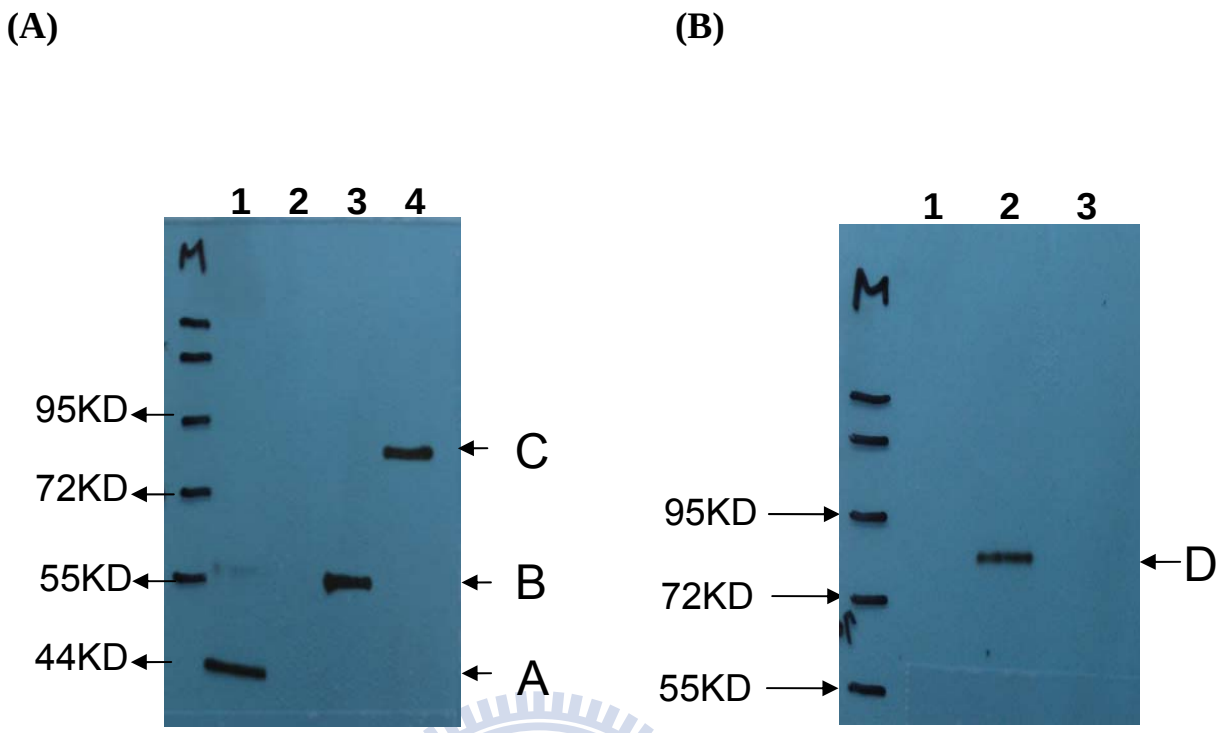
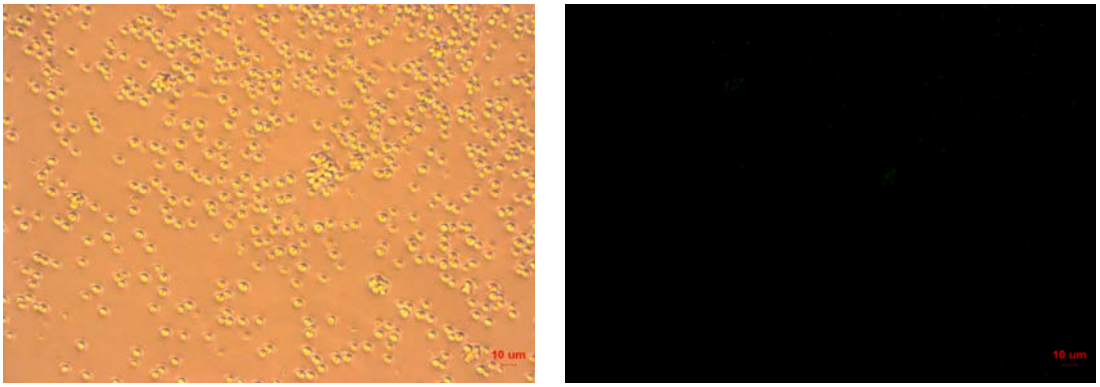


Figure 8. The western blot of supernatant samples of CaENO1-EGFP fusion proteins from the supernatant of cultured medium.

The protein samples of supernatant were detected by antibody. Each lane was loaded 30 μl of protein sample mixture. (A) The result of western bolt with anti-HA antibody. Lane 1: E-tag sample. (E-tag construct tagged with HAHIS tag was about 40 kDa, but it also can be detected to be about 55 kDa and 80 kDa in this study). Lane 2: the protein sample from 10560-2B-f0. Lane 3: the protein sample (eno-tp) from 10560-2B-f1. Lane 4: the protein sample (eno-EGFP-tp) from 10560-2B-f2. (B) The result of western bolt with anti-EGFP antibody. Lane 1: the protein sample from 10560-2B-f0. Lane 2: the protein sample (eno-EGFP-tp) from 10560-2B-f3. Lane 3: the protein sample (eno-EGFPp) from 10560-2B-f2.

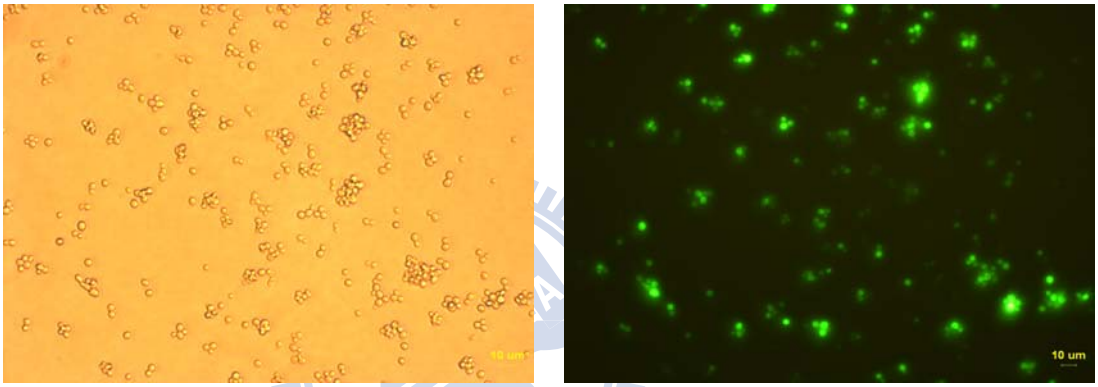
A: 40 kDa of E-tag. B: 53 kDa protein of eno-tp. C: 79kDa protein of eno-EGFP-tp. D: 75 kDa protein of eno-EGFPp . M: Prestain Protein marker (Cat. No.0901)

(A) 10560-2B-f0



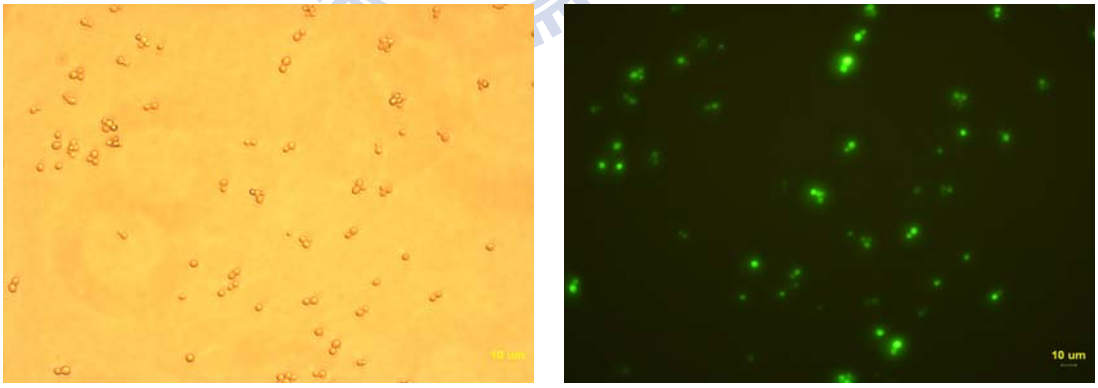
visible light UV light

(B) 10560-2B-f2



visible light UV light

(C) 10560-2B-f3



visible light UV light

Figure 9. The microscopy observation of CaENO1-EGFP fusion protein.

The transformed cells were incubated for 48 hours and observed under fluorescence microscopy with 400X maganification.

(A) the visible light and UV light of 10560-2B-f0 . (B) the visible light and UV light of 10560-2B-f2 expressing eno-EGFP-tp under uv light. (C) the visible light and UV light of 10560-2B-f3 expressing eno-EGFP under uv light.

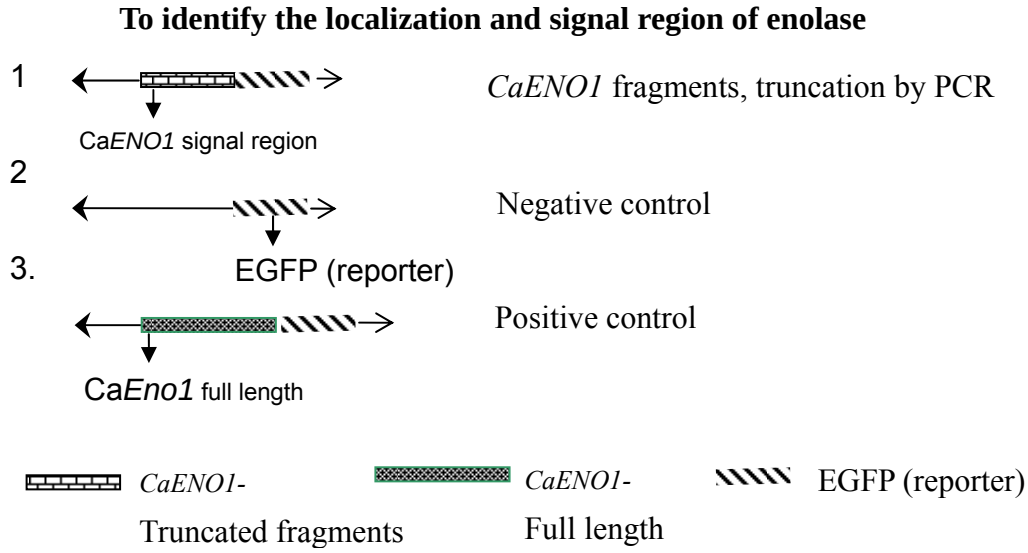


Figure 10. The diagram of the strategy in this experiment. To identify the localization and signal region of enolase

