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Functional analysis of a gene encoding Anthranilate phosphoribosyltransferase from rice

Md. Shafiqul Islam Sikdar
Department of Agronomy
Hajee Mohammad Danesh Science and Technology University
Dinajpur-5200, Bangladesh

Jung-Sup Kim
Faculty of Biotechnology
Jeju National University
Jeju, 690-756, Korea
e-mail: biotech2020@jejunu.ac.kr

***Abstract*—Anthranilate phosphoribosyltransferase (EC 2.4.2.18) from the Oryza sativa (OsAnPRT) was expressed, analyzed and characterized in Escherichia coli. Nucleotide analysis of a cDNA encoding OsAnPRT, subsequent homology comparisons and complementation of trpD mutant strain of E. coli were performed. The properties of the AnPRT enzyme have been reported in many bacteria and a few plants. Sequence analysis of an EST clone from rice revealed that it harbors a full-length open reading frame for OsAnPRT encoding for 395 amino acids, corresponding to a protein of approximately 41.6 kD. The predicted amino acid sequence of OsAnPRT is highly homologous to that of Arabidopsis AnPRT and many bacterial AnPRT encoded by trpD gene. OsAnPRT expression was correlated with survival of the trpD mutant strain of E. coli, which is affected by the supplementation of the tryptophan. These results indicated that OsAnPRT encodes for a protein of anthranilate phosphoribosyltransferase in rice.**

Keywords-anthranilate phosphoribosyltransferase; rice (Oryza sativa); tryptophan; functional complementation

I. INTRODUCTION

Plants are able to synthesize all the amino acids including tryptophan (Trp) from inorganic materials. Their metabolism is largely based on the reversible transfer of a phosphoribosyl group to aromatic bases [1]. Trp is an essential aromatic amino acid that must be obtained from their diets in animals. Within the eukaryotes, Trp is synthesized either in the chloroplasts, as in photoautotrophs, or in the cytosol, as in fungi and oomycetes. All other eukaryotic heterotrophs obtain aromatic amino acids from their food [2].

The members of the phosphoribosyltransferase (PRT) enzyme class catalyze the transfer of a phosphoribosyl group to an aromatic base. PRTs have an important role in the metabolism of nucleotides and amino acids [3]. The pathway of Trp biosynthesis from chorismate in bacteria and plants consists of seven enzymatic reactions [4]. Anthranilate phosphoribosyltransferase (AnPRT; EC 2.4.2.18; the TrpD gene product) is involved in the biosynthesis pathway of the aromatic amino acid Trp which catalyzes the second reaction specific to the pathway of Trp biosynthesis (Fig. 1) and belongs to the functional superfamily of phosphoribosyltransferases, which catalyze the transfer of the ribose-5-phosphate moiety from 5-phosphoribosyl-1-pyrophopsphate (PRib-PP) to various acceptors. This reaction involves the practically irreversible replacement of the pyrophosphate moiety of PRib-PP by a nucleophile with accompanying anomeric inversion of the ribofuranose ring [5]. The pathway products are precursors for the synthesis of plant hormones such as indole acetic acid (IAA), phytoalexins, glucosinolates, and indole- and antharanilate-derived alkaloids. The plant enzymes are all monofunctional and all identified domains correspond to those found in microbial homologues [6].

