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Study on dsRNA Mediated Gene Silencing in *Saccharomyces cerevisiae*: Suppressing The Expression of *GRE3* gene*

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Abstract RNA silencing is increasingly employed as an experimental strategy to probe for gene function in several organisms. The purpose of the present study was to test the effect of gene silencing by dsRNA in budding yeast *Saccharomyces cerevisiae*. *GRE3* gene encoding an NADPH-dependent aldose reductase was chosen as an example. A recombinant plasmid psiLENT-*GRE3* was constructed based on the pESC-LEU backbone and used to transform *S. cerevisiae* YPH499. The down regulation of *GRE3* gene expression by inducing 1 kb RNA duplex and a 136 bp loop was investigated using reverse transcription - PCR. The results showed that double-stranded RNA mediated gene silencing could be used as a functional tool to decrease the expression level of a specific gene in *S. cerevisiae*, which would contribute to the understanding of RNA interference in budding yeast.

Key words RNA interference, *Saccharomyces cerevisiae*, *GRE3* gene, aldose reductase

The success of genome sequencing projects is a milestone for access to the post-genome era of the research on life science. As the mass gene information increasing, analysis of gene function is absolutely necessary for understanding the complexity of biological processes. Genomics study has already completed the transition from structural genomics to functional genomics. Several experiment approaches have recently been developed for the purpose of probing genome function and the regulation mechanisms[1].

Double stranded RNA (dsRNA) mediated gene silencing, known as RNA interference (RNAi), is spread rapidly to various organisms for molecular and cellular research[2~4] since its discovery by Fire *et al*, in 1998 [5]. The potential of using this mechanism to explore gene function, validate candidate drug targets and perhaps even treat disease[6] attracts a number of researchers focusing on RNAi. Without manipulation of the genetic material, the temporal induction of RNAi allows fast and specific disruption of gene expression during different developmental stages of the organisms. After the amount of the interested protein begins to decline, direct effects can be observed, especially important for lethal genes. In this sense, this method is more informative, offering a strong advantage over the gene knockout method in the case that complete loss of the gene may affect viability or for a specific reason that a knockdown effect is needed.

The gene silencing phenomena have also been observed in several species of fungi, such as *Neurospora crassa*, *Schizosaccharomyces pombe*, *Magnaporthe oryzae* and *Aspergillus nidulans* [7]. However, situations are not quite the same for *Saccharomyces cerevisiae*, one of the most important model organisms with sophisticated genetic system for basic research. Although Chen *et al*.[8] reported that proteasome inhibition was induced using RNAi approach in *S. cerevisiae*, it still remains a disputation if *S. cerevisiae* possesses the silencing machinery since it lacks the essential and evolutionarily conserved protein components including RNA-dependent RNA polymerase (RDRP) and argonaute of the

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post-transcriptional gene silencing (PTGS) pathway[9].

S. cerevisiae aldose reductase (AR, EC 1.1.1.21) encoded by *GRE3* gene is a monomeric NADPH-dependent oxidoreductase with overlapping substrate specificity and classified as a member of the aldo-keto reductase (AKR) superfamily[10]. Expression of *GRE3* gene is induced not only in response to osmotic stress but also to ionic, oxidative and heat stress, which suggests that it may be under the control of a general stress response network, including the high-osmolarity glycerol (HOG) pathway [11]. The aldose reductase from *S. cerevisiae* was also found to participate in the degradative pathway of methylglyoxal(MG), which must be strictly controlled in order to prevent the accumulation of this 2-oxoaldehyde to toxic levels *in vivo* [12]. Moreover, interest in this enzyme stems largely from the desire to improve bioconversion efficiency of pentose sugars in biomass for obtaining greater economic value, as this endogenous enzyme is thought to play a significant role in production of the main byproduct xylitol during pentose fermentation. Over-expression of *GRE3* gene greatly increased the formation of ethanol but also that of xylitol [13]. While deleting it led to the decrease of xylitol, companied with the reduction of biomass [14], which was unfavorable for industrial applications. Up to the present, the regulation mechanism of *GRE3* gene and the physiological function of AR still remain unclear.