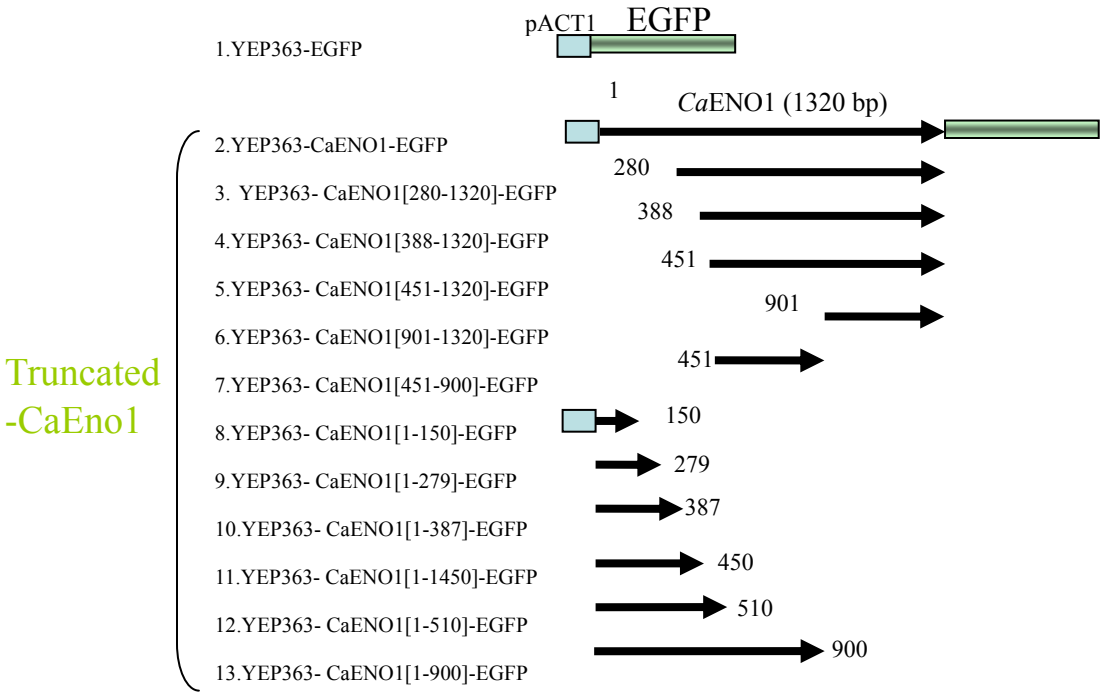
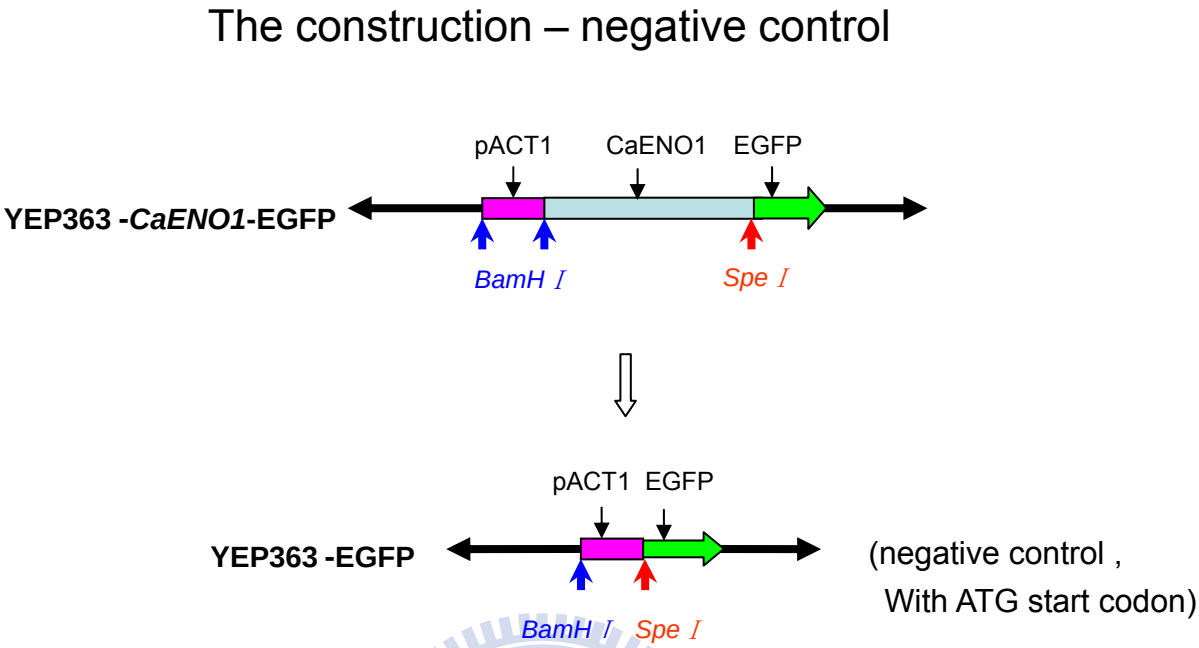


Figure 11. The diagram of the constructs of truncated *CaENO1*.
The constructs were transformed into *S.cerivisiae* for analysis of secretion.

(A)



(B)

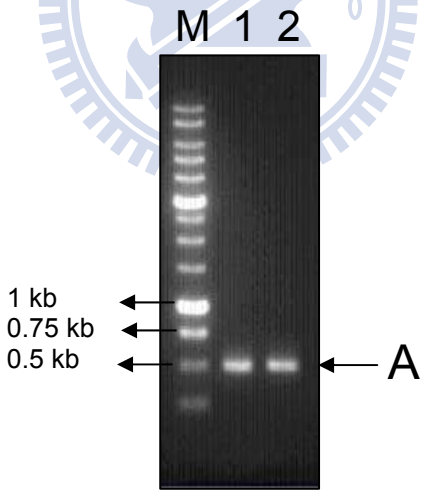
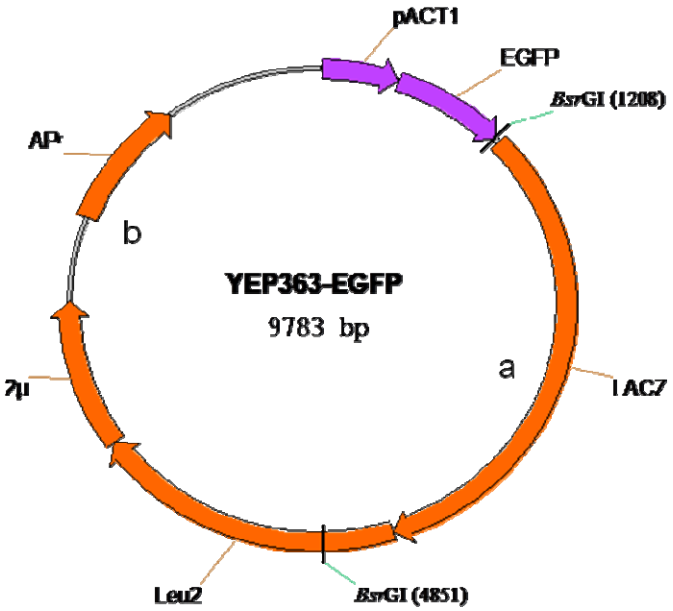


Figure 12. The construction of the plasmid expressing EGFP protein in *S. cerivisiae* cell.
(A) The strategy of the construction. (B) The PCR product of pACT1 on agarose gel. lane1. 2 : PCR products of pACT1. A: 500 bp DNA fragment of pACT1. M : 1kb DNA marker

(A)



(B)

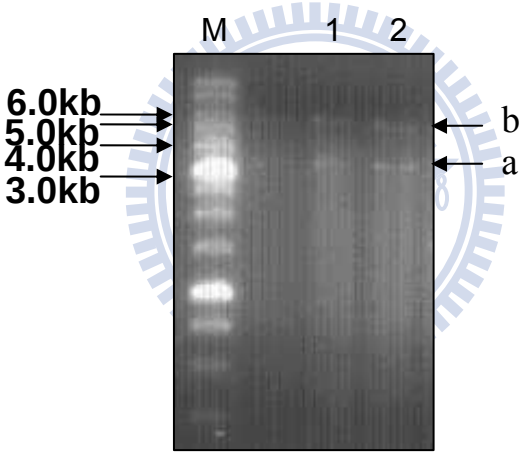


Figure 13. Restriction digestion of the plasmids expressed EGFP genes.

(A) The maps of the plasmids YEP363 -EGFP (B) The result of *BsrGI* digestion on plasmid YEP363-EGFP. Lane 1.2: the plasmid was digested into 3643 bp and 6140 bp DNA fragments (band a and b). M: 1kb DNA leader.

