Doctoral Dissertation

Breeding of *Saccharomyces cerevisiae* with high RNA content by molecular and genome engineering technology

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CHAPTER 1

General Introduction

1.1 *Saccharomyces cerevisiae* as an important component of industrial biotechnology

Saccharomyces cerevisiae (baker's yeast), an important species of yeast, is one of the premier microorganisms to be used in diversified field of industrial biotechnology. With increasing knowledge about the functionality of *S. cerevisiae*, it became more common to employ them for biotechnological processes. A new industry developed on the principle of using microorganisms as bioreactors – the "White Biotechnology". *S. cerevisiae* is one of the important components of so-called "White Biotechnology" for its attractive qualities. It plays a major role in applied research due to its outstanding capacity to produce ethanol and carbon dioxide from sugars with high productivity. Baking, wine making, brewing, and production of bioethanol constitute the majority of the *S. cerevisiae* biotechnological industry. It is relatively tolerant to low pH values and high sugar and ethanol concentrations, i.e., properties which lower the risk of contamination in industrial fermentation. Moreover, this yeast is resistant to inhibitors present in biomass hydrolysates and is able to grow anaerobically. *S. cerevisiae* also plays a major role in industrial bioethanol production due to its high ethanol productivity as well as its high ethanol tolerance. These have been the major reasons for increasing *S. cerevisiae* exploration in industrial ("white") biotechnology.

S. cerevisiae is the most employed microorganism in food and beverage production. The utilization of *S. cerevisiae* for food production has been part of the cultural evolution of humans for centuries. Early in the history of man, yeasts were used to prepare foodstuffs such as beer, wine, bread and cheese. In the 17th century, Van Leeuwenhoek used his microscope to observe yeast in beer. In the 19th century, Pasteur established that yeasts are living organisms. They

reproduce themselves using simple nutrients such as sugar, nitrogen and phosphorus (Demain et al., 1998). These nutrients present, for example in grape must be used for the production of wine. At the moment many kinds of yeasts are distinguished based on their origin, composition and properties. The industrial production of active (live) yeast, "*S. cerevisiae*", began in 1850. This yeast was used in the production of bread. During the first half of the 20th century, the Germans started to consume inactivated yeast as a food ingredient. Expedient culture technique, easy handling and thereby reaching an unlimited number of cells within short time are major benefits when working with these cells for the production of food and nutrition additives. Many valuable food ingredients are derived from yeast and considered as a safe source of ingredients and additives for food processing (Demain et al. 1998). In 1974, the first commercial yeast extract containing 5'-GMP, a natural nucleotide coming from the yeast RNA, was produced on an industrial scale (Bernett, 2003). 5´- ribonucleotides are used as flavor enhancers in the food industry ((Nagodawithana, 1992; Olmedo et al., 1994). It thereby follows that yeasts with high RNA content, is highly desirable in the food industry.

1.2 Different aspects of ribonucleic acid consumption in food industry with special reference to *S. cerevisiae*

In food industry, there are couples of microorganisms, which are used as source of ribonucleic acid, especially for strong flavor enhancers 5'-ribonucleotides, inosine 5'-monophosphate (5'-IMP) and guanosine 5'-monophosphate (5'-GMP) (Nagodawithana, 1992). Although the direct way to produce inosine and guanosine from carbon source by fermentation using industrial bacteria such as *Bacillus subtilis, Corynebacterium ammoniagenes* to achieve

production of IMP and GMP (Mori et al., 1997; Asahara et al., 2010) is already reported, but consumption of yeast cells is well-known not only for its safety to human health but also part of a normal, healthy human diet (Barnett et al., 2000). A variety of yeast species including *S. cerevisiae*, *S. uvarum*, *S. pastorianus*, *Kluyveromyces fragilis* and *Candida utilis* were used as sources to extract RNA and then decomposed either chemically or by enzymatic method to produce IMP and GMP (Geoffrey, 2009). Among all the yeast species, without any doubt, *S. cerevisiae* is mostly used for various forms of processed yeast products. Yeast extracts are mainly used in the fermentation industry as substrates and in the food industry as flavor enhancers. However, flavor enhancing properties have only been found in 5'-GMP (Edens et al., 2002). By use of adenylic deaminase, adenine-5'-monophosphate (5'-AMP) is converted to inosine-5'- monophosphate (5'-IMP). Yeast extracts containing 5'-IMP have significantly increased flavor enhancing properties (Edens et al., 2002). These products can mask bitterness or sour taste, increase aroma and serve as coloring agents or antioxidants. In 5'- IMP and 5'-GMP enriched foods (ratio of the ribonucleotides: 1: 1), sweet and salty tastes are only slightly enhanced, whereas sour and bitter tastes are suppressed (Dziezak, 1987). The flavor improving properties come from the interaction of various amino acids (the most important one is glutamic acid) in combination with 5' nucleotides, peptides and reaction products. To put it simply, the flavor enhancing effect of 5'- GMP, 5'- IMP and glutamic acid is a continuous stimulation of the receptors in the taste buds which creates a greater sensory potential for flavors (Geoffrey, 2009). Further applications of salt-free yeast extracts containing 5'-nucleotides are children foods, oncological preparations and cancer therapy. The antioxidant properties of yeast extracts come from their contents of glutathione, and sulfur containing amino acids. In future, the demand for

yeast nucleotides with special components to support defined functions/reactions will increase rapidly. In this study, I attempted to construct a yeast strain with higher RNA content, an important source of guanosine and inosine by increasing the transcription of rDNA gene since most cellular RNA originates from rRNA.

1.3 RNA polymerase I mediated rDNA transcription in *S. cerevisiae*

The genes for rRNA (rDNA) in all eukaryotes are transcribed by RNA polymerase I (Pol I). Like other eukaryotic rDNA promoters, the yeast rDNA promoter for the 35S precursor rRNA gene consists of two cis elements, the upstream element and the core element. The upstream element is required for high level transcription whereas the core promoter is essential for accurate transcription initiation (Musters et al.; 1989, Kulkens et al., 1991; Choe et al., 1992). Initiation of yeast rDNA transcription by Pol I uses four factors in addition to Pol I: upstream activation factor (UAF), core factor (CF), TATA-binding protein and Rrn3p (Keener et al., 1998). UAF is a multiprotein transcription factor containing three Pol I-specific proteins, Rrn5p, Rrn9p, an Rrn10p (encoded by *RRN5*, *RRN9*, and *RRN10*, respectively), histones H3 and H4, and protein Uaf30p (Keys et al., 1996; Keener et al., 1997). *In vitro*, UAF interacts with the upstream element of the rDNA promoter, forming a stable UAF template complex and committing the template to transcription. UAF is required for high level transcription mediated by the upstream element (Keener et al., 1998; Keys et al., 1996). CF consists of three proteins; Rrn6p, Rrn7p, and Rrn11p. Rrn3p interacted directly with Pol I and stimulated initiation transcription. This stimulation is meditated through interactions with A43 subunit of Pol I and

with Rrn6p subunit of CF, facilitating binding of Pol I to the promoter. High level of *in vitro* transcription from complete rDNA promoter requires UAF and TATA binding protein (TBP) in addition to Pol I, Rrn3p, and CF. TBP interacts with Rrn6p subunit of CF and Rrn9p subunit of UAF which is essential for the TBP function in Pol I transcription of rDNA *in vivo*. Summary of arrangement of these components within ribosomal gene pre-initiation complex (PIC) is shown in Fig. 1.

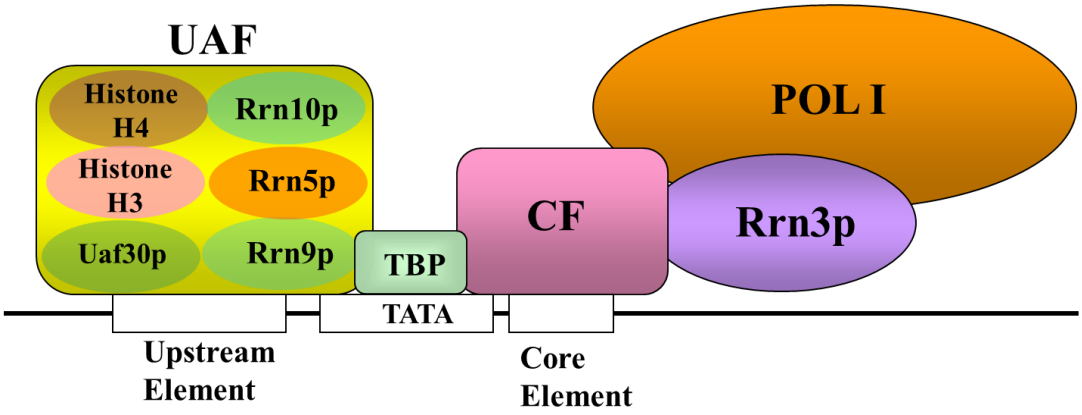

Fig. 1 Pre-initiation complex for transcription of rDNA in S. cerevisiae. UAF locates the promoter and binds stably in a sequence-specific manner. Pol I-Rrn3p and CF of pre-initiation complex is recruited by UAF. TBP is also required for this recruitment but the order of their arrangement is not yet determined. Upon initiation of transcription, pre-initiation complex and TBP leave the promoter. UAF remains bound as a scaffold for further rounds of recruitment and transcription.

1.4 Correlation between rDNA copy number and rDNA transcription in *S. cerevisiae*

The vital role played by ribosomal RNA (rRNA) in protein synthesis is reflected by its ubiquity and high degree of functional and sequence conservation. rDNA repeats are tandemly arranged on chromosome XII and consist of approximately 150 repeated copies of a 9.1 kb unit

of the rDNA gene where a single 9.1 kb unit consists of two transcribed regions, the 35S precursor rRNA and 5S rRNA respectively, as well as two non-transcribed regions, IGS1 and IGS2 (Petes, 1979). The current level of knowledge of rDNA is directly attributable to its high genomic copy number relative to other genes, which has facilitated its use in addressing questions from transcription regulation to phylogenetic. However, most organisms are able to vary the number of repeats of the rDNA gene in response to intracellular as well as extracellular conditions. It is already reported that yeast strains, in which rDNA copy numbers are altered, can be maintained stably and that such strains have been proven to be useful for studies concerning the regulation of rDNA transcription by RNA polymerase I (Nomura, 1999). While the high gene copy numbers observed in eukaryotic rDNA clusters has been interpreted to reflect the demand for large quantities of ribosomes, it is also known, paradoxically, that about half of the rDNA copies are not transcribed in yeast (French et al., 2003). It is also reported that, there is a clear negative correlation between copy number and sensitivity to DNA damaging factors indicate that the damage sensitivity of the cell is determined by rDNA copy number (Kobayashi, 2011). Previously, it was also reported that rDNA transcription in two wild-type strains showed approximately the same level regardless of their 3- to 3.4-fold lower rDNA gene number due to a feedback inhibition of rDNA transcription system that may exist to stabilize rDNA transcription (French et al. 2003, Nomura 2001, Nomura 1999). As a result, it was found that rDNA transcription levels remain constant in spite of a difference of rDNA gene copy number (French et al., 2003; Nomura et al., 1984; David and Peter, 1987). In this study, I aimed to construct a *S. cerevisiae* strain with a high rDNA copy number for improving its RNA content considering the

possibility that increased copy number of the rDNA gene would be effective to increase RNA content in the Δ*rrn10 SUPE* background of *S. cerevisiae*.

1.5 Objectives of this study

According to a very recent report (Chuwattanakul et al.; 2011), it is found that disruption of *RRN10* gene drastically reduced the growth rate and RNA content in *S. cerevisiae* and subsequently seven suppressor (SupA-SupG) strains were isolated by EMS mutagenesis from Δ*rrn10* disruptant strain. In this study, I focused on a suppressor strain, SupE, which showed the ability to suppress growth defect and to recover reduced RNA content. SupE strain harbors dominant and multiple mutations (Chuwattankul et al.; 2011) which is termed collectively as *SUPE*. In this study, I attempted to investigate the mechanism involved in suppressing the growth defect and recovering reduced RNA content by *SUPE* mutation. To elucidate the mechanism of suppression by *SUPE*, a gene library prepared from genomic DNA of SupE strain was introduced into the Δ*rrn10* disruptant strain to screen for the transformants showing faster growth and bigger colony size. After genome sequencing of the transformant, I have obtained a plasmid insert which contain *RPL40A* gene and part of *MLP2* and *SLN1* gene. Sub cloning analysis revealed that, additional copy of *RPL40A* was responsible for the partial suppression of the growth defect caused by Δ*rrn10* disruption. I also found that *SUPE* mutation facilitate the transcription of ribosomal protein (RP) coding genes such as *RPL40A* and *RPL40B*. I also found that increased transcription of *RPL40A* and *RPL40B* not only enhance the growth rate but also increase the rRNA content and total RNA content in the SupE strain.

Furthermore, I also expected that an increased rDNA copy number would be effective at increasing RNA content based upon a previous finding that rDNA transcription increased under a Δ*rrn10 SUPE* background despite not to be changing under a wild-type background (Chuwattanakul et al., 2011). According to this idea, in this study, I constructed a *S. cerevisiae* strain possessing two copies of the rDNA cluster constituting approximately 300 copies of the rDNA gene under a Δ*rrn10 SUPE* background and I found that this strain showed 40% higher RNA content compared to the wild-type strain having two copies as well. Further increases in RNA content by 47% was achieved from the strain by introducing multicopy *RPL40A* genes, thus reaffirming its efficiency. I concluded that this strategy is a useful approach for construction of a yeast strain with higher RNA content.

1.6 Overview of the thesis

The thesis consists of four chapters

Chapter 1 is the general introduction of this research. It contains the background of current research, and review of literature regarding importance of *S. cerevisiae* as industrial microorganism especially in food industry, structural and transcriptional characteristics of ribosomal DNA (rDNA) and their coordination for construction of an industrially important *S. cerevisiae* strain.

Chapter 2 describes the strategy for increasing the RNA production in yeast by elucidating the mechanism responsible for suppression of defect caused by disruption of an important

constituent of UAF, named *RRN10*. The results indicated that by increasing transcription of *RPL40A* and *RPL40B*, it is possible to improve RNA production in *S. cerevisiae*.

Chapter 3 describes a mechanism for further increase of RNA content by double copying of rDNA cluster constructed using PCR mediated splitting method. The results indicated that high rDNA copy number is effective to increase rDNA transcription thereby increase total RNA content in suppressor background of Δ*rrn10* disruptant of *S. cerevisiae*.

Chapter 4 summarizes the results obtained in this research and discusses further scope of this research.

2. CHAPTER 2

Increased transcription of *RPL40A* and *RPL40B* is important for the improvement of RNA production in *Saccharomyces cerevisiae*

2.1 Introduction

RNA has the potential for many beneficial uses. It is an attractive source of 5´ ribonucleotides that can be used as flavor enhancers in the food industry (Nagodawithana, 1992; Olmedo et al., 1994; Boekhout and Robert, 2003). It has also become an interesting dietary source of pyrimidines and purines for human immune functions (Usami and Saitoh, 1996; Julian and Antonio, 2004). In addition, RNA and ribonucleotides are used in various medical fields, for example, for the improvement of energy metabolism in patients with liver and heart disease (Usami and Saitoh, 1996; Julian and Antonio, 2004), and for cancer and antiviral therapies (Perigaud et al., 1992). Therefore, an organism, with high RNA content, such as yeast is highly desirable as a source of RNA.

S. cerevisiae contains three species of RNAs, rRNA, tRNA and mRNA. Among these species, rRNA is our main target for increasing RNA content because it comprises 80% of the total RNA (Warner, 1999). It hereby follows that, efficient transcription of rDNA is very important for the construction of a yeast strain with high RNA content (Nomura et al., 1985). Transcription of the rDNA gene in yeast begins with the formation of a Polymerase I (Pol I) pre-initiation complex at the promoter, and requires four major transcription factors: upstream activation factor (UAF), core factor (CF), TATA-binding protein (TBP), and Rrn3p (Nomura et al., 2004). UAF contains six subunits, i.e., *RRN5*, *RRN9*, *RRN10*, Uaf30p, and histones H3 and H4, and it directly associates with a region of rDNA located ~100 bp upstream of the transcription initiation site called the upstream element (UE) (Keener et al., 1997). CF is a complex of three subunits, i.e., *RRN6*, *RRN7* and *RRN11*, and centrally localizes to the core element of the promoter (Bedwell et al., 2011). TBP interacts with both UAF and CF, bridging

the two factors (Bedwell et al., 2011). Rrn3p is involved in recruitment of Pol I to the rDNA cluster (Bedwell et al., 2011).

