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Influence of Different Gene Integration Strategies on Transcriptional Silencing at rDNA Gene in *Saccharomyces cerevisiae*

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ABSTRACT

Chromatin silencing at ribosomal DNA (rDNA silencing) is the crucial phenomenon for prevention of homologous recombination of repetitive rDNA sequences. In the previous study, rDNA silencing was shown to play a role in preservation of stability of rDNA (*RDN1*) locus contributing to enhance yeast lifespan. The silencing efficiency of rDNA locus can be detected by integration of reporter gene into rDNA locus. However, the proper strategy for integration of a reporter gene into rDNA locus has been controversial. This study showed that different type of gene integration strategies, gene insertion and gene replacement, affect silencing capability at rDNA locus of yeast *Saccharomyces cerevisiae* AS14. A reporter gene, *GIN11M86* growth-inhibitory sequence under the control of galactose-inducible promoter, was inserted into external transcribed spacer1 (*ETS1*) gene and replaced into ETS1-18S (*ETS1-RDN18*) gene of rDNA locus. The results showed that *ETS1-RDN18*-replaced strain exhibited a reduced growth rate significantly in comparison with that of the *ETS1*-inserted and wild-type strain grown under galactose-induced condition. This suggested that gene insertion is the appropriate integration strategy for assessment of rDNA silencing together with study of the critical factor to silencing at rDNA locus.

Keywords: Heterochromatin, Gene integration, rDNA silencing, *Saccharomyces cerevisiae*

INTRODUCTION

Heterochromatin is a type of eukaryotic chromosome which DNA is assembled into specialized chromatin domains (Sharp and Kaufman, 2003). Heterochromatin plays role in stabilization of repetitive DNA at centromeres, telomeres, and any positions in the genome by inhibiting recombination among homologous repeats to maintain genome stability (Grewal and Moazed, 2003). Furthermore, it also regulates the expression of genes during development and cellular differentiation or called heterochromatin-mediated transcriptional gene silencing (Johnson and Straight, 2017). The critical process associated with the formation of heterochromatin is deacetylation of histone protein at lysine residues, which conserved from yeast to human. In mammals, heterochromatin promotes genomic stability and regulates the progression of genetic diseases (Hendrich and Bickmore, 2001). Heterochromatin formation can extend lifespan and controls ribosomal RNA synthesis, whereas the decline of heterochromatin levels shows a dramatic shortening of lifespan in *Drosophila* (Larson *et al.*, 2012). In budding yeast, the major silencing targets consist of mating-type (*HML* and *HMR*), rDNA (*RDN1*) loci and telomere. The rDNA repeat is the repetitive nucleotide sequence, which locates in chromosome 12 of *S. cerevisiae*. This locus comprises of the repetitive nucleotide sequence of 9.1 kb rDNA repeat around 100-200 tandem copies as shown in figure 1A. Therefore, homologous recombination among rDNA repeats can occur easily during the loss of heterochromatin of chromosome and result in formation of extrachromosomal rDNA circle (ERC), which induces nucleolar fragmentation, cessation of cell division, and cellular senescence in yeast (Sinclair and Guarente, 1997). Normally, gene silencing requires a larger protein complex. The RENT (regulator of nucleolar silencing and telophase exit) (Ryu and Ahn, 2014) and Tof2-Lrs4/Csm1 complexes are found as the main rDNA-silencing complex in *S. cerevisiae* (Srivastava *et al.*, 2016). These complexes play a crucial role to prevent the exponential accumulation of ERC, resulting in expansion of yeast lifespan (Huang and Moazed, 2003).

Akada *et al.* (2002) developed a novel counter-selection system for repeated gene disruptions using a galactose-inducible growth-inhibitory sequence, named *GAL10p-GIN11M86*. As it was compared to previous counter-selection markers, the advantages of this counter-selection marker are that it can be performed by an inexpensive galactose medium, combined with any transformation markers for gene introduction, and no requirement of specific mutations in the host strains (Akada *et al.*, 2002).