In this study, we chose *GRE3* gene as an example to test the effective and feasible knockdown using RNAi treatment in *S. cerevisiae*. An RNAi vector psiLent-*GRE3* was constructed and transformed to *S. cerevisiae* YPH499. Expression profiles of *GRE* of transformants under or without induction of RNAi were analyzed by RT-PCR at mRNA level. Our data indicate that RNA silencing provides an available approach for exploring function of specific genes in *S. cerevisiae*.

1 Materials and methods

1.1 Strains, plasmids and genes

S. cerevisiae YPH499 (*ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1,*α) and pESC-LEU vector were purchased from Stratagene (USA) for yeast transformation and plasmid reconstruction. *E. coli* DH5α was routinely used for gene cloning and manipulation. Plasmids T-vector-*GRE3* and T-vector -*xylA* were constructed for sequencing and stored in our laboratory, with *GRE3* gene amplified from *S. cerevisiae* CICC1747 and *xylA* gene encoding xylose isomerase from *E. coli* K12 respectively. *GRE3* gene and the DNA template for producing loop of mRNA were amplified from these two plasmids as mentioned in **2.1**.

1.2 Enzymes and chemicals

Restriction endonuclease, T4 DNA ligase, SuperScript Ⅲ Reverse Transcriptase and TRIzol reagent were obtained from New England Biolabs (UK) and Invitrogen (USA). Plasmid DNA was prepared with a BioDev plasmid rapid isolation kit (China). The QIAquick gel extraction kit (Qiagen, Germany) was used for DNA extraction from agarose gel. Reaction conditions were as recommended by the manufacturers. Ampicillin, yeast extract and peptone were obtained from OXOID (UK). Other general chemicals were in-country analytical reagents.

1.3 Transformation

Competent cells of *E. coli* DH5α were prepared and transformed as standard method [15]. *E. coli* transformants were selected on Luria-Bertani (LB) medium plates with 100 mg/L ampicillin.

Yeast transformation was made according to the instruction manual of pESC yeast epitope tagging vectors supplied by Stratagene (USA). *S. cerevisiae* transformants were selected on SD dropout medium plates.

1.4 Media and cultivation

Yeast transformants were cultivated in 50 ml of SD (-LEU) medium (6.7 g/L Yeast Nitrogen Base without amino acids plus 20 g/L of glucose and 1.3 g/L amino acid mixture lacking leucine) in a 250 ml flask shaken at 200 r/min and 30℃ for 38 h. Then cells were harvested and transferred to the SG (-LEU) medium (6.7 g/L Yeast Nitrogen Base without amino acids plus 20 g/L of galactose and 1.3 g/L amino acid mixture lacking leucine) of equal volume and incubated for another 48 h. Finally, 0.5 ml culture was inoculated into 50 ml of SD (-LEU) medium for cultivation of 48 h without induction.

1.5 RNA preparation and RQ1 RNase-Free DNase treatment

Total RNA was isolated from 5 ml yeast cultures at different time points after transfer to SG (-LEU) medium. Cells were grinded with liquid nitrogen, followed by TRIzol reagent (Invitrogen) method. Then the total RNA was treated with RQ1 RNase-Free DNase to eliminate DNA contamination. In brief,

about 5 μg of RNA in 10 μl DEPC water was mixed with 1.5 μl of 10×RQ1 RNase-Free DNase reaction buffer and 3.5 U RQ1 RNase-Free DNase to final volume of 15 μl and incubated at 37℃ for 1 h. The reaction was stopped by the addition of 1.5 μl 20 mmol/L EGTA, pH 8.0, followed by inactivation of RQ1 RNase-Free DNase at 65℃ for 10 min. RNA was quantified by spectrophotometer and its quality was checked by gel electrophoresis.

