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Full Length Research Paper

Functional analysis of a gene encoding threonine synthase from rice

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Threonine synthase (TS) is a pyridoxal phosphate dependent enzyme that catalyzes the formation of threonine (Thr) through *O*-phosphohomoserine (OPH) from the aspartate family pathway in plants. The properties of the TS enzyme have been evaluated in many bacteria and few plants. Sequence analysis of the cDNA from rice revealed that it harbors a full-length open reading frame for *OsTS* encoding for 521 amino acids, corresponding to a protein of approximately 57.2 kD. The predicted amino acid sequence of OsTS is highly homologous to that of *Arabidopsis* TS and many bacterial TS encoded by *thrC* gene. The OsTS protein harbors a signature binding motif for pyridoxal- 5' -phosphate at the amino terminus. A *thrC* mutant strain of *Escherichia coli* was complemented by *OsTS* expression. *OsTS* expression was correlated with the survival of the *thrC* mutant, which is affected by the supplementation of an aspartate pathway metabolite, methionine.

Key words: *Oryza sativa*, methionine, threonine, threonine synthase.

INTRODUCTION

Threonine (Thr) is an essential amino acid in animals, including humans. The biosynthetic pathway of Thr is initiated from aspartate (Asp) and is called the Asp family pathway in plants. The aspartate-derived amino-acid pathway from plants is well suited for analyzing the function of the allosteric network of interactions in branched pathways (Curien et al., 2009).

Thr is synthesized via a branched pathway that includes lysine (Lys) and methionine (Met) (Azevedo et al., 1997; Bryan, 1980). The carbon skeleton of Thr is derived from Asp as the amino acids Lys and Met. The common precursor for the synthesis of Thr and Met in the branching point is *O*-phosphohomoserine (OPH) (Figure 1). In plants and microorganisms, Thr synthesis is a component of the multibranched biosynthetic pathway originnating with Asp and resulting in the synthesis of Lys, Met, Thr and isoleusine (Curien et al., 1996). Threonine synthase (TS: EC 4.2.99.2) is a fold type II pyridoxal 5' -phosphate (PLP)-dependent enzyme and catalyses the final step of Thr formation (Curien et al., 2008; Mas-Droux et al., 2006). TS is involved in the essential amino acids pathway derived from aspartate and catalyzes the conversion of *O*-phosphohomoserine (OPH) into Thr and inorganic phosphate via a PLP dependent reaction (Mas-Droux et al., 2006; Casazza et al., 2000). The TS activity has been identified, purified and described in a variety of microorganisms, such as *Neurospora crassa* (Flavin and Slaughter, 1960), *Escherichia coli* (Farrington et al., 1993; Parsot et al., 1983), *Corynebacterium glutamicum* (Eikmanns et al., 1993), *Crytococcus neoformans* (Kingsbury and McCusker, 2008), *Streptococcus* sp. (Tang et al., 2007) and *Mycobacterium tuberculosis* (Covarrubias et al., 2008). The corresponding gene was isolated from a number of bacteria (Parsot, 1986; Han et al., 1990; Motoyama et al., 1994; Omori et al., 1993; Clepet et al., 1992). The characterization and analysis of several plant genes have been reported, including those of *Arabidopsis thaliana* (Curien et al., 1998; Avraham and

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Abbreviations: TS, Threonine synthase; ***OsTS*,** *Oryza sativa* threonine synthase gene; **Thr,** threonine; **Met,** methionine; **Lys,** lysine; **OPH,** O-phosphohomoserine; **cDNA,** complementary deoxyribonucleic acid; **SAM,** S-adenosylmethionine; **CGS,** cystathionine-γ-synthase; **EST,** expressed sequence tag; **PCR,** polymerase chain reaction; **ORF,** open reading frame; **IPTG,** isopropyl β-D-thiogalactopyranoside; **BLAST,** basic local alignment search tool; **EMBL,** European molecular biology laboratory.