. Rrn10p is a structural constituent of UAF and an important protein in the rDNA transcription process. The transcript level of rRNA is drastically reduced, and growth is markedly slowed down by the disruption of *RRN10* (Chuwattanakul et al., 2011). By using EMS mutagenesis, seven suppressor (SupA to SupG) strains were isolated that showed the ability to suppress the growth defects and recover the reduced RNA content in the Δ*rrn10* disruptant strain (Chuwattanakul et al., 2011). In this study, I concentrated on the SupE strain. Suppressor mutations in SupE strain are dominant and consist of multiple mutations (Chuwattanakul et al., 2011) collectively designated here as *SUPE*. To uncover the mechanism of suppression against the defect in Pol I transcription of rRNA genes in the Δ*rrn10* disruptant, the genomic library of the SupE strain was introduced into the Δ*rrn10* disruptant strain to screen for transformants showing faster growth and bigger colony size. Subcloning analysis indicated that *RPL40A*, which encodes a protein of the large (60S) ribosomal subunit (Lecompte et al., 2002), was responsible for the partial suppression of the growth defects caused by Δ*rrn10* disruption. I also found that *SUPE* mutation has a positive effect in increasing the transcription of RP genes such as *RPL40A* and *RPL40B*, and this increased transcription was found to have an important role not only in enhancing the growth rate but also in producing RNA in the Δ*rrn10* disruptant and SupE strain.

2.2 Materials and Methods

2.2.1 Strains and oligonucleotide primers

The *S. cerevisiae* strains used in this study are listed in Table 1. The yeast strain SH6976 (*rrn10*::Cg*LEU2 SUPE*), which was termed as SupE strain, was used to analyze the suppression mechanism of the growth defect caused by the disruption of *RRN10*. SupE strain was selected for its ability to suppress the slow growth defect of the Δ*rrn10* disruptant (SH6789) strain, although the segregation pattern of tetrads obtained from the cross between SH6789 and SH6976 did not show a single nuclear mutation (Chuwattanakul et al., 2011). *E .coli* strain DH5α was used as a host for plasmid preparation. The oligonucleotide primers used in this study are shown in Table 2.

Table 1. Strains and plasmids used in this chapter

Strain	Genotype	Remarks
S. cerevisiae		
SH6446	*MAT*a *ura3-52 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 Δfob1::HIS3*	Kim et al., 2006
SH6789	*MAT*α *ura3-52 or ura3Δ851 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 Δfob1::HIS3 Δrrn10::CgLEU2*	Chuwattanakul et al., 2011
SH6812 (SupE)	*MAT*α *ura3-52 or ura3Δ851 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 Δfob1::HIS3 Δrrn10::CgLEU2 SUPE*	Chuwattanakul et al., 2011
SH6976 (SupE)	*MAT*a *ura3-52 or ura3Δ851 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 Δrrn10::CgLEU2 SUPE*	*MAT**a*** convertant of SH6812, transformed with *HO* gene
E.coli	*φ80dlacZΔM15, recA1, endA1, gyrAB, thi-1,*	NBRP-Yeast, Japan[a]

DH5α	*hsdR17(r_k-,m_k+), supE44, relA1, deoR, Δ(lacZYA-argF)U169, phoA*	
Plasmid		
p560 (pRS314)	YCp+*TRP1*	NBRP-Yeast, Japan[a]
p562 (pRS316)	YCp50+*URA3*	Sikorski and Hieter, 1989
p617	YEp+*URA3*	NBRP-Yeast, Japan[a]
p1498	YCp+*HO+TRP1+LYS2*	NBRP-Yeast, Japan[a]
p3371	YCp50+*URA3*+Part of chromosome IX containing *RPL40A* gene and part of *MLP2* and *SLN1* gene	This study
p3372	YCp50+*URA3*+ partial fragment of *MLP2* and *SLN1* gene	This study
p3373	YCp50+*URA3+RPL40A*	This study
p3374	YEp+*URA3+RPL40A*	This study
p3398	YCp+*TRP1+RRN10*	This study
p3399	YEp+*URA3+RPL40B*	This study

[a]National Bio-Resource Project-Yeast, Japan: http://yeast.lab.nig.ac.jp/nig/index_en.html.

Table 2. Oligonucleotide primers used in this chapter

Name	Sequence (5'- 3')
T7	GTAATACGACTCACTATAGGGC
RRN10 cf-R2	GTCCATCTTTACAGTCCTGT
ACT1-F	CGCTCCTCGTGCTGTCTTC
ACT1-R	TTGACCCATACCGACCATGATA
18S-F	CCTGAGAAACGGCTACCA
18S-R	ATTGTCACTACCTCCCTGAATTAAGGA

PGK1-F	AATCGGTGACTCCATCTTCG
PGK1-R	GTGTTGGCATCAGCAGAGAA
GAPDH-F	AGACTGTTGACGGTCCATCC
GAPDH-R	CCTTAGCAGCACCGGTAGAG
RT-RPL40A-F	GGCTTCCAAGTACAACTGTGACAA
RT-RPL40A-R	GCTCTTGGTGGCAATCTAGCA
RT-RPL40B-F	AATCCACTTTACATTTGGTCTTGAGA ’
RT-RPL40B-R	GCCAAGGCTTTCAAAGATGGT
RT-RPL5-F	CGGTGCTCTAAAGGGTGCTT
RT-RPL5-R	CCCAACCTGGGAATCTGTTTT
RT-RPS31-F	GGTAAGCAATTGGAAGATGGTAGAA
RT-RPS31-R	CCACCACCTCTCAATCTCAAGAC
SEQ-RPL40A-F1	TCCCATAGTTGAGACGACCAAGATTCAAAC
SEQ-RPL40A-R1	AAATGTATAGATTGATTGGGCGAAACAGAT
SEQ-RPL40A-F2	CTCATTGCCTAAGAAATATCCAAATTTGTG
SEQ-RPL40A-R2	TCAGGTGGGATACCTTCCTTGTCTTGAATC
SEQ-RPL40A-F3	GATTCAAGACAAGGAAGGTATCCCACCTGA
SEQ-RPL40A-R3	CACAAATTTGGATATTTCTTAGGCAATGAG
SEQ-RPL40A-PF1	TCGATCAACTCTATCCAACAATTCTATAAT
SEQ-RPL40A-PR1	GTTTGAATCTTGGTCGTCTCAACTATGGGA
SEQ-RPL40A-PF2	CATAGGCGGAGCATATTCCTCCTATGGGAT
SEQ-RPL40A-PR2	TCCAGGCGGAGTGCTAAGAGGTTCCGCATC
SEQ-RPL40A-TF	ATCTGTTTCGCCCAATCAATCTATACATTT
SEQ-RPL40A-TR	GCGTTGACTTATATGCATAATATACAAATA
DR-RPL40A-F	TATTACCGCTTATTATCCCATAGTTGAGACGACCAAGATTCAA ACCTTCGTACGCTGCAG
DR-RPL40A-R	AAGTATACAGTAAATAAATGTATAGATTGATTGGGCGAAACA GATGCCACTAGTGGATCT

CDR-RPL40A-F	CTCTCTAGACAGGACCTCCG
CDR-RPL40A-R	CCACCGCTACGCGTTGACTT
DR-RPL40B-F	CACTTTTTCCCGTTCAGCAAGAGGTAAAGCCACCAAAGGTTCAAACTTCGTACGCTGCAG
DR-RPL40B-R	GTGATGTATACAAACTTTGGATTTGTGGAGATCGTAATAAATCGAGCCACTAGTGGATCT
CDR-RPL40B-F	TGAAGTACCGCAATAGGATA
CDR-RPL40B-R	GTCCTCTTCAGGCTCACTGT
SUPE-RPL40A-F1	CTCCTTAAGTCGATCAACTCTATCCAACA (Underlined represent the recognition sequence for *Afl*II)
SUPE-RPL40A-R1	CTCCTTAAGCCACCGCTACGCGTTGACTT (Underlined represent the recognition sequence for *Afl*II)
SUPE-RPL40A-F2	CTCGGATCCTCGATCAACTCTATCCAACA (Underlined represent the recognition sequence for *BamH*I)
SUPE-RPL40A-R2	CTCGAATTCCCACCGCTACGCGTTGACTT (Underlined represent the recognition sequence for *EcoR*I)
SUPE-RPL40B-F2	CTCGGATCCGGGCAATGCA ATTTGGCGTT (Underlined represent the recognition sequence for *BamH*I)
SUPE-RPL40B-R2	CTCGAATTC GTCCTCTTCAGGCTCACTGT (Underlined represent the recognition sequence for *EcoR*I)
YCp50NruI-F1	GACGCGCTGGGCTACGTCTTGCTGGCGTTC
YCp50NruI-R1	GCGAGAAGAATCATAATGGGGAAGGCCATC
RRN10-ORF-F	CTCGGTACCGAATTATGCAGTCTAT
RRN10-ORF-R	CTCGAGCTCCCTTATAGTGTATTCT

2.2.2 Culture conditions, media preparation and transformation

Yeast strains were grown in either a YPDA medium or a synthetic complete (SC) medium as previously described (Amberg et al., 2005) and were cultured at 30°C. *E. coli* strains were cultured at 37°C in a LB medium as previously described (Sambrook and Russel, 2000). Yeast transformation was carried out by the method of high efficiency transformation (Amberg et al., 2005), and the Z-competent *E. coli* transformation kit system (Zymo Research, USA) was used for *E. coli* transformation. In the cloning experiment, cells were cultured in a SC medium without uracil at 37°C to screen for yeast transformants.

2.2.3 Plasmid construction

Plasmid p3371 was constructed by using the insert isolated from a transformant containing the *RPL40A* gene and part of the *MLP2* and *SLN1* genes. After restriction digestion of p3371 by *Afl*II and removal of the *RPL40A* gene with its 500-bp upstream and 199-bp downstream regions, the remaining part of the plasmid was self-ligated and designated as p3372 (YCp+*URA3*+partial fragment of *MLP2* and *SLN1* gene). Plasmid p3373 (YCp+*URA3*+*RPL40A*) was constructed by ligating an *Afl*II-linearized pRS316 vector (Sikorski and Hieter, 1989) with a PCR fragment of the *RPL40A* gene with its 500-bp upstream and 199-bp downstream regions amplified using SUPE-RPL40A-F1 and SUPE-RPL40A-R1 as primers and p3371 as a template (Table 2). p3374 (YEp+*URA3*+*RPL40A*) was constructed by cloning a fragment of *RPL40A* gene amplified using plasmid p3371 as a template and SUPE-RPL40A-F2 and SUPE-RPL40A-R2 as primers (Table 2) into a *Bam*HI-*Eco*RI linearized p617 vector. Plasmid p3398 (YCp+*TRP1*+*RRN10*) was constructed by ligating a SmaI-linearized pRS314

vector (16) with a PCR product (1.5 kb) harboring the *RRN10* gene that was amplified using genomic DNA of SH6446 as a template and RRN10-ORF-F and RRN10-ORF-R as forward and reverse primers, respectively (Table 2). Plasmid p3399 (YEp+*URA3*+*RPL40B*) was constructed by cloning a fragment of *RPL40B* gene amplified using genomic DNA of SH6976 as a template and SUPE-RPL40B-F2 and SUPE-RPL40B-R2 as primers (Table 2) into a *Bam*HI-*Eco*RI linearized p617 vector.

2.2.4 Construction of the genomic library, cloning, and sequencing

A genomic library of the SupE (SH6976) strain was constructed by Takara Bio Inc., Shiga. Genomic DNA of SH6976 was randomly sheared mechanically and fractioned by agarose gel electrophoresis. DNA fragments greater than 5 kb were purified from the agarose gel and were treated with T4 DNA polymerase to convert both ends into blunt ends. The blunt-ended DNA fragments were ligated with bacterial alkaline phosphatase (BAP)-treated YCp50, a centromere-based vector that had been digested by *Nru*I with *URA3* as a marker. The ligation mixture was introduced into *E. coli* DH10B competent cells (Invitrogen) by electroporation with a GenePulser (Bio-Rad Laboratories Inc.). Cells of ampicillin-resistant colonies were scraped off and pools of plasmid DNAs were prepared. The Δ*rrn10* disruptant strain (SH6789) showing the growth defect phenotype was used as the host. Sequencing was performed as described in the protocol of the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem).

2.2.5 rRNA quantification and measurement of total RNA

For RNA isolation, yeast cells were collected when the Optical Density (OD) at 660nm reached 1.0. For the preparation of cDNA, extracted RNA was reverse transcribed using a High Capacity cDNA Archive kit (Applied Biosystems) following the manufacturer's instructions. rRNA was quantified in triplicates by quantitative real-time PCR using SYBR green PCR Master-Mix (Applied Biosystems) and an Applied Biosystems 7300 real-time PCR system (Applied Biosystems. For each strain, RNA was independently extracted three times by the previously described method (Hermansyah et al., 2009). Relative mRNA levels were normalized to *ACT1* mRNA levels. The primers 18S-F and 18S-R (Table 2) were used to detect 18S rRNA. To detect *ACT1* mRNA as an internal control, ACT1-F and ACT1-R (Table 2) were used as primers. Total RNA from each strain was measured by the Perchloric Acid (PCA) method as previously described (Chuwattanakul et al., 2011).

2.3 Results

2.3.1 Identification of *RPL40A* as a single-copy suppressor of the defect caused by the disruption of *RRN10*

To elucidate the molecular mechanism underlying suppression of the defect in pol I transcription of rRNA genes in the Δ*rrn10* disruptant strain, a genomic library (insert size is approximately 5kb) of the SupE strain (SH6976) was constructed using the single-copy vector YCp50. The library's DNA was introduced into the Δ*rrn10* disruptant (SH6789) strain, and Ura^+ transformants were screened on uracil-depleted plates supplemented with essential amino acids. The plates were incubated at 37°C for 7 days. Of the approximately 100,000

transformants that were screened, initially 50 candidates were drawn on the basis of faster growth and bigger colony size as compared with the host Δ*rrn10* disruptant. Among these 50 candidates, 3 clones were finally selected that showed significantly faster growth and bigger colony size. The plasmid was extracted from each of these 3 clones and subjected to restriction analysis. All 3 plasmids showed the same restriction pattern; as a result, one of these plasmids was designated as p3371 and used in the subsequent work.

The Δ*rrn10* disruptant strain (SH6789) was retransformed with p3371, and cells of the retransformant were streaked on uracil-deficient selection plates for comparison with the host Δ*rrn10* disruptant strain for the growth phenotype. The growth phenotype of this strain was shown in Fig. 2A. The plasmid was then extracted from the retransformant, where the insert was subjected to sequencing. Sequencing analysis indicated that the insert consisted of a sequence from chromosome IX containing the gene *RPL40A* and part of two other genes, *MLP2* and *SLN1* (Fig. 2B). *RPL40A* encodes a fusion protein consisting of ribosomal 60S subunit protein Rpl40Ap-ubiquitin; in which ubiquitin serves as a chaperon in assembly of the ribosomal protein into ribosomes (Lecompte et al., 2002). Mlp2p is a myosin-like protein that is associated with the nuclear envelope and is involved in the regulation of telomere length (Hediger et al., 2002). Lastly, Sln1p is a histidine kinase osmosensor that regulates the MAP kinase cascade (Reiser et al., 2003).