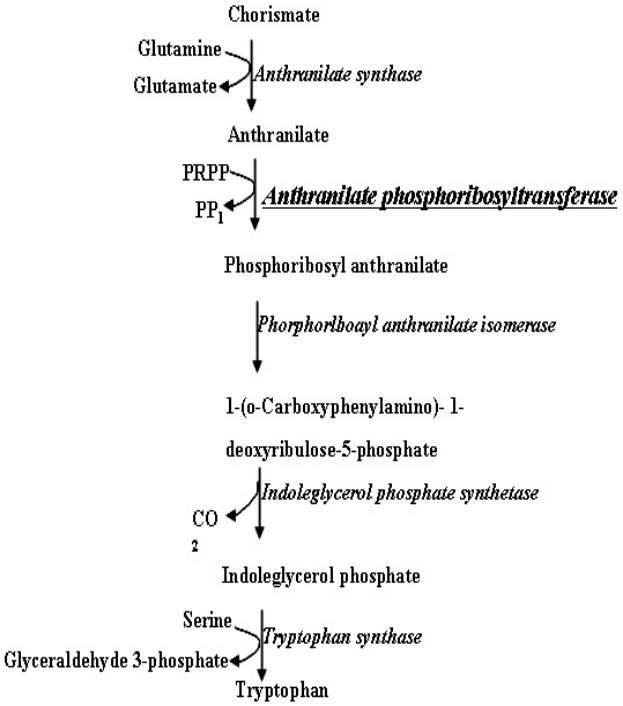

Fig. 1. The tryptophan biosynthetic pathway in Bacteria and plants. The figure was adopted and slightly modified from (Dutcher *et al.*, 1992 and Zhao and Last, 1995).

The AnPRT activity has been identified, purified, and described in a variety of microorganisms and plant , such as Chlamydomonas reinhardtii [7];Pectobacterium carotovorum [8];Sulfolobus solfataricus [1, 9]; Mycobacterium tuberculosis [10]; Salmonella typhimurium [11]; Escherichia coli [12]; Saccharomyces cerevisiae [13, 14]; *Hafnia alvei* [15]; and Arabidopsis thaliana [16]. Little is known about Trp biosynthesis in photosynthetic eukaryotes, such as land

plants and algae. However, it is clear that the pathway has been relocated to the primary chloroplast [6]. The pathway is located in the chloroplasts of plants, with all involved enzymes being separately nuclear-encoded and posttranslationally targeted to the plastid [17]. Here, we made use this experience in this study, in which we describe the functional analysis and characterization of a gene of TrpD from *Oryza sativa* (OsAnPRT) an important crop plant.

II. METHODS AND MATERIALS

A. Strains and *Plasmids*

Two *E. coli* strains were used in the study namely *2032* and *Gif41*. The source of *2435* strain was the KCTC (Korean Collection for Type Cultures) and *Gif41* strain was the *E. coli* Genetic stock Center (CGSC) in Yale University, USA. The Accession Number of used plasmid is AK121680, Clone name J033069P19, Clone ID: 216399 and the protein is Anthranilate phosphoribosyltransferase. Besides, more two plasmids were used as control plasmids in this study.

B. DNA *sequence* analysis

An expressed sequence tag (EST) clone (GenBank accession number AK120254) was obtained from the Rice Genome Resource Center (RGRC), Japan. The clone was derived from a rice cDNA library [18] from shoots. DNA sequencing and sequence analysis were described previously [19]. The nucleotide sequences and amino acid sequences were compared with sequences present in the GenBank and EMBL databases including complete rice genome, then analyzed using BLAST [20] and Clustal W multiple sequence alignment programs [21] or Biology WorkBench 3.2 (http://workbench.sdsc.edu, San Diego Supercomputer Center, USA). Sequence comparison was conducted at the nucleotide and amino acid levels. Protein localization was predicted by the iPSORT program (http://ipsort.hgc.jp) and motifs were searched by the GenomeNet Computation Service at Kyoto University (http://www.genome.ad.jp). The phylogenetic tree with bootstrap value was prepared via the Mega 4.1 neighbor-joining program [22].

C. Polymerase chain reaction (PCR) and recombinant constructs

Our sequence analysis showed the presence of an ATG start codon located in-frame at -32 positions upstream from the translation-starting site. Therefore, the specific primers were designed from the sequence information around the translational start and stop codons of OsAnPRT to amplify the full-length open reading frame (ORF) and to overexpress the gene product in E. coli. Polymerase chain reaction (PCR) [23] was conducted to amplify the full-length ORF. After the EST was purified from a pellet harvested from a liquid culture containing ampicillin (Amp), the ORF of OsAnPRT was amplified from the EST clone as a template, and the following primers were designed from the OsAnPRT sequence: OsAnPRT-F (5'- AAAGCTTATGGCGGCGGCG TCGATCAA-3') and OsAnPRT-R (5'- AAAGCTTGATCG TTGATAACCTTTAGA-3') using AmpliTaqGold polymerase (Perkin-Elmer, U.S.A). The underlined bases in the OsAnPRT -F and OsAnPRT-R primers are the designed restriction sites for *Hin*dIII to facilitate subcloning, respectively. The polymerase chain reaction was conducted using a MYCyler™ PCR system (BioRad, U.S.A) for 35 cycles with 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with 10 μM primers. The PCR products were analyzed on 1% (w/v) agarose gel. The amplified fragment (1.5 kb) was then subcloned into pGEM-T-easy vector (Promega) and finally subcloned into pBluescript II KS+ (Stratagene Inc., U.S.A) as a *Hin*dIII fragment, to give *pB::OsAnPRT*. Restriction analysis was conducted in an effort to confirm the recombinant DNA construct of *pB::OsAnPRT* with the right orientation for overexpression.

