Construction of *hsf1* Knockout-mutant of a Thermotolerant Yeast Strain *Saccharomyces cerevisiae* KNU5377

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HSF1 is the heat shock transcription factor in *Saccharomyces cerevisiae*. *S. cerevisiae* KNU5377 can ferment at high temperature such as 40°C. We have been the subjects of intense study because Hsf1p mediates gene expression not only to heat shock, but to a variety of cellular and environmental stress challenges. Basing these facts, we firstly tried to construct the *hsf1* gene-deleted mutant. PCR-method for fast production of gene disruption cassette was introduced in a thermotolerant yeast *S. cerevisiae* KNU5377, which allowed the addition of short flanking homology region as short as 45 bp suffice to mediate homologous recombination to *kanMX* module. Such a cassette is composed of linking genomic DNA of target gene to the selectable marker *kanMX4* that confers geneticin (G418) resistance in yeast. That module is extensively used for PCR-based gene replacement of target gene in the laboratory strains. We describe here the generation of *hsf1* gene disruption construction using PCR product of selectable marker with primers that provide homology to the *hsf1* gene following separation of haploid strain in wild type yeast *S. cerevisiae* KNU5377. Yeast deletion overview containing replace cassette module, deletion mutant construction and strain confirmation in this study used *Saccharomyces* Genome Deletion Project (http::/www-sequence.standard.edu/group/yeast_deletion_project). This mutant by genetic manipulation of wild type yeast KNU5377 strain will provide a good system for analyzing the research of the molecular biology underlying their physiology and metabolic process under fermentation and improvement of their fermentative properties.

Key words – Gene disruption, *hsf1* gene, *Saccharomyces cerevisiae* KNU5377

The characteristics of wild and industrial yeast and the complex metabolic networks influencing fermentation and the quality of products might be approached by investigating the standard and recombinant genetic techniques which have been successfully used with a laboratory strain *S. cerevisiae* S288C or other strains derived from S288C[1]. The recombinant genetic techniques are proved to be powerful methods to investigate the regulation of metabolic pathway or the studies of stress responses because stress research has a large impact on medical issues. To date, most studies for genetic manipulation in yeasts have been achieved in laboratory strains because these laboratory strains are haploid or diploid, have good mating ability, readily take up exogenous DNA and contain convenient selectable markers. However, wild type and industrial yeast strains of *S. cerevisiae* are far less easily accessible for genetic analysis and manipulation than their laboratory strains because of homothallic type, low sporulation efficiency, low spore viability, instability of mating types and poor mating efficiencies, and the absence of easily tractable auxotraphic markers[2]. Unfortunately, these studies to wild type and industrial strains of *S. cerevisiae* is somewhat questionable since in many cases the genomics of strains used in industry is significantly different from that of typical laboratory strains[2]. In addition to this fact, we also confirmed considerable differences in DNA genome between KNU5377 strain and laboratory strain such as S288C, W303, and ATCC24858 by using a various DNA fingerprinting. Nevertheless, the important information and experimental analysis of wild and industrial yeasts such as *S. cerevisiae* KNU5377 have been obtained from genomic exploration of the laboratory strain S288C that finishes genome sequencing. Recently, most of the industrial yeasts of *S. cerevisiae* are prototrophic, which are difficult to apply vectors and transformation system designed for auxotrophic laboratory strain [4]. This problem can be achieved by using positive selection marker genes such as

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kanMX module, a completely heterologous dominant resistance marker. These *kanMX* modules are hybrids of the coding sequences of the kanr gene of tranposon Tn903 coding for aminoglycoside phosphotransferase and transcriptional and translational control sequences from the TEF gene of the filamentous fungus *Ashbya gossypi* [11]. Aminoglycoside phosphotransferase renders *S. cerevisiae* resistant to the drug geneticin (G418). Due to the heterology of the *kanMX* module, gene deletion of wild and industrial yeast haploid strain in *S. cerevisiae* was possible, which flanked to the *kanMX* module at either side of yeast homologous DNA. However, when a primer is designed, sequence of a target gene must be preceded because of the difference from DNA genome between KNU5377 and S288C.