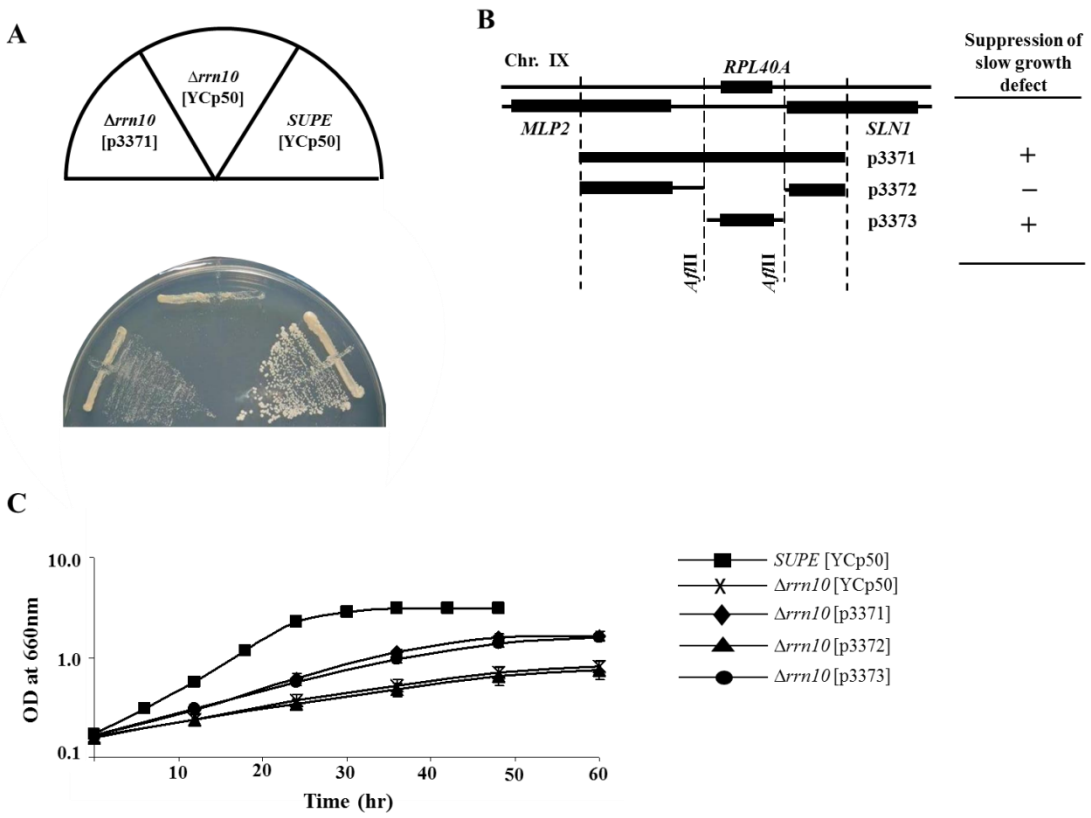

Fig. 2 Identification of *RPL40A* as a suppressor of the Δ*rrn10* disruptant strain. (A) Comparison of growth phenotype among strains having Δ*rrn10* [YCp50], *SUPE* [YCp50] and Δ*rrn10* [p3371] backgrounds. Photograph was taken after 7 days of incubation at 37ºC on an SD-Ura plate. (B) Structural outline of the plasmid insert. The insert consisted of a sequence from chromosome IX containing the gene *RPL40A* and parts of two other genes, *SLN1* and *MLP2*. Black box represents the ORF of the respective gene. Three plasmids p3371, p3372 and p3373, were constructed using different regions of the plasmid insert. Plasmid p3371 contained the whole plasmid insert, p3372 contained part of the *MLP2* and *SLN1* genes, and p3373 contained the *RPL40A* gene with its 500-bp upstream and 199-bp downstream region. (C) Growth phenotype of the strains having Δ*rrn10* [YCp50], *SUPE* [YCp50], Δ*rrn10* [p3371], Δ*rrn10* [p3372] and Δ*rrn10* [p3373] backgrounds. Growth rate was monitored at OD660 nm. Three independent experiments using 3

independent samples were performed and mean±SD values are presented. Filled square represents *SUPE* [YCp50], cross-mark represents Δ*rrn10* [YCp50], filled diamond represents Δ*rrn10* [p3371], filled triangle represents Δ*rrn10* [p3372], and filled circle represents Δ*rrn10* [p3373] strains.

To delimit the gene(s) involved in restoring growth of the Δ*rrn10* disruptant (SH6789) strain, two plasmids were constructed, one containing the *MLP2* and *SLN1* genes (p3372) with the other only containing *RPL40A* (p3373) and these were separately introduced into the Δ*rrn10* disruptant (SH6789) strain. The growth phenotype of these transformants was analyzed by culturing transformants in 50 ml of liquid SD-Ura media at 37°C. Comparison of the growth phenotype revealed that the Δ*rrn10* disruptant strain which was transformed with part of *MLP2* and *SLN1* genes (p3372) showed growth rate similar to that of the Δ*rrn10* disruptant strain. This result suggested that *MLP2* and *SLN1* genes are not involved in restoration of the growth of the Δ*rrn10* disruptant strain. By contrast, introduction of plasmid p3373 harboring the *RPL40A* gene into the Δ*rrn10* disruptant strain led to faster growth as compared with the Δ*rrn10* disruptant, and the growth behavior was similar to that of the Δ*rrn10* disruptant transformed with plasmid (p3371) harboring all three genes (Fig. 2C). These observations suggested that the *RPL40A* gene plays an important role in restoring the growth defect of the Δ*rrn10* disruptant strain.

To determine whether there was a base change in the *RPL40A* gene originating from SupE strain, the sequence of the cloned *RPL40A* gene was compared with that of *RPL40A* from the Δ*rrn10* disruptant (SH6789) strain. I did not find any base changes in sequencing even though introduction of *RPL40A* led to suppression of the slow growth defect of the Δ*rrn10* disruptant (SH6789) strain. This result suggested that the double copies of the *RPL40A* gene

present in transformants of the Δ*rrn10* disruptant might improve the growth rate of the Δ*rrn10* disruptant strain.

2.3.2 A double copy of the *RPL40A* gene can increase RNA content in disruptant and suppressor strains

To explore an idea described in previous section that suppression of the growth defect of the Δ*rrn10* disruptant was caused by introduction of an additional copy of *RPL40A* (Figs. 2C, 3A), I investigated the effect of a double copy of the *RPL40A* gene on the growth and RNA content of the SupE and wild-type strains. To confer the effect of an additional copy of *RPL40A*, I introduced the single-copy plasmid p3373 (YCp50+*URA3*+*RPL40A*) into these strains. When the growth rate of the transformant of SupE and wild-type strains harboring p3373 was measured, the SupE transformant with a double copy of the *RPL40A* gene showed a growth rate faster than that of SupE strain with a single copy of *RPL40A*, while the wild-type transformant did not show any change in growth rate (Fig. 3B).

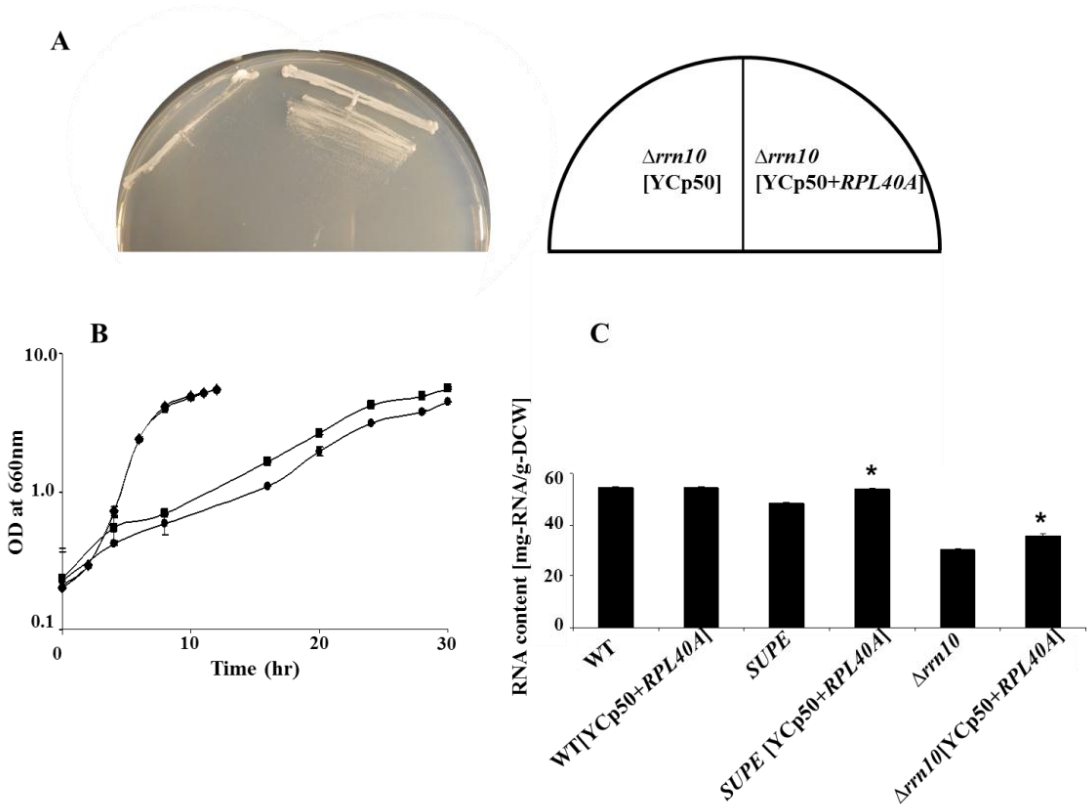

Fig. 3 Effect of an additional copy of *RPL40A* on the growth and RNA content of wild-type, SupE and Δ*rrn10* disruptant strains. (A) Comparison of growth phenotype between Δ*rrn10* [YCp50+*RPL40A*] and Δ*rrn10*[YCp50] strains. Photograph was taken after 6 days of incubation at 37ºC on SD-Ura plate. (B) Comparison of growth rate among strains having wild-type (WT), WT [YCp50+*RPL40A*], SUPE, and SUPE [YCp50+*RPL40A*] background. Growth rate was monitored at OD660 nm. Three independent experiments using 3 independent samples were performed and mean±SD values are presented. Cross-mark represents wild-type, filled square represents WT [YCp50+*RPL40A*], asterisk represents *SUPE*, and filled triangle represents *SUPE* [YCp50+*RPL40A*] strains. (C) Total RNA content of strains having wild-type, WT [YCp50+*RPL40A*], *SUPE*, *SUPE* [YCp50+*RPL40A*], Δ*rrn10* and Δ*rrn10* [YCp50+*RPL40A*] backgrounds. Cells were collected at OD660=1.0. Total RNA of each strain was extracted by the perchloric acid method (PCA) as described in Materials and methods. Statistical analysis was performed by one-way ANOVA test with IBM SPSS ver.21 Software (IBM, US). Asterisks (*) indicate significantly different group means at an alpha level of 0.05. Each experiment was repeated individually three times starting from three independent starter cultures, and

the mean of the three samples was compared among groups. Tukey test was performed as a post-hoc range test.

I also measured the RNA content of the Δ*rrn10* disruptant, SupE and wild-type strains with a single copy (resident) and a double copy (resident and introduced) of *RPL40A*. The single copy SupE strain displayed 11% lower RNA content as compared to the wild-type strain whereas RNA content of the Δ*rrn10* disruptant strain was 44% lower than that of the wild-type strain. In the case of the Δ*rrn10* disruptant and SupE strains containing double copies of *RPL40A*, RNA content was increased 18% (*$p<0.05$) in the Δ*rrn10* disruptant strain and 11% (*$p<0.05$) in the SupE strain in comparison to their counterparts which had a single copy. On the other hand, wild-type strain with a double copy of *RPL40A* did not show a significant change in RNA content (Fig. 3C) as compared with the wild-type strain containing a single copy of *RPL40A*. The above findings reveal that a double copy of the *RPL40A* gene suppresses not only the growth defect but also increases RNA content of both the Δ*rrn10* disruptant and SupE strain without showing any effect on the wild-type strain.

2.3.3 A multicopy plasmid containing *RPL40A* further increases the RNA content of the Δ*rrn10* disruptant and SupE strains on a *RRN10*$^+$ background

Because a double copy of the *RPL40A* gene had the effect of suppressing the growth defect and recovering the reduced RNA content of the Δ*rrn10* disruptant and SupE strain harboring *rrn10* disruption mutation, I investigated whether a further increase in the copy number of *RPL40A* would lead to a faster growth rate and higher RNA content. For this purpose, I introduced a multicopy plasmid, p3374 [YEp+*URA3*+*RPL40A*] into the Δ*rrn10* disruptant, SupE and wild-type strains. Since *RPL40A* has a paralog, *RPL40B*, (*Saccharomyces* Genome

Database), I became interested in investigating the effect of *RPL40B* on the growth rate, on the transcription levels of *RPL40A* and *RPl40B*, and on the total RNA content. For this purpose, I introduced the multicopy plasmid p3399 [YEp+*URA3*+*RPL40B*] into the Δ*rrn10* disruptant, SupE and wild-type strains.

First, I compared growth performance among the Δ*rrn10* disruptant strains harboring single copy of *RPL40A* and *RPL40B* against the Δ*rrn10* disruptant strains transformed with multicopies of *RPL40A* and *RPL40B*, respectively. I observed that transformants with either multicopies of *RPL40A* or multicopies of *RPL40B* showed better growth phenotypes as compared with the Δ*rrn10* disruptant strains harboring single copy *RPL40A* and *RPL40B*, respectively (Fig. 4A). I then examined the transcription level of *RPL40A* and found that the transcription level of *RPL40A* was significantly increased by 30% (*$p<0.05$) in the Δ*rrn10* disruptant and SupE strains with multicopies of *RPL40A* compared to the Δ*rrn10* disruptant and SupE strain having a single copy. On the other hand, I did not observe any significant change in *RPL40A* transcription in wild-type strain (Fig. 4B). Furthermore, I measured the transcription level of *RPL40A* in the Δ*rrn10*, SupE and wild-type strains having multicopies of *RPL40B*. I did not observe any change in the transcription level of *RPL40A* gene whereas transcription level of *RPL40B* was significantly increased in the Δ*rrn10* disruptant and SupE strain while, again, wild-type strain did not show any significant difference. (Figs. 4B, 5).

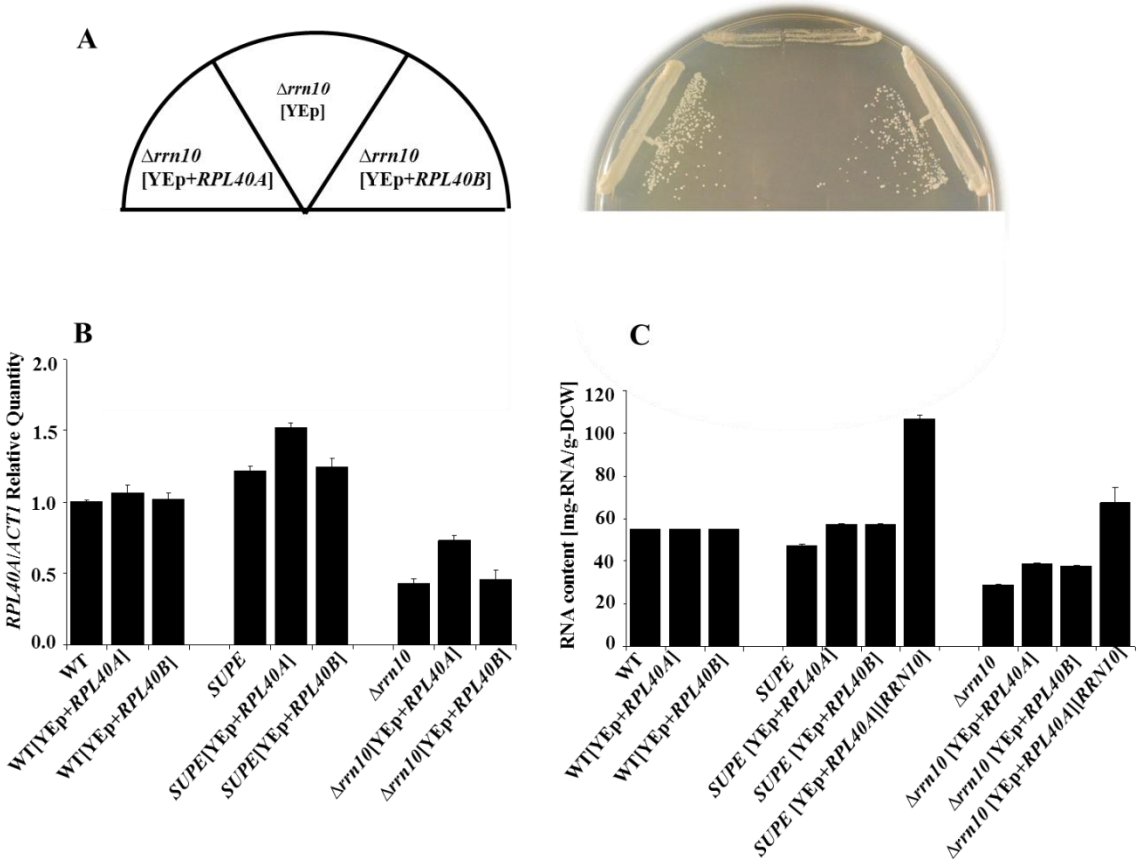

Fig. 4 Effect of the multicopy RPL40A gene on wild-type, SupE and Δ*rrn10* disruptant strains. (A) Comparison of growth phenotype between strains having Δ*rrn10*[YEp], Δ*rrn10*[YEp+*RPL40A*] and Δ*rrn10*[YEp+*RPL40B*] background. Photograph was taken after 4 days of incubation at 37ºC on SD-Ura plate. (B) Measurement of the mRNA level of *RPL40A* in strains having wild-type [WT], WT[YEp+*RPL40A*], WT[YEp+*RPL40B*], *SUPE*, *SUPE*[YEp+*RPL40A*], *SUPE*[YEp+*RPL40B*], Δ*rrn10*[YEp], Δ*rrn10*[YEp+*RPL40A*] and Δ*rrn10*[YEp+*RPL40B*] backgrounds. Cells were collected at OD660=1.0 and RNA was extracted as described in Materials and Methods. *RPL40A* transcript was measured by real-time RT-PCR using *ACT1* as an endogenous control. Statistical analysis was performed as described in Fig. 3C. (C) Total RNA content of strains having wild-type (WT), WT[YEp+*RPL40A*], WT[YEp+*RPL40B*], *SUPE*, *SUPE*[YEp+*RPL40A*], *SUPE*[YEp+*RPL40B*], *SUPE*[YEp+*RPL40A*][*RRN10*], Δ*rrn10*, Δ*rrn10*[YEp+*RPL40A*], Δ*rrn10*[YEp+*RPL40B*] and Δ*rrn10*[YEp+*RPL40A*][*RRN10*] backgrounds. The experiment and statistical analysis were performed as described in the legend of Fig. 3C. Asterisks (*) indicate significantly different group means at an alpha level of 0.05.