Currently, integration of a reporter gene into rDNA array was used for detection of silencing at rDNA (Smith *et al.*, 1999), but the searching for appropriate integration strategy has been continued. Even if gene insertion and gene replacement are popular strategies for gene integration. But gene insertion strategy is widely used for assessment of rDNA silencing. Thus, rDNA silencing might be required a unique gene integration strategy. To evaluate this hypothesis, the growth-inhibitory sequence *GIN11M86*, whose expression is under the control of the galactose-inducible promoter, was used as the reporter gene. The reporter gene was integrated into rDNA gene with the two different strategies, insertion and replacement, for evaluation of rDNA silencing.

MATERIALS AND METHODS

Strains and cultivation media

Saccharomyces cerevisiae strain AS14 (genotype: *MATa trp1-1 leu2-3,112 his3-11,15 ura3-1 ade2-1 can1-100 yrs1::HIS3 yrr1::*loxP *pdr1::hisG pdr3::hisG erg3::*loxP*)*, kindly provided by Anyaporn Sangkaew, Department of Microbiology, Faculty of Science, Chulalongkorn University, used for integration of a reporter gene into rDNA region and testing of rDNA silencing. *E. coli* DH5α was used for plasmid construction.

Yeast and *E. coli* media were used as described (Burke *et al.*, 2000; Chen *et al.*, 2001). Yeast cells were grown in yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% glucose) at 30°C. Synthetic complete drop out histidine (SC-His) medium consisted of 2% glucose, 0.67% yeast nitrogen base without amino acids and essential nutrients as required except histidine. SGal-His is synthetic galactose medium in which 2% glucose of SC-His is substituted by 2% galactose. SR-His is synthetic raffinose medium in which 2% raffinose is substituted for 2% glucose in SC-His.

Construction of rDNA gene integrating cassettes and plasmids

1) **Construction of pBGG11H18s plasmid containing *ETS1-RDN18* integrating cassette**

ETS1-RDN18 rDNA integrating cassette was constructed by amplification of *GAL10p-GIN11M86* fragment from plasmid pGG116 (a kind gift from Prof. Rinji Akada, Yamaguchi University) as DNA template with a primer pair no. 3 and 8 (Table 1). The PCR condition was as follows: initial step 94 °C for 3 min, followed by 35 cycles of 98 °C for 10 sec, 52 °C for 30

sec, 72 °C for 1 min, and final extension 72 °C for 5 min. The PCR product was then ligated into *Kpn*I-*Hind*III gap of pBluescript SK+ vector (Addgene, USA) named pBGG11. A gene integrating marker, hygromycin resistance gene (*hphMX6*), was amplified from plasmid pCfB2513 (Addgene, USA) using primer no. 10 and 11 (Table 1). The PCR reaction was the same as that of the amplification of *GAL10p-GIN11M86* fragment. PCR product was then ligated into *Hind*III-*Bam*HI gap of pBGG11 plasmid to obtain pBGG11Hr18s plasmid. The *ETS1-RDN18* integrating cassette in pBGG11Hr18s was flanked by 50 homologous nucleotide sequences of 5' upstream and 3' downstream of ETS1-18S rDNA region in the yeast chromosome.

2) Construction of pBGG11HrE plasmid containing *ETS1* integrating cassette

For insertion of a reporter gene into ETS1 region of rDNA, ETS1 integrating cassette was flanked by 350 bp of 5' and 3' ETS1 homologous sequence. The 350 bp of first 5' and last 3' of ETS1 region were generated via PCR using primer pairs no. 4 and 5, and no. 1 and 2 (Table 1). The PCR condition used was the same as in the construction of pBGG11Hr18s plasmid except for annealing step at 60 ºC. The PCR product of 5'-ETS1 was digested by *Kpn*I and *Sal*I enzyme. The *Kpn*I-*Sal*I fragment containing 350 bp of 5'-ETS1 gene was ligated into the corresponding site of pBluescript SK+ vector, pBE1 was obtained. The *Spe*I-*Sac*I fragment of 350 bp of 3' ETS1 gene was ligated into the corresponding site of pBE1 plasmid, pBE2 was obtained. The pBGG11Hr18s was used as a template for amplification of *GAL10p-GIN11M86-hphMX6* fragment using primer pair no. 7 and 13 (Table 1). The PCR condition used was the same as in the construction of pBGG11Hr18s plasmid except for annealing temperature 68 ºC and extension time 1.5 min. The PCR product of *GAL10p-GIN11M86-hphMX6* fragment was digested with *Sal*I and *Spe*I and ligated into the corresponding site of pBE2 plasmid, pBGG11HrE was obtained. The KOD-Plus-Neo DNA polymerase (Toyobo, Japan) was used for all the PCR amplification.

