Expression of a Yeast Superkiller Gene(*SKI3*) in *Saccharomyces cerevisiae*

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*Saccharomyces cerevisiae*에서 효모 Superkiller 유전자(*SKI3*)의 발현

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ABSTRACT: A yeast chromosomal superkiller gene (*SKI3*) was cloned and expressed in *ski*⁻ *Saccharomyces cerevisiae* strains. The gene was fused to the structural region of *E. coli lacZ* gene at its C-terminus in a yeast-*E. coli* shuttle vector, pSR605. The fused gene complemented *ski3*⁻ strains with *SKI3* activity and the quantitative level of expression was measured as determined by assaying β-galactosidase activity. The SDS-polyacrylamide gel electrophoresis and the Western blot analysis of this fused protein showed the immuno-reacted bands with a protein of the estimated molecular size (ca.250 Kd).

KEY WORDS□*SKI3*, *S. cerevisiae*, *lacZ*, gene fusion

Strains of the yeast *Saccharomyces cerevisiae* carry as many as five nonhomologous species of double-stranded RNA (dsRNA), called L-A, L-BC, T, W and M. (Wickner, 1980). Among these dsRNAs, L-A, L-BC and M are found in intracellular virus-like particles. M dsRNA encodes a secreted protein toxin, called a killer toxin, and immunity to the homologous toxin. Thus, sensitive yeast strains lacking M dsRNA are killed by this toxin. The high titer of M dsRNA in killer strains, however, causes cell pathology by over-utilizing some host proteins needed by M dsRNA for its replication (Ridley *et al.*, 1984). Mutant strains defective in the *SKI* genes (*ski*⁻) were first isolated by their superkiller phenotype and found to have a higher M dsRNA copy number than the wild type (Toh-e *et al.*, 1978; Toh-e and Wickner, 1979). As a consequence, the proteins encoded by *SKI* genes were suggested to be repressors of the M dsRNA replication, protecting the host from the otherwise lethal effects of M dsRNA. Among the six known *SKI* genes (*SKI2*, *SKI3*, *SKI4*, *SKI6*, *SKI7* and *SKI8*), only *SKI3* (Rhee *et al.*, 1989) and *SKI8* (Sommer and Wickner, 1987) genes have been cloned. The expression of these cloned genes, however, was confirmed only by the phenotypic variations. In the present investigation, we fused *SKI3* gene to the structural region of *lacZ* gene at the C-terminus for the quantitative determination of the level of gene expression.

MATERIALS AND METHODS

Strains, plasmids and media

The strains of *S. cerevisiae* and *E. coli* employed in this study are listed in Table 1 along with plasmids. For *S. cerevisiae*, the YPAD medium consisting of 10g yeast extract, 0.4g adenine sulfate, 20g peptone and 20g dextrose per liter (pH 6.0) was used as a non-selective medium and the H synthetic complete medium lacking uracil (H-ura) as a selective medium (Wickner, 1980). The MB medium for complementation test was the YPAD medium buffered at pH 4.7 with 0.1M sodium citrate and contained 0.003% methylene blue. For *E. coli*, the LB medium supplemented with amplicillin (50 μg/ml) was used for the selection of specific markers (Davis *et al.*, 1980).

Table 1. *Strains and plasmids used*

Strains	Genotype	
S. cerevisiae		
2602	*MAT α*	*ura3-52 his6 leu2 ski3-11*
2634	*MAT α*	*leu2 his5 ura3 ski3-11 mkt1*
5×47	*MATa/α*	*trp/+ ura3/+ his1/+* [KIL-0]
E. coli		
HB 101	*hsdR hsdM recA13 supE44 lacZ4 leuB6 proA2 thi-1 rpsL*	

