Cloning and sequencing analysis of TRP1 gene of \textit{Flammulina velutipes}

Ryohsuke Nakai 1, Kikuo Sen *, Shin-ichi Kurosawa 2, Hiroshiro Shibai

Department of Bioscience and Biotechnology, Faculty of Agriculture, Shinshu University, 830 Minaminomura, Nagano 399-4598, Japan

Received 23 November 1999; received in revised form 5 May 2000; accepted 10 May 2000

Abstract

The genomic TRP1 gene from basidiomycete \textit{Flammulina velutipes} was cloned by complementation of yeast \textit{Saccharomyces cerevisiae} trp1 mutation. Sequencing analysis revealed that the TRP1 gene encoded a single protein consisting of three catalytic functional domains; glutamine amidotransferase, indole-3-glycerol phosphate synthase) and N(5'-phosphoribosyl)anthranilate isomerase, in order of NH$_2$-glutamine amidotransferase-indole-3-glycerol phosphate synthase-N(5'-phosphoribosyl)anthranilate isomerase-COOH. The coding sequence of the TRP1 gene was interrupted by a single intron of 48 bases, the position and flanking sequences of which were highly homologous to those of basidiomycete \textit{Phanerochaete chrysosporium} trpC.

Keywords: \textit{Flammulina velutipes}; Basidiomycete; TRP1; Anthranilate synthase; Gene cloning

1. Introduction

Although the biosynthetic pathway of tryptophan appears to be identical in all organisms studied so far and involves five sequential reactions from chorismic acid [1], the organization of genes encoding enzymes on the pathway is not always conserved. In \textit{Escherichia coli}, a single operon contains five genes encoding all enzymes [2], whereas in the yeast \textit{Saccharomyces cerevisiae}, five unlinked genes specify the tryptophan biosynthetic pathway [3]. At this point, filamentous fungi exhibit an intermediate position between \textit{E. coli} and \textit{S. cerevisiae}, that is, in ascomycete \textit{Neurospora crassa}, four unlinked genes drive the pathway. One of the four genes, the \textit{trp-1}, encodes a tri-functional enzyme anthranilate synthase (AS) with domains for glutamine amidotransferase (GAT), indole-3-glycerol phosphate synthase (IGPS) and anthranilate isomerase (PRAI) activities [1]. Although basidiomycetes also include equivalent genes, sequence data are available for only trpC from \textit{Phanerochaete chrysosporium} [4].

Transformation systems have been developed in many basidiomycetous fungi, based on auxotrophic [5–8] and drug-resistant markers [9–12]. Heterologous promoters have been used for expression of drug-resistant marker genes, giving insufficient transformation. One of the important points for a transformation system is sufficient expression of a selectable marker gene, which includes recognition of a promoter sequence by transcriptional machinery of host cells. Therefore, endogenous promoters are expected to contribute to an efficient transformation system.

Despite being of economic importance, the edible basidiomycete \textit{Flammulina velutipes} has no transformation system. In this study, we have cloned and sequenced the TRP1 gene of \textit{F. velutipes} aiming at its application as a marker gene for a transformation vector.

2. Materials and methods

2.1. Strains and media

\textit{F. velutipes} dikaryotic strain R15 was obtained from Nagano Vegetable and Ornamental Crops Experiment Station, and was grown at 25°C on peptone–suarcose medium (PSM: 2% sucrose, 0.5% peptone, 0.2% yeast extract, 0.1% KH$_2$PO$_4$, 0.05% MgSO$_4$·7H$_2$O, pH 5.5) to
maintain it and to prepare its DNA, and also on complete yeast medium (CYM; 2% glucose, 0.2% yeast extract, 0.2% peptone, 0.046% KH$_2$PO$_4$, 0.1% K$_2$HPO$_4$, 0.05% MgSO$_4$·H$_2$O) to prepare its RNA. E. coli strains HB101 (F$^-$, supE44, hisD20, ara-14, galK2, lacY1, proA2, rpsL20, xyl-5, mtl-1, recA13, $\lambda^-$) [13] and DH10B (F$^-$, mer$^C_A$, $\Delta$ (mer-hsdRMS-merBC), $\bigcirc\bigcirc$ lacUV5, lacX74, deoR, recA1, endA1, araD139, $\Delta$ (ara, leu) 7697, galU, galK, $\lambda^-$, rpsL, mprG) were used for construction of an F. velutipes genomic library and routine plasmid preparations. S. cerevisiae strain SP1 (MATa, trp1, his3, ade8, leu2, can1) was the host for complementation experiments.

2.2. Vectors and general procedure

Yeast expression vector pAAH5 containing a HindIII restriction site flanked by yeast ADC1 promoter and terminator was used to construct the F. velutipes genomic library. Plasmid vector pUC118 [13] was used for subcloning and sequencing of an F. velutipes gene. General manipulations of DNA, such as restriction enzyme treatment, ligation and dephosphorylation of plasmid were done according to the procedures of Sambrook et al. [13]. Yeast transformation was carried out by the lithium acetate technique [14].

