Isolation of the Phosphoribosyl Anthranilate Isomerase Gene (TRP1) from Starch-Utilizing Yeast Saccharomycopsis fibuligera

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The nucleotide sequence of the TRP1 gene encoding phosphoribosyl anthranilate isomerase in yeast Saccharomycopsis fibuligera was determined by degenerate polymerase chain reaction and genome walking. Sequence analysis revealed the presence of an uninterrupted open-reading frame of 759 bp, including the stop codon, encoding a 252 amino acid residue. The deduced amino acid sequence of Trp1 in S. fibuligera was 43.5% homologous to that of Komagataella pastoris. The cloned TRP1 gene (SfTRP1) complemented the trp1 mutation in Saccharomyces cerevisiae, suggesting that it encodes a functional TRP1 in S. fibuligera. A new auxotrophic marker to engineer starch-degrading yeast S. fibuligera is now available. The GenBank Accession No. for SfTRP1 is KR078268.

Keywords: Phosphoribosyl anthranilate isomerase, TRP1, Saccharomycopsis fibuligera

Gene transformation or disruption would be a useful tool to analyze the molecular mechanisms in yeast [16, 21]. Genetic transformation systems employing auxotrophic markers, such as URA3, TRP1, HIS3, and LEU2, have been developed for different yeasts, because transformants can be easily selected on drop-out media [19, 23]. The TRP1 gene encodes phosphoribosyl anthranilate isomerase, which catalyzes the third step in tryptophan biosynthesis [2] and is commonly used as a selectable marker in yeasts [6, 8, 15].

Saccharomycopsis fibuligera is the major amylolytic yeast found in starchy substrates, such as bread dough, alcoholic beverages, and rice cake starters [5, 24]. Co-cultures of S. fibuligera with Saccharomyces cerevisiae, Candida utilis, or Zymomonas mobilis are used to produce ethanol from starch [4, 11]. These co-cultures produce amylase, protease, β-glucosidase, and trehalase, and have applications in food, biofuel, and pharmaceutical industries [12, 17, 18, 27]. In our previous study, a thermotolerant S. fibuligera strain was isolated from nuruk, a traditional Korean starter for rice wine fermentation [5].

In this study, we isolated and sequenced the TRP1 gene from S. fibuligera as the first step in the construction of a host-vector tool for an auxotrophic transformation system for this starch-utilizing yeast. The functionality of the cloned SfTRP1 was demonstrated by complementation of a TRP1-negative S. cerevisiae strain. S. fibuligera [5] and S. cerevisiae YPH499 [MATa ura3-52 lys2-801 amber ade2-101 ochre trp1-∆63 his3-∆020 leu2-∆1] (Clontech, Palo Alto, CA, USA) were used. Escherichia coli TOP10 (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for plasmid DNA preparation and was grown at 37°C in LB medium (5 g/l bacto-yeast extract, 10 g/l bacto-tryptone peptone, and 10 g/l NaCl) supplemented with ampicillin (100 mg/l). To prepare genomic DNA, S. fibuligera was grown in YEPD (10 g/l bacto-yeast extract, 20 g/l bacto-proteose peptone, and 20 g/l glucose) at 30°C.

Degenerate oligonucleotide primers TRP3F (5'-GTNGTNTTNMGCAAYCARWSN-3') and TRP3R (5'-NGTGTNTTNMGCAAYCARWSN-3') were designed based on the core consensus conserved regions VGVRNQS and DVSGGVET [3, 6]. The PCR was performed using 2.5 units of TOPSimple nTaq polymerase (Enzynomics, Daejeon, Korea). The reaction condition was 5 min at 94°C, followed by 30 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 58°C, and extension for 30 sec at 72°C, with a final 5 min elongation at 72°C. The amplified fragment (approximate 400 bp) was cloned to the pTOP TA V2 vector (Enzynomics) and sequenced. E. coli cells were transformed as described previously [25]. The complete open reading frame (ORF), promoter, and terminator regions were obtained.
by genomic walking, which was performed with the DNA Walking SpeedUp Kit (Seegene, Seoul, Korea) according to the manufacturer’s protocol.

Nucleotide and protein sequence similarity searches were performed using the Web-based BLAST algorithm of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/blast). Multiple amino acid sequence alignment was performed by using San Diego Supercomputer Center (SDSC) Biology Workbench (http://workbench.sdsc.edu/) [7]. The C-DART program (Conserved Domain Architecture Retrieval Tool) from NCBI was used to compare the amino acid sequence of the protein with database sequences.