Table 1. Primers used in this study.

No.	Primers name	Primers sequences (5'-3')	References
1	ETS1 down (*Spe*I)_F[(b)]	GACTAGTCATAGCCGGTCGCAAGA CTGTG (29-mers)	This study
2	ETS1 down (*Bam*HI, *Sac*I)_R[(b)]	CGAGCTCGGATCCACTATCTTAAAA GAAGAAGC (33-mers)	This study
3	ETS1-GAL10p_F[(a)]	GGGGTACCCCGTTTAGTCATGGAGT ACAAGTGTGAGGAAAAGTAGTTGG GAGGTACTTCGCGGAGCAGTGCGG CGCGAGG (80-mers)	This study
4	ETS1 up(*Kpn*I, *Bam*HI)_F[(b)]	CCGGTACCGGATCCATGCGAAAGC AGTTGAAGAC (34-mers)	This study
5	ETS1 up(*Sal*I)_R[(b)]	GACGTCGACTCAACAAGGCATTCC CCCAAG (30-mers)	This study
6	ETS1 upstream_F[(c)]	GGGCACCTGTCACTTTGG (18-mers)	This study
7	Gal10p(*Sal*I)_F[(b)]	GCGTCGACGGAGCAGTGCGGCGCG AG (26-mers)	This study
8	GIN11_R[(a,c)]	CCCAAGCTTGGGACTAGATGCACTC ATATCAT (32-mers)	This study
9	ITS1_R[(c)]	GCAAGACCGCGCACTTAAGC (20-mers)	This study
10	loxP_F[(a)]	CCCAAGCTTGGGATAACTTCGTATA ATGTATGC (33-mers)	This study
11	loxP-ITS1_R[(a)]	CGGGATCCCGATGCTCTTGCCAAAA CAAAAAAATCCATTTTCAAAATTAT TAAATTTCTTCACCTAATAACTTCG TATAG (80-mers)	This study
12	*Not*I-hphMX_F[(c)]	ATAAGAATGCGGCCGCTAAACTAT GACATGGAGGCCCAGAATAC (44-mers)	RL
13	*Spe*I-hphMX_R[(b)]	GGACTAGTCCCAGTATAGCGACCA GCATTCAC (32-mers)	RL

[(a)], [(b)], [(c)]: primers used for construction of *ETS1-RDN18*, *ETS1* integrating cassette, and confirmation of correct gene integration, respectively.

RL: primers were provided by Rittirat Lengwittaya, Department of Microbiology, Faculty of Science, Chulalongkorn University.

Gene integration

Firstly, the two expression cassettes of reporter gene would be linearized via restriction digestion. The *ETS1-RDN18* integrating cassette in pBGG11Hr18s was digested by *Kpn*I and *Bam*HI, while *ETS1* integrating cassette in pBGG11HrE was cut by *Bam*HI (flanked on left and right end of the cassette) and transformed into yeast cells using a high-efficiency transformation method (Gietz and Schiestl, 2007). Both cassettes were integrated into the yeast genome by homologous recombination. Yeast transformants were plated on YPD medium supplemented with 250 µg/ml hygromycin and incubated at 30 ºC for 3 days. Each transformant was confirmed that the integrating cassette was correctly integrated into the genome by PCR using combination primer pairs no.6 and 8, and no.9 and 12. PCR reaction was performed as 94 ºC for 3 min, followed by 35 cycles of 98 ºC for 10 sec, 53 ºC for 30 sec, 72 ºC for 2 min, and final extension 72 ºC for 5 min. The PCR amplicons were separated by 1% agarose gel electrophoresis.