Next, total RNA content of the wild-type, Δ*rrn10* disruptant and SupE strains, each harboring a single or a multicopy of *RPL40A*, or *RPL40B* was measured. The results revealed that the Δ*rrn10* disruptant strain with multicopy *RPL40A* and *RPL40B* showed 32% (*p<0.05) and 30% (*p<0.05) higher RNA content, respectively against the Δ*rrn10* disruptant strains with single copy of *RPL40A* and *RPL40B*. On the other hand, SupE strain with multicopies of *RPL40A* and *RPL40B* showed 21% (*p<0.05) and 20% (*p<0.05) higher RNA content, respectively against other SupE strains with single copy of *RPL40A* and *RPL40B* gene (Fig. 4C).

In this part of the study, the effect of multicopy *RPL40A* into the Δ*rrn10* disruptant and SupE strains under $RRN10^{+}$ background was investigated based on a previous findings that introduction of *RRN10* gene into the Δ*rrn10* disruptant and SupE strains harboring Δ*rrn10* disruption mutation led to higher RNA content as compared with the wild-type strain (Chuwattanakul et al., 2011).

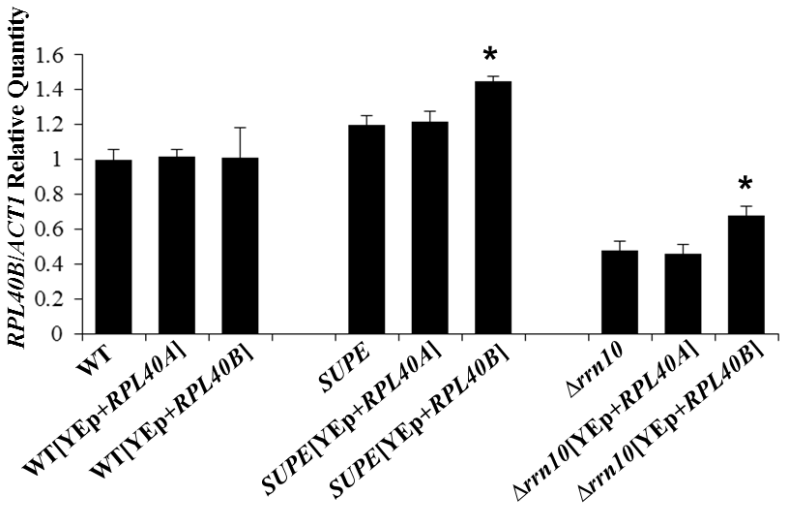

Fig. 5 Measurement of the mRNA level of RPL40B in strains having wild-type [WT], WT [YEp+*RPL40A*], WT [YEp+*RPL40B*], *SUPE*, *SUPE* [YEp+*RPL40A*], *SUPE* [YEp+*RPL40B*], Δ*rrn10* [YEp] and Δ*rrn10* [YEp+*RPL40A*] and Δ*rrn10* [YEp+*RPL40B*] backgrounds. Cells were collected at OD660=1.0 and RNA were extracted as described in Materials and Methods. *RPL40B* transcript was measured by real-time RT-PCR using *ACT1* as an endogenous control. Statistical analysis was performed as described in Fig. 3C

The plasmid p3398 (YCp+*TRP1*+*RRN10*) was introduced into the Δ*rrn10* disruptant and SupE strains harboring a multicopy of *RPL40A* and measured total RNA content of each of the transformed strains. RNA content was further increased by 85% (*$p<0.05$) after introduction of *RRN10* into the SupE strain harboring a multicopy of *RPL40A* and 76% (*$p<0.05$) higher RNA content was observed in the Δ*rrn10* strain harboring a multicopy of *RPL40A* under a *RRN10*$^{+}$ background (Fig. 4C). In this chapter, further improvement in RNA content was obtained by combining the Δ*rrn10 SUPE* background with multicopies of *RPL40A* and *RRN10* genes and this

amount was 92% (*p<0.05) higher than the wild-type strain. By contrast, in wild-type strain, RNA content was increased by only 0.08%, a non-significant change, in response to the increased copy number of *RPL40A* and *RPL40B* (Fig. 4C). In addition, when multiple copies of the *RPL40A* were combined with *SUPE* mutation on the $RRN10^{+}$ background, the resultant strain showed significantly higher RNA content than the wild-type strain. These findings suggest that increasing the copy number of both *RPL40A* and *RPL40B* improve the growth rate and increase RNA content in the strains harboring *SUPE* mutation along with $RRN10^{+}$ background.

2.3.4 Deletion of *RPL40A* and *RPL40B* gene decreases the growth rate and RNA content of Δ*rrn10* disruptant and suppressor strains

Because the above results showed that introduction of multicopies of *RPL40A* and *RPL40B* genes increased growth rate and RNA content of the Δ*rrn10* disruptant and SupE strains, I became interested in investigating the deletion effect of *RPL40A* and *RPL40B* on both the growth rate and RNA content in Δ*rrn10* disruptant, *SUPE* and wild-type strains. For this purpose, I disrupted *RPL40A* and *RPL40B* in the above mentioned strains. Growth phenotype and RNA measurement data (Fig. 6) showed that deletion of *RPL40A* and *RPL40B* had no effect on the growth rate and RNA content of the wild-type strain, but it caused significant decrease (*p<0.05) in the growth rate and RNA content in both Δ*rrn10* disruptant and SupE strains. Based on the above data, I suggest that the requirement of both *RPL40A* and *RPL40B* for maintaining optimum growth performance and RNA content differ in the strains having an Δ*rrn10* background compared to wild-type strains if either one is knocked out.

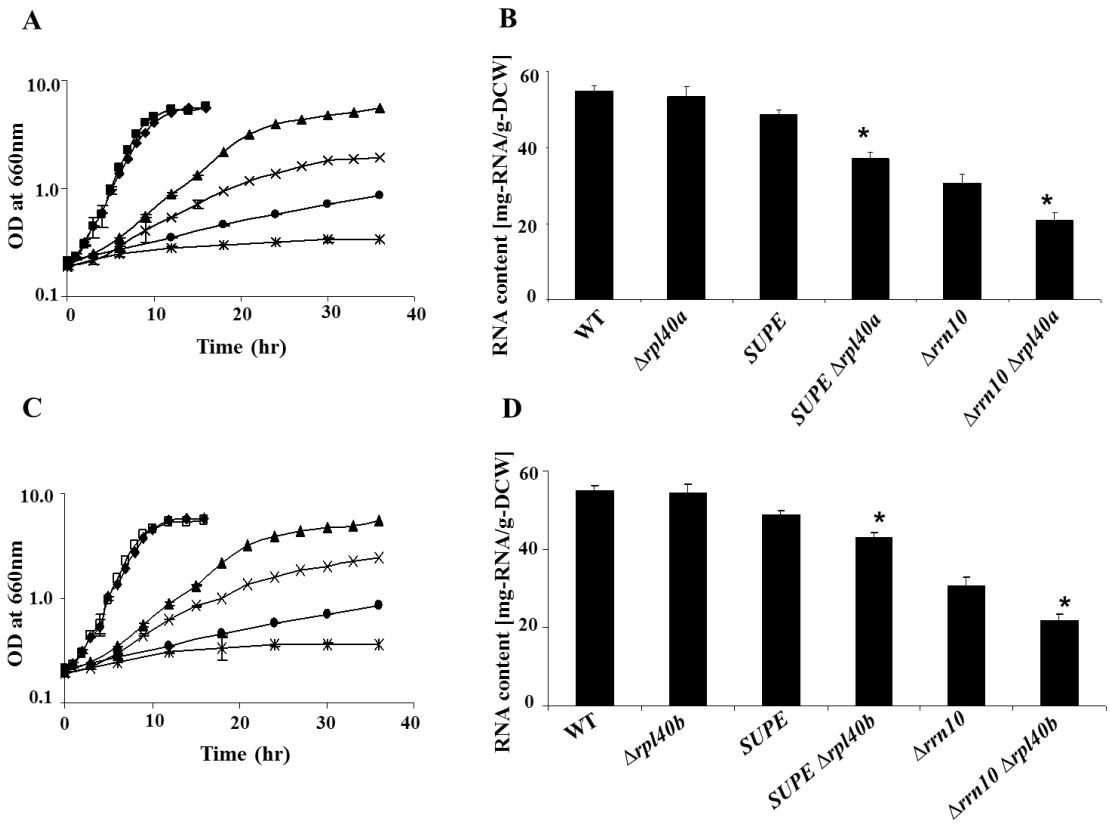

Fig. 6 Deletion effect of RPL40A and RPL40B on the growth and RNA content of wild-type, Δrrn10 disruptant and SupE strains. (A) Comparison of growth rate among strains on wild-type (WT), Δrpl40a, SUPE, SUPE Δrpl40a, Δrrn10 and Δrrn10 Δrpl40a backgrounds. Growth rate was monitored at OD660 nm. Three independent experiments using 3 independent samples were performed and mean±SD values are presented. Filled square represents WT, filled diamond represents Δrpl40a, filled triangle represents SUPE, cross-mark represents SUPE Δrpl40a, filled circle represents Δrrn10 disruptant, and asterisk represents Δrrn10 Δrpl40a strains. (B) Total RNA content of strains having wild-type, Δrpl40a, SUPE, SUPE Δrpl40a, Δrrn10 and Δrrn10 Δrpl40a backgrounds. The experiment and statistical analysis were performed as described in Fig. 3C. (C) Comparison of growth rate among strains on WT, Δrpl40b, SUPE, SUPE Δrpl40b, Δrrn10 and Δrrn10 Δrpl40b backgrounds. Growth rate was monitored at OD660 nm. Three independent experiments using 3 independent samples were performed and mean±SD values are presented. Square represents wild-type, filled diamond represent Δrpl40b, filled triangle represents SUPE, cross-mark represents SUPE Δrpl40b, filled circle represents Δrrn10 disruptant, and asterisk represents Δrrn10 Δrpl40b strains. (D) Total

RNA content of strains on WT, Δrpl40b, SUPE, SUPE Δrpl40b, Δrrn10 and Δrrn10 Δrpl40b backgrounds. The experiment and statistical analysis were performed as described in the legend of Fig. 3C. Asterisks (*) indicate significantly different group means at an alpha level of 0.05.

I also investigated whether extra copies of *RPL40A* or *RPL40B* can restore the growth defect in the strains having *SUPE* Δ*rpl40b* and *SUPE* Δ*rpl40a* backgrounds, as well as for the strains having Δ*rrn10* Δ*rpl40b* and Δ*rrn10* Δ*rpl40a* backgrounds, respectively (Fig. 7).

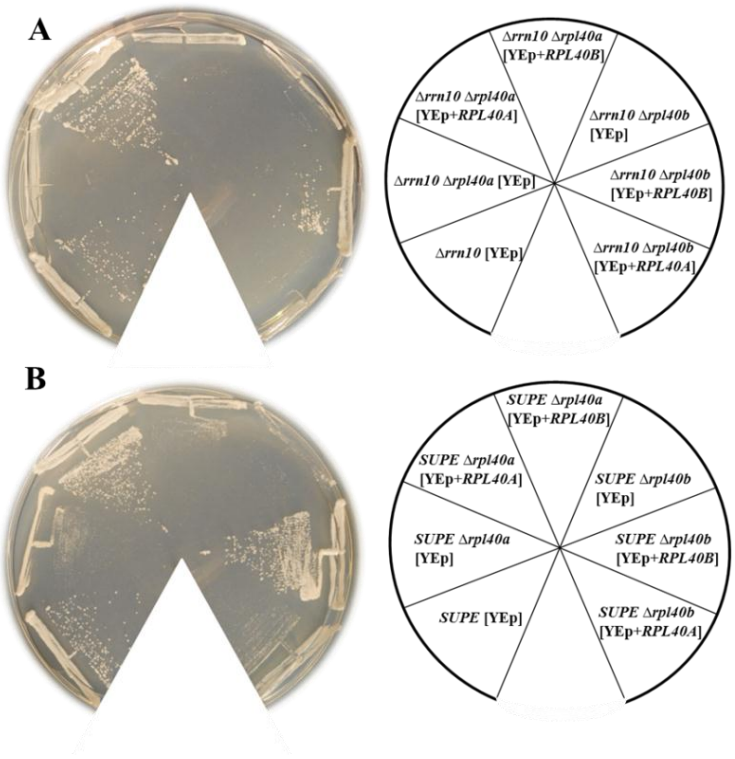

Fig. 7 A) Comparison of growth phenotype between strains having Δ*rrn10* [YEp], Δ*rrn10* Δ*rpl40a* [YEp], Δ*rrn10* Δ*rpl40a* [YEp+*RPL40A*], Δ*rrn10* Δ*rpl40a* [YEp+*RPL40B*], Δ*rrn10* Δ*rpl40b* [YEp], Δ*rrn10* Δ*rpl40b* [YEp+*RPL40B*], Δ*rrn10* Δ*rpl40b* [YEp+*RPL40A*] backgrounds. Photograph was taken after 7 days of incubation at 37ºC on SD-Ura plate. B) Comparison of growth phenotype between strains having *SUPE* [YEp], *SUPE* Δ*rpl40a* [YEp], *SUPE* Δ*rpl40a* [YEp+*RPL40A*], *SUPE* Δ*rpl40a* [YEp+*RPL40B*], *SUPE* Δ*rpl40b*

[YEp], *SUPE* Δ*rpl40b* [YEp+*RPL40B*], *SUPE* Δrpl40b [YEp+*RPL40A*] backgrounds. Photograph was taken after 4 days of incubation at 37ºC on SD-Ura plate.

Growth phenotype of *SUPE* Δ*rpl40a* [YEp+*RPL40A*] and *SUPE* Δ*rpl40b* [YEp+*RPL40B*] as positive controls and *SUPE* Δ*rpl40a* [YEp] and *SUPE* Δ*rpl40b* [YEp] as negative controls for SupE strain was compared. In the case of the Δ*rrn10* disruptant strain, Δ*rrn10* Δ*rpl40a* [YEp+*RPL40A*] and Δ*rrn10* Δ*rpl40b* [YEp+*RPL40B*] were used as positive controls and Δ*rrn10* Δ*rpl40a* [YEp] and Δ*rrn10* Δ*rpl40b* [YEp] were used as negative controls. Results revealed that suppression did not occur in the *SUPE* Δ*rpl40b* background by introducing extra copies of *RPL40A* or in the *SUPE* Δ*rpl40a* background by providing extra copies of *RPL40B* (Fig. 7). The same result was obtained for the Δ*rrn10* disruptant strain. Although there is no functional distinction between *RPL40A* and *RPL40B* and they perform in a similar way to compensate the defect caused by *rrn10* disruption, the above results suggest that individual function of both the *RPL40A* and *RPL40B* are required for the strains having a Δ*rrn10* background.

2.3.5 *SUPE* mutation increases the transcription of both *RPL40A* and *RPL40B* as well as other ribosomal protein coding genes

The above results showed that a combination of increased copy number of either *RPL40A* or *RPL40B* with *SUPE* mutation synergistically increases RNA content; therefore, I assumed that *SUPE* mutation has the ability to increase the transcription level of the *RPL40A* and *RPL40B* genes. To explore this idea, I investigated the effect of *SUPE* mutation on the transcription of

RPL40A and *RPL40B*. I conducted real-time RT-PCR using RNA extracted from the wild-type (SH6446), SupE (SH6976) and Δ*rrn10* disruptant (SH6789) strains. Transcription of *PGK1* and *GAPDH* was measured as a control (Fig. 8A-B). The results of real-time RT-PCR revealed that the amount of *RPL40A* transcript in comparison to the wild-type strain was reduced 2.0-fold in the Δ*rrn10* disruptant strain whereas the amount in SupE strain increased by 1.5 times (Fig. 8C), thereby suggesting that *SUPE* mutation may increase the transcription efficiency of *RPL40A* as compared with the Δ*rrn10* disruptant strain. Transcription of *RPL40B* was also reduced 2.0-fold in the Δ*rrn10* disruptant and increased about 1.5 fold in the SupE strain as compared with the wild-type strain (Fig. 8D).