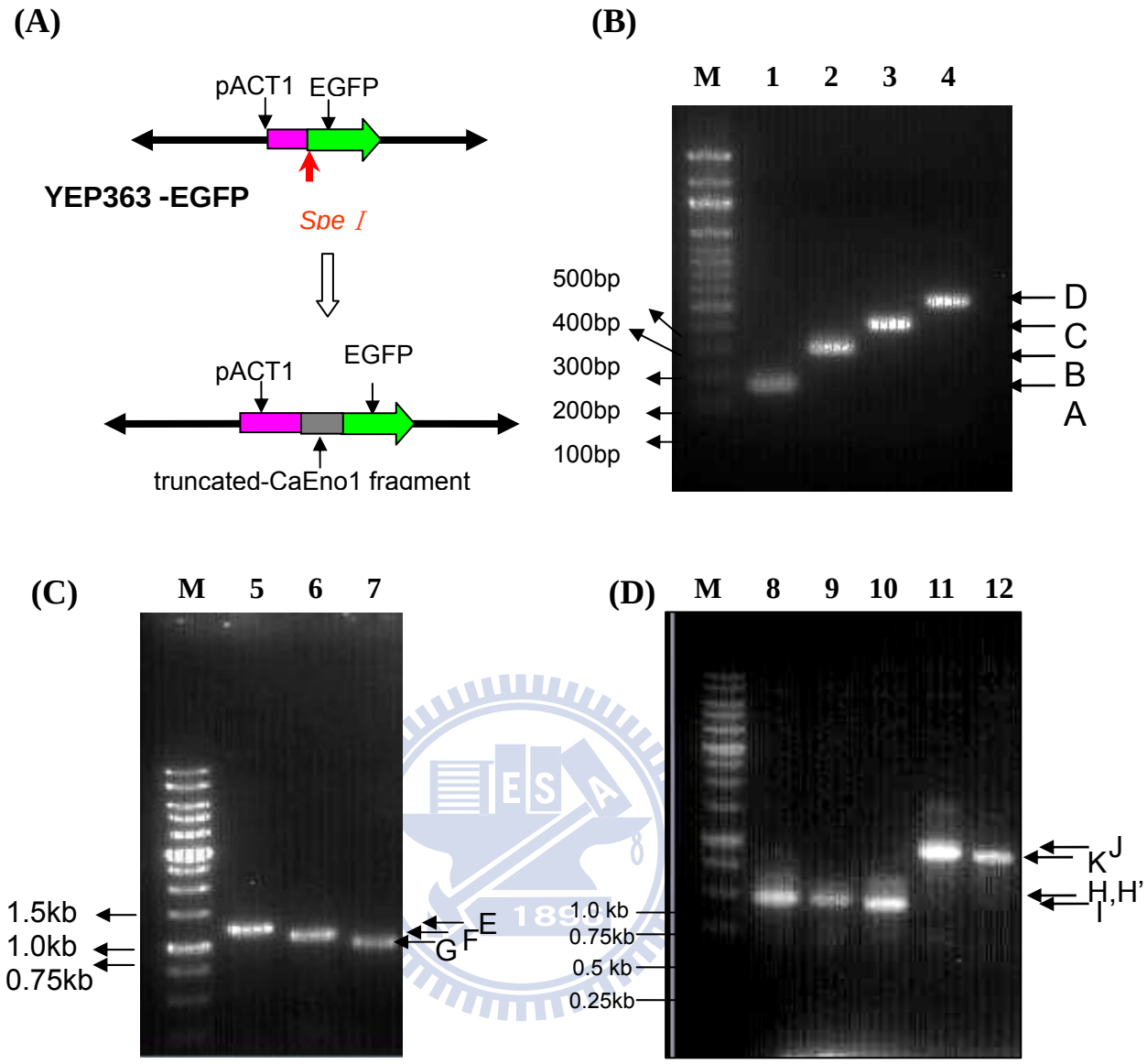
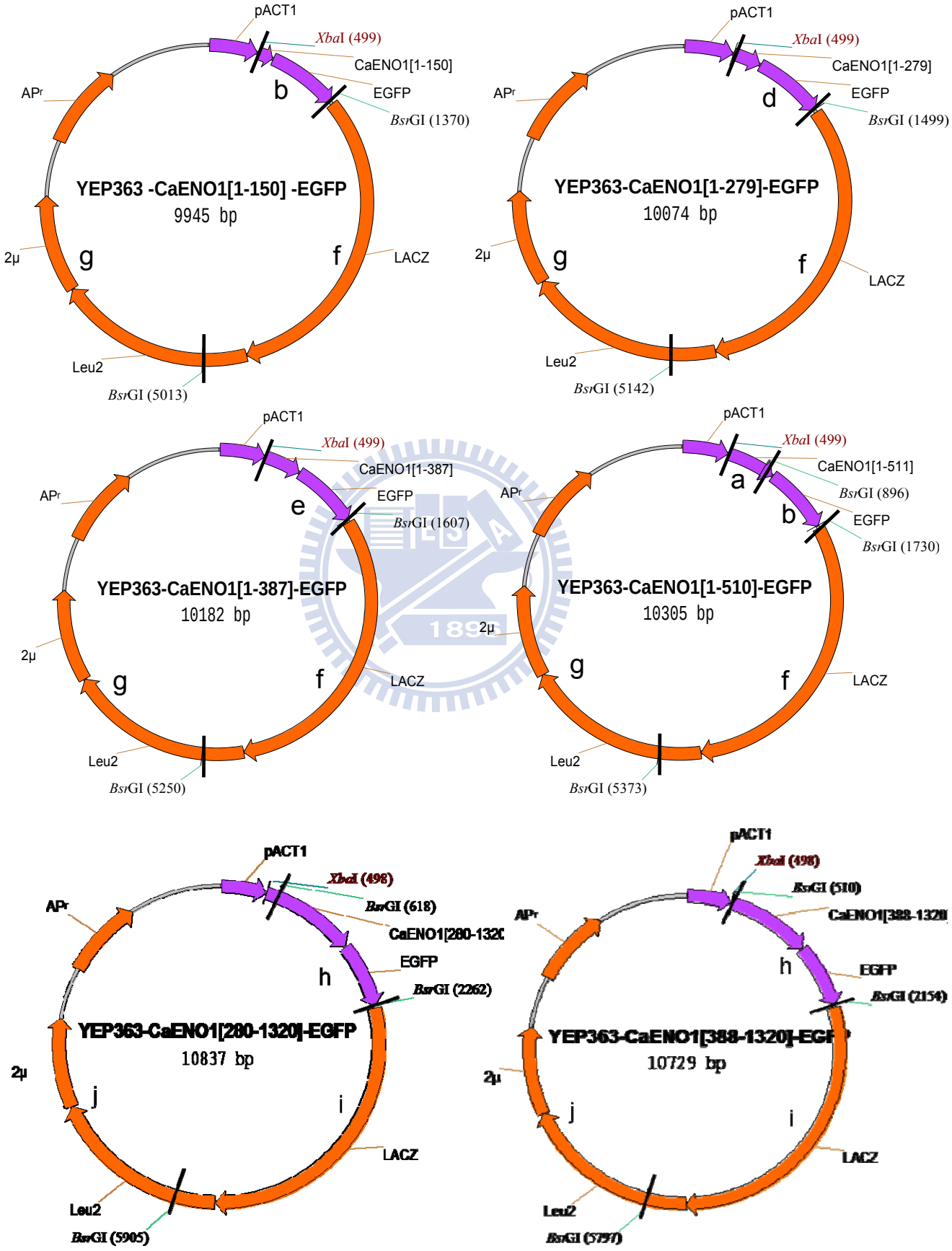
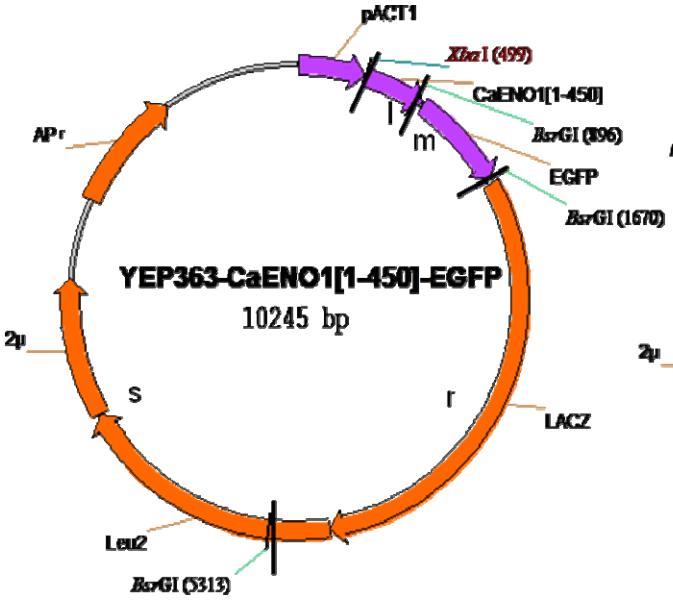


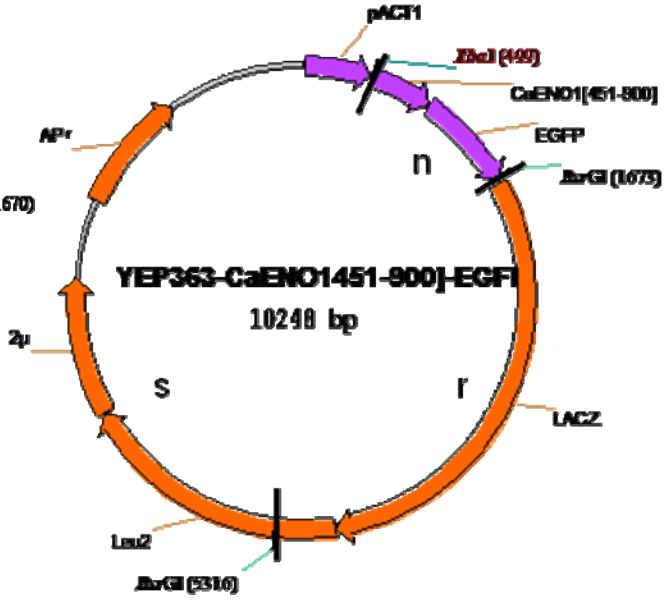
Figure 14. The construction of the plasmids expressing truncated CaENO1-EGFP protein in *S. cerivisiae* cell.

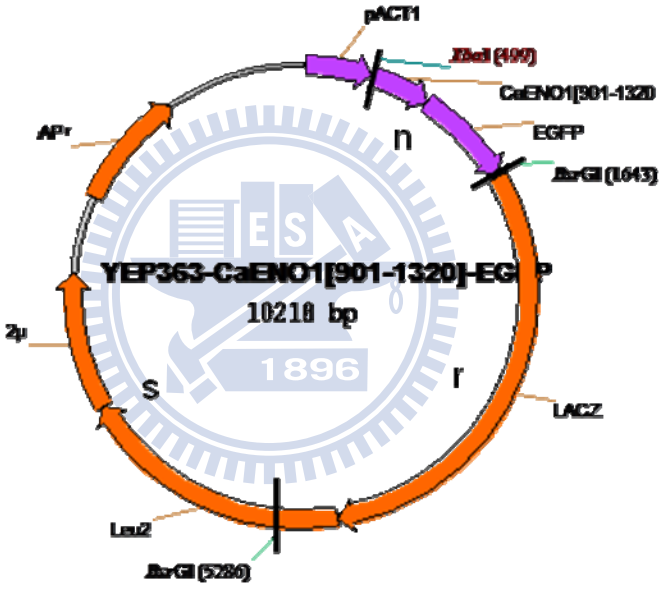
(A) The strategy of the construction. (B) The PCR products of truncated CaENO1 on agarose gel. Lane 1: CaENO1/1-150 (150 bp, band A). Lane 2: CaENO1/1-279 (279 bp, band B). Lane 3: CaENO1/1-387(387 bp, band C). Lane 4: CaENO1/1-510(510 bp, band D). M: 100bp DNA marker (C) The PCR products of truncated CaENO1 on agarose gel. Lane 5: CaENO1 gene (1320 bp, band E). Lane 6: CaENO1/280-1320 (1041 bp, band F). Lane 7: CaENO1/389-1320 (933 bp, band G). M: 1kb DNA marker (D) The PCR products of truncated CaENO1 on agarose gel. Lane 8: CaENO1/1-451(450bp, band H). Lane 9: CaENO1/451-900 (450bp, band H'). Lane 10: CaENO1/900-1320 (420 bp, band I). Lane 11: CaENO1/1-900 (900 bp, band J). Lane 12: CaENO1/451-1320 (869 bp, band K). M: 1kb DNA marker.

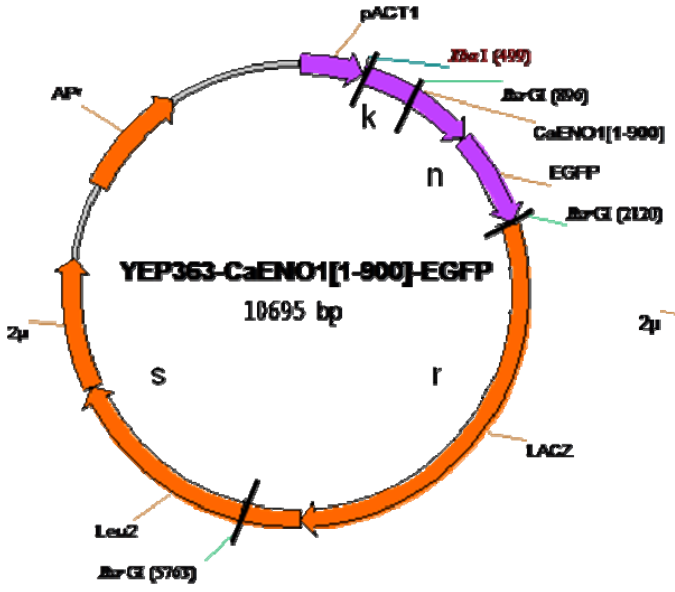
(A)

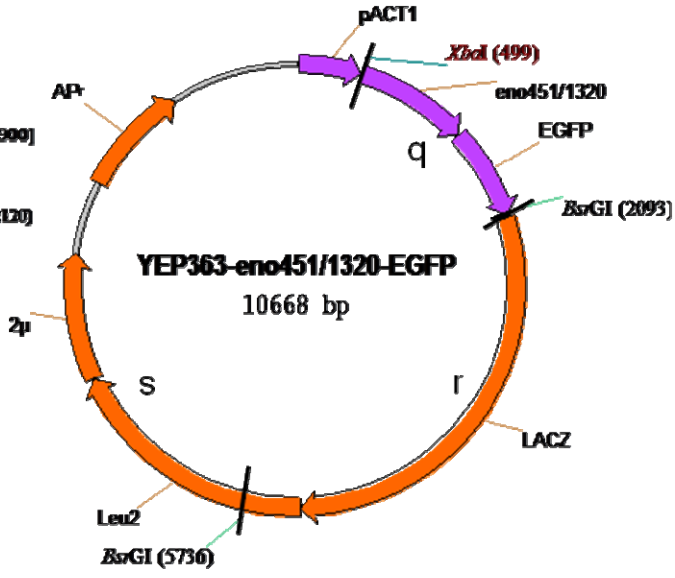

pACT1
XbaI (499)
CaENO1[1-150]
EGFP
BsrGI (1370)
APr
b
YEP363 -CaENO1[1-150] -EGFP
9945 bp
2μ
g
f
LACZ
Leu2
BsrGI (5013)
pACT1
XbaI (499)
CaENO1[1-279]
EGFP
BsrGI (1499)
APr
d
YEP363-CaENO1[1-279]-EGFP
10074 bp
2μ
g
f
LACZ
Leu2
BsrGI (5142)
pACT1
XbaI (499)
CaENO1[1-387]
EGFP
BsrGI (1607)
APr
e
YEP363-CaENO1[1-387]-EGFP
10182 bp
2μ
g
f
LACZ
Leu2
BsrGI (5250)
pACT1
XbaI (499)
CaENO1[1-511]
BsrGI (896)
EGFP
BsrGI (1730)
APr
a
b
YEP363-CaENO1[1-510]-EGFP
10305 bp
2μ
g
f
LACZ
Leu2
BsrGI (5373)
pACT1
XbaI (498)
BsrGI (618)
CaENO1[280-1320]
EGFP
BsrGI (2262)
APr
h
YEP363-CaENO1[280-1320]-EGFP
10837 bp
2μ
j
i
LACZ
Leu2
BsrGI (5905)
pACT1
XbaI (498)
BsrGI (510)
CaENO1[388-1320]
EGFP
BsrGI (2154)
APr
h
YEP363-CaENO1[388-1320]-EGFP
10729 bp
2μ
j
i
LACZ
Leu2
BsrGI (5797)