Gene silencing assays

Cells were grown at 30 ºC in SR-His medium to an OD_{660} of 1 (mid-log phase). To test silencing assay on agar plate, the cells were washed with sterile H_2O 1 time. After that, the cells were normalized to an OD_{660} of ~0.68 (1×10^7 cells/ml) in microfuge tube and then diluted with 10-fold serial dilutions by sterile H_2O. Then 5 µl of each dilution was spotted onto SC-His and SGal-His plate. The plates were incubated at 30 °C for 3-4 days before imaging. For silencing assay in liquid medium, the cells were washed with sterile H_2O 1 time and resuspended by SC-His or SGal-His medium. The cells were then diluted with SC-His or SGal-His medium to an OD_{660} of ~0.1 and final volume 3 ml/tube. The tubes were incubated with shaking at 200 rpm, 30 °C. Growth was assessed by measuring absorbance at 660 nm for 4 days using a UV-Visible spectrophotometer (BioMate 3S UV-Visible spectrophotometer, Thermo Scientific). Each experiment was performed in triplicate.

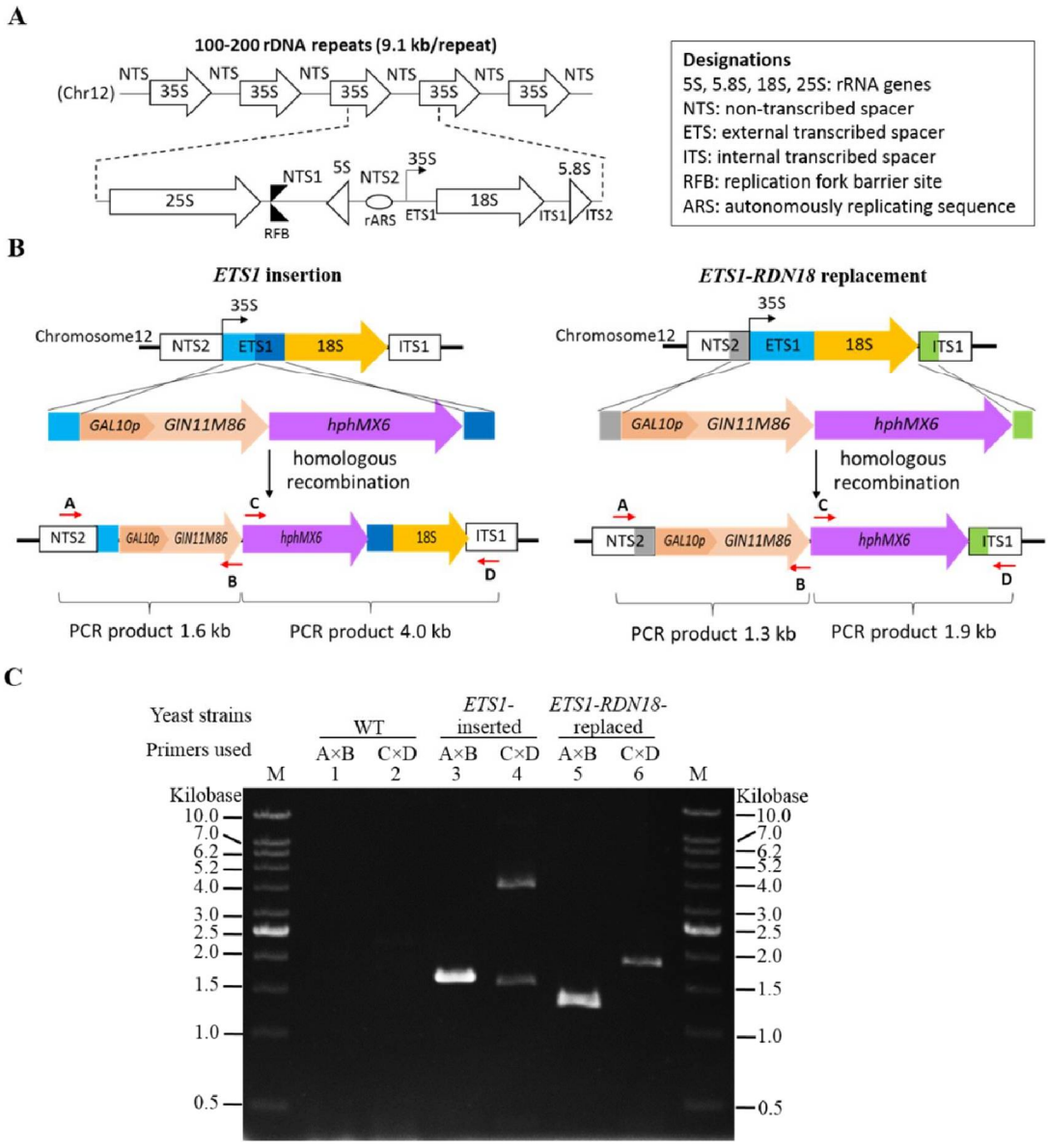
Statistical analysis

Growth curves and statistical analyses were performed with GraphPad Prism 5.01 (GraphPad Software, Inc., San Diego, CA). Yeast growth of each group was compared using two-way analysis of variance (ANOVA) and Bonferroni post-hoc test. All data were represented as means ± SEM (n = 3). A p-value of less than 0.05 was considered statistically significant.

RESULTS

Verification of correct clone/gene integration by PCR

The successful integration of each strain was confirmed the correct insertion or replacement of a reporter gene into yeast genome by PCR using two primer pairs: no. 6 and 8, and no. 9 and 12. The PCR products were separated by 1% agarose gel electrophoresis and detected by ethidium bromide staining. The result of gel electrophoresis showed the correct size of each PCR product as expected. The correct expected PCR product size of *ETS1*-inserted and *ETS1-RDN18*-replaced strains are 1.6 and 1.3 kb for primer pair no. 6 and 8, and 4.0 and 1.9 kb for primer pair no. 9 and 12, respectively, while wild-type strain was not found the PCR products for both amplifications (Figure 1C).



of *ETS1*-inserted and *ETS1-RDN18*-replaced yeast strain. (A) Schematic