D. Functional complementation and growth inhibition assay of OsAnPRT in E. coli

The competent TrpD mutant of the *E. coli* strain 2032 and wild type strain Gif41 were transformed with *pB::OsAnPRT* or control via electroporation (ECM399, BTX, USA) after producing competent cells by washing with water and glycerol [24] using a cuvette with a 0.1 cm electrode gap, then plated on LB (20 g/L) with Amp (100 μg/ml). The growing culture was tested for growth retardation in M9 minimal (MM) medium containing Amp (25 μg/ml), 20% glucose, 1 mM isoptopyl β-D-thiogalactopyranoside (IPTG) and 19 amino acids (Sigma, Germany) each at a concentration of 25 μg/ml, excluding Trp. Nineteen amino acids were supplemented to MM medium because the bacterial growth is not limited from the amino acid nutrients except Trp even though bacteria could synthesize these amino acids by themselves. The plates were incubated overnight at 37°C. The growing colonies were then retested for growth on MM agar medium without Trp [24].

Bacterial growth was then assessed by measuring optical density at 595nm at one-hour intervals. After 12 hrs, the diluted culture was plated and incubated overnight at 37°C. The bacterial growth was monitored via optical density measurements every hour using a spectrophotometer (UV1101, Biochrom, England) at 595 nm (OD_{595}). The *trpD* mutant *E. coli* strain harboring the *pB::OsAnPRT* construct, or the control plasmid and wild-type were grown at 37°C in MM liquid medium with 1 mM IPTG, 20% glucose (20 ml/L), Amp (25 μg/ml) and 19 amino acids excluding Trp. Bacterial growth was monitored hourly via measurements of optical density using a spectrophotometer (UV1101, Biochrom, England) at 595 nm (OD_{595}).

III. RESULTS AND DISCUSSION

A. Sequence analysis of OsAnPRT

An expressed sequence tag (EST) clone (GenBank Accession Number AK121680, Clone name J033069P19, Clone ID: 216399) obtained from the Rice Genome Resource Center (RGRC) was analyzed to determine the nucleotide sequence using the designed primers. The cDNA (*OsAnPRT)* sequence harbored a full-length open reading frame consisting of 1188bp, encoding for a protein of approximately 41.6 kdal. The expected isoelectric point of

the protein was 8.00. Data analysis revealed that the *OsAnPRT* sequence was identical to the genomic region located in chromosome V. Comparisons of the amino acid sequence of the *OsAnPRT* and the homologous sequences from *Arabidopsis thaliana* and *Escherichia coli* revealed high identity, at 72% and 37% respectively (Fig. 2).Analysis of the amino acid sequence of *OsAnPRT* revealed that there are many signature and binding site found in that amino sequence such as pyrimidine-nucleoside phosphorylases which are located at (93..126), (135..146) and (156..182), respectively (Fig. 2). Phylogenic analysis based on comparison of the related sequence indicated further that OsAnPRT is divergent and evolved from ancestor bacterial AnPRT. Number at nodes indicate levels of bootstrap support based at neighbor-joining analysis of 1000 re-sampled data set by using Mega 4.1(Kumer *et al*., 2008). Numbers on branches are percentage of bootstrap analysis supporting the grouping of each branch (Fig. 3).