To obtain SfTRP1 with its own promoter and terminator regions, approximately 300 bp of 5’ and 3’ regions of the SfTRP1 were amplified by PCR using SfTRP1-F (5’-AATGAGCTCTCTGGGCCTATTGATAACTCATC-3’) and SfTRP1-R (5’-AATTGTCGACTGCGAGTTTTAGCGACA AACTTT-3’). The PCR product of the expected size (approximate 1.4 kb) was digested with the SacI and SalI restriction enzymes and inserted into the LEU2-marked plasmid pRS315 (ATCC77144, 6.02 kb) to construct plasmid

![Fig. 1. Alignment of multiple amino acid sequences from S. fibuligera Trp1 and other homologous yeast Trp1.](image)

Fully conserved residues are marked in black. The conserved regions on which degenerate primers were designed are marked with asterisks and hydrophobic conserved regions are in boxes. The numbers on the left and right indicate the positions of the amino acids. Saccharomycopsis fibuligera (GenBank Accession No. KR078268); Candida albicans (XP_718951); Candida glycerinogenes (ABU53939); Candida orthopsilosis (EAZ63723); Komagataella pastoris (CAA04452); Saccharomyces cerevisiae (CAA24634); Scheffersomyces stipitis (EAZ63723); Wickerhamomyces anomalus (AAO19636).
pME1376 (7.3 kb). *S. cerevisiae* was transformed by the lithium acetate method [10]. *S. cerevisiae* cells carrying these plasmids were grown in synthetic complete medium lacking tryptophan (SC-TRP) or leucine (SC-LEU).

The *S. cerevisiae* YPH499 transformants harboring plasmid pRS315 (vector control) or pME1376 (pRS315-*SfTRP1*) were grown in selective medium overnight and diluted to an OD$_{600}$ of 0.2 with sterile SC-LEU medium. Afterwards, 5-fold serial dilutions were prepared, and 20 µl of each was spotted on the SC-TRP plate.

Primers for degenerate PCR were designed on the basis of a multiple alignment of published yeast *TRP1* gene sequences [3, 6, 8, 22]. A PCR product with an approximate size of 0.4 kb was amplified, TA-cloned, and sequenced. The alignment of the deduced amino acid sequence encoded by the amplified fragment with those of Trp1 homologs suggested that the cloned fragment contains a conserved *TRP1* sequence, which was named *SfTRP1*. On the basis of the sequenced gene fragment, primers for genome walking were designed to isolate the 5'- and 3'-untranslated regions of the *SfTRP1* gene (data not shown). The entire nucleotide sequence of *SfTRP1*, including 300 bp of the 5' and 3' regions, was obtained by genome walking and sequence assembly, and deposited in the GenBank under Accession No. KR078268.

Analysis of the *SfTRP1* upstream sequence revealed two TATA-like sequences [14] at nucleotide positions -200 and -53. Potential transcription initiation sites [13] were found, which had consensus motifs TC(G/A)A (at nucleotide positions -90 and -97) or RRYRR (a pyrimidine surrounded by two purines; positions -136 and -270). A possible CAAT box [26] was found at nucleotide position -135.

The *SfTRP1* gene consists of an ORF of 759 bp and encodes a putative 252-amino-acid polypeptide with an estimated molecular mass of 27.6 kDa, which is larger than the 24.1 kDa protein in *S. cerevisiae* [14]. The calculated pI was 5.7, similar to 5.34 in *S. cerevisiae* [1]. Sequence analysis indicated that the *SfTRP1* gene does not contain introns. Sequence alignment with other Trp1 proteins (Fig. 1) indicated that *S. fibuligera* Trp1 shares a conserved structure with the *PRAI* family [9].

The ability of *SfTRP1* to complement the trp1 phenotype was tested by transformation of the *S. cerevisiae* strain YPH499. The cloned *SfTRP1* gene with its own promoter and terminator was inserted into the low-copy plasmid pME1376. As shown in Fig. 2, the *S. cerevisiae trp1* strain harboring pRS315 (vector control) was unable to grow on a SC-TRP plate. However, the *S. cerevisiae* transformant harboring the plasmid pME1376 grew successfully on SC-TRP medium. These results indicated that the *TRP1* gene from *S. fibuligera* complements the *trp1* mutation in *S. cerevisiae*.

In conclusion, we successfully isolated the full-length *SfTRP1* gene through degenerate PCR and genome walking. The functionality of the cloned *TRP1* gene was confirmed by complementation of trp1 auxotrophy in a *S. cerevisiae* strain. Thus, a new marker gene to engineer starch-utilizing yeast *S. fibuligera* is now available.

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**References**

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