Figure 1. Construction of ETS1-inserted and ETS1-RDN18-replaced yeast strain. (A) Schematic representation of the rDNA locus of S. cerevisiae. (B) Schematic representation of insertion and replacement of the GIN11M86 expression cassette into ETS1 and ETS1-RDN18 genes. Red arrows indicate the position and direction of primers used for PCR confirmation of correct gene integration. (C) PCR confirmation of GIN11M86

expression cassette integration of ETS1-inserted, ETS1-RDN18-replaced and wild-type strains. The A×B and C×D represented combination primer pairs no.6 and 8, and no.9 and 12, respectively.

Effect of different gene integration strategies on transcriptional silencing at the *S. cerevisiae* rDNA locus

Heterochromatin associated with repression of rRNA transcription. To assess the effect of different gene integration strategies on transcriptional silencing in yeast, the *GAL10p-GIN11M86* reporter was inserted into *ETS1* gene (*ETS1*::*GAL10p-GIN11M86-hphMX6*) and replaced *ETS1-RDN18* gene (*ETS1-RDN18*::*GAL10p-GIN11M86-hphMX6*) of *S. cerevisiae* rDNA locus (Figure 2A).The transcriptional silencing of GIN11M86 expression under the control of galactose-inducible promoter at rDNA locus was investigated by growing yeast strains in selective media containing different carbon source, glucose (SC-His) and galactose (SGal-His). When three yeast strains (wild-type, *ETS1*-inserted, and *ETS1-RDN18*-replaced) were cultivated in medium containing glucose which was the repressed condition for *GIN11M86* reporter gene expression (gene expression turned off). It was found that there was no different on their growth rate (Figure 2A). On the other hand, under 2% galactose induction (gene expression turned on), the *ETS1-RDN18*-replaced strain exhibited the reduced growth rate significantly when compared with that of wild-type as well as that of the *ETS1*-inserted strain (Figure 2B). Therefore, transcriptional silencing activity of GIN11M86 expression at rDNA locus of *ETS1-RDN18*-replaced strain is worse than *ETS1*-inserted strain. The rDNA silencing of each strain was confirmed on solid media, SC-His and SGal-His. Growth in solid medium was consistent with liquid medium. Nevertheless, in SGal-His agar in which containing 2% galactose, the difference of growth between *ETS1-RDN18*-replaced strain and *ETS1*-inserted strain were subtle to distinguish by eyesight (Figure 2C).