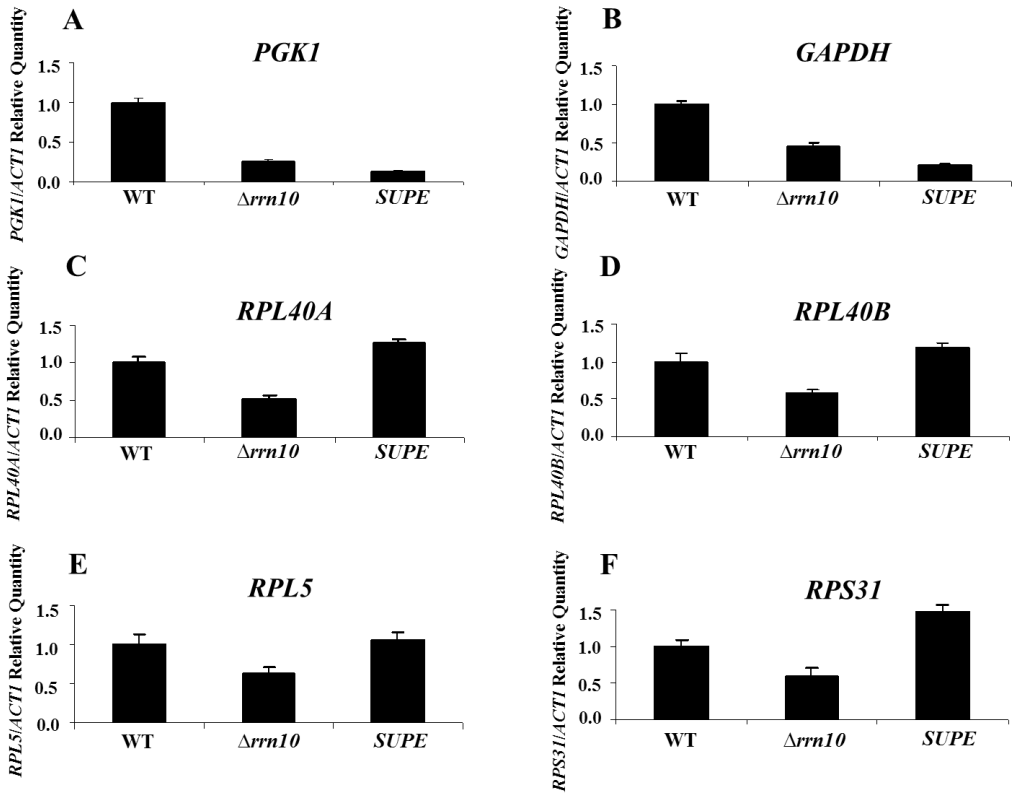

FIG. 8. Measurement of transcription level in Ribosomal protein (RP) genes. (A-B) Measurement of transcription level of PGK1and GAPDH as a control in strains having WT, Δrrn10 disruptant and SUPE backgrounds. (C-D) Measurement of transcription level of RPL40A and RPL40B in strains having WT, Δrrn10 disruptant and SUPE backgrounds. (E-F) Measurement of transcription levels of RPL5 and RPS31 in strains having WT, Δrrn10 disruptant and SUPE backgrounds. The experiment was conducted as described in the legend of Fig. 4B.

Because transcription of *RPL* and *RPS* genes is important for ribosome biogenesis (Zhao et al., 2003), I also investigated the effect of *SUPE* mutation on transcription of *RPL5* as a

representative of *RPL* genes and *RPS31* as a representative of *RPS* genes. The results showed that the amount of *RPL5* and *RPS31* transcripts was reduced 2.0-fold in the Δ*rrn10* disruptant while increasing about 1.5-fold in SupE strain as compared with wild-type strain, similar to *RPL40* gene transcription (Fig. 8E-F). These observations suggested that *SUPE* mutation increased the transcription of not only *RPL40A* and *RPL40B* genes, but also other ribosomal protein coding genes in the SupE (SH6976) strain.

2.4 Discussion

In this chapter, the effect of increased transcription of *RPL40A* and *RPL40B* on growth phenotype and RNA content in *S. cerevisiae* was investigated. It is reported that the protein products encoded by *RPL40A* and *RPL40B*, rather than mRNA, is pre-requisite for subunit joining, and that Rpl40Ap and Rpl40Bp also contribute to optimal 27SB pre-rRNA maturation and proper functioning of the translocation process (Fernández-Pevida et al.; 2012). Two categories of strains having different genetic backgrounds i.e., strain (SH6446) having a wild-type background and strains [SH6789 (Δ*rrn10* disruptant) and SH6976 (Δ*rrn10 SUPE*)] having a Δ*rrn10* disruptant background was used to evaluate the effect of *RPL40A* and *RPL40B* on the growth rate and RNA content in *S. cerevisiae.*

It has been reported that Δ*rrn10* disruption caused severe growth defects and reduced RNA content and that these defects were restored by *SUPE* mutation (Chuwattanakul et al.; 2011). *SUPE* mutation not only restored the reduced RNA content but also increased the amount to levels near the wild-type strain (Figs. 3, 4). It was also found that *SUPE* mutation increases the transcription level of ribosomal protein genes such as *RPL40A, RPL40B, RPL5* and *RPS31* (Fig.

8). Although the identities of genes involved in *SUPE* mutation are still unknown, It was assumed that mutations in the SupE strain may occur in nucleosome components or in other components of the UAF complex which could function to compensate the defect caused by Δ*rrn10* disruption (Chuwattanakul et al.; 2011). The possible involvement of *SUPE* mutation was assumed in the following mechanisms. The first possible mechanism is that suppressor mutation might occur in some of the nucleosome components. It was reported that actively transcribed gene was found in decondensed chromatin corresponding to extended chromatin fibers (Cooper and Housman, 2004). It is difficult for transcription factors and RNA polymerase to interact with chromatin when chromatin is condensed. Although the precise mechanism of the role of the Rrn10p is not known, it might be possible to imagine that Rrn10p plays an important role in relaxing the nucleosome and thus in promoting efficient Pol I transcription (Chuwattanakul et al.; 2011). Therefore, if the *SUPE* mutation occurs in a nucleosome component, this might lead to relaxation of the nucleosome and chromatin structure and consequently to increased access of Pol I to promoter of rDNA thereby accelerating rDNA transcription. The second possibility is that suppressor mutations occurred in other components of the UAF complex. In the Δ*rrn10* disruptant strain, UAF does not function normally due to the absence of Rrn10p. Because Rrn10p is believed to work with other components of the UAF complex (Keener et al.; 1998), however, it is possible that *SUPE* mutation that occurs in other components of the UAF complex could function to compensate for the defect caused by the Δ*rrn10* mutation. If that is the case, decreased rRNA transcription in the Δ*rrn10* disruptant might be restored by *SUPE* mutation. However, it also speculated that *SUPE* mutation may occur in some component of the rDNA transcription system which also largely influences the

transcription level of RP genes such as *RPL40A* and *RPL40B*. As rDNA transcription, rRNA processing, and RP gene transcription are strictly coordinated, increased production of the ribosomal protein may help to compensate for the defect caused by Δ*rrn10* disruption and thereby increased the total RNA content in the strains having a Δ*rrn10 SUPE* background.

In response to increased or decreased transcription of *RPL40A* and *RPL40B*, wild-type strain did not show any significant alteration in growth phenotype or in RNA content among transformants with single copy, double copy, further additional copies nor absent copies of the *RPL40A* and *RPL40B* genes (Figs. 3, 4, 6). By contrast, in the strains having a Δ*rrn10* background, additional copies of *RPL40A* and *RPL40B* significantly increased the growth rate and RNA content (Fig. 2C, Figs. 3, 4). Transcription of the rDNA is a vast process which maintains a harmony between all the factors involved in the rDNA transcription process ranging from pre-initiation complex formation to termination for completion of synthesis of mature rRNA species. If any defect occurred in a particular process, all other components take part to recover the defect (Zhao et al.; 2003). In the wild-type strain, Rpl40Ap and Rpl40Bp are able to complement mutual defect as reported by Fernandez-Pevida, et al. (2012) but in the strains where rDNA transcription is severely affected by disruption of *RRN10*, one of the important factor of upstream activation factor, these two proteins seems to exert their effect in a different fashion (Figs. 4, 6) and failed to compensate the defect caused by the absence of their counterpart in the SupE or Δ*rrn10* strain (Fig. 7) indicating the difference in their necessity in two different types of strain. I assumed that growth phenotype and RNA content of the strains having Δ*rrn10* disruptant background significantly depend on the cumulative activities of all the factors

involved in rDNA transcription and distinct function of each component seems to be important to compensate the loss of defective rDNA transcription. Moreover, Rpl40Ap and Rpl40Bp are important for ribosome biosynthesis especially for the ribosomal subunit joining process and ribosomal translocation process (Fernández-Pevida et al.; 2012) which is in turn expected to facilitate the efficient transcription of rDNA, processing of pre-RNA and formation of mature rRNA. The reason behind this prediction is based on many scientific reports which mentioned that ribosome biosynthesis, rDNA transcription, pre-rRNA processing and mature rRNA formation occurred concomitantly where more than 80 ribosomal proteins and 250 non-ribosomal proteins are involved in different steps (Kressler et al.; 1999, Venema and Tollervey, 1999, Warner, 2001, Nazar, 2004, Rudra et al.; 2007, Gallagher et al.; 2004). It can be assumed that, being important factor for ribosome biosynthesis, individual function of Rpl40Ap and Rpl40Bp has significant impact on rRNA production of Δ*rrn10* disruptant and SupE strain. Thereby, I predicted that, despite being identical in structure and function, these two proteins failed to complement the defect caused by the absence of their counterpart in the strains having Δ*rrn10 SUPE* or Δ*rrn10* background (Fig. 7). Indeed, there are many reports describing that, the specific role of a ribosomal protein, in terms of translational activity, is extremely difficult to explain properly because of their highly cooperative nature of the interactions between rRNA and ribosomal proteins and between the ribosomal proteins themselves (Petrow et al.; 2008, Briones and Ballesta, 2000, Meskauskas et al.; 2008).

In this study, I found that *SUPE* mutation helps to improve the transcription of *RPL40A*, *RPL40B* and other RP genes (Figs. 8C-8F). On the basis of these observations, a model is

proposed to explain how *SUPE* mutation affects activities of *RPL40A* gene as well as other ribosomal protein genes. Our hypothesis highlights an important complex named CURI which is composed of the following four protein components: Casein Kinase II, Utp22p, Rrp7p and Ifh1p.

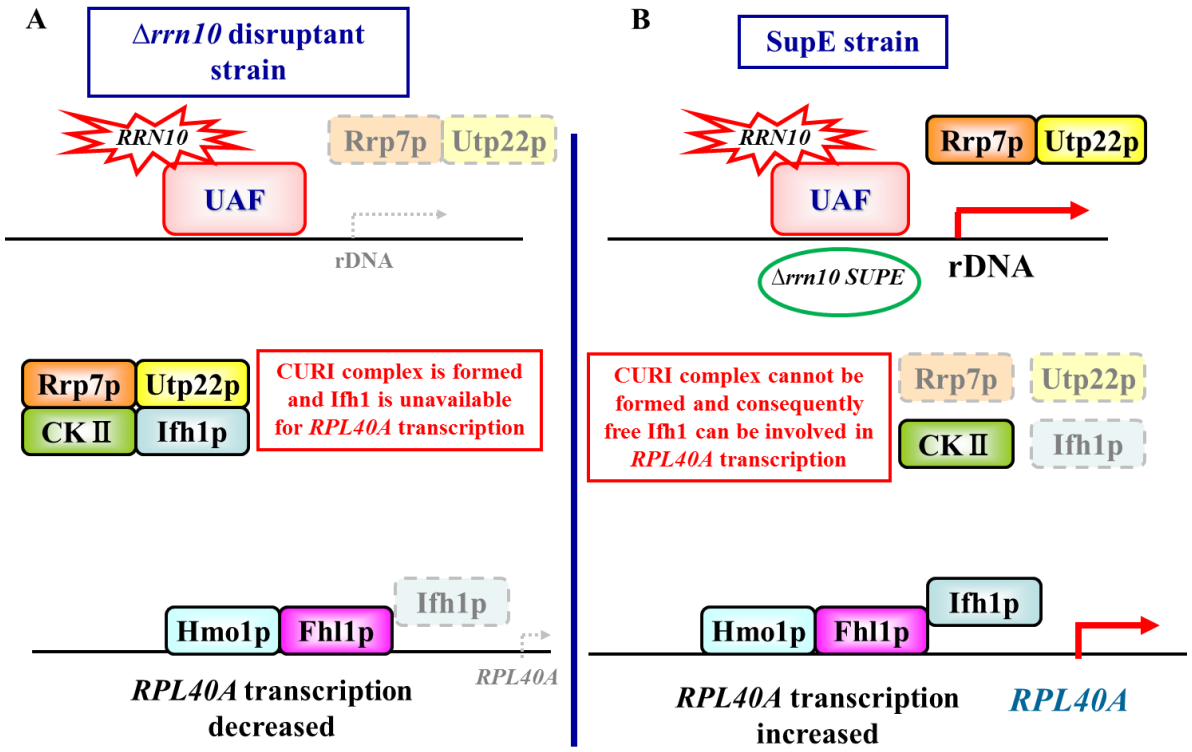

Fig. 9 Possible mechanism underlying suppression of the defect caused by Δrrn10 disruption in Pol I-mediated rRNA transcription. (A) Disruption of RRN10 causes reduced rDNA transcription; as a result, unused rRNA processing factors Rrp7p and Utp22p can form the CURI complex with Ifh1p, which decreases RPL40A transcription. (B) SUPE mutation can restore the rRNA transcriptional defect by utilizing Rrp7p and Utp22p, thereby allowing free Ifh1p to increase RPL40A transcription. [UAF, Upstream Activation Factor; Rrp7p, Essential protein involved in rRNA processing; Utp22p, Protein involved in maturation of pre-18S rRNA; CKII (Casein Kinase II), Subunit of CURI complex which plays an important role in regulation of enzyme activity; Ifh1p, Essential protein involved in transcription of RP gene and subunit of CURI complex which co-

ordinates RP production and pre-rRNA processing; Fhl1p, Regulator of ribosomal protein transcription; Hmo1p, A high mobility group protein (HMG) that directly influences transcription and maturation of rRNA by Pol I.]

Rrp7p and Utp22p function as rRNA processing factors and Ifh1p functions as an important transcription factor for ribosomal protein genes. It is already reported that the CURI complex plays an important role in the coordination of rRNA processing and transcription of ribosomal protein genes (Rudra et al.; 2007). Disruption of *RRN10* caused defective transcription of rDNA, and thereby produced less amount of rRNA transcripts (Chuwattanakul et al.; 2011). Rrp7p and Utp22p, which are involved in rRNA processing, receive less amount of rRNA transcripts for further processing. As a result, Rrp7p and Utp22p are assumed to remain unused and consequently become available to form the CURI complex and thereby, may block Ifh1p to take part in RP gene transcription (Fig. 9A). Unavailability of Ifh1p decreases transcription of *RPL40A* and other RP genes. *SUPE* mutation was shown to restore defective rDNA transcription and increased rRNA content compared to the Δ*rrn10* disruptant strain (Chuwattanakul et al.; 2011). It was assumed that as rRNA processing factors receive adequate amounts of rRNA transcripts in the SupE strain for further processing, they become involved in their regular function resulting in failure to form the CURI complex; which in turn, makes Ifh1p available to take part as a transcription factor for ribosomal protein genes. As a result, it was believed that transcription of *RPL40A* gene along with other ribosomal protein genes increased (Fig. 9B). Although further analysis is required to determine how *SUPE* mutation helps to improve the transcription of *RPL40A*, as well as *RPL40B* and other RP genes which

consequently increases rRNA content leading to higher RNA content in the SupE strain (SH6976), our findings might be useful for designing strategies for further increase in RNA content of yeast.

2.5 Summary

Yeast (*Saccharomyces cerevisiae*) RNA is an important source of 5´-ribonucleotides that is used in both the food and pharmaceutical industries. Efficient transcription of rDNA is very important to construct yeast strains with high RNA content. The gene *RRN10*, which encodes, a component of the upstream activation factor (UAF), is essential to promote high-level transcription of rDNA. In a previous study, it was reported that SupE strain harbors a dominant and multiple mutations, named *SUPE,* which showed the ability to restore the severe growth defects and reduced RNA content caused by disruption of the *RRN10* gene. Further analysis on *SUPE* mutation and subsequent genome sequencing results revealed that, there were no base changes in *RPL40A* gene gene as compared with the parental Δ*rrn10* strain, thus suggesting that an additional copy of *RPL40A* suppress the defects caused by Δ*rrn10* disruption, and that, in SupE strain. When multiple copies of *RPL40A* were combined with *SUPE* mutation on an *RRN10*$^{+}$ background, the resultant SupE strain had significantly higher RNA content than wild-type strain. In addition, increased transcription of *RPL40B* also showed significant effect to restore the growth defect and reduced RNA content caused by Δ*rrn10* disruption. A model is proposed to explain the probable mechanism regarding how *SUPE* mutation increases the transcription of ribosomal protein (RP) genes such as *RPL40A* and *RPL40B* in the SupE strain, resulting in an increase in RNA content.

