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Full Length Research Paper

Functional analysis of a gene encoding homoserine kinase from rice

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Homoserine kinase (HSK) is an enzyme that catalyzes the common pathway in threonine and methionine biosynthesis in plants. The genes encoding HSK have been reported in many bacteria and some plants including Arabidopsis. Our group recently reported a gene for threonine synthase (TS) from rice. In this study, we functionally characterized a gene encoding HSK from rice (*OsHSK*). Analysis of a cDNA sequence and genome of rice revealed that a full-length open leading frame of OsHSK consisted of 378 amino acids, which corresponded to a protein with the molecular weight of approximately 37.8 kDa and with the predicted isoelectric point of 6.86. The predicted amino acid sequence of OsHSK harbored a distinct signature motif for ATP binding and was highly homologous to that of enzymes of plant and bacterial HSKs. Expression of OsHSK in the *thrB* mutant of *Escherichia coli* showed that the gene was able to functionally complement the mutant. These results suggest that OsHSK encodes a protein for HSK in rice.

Key words: Homoserine, homoserine kinase, rice (*Oryza sativa*), *thrB*.

INTRODUCTION

Animals are not able to synthesize ten essential amino acids, which must be acquired through their diet (Galili et al., 2002). Among the essential amino acids, lysine (Lys), methionine (Met), threonine (Thr) and isoleucine (Ile) are synthesized from aspartate (Asp). Therefore, they are commonly called Asp-derived amino acids. The Asp-derived amino acids pathway in plants is well suited for analyzing the function of the allosteric network of interactions in branched pathways (Curien et al., 2009). The common precursor for the synthesis of Met, Thr, and Ile in the branching point is *O*-phospho-L-homoserine (OPH) (Figure 1). The availability and partitioning of OPH has been shown to play a role in the regulation of the S-adenosylmethionine (SAM) and Thr pathways (Azevedo et al., 1997; Ravanel et al., 1998; Avraham and Amir, 2005). OPH is either directly converted to Thr by threonine synthase (TS) or to Met, in a three-step mechanism, through condensation of cysteine and OPH to cystathionine, which is subsequently converted to homocysteine and then to Met by the enzymes cystathionine γ-synthase (CGS), cystathionine β-lyase (CBL), and methionine synthase (MS), respectively (Matthews, 1999; Hesse and Höfgen, 2003).

Homoserine kinase (HSK, product of the *thrB* gene; EC 2.7.1.39) catalyzes the formation of OPH from homoserine in the Asp family pathway in plants as well as many bacteria and fungi. The next step in the pathway leads to the formation of Thr by TS. OPH can also be converted to Met by CGS (Lee and Leustek, 1999; Lee et al., 2005; Jander and Joshi, 2010). HSK belongs to a large, unique class of small metabolite kinases, the GHMP kinase superfamily. Members in the GHMP superfamily participate in several essential metabolic pathways, such as amino acid biosynthesis, galactose metabolism, and the mevalonate pathway (Bork et al.,

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Abbreviations: Lys, Lysine; **Met,** methionine; **Thr,** threonine; **Ile,** isoleucine; **Asp,** aspartate; **OPH,** *O*-phospho-L-homoserine; **SAM,** S-adenosylmethionine; **TS,** threonine synthase; **CGS,** cystathionine γ-synthase; **CBL,** cystathionine β-lyase; **MS,** methionine synthase; **HSK,** homoserine kinase; **BLAST,** basic local alignment search tool; **PCR,** polymerase chain reaction; **Amp,** ampicillin; **MM,** minimal medium; **IPTG,** isopropyl-β-D-thio-galactoside.