1.6 Reverse transcriptase PCR

About 2 μg of total RNA was used for reverse transcription using SuperScript™ Ⅲ reverse transcriptase (Invitrogen) according to the manufacturer's recommendations, together with negative control reactions. Amplification of cDNA by PCR using each of the appropriate primers (Table 1) was performed for 60 s at 94℃, 50 s at 60℃ and 90 s at 72℃ for 35 cycles after an initial denaturation step for 5 min at 94℃, then 1 cycle at 72℃ for 10 min. 2 μl of each RT-PCR reaction was run on 0.8% agarose gel with ethidium bromide in 1×TBE buffer, pH 8.0.

In order to avoid that the encoding *GRE3* gene inserted in the recombinant plasmid intervened analysis results, one of the primers adopted part of the non-ORF sequence for analyzing *GRE3* mRNA transcripted from genomic DNA.

Table 1 Primers used in RT-PCR analysis

Target mRNA	Primer sequence	Amplicon size /bp
GRE3	Forward, 5′ ACGAGAAGAAAGGTCACATCAC 3′	611
	Reverse, 5′ TCGTCGTTGAGTATGGATTTTA 3′	
PDA1	Forward, 5′ GGTCAGGAGGCCATTGCTGT 3′	673
	Reverse, 5′ GACCAGCAATTGGATCGTTCTTGG 3′	
TDH1	Forward, 5′ TCGCTACCTACCAAGAAAGAGACC 3′	643
	Reverse, 5′ ACCCAAGAAATCAGAGGAGACAAC 3′	

2 Results

2.1 Construction of recombinant psiLENT-*GRE3*

Plasmids T-vector-*GRE3* and T-vector -*xylA* were used as DNA templates for PCR. The primers (Table 2) were designed to include sequences recognized by different restriction enzyme (underlined) so that the resulting PCR fragments could be digested and ligated correctly to the pESC-LEU vector as Figure 1. The recombinant vector psiLENT-*GRE3* contained two fragments of full-length coding region of *GRE3* gene ligated in reverse direction under the control of the repressible GAL1 promoter, which was repressed when transformed cells were grown in SD dropout medium and was induced when the cells were grown in SG dropout medium.

Table 2 Primers used for construction of psiLENT-*GRE3*

Primer name	Primer sequence	DNA template	Amplicon size /bp	Restriction enzyme sites
*GRE3*sen-f	5′ CCGCTCGAGATGTCTTCACTGGTTA 3′	T-vector-*GRE3*	1 001	*Xho* Ⅰ -> *Bgl* Ⅱ
*GRE3*sen-r	5′ GAAGATCTTCAGGCAAAAGTGGGGAA 3′			
*GRE3*anti-f	5′ CTAGCTAGCATGTCTTCACTGGTTACTCTT 3′	T-vector-*GRE3*	1 000	*Eco*R Ⅰ <- *Nhe* Ⅰ
*GRE3*anti-r	5′ GGAATTCTCAGGCAAAAGTGGGGAA 3′			
*GRE3*loop-f	5′ GAAGATCTCACTTGCGTTTTGCCG 3′	T-vector-*xylA*	136	*Bgl* Ⅱ --- *Eco*R Ⅰ
*GRE3*loop-r	5′ CGGAATTCCTTCGCCAACGCCA 3′			