pACT1
XbaI (499)
CaENO1[1-450]
BsrGI (896)
EGFP
BsrGI (1670)
l
m
APr
YEP363-CaENO1[1-450]-EGFP
10245 bp
2μ
s
r
LACZ
Leu2
BsrGI (5313)


pACT1
XbaI (499)
CaENO1[451-800]
EGFP
BsrGI (1673)
n
APr
YEP363-CaENO1451-900]-EGFP
10248 bp
2μ
s
r
LACZ
Leu2
BsrGI (5316)


pACT1
XbaI (499)
CaENO1[901-1320]
EGFP
BsrGI (1643)
n
APr
YEP363-CaENO1[901-1320]-EGFP
10218 bp
2μ
s
r
LACZ
Leu2
BsrGI (5286)


pACT1
XbaI (499)
BsrGI (896)
CaENO1[1-900]
EGFP
BsrGI (2120)
k
n
APr
YEP363-CaENO1[1-900]-EGFP
10695 bp
2μ
s
r
LACZ
Leu2
BsrGI (5763)


pACT1
XbaI (499)
eno451/1320
EGFP
BsrGI (2093)
q
APr
YEP363-eno451/1320-EGFP
10668 bp
2μ
s
r
LACZ
Leu2
BsrGI (5736)

(B)

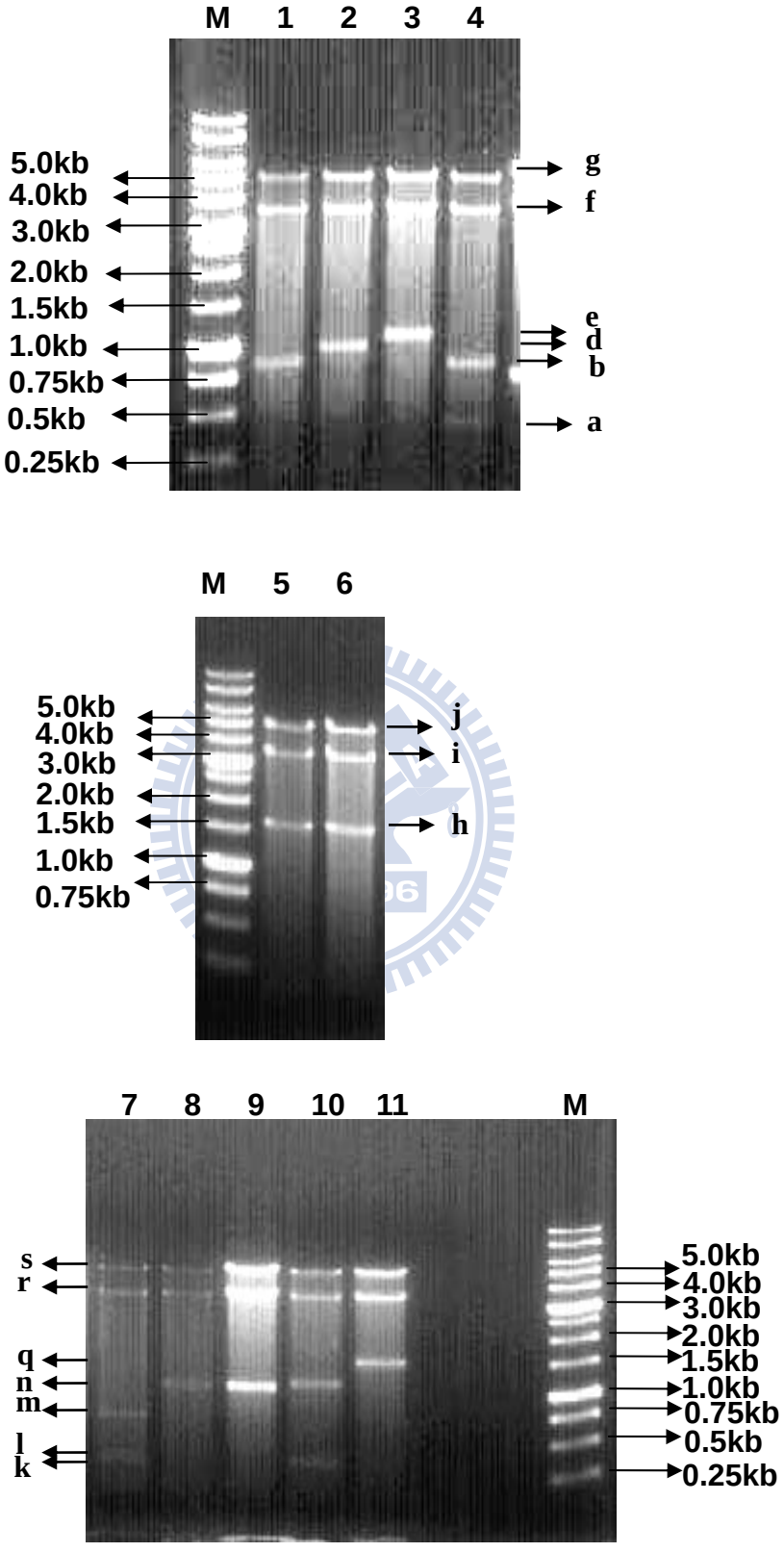

M 1 2 3 4
5.0kb
4.0kb
3.0kb
2.0kb
1.5kb
1.0kb
0.75kb
0.5kb
0.25kb
g
f
e
d
b
a
M 5 6
5.0kb
4.0kb
3.0kb
2.0kb
1.5kb
1.0kb
0.75kb
j
i
h
7 8 9 10 11 M
s
r
q
n
m
l
k
5.0kb
4.0kb
3.0kb
2.0kb
1.5kb
1.0kb
0.75kb
0.5kb
0.25kb

Figure 15. Restriction digestion of the plasmids expressing truncated CaENO1-EGFP fusion gene.

(A) The maps of the plasmids. (B) The result of restriction enzyme digestion.

Lane 1: the plasmid YEP363-CaENO1[1-150]-EGFP was digested by *XbaI* and *BsrGI* (the arrows showed b, f and g).

Lane 2: the plasmid YEP363-CaENO1[1-279]-EGFP was digested by *XbaI* and *BsrGI* (the arrows showed d, f and g).

Lane 3: the plasmid YEP363-CaENO1[1-387]-EGFP was digested by *XbaI* and *BsrGI* (the arrows showed e, f and g).

Lane 4: the plasmid YEP363-CaENO1[1-510]-EGFP by *XbaI* and *BsrGI* (the arrows showed a, b, f and g).

Lane 5: the plasmid YEP363- CaENO1[280-1320]-EGFP was digested by *BsrGI* (the arrows showed h, I and j).

Lane 6: the plasmid YEP363- CaENO1[388-1320]-EGFP was digested by *BsrGI* (the arrows showed h, I and j).

Lane 7: the plasmid .YEP363- CaENO1[1-1450]-EGFP was digested by *XbaI* and *BsrGI*(the arrows showed l, m, r and s).

Lane 8: the plasmid YEP363-CaENO1[451-900]-EGFP was digested by *XbaI* and *BsrGI* (the arrows showed n, r and s).

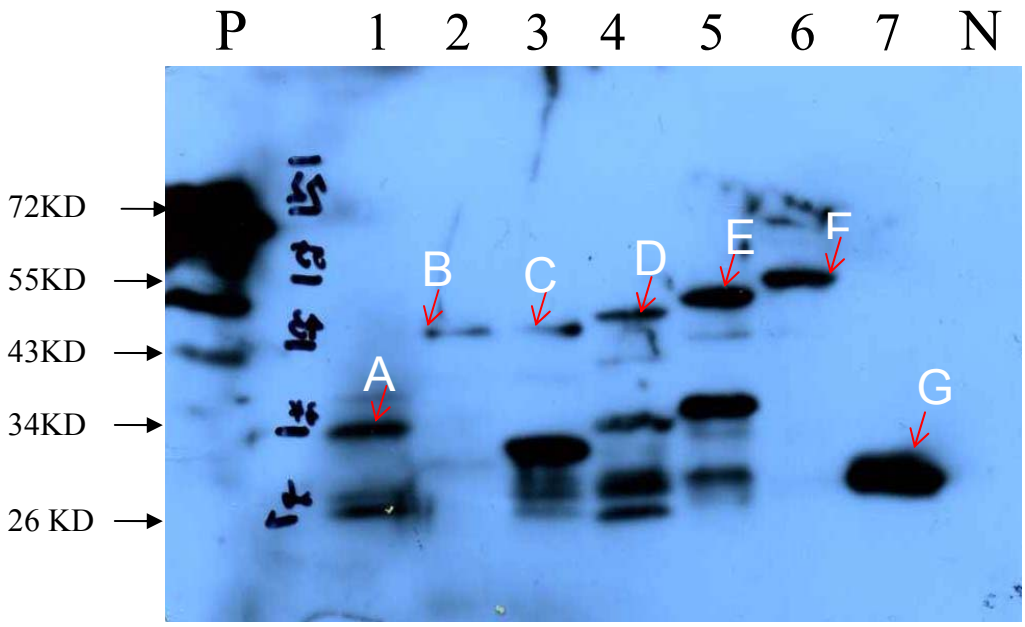
Lane 9: the plasmid YEP363-CaENO1[901-1320]-EGFP was digested by *XbaI* and *BsrGI* (the arrows showed n, r and s).

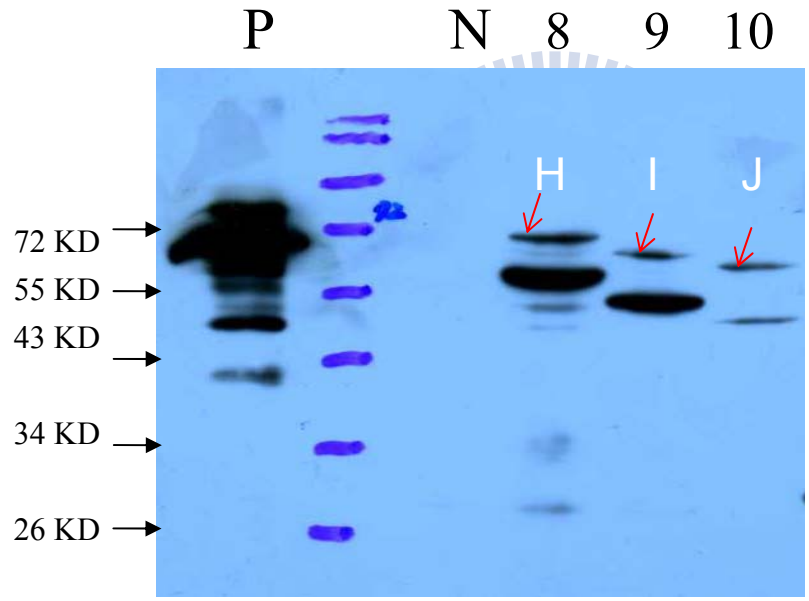
Lane 10: the plasmid YEP363-CaENO1[1-900]-EGFP was digested by *XbaI* and *BsrGI* (the arrows showed n, r and s).

Lane 11: the plasmid YEP363-CaENO1[451-1320]-EGFP was digested by *XbaI* and *BsrGI*(the arrows showed k, q, r and s).