Plasmids	Designation	Source
pSR605	A shuttle vector carrying 4.3 Kb of *SKI3* fused to *lacZ*	This work
pSR308	pTI15 inserted by whole sequence of *SKI3*	This work
pLG669-Z	A derivative of YEp24-B carrying *lacZ* under the control of *CYC1* promoter	Guarente and Ptashne (1981)
YEp356	A shuttle vector carrying *lacZ* and multiple cloning site	Myers *et al.* (1986)
pTI15	A derivative of *CEN* vector YCp50 carrying multiple cloning site of pUC18	Icho and Wickner (1988)
pSKI3:5-1	pTI15 inserted by 7 Kb fragment containing *SKI3*	Rhee *et al.* (1989)

***In vitro* construction of fusion plasmids**

The fusion of *SKI3* gene with *lacZ* was constructed as shown in Fig. 1. The gene was isolated from the plasmid pSKI3:5-1 by treating this DNA with Bam HI and Sal I endonucleases. The Bam HI-Sal I fragment containing the 4.3 Kb of the whole *SKI3* gene was inserted to the *CEN* vector, pTI15 (Icho and Wickner, 1988), which was cut with the same enzymes. The resulting plasmid, pSR308, was subsequently cleaved with Pst I endonuclease. A yeast-*E. coli* shuttle vector, YEp356 (Myers *et al.*, 1986) with unique multiple restriction sites was also digested with Pst I and a 4.3 Kb fragment isolated was then inserted into this site of the plasmid to construct a new plasmid, pSR605.

Assay of *SKI3* activity

The activity of *SKI3* gene was determined indirectly but quantitatively by measuring β-galactosidase activity as described by Miller (1972).

The direct but qualitative assay was made by complementation test according to Wickner (1980).

Transformation

The transformation methods were followed as described by Maniatis *et al.* (1982) for *E. coli* and Ito *et al.* (1983) for yeast.

Preparation of yeast cell extracts

Yeast cells were grown overnight at 30 °C and chilled on ice for 15 min. Cells were collected by centrifugation at 7000 rpm and resuspended in 0.1 volume of the buffer solution consisting of 0.1 M Tris-HCl (pH 7.8), 20% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (Sigma). Glass beads were added to the meniscus, and the mixture was frozen at -20 °C. Cell extracts were made by vigorous shaking on a Vortex mixer for six times, 15 sec each at 4 °C. An equal volume of buffer solution was added and the supernatant was collected for SDS-polyacrylamide gel electrophoresis after centrifugation for 15 min at 4 °C.

SDS-polyacrylamide gel electrophoresis

7.5% acrylamide gels containing 0.1% SDS were run according to Laemmli (1970). Gels were stained in Coomassie brilliant blue dye solution for 3 hrs. Photographs were taken using Kodak electrophoresis duplicating paper EDP.

Western blot analysis

After separation by SDS-polyacrylamide gel electrophoresis, protein samples were electroblotted onto a nitrocellulose sheet at 1.5 amps for 1 hr in TAE buffer. The sheet was incubated with 1% bovine serum albumin in TBST buffer containing 10 mM Tris-HCl (pH 8.0), 0.15 M NaCl and 0.05% Tween 20 for 30 min. Then the sheet was incubated with β-galactosidase antibody in TBST buffer for 30 min. The sheet was washed 3 times with TBST buffer, incubated with alkaline phosphatase-conjugated se-