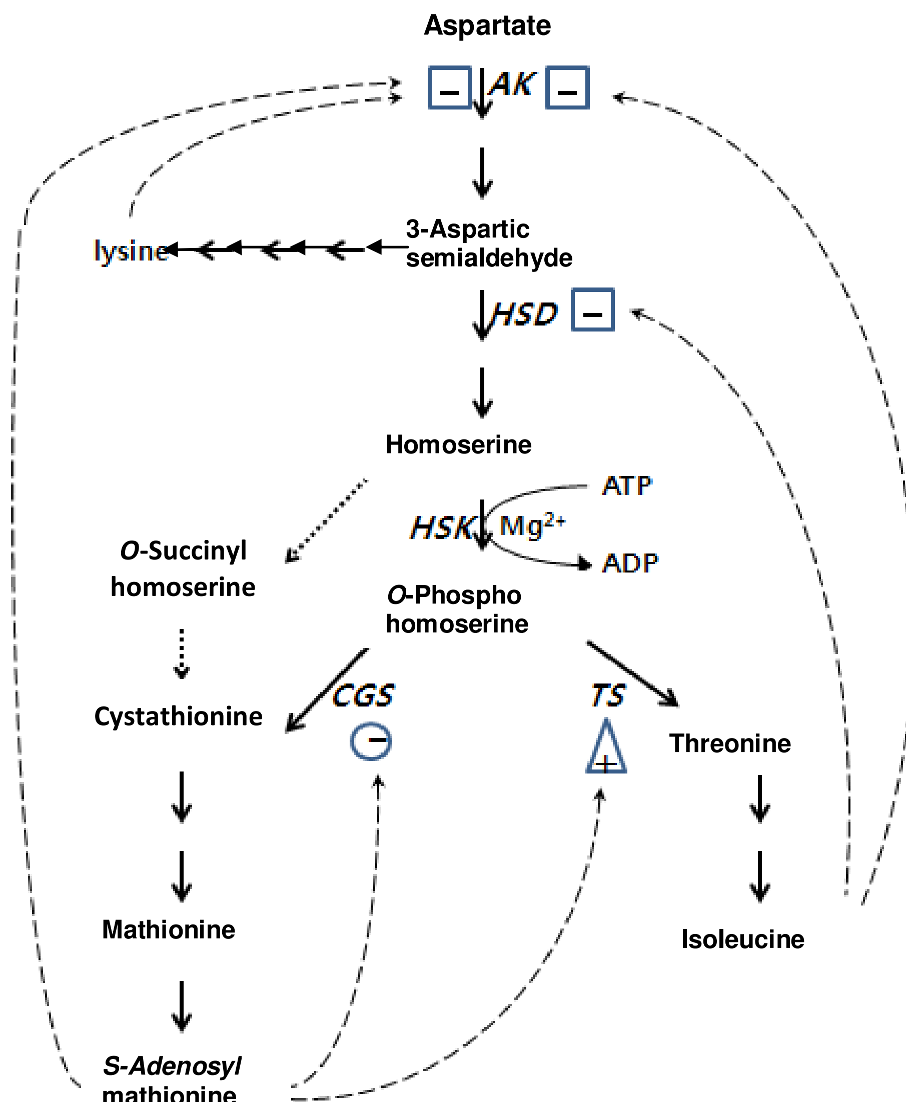

Figure 1. Schematic of the threonine biosynthesis pathway and regulation of the aspartate family pathway in plants. The abbreviations are AK, Aspartate kinase; HSD, homoserine dehydrogenase; HSK, Homoserine kinase: CGS, cystathionine γ-synthase; TS, threonine synthase. Symbols are indicated: ; allosteric activation, ; feedback repression, ; feedback inhibition, •••> bacterial pathway via *O*-succinylhomoserine (Modified from Sikdar and Kim, 2010a).

1993). This enzyme has been found to have broad substrate specificity, including the phosphorylation of L-homoserine analogs, where the carboxyl functional group at the R-position is replaced by an ester or hydroxymethyl group (Huo and Viola, 1996). The enzyme is a homodimer and the molecular weight of the subunit is approximately 33 kDa (Cossart et al., 1981).

The gene encoding HSK (*thrB* in bacteria) has been cloned or characterized from many microorganisms and some plants, including *Escherichia coli* (Theze et al., 1974; Burr et al., 1976; Shames and Wedler 1984; Huo and Viola, 1996), *Corynebacterium glutamicum* (Follettie et al., 1988), *Saccharomyces cerevisiae* (Schultes, et al., 1990); *Methylobacillus flagellatus* (Marchenko et al., 1999), *Candida albicans* (Joanne and John, 2010), *Rhodospirillum rubrum* (Finkelnburg and Klemme, 1987), *Pisum sativum* (Thoen et al., 1978), *Hordeum vulgare* (Aarnes, 1976) (*Triticum aestivum* (Riesmeier et al., 1993) and *Arabidopsis thaliana* (Lee and Leustek, 1999).

To meet the essential amino acid requirements in animal feeds, supplements may be added or various plant sources may be combined. The manipulation of essential amino acid levels in crops is, therefore, of high interest to feed producers, plant breeders, and, eventually, the consumers (Galili and Höfgen, 2002). For such manipulations to be effective, it is essential to

understand the underlying metabolic regulation of these amino acids in plants. Previous, we examined the functional role of TS (Sikdar and Kim, 2010a) and dihydrodipicolinate synthase (Sikdar and Kim, 2010b) in rice. In this study, we functionally analyzed a gene encoding HSK from rice to better understand the Asp family pathway.