M: 1kb DNA leader.

a : 397 bp DNA fragment, b: 843 bp and 871 bp, d: 1000 bp, e: 1108 bp, f : 3643 bp, g: 5431 bp, h: 1644bp, i: 3643bp, j: 5550 bp and 5442bp, k: 397 bp, l: 447 bp, m: 774 bp, n:1144 bp, 1174 bp and 1224 bp, q: 1594 bp, r: 643 bp, s:~5431 bp.


P
1
2
3
4
5
6
7
N
72KD
55KD
43KD
34KD
26 KD
A
B
C
D
E
F
G


P
N
8
9
10
72 KD
55 KD
43 KD
34 KD
26 KD
H
I
J

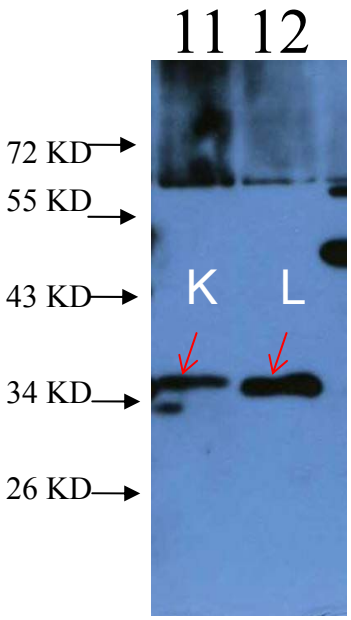


Figure 16. The detection of truncated CaENO1-EGFP protein in *S. cerivisiae* cell with western blot analysis.

The transformed cells were incubated for 48 hours and broken to extract the protein. Then the protein samples were analyzed by anti-EGFP antibody by western blot analysis. Each lane was loaded 10 μl of protein sample mixture.

N (negative control) : the protein sample from 10560-2B-f0

P (positive control) : the protein sample (eno-EGEPp) from 10560-2B-f3.

truncated CaENO1-EGFP fusion protein samples :

1.eno1[1-150-]EGFP p (band A)　2.eno1[1-279]-EGFP p (band B)

3.eno1[1-387]-EGFP p (band C)　4. eno1[1-450]-EGFP p (band D)

5.eno1[1-510]-EGFP p (band E)　6.eno1[1-900]-EGFP p (band F) 7. EGFP (band G)

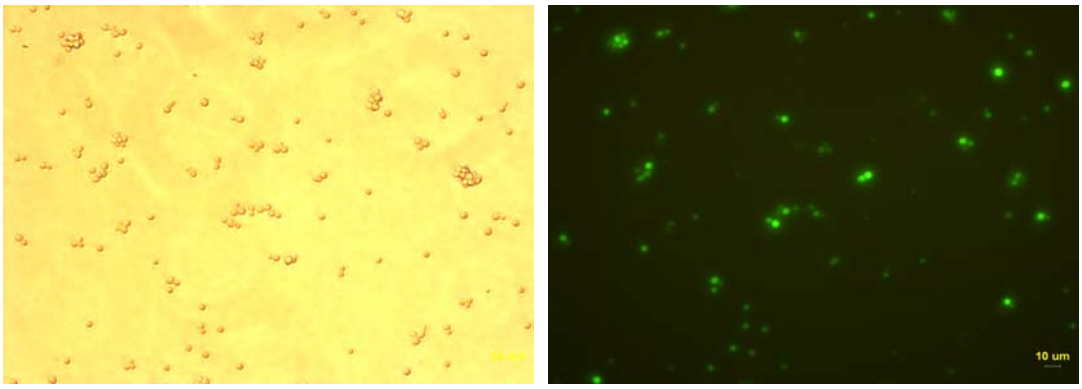
8.eno1[280-1320]-EGFPp(band H) 9.eno1[389-1320]-EGFPp(band I)

10.eno1[451-1320]-EGFPp(band J) 11.eno[451-900]-EGFPp (band K)

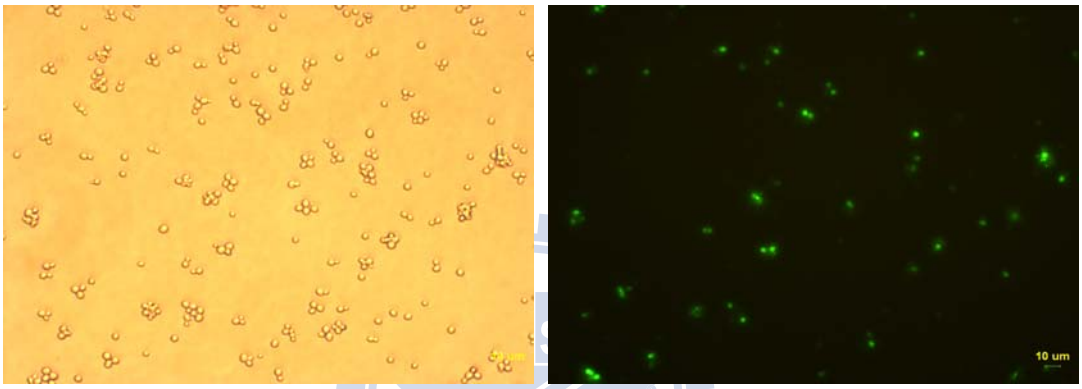
12. eno[901-1320]-EGFPp (band L)

A: ~31kDa protein　B : ~43kDa C: ~40kDa D: ~43kDa E: ~45kDa F: ~59kDa kDa G: ~26kDa H: ~65kDa I: ~62kDa J: ~58kDa K: ~34kDa L:~34kDa M: Prestain Protein marker (Cat. No.0901)

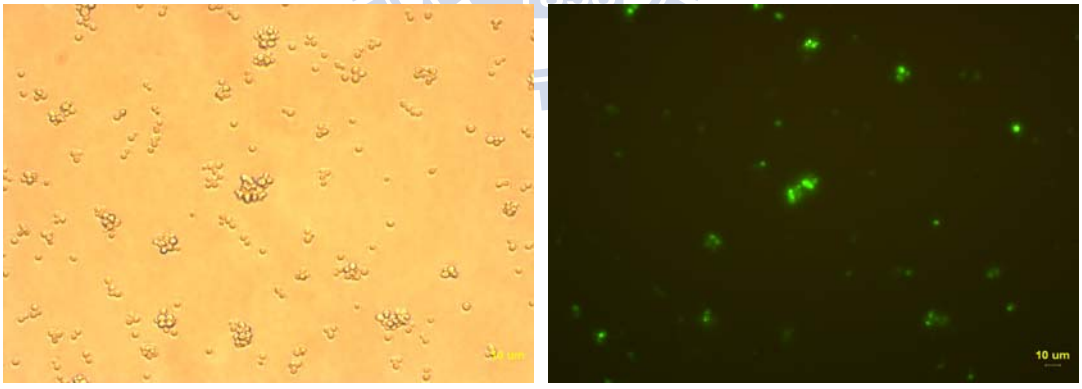
1.10560-2B.- EGFP



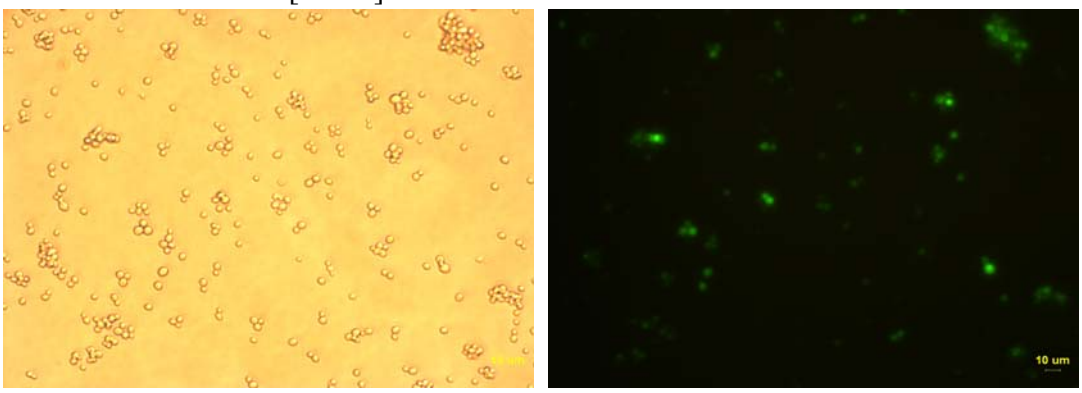
2. 10560-2B-CaENO1[1-150]



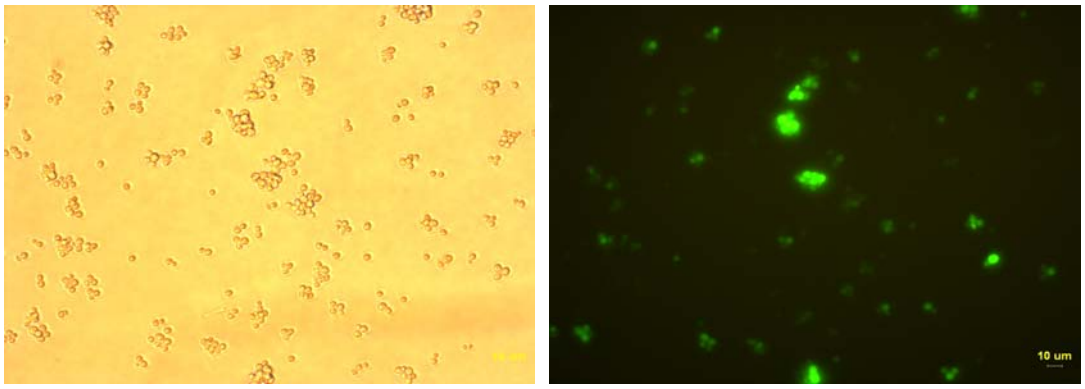
3.10560-2B-CaENO1[1-279]



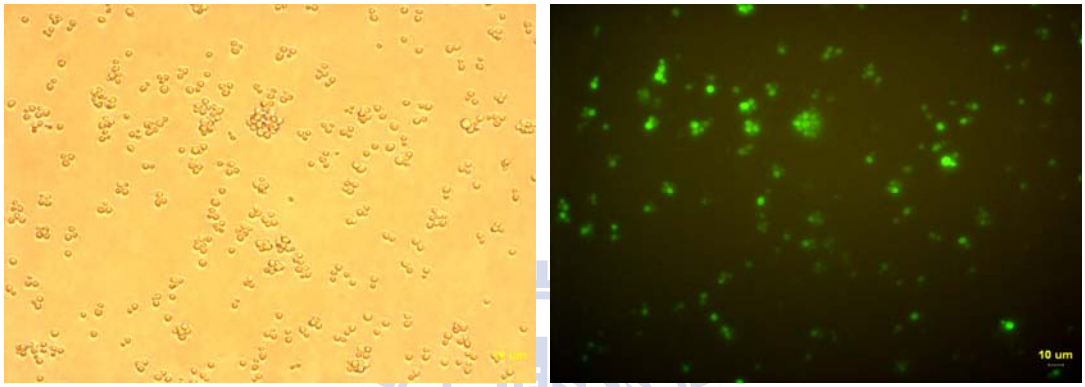
4.10560-2B-CaENO1[1-387]



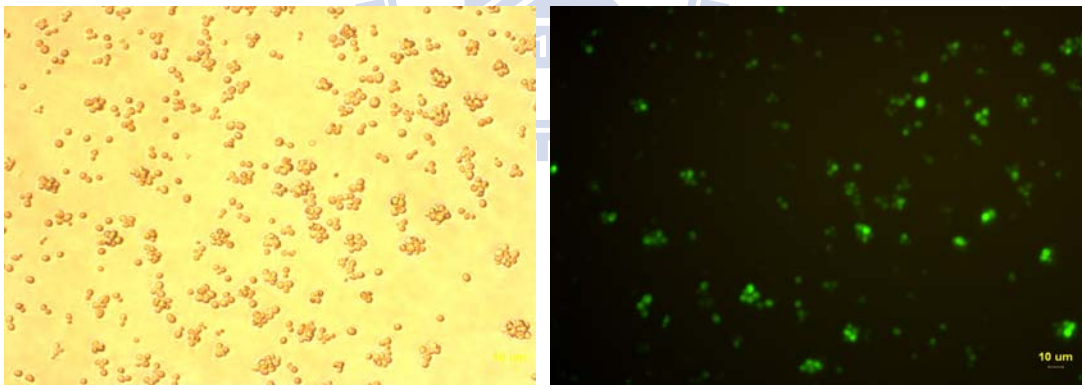
5.10560-2B-CaENO1[1-450]