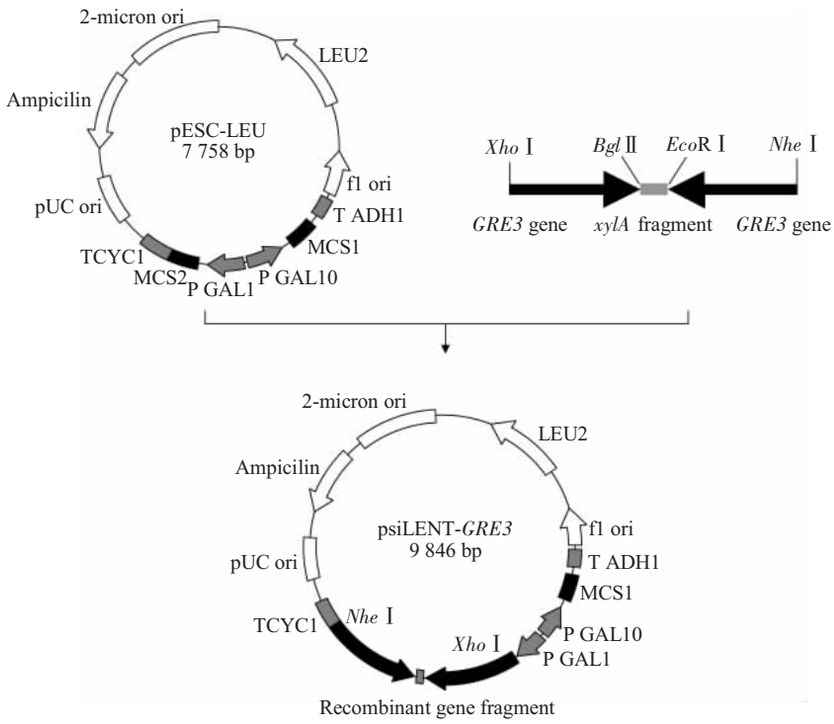

Fig. 1　Construction of recombinant psiLENT-*GRE3*

The PCR fragments using plasmids T-vector-*GRE3* and T-vector-*xylA* as DNA templates and the primers as Table 2, were digested and ligated to the pESC-LEU vector which was linearized with *Xho* Ⅰ and *Nhe* Ⅰ, allowing two fragments of full-length coding region of *GRE3* gene ligated in reverse direction under the control of the repressible GAL1 promoter in the recombinant psiLENT-*GRE3*.

2.2　Prediction of mRNA secondary structure

We used RNA Structure 4.3 [16,17] to predict the structure of mRNA. RNAstructure is a Windows program for the prediction and analysis of RNA secondary structure, based on the Zuker algorithm for free energy minimization using the nearest neighbor parameters of Doug Turner and co-workers. A recursive algorithm is used that generates an optimal structure and a series of suboptimal structures with free energy similar to the lowest free energy structure. By means of forcing single stranded of loop, we could still obtain one structure of relatively low free energy as Figure 2 using the recombinant sequence. This result suggested that intramolecular hybridization probably generated the RNA with 1 kb RNA duplex and a 136 bp loop.

Fig. 2　mRNA secondary structure was predicted using RNA Structure 4.3 with default values

2.3　Identification of psiLENT-*GRE3*

psiLENT-*GRE3* was digested by *Xho* Ⅰ / *Nhe* Ⅰ and *Eco*R Ⅴ respectively for identification. The DNA fragments were run on 0.8% agarose gel with ethidium bromide in 1×TBE buffer, pH 8.0. The recombinant plasmid was cut out of a fragment of 2.1 kb with *Xho* Ⅰ and *Nhe* Ⅰ (Figure 3), which equaled double sizes of *GRE3* gene plus the loop. As to the products digested by *Eco*R Ⅴ, three fragments were seen on the electrophoresis map due to one same restriction enzyme site in pESC-LEU backbone. The smallest stripe was approximately 0.8 kb (Figure 4), obtained

from the inserted recombinant *GRE3* gene fragments. Therefore, the electrophoresis pattern confirmed the constructed vector.

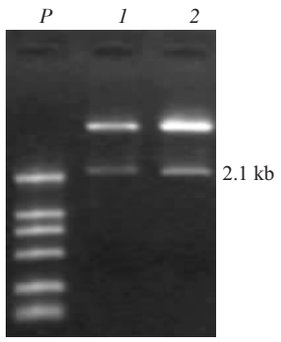

Fig. 3 The electrophoresis map for identification of recombinant psiLENT-*GRE3* digested with *Xho* Ⅰ and *Nhe* Ⅰ

1,*2*: psiLENT-*GRE3*, *Xho* Ⅰ/*Nhe* Ⅰ; *P*: DNA marker DL 2000 (Takara, Japan).