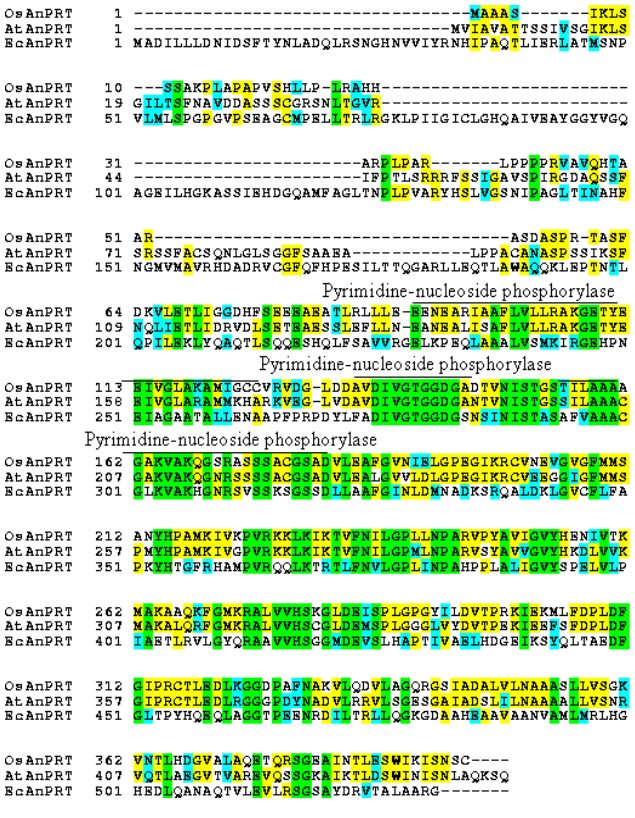

Fig.2. Amino acid sequence alignment of AnPRT from *Oryza sativa* (OsAnPRT), *Arabidopsis thaliana* (AtAnPRT), and *Escherichia* coli (EcAnPRT). Shaded residues represent amino acids that are identical among at least three of the three amino acids. GenBank accession numbers; AK121680, NP_197300 and ZP_06657245, respectively.

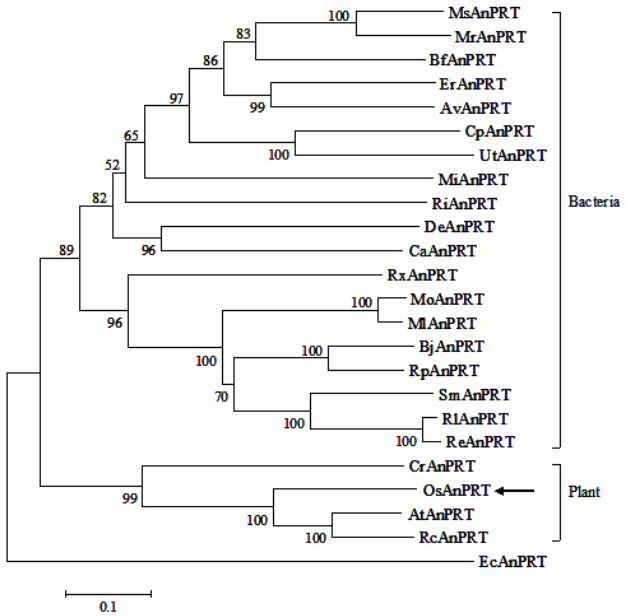

Fig.3. Phylogenetic tree: Phylogenetic analysis of OsTS related proteins using Clustal W and Mega 4.1. Accession numbers are as follows: YP_001273717 (MsAnPRT from *Methanobrevibacter smithii*), YP_003422954 (MrAnPRT from *Methanobrevibacter ruminantium*), ZP_05347396 (BfAnPRT from *Bryantella formatexigens*), CBK89975 (ErAnPRT from *Eubacterium rectale*), ZP_03945848 (AvAnPRT from *Atopobium vaginae*), ZP_05496790 (CpAnPRT from *Clostridium papyrosolvens*), YP_003615923 (MiAnPRT from *Methanocaldococcus infernus*), CBL11730 (RiAnPRT from *Roseburia intestinalis*), YP_182183 (DeAnPRT from *Dehalococcoides ethenogenes*), YP_001635357 (CaAnPRT from *Chloroflexus aurantiacus*), YP_644851 (RxAnPRT from *Rubrobacter xylanophilus*), ZP_05807498 (MoAnPRT from *Mesorhizobium opportunistum*), NP_102383 (MlAnPRT from *Mesorhizobium loti*), NP_771449 (BjAnPRT from *Bradyrhizobium japonicum*), YP_532337 (RpAnPRT from *Rhodopseudomonas palustris*), YP_001327089 (SmAnPRT from *Sinorhizobium medicae*), YP_768077 (RlAnPRT from *Rhizobium leguminosarum*), ZP_03524127 (ReAnPRT from *Rhizobium etli*), XP_001702699 (CrAnPRT from *Chlamydomonas reinhardtii*), AK121680 (OsAnPRT from *Oryza sativa* in this study), NP_197300 (AtAnPRT from *Arabidopsis thaliana*), XP_002511378 (RcAnPRT from Ricinus communis), ZP_06657245 (EcAnPRT from Escherichia coli).