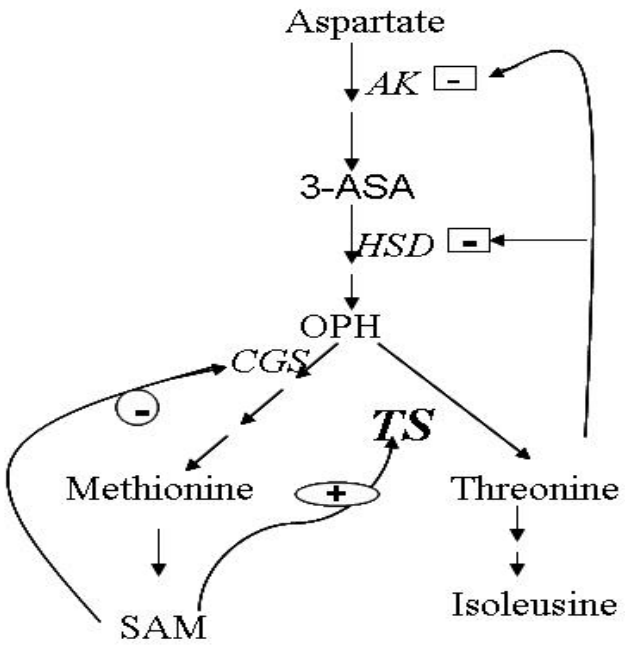

Figure 1. Scheme of the threonine biosynthesis pathway of aspartate family in plants. The abbreviations are AK, aspartate kinase; 3-ASA, 3-aspartic semialdyde; HSD, homoserine dehydrogenase; OPH, *O*-phosphohomoserine; SAM, *S*-adennosylmethionine, CGS, cystathionine g-synthase; TS, threonine synthase; TDH, threonine dehydratase. Symbols are indicated: ⊕; allosteric activation, ⊙; feedback repression and ⊟; feedback inhibition.

Amir, 2005, Lee et al., 2005; Laber et al., 1999), *Solanum tuberosum* L (Casazza et al., 2000) and *Sorghum bicolor* (Ferreira et al., 2006).

The synthesis of aspartate-derived amino acids is subject to complex regulation. The key to pathway control is feedback inhibition of aspartate kinase by Lys and/or Thr, or by Lys in concert with S-adenosylmethionine (SAM) (Rinder et al., 2008). Aspartate kinase, the first enzyme in the pathway, is inhibited allosterically by Lys and Thr (Lee et al., 2005). TS compete with the first enzyme required for subsequent Met biosynthesis, cystathionine-γ-synthase (CGS), for their common substrate OPH (Thompson et al., 1982). TS enzyme activity is activated by S-adenosyl-methionine (SAM) and inhibited by cysteine (Madison and Thompson, 1976; Giovanelli et al., 1984; Curien et al., 1996). SAM is, in turn, directly synthesized from Met; therefore, increasing Met levels will result in increases in the concentration of SAM and subsequently affect TS activity (Casazza et al., 2000). *In vitro* studies have showed that SAM stimulates TS activity in an allosteric manner (Curien et al., 1998). A number of studies have also documented a dynamic interaction between CGS and TS in the control of Met biosynthesis (Amir et al., 2002; Hesse and Hoefgen, 2003). Over expression of CGS resulted in elevated free Met levels, but did not significantly affect Thr levels (Chiba et al., 2003; Inaba et al., 1994; Kim et al., 2002). Here, we report the analysis and characterization of a gene for the TS enzyme from rice (*Oryza sativa*), an important crop plant and the influence on its activities by an Asp pathway metabolite, probably SAM.

METHODS AND MATERIALS

Strains and plasmids

Two *E. coli* strains were used in this study, Gif41 [*thrC1001, λ14-, e14-, relA1, spoT1, th-1 thi-1*] (Theze et al., 1974) and Sφ415 [*Udk-2*, *upp-11*, *rclA1*, *rpsL254* (*strR*), *metB1*] (Hammer-Jespersen and Munch-Petersen, 1973). The source of both strains was the *E. coli* Genetic stock Center (CGSC) at Yale University, USA. An EST clone (Genbank Accession Number AK101669 and clone name J033058D04) was used in this study.