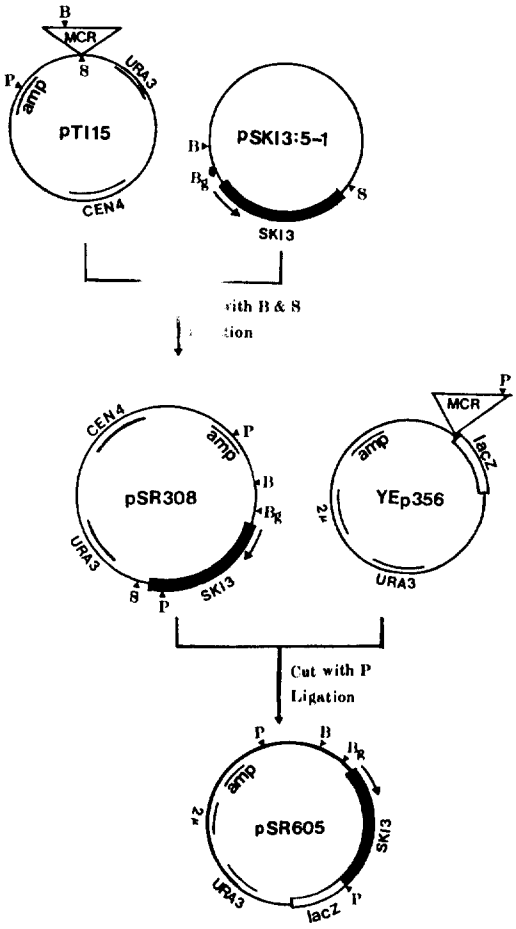

Fig. 1. *Construction of a SKI3::lacZ fusion vector, pSR605.*
Abbreviations: B, Bam HI; Bg, Bgl II; P, Pst I; S, Sal I; MCR, Multiple Cloning Region; Arrow, direction of transcription.

cond antibody (Promega), and developed according to the manufacturer's recommendations.

RESULTS

In-frame fusion of *SKI3* to *lacZ*

Owing to the unknown biological functions of *SKI3* gene product, the *in vitro* assay system of this protein has not been established. Hence, the strategy was to measure its activity indirectly as the corresponding β-galactosidase activity by fusing *SKI3* gene to *lacZ* gene. A *lacZ* fusion vector, YEp356, was used as a cloning vehicle since this plasmid allows the fusion of *SKI3* to the *lacZ* structural gene in frame in its multiple cloning region upstream from the *lacZ* gene (Fig. 2). The inserted DNA fragment of yeast includes 5′ upstream regions and the coding regions of the total of 4.3 Kb *SKI3* gene lacking only 27 bp in the C-terminus. Since YEp356 could express β-galactosidase neither in yeast nor in *E. coli* in the absence of inserted promoter sequences, and the *lacZ* gene of *E. coli* fused to *SKI3* lacks its own promoter sequences, the translational signals and the first seven codons, it was clear that 5′-nontranslated sequences must contain the *SKI3* promoter. The 16 Kb fusion vector thus constructed was named as pSR605 (Fig. 1) and has two replication origins from both of *E. coli* ColE1 plasmid and of the yeast 2μ circle together with Ap^r and *URA3* genes for the selection of both *E. coli* and yeast transformants.

Expression of *SKI3* in *ski3*⁻ yeast mutants

The fusion vector pSR605 was transformed into *ski3*⁻ yeast strains of 2602 and 2634 with the high transformation frequency. These cells were grown on H-ura medium for 72 hrs at 30°C. Among 100-200 transformants obtained in each plate, the colonies were randomly picked up and transferred into the liquid H-ura medium. The overnight cultures were subjected to the assay of β-galactosidase activity. As shown in Table 2, the transformants of each strain yielded β-galactosidase activity indicating that the *SKI3* gene was expressed exclusively under the control of the *SKI3* promoter in *ski3*⁻ mutant strains. In order to confirm this, the complementation tests were also undertaken using the cold-sensitive phenotype of *ski3*⁻ mutants at 20°C and the suppression of M2 dsRNA exclusion by L-A-HN in *mkt1 SKI3*⁺ strains (Toh-e and Wickner, 1980). The *MKT1* gene is needed specifically by M2 dsRNA if L-A-HN is present (Wickner, 1980). This requirement is temperature dependent, with the loss of M2 dsRNA at 32°C but not at 20°C. The *ski*⁻ mutations also suppress this effect of *mkt1* mutations so that an *mkt1 ski3*⁻ L-A-HN M2 strain can stably maintain M2 dsRNA even at 32°C (Ridley *et al.*, 1984). Consequently, the transformants of each strains were streaked onto H-ura plates for the single colony isolation and incubated at 32°C and 20°C. When the full size colonies were developed, the plate was replica-plated on MB plates which were previously inoculated with a killer toxin sensitive *S. cerevisiae* strain 5×47. All the plates were further incubated at 20°C for 8-10 days to determine the killer activity. If the transformed plasmid complements the *SKI3* activity to the *ski3*⁻ mutant strains, the transformants should become cold resistant at 20°C and non-killers owing to the *mkt1* exclusion of M2 dsRNA (Sommer and Wickner, 1987). As shown in Table 3, the plasmid