2.3. Construction of the genomic library

Genomic DNA was isolated from lyophilized mycelia by the method of Lichtenstein and Draper [15] and partially digested with a restriction enzyme HindIII to give a mixture of fragments ranging from 5 to 10 kb, which were electrophoretically fractionated. Then the F. velutipes DNA fragments were inserted into the HindIII site in pAAH5, and recombinant plasmids constructed were used to transform the E. coli HB101 strain, resulting in $3.8 \times 10^4$ independent transformants.

2.4. Polymerase chain reaction (PCR)

Two primers were designed, a degenerate primer T323mix from a highly conserved region (sense, at nucleotides 395-413) of amino acid sequences in several fungal AS and a T304A primer (5'-CATCTAGAAAGTCGTGAGGGTTGGGC-3', antisense for nucleotides 1380-1396) from a 5'-truncated F. velutipes TRP1 gene originally cloned in this study, and their binding positions are shown in Fig. 1. The T323mix and the T304A primers were used in the PCR amplification of the F. velutipes TRP1 DNA containing its 5'-region. The reaction sample contained 10 mM Tris–HCl at pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$, 0.2 mM dNTPs, 60 ng of F. velutipes genomic DNA, 0.9 g of T323mix, and 0.2 g of T304A. After heating at 94°C for 2 min and addition of 2.5 units of Taq DNA polymerase (Takara Shuzo, Kyoto, Japan), the reaction was cycled 36 times at 94°C for 0.5 min, 60°C for 1 min, and 72°C for 1.3 min.

2.5. DNA sequencing

Nucleotide sequences of the F. velutipes TRP1 gene were determined from both strands of the plasmid clones using DSSQ1000L sequencer (Shimadzu, Kyoto, Japan). Nucleotide and amino acid sequences were analyzed using the GENETYX software system (Software Development, Tokyo, Japan).

2.6. RNA preparation, Northern analysis and reverse transcription (RT)-PCR

Total RNA was extracted from mycelia according to the standard method using phenol and guanidine thiocyanate. Northern blotting was done with approximately 5 μg of total RNA per sample. A probe of the F. velutipes TRP1 gene was digoxigenin-labeled using DNA Labeling and Detection Kit (Boehringer Mannheim, Mannheim, Germany). Detection of specific RNA was done following the supplier’s instructions.

Complementary DNA was created from total RNA using RNA LA PCR® Kit (AMV) (Takara Shuzo) containing avian myeloblastosis virus (AMV) reverse transcriptase and oligo dT-adaptor primer. Two primers, T5’22S (5’-GGGGTACCGTCTACGCGTCGACG-3’, sense for nucleotides -28 to -8) and T3’93A (5’-GGCTAAGACGCTTTCAATTTTCGACG-3’, antisense for nucleotides 2749-2769), whose binding positions are shown in Fig. 1, were used for RT-PCR; and the thermal cycling reaction described above was done, except for annealing at 54°C for 1 min, and extension at 72°C for 3 min.

2.7. Primer extension analysis

About 0.5 μg of the fluorescein isothiocyanate-labeled primer (5’-TGCGAATGACTTCGACTTTTGCC-3’, antisense at nucleotides 126-148), whose binding position is shown in Fig. 1, was annealed to 3 μg of total RNA and extended by the AMV reverse transcriptase (Takara Shuzo) at 42°C for 1 h in reaction buffer recommended by the manufacturer. The reaction was terminated by chloroform treatment and resulting molecules of nucleic acids were recovered by ethanol precipitation. The extension products were analyzed by the DSSQ1000L sequencer.

3. Results

3.1. Isolation of the F. velutipes TRP1 gene

The genomic library of F. velutipes was used to transform the Trp$^-$ strain of S. cerevisiae SP1 to Trp$^+$, Two yeast transformants were confirmed on the minimal me-
Fig. 1. The complete nucleotide sequence of coding and flanking regions of F. velutipes TRP1 and the sequence of 875 amino acid residues of the gene product. The position of intron is indicated by lower-case letters in the nucleotide sequence. The position of predicted transcription start point is indicated by a single-underline, the CAAT-box by a box, and the stop codon by an asterisk. Overlying arrows show the binding positions and directions of primers used. This sequence data will appear in DDBJ/EMBL/GenBank nucleotide sequence database with accession number AB028647.
Fig. 2. Comparison of the amino acid sequence deduced for the F. velutipes TRP1 protein with the corresponding sequences from other filamentous fungi. Fv, F. velutipes TRP1; Pc, P. chrysosporium trpC; Ad, A. nidulans trpC; Ag, A. niger trpC; Nc, N. crassa trp-1; Pb, P. blakesleeanus trp1. Completely matched residues are indicated by asterisks.
dium lacking tryptophan, and they had the same recombi-
nant plasmids, designated pFTT1, containing an insert of
approximate 7.4 kb, which was then subcloned to
pUC118. Partial DNA sequencing analysis of this insert
and comparison of its deduced amino acid sequence to
those of other fungal protein homologues revealed that
the cloned TRP1 gene did not contain an entire sequence.