DNA sequence analysis

An EST clone (GenBank Accession No. AK101669 and clone name J033058D04) used in this study was obtained from the Rice Genome Resource Center (RGRC), National Institute of Agrobiological Science (NIAS), Japan. The clone was derived from a rice cDNA library (Osato et al., 2002) from developing seeds prepared in pBluescript SK-. DNA sequencing was conducted using an automatic sequencer (A1Fexpress DNA sequencer, Pharmacia Biotech. Inc., UK) with synthetic oligonucleotide primers. Nucleotide sequences and amino acid sequences were compared with the sequences in the GenBank and EMBL databases and analyzed via BLAST (Wheeler et al., 2003) and the Clustal W multiple sequence alignment program (Thompson et al., 1994) or Biology WorkBench 3.2 (http://workbench.sdsc.edu; San Diego Supercomputer Center; University of California San Diego, USA). Sequence comparisons were conducted at the nucleotide and amino acid levels. Motifs were searched by the GenomeNet Computation Service at Kyoto University (http://www.genome.ad.jp) and phylogenic tree with bootstrap value prepared by the Mega 4.1 program (Kumer et al., 2008).

Polymerase chain reaction (PCR) and recombinant constructs

Our sequence analysis showed the presence of an ATG start codon located in-frame at -99 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 OsTS to amplify the full-length open reading frame (ORF) and to over express the gene product in *E. coli*. Polymerase chain reaction (PCR) (Sambrook and Russell, 2001) 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 OsTS was amplified from the EST clone as a template and the following primers were designed from the *OsTS* sequence: OsTS-F (5'- AAA GCT TTC ACT CAC TCC CTA AAA CCC-3') and *OsTS*-R (5' AAA GCT TCA CAC TTC AGA GCT TAC CCT -3') using Ampli TaqGold polymerase (Perkin-Elmer, U.S.A). The underlined bases in the OsTS-F and OsTS-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℃ for 1 min, 55℃ for 1 min and 72℃ 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::OsTS*. Restriction analysis was conducted in an effort to confirm the recombinant DNA construct of *pB::OsTS* with the right orientation for over expression.

Functional complementation and growth assay

The competent *thrC* mutant of *E. coli* strain, Gif41, was transformed with *pB::OsTS* via electroporation (ECM399, BTX, USA) using a cuvette with a 0.1 cm electrode gap, then plated on LB medium (20 g/l) with Amp (100 µg/ml). The growing culture was tested for growth retardation in M9 minimal medium containing Amp (25 µg/ml), 20% glucose, 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and 19 amino acids (Sigma, Germany) each at a concentration of 25 µg/ml, excluding Thr. Bacterial growth was then assessed by measuring optical density at 595 nm at one-hour interval. After 12 h, the diluted culture was plated and incubated overnight at 37°C.

Growth inhibition assay of OsTS in *E. coli*

The *E. coli* mutants harboring the *pB::OsTS* construct, control plasmid and wild-type with control plasmid were grown at 37°C in M9 minimal medium (5 x M9 salts (200 ml/l), 1 M $MgSO_4$ (2 ml/l), 1 M $CaCl_2$, 0.1 ml/l IPTG, 20% glucose (20 ml/l), containing 19 amino acids and Amp (25 µg/ml), excluding Thr and the same medium was used with all the reagents kept constant, but an additional supplementation of 10-fold high Met. The bacterial growth was monitored via optical density measurements every hour using a spectrophotometer (UV1101, Biochrom, England) at 595 nm (OD_{595}).

RESULTS AND DISCUSSION

Sequence analysis of *OsTS*

An expressed sequence tag (EST) clone (GenBank Accession number AK101669, clone name J033058D04 and clone ID 212512) obtained from the Rice Genome Resource Center (RGRC) was analyzed to determine the nucleotide sequence using the designed primers. The cDNA (*OsTS)* sequence harbored a full-length open reading frame consisting of 1563bp, encoding for a protein of approximately 57.2 kDa. The expected isoelectric point of the protein was 6.60. Data analysis revealed that the *OsTS* sequence was identical to the genomic region located in chromosome V. Comparisons of the amino acid sequence of the *OsTS* and the homologous sequences from maize (*Zea mays*) and Arabidopsis (*A. thaliana*) revealed high identity, at 91 and 71%, respectively (Figure 2).

Analysis of the *OsTS* amino acid sequence revealed a signature binding motif for PLP in the N-terminal region (189-203) (Figure 2). The motif sequence (HCGISHTGSF KDLGM) was highly homologous to the consensus [DESH]-x(4,5)-[STVG]-{EVKD}-[AS]-[FYI]-K-[DLIFSA]-[RLVMF]-[GA]-[LIVMGA], where the underlined amino acids were well conserved. The binding motif for PLP is present in bacterial TSs and Serine/threonine dehydratases that utilize PLP as a cofactor. The exact PLP binding site seemed to be K-199 and was identified via comparison with the binding site of bacterial TS. This result indicates that the OsTS product utilizes PLP as a co-factor. Phylogenic analysis of the related sequence further indicated that OsTS is grouped with several plant sequences and is divergent and evolved from ancestor bacterial TS (Figure 3).