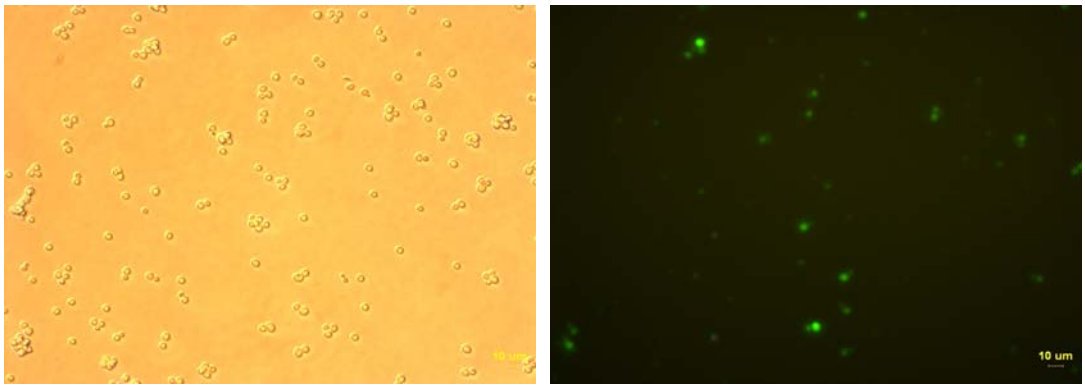
6.10560-2B-CaENO1[1-510]



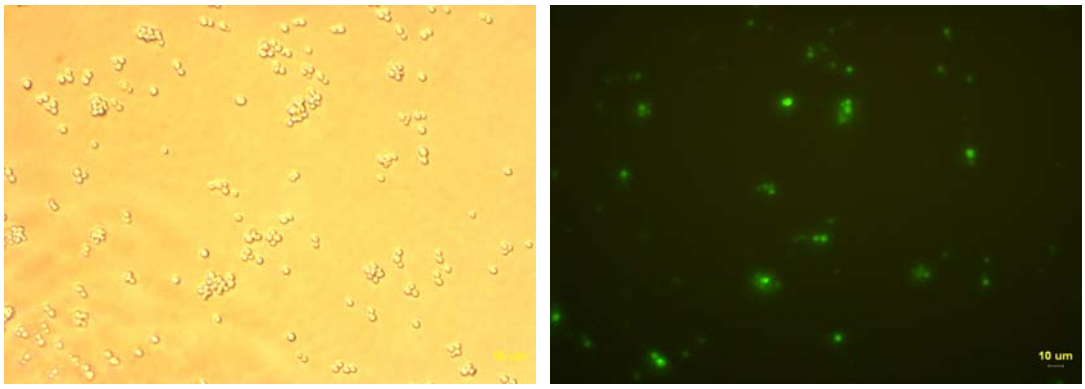
7.10560-2B-CaENO1[1-900]



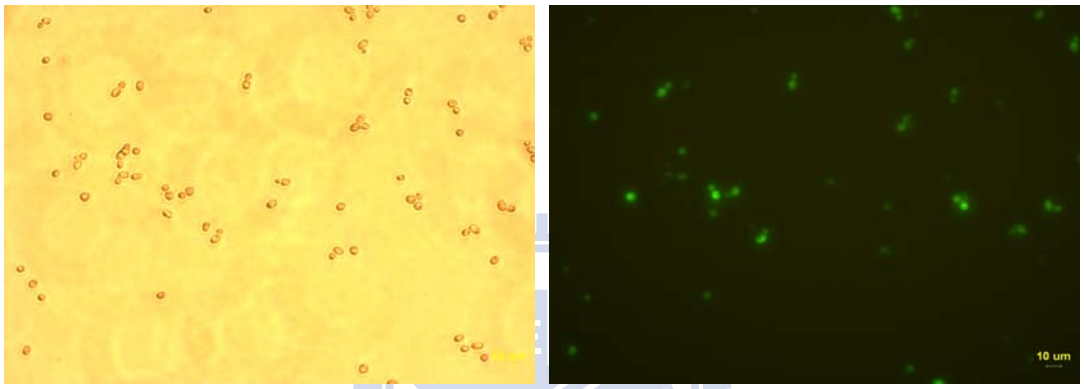
8.10560-2B-CaENO1[280-1320]



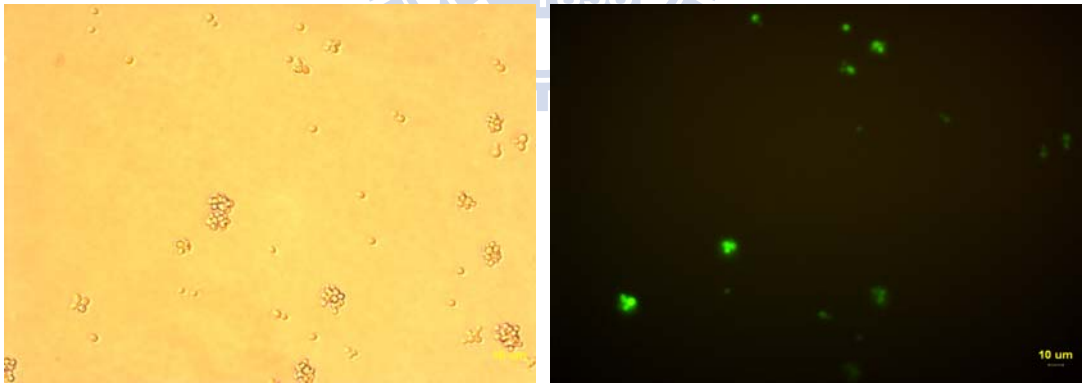
9.10560-2B-CaENO1[388-1320]



10.10560-2B-CaENO1[451-1320]



11.10560-2B-CaENO1[901-1320]



12.10560-2B-CaENO1[451-900]

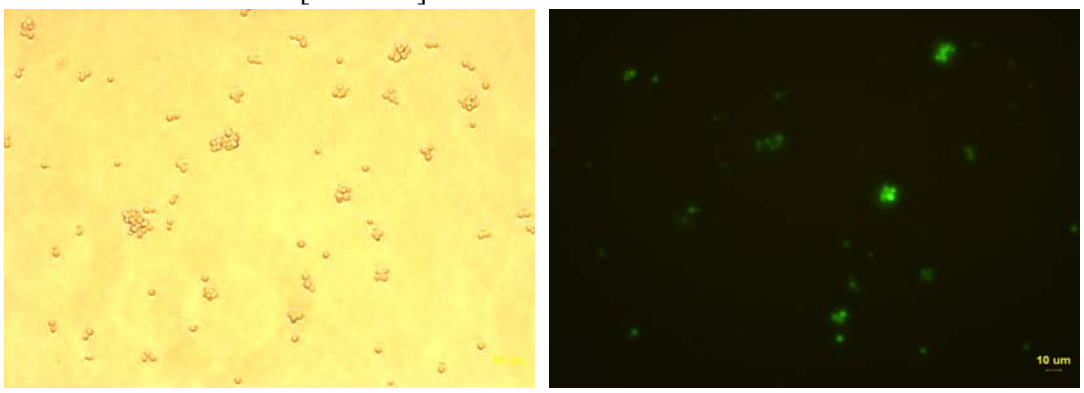


Figure 17. The microscopy observation of truncated CaENO1-EGFP proteins in *S. cerivisiae* cells.

S. cerivisiae cells were incubated for 48 hours and observed under fluorescence microscopy with 400X magnification.

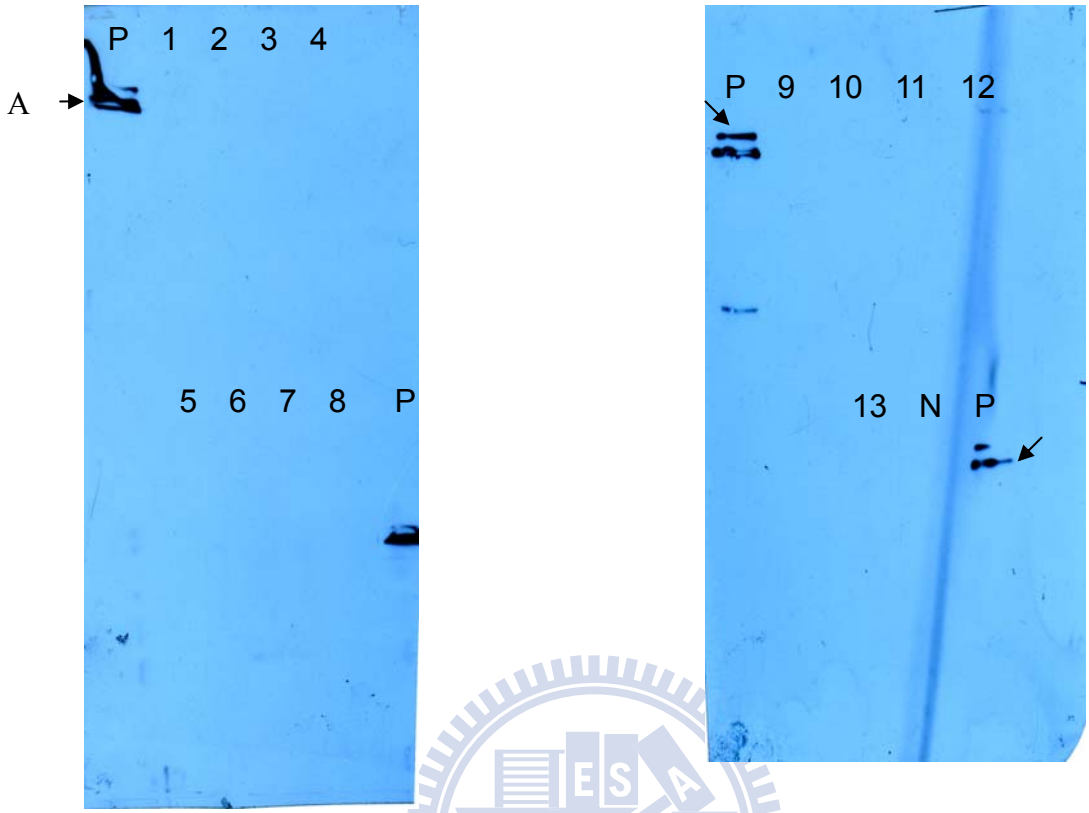


Figure 18. The western blot of truncated CaENO1-EGFP fusion proteins from cultured medium.

The protein samples of supernatant were detected by anti-EGFP antibody with western blot analysis. Each lane was loaded 30 μl of protein sample mixture.

Western blot of truncated CaENO1-EGFP proteins.

N: protein sample as negative control, extracted from 10560-2B-f0

P: positive control, eno-EGFPp sample extracted from cells of 10560-2B-f3.

1.eno1[1-150-]EGFP p (~31kDa) 2.eno1[1-279]-EGFP p (~36kDa)

3.eno1[1-387]-EGFP p (~40kDa) 4. eno1[1-450]-EGFP p (~43kDa)

5.eno1[1-510]-EGFP p (~45kDa) 6.eno1[1-900]-EGFP p (~59kDa) 7.EGFP (~26kDa) 8.eno-EGFPp(~75kDa) 9.eno1[280-1320]-EGFPp(~65kDa).

10.eno1[389-1320]-EGFPp (~62kDa) 11.eno1[451-1320]-EGFPp (~58kDa).