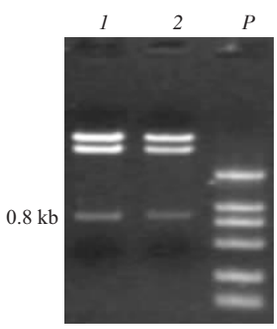

Fig. 4 The electrophoresis map for identification of recombinant psiLENT-*GRE3* digested with *Eco*RⅤ

1,*2*: psiLENT-*GRE3*, *Eco*RⅤ; *P*: DNA marker DL 2000 (Takara, Japan).

2.4 Detection of RNA silencing towards *GRE3* gene

Compared with permanent knockout technologies, the effect of RNAi is also called "knockdown" because the protein is not necessarily eliminated from the cells completely. If a small amount of mRNA escape degradation or the rate of protein turnover is slow, some protein can remain in the cell for a long time so that the true phenotype is weaken or masked and difficult to detect. However, RNAi uses gene-specific double-stranded RNA (dsRNA) to disrupt gene expression at the level of messenger RNA (mRNA) in organisms. Reverse transcription-polymerase chain reaction (RT-PCR) is a sensitive technique for the detection of low-abundance mRNA. Although mainly used for qualitative studies, this method may be adequate to determine the significant variation in mRNA expression patterns.

TDH1 gene is the housekeeping gene encoding glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) at the stationary growth phase [18,19]. Transcription of *PDA1* gene is also stable in stationary phase cultures and the *PDA1* mRNA is easily detected in total RNA samples, making it an excellent standard for mRNA quantification [20,21]. In our presented study, both *TDH1* and *PDA1* genes were used as standard internal controls for normalization, so that the measured differences reflected true changes in expression rather than sample loading.

For experimentation, psiLENT-*GRE3* and pESC-LEU were used to transform *S. cerevisiae* YPH499. Individual colonies were grown on SD (-LEU) medium for 38 h, then washed with sterilized water and shifted to SG (-LEU) medium. After continual induction for 48 h, 0.5 ml culture was inoculated into 50 ml of SD (-LEU) medium for another cultivation of 48 h without induction. 5 ml cultures were taken out for RT-PCR analysis after induction of 8 h, 24 h and 48 h, or transfer to SD (-LEU) medium for 48 h respectively. The profiles of all the samples were as Figure 5. mRNA levels of cells transformed by psiLENT-*GRE3* for *GRE3* gene showed a remarkable drop after cells growing in SG (-LEU) medium for 8 h(Figure 5-*5*), which was barely detected, compared with that by pESC-LEU (Figure 5 -*1*). While after the inducible factor of galactose was removed, transcript of *GRE3* gene could be observed

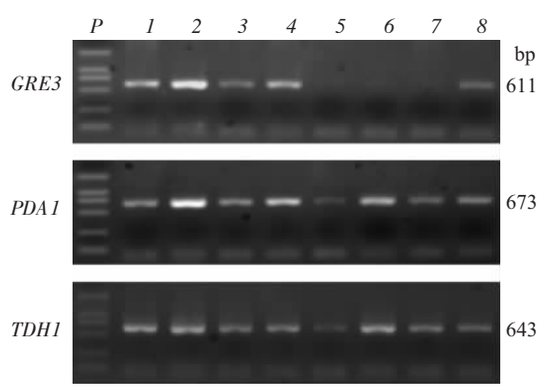

Fig. 5 RT-PCR detection of *GRE3* mRNA from *S. cerevisiae*

Total RNA isolated from yeast cells transformed with pESC-LEU and psiLENT-*GRE3* was treated with RQ1 RNase-Free DNase and amplified by RT - PCR using the primers listed in Table 1. 3 μl of RT-PCR product was run on a 0.8% agarose gel with ethidium bromide. *PDA1* mRNA and *TDH1* mRNA were used as gene expression control. *1*～*4*: pESC-LEU / 8 h, 24, 48 h in SG and another 48 h in SD; *5*～*8*: psiLENT-*GRE3* / 8 h, 24, 48 h in SG and another 48 h in SD; *P*: DNA marker DL 2000 (Takara, Japan). At least three independent colonies were assayed for each strain.

again (Figure 5-*8*). These data indicted that replacement of cells transformed with psiLENT-*GRE3* in SG (-LEU) medium allowed the induction of the phenomena of RNAi, leading to the silencing of *GRE3* gene expression.