OsTS expression in *E. coli*

The recombinant DNA, *pB::OsTS*, was constructed using the ORF of a PCR-amplified *OsTS* fragment. After the transformation of *E. coli* with the recombinant DNA, *OsTS* activity was monitored *in vivo* in a medium containing IPTG and 19 amino acids, excluding Thr. Functional complementation was performed using the *TS* mutant of *E. coli* to confirm the enzyme activity of the gene product of *OsTS*. To assess the viability of *E. coli* cells by OsTS activity, the *OsTS*-expressing cells were cultured for 12 h with shaking and the diluted portion was plated on agar medium containing the 19 amino acids and Amp (25 µg/ml) without Thr (Figure 4). The *thrC* mutant of *E. coli* with the *OsTS* construct could grow under the conditions above in which the mutant without *OsTS* could not. The result revealed that the OsTS is able to complement with functional TS activity.

Expression of *OsTS* can complement the *thrC* mutant of *E. coli*

A growth study was performed to determine whether the *OsTS* gene would increase the sensitivity of bacterial cells to Thr. The *pB::OsTS* construct was transformed into the *thrC* mutant *E. coli* Gif41. A control plasmid was also transformed into wild-type (Sφ415) and the *thrC* mutant Gif41. The *pB::OsTS* activity was monitored via a growth assay in the absence of Thr. Bacterial cells were grown in M9 minimal medium with IPTG and Amp and 19 amino acids excluding Thr. The wild-type *E. coli* strain Sφ415 harboring the control plasmid grew normally and evidenced an S-shaped classical growth curve in the medium without Thr (Figure 5A). The Sφ415 strain could synthesize Thr itself and thus grew normally in the medium. The *thrC* mutant strain Gif41 expressing *pB::OsTS* 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 (Figure 5A), although the Gif41strain harboring the control plasmid in the same medium without Thr evidenced dramatically retarded growth. In this case, the *thrC* mutant *E. coli* strain Gif41 could not synthesize Thr itself and thus grew dramatically less rapidly; however, the same *E. coli* strain Gif41 containing *pB::OsTS* grew well because the *thrC* mutant *E. coli* was able to synthesize Thr using TS expressed by the *pB::OsTS* plasmid (Figure 5A). This is a consequence of *pB::OsTS* activity.