HSF1 is the heat shock transcription factor in *S. cerevisiae*. *S. cerevisiae* KNU5377 can ferment at high temperature such as 40°C. We have focused on the subjects of intense study because Hsf1p mediates gene expression not only to heat shock, but to a variety of cellular and environmental stress challenges. Hsf1p have control site (heat shock element; HSE) and consensus motif (nGAAnnTTCnnGAAn). The activation of the regulated genes such as *SSA1*, *SSA3*, *HSP82* and *UBI4* through Hsf1p increase a tolerance to stress. Basing these facts, we firstly tried to construct the *hsf1* gene-deleted mutant to study whether HSF1 affects a gene expression as a mediator and functions a physiological role under stressful conditions or fermentation processes at 40°C.

To construct the mutant, we first performed random spore analysis to separate haploid cells. After sporulation, each single band indicating MATa or MATα was obtained by colony-PCR with growing colony by random spore analysis. PCR product size of MATa was 544 bp, while that of MATα was 404 bp. For cells expressing simultaneous MATa/MATα, double bands were shown (data not shown). In addition, efficiency of sporulation was lower than 10%, and that of liquid sporulation media was near to zero percent (data not shown).

E. coli DH5α containing plasmid pFA6-KanMX6 was cultures at 37°C overnight with shaking. After cell harvest by centrifugation, plasmid DNA of pFA6-kanMX6 was isolated by Plasmid Purification Kit (Nucleogen). The isolated plasmid DNA was used as a template for PCR reaction. A PCR-generated deletion strategy was used to systematically replace each open reading frame from its start- and stp-codon with a *kanMX* module and two unique 20 mer molecular bar codes. Deletion cassette was constructed using two sequential PCR reactions. In the first amplication, 74 bp UPTAG and DNTAG primers amplify the *KanMX* gene from pFA6-kanMX DNA whose *KanMX* expression confers dominant selection to yeast. For first PCR reaction, the mixture (50 μl) contained 100 ng of plasmid template DNA, 5 μl of 10X *Taq* buffer, 4 μl of 10 mM dNTP mix, 5 μl of 10 pmol of each primer, 0.5 μl of 10 mg/ml BSA, and 1 μl of Taq polymerase. After initial denaturation at

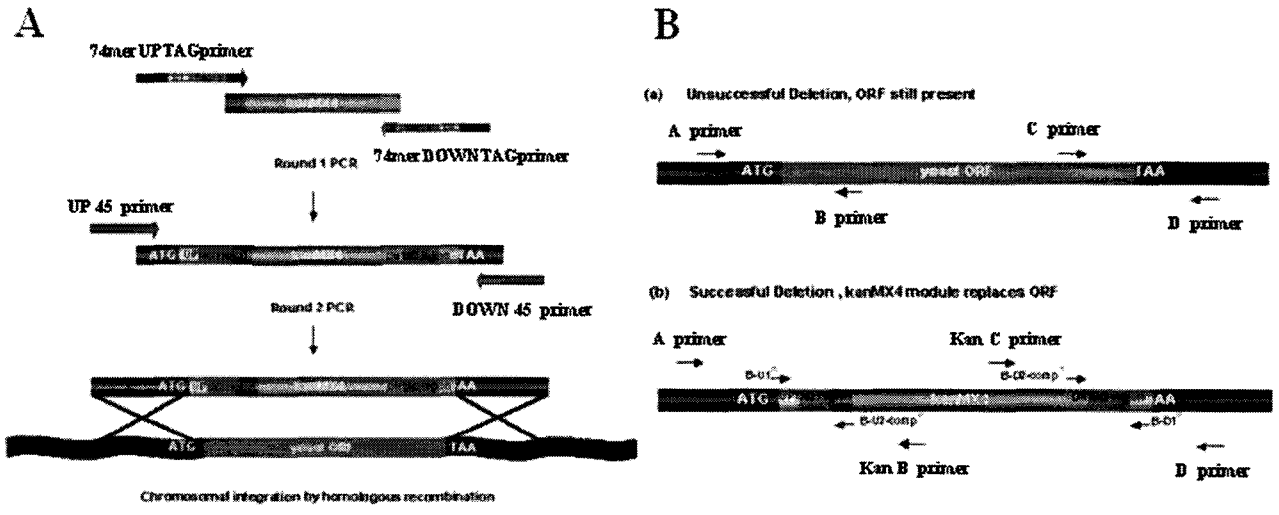

Fig. 1. The scheme of new heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. (A) Deletion cassette module. (B) Confirmation of deletion mutant.