12.eno[451-900] (~43kDa) 13.eno[901-1320] (~43kDa) .

A: ~75 kDa protein; M: Prestain Protein marker (Cat. No.0901)

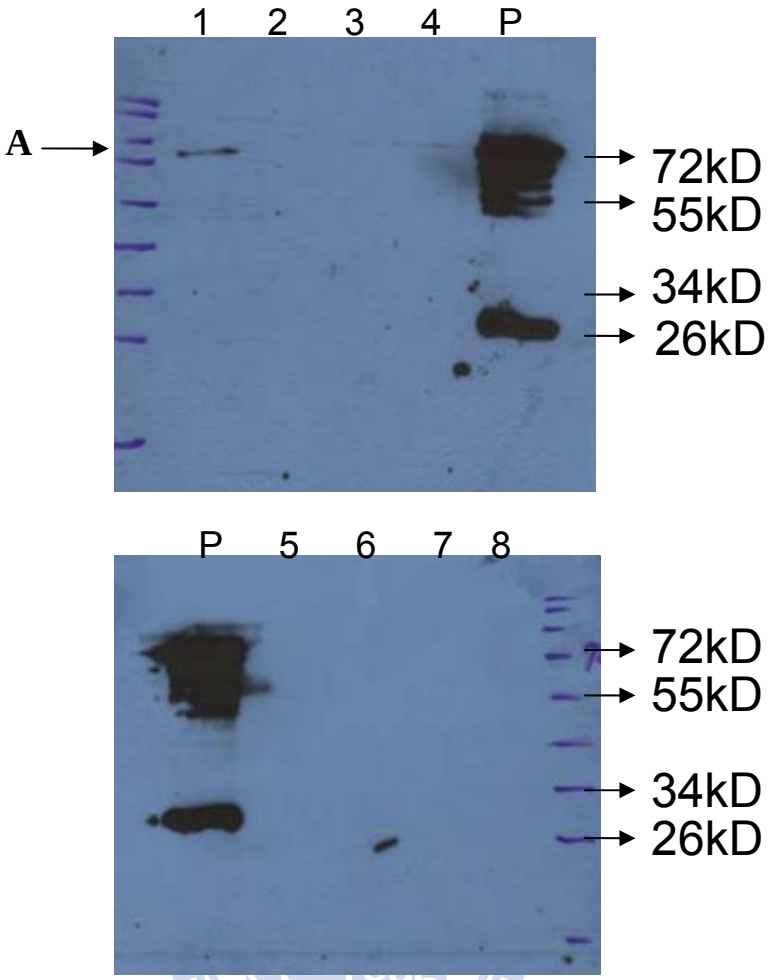


Figure 19. The western blot of concentrated supernatant.

The 5 ml supernatants of cultured media for carrying truncated CaENO1-EGFP fusion gene were concentrated to 250 μl and analyzed by anti-EGFP antibody with western blot analysis. Each lane was loaded 30 μl of protein sample mixture.

P : eno-EGFPp protein sample extracted from cells.

1. eno-EGFPp (~75kDa)
2. protein sample as negative control from 10560-2B-f0
3. EGFP (~26kDa)
4. eno1[1-450]-EGFP p (~43kDa)
5. eno[451-900]-EGFPp (~43kDa)
6. eno[901-1320]-EGFPp(~43kDa)
7. eno1[1-900]-EGFPp(~59kDa)
8. eno1[451-1320]-EGFPp(~58kDa)

A: ~75 kDa protein

M: Prestain Protein marker (Cat. No.0901)

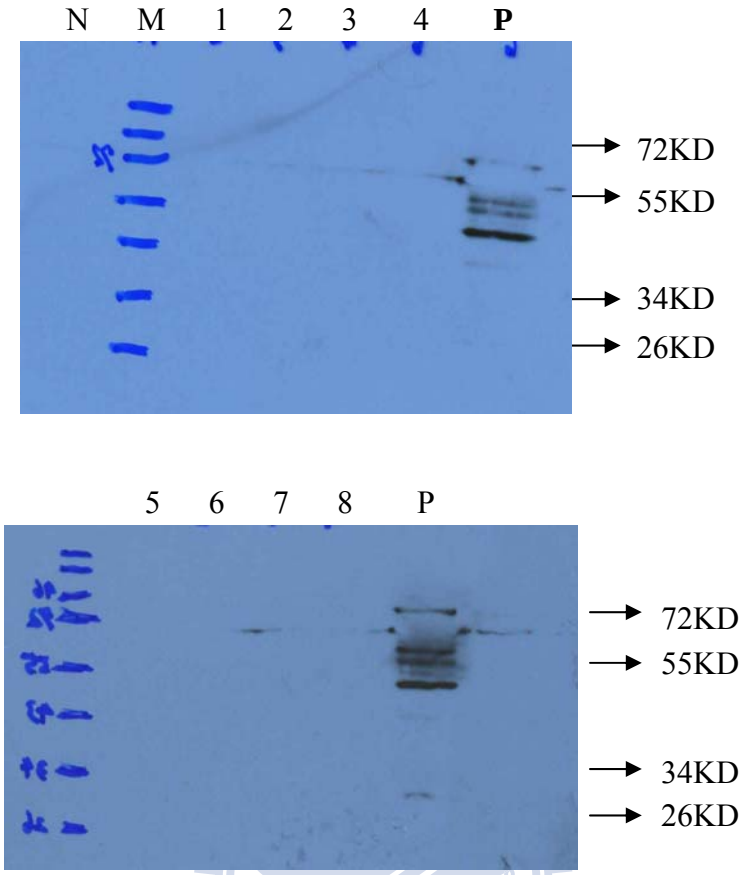


Figure 20. The western blot of concentrated supernatants.

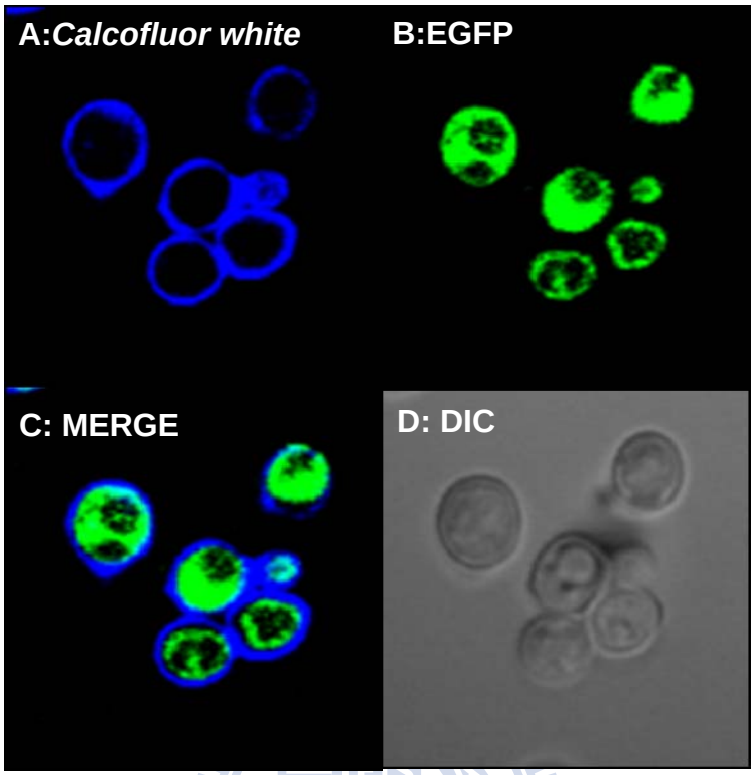
The 50 ml supernatants of cultured media for truncated CaENO1-EGFP fusion gene were concentrated to 250 μl and analyzed by anti-EGFP antibody with western blot analysis. Each lane was loaded 30 μl of protein sample mixture.

P : eno-EGFPp sample extracted from cell.

1. eno-EGFPp (~75kDa).
2. protein sample as negative control, extracted from 10560-2B-f0.
3. EGFP (~26kDa).
4. eno1[1-450]-EGFP p (~43kDa)
5. eno[451-900]-EGFPp (~43kDa).
6. eno[901-1320]-EGFPp(~43kDa)
7. eno1[1-900]-EGFPp (~59kDa).
8. eno1[451-1320]-EGFPp(~58kDa)

M: Prestain Protein marker (Cat. No.0901)

(A)


A:Calcofluor white
B:EGFP
C: MERGE
D: DIC

(B)

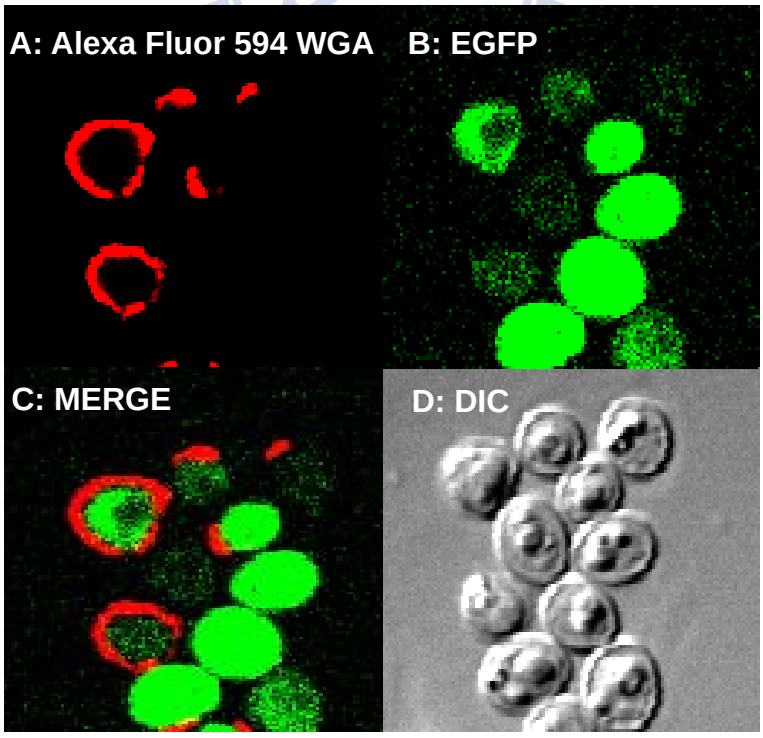

A: Alexa Fluor 594 WGA
B: EGFP
C: MERGE
D: DIC

Figure 21. The localization of CaENO1p in *S. cerevisiae* cell.

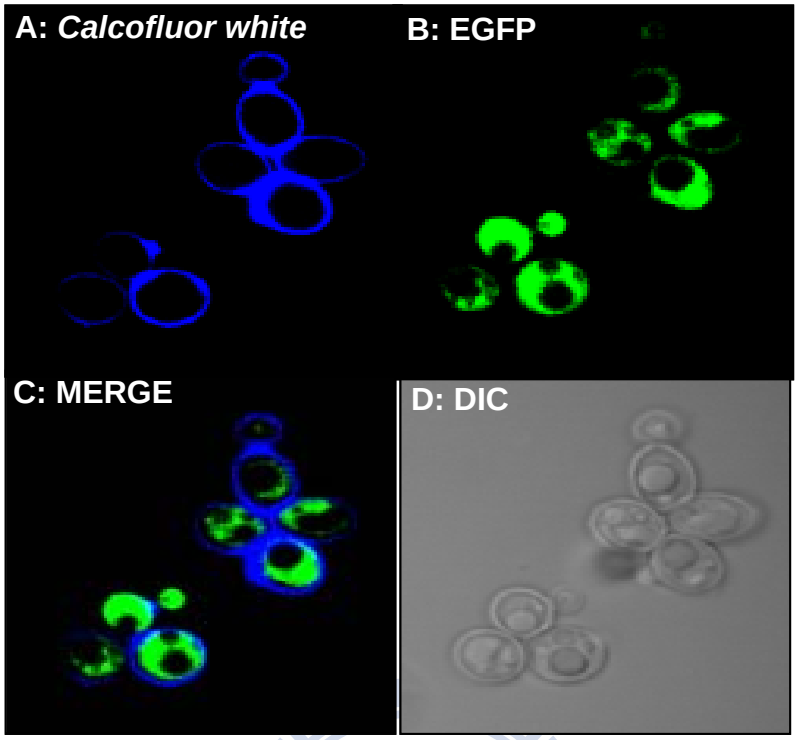
The *S. cerevisiae* cells were stained with Alexa Fluor 594 WGA (which binds to sialic acid and N-acetylglucosaminyl residues) for cell membrane labeling and Calcofluor white (which binds to beta-1,3 and beta-1,4 polysaccharides) for cell wall labeling. The treated cell was fixed on slide to observe under confocal microscope with 1000X magnification.