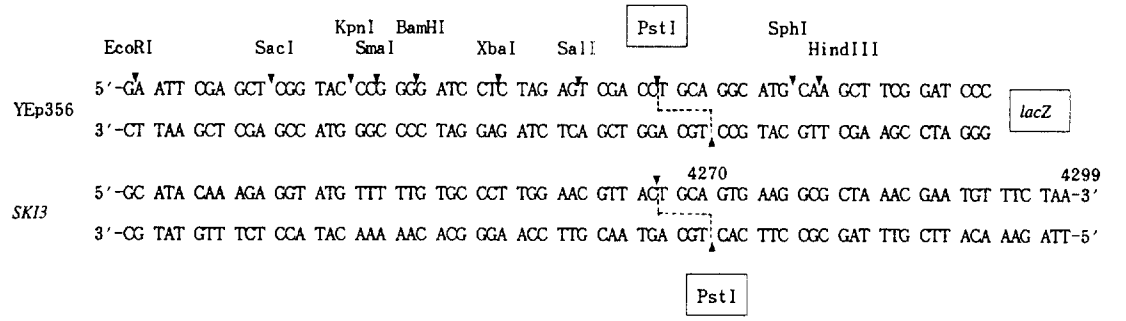

Fig. 2. *Sequence and reading frame of SKI3 and the multiple cloning region of YEp356.*

Table 2. *β-galactosidase activity of ski3⁻ strains transformed with pSR605*

Strains	β-galactosidase activity(U)*	
	Transformant	Non-transformant
2602	28.2	0
2634	51.1	0

*Normalized unit as described by Miller (1972).

Table 3. *Complementation of ski3⁻ strains with SKI3 activity*

Plasmids	Phenotypes*	
	2602	2634
pSR605	K^+R^+	K^+R^+
pSR308	K^-R^-	K^-R^-

*K^+ or K^- stands for ability or inability of a strain to secrete an active killer toxin. R^+ or R^- refers to resistance or sensitivity to the killer toxin.

pSR605 did not complement the mutant strains with the *SKI3* gene function. All the colonies developed were killers and showed the resistance to the killer toxin. This is most likely due to the deletion of 27 bp from the inserted *SKI3* gene in pSR605. This fact strongly suggested that the full length open reading frame of *SKI3* gene might be required to recover the *SKI3* activity. This was confirmed by using another plasmid, pSR308, which contains the whole open reading frame of *SKI3* (Fig. 1). Both mutant strains were complemented by this plasmid to become non-killers.

Molecular characterization of *SKI3*::*lacZ* fusion protein

The *SKI3*::*lacZ* fusion protein was determined by SDS-polyacrylamide gel electrophoresis and the Western blot analysis. As a control, another fusion vector, pLG669-Z, was transformed into the strain 2634. This plasmid contains the initiation codon of the iso-1-cytochrome C (*CYC1*) gene of *S. cerevisiae* fused to *lacZ* gene (Guarente and Ptashne, 1981). The fused gene is preceded by the 1.1 Kb nucleotides that lie upstream from *CYC1*, hence the synthesis of β-galactosidase is directed under the control of *CYC1* gene promoter. From the size of the *SKI3*::*lacZ* fused gene (ca. 7.3 Kb), it was assumed that the fusion protein of ca. 270 Kd would be obtained. The Western blot analysis which was carried out by use of antibodies raised against β-galactosidase showed a number of degraded bands together with two major bands of ca. 250 Kd (Fig. 3). It seems probable that the degradation was owing to the large size of the *SKI3*::*lacZ* fusion protein. In contrast, the same yeast strain transformed with pLG669-Z showed intact single band of β-galactosidase (116 Kd).