To obtain upper stream of the 5’-truncated TRP1 gene,
a 1.0-kb fragment amplified with T304A and T323mix
primers was cloned from F. velutipes genomic DNA being
used as a template. Using the 1.0-kb fragment as a hybrid-
ization probe, another 4.7-kb DNA fragment was screened
from the F. velutipes genomic DNA library, subcloned and
sequenced.

Expression of the TRP1 gene was confirmed by dot blot
analysis of total RNA extracted from F. velutipes vegeta-
tive mycelia grown in CYM (data not shown).

3.2. Nucleotide sequence of the F. velutipes TRP1 gene

Sequencing analysis of the clones obtained from the
genomic DNA library and the PCR clone showed their
similarities to sequences of tryptophan biosynthesis genes
previously published [1–4, 16–18]. The resulting nucleotide
sequence could be translated to an amino acid sequence
similar to those deduced from the other fungal genes.

3.3. Sequence homology in fungal polypeptide

The deduced amino acid sequence of the F. velutipes
TRP1 gene (Fig. 1) was compared to the trpC genes of
P. chrysosporium [4], Aspergillus nidulans [16] and Asper-
gillus niger [17] together with the trp-1 gene of N. crassa [1]
and the TRP1 gene of Phycomyces blakesleeanus [18] cod-
ing for ASs (Fig. 2). These sequences exhibited remarkable
similarity including identical stretches, strongly suggesting
that the TRP1 gene in F. velutipes encodes a trifunctional
polypeptide consisting of three domains which correspond

3.4. Analysis of intron sequence of TRP1 gene

The single presumable intron was found to locate at the
position of nucleotides 342–390 (Fig. 1), and its length,
position, splice junctions, inside termination codon and
flanking sequences were highly homologous to those of
P. chrysosporium trpC (Fig. 3). The restriction fragments
of genomic- and RT-PCR products using T3’22S and T3’93A primers were loaded and separated
on a 1.2% agarose gel. Arrow shows the DNA fragments differently mi-
grated. The numbers to the left of figure indicate the molecular sizes (in base pairs).

4. Discussion

The TRP1 gene from F. velutipes in this study exhibited
striking similarity to the corresponding genes of other fil-
amentous fungi coding for ASs [1, 4, 16–18], indicating that
F. velutipes had AS conserved among filamentous fungi.

So far, only the one gene encoding AS from basidiomy-
cetes is sequenced [4] other than the F. velutipes TRP1
gene. The Sall-digests of RT-PCR (lane 1) and genomic-PCR (lane 2)
products using T3’22S and T3’93A primers were loaded and separated
on a 1.2% agarose gel. Arrow shows the DNA fragments differently mi-
grated. The numbers to the left of figure indicate the molecular sizes (in base pairs).

Fig. 4. PCR analysis of the presumable intron of F. velutipes TRP1
gene. The Sall-digests of RT-PCR (lane 1) and genomic-PCR (lane 2)
products using T3’22S and T3’93A primers were loaded and separated
on a 1.2% agarose gel. Arrow shows the DNA fragments differently mi-
grated. The numbers to the left of figure indicate the molecular sizes (in base pairs).

Fig. 3. Comparison of the intron and flanking regions of F. velutipes
TRP1 with those of P. chrysosporium trpC for nucleotide and amino
acid sequences. Identical residues are indicated by asterisks. Underlines
represent a stop codon.
in the former, \textit{P. chrysosporium} \textit{trpC} gene [4], and their introns were specific to basidiomycetes, not to ascomycetes.

Functional analysis of the \textit{AS} genes from filamentous fungi must also be done in addition to their structural analysis, especially those from basidiomycetes. While sequencing data of the \textit{AS} from filamentous fungi show that each of the proteins is the trifunctional enzyme related to tryptophan synthesis, basidiomycete \textit{Schizophyllum commune} \textit{TRP1} gene encoding \textit{AS} has another function related to mating and fruiting [19]. Moreover the promoter and terminator regions for sufficient expression of the \textit{F. velutipes} \textit{TRP1} gene have not yet been specified in this study. Further investigation is now under way to develop the efficient transformation system of the edible mushroom \textit{F. velutipes}.

Acknowledgements

We wish to thank Dr. M. Nakafuku (the University of Tokyo) for his kind supplies of an \textit{S. cerevisiae} strain and a yeast expression vector, and his valuable discussion and suggestions; to Mr. K. Akahane for his kind supply of \textit{F. velutipes} R15 strain; and to Dr. S. Eda, Mr. S. Habutsu and Mr. T. Togami for their technical support. This investigation was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and by the Agricultural Chemical Research Foundation.

References