B. OsAnPRT *expression in* E. coli *and* in vivo *activity*

The recombinant DNA, *pB::OsAnPRT*, was constructed using the ORF of a PCR-amplified *OsAnPRT* fragment. After the transformation of *E. coli* with the recombinant DNA, *OsAnPRT* activity was monitored *in vivo* in a medium containing IPTG and 19 amino acids, excluding Tryp. Functional complementation was performed using the *AnPRT* mutant of *E. coli* to confirm the enzyme activity of the gene product of *OsAnPRT*. To assess the viability of *E. coli* cells by *OsAnPRT* activity, the *OsAnPRT* -expressing cells were cultured for 12 hrs with shaking, and the diluted portion was plated on agar medium containing the 19 amino acids and Amp (25mg/ml) excluding Trp (Fig. 4). The viable colonies greatly differed among the plasmids. The TrpD mutant of *E. coli* with *OsAnPRT* could grow under

conditions in which the mutant without OsAnPRT could not. This showed that the *OsAnPRT* was capable of functioning as a complement, and evidenced functional AnPRT activity.

Fig.4. Functional complementation assay. The TrpD mutant E. coli strain 2032 containing pB::OsAnPRT and control and Gif41 wild-type E. coli containing control.

Expression of *OsAnPRT* can complement the *TrpD* mutant of *E. coli.*

A growth study was performed to determine whether the *OsAnPRT* gene would increase the sensitivity of bacterial cells to Trp. The *pB::OsAnPRT* construct was transformed into the *trpD* mutant *E. coli* strain2032. A control plasmid was also transformed into wild-type (Gif41) and the TrpD mutant 2032. The *pB:: OsAnPRT* activity was monitored via a growth assay in the absence of Trp. Bacterial cells were grown in MM with 19 amino acids excluding Trp, containing IPTG and Amp. The wild-type *E. coli* strain Gif41harboring the control plasmid grew normally and evidenced an S-shaped classical growth curve in the medium without Trp (Fig. 5). The Gif41strain could synthesize Trp itself, and thus grew normally in the medium. The TrpD mutant strain 2032 expressing *pB:: OsAnPRT* also grew normally and evidenced an S-shaped classical growth curve in the same medium, but grew slightly more slowly than the wild-type strain containing the control plasmid (Fig. 5), although the 2032 strain harboring the control plasmid in the same medium without Trp evidenced dramatically retarded growth. In this case, the TrpD mutant *E. coli* strain 2032 could not synthesize Trp itself, and thus grew dramatically less rapidly; however, the same *E. coli* strain 2032 containing *pB::OsAnPRT* grew well because the TrpD mutant *E. coli* was able to synthesize Trp using AnPRT expressed by the *pB::OsAnPRT* plasmid (Fig. 5). This is a consequence of *pB::OsAnPRT* activity. From the above finding, it was concluded that OsAnPRT expression can functionally complement the *trpD* mutant *E. coli*. Our attention was drawn to obtain some important information about the substrate specificity of the enzyme by purifying recombinant *Os*AnPRT in *E. coli,* and to assess the physiological functions of this novel enzyme for Trp metabolism by screening T-DNA insertion mutants in which the *Os*AnPRT gene is knocked out in rice. Our reports regarding the cloning and characterization of the cDNA encoding for AnPRT from rice have generated bioinformatic predictions, as well as motifs and complementation, in a trpD mutant of *E. coli*. These results may constitute a starting point for investigations at the molecular level to investigate Trp biosynthesis in rice, which might eventually be applied to modify the nutritional compositions of crop plants.