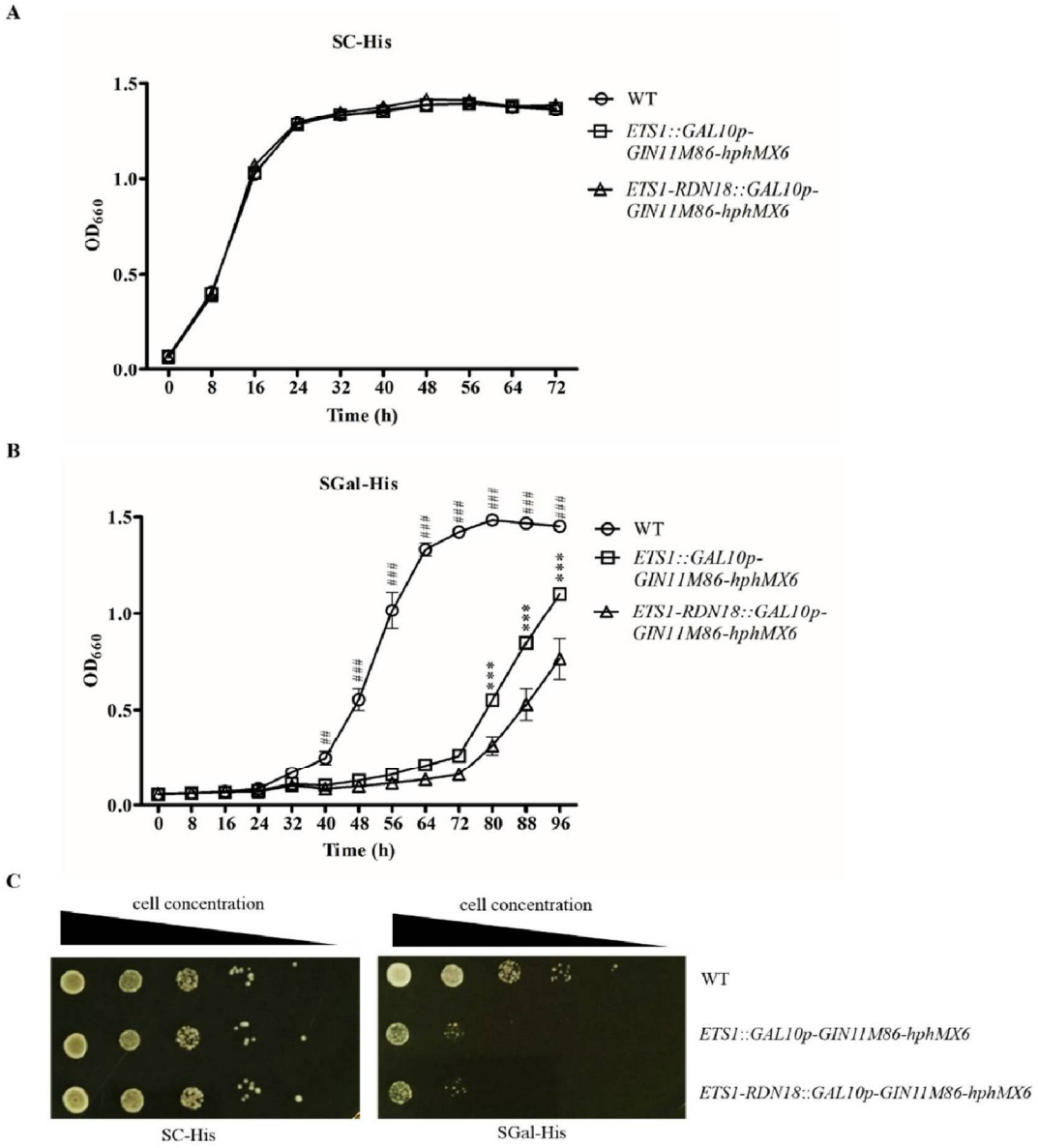


Figure 2. Effect of *GIN11M86* reporter gene expression on yeast growth in liquid and solid media. Growth curves of *S. cerevisiae* AS14 wild-type, *ETS1*-inserted, and *ETS1-RDN18*-replaced strains on liquid synthetic complete lacking histidine when reporter gene expression was turned-off (A) or turned-on (B). The experiment data were represented as means ± SEM ($n = 3$). Significance of the data was determined by two-way ANOVA-Bonferroni post-hoc test, $^{***}p < 0.001$: *ETS1-RDN18*-replaced vs *ETS1*-inserted strain; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$: *ETS1-RDN18*-replaced vs wild-

type strain. (C) Serial spot dilution assays of *S. cerevisiae* AS14 wild-type, *ETS1*-inserted and *ETS1-RDN18*-replaced strains on solid synthetic complete drop out histidine in the condition for reporter gene expression turned off (left panel) or turned on (right panel) of galactose.

DISCUSSION

The fundamental mechanisms of rDNA silencing are generally conserved in eukaryote ranging from yeast to humans (Merz *et al.*, 2008). In *Drosophila* study, deletion of rDNA array result in reduced heterochromatin-induced gene silencing elsewhere in the genome, and the range of the rDNA deletion relates to the loss of silencing (Paredes and Maggert, 2009). Alteration of copy number of rDNA array is a cause of genome-wide variation in gene expression and may lead to biologically relevant phenotypic variation (Paredes *et al.*, 2011). In yeast researches, complete deletion of rDNA brought about growth defect and altered nucleolar structures as compared with those of the wild-type strain. The altered nucleolar structures may cause by the inefficiency in rDNA transcription, but the reduction in rRNA synthesis maybe not a direct consequence of the disrupted nucleolar structure rather than an indirect consequence of some other defect, e.g. inefficient rRNA processing/ ribosome assembly or possible defects in regulation of the cell cycle (Wai *et al.