3. CHAPTER 3

Increase in rRNA content in a *Saccharomyces cerevisiae* Δ*rrn10* suppressor strain by rDNA cluster duplication

3.1 Introduction

The transcription of ribosomal DNA (rDNA) has been investigated for over half a century. Recognition of the importance of rDNA as a nucleolar organizer initiated studies of rDNA transcription, rRNA processing and ribosome assembly in eukaryotes, processes that serve as major functions of the nucleolus (Brown and Gurdon 1964; Ritossa and Spiegelman 1965; Wallace and Birnstiel 1996; Kobayashi 2011). Although earlier studies of rDNA transcription were mostly done in metazoan cells, the yeast species *Saccharomyces cerevisiae* is now being utilized as a model system because it offers the ability to combine powerful genetic approaches with various modern molecular techniques (Nomura 1999; Nomura et al. 2004). A recent investigation suggests that rDNA transcription rates are important for the proper growth performance of yeast cells because rDNA is central to cellular physiology due to its role in mRNA translation (Kobayashi 2011).

rRNA is also an important component in the food industry as a major source of RNA because it accounts for about 80% of the total RNA content in *S. cerevisiae* (Warner 1999). Yeast RNA is a valuable source of nucleotides that act as strong flavor enhancers and that are highly used as additives in the food industry (Dziezak 1987; Nagodawithana 1992; Stam et al. 1998). Many valuable food ingredients are derived from *S. cerevisiae*, which is considered to be a safe food source in food processing (Demain et al. 1989).

In *S. cerevisiae*, rDNA repeats are tandemly arranged on chromosome XII and consist of approximately 150 repeated copies of 9.1-kb units of the rDNA gene; each 9.1-kb unit consists of two transcribed regions, the 35S precursor rRNA and 5S rRNA, as well as two intergenic spacers, IGS1 and IGS2 (Petes 1979). The number of repeats seems to be maintained at an

appropriate level for each organism, and most organisms are able to vary the number of repeats of the rDNA gene in response to intracellular as well as extracellular conditions (Nomura et al. 2004). Yeast strains in which rDNA copy numbers are altered can be maintained stably and have been proven to be useful for studies concerning the regulation of rDNA transcription by RNA polymerase I (Nomura 1999). Although it is thought that the high gene copy numbers observed in eukaryotic rDNA clusters reflect the demand for large quantities of ribosomes, it is also known, paradoxically, that about half of the rDNA copies in yeast are not transcribed (French et al. 2003). Nevertheless, there is a clear negative correlation between copy number and sensitivity to DNA damaging factors, which indicates that the damage sensitivity of the cell is determined by rDNA copy number (Kobayashi 2011).

In *S. cerevisiae*, formation of the pre-initiation complex is very important for the efficient transcription of rRNA, which requires four major transcription factors: upstream activation factor (UAF), core factor (CF), TATA-binding protein (TBP), and Rrn3p (Hontz et al. 2008). UAF contains six subunits, i.e., Rrn5p, Rrn9p, Rrn10p, Uaf30p, histone H3 and histone H4 (Keys et al. 1996). In our previous study, I constructed an *rrn10* disruptant strain that showed severe growth defects and reduced RNA content (Chuwattanakul et al. 2011). After ethyl methane sulfonate (EMS) mutagenesis, seven suppressor strains (SupA-SupG) from the *rrn10* disruptant strain were isolated that showed the ability to suppress the growth defect and to recover reduced RNA content caused by *rrn10* disruption (Chuwattanakul et al. 2011).

In this chapter, I have focused on a SupE strain in which the mutation is dominant and multiple, collectively designated as *SUPE* (Chuwattanakul et al. 2011). *SUPE* restored the growth defect caused by *rrn10* disruption and demonstrated significantly higher rRNA content as

compared with the wild-type strain (Chuwattanakul et al. 2011). Here, I aimed to construct a SupE strain with a higher rDNA copy number to further improve its RNA content. Previously, the rRNA levels in two wild-type strains were found to be approximately the same regardless of a 3- to 3.4-fold difference in rDNA gene number, presumably due to a feedback inhibition system that may exist to stabilize rDNA transcription (French et al. 2003; Nomura 1999; Nomura 2001). I hypothesized that the feedback inhibition mechanism of rDNA transcription might be interrupted by *SUPE*, and thus additional copies of the rDNA gene would be effective in increasing the rRNA content of the SupE strain. According to this idea, here we constructed a SupE strain possessing two copies of the rDNA cluster constituting approximately 300 copies of the rDNA gene and it was found that this strain showed 41% higher RNA content compared to the wild-type strain having two copies of rDNA cluster as well. Further increase in RNA content of 47% was achieved from this strain by introducing multicopy *RPL40A* genes, thus confirming the positive effect of *RPL40A* gene on RNA content of the SupE strain observed in a previous report (Khatun et al. 2013). I concluded that this strategy is a useful approach for construction of a yeast strain with higher RNA content.

3.2 Materials and Methods

3.2.1 Strains, plasmids and oligonucleotide primers

The *S. cerevisiae* strains and plasmids used in this study are listed in Table 3. The yeast strain SH6976 (Δ*rrn10*::*CgLEU2 SUPE*) was used for analyzing the effect of rDNA copy number on RNA content in the haploid strain on a Δ*rrn10 SUPE* background and the wild-type

strain SH6471 was used as a control. The oligonucleotide primers used in this study are shown in Table 4.

Table 3. Strains and plasmids used in this chapter

	Description	**Source/Reference**
Strains:		
SH6446	*MAT*a *ura3-52 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 Δfob1::HIS3*	Kim et al., (2006)
SH6471 (WT)	*MATα ura3 Δ851 his3Δ 200 leu2Δ1 trp1Δ63 ade2-661*	NBRP-Yeast, Japan[a]
SH6623	*MATα ura3-52 his3Δ200 lys2Δ202 trp1Δ63 Δ fob1::HIS3* [1500 kb rDNA cluster: *TRP1*]	Kim et al., (2006)
SH6789	*MATα ura3-52 or ura3Δ851 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 Δfob1::HIS3 Δrrn10::CgLEU2*	Chuwattanakul et al., (2011)
SH6812 (SupE)	*MATα ura3-52 or ura3Δ851 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 Δfob1::HIS3 Δrrn10::CgLEU2 SUPE*	Chuwattanakul et al., (2011)
SH6976 (SupE)	*MAT*a *ura3-52 or ura3Δ851 his3Δ200 leu2Δ1 trp1Δ63Δrrn10::CgLEU2 SUPE*	*MAT*a convertant of SH6812; transformed with *HO* gene
SH6980	Trp^+ transformant of SH6471 with p564	This study
SH7992	Ura^+ transformant of SH6976 with p3336, *RRN10*	Kim et al., (2006)
SH30007	SH6471 [Chr.XII/*URA3* +rDNA-L roximal:450 kb] [rDNA	This study

(WT-RL)	cluster+*TRP1*] [Chr. XII/rDNA-R distal:610 kb]	
SH30012 (WT-2X)	*MATα ura 3 Δ851 his3Δ 200 leu2Δ1 trp1Δ63 ade2-661 Δfob1::HIS3* [rDNA cluster:: *TRP1*]	This study
SH30009 (SupE-R)	SH6976 [Chr. XII/rDNA-R distal:610 kb] [Chr. XII/rDNA cluster-L distal: 1900 kb]	This study
SH30010 (SupE-RL)	SH6976 [Chr.XII/*URA3* +rDNA-L proximal:450kb] [rDNA cluster+*TRP1*] [Chr. XII/rDNA-R distal:610 kb]	This study
SH30011 (SupE-2X)	*MATα ura3-52 or ura3Δ851 his3Δ200 leu2Δ1 trp1Δ63 Δfob1::HIS3 rrn10::CgLEU2 SUPE* [rDNA cluster:: *TRP1*]	This study
SH30013 (SupE-2X[YEp-*RPL40A*])	*MATα ura3-52 or ura3Δ851 his3Δ200 leu2Δ1 trp1Δ63 Δfob1::HIS3 rrn10::CgLEU2 SUPE* [rDNA cluster:: *TRP1*][YEp-*URA3- RPL40A*]	This study
Plasmids: p564	YEp+*TRP1*	NBRP-Yeast, Japan[a]
p617	YEp+*URA3*	NBRP-Yeast, Japan[a]
p1498	YCp+*HO+TRP1+LYS2*	NBRP-Yeast, Japan[a]
p3010	A derivative of pUG6 carrying *loxP-CgTRP1-loxP*	Sugiyama et al., (2005)

p3121	A derivative of pUG6 carrying *CEN4*	Sugiyama et al., (2005)
p3151	A derivative of YCplac33 carrying *FOB1* gene (YCp-*FOB1*)	Kobayashi et al. (1998)
p3276	A derivative of pUG6 carrying *URA3*	Sugiyama et al., (2008)
p3336	pRS306+*RRN10*=YIp+*URA3*+*RRN10*	Chuwattanakul et al., (2011)
p3374	YEp+*URA3*+*RPL40A*	Khatun et al., (2013)

[a]National Bio-Resource Project-Yeast, Japan: http://yeast.lab.nig.ac.jp/nig/index_en.html

Table 4. Oligonucleotide primers used in this chapter

Name	Sequence (5'- 3')
rDNAr-1	GTTGTTTTTTTTTCGCGCA
rDNAr-2	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCATGTGCCAGTTAAGCTATTT
rDNAr-3	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCATGAAAAGGATGTAGAAAT
rDNAr-4	AACGAACAACTTTATGAAGA
rDNAl-1	TGTATATCACGTAATACACA
rDNAl-2	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCGTTCTATAGCAAACATGAGGAAATATCC
rDNAl-3	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCGTTCTATACGCTCTGATGGTGCGGAAAA
rDNAl-4	TTTTTATTTCTTTCTAAGTG

Tr6-4	$(CCCCAA)_6$AGGCCACTAGTGGATCTGAT
loxP-F1	GGCCGCCAGCTGAAGCTTCG
rDNA5S-1	GCGGCCATATCTACCAGAAA
rDNA5S-2	TGCGGAGTTGTAAGATGTAC
HaY12P3F	CGGAATTCGAACATGTTTGCGCCTT
HaY12P3R	ATAGCGTCATTTGGGCCGCTAGTAT
HaY12P5F	CGGTAGTGCTGTTGTTTGTCAGG
HaY12P5R	TTTCTCTTCTTCCAACCAGGC
18S-F	CCTGAGAAACGGCTACCA
18S-R	ATTGTCACTACCTCCCTGAATTAGGA
rDNA 5S-F	GCGGCCATATCTACCAGAAA
rDNA 5S-R	TGCGGAGTTGTAAGATGTAC
ACT1-F	TGGTATGTGTAAAGCCGGTTTTG
ACT1-R	CATGATACCTTGGTGTCTTGGTCTA
MKR1-F	GACCCAAGAG ATTCGACTCA
MKR1-R	TGTCTCAAGCACTCATCAGG

3.2.2 Culture conditions, media preparation and yeast transformation

Yeast strains were grown in either a YPDA medium or a synthetic complete (SC) medium as previously described (Amberg et al. 2005) and were cultured at 30°C. Yeast transformation was carried out by the lithium acetate method for transformation of yeast

(Amberg et al. 2005). To screen for yeast transformants, cells were cultured in SC medium without histidine and tryptophan at 30°C.

3.2.3 Construction of a segmental aneuploid strain with a 1,500-kb region of chromosome XII harboring a rDNA cluster

The PCR-mediated chromosome splitting method was used to split chromosome XII (Sugiyama et al. 2005). The *Saccharomyces* Genome Database (www.yeastgenome.org) was used to design the splitting site and the oligonucleotide primers. Construction of the fragments used for the splitting was done as previously described by Kim et al. (2006). To split the 610-kb region from the right end of chromosome XII, two splitting DNA fragments, RI and RII, were prepared using genomic DNA of the SupE (SH6976) strain, plasmids p3010 and p3121 as templates for amplification of the target site containing marker *CgTRP1* and *CEN4*, respectively, and the primers rDNAr-1, rDNAr-2, rDNAr-3, rDNAr-4, loxP-F1 and Tr6-4 (Table 2). Next, two splitting fragments, L-I and L-II, were prepared for splitting 450 kb from the left end of chromosome XII using genomic DNA of strain SH6976, plasmids p3121 and p3276 as templates and the primers rDNAl-1, rDNAl-2, rDNAl-3, rDNAl-4, loxP-F1 and Tr6-4 (Table 2). Primers rDNA5S-1, rDNA5S-2, HaY12P3F, HaY12P3R, HaY12P5F and HaY12P5R (Table 2) were used for the amplification of probe-5S (5S), probe-R (R) and probe-L (L), respectively. First, the right side of the rDNA cluster was split by introducing the two splitting fragments RI and RII into strain SH6976, and transformants harboring the split chromosomes were verified by CHEF electrophoresis, followed by Southern hybridization. Next, the left side of the rDNA

cluster was split in one of these transformants by introducing the fragments LI and LII. The successful transformant was verified by CHEF electrophoresis, followed by Southern hybridization. Lastly, the resulting transformant with a split at both sides of chromosome XII was also checked for the presence of the rDNA cluster by CHEF electrophoresis, followed by Southern hybridization using the 5S region as a probe.

3.2.4 Pulsed-field gel electrophoresis and Southern blot analysis

Chromosomal DNAs of *S. cerevisiae* embedded in agarose plugs were prepared as described by Sheehan and Weiss (1990) after the transformants were cultivated overnight in 5 ml of YPAD medium. Chromosomal DNAs were separated in a 1% agarose gel (pulsed-field certified agarose; Bio-Rad Laboratories, Richmond, CA, USA) using a CHEF Mapper^{TM} system (Bio-Rad Laboratories, CA, USA) in 0.5XTBE buffer. CHEF gel electrophoresis was performed for 15 h at 6.0 V/cm and 14°C with switching intervals of 60s, followed by a 9-h run with 90-s switching intervals. Following electrophoresis, the gel was stained in 0.1µg/ml of ethidium bromide and photographed under UV light. Next, the DNA was transferred onto Hybond™-N^{+} membranes (Amersham Biosciences, Piscataway, NJ, USA) in 20XSSC by standard methods, and the membranes were hybridized with probes generated by PCR using specific primer pairs. I did not observe a hybridization signal in the wells because the DNA plugs became separated from the well during gel processing before the blotting step. Probe labeling, hybridization, and hybridization signal detection were carried out using the ECL direct™ nucleic acid labeling and detection system (Amersham Biosciences).

3.2.5 Estimation of the rDNA copy number by quantitative real-time PCR

To determine the copy number of the rDNA gene, quantitative real-time PCR using SYBR green PCR Master-Mix (Takara) was performed in triplicate using an Applied Biosystems 7300 real-time PCR system. Genomic DNA from the yeast strain was isolated using a Dr. GenTLE® (from Yeast) High Recovery kit (Takara Biotechnology, Dalian. Co., LTD). Each reaction mixture was prepared in a total volume of 20 μl with a concentration of genomic DNA of 10 ng for every 20 μl of PCR mixture. The thermal cycling protocol was performed in accordance with the SYBR® *Premix EX Taq*^{TM} II (Takara) kit. The primer sets for the detection of 18S rRNA (Chromosome accession number: NC_001144.5, SGDID: S000006482), 5S rRNA (Chromosome accession number: NC_001144.5, SGDID: S000006479), *ACT1* (Gene accession number: NC_001138) and *MKR1* (Gene accession number: NC_001136) gene are shown in Table 4. The copy number of each gene was estimated according to the method of Herrera et al. (2009). 18S rDNA and 5S rDNA were used as the test genes, whereas *ACT1* and *MKR1* were used as reference genes. The threshold cycle (C_T) values were used for relative calculation, and copy numbers of the target genes were determined using the relative quantification ($^{\Delta\Delta}C_T$) $2^{-\Delta\Delta CT}$ method (Herrera et al. 2009).

3.2.6 Quantification of 18S rRNA and the measurement of total RNA

For RNA isolation, yeast cells were collected at an optical density (OD) of 1.0 at 660 nm. For the preparation of cDNA, extracted RNA was reverse-transcribed using a High Capacity cDNA Archive kit (Applied Biosystems) in accordance with the manufacturer's instructions. 18S rRNA was quantified in triplicate by quantitative real-time PCR using SYBR green PCR

Master-Mix (Applied Biosystems) and an Applied Biosystems 7300 real-time PCR system (Applied Biosystems). For each strain, RNA was independently extracted three times by a previously described method (Hermansyah et al. 2009). The amount of 18S rRNA was divided by that of *ACT1* to obtain a normalized 18S rRNA value. The primers 18S-F and 18S-R (Table 2) were used to detect 18S rRNA. *ACT1* transcription was measured as an internal control using the primers ACT1-F and ACT1-R (Table 2). Total RNA from each strain was measured by the Perchloric Acid (PCA) method as previously described (Chuwattanakul 2011).

3.3 Results

3.3.1 **Construction of a haploid SupE strain with two copies of the rDNA cluster**

To construct a SupE strain with two copies of the rDNA cluster, I performed the following steps. First, construction of a haploid SupE strain with the rDNA cluster split at the right and the left side of chromosome XII. Second, crossing of the resulting SupE strain with another SupE strain with an intact chromosome XII, followed by sporulation. Third, selection of the expected candidate based on the growth phenotype on selection plates. Fourth, final confirmation by CHEF electrophoresis, followed by Southern hybridization and quantitative real-time PCR. As a control, I followed the above steps to construct a wild-type strain with two copies of the rDNA cluster. Below, I describe these sequential steps in detail.