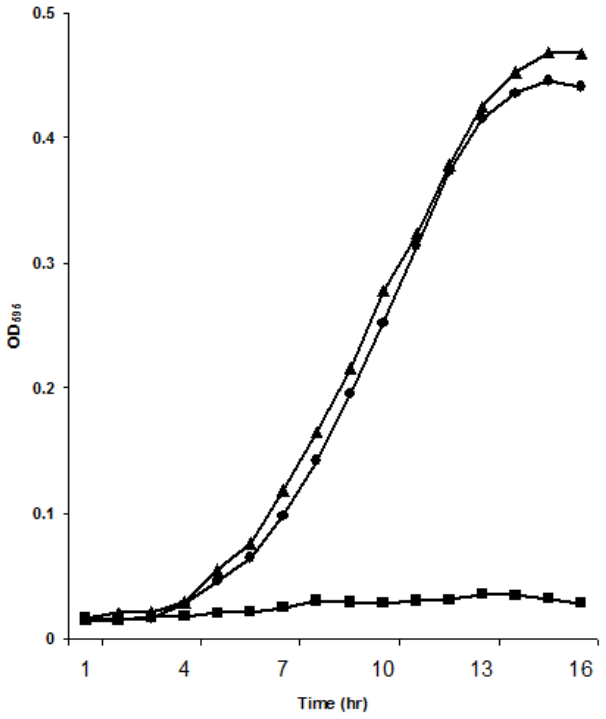

Fig.5. Growth curves of *E. coli* mutant *2032* harboring *pB::* OsAnPRT and and control plasmid; and *Gif41* containing control plasmid. Bacterial cells were grown at 37°C in MM containing all amino acids excluding Tryp. Growth was monitored via optical density measurements at 595nm (OD_{595}). Symbols: ●, *2032* + *pB::OsAnPRT*; ▲, Wild type + control; ■, *2435* + control.

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REFERENCES

[1] Marino, M., Deuss, M., Svergun, D. I., Konarev, P. V., Sterner, R., and Mayans, O., Structural and mutational analysis of substrate complexation by anthranilate phosphoribosyltransferase from Sulfolobus solfataricus, J. Biol. Chem., 2006, vol. 281, pp. 21410–21421.

[2] Jiroutová, K., Horák, A., Bowler, C., and Oborník, M., Tryptophan biosynthesis in stramenopiles: eukaryotic winners in the diatom complex chloroplast, J. Mol. Evo., 2007, vol. 65, no. 5, pp. 496-511.

[3] Schwab, T., Skegro, D., Mayans, O., and Sterner, R., A Rationally designed monomeric variant of anthranilate phosphoribosyl transferase from Sulfolobus solfataricus is as active as the dimeric wild-type enzyme but less thermostable, J. Mol. Biol., 2008, vol. 376, pp. 506-516.

[4] Crawford, I. P., Evolution of a biosynthetic pathway: the tryptophan paradigm, Annu. Rev. Microbiol. , 1989, vol. 43, pp. 567-600.

[5] Chelsky, D., and Parsons, S. M., Stereochemical course of the adenosine triphosphate phosphoribosyl transferase reaction in histidine biosynthesis, J. Biol. Chem., 1974, vol. 250, pp. 5669-5673.

[6] Radwanski, E. R., and Last, R. L., Tryptophan biosynthesis and metabolism: biochemical and molecular genetics, Plant Cell., 1995, vol. 7, pp. 921–934.

[7] Dutcher, S. K., Galloway, R. E., Barclay, W. R., and Poortinga, G., (1992) Tryptophan analog registance mutations in Chlamydomonas reinhardtii, Genetics.,1992, vol. 131, pp. 593-607.

[8] Kim, C., Xuong, N., Edwards, H., Madhusudan, S., Yee, M. C., Spraggon, G., and Mills, S. E., The crystal structure of anthranilate phosphoribosyltransferase from the enterobacterium Pectobacterium carotovorum, FEBS Lett., 2002, vol. 523, pp. 239–246.

[9] Ivens, A., Mayans, O., Szadkowski, H., Wilmanns, M., and Kirschner, K., Purification, characterization and crystallization of thermostable anthranilate phosphoribosyltransferase from Sulfolobus solfataricus, Eur. J. Biochem., 2001, vol. 268, pp. 2246–2252.