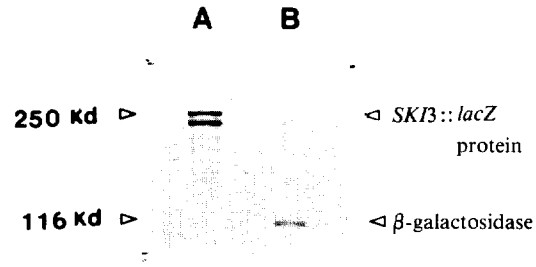

Fig. 3. *Identification of SKI3::lacZ fusion protein and β-galactosidase by Western blot analysis in cell extracts of S. cerevisiae 2634 transformed with pSR605 (Lane A) and pLG669-Z (Lane B).*

DISCUSSION

Although *SKI3* gene seems to have the repression of M dsRNA replication as their sole essential function, the molecular function of the gene is yet to be

elucidated (Rhee *et al.*, 1989). However, the localization of the *SKI*3 protein in the yeast nuclei strongly suggested that it might indirectly repress the M dsRNA replication by modifying the activity of some cellular components needed by M dsRNA for its replication (Rhee *et al.*, 1989).

The physiological importance of *SKI* genes was first suggested by the finding that *ski*$^-$ mutants were cold sensitive for growth at 8 °C if an M replicon was present (Ridley *et al.*, 1984). It has also been found that if a cytoplasmic element [D] is present with M dsRNA in a *ski*$^-$ strain, the cytopathology is evident not only at 8 °C but also at 20,30 and 37 °C (Esteban and Wickner, 1987). Thus, the *SKI* proteins are important to the cell to control M copy number in order to prevent cytopathology induced by M dsRNA. Since the *ski*3$^-$ mutation suppresses the *mkt*1$^-$ mutation and causes the loss of M2 at 32 °C, transformant yeast cells carrying *SKI*3 gene on a plasmid should become K$^-$ at this temperature while the parent strain remains K$^+$. We have used this phenotypic change as a selective marker for the complementation test. However, a heavy load of work is involved in this method and the long period of incubation time is needed for the colony development at low temperature. Consequently, the necessity of the simple and the quantitative way of *SKI*3 gene expression was arisen. As gene fusion is a powerful tool for the analysis of the expression, regulation and structure of a gene (Rose and Botstein, 1983), we tried the fusion of *SKI*3 gene with the truncated *lacZ* gene encoding β-galactosidase (β-D-galactoside galactohydrolase, E.C. 3.2.1.23). The *lacZ* fragment was put into a plasmid, pSR605, that can be selected for and maintained in both *E. coli* and *S. cerevisiae,* and the *SKI*3 gene was placed in front. As shown in Table 2 and Fig. 3, the expression of *SKI*3 gene was able to be determined by assaying on the basis of their ability to produce β-galactosidase in yeast. This method was proven to be simple and easy to quantitize the level of *SKI*3 gene expression.

In the analysis of Western blot experiment, it was clear that the fusion protein was degraded most possibly due to its large molecular size (ca. 270 Kd) as well as the action of proteases. The degree of degradation is likely to be minimized by deleting a fair portion of *SKI*3 and/or *lacZ* genes in the fusion or by using protease negative *pep*4 strains as host cells.

적 요

효모 *Saccharomyces cerevisiae*의 염색체상에 존재하는 superkiller 유전자인 *SKI*3 유전자를 cloning 시켜 *ski* 변이주내에서 발현시켰다. 이 유전자의 C-말단부위에 *E. coli*의 *lacZ* 구조 유전자를 융합시켜 효모와 *E. coli*의 shuttle vector인 pSR605를 제조하고 이를 효모에 형질전환 시킨 후 나타나는 β-galactosidase의 활성에 의해 *SKI*3 유전자의 발현 정도를 정량적으로 측정할 수 있었다. 또한 SDS-polyacrylamide 전기영동과 Western blot 방법을 이용하여 약 250 Kd의 크기를 갖는 *SKI*3와 β-galactosidase의 융합단백질을 확인할 수 있었다.

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(Received February 8, 1990)
(Accepted May 15, 1990)