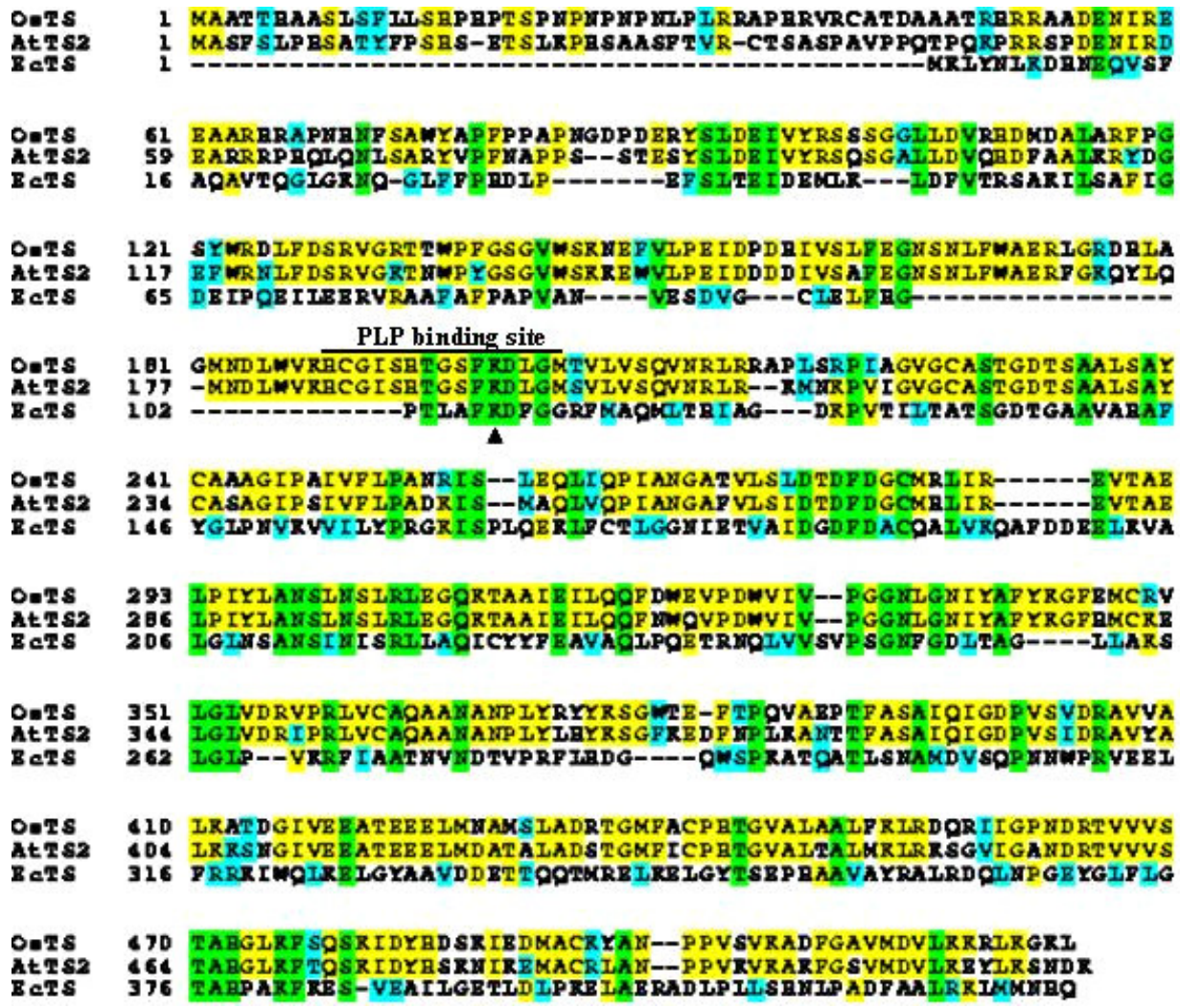

Figure 2. Amino acid sequence alignment of TS from *Oryza sativa* (OsTS), *Arabidopsis thaliana* (AtTS2) and *Escherichia coli* (EcTS). Shaded residues represent amino acids that are identical among at least three of the three amino acids. GenBank Accession Numbers; AK101669 (OsTS), Q9SSP5 (AtTS2) and NP_414545 (EcTS).

From the above finding, it was concluded that *OsTS* expression can functionally complement the *thrC* mutant *E. coli*.

The growth of the *thrC* mutant of *E. coli* was influenced by the expression of *OsTS* in high levels of methionine

The growth pattern of the *thrC* mutant of *E. coli* complemented with *pB::OsTS* was also assessed in the presence of high Met levels. The wild-type *E. coli* strain Sφ415 harboring the control plasmid grew normally and evidenced an S-shaped classical growth curve in M9 minimal medium with 19 amino acids (excluding Thr, containing 1 mM IPTG and supplemented with additional 10-fold high Met). The *E. coli* strain Gif41 grew and evidenced an S-shaped classical growth curve in the same medium, but the growth pattern was much more vigorous than in the medium without Met (Figure 5B). In this case, when a high level of Met was added, the Met was converted to SAM and the SAM allosterically activated TS activity--this is why the *thrC* mutant of *E. coli* grew so vigorously. This result is consistent with previously reported results in studies of bacteria and plants (Giovanelli et al., 1984; Curien et al., 1996, Casazza et al., 2000 and Ferreira et al., 2006). The principal feature of plant TS, in contrast to its bacterial counterpart, may be allosteric regulation by SAM, which induces a dramatic stimulation of TS activity (Hesse et al., 2004). However, the Gif41 strain harboring the control plasmid also evidenced dramatically retarded growth in the same medium owing to a lack of Thr, even when 10-fold high Met was added (Figure 5B). This finding indicates that Met has a marked influence on *OsTS* activity in rice plants.