94°C for 4 min, the PCR reaction was cycled 30 times. PCR conditions are the following: 94 °C for 45 s, 52°C for 45 s, 72°C for 2 min. After 30 cycles, a final extension was followed at 72°C for 10 min and subsequently cooled to 4°C. The oligonucleotides used for the PCR disruption of *hsf1* were Uptag and Downtag, the sequences of which were: 5′-TGTATTGTTGGCGCCATGGATGTCCACGAGGTCTCT CGGGTGTAATACTATATCCGCGTACGCTGCAGGTC GAC-3′ and 5′-ACCTTGCCCTGTGTACTACGGTGTCGGT CTCGTAGCAGGACCGCTTACTTCATTTATCGATGAATT CGAGCTCG-3′, respectively. The underlined regions correspond to *kanMX4* sequences. These primers consist of (5′ to 3′): 18 bp of genomic sequence that flank either 5′ or 3′ end of the ORF (directly proximal and distal to the start and stop codons respectively), 18 and 17 bp of sequence common to all gene disruptions (U1: 5′-GATGTCCACGAG GTCTCT-3′ or DI: 5′-CGGTGTCGGTCTCGTAG-3′), a 20 bp unique (the 'molecular bar-code' TAG) and 18 and 19 bp of sequence, respectively, homologous to the KanMX cassette (U2: 5′-CGTACGCTGCAGGTCGAC-3′ or D2: 5′-ATC GATGAATTCGAGCTCG-3′). After electrophoresis, the PCR product was purified by Gel Elution Kit (Omega). The purified DNA was used as a template for the second PCR reaction. In thee second PCR reaction, two ORF specific 45-mer oligonucleotides (UP_45 and DOWN_45) was used to extend the ORF specific homology to 45 bp, increasing the targeting specificity during recombination of the gene disruption cassette. For the second PCR, PCR mixture was the same components except primer pairs. PCR conditions are the following: 94°C for 1 min, 50°C for 1 min, and 72 °C for 2 min. The oligonucleotides used for the second PCR was as the follows: Upstream 45 primer (5′-AGGAAA CAAAAAAGACAAAAAGACAGCTGTATTGTTGGCGCC ATG-3′) and Downstream primer 45 (5′-TTAAATGATTAT ATACGCTATTTAATGACCTTGCCCTGT GTACTA-3′).

The PCR product was used to transform the haploid cells of *S. cerevisiae* KNU5377 for geneticin resistance. The transformation was performed by CiCl2 method [3]. The transformed cells were selected for kanr recombinants on YPED plus G418 plates. The transformation efficiency was less than 20% (data not shown). The deletion confirmation for target gene was verified by colony-PCR [12] using confirmation primer designed by Yeast Deletion Project (http://sequence-www.stanford.edu/group/yeast/yeast_deletion_project/) on the basis of genomic DNA sequence in a laboratory strain *S. cerevisiae* S288C. The correct replacement of the gene with KanMX was verified in the mutants by the appreance of PCR products of the expected size using primers that span the left and right junctions of the deletion module within the genome. The "A" and "D" primers were positioned 200-400 bp from the start and stop codons of the target gene, respectively. The KanB and KanC primers are internal to the KanMX module. For haploid isolates, the junctions of the disruption were verified by amplication of genomic DNA using primers "A" and "KanB" and primers "KanC" and "D". The deletion mutant was checked for a PCR product of the proper size using the primers flanking the gene. For the 5′ end verification, a PCR was performed with the following primers: HSF1-A (5′-TGCAGTTCATGCATATTAAG TGAGT-3′) and KanB (5′-CTGCAGCGAGGAGCCGTAAT-3′). For the 3′ end verification, PCR was carried out with the primers: HSF1-D (5′-AGTCAATATAAGTAC GCCAACTTGC-3′) and KanC (5′-TGATTTTGATGACGAGCGTA AT-3′). As shown in Fig. 2A, the correct gene-deleted cells showed the higher size (2140 bp) than those of the untransformed cells (3058 bp). We verified the deep and clear confirmation with KanB and KanC primers. PCR product sizes of HSF1-A and KanB and HSF1-D and KanC in the transformed cells were 575 bp and 907 bp, respectively. (Fig. 2B). However, the untransformed cells did not detect any bands.