First, I applied PCR-mediated chromosome splitting technology (PCS) to chromosome XII because this chromosome contains a cluster of rDNA repeats. The PCS method combines a two-step PCR with overlap extension for preparing the DNA fragments and one transformation

per splitting event (Sugiyama et al. 2005). Here I constructed two sets of DNA fragments: RI and RII for splitting 610 kb from the right side of the rDNA cluster, and LI and LII for splitting 450 kb from the left side of the rDNA cluster. Candidate transformants after successful splitting were confirmed by CHEF gel electrophoresis followed by Southern hybridization. The splitting site from the left and right ends, and the location of hybridization probes are shown in Fig. 10A. The SupE strain split at the right side of the rDNA cluster was designated as SupE-R (SH30009) (Fig. 10B). The SupE-R strain was then used to split the left side of the rDNA cluster, and the successful transformant was designated as SupE-RL (SH30010) (Fig. 10C). The SupE-RL strain was then checked for the presence of the rDNA cluster by Southern blot analysis using the 5S region as a probe (Fig. 10D). The wild-type SH6471 strain was split in the same way to construct WT-RL (SH30007). The SupE-RL and WT-RL strains were then used in the second step to construct, respectively, a SupE strain and a wild-type strain with two copies of the rDNA cluster.

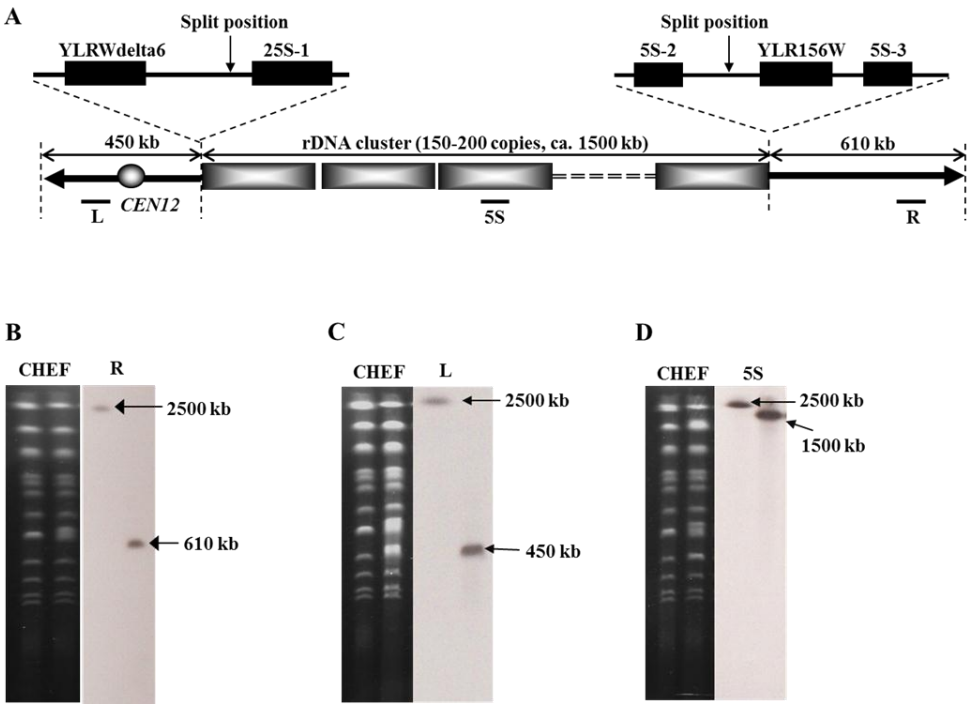

Fig. 10 Splitting of the right end and the left end of the rDNA cluster in chromosome XII of SupE strain (SH6976). (A) Outline of chromosome XII with 150-200 copies of rDNA repeats in S. cerevisiae. The locations of the hybridization probes L, 5S and R are also shown. (B, C, D) Analysis of split-chromosomes by CHEF gel electrophoresis and Southern hybridization. Signals in 610 kb, 450 kb, 1,500 kb and 2,500 kb indicate the right and left side of the rDNA cluster, only the rDNA cluster and chromosome XII, respectively. R, right side; L, left side; 5S, 5S rDNA.

In the second step, a diploid strain was constructed by crossing SH30010 (MATa Δrrn10 SUPE) with SH6812 (MATα Δrrn10 SUPE). Chromosome XII of SH30010 was divided into three split chromosomes: that is, a 1,500-kb rDNA cluster, as well as a 450-kb fragment from the left side and a 610-kb fragment from the right side of the rDNA cluster. By contrast, chromosome XII of strain SH6812 was intact. The resulting diploid SupE strain was subjected to sporulation. With respect to viability, five asci showed a segregation pattern of 3:1, four asci

showed a pattern of 2:2, two asci showed a pattern of 4:0 and one ascus showed a pattern of 1:3 (Fig. 11B). Although it is difficult to predict quantitatively the exact segregation patterns of the three split chromosomes, they are likely to segregate independently into tetrad segregants because each of the chromosomes has a centromere. If any of the three split chromosomes were to not segregate normally into the segregant, such tetrad segregants would lack part of chromosome XII and consequently would become inviable; in that case, segregation patterns of 3:1 or 2:2 viable/inviable segregants would be observed. Moreover, the germination rate is not always 100% even if all the chromosomes segregate normally. Considering the above facts, I might expect 1:3 and 0:4 viable/inviable segregation patterns, although the latter was not observed in this experiment.

In the third step, to identify a yeast strain with two copies of the rDNA cluster in addition to a normal chromosome XII, expected segregants were screened on the basis of their growth phenotype on a selection plate (SC-Trp-His) (Fig. 11C). On the one hand, the Trp+ phenotype indicates the presence of an additional rDNA cluster because the rDNA cluster was integrated with the CgTRP1 marker after successful splitting (Fig. 11A). On the other hand, the His+ phenotype confirmed the presence of fob1 deletion, which is necessary for maintaining a fixed number of rDNA copies in S. cerevisiae (Kobayashi et al. 1998; Johzuka and Horiuchi 2002; Kim et al. 2006).

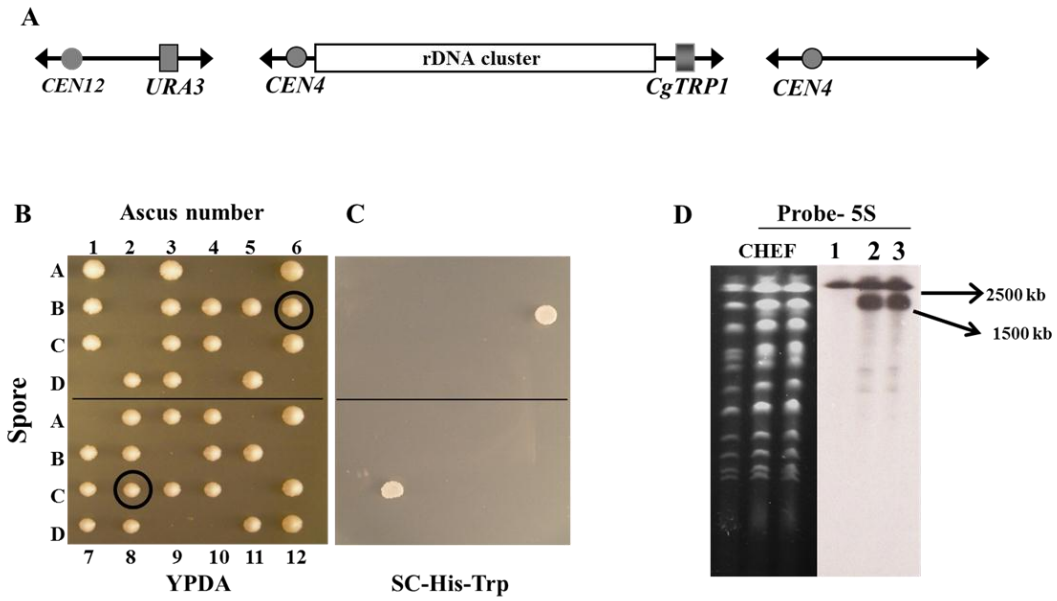

Fig. 11 Construction of haploid strains with double copies of the rDNA cluster (A) Schematic illustration of split chromosomes and the rDNA cluster (B) Tetrad analysis of diploid constructed by crossing SupE-RL strain (SH30010) with SH6812. Tetrad segregants were grown on YPDA solid medium at 30ºC for 3 days. (C) Tetrad segregants grown in selection medium (SC-His-Trp) at 30ºC for 3 days to screen for expected segregants. (D) Confirmation of the presence of double copy of rDNA cluster by CHEF electrophoresis followed by Southern hybridization. Chromosomal DNA was isolated from the following strains: lane 1, WT (SH6471) strain; lane 2, WT-2X (SH30012) strain; lane 3, SupE-2X (SH30011) strain. The 2,500 kb band indicates intact Chromosome XII and the 1,500 kb band indicates Chromosome XII containing an rDNA cluster split at its right end and left end. The positions of the probes are shown in Fig. 10A.

In the last step, I confirmed two expected candidates out of 32 segregants (Fig. 11C) by CHEF electrophoresis, followed by Southern hybridization (Fig. 11D). The 2500-kb band indicates the position of chromosome XII and the 1500-kb band indicates the presence of an additional rDNA cluster (Fig. 11D). I designated the haploid SupE strain with two copies of the

rDNA cluster as SupE-2X (SH30011), and the wild-type strain with two copies of the rDNA cluster as WT-2X (SH30012). To quantify the copy number of the rDNA gene in the SupE-2X and WT-2X strains, quantitative real-time PCR was performed (Table 5). The copy number of the rDNA gene varied on the FOB1 background of the SupE-2X strain (Table 5), indicating that the Δfob1 background is important for stabilizing a high copy number of the rDNA gene on the Δrrn10 SUPE background. qPCR analysis revealed that WT-2X (SH30012) had about a two-fold higher rDNA gene copy number as compared with SH6446; similarly, SupE-2X (SH30011) had about a two-fold higher rDNA gene copy number as compared with SH6976 (Table 5). These results indicated that I had successfully constructed haploid S. cerevisiae strains possessing an additional rDNA cluster and thereby a higher copy number (ca. 300) of the rDNA gene.

3.3.2 An additional copy of the rDNA cluster increases RNA content on a Δ*rrn10 SUPE* background

To determine the effect of the additional copy of the rDNA cluster, I compared the rRNA level and total RNA content of the SupE-2X (SH30011) strain with those of the SupE strain (SH6976) with a single rDNA cluster. I similarly compared the rRNA level and total RNA content of WT-2X (SH30012) with those of the wild-type strain (SH6446) with a single copy of the rDNA cluster (Fig. 12A). The SupE-2X (SH30011) strain showed 30% (*$p<0.05$) higher rRNA content as compared with the SupE strain (SH6976), whereas the two wild-type strains did not show any difference in rRNA content.

The total RNA content of these strains was then measured (Fig. 12B). The SupE-2X strain showed a 61% (*p<0.05) higher RNA content as compared with the SupE (SH6976) strain, whereas the RNA content of the wild-type strains was similar regardless of the difference in rDNA copy number. Furthermore, the total RNA content was 40% higher (*p<0.05) in the SupE-2X strain than in the wild-type strain (Fig. 12B). This observation suggests that the SUPE mutation affects the rDNA transcription system differently as compared with the wild-type background.

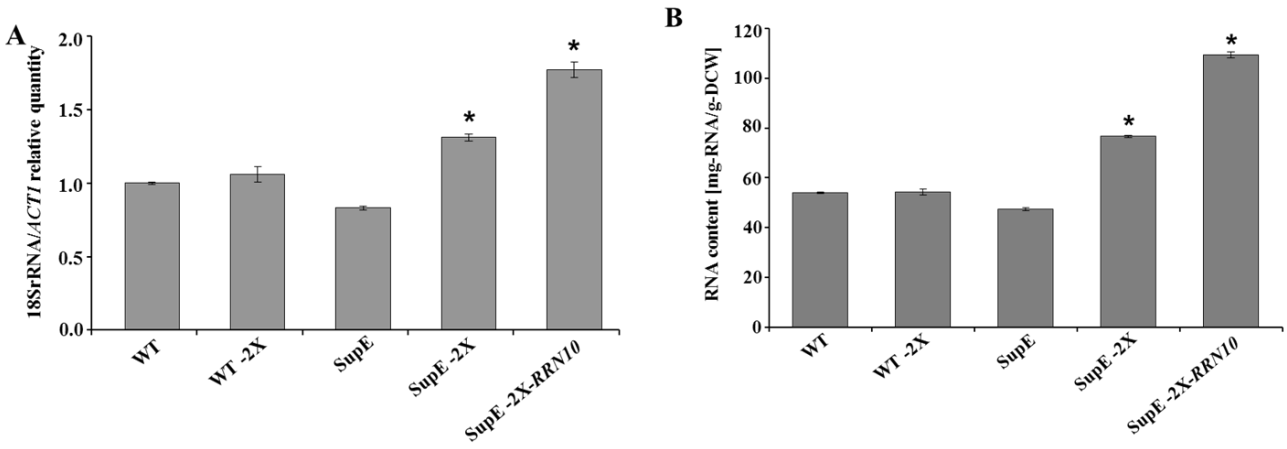

Fig. 12 Additional copy of the rDNA cluster increase rRNA content and total RNA content in the SupE strain (A) Relative quantification of 18S rRNA using real-time PCR in WT (SH6471), WT-2X (SH30012), SupE (SH6976), SupE-2X (SH30011) and SupE-2X-*RRN10* strains. Cells were collected at OD660=1.0 and RNA were extracted as described in the Materials and Methods section. The 18S rRNA amount is divided by the *ACT1* amount to normalize the 18S rRNA value and the normalized amount of 18S rRNA in WT (SH6471) is defined as 1.0 to compare the relative amount of 18S rRNA in different strains. (B) Comparison of total RNA content among WT (SH6471), WT-2X (SH30012), SupE (SH6976), SupE-2X (SH30011) and SupE-2X-RRN10 strains. Cells were collected at OD660=1.0. Total RNA of each strain was extracted by the perchloric acid method as described in Materials and Methods section. Three independent experiments were

performed using 3 independent samples and average values with ±SD are presented. Statistical analysis was performed by a one-way ANOVA test with IBM SPSS ver.21 Software (IBM, US). Asterisks (*) indicate significantly different group means at an alpha level of 0.05.

Next, I introduced the RRN10 gene into the SupE-2X strain and measured the rRNA level and total RNA content. The rRNA level increased by 35% and the total RNA content increased by 43% as compared with the SupE-2X strain without RRN10. As shown in Fig. 12, the positive effect of the additional rDNA cluster was still observed when the RRN10 gene was introduced in the SupE-2X strain. Considering all of the data in Fig. 12 together, I assume that the effect of the additional rDNA cluster on rRNA content depends on the SUPE mutation.

3.3.3. Introduction of a multicopy *RPL40A* gene further increases RNA content in the SupE-2X strain

I previously reported that introduction of a multicopy RPL40 gene, which encodes one of 60S subunit proteins, is effective at increasing RNA content on a Δrrn10 background (Δrrn10 and SupE strains), but not on a wild-type background (Khatun et al. 2013). Based on these findings, I introduced a multicopy RPL40A gene via plasmid p3374 (YEp+URA3+RPL40A) into the SupE-2X (SH30011) strain and investigated the effect of multicopy expression of the RPL40A gene on rRNA content and total RNA content on a Δrrn10 SUPE background (Fig. 13).