*, 2000). Besides, silencing efficiency in rDNA repeat also depend on specific extent, NTS2/18S and NTS1 region have strong silencing activity (Huang and Moazed, 2003).

In this work, the researcher could not conclude that insertion or replacement strategy of the reporter gene into rDNA locus affect the alteration of heterochromatin structure because of DNA topological analysis at rDNA region has not been performed. However, the results showed gene insertion strategy exhibited a less effect on the silencing of the reporter gene at rDNA locus (Figure 2B). Silencing of *GIN11M86* reporter gene can be distinguished by the difference on growth of yeast strain with *ETS1*-insertion from most of *ETS1-RDN18*-replaced strain growth in liquid galactose-containing medium. Nevertheless, the growth of these two yeast strains were indistinguishable when were cultivated on solid medium because the subtle change of cell growth on agar plate could not differentiate by eyesight as compare to measuring cell growth in liquid medium by spectrophotometer

CONCLUSION

This study aimed to evaluate the effect of different gene integration strategies influence on the transcriptional silencing activity at rDNA gene of yeast *S. cerevisiae* which is a model organism for studying eukaryotic biological process. We showed that the different type of gene integration strategies gene insertion and gene replacement, affected rDNA silencing. The yeast strain with a reporter gene inserted into rDNA locus exhibited silencing on the expression of *GIN11M86* reporter gene inducing growth inhibition better than the strain with a reporter gene integrated into rDNA locus by replacing of some of rRNA genes.

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