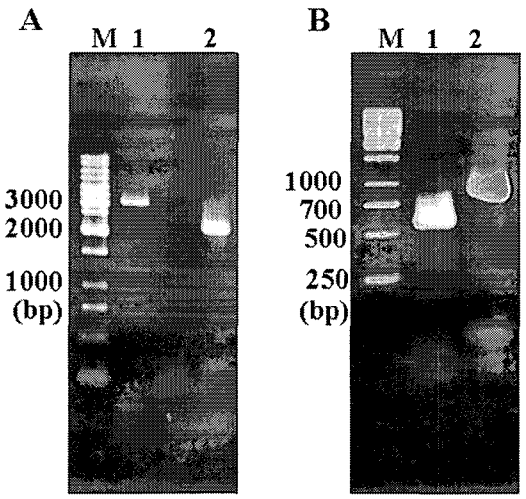

Fig. 2. *hsf* gene deletion. (A) PCR profiles using HSF1-A and HSF1-D primer. M: 100 bp DNA ladder marker (Bioneer), lane 2: the correct deletion strain with 2140 bp amplicons, and lane3: wild type strain with 3058 bp amplicons. (B) PCR profile using HSF1-A and Kan B primer (lane2) and HSF1-D and Kan C primer (lane3) with 575 bp and 907 bp amplicon, respectively. M: 100 bp DNA ladder marker (Bioneer).

In this study, we had many difficulties in deleting the target gene in wild type yeast strain *S. cerevisiae* KNU5377. There is a good reason. Up to date, the properties (strain improvement suited to industry) of wild, and industrial yeast such as yeasts in food and beverage, wine yeast and brewing yeast and the complex metabolic networks influencing fermentation and the quality of products might be approached by investigating standard and recombinant genetic techniques which have been successfully used with the laboratory strain *S. cerevisiae* S288C or other strains derived from S288C[1,5,8,9]. These laboratory strains are haploid or diploid, have good mating ability, readily take up exogenous DNA and contain convenient selectable markers such as *URA*, *HIS* and *LEU*[7,13], which is able to make double mutant or more. However, application of these techniques to wild type strains has been restricted because such strains are typically prototrophic, aneuploid or polyploid, homothallic, poor sporulation, production of few viable spores, and a choice restriction of the selection marker. Hence, the shortage of genetic backgrounds of wild type yeast such as KNU 5377 strain and the genetic differences as described above make it more difficult because these strains lack of these properties of their characteristics and optimum standard protocol. By these facts, most of studies in terms of the construction of industrial valuable yeasts have recently been performed by traditional breeding method. These methods are time consuming and labor intensity. It is also difficult to obtain the valuable and useful gene by the precise molecular genetic method.

In the spite the existence of these various limitations, we could overcome the limitations of molecular genetic techniques in wild type yeast. The successful construction of knocked-out mutant for *hsf1* gene in *S. cerevisiae* KNU5377 strain that posses a excellent stress tolerance against a variety of stresses caused during fermentation such as heat shock, oxidative stress, ethanol shock or acid stress[6,10,14] make us study a molecular physiological role of HSF1 as a regulator of gene expression under environmental stress conditions because Hsf1p mediates gene expression not only to heat shock, but to a variety of cellular and environmental stress challenges.

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초록 : 고온내성 연료용 알코올 효모균주 *Saccharomyces cerevisiae* KNU5377에서 HSF1 유전자의 변이주 구축

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출아효모인 *Sacharomyces cerevisiae* S288C 균주를 이용한 효모의 게놈이 완성된 후 S. cerevisiae는 다양한 연구 모델로 이용되어져 왔다. 현재까지 효모를 이용한 기능 유전체학 측면에서의 연구는 laboratory strain인 S288C 균주 또는 그 유래의 균주들이다. 그러나 자연에서 분리된 효모 또는 산업적으로 이용되어지고 있는 S. cerevisiae의 유전학 측면에서의 연구는 낮은 포자형성률 및 형질전환률, 그리고 S288C 균주와의 게놈상의 상이성 때문에 거의 이루어지지 않고 있다. 여기서 우리 연구진은 자연에서 분리된 *Saccharomyces cerevisiae* KNU5377 균주를 이용하여 random spore analysis를 통해 MATa 및 MATα 타입의 각각의 haploid cell을 분리 후 이미 보고된 KanMX module를 가지고 round PCR기법에 의한 short flanking homology 기법을 이용하여 전사조절인자인 HSF1 유전자가 치환된 변이주를 구축할 수 있었다. 덧붙여, 모든 유전자에 이 기법을 적용할 수는 없다는 것을 확인하였다. 앞으로 이 변이주를 통해 기능 유전체학적인 측면에서 이 유전자의 스트레스와의 관련성을 연구하고자 한다.