[10] Lee, C. E., Goodfellow, C., Javid, M. F., Baker, E. N., and Shaun, L. J., The crystal structure of TrpD, a metabolic enzyme essential for lung colonization by Mycobacterium tuberculosis, in complex with its substrate phosphoribosylpyrophosphate, J. Mol. Biol., 2006, vol. 355, pp. 784–797.

[11] Bauerle, R., Hess, J., and French, S. Anthranilate synthase± anthranilate phosphoribosyltransferase complex and subunits of Salmonella typhimurium, Methods Enzymol., 1987, vol. 142, pp. 366-386.

[12] Yanofsky, C., Platt, T., Crawford, I. P., Nichols, B. P., Christie, G. E., Horowitz, H., van Cleemput, M., and Wu, A. M., The complete nucleotide sequence of the tryptophan operon of Escherichia coli, Nucleic Acids Res., 1981, vol. 9, pp. 6647-6668.

[13] Hommel, U., Lustig, A., and Kirschner, K., Purification and characterization of yeast anthranilate phosphoribosyltransferase, Eur. J. Biochem., 1989, vol. 180, pp. 33-40.

[14] Braus, G. H., Aromatic amino acid biosynthesis in the yeast Saccharomyces cerevisiae: a model system for the regulation of a eukaryotic biosynthetic pathway, Microbiol Rev., 1991, vol. 55, pp. 349-370.

[15] Edwards, S. L., Kraut, J., Xuong, N., Ashford, V., Halloran, T. P., and Mills, S. E., Crystallisation and purification of the enzyme anthranilate phosphoribosyl transferase, J. Mol. Biol., 1988, vol. 203, pp. 523-524.

[16] Rose, A. B., Casselman, A. L., and Last, R. L., A Phosphoribosylanthranilate Transferase Gene Is Defective in Blue Fluorescent Arabidopsis thaliana Tryptophan Mutants, Plant Physiol., 1992, vol. 100, pp. 582-592.

[17] Zhao, J., and Last, R. L., Immunological characterization and chloroplast import of the tryptophan biosynthetic enzymes of the flowering plant Arabidopsis thaliana, J. Biol. Chem., 1995, vol. 270, pp. 6081– 6087.

[18] Osato, N., Itoh, M., Konno, H., Shibata, K., Carninci, P., Shiraki, T., Shinagawa, A., Arakawa, T., Kikuchi, S., Sato, K., Kawai, J., and Hayashizaki, Y., A Computer-Based Method of Selecting Clones for a Full-length cDNA Project: Simultaneous Collection of Negligibly Redundant and Variant cDNAs, Genome Res., 2002, vol.12, pp. 1127-1134.

[19] Sikdar, M.S.I., and Kim, J.S., Functional Analysis of a Gene Encoding Threonine Synthase from Rice, Afr. J. Biotechnol., 2010, vol. 9, pp. 1122-1129.

[20] Wheeler, D.L., Church, D.M., Federhen, S., Lash, A.E., Madden, T.L., Pontius, J.U., Schuler, G.D., Schriml, L.M., Sequeira, E., Tatusova, T.A., and Wagner, L., Database Resources of the National Center for Biotechnology, Nucleic Acids Res., 2003, vol. 31, pp. 28-33.

[21] Thompson, J.D., Higgins, D.G., and Gibson, T.J., CLUSTAL W: Improving the Sensitivity of Progressive Multiple Sequence Alignment through Sequence Weighting, Position-specific Gap Penalties and Weight Matrix Choice, Nucleic Acids Res., 1994, vol. 22, pp. 4673-4680.

[22] Kumar, S., Nei, M., Dudley, J., and Tamura, K., MEGA: A Biologist-centric Software for Evolutionary Analysis of DNA and Protein Sequences, Briefings Bioinform., 2008, Vol. 9, no. 4, pp. 299-306.

[23] Sambrook, J., and Russell, D.W., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press. Cold Spring Harbor, 2001.

[24] Kim, J., and Leustek, T., Cloning and Analysis of the Gene for Cystathionine γ- Synthase from Arabidopsis thaliana, Plant Mol. Biol., 1996, vol. 32, pp. 1117-1124.