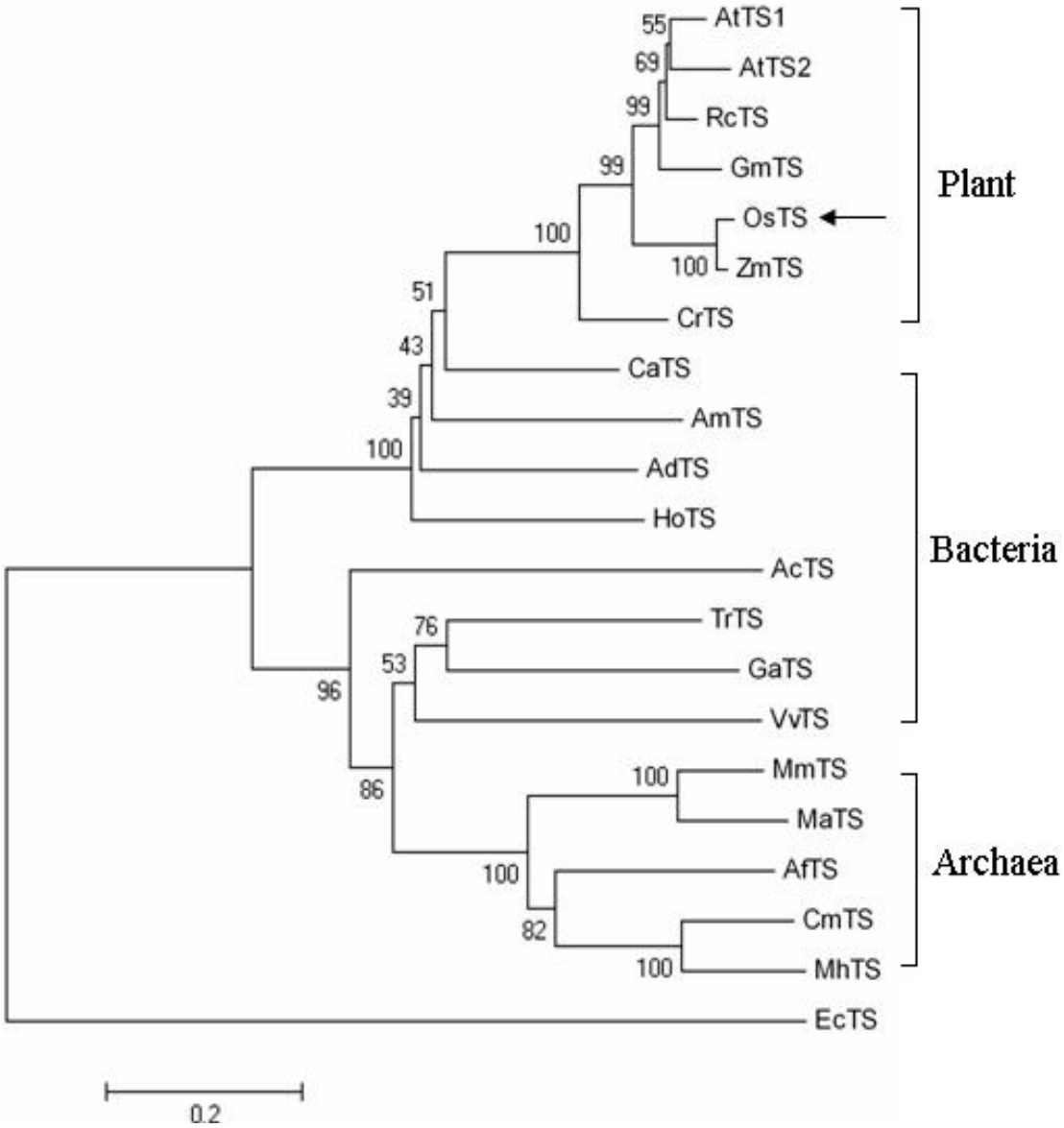

Figure 3. Phylogenetic tree: Phylogenetic analysis of OsTS related proteins using Clustal W and Mega 4.1 program. GenBank accession numbers are as follows: AK101669 (OsTS from *Oryza sativa*), XP_002514088 (RcTS; *Ricinus communis*), Q9S7B5 (AtTS1; *Arabidopsis thaliana*), Q9SSP5 (AtTS2; *A. thaliana*), ABC00741(GmTS; *Glycine max*), ACG39080 (ZmTS; *Zea mays*), XP_001698517 (CrTS; *Chlamydomonas reinhardtii*), YP_001515596 (AmTS; *Acaryochloris marina*), YP_002463167(CaTS; *Chloroflexus aurantiacus*), YP_003264969 (HoTS; *Haliangium ochraceum*), YP_002492618 (AdTS; *Anaeromyxobacter dehalogenans*), YP_002753372 (AcTS; *Acidobacterium capsulatum*), YP_002522459 (TrTS: *Thermomicrobium roseum*), YP_002760880 (GaTS; *Gemmatimonas aurantiaca*), ZP_01923848 (VvTS; *Victivallis vadensis*), YP_001330351 (MmTS; *Methanococcus maripaludis*), NP_070145 (AfTS; *Archaeoglobus fulgidus*), YP_002466596 (CmTS; *Candidatus Methanosphaerula*), YP_503069 (MhTS; *Methanospirillum hungatei*) and NP_414545 (EcTS; *Escherichia coli*).