3 Discussion

Prior to the present study, the yeast *S. cerevisiae* appears to be devoid of typical two ribonuclease machines known as pivotal components of RNAi pathway. The ribonuclease Ⅲ enzyme Dicer initiates the RNAi pathway by generating the active short interfering RNA trigger. Silencing is effected by the RNA-induced silencing complex and its RNaseH core enzyme Argonaute[22]. However, Nathan *et al*. [23] have firstly identified a new gene silencing mechanism of *S. cerevisiae* recently. SUMO is attached to a large number of histone lysines to repress transcription and exerts its negative genomic regulatory effect in chromatin. If RNAi in *S. cerevisiae* stems from histone sumoylation or other RDRP and Argonaute independent mechanisms is still unknown.

RNAi-related phenomena play a significant role in the bioprocess of eukaryotes, revealing a new kind of gene expression regulation. Compared with conventional reverse genetic tools, RNAi operates in a sequence-specific manner, allowing to silencing single gene or multiple genes at the same time for gene function analysis, and uncovering interaction and cooperative effects of them. Although the knockout strategy based on homologous recombination is the most direct way to assess gene function at the DNA level in genome. About 6 000 *S. cerevisiae* deletion strains were generated using this method [24]. Information on function of essential genes in various aspects of biological processes, which remains largely unavailable and can not be achieved by gene deletion strategies, can partially clarified by RNA silencing with an inducible promoter or transient silencing by siRNA since it is useful for study of gene expression at a specific stage during development or before lethality. Some observations supported the notion that dsRNA and antisense RNA pathways might share a common mechanism. But the level of gene silencing was much higher in the panhandle strain than that in the antisense strain[25,26].

On the other side, RNAi silencing gene expression is a direct approach to perturb metabolic networks at the transcriptional level. In view of the available *S. cerevisiae* genome, combination of RNAi-based technology with analysis methods of bioinformatics applying in the molecular perturbing of yeast metabolism provides an approach for understanding the complexity of systems biology, and will contribute to strain optimization and even biotechnology.

In this paper, we validated the expression silencing towards *GRE3* mRNA mediated by dsRNA. The feasibility and potential of RNAi as a new tool for the research of functional enzymes such as aldose reductase was also tested by this inducible RNAi treatment. The results with experimental data revealed that RNA silencing was functional in *S. cerevisiae* and supported the previous report by Chen *et al*[8]. On the basis, we'll study how the course of RNAi effect or the different degrees of knockdown affect the function of some specific enzymes of *S. cerevisiae* in the future work.

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酿酒酵母中双链 RNA 介导基因沉默技术研究 *GRE3* 基因表达抑制 *

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摘要 RNA 沉默技术作为探索基因功能的实验手段应用于多种生物. 以编码酿酒酵母 NADPH 依赖型醛糖还原酶的 *GRE3* 基因为对象，检测酿酒酵母双链 RNA 介导的基因沉默效应. 以 pESC-LEU 为骨架，构建重组质粒 psiLENT-*GRE3* 并用于转化酿酒酵母 YPH499. 用 RT-PCR 检测到诱导 1 kb RNA 双螺旋和 136 bp loop 结构引起的 *GRE3* 基因表达下调. 结果表明，双链 RNA 介导的基因沉默技术，能够用作降低酿酒酵母某一特定基因表达水平的工具. 并有助于理解芽殖酵母的 RNA 干扰现象.

关键词 RNA 干扰，酿酒酵母，*GRE3* 基因，醛糖还原酶

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