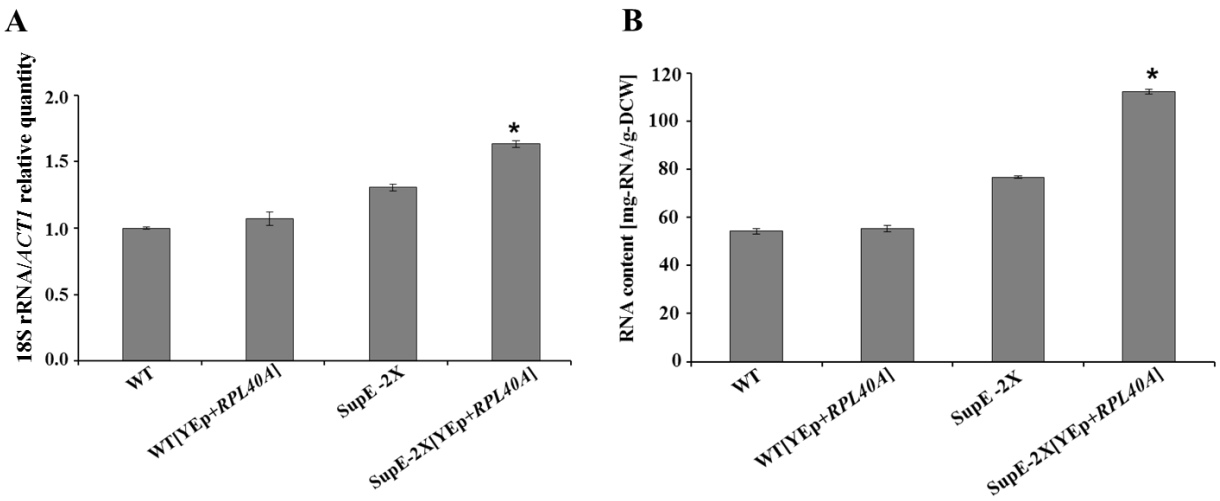

Fig. 13 Combination of multicopy *RPL40A* and double copies of the rDNA cluster under Δ*rrn10 SUPE* background increase RNA content (A) Relative quantification of 18S rRNA using real-time PCR in WT (SH6471), WT [YEp+*RPL40A*], SupE-2X (SH30011) and SupE-2X[YEp+*RPL40A*] (SH30013) strain. Cells were collected at OD660=1.0 and RNA were extracted as described in Materials and methods section. The 18S rRNA amount is divided by the *ACT1* amount to normalize 18S rRNA value and the normalized amount of 18S rRNA in WT (SH6471) is defined as 1.0 to compare the relative amount of 18S rRNA in different strains. (B) Comparison of total RNA content among WT (SH6471), WT [YEp+*RPL40A*], SupE-2X (SH30011) and SupE-2X [YEp+*RPL40A*] (SH30013). Cells were collected at OD660=1.0. Total RNA of each strain was extracted by the perchloric acid method as described in Materials and methods section. Three independent experiments were performed using 3 independent samples and average values with ±SD are presented. Statistical analysis was performed by a one-way ANOVA test with IBM SPSS ver.21 Software (IBM, US). Asterisks (*) indicate significantly different group means at an alpha level of 0.05.

The data revealed that rRNA content was increased by 25% (*$p<0.05$) (Fig. 13A) and the total RNA content was increased by 47% (*$p<0.05$) in the SupE-2X (SH30011) strain harboring the multicopy RPL40A gene as compared with the SupE-2X (SH30011) strain (Fig. 13B). As

compared with the wild-type strain, the SupE-2X [YEp+*RPL40A*] strain displayed a 1.6-fold higher rRNA content and 2-fold higher total RNA content (Fig. 13). These findings suggest that expression of the multicopy RPL40A gene on a Δ*rrn10 SUPE* background exerts a novel effect by increasing the RNA content in *S. cerevisiae*.

3.4 Discussion

In this chapter, I investigated the effect of differences in the copy number of the rDNA gene on rRNA level and total RNA content of *S. cerevisiae* strains on two different genomic backgrounds: the wild-type strain and the SupE strain on a Δ*rrn10 SUPE* mutant background. Both wild-type and SupE strains were constructed with two copies of the rDNA cluster by PCR-mediated chromosome splitting (Fig. 10 and 11). The additional copies of the rDNA gene were effective at increasing RNA content on the Δ*rrn10 SUPE* background but not on the wild-type background (Fig. 12). I also measured the doubling time of the wild-type, WT-2X, SupE and SupE-2X strains (126 min, 122 min, 172 min and 175 min, respectively) and found that the additional copies of the rDNA gene did not affect the doubling time of strains on either the wild-type or the Δ*rrn10 SUPE* background (data not shown).

I can propose a couple of reasons for the difference in rRNA level and total RNA content observed between the wild-type and the SupE strain. As compared with wild type, the SupE strain has two differences in its genotype: disruption of *rrn10*, which affects the UAF of RNA polymerase I; and the *SUPE* mutation, which restores the defects caused by *rrn10* disruption. As mentioned above, variations in rDNA gene copy number do not affect total RNA content in wild-type strains, due to feedback inhibition of rDNA transcription (French et al. 2003; Nomura 1999;

Nomura 2001). Our findings in the wild-type strain in this study support this idea; by contrast, the SupE strain showed higher RNA content when its rDNA gene copy number was increased (Fig. 12). It has been reported that Pol I transcription is a finely tuned process based on the highly cooperative nature of interactions among rDNA transcription, rRNA processing and ribosomal protein (RP) synthesis and that any defect in pre-initiation of the rDNA transcription process can alter the overall transcription process (Wai et al. 2001). Considering the above facts, I assume that there are some irregularities in the normal rDNA transcription process in a strain on the Δ*rrn10 SUPE* background. Specifically, one such irregularity might lie in the feedback inhibition of the rDNA transcription process, which would help to increase the RNA content in the SupE strain by increasing the transcription level of the additional copies of the rDNA gene. I also observed that exogenous multicopy expression of the *RPL40A* gene, which encodes a protein of the large ribosomal subunit that is required for subunit joining, optimal 27SB pre-rRNA maturation, and proper functioning of the translocation process (Fernández-Pevida et al. 2012), led to a further increase in rRNA and total RNA content in the SupE-2X strain. It has been previously reported that the SupE strain produces higher levels of mRNA of ribosomal protein genes such as the 60S subunit genes *RPL40B* and *RPL5*, and the 40S subunit gene *RPS31* (Khatun et al. 2013).

Table 5. Copy number estimation of rDNA genes in wild-type and SupE strain

Strains	***ACT1*** **avg. C_T**	***ACT1*** **Copy no.**	***MKR1*** **avg. C_T**	***MKR1*** **Copy no.**	**18S avg. C_T**	**18S Copy no.**	**5S avg. C_T**	**5S Copy no.**
SH6446 (WT-haploid)	21.55±0.24	1	21.49±0.42	1	14.51± 0.21	131.28	14.53±0.36	129.10
SH30012 (WT-2X, haploid with two copies of rDNA cluster)	21.57±0.19	1	20.98±0.18	1	13.42±0.15	285.45	13.43±0.22	283.79
(WT- 2X -*FOB1*)	20.57±0.20	1	20.64±0.18	1	13.32±0.53	152.21	13.44±0.61	137.19
SH6976 (SupE-haploid)	21.41±0.408	1	21.39±0.175	1	14.36±0.515	132.84	14.30±0.414	137.91
SH30011 (SupE-2X, haploid with two copies of rDNA cluster)	21.45±0.314	1	21.56±0.371	1	13.44±0.230	257.91	13.41±0.304	262.69
(SupE-2X-*FOB1*)	21.09±0.81	1	20.98±0.371	1	13.90±0.23	146.02	13.87±0.54	149.09

In *S. cerevisiae*, among the ~150 copies of rDNA repeats in chromosome XII, a certain number are usually not transcribed under normal conditions due to regulation of Pol I activity by the pre-initiation complex of rDNA transcription (French et al. 2003). It has been reported that any failure in one of the major systems such as DNA replication, membrane biosynthesis, rDNA transcription and ribosome formation leads to severe alteration of the transcription of rRNA genes (Wai et al. 2001; Nierras and Warner 1999). As the SupE strain is on the Δ*rrn10 SUPE* background, the pathways of rDNA transcription and RP synthesis are likely to be altered from that of the wild-type strain (Khatun et al. 2013), which might activate the transcription of additional copies of the rDNA gene. As rDNA transcription, rRNA processing, and RP gene transcription are strictly coordinated, I propose that a combination of two copies of the rDNA cluster on the *SUPE* background, possibly with further addition of the *RRN10* gene, might be an effective way to improve rRNA production in the SupE-2X strain, and thereby represents a novel strategy for constructing *S. cerevisiae* strains producing a higher content of RNA. These achievements would be beneficial not only in the field of yeast food biotechnology but also in that of transcription regulation of the rDNA gene.

3.5 Summary

Breeding of yeast strains with higher RNA content is important because yeast RNA is a significant source of 5´-ribonucleotides, which have considerable use in both the food and pharmaceutical industries. Ribosomal RNA (rRNA) is an important source of yeast RNA as it accounts for about 80% of total RNA content. I previously reported a dominant suppressor mutant of an *rrn10* disruptant named SupE, which displays the ability not only to restore diminished RNA content caused by *rrn10* disruption, but also to increase the transcription level of ribosomal protein (RP) genes on an Δ*rrn10* background in *Saccharomyces cerevisiae*.

Here, to construct an *S. cerevisiae* strain with higher RNA content, I investigated the effect of increasing the copy number of the rDNA gene on a Δ*rrn10 SUPE* background. I successfully constructed a SupE strain with two copies of the rDNA cluster (ca. 300 rDNA genes) by using chromosome splitting technology. The RNA content of this strain was 61% higher than that of the SupE strain with a single copy of the rDNA cluster (ca. 150 rDNA genes), and 40% higher than that of the wild-type strain with two copies of the rDNA cluster. A further increase in RNA content of 47% was achieved by multicopy expression of the *RPL40A* gene in the SupE strain with two copies of the rDNA cluster. These observations suggest that I have constructed an *S. cerevisiae* strain with two copies of the rDNA cluster, which has achieved a considerably higher RNA content. Furthermore, the strategy taken in this study provides an effective approach to constructing *S. cerevisiae* strains with high potential for yeast food biotechnology.

CHAPTER 4

General Discussion and Conclusion

Yeast is known to human for thousands of years as they have been used in traditional fermentation processes like wine, beer and bread making. The significance of yeasts in food technology as well as in human nutrition, as alternative sources of protein to cover the demands in a world of low agricultural production and rapidly increasing population, makes the production of food grade yeasts extremely important. A large part of the earth's population is malnourished, due to poverty and inadequate distribution of food. Scientists are concerned whether the food supply can keep up with the pace of the world population increase, with the increasing demands for energy, the ratio of land area required for global food supply or production of bioenergy, the availability of raw materials, as well as the maintenance of wild biodiversity (Pimentel et al., 1994; Pimentel and Morse, 2003; Gilland, 2002; Wolf et al., 2003). Therefore, the production yeast strain with food grade quality is a main concern for the industry and the scientific community.

The most common food grade yeast is *S. cerevisiae*, also known as baker's yeast, which is used worldwide for the production of bread, baking and other food products. Today, *S. cerevisiae* strains are also used as alternative sources of high nutritional value proteins, enzymes and vitamins, and have numerous applications in the food industry as food additives, conditioners and flavoring agents (Demain et al. 1998; Stam et al. 1998; Nagodawithana 1992). Commercial *S. cerevisiae* strains are an important subject matter for the improvement of its efficiency so that these strains are capable enough to cover the demand of the food industries or for use as nutritional supplements for humans and/or animals.

Modern techniques like DNA recombination, induced mutations, and selection methodologies generally employed to obtain new specialized *S. cerevisiae* strains with improved properties, according to the manufacturer's demands for fermentation efficiency and productivity (Randez-Gil et al., 1999; Angelov et al., 1996; Petsas et al., 2002; Olsson

and Nielsen, 2000). For example, the modern food industry demands the production of yeast strains, with higher RNA content (Nevoigt, 2008; Ringbom et al., 1996). Genetic engineering has made possible the creation of such yeast strains, with improved properties for maximum benefit. Therefore, genetic engineering can lead to the construction of *S. cerevisiae* strain with higher RNA content (Nevoigt, 2008). RNA content of various yeast species are reported by using different culture media and also by using different methods of RNA measurement to investigate the ability of yeast species to produce RNA. Although the comparison is not so precise based on the difference of culture condition and methods of RNA measurement (Jeong-Hoon et al., 2010), I made a comparison of the RNA content of those yeast strains with the *S. cerevisiae* strains that I constructed in this study as shown in Table 6. The strain *S. cerevisiae [*Δ*rrn10 SUPE*-rDNA cluster (2X)-*RRN10*] showed the highest RNA content (2.97 mg/ml-culture) and the highest RNA productivity (56.88 mg-RNA/g-DCW/hr).

Table 6. Growth and RNA yields of various yeast strains

Yeast	Growth (OD_{660})	RNA yield (mg/ml-culture)	RNA productivity (mg-RNA/g-DCW/hr)
Candida spp. (group III) OE-25	0.37	0.20	7.4
Saccharomyces spp. S-3	0.49	0.41	20.09
Kluyveromyces spp. S-7	0.23	0.54	12.42
Hansenula spp. S-9	0.25	0.57	14.25
Zygosaccharomyces rouxii S-10	0.36	0.76	27.36
S. cerevisiae T-71	0.32	1.04	33.38
S. cerevisiae HG-7	0.63	0.73	45.99
S. cerevisiae HG-10	0.56	0.99	55.04
S. cerevisiae (SupA)*	1.15	2.41	55.09
S. cerevisiae (Δ*rrn10 SUPE-RPL40A-RRN10*)**	1.08	2.67	55.59
S. cerevisiae [Δ*rrn10 SUPE*-rDNA cluster (2X)-*RPL40A*]**	1.10	2.80	46.04
*S. cerevisiae [*Δ*rrn10 SUPE*-rDNA cluster (2X)-*RRN10*]**	1.12	2.97	56.88

(Jeong-Hoon et al., 2010), ∗ Chuwattanakul et al., (2011), ** This study

In this study, I followed two general strategies to breed a *S. cerevisiae* strain with high amount of RNA, a) By increasing the transcription level of rDNA gene b) By increasing the copy number of the rDNA gene. However, it is known that transcription of rDNA is not increased in the wild-type strain up to a certain limit due to regulation of Pol I activity by pre-initiation complex of rDNA transcription (French et al. 2003). I found that *SUPE* is effective to increase rRNA level in the SupE strain (Chuwattanakul et al. 2011; Khatun et al. 2013) assuming that *SUPE* might affect the pre-initiation complex of Pol I mediated rDNA transcription. To investigate the second possible strategy of increasing RNA content, I increased the copy number of the rDNA genes in the SupE strain. Although it is reported that copy number variation of the rDNA genes in the wild-type strain did not affect rRNA level due to feedback inhibition of rDNA transcription (French et al. 2003; Nomura 1999; Nomura 2001), I expected that *SUPE* might be effective to interrupt the feedback inhibition system and will help to increase rRNA level in the SupE strain with increased copy number of the rDNA gene. Although the identities of genes involved in *SUPE* mutation are still unknown, but I can assume that the genes involved in *SUPE* mutation might affect both pre-initiation complex and also feedback inhibition mechanism of rDNA transcription.

Based on the above mentioned striking features of the SupE strain, I investigated both strategies to increase RNA content in the SupE strain and the results revealed that *SUPE* is responsible for not only increase the rRNA level but also mRNA level of other ribosomal protein genes such as *RPL40A* and *RPL40B*. Overexpression of these ribosomal protein genes further increase RNA content in the SupE strain. As rDNA transcription, rRNA processing, and ribosomal protein gene transcription are strictly coordinated, increased activities of the ribosomal protein may help to compensate for the defect caused by Δ*rrn10* disruption and afterward a combination of increased copy number of either *RPL40A* or

RPL40B with *SUPE* mutation significantly increased RNA content in the SupE strain. For further increase of RNA content, I investigated the efficiency of the second strategy of increasing RNA content by increasing the copy number of the rDNA gene. I observed that increased copy number of the rDNA genes significantly increased RNA content under Δ*rrn10 SUPE* background although it did not increase RNA content in the wild-type strain due to probable feedback inhibition of rDNA transcription system (French et al., 2003, Nomura et al., 2001). Considering the above facts, I assume that some irregular behavior might occur in the regular rDNA transcription process, which in turn helps to improve the RNA content in SupE strain by increasing the number of active rDNA genes and the transcription level of the active rDNA genes. Further investigations on the genes involved in *SUPE* mutation will be helpful to confirm possibility of exploiting this technique commercially for industrial applications.

Finally, I conclude that the strategies followed in this study for construction of *S. cerevisiae* strain with high level of RNA content and the knowledge of suppressor mutation will be useful to upgrade the strategy for molecular breeding of industrially used strain with further increased level of RNA content in *S. cerevisiae*.

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LIST OF PUBLICATIONS

1. **Khatun, F.,** Kurata, K., Chuwattanakul, V., Sugiyama, M., Kaneko, Y., and Harashima, S.: Increased transcription of *RPL40A* and *RPL40B* is important for the improvement of RNA production in *Saccharomyces cerevisiae*. ***J. Biosci. Bioeng.*** (In press; http://dx.doi.org/10.1016/j.jbiosc.2013.04.006)

2. **Khatun, F.,** Sasano, Y., Sugiyama, M., and Harashima, S.: Increase in rRNA content in a *Saccharomyces cerevisiae* Δ*rrn10* suppressor strain by rDNA cluster duplication. ***Appl. Microbiol. Biotechnol.*** (In press) (2013)

3. Chuwattanakul, V., Sugiyama, M., **Khatun, F.**, Kurata, K., Tomita, I., Kaneko, Y., Harashima, S.: Increased transcription of *NOP15*, involved in ribosome biogenesis in *Saccharomyces cerevisiae*, enhances the production yield of RNA as a source of nucleotide seasoning. ***J. Biosci. Bioeng.*** 114: 17-22 (2012)

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