Attempts are currently underway to obtain some important information about the substrate specificity of the enzyme by purifying recombinant *OsTS* in *E. coli and* to assess the physiological functions of this novel enzyme for Thr metabolism by screening T-DNA insertion mutants in which the *OsTS* gene is knocked out in rice. Our reports regarding the cloning and characterization of the cDNA encoding for TS from rice have generated bioinformatic predictions, as well as motifs and complementation, in a *thrC* mutant of *E. coli*. These results may constitute a starting point for investigations at the molecular level to investigate Thr biosynthesis in rice, which might eventually be applied to modify the nutritional compositions of crop plants.

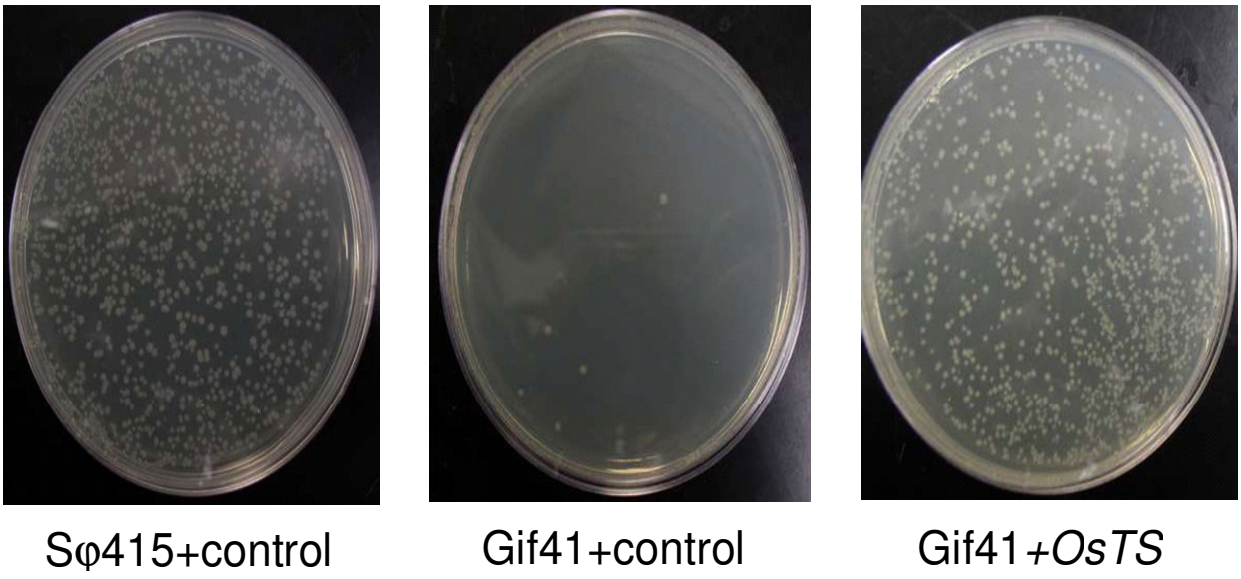

Figure 4. Functional complementation assay. The *thrC* mutant strain of *E. coli* Gif41 containing *pB::OsTS* and pBluescript II KS+ and wild-type *E. coli* Sφ415 containing pBluescript II KS+ as a control plasmid.