(A) The transformed cell (10560-2B-f2) stained with Calcofluor white (blue)for cell wall labeling .

(B) The transformed cell (10560-2B-f2) stained with Alexa Fluor 594 WGA (red) for cell membrane labeling.

A: Dye of Alexa Fluor 594 WGA. B: Dye of EGFP. C: A and B merged. D: image of DIC (Differential Interference Contrast)

(A)


A: *Calcofluor white*
B: EGFP
C: MERGE
D: DIC

(B)

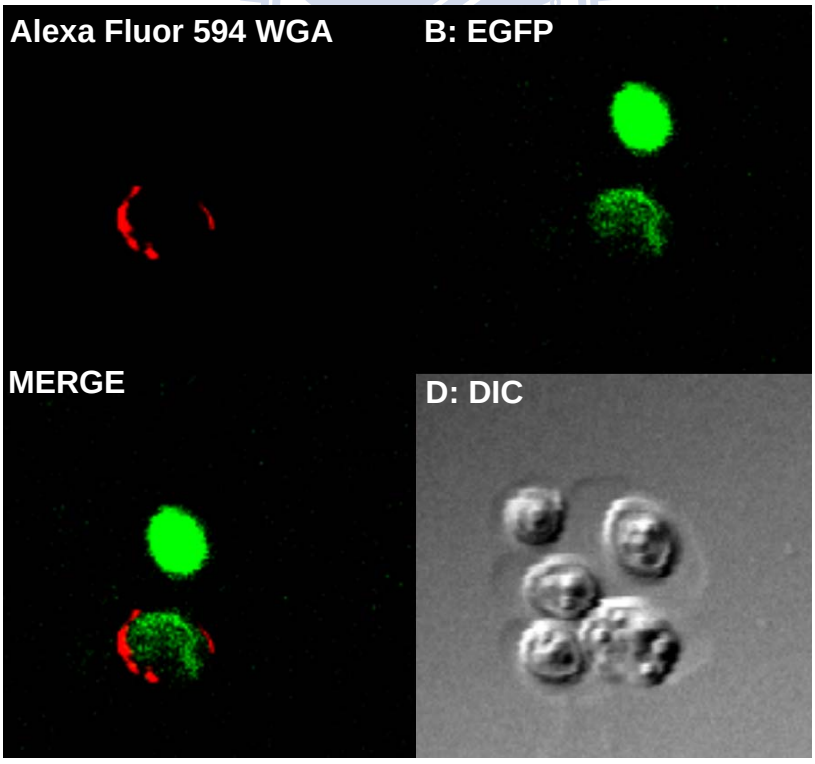

Alexa Fluor 594 WGA
B: EGFP
MERGE
D: DIC

Figure 22. The localization of EGFP in *S. cerevisiae* cell.

The *S. cerevisiae* cells were stained with Alexa Fluor 594 WGA (which binds to sialic acid and N-acetylglucosaminyl residues) for cell membrane labeling and Calcofluor white (which binds to beta-1,3 and beta-1,4 polysaccharides) for cell wall labeling. The treated cell was fixed on slide to observe under confocal microscope with 1000X magnification.

(A) The transformed cell (10560-2B- EGFP) stained with Calcofluor white (blue) for cell wall labeling .

(B) The transformed cell (10560-2B- EGFP) stained with Alexa Fluor 594 WGA (red) for cell membrane labeling.

A: Dye of Alexa Fluor 594 WGA. B: Dye of EGFP. C: A and B merged. D: image of DIC (Differential Interference Contrast)

(A)

```
 Score =  656 bits (1692),  Expect = 0.0
 Identities = 332/436 (76%), Positives = 383/436 (87%), Gaps = 2/436 (0%)

Query  5    TKIHARYVYDSRGNPTVEVDFTTDKGLFRSIVPSGASTGVHEALELRDGDKSKWLGKGVL  64
            +K++AR VYDSRGNPTVEV+ TT+KG+FRSIVPSGASTGVHEALE+RD DKSKW+GKGV+
Sbjct  4    SKVYARSVYDSRGNPTVEVELTTEKGVFRSIVPSGASTGVHEALEMRDEDKSKWMGKGVM  63

Query  65   KAVANVNDIIAPALIKAKIDVVDQAKIDEFLLSLDGTPNKSKLGANAILGVSLAAANAAA  124
             AV NVN++IA A +KA +DV DQ  +D+FLLSLDGT NKSKLGANAILGVS+AAA AAA
Sbjct  64   NAVNNVNNVIAAAFVKANLDVKDQKAVDDFLLSLDGTANKSKLGANAILGVSMAAARAAA  123

Query  125  AAQGIPLYKHIANISNAKKGKFVLPVPFQNVLNGGSHAGGALAFQEFMIAPTGVSTFSEA  184
            A + +PLY+H+A++S +K    +VLPVPF NVLNGGSHAGGALA QEFMIAPTG  TF+EA
Sbjct  124  AEKNVPLYQHLADLSKSKTSPYVLPVPFLNVLNGGSHAGGALALQEFMIAPTGAKTFAEA  183

Query  185  LRIGSEVYHNLKSLTKKKYGQSAGNVGDEGGVAPDIKTPKEALDLIMDAIDKAGYKGKVG  244
            +RIGSEVYHNLKSLTKK+YG SAGNVGDEGGVAP+I+T +EALDLI+DAI  AG+ GKV
Sbjct  184  MRIGSEVYHNLKSLTKKRYGASAGNVGDEGGVAPNIQTAEEALDLIVDAIKAAGHDGKVK  243

Query  245  IAMDVASSEFYKDGKYDLDFKNPESDPSKWLSGPQLADLYEQLISEYPIVSIEDPFAEDD  304
            I +D ASSEF+KDGKYDLDFKNPESD SKWL+G +LAD+Y  L+  YPIVSIEDPFAEDD
Sbjct  244  IGLDCASSEFFKDGKYDLDFKNPESDKSKWLTGVELADMYHSLMKRYPIVSIEDPFAEDD  303

Query  305  WDAWVHFFERVGDKIQIVGDDLTVTNPTRIKTAIEKKAANALLLKVNQIGTLTESIQAAN  364
            W+AW HFF+  G  IQIV DDLTVTNP RI TAIEKKAA+ALLLKVNQIGTL+ESI+AA
Sbjct  304  WEAWSHFFKTAG--IQIVADDLTVTNPARIATAIEKKAADALLLKVNQIGTLSESIKAAQ  361

Query  365  DSYAAGWGVMVSHRSGETEDTFIADLSVGLRSGQIKTGAPARSERLAKLNQILRIEEELG  424
            DS+AA WGVMVSHRSGETEDTFIADL VGLR+GQIKTGAPARSERLAKLNQ+LRIEEELG
Sbjct  362  DSFAANWGVMVSHRSGETEDTFIADLVVGLRTGQIKTGAPARSERLAKLNQLLRIEEELG  421

Query  425  SEAIYAGKDFQKASQL  440
             +A+YAG++F    +L
Sbjct  422  DKAVYAGENFHHGDKL  437
```

(B)

```
 GENE ID: 853169 ENO1 | Eno1p [Saccharomyces cerevisiae] (Over 10 PubMed links)

 Score =  694 bits (1790),  Expect = 0.0, Method: Compositional matrix adjust.
 Identities = 339/436 (77%), Positives = 385/436 (88%), Gaps = 2/436 (0%)

Query  5    TKIHARYVYDSRGNPTVEVDFTTDKGLFRSIVPSGASTGVHEALELRDGDKSKWLGKGVL  64
            +K++AR VYDSRGNPTVEV+ TT+KG+FRSIVPSGASTGVHEALE+RDGDKSKW+GKGVL
Sbjct  4    SKVYARSVYDSRGNPTVEVELTTEKGVFRSIVPSGASTGVHEALEMRDGDKSKWMGKGVL  63

Query  65   KAVANVNDIIAPALIKAKIDVVDQAKIDEFLLSLDGTPNKSKLGANAILGVSLAAANAAA  124
             AV NVND+IAPA +KA IDV DQ  +D+FL+SLDGT NKSKLGANAILGVSLAA+ AAA
Sbjct  64   HAVKNVNDVIAPAFVKANIDVKDQKAVDDFLISLDGTANKSKLGANAILGVSLAASRAAA  123

Query  125  AAQGIPLYKHIANISNAKKGKFVLPVPFQNVLNGGSHAGGALAFQEFMIAPTGVSTFSEA  184
            A + +PLYKH+A++S +K    +VLPVPF NVLNGGSHAGGALA QEFMIAPTG  TF+EA
Sbjct  124  AEKNVPLYKHLADLSKSKTSPYVLPVPFLNVLNGGSHAGGALALQEFMIAPTGAKTFAEA  183

Query  185  LRIGSEVYHNLKSLTKKKYGQSAGNVGDEGGVAPDIKTPKEALDLIMDAIDKAGYKGKVG  244
            LRIGSEVYHNLKSLTKK+YG SAGNVGDEGGVAP+I+T +EALDLI+DAI  AG+ GK+
Sbjct  184  LRIGSEVYHNLKSLTKKRYGASAGNVGDEGGVAPNIQTAEEALDLIVDAIKAAGHDGKIK  243

Query  245  IAMDVASSEFYKDGKYDLDFKNPESDPSKWLSGPQLADLYEQLISEYPIVSIEDPFAEDD  304
            I +D ASSEF+KDGKYDLDFKNP SD SKWL+GPQLADLY  L+  YPIVSIEDPFAEDD
Sbjct  244  IGLDCASSEFFKDGKYDLDFKNPNSDKSKWLTGPQLADLYHSLMKRYPIVSIEDPFAEDD  303

Query  305  WDAWVHFFERVGDKIQIVGDDLTVTNPTRIKTAIEKKAANALLLKVNQIGTLTESIQAAN  364
            W+AW HFF+  G  IQIV DDLTVTNP RI TAIEKKAA+ALLLKVNQIGTL+ESI+AA
Sbjct  304  WEAWSHFFKTAG--IQIVADDLTVTNPKRIATAIEKKAADALLLKVNQIGTLSESIKAAQ  361

Query  365  DSYAAGWGVMVSHRSGETEDTFIADLSVGLRSGQIKTGAPARSERLAKLNQILRIEEELG  424
            DS+AAGWGVMVSHRSGETEDTFIADL VGLR+GQIKTGAPARSERLAKLNQ+LRIEEELG
Sbjct  362  DSFAAGWGVMVSHRSGETEDTFIADLVVGLRTGQIKTGAPARSERLAKLNQLLRIEEELG  421

Query  425  SEAIYAGKDFQKASQL  440
              A++AG++F    +L
Sbjct  422  DNAVFAGENFHHGDKL  437
```

Figure 23. The blast result of enolase between *S.C.* and *C.A.*

(A) Query: CaEno1p, sbjct : ScEno2p

(B) Query: CaEno1p, sbjct : ScEno1p

簡歷

許淑貞

Shiu, Shu - Jen

主要求學歷程:

2006-2008 國立交通大學

生物科技學院 生化工程所

2002-2006 國立高雄大學

生命科學系

1999-2002 私立永年高級中學