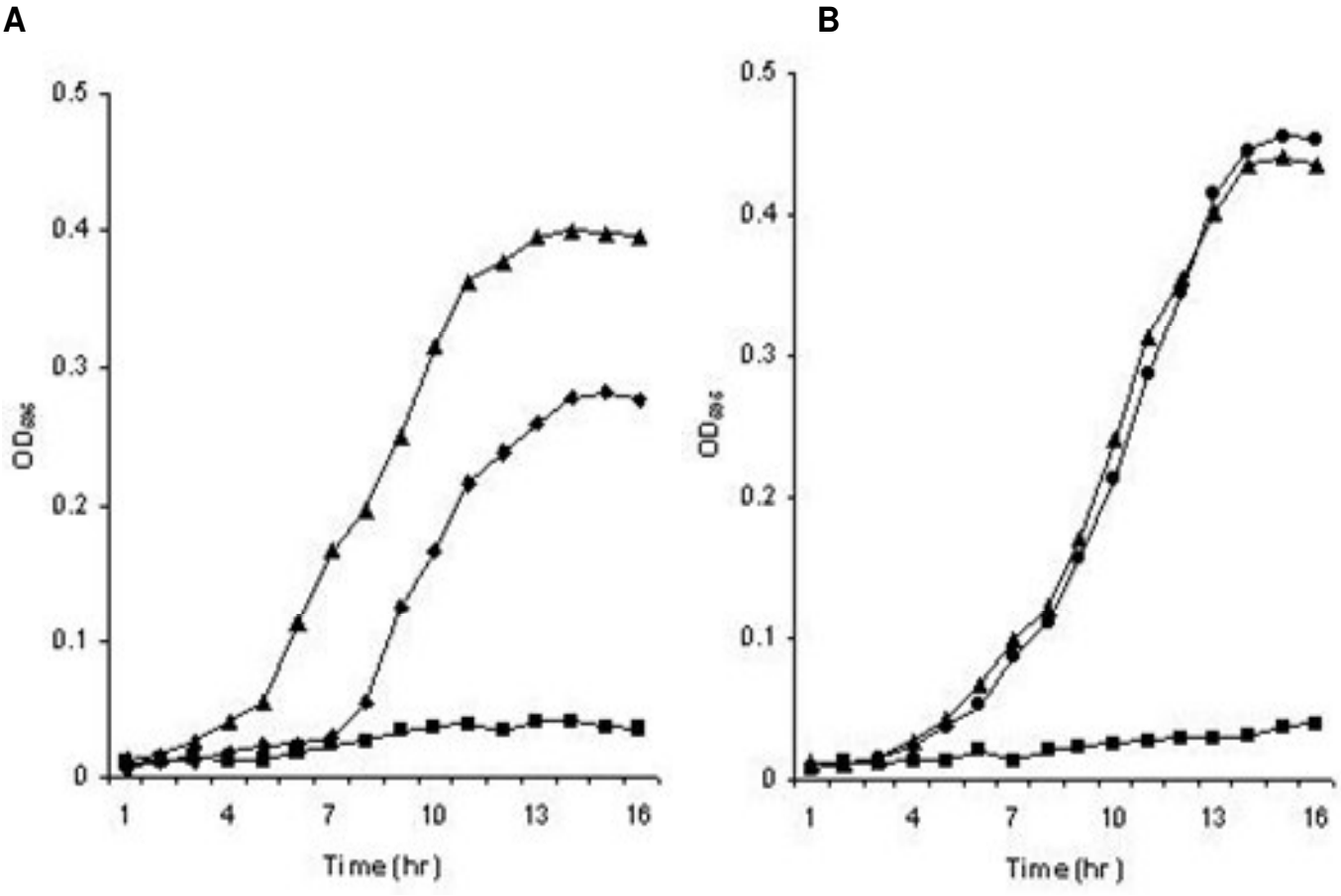

Figure 5. Growth curves of *E. coli* mutant Gif41 and Sφ415 harboring *OsTS* without Met (A) and supplementing Met (B). Bacterial cells were grown at 37℃ in M9 minimal medium containing 19 amino acids except Thr (A) or the same medium supplemented with an additional 10 times high Met. Growth was monitored via optical density measurements at 595 nm (OD_{595}). Symbols: ♦, Gif41+ *pB::OsTS*; ▲, Sφ415+ pBluescript II KS+; ■, Gif41+ pBluescript II KS+.

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