MATERIALS AND METHODS

Strains and plasmids

Two *E. coli* strains were used in this study, KCTCT 2435 and KCTC 2323, which were obtained from Korean Collection for Type Cultures. The genotype of the strains was KCTC 2435 [*recA441, sulA11, lacU169, thr1, leu6, his4, argE3, ilv (ts), galK2 rp*] and KCTC 2323 [*ApRam, TcR, tr/argEam, thi, lac-proDEX111/F'lac-ProAB, traD36, lac,* parent: Seed. Plasmid: P3 (RP1)], respectively. The KCTC 2435 and KCTC 2323 have been used as a mutant to Thr auxotroph and wild type strain, respectively.

DNA sequence analysis

The expressed sequence tag (EST) clone (GenBank Accession No. AK060519, clone name 001-020-B10) 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. DNA sequencing and sequence analysis were described previously (Sikdar and Kim, 2010a). Nucleotide sequences and amino acid sequences were compared with the sequences in the GenBank databases and analyzed via the Basic Local Alignment Search Tool (BLAST) (Wheeler et al., 2003), Biology WorkBench 3.2 (http://workbench.sdsc.edu; San Diego Supercomputer Center; University of California San Diego, USA) or the Clustal W multiple sequence alignment program (Thompson et al., 1994). 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 the Phylogenic tree with bootstrap values were determined using the MEGA 4.1 program (Kumar et al., 2008). Protein localization was predicted by iPSORT program (http://ipsort.hgc.jp).

Polymerase chain reaction (PCR) and recombinant constructs

The specific primers were designed from the sequence information around the translational start and stop codons of *OsHSK* and the full-length open reading frame (ORF) was amplified by the PCR and expressed in *E. coli* (Sambrook and Russell, 2001). After the EST was purified from a pellet harvested from a liquid culture containing ampicillin (Amp), the ORF of *OsHSK* was amplified from the EST clone as a template, and the following primers were designed from the *OsHSK* sequence: OsHSK-F (5'-CGGTACCCCACCACTACCGAGTACC-3') and OsHSK-R (5'-CGAGCTCCCCTTCTTCTCCTCCCAC-3'). The underlined bases in the OsHSK-F and OsHSK-R primers were the designed restriction sites for *Kpn*I and *Sac*I to facilitate subcloning, respectively. The PCR was conducted using a MYCyler^{TM} PCR system (BioRad, U.S.A) for 40 cycles with 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with 10 µM primers. The PCR products were analyzed on 1% (w/v) agarose gel. The amplified fragment (1.13 kb) was subcloned into pBluescript II KS+ (Stratagene Inc., U.S.A) to give *pB::OsHSK*. Restriction analysis was conducted to confirm the construct for expression.

Functional complementation and growth inhibition assay of *OsHSK* in *Escherichia coli*

The competent thrB mutant of the *E. coli* strain KCTC 2435 and wild type strain KCTC 2323 were transformed with *pB::OsHSK* or pBluescript II KS+ as a control via electroporation (ECM399, BTX, USA) using a cuvette with a 0.1 cm electrode gap. During this process, competent cells were first produced by washing with water and glycerol (Kim and Leustek, 1996). The resulting competent cells after electroporation were plated on LB medium (20 g L^{-1}) with Amp (100 µg mL^{-1}). The growing colonies were tested for growth retardation in M9 minimal medium (MM) [(5 × M9 salts (200 ml L^{-1}), 1 M $MgSO_4$ (2 ml L^{-1}), 1 M $CaCl_2$ (0.1 ml L^{-1})] plates containing Amp (25 µg mL^{-1}), 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), 20% glucose, 19 amino acids (Sigma, Germany) at concentrations suggested by the manufacturer and excluding Thr. The MM medium was supplemented with nineteen amino acids since bacterial growth was not limited by the amino acid nutrients except Thr. The plates were incubated overnight at 30°C and the growing colonies were confirmed to harbor the construct by restriction digestion followed by PCR after plasmid purification.

Bacterial growth was then assessed by measuring the optical density at one-hour intervals using a spectrophotometer (UV1101, Biochrom, UK) at 595 nm (OD_{595}). The *thrB* mutant *E. coli* strain harboring the *pB::OsHSK* construct, or the control plasmid and wild-type were tested.

RESULTS AND DISCUSSION

Sequence analysis of *OsHSK*

An expressed sequence tag (EST) clone (GenBank Accession number AK060519, clone name 001-020-B10 and clone ID 102784) obtained from the RGRC was analyzed to determine the nucleotide sequence using the designed primers. The cDNA (*OsHSK)* sequence harbored a full-length ORF consisting of 1137 bp, encoding for a protein with a molecular weight of approximately 37.8 kDa. The expected isoelectric point of the protein was 6.86. Data analysis revealed that the *OsHSK* sequence was identical to the genomic region located in chromosome II, Os02g0831800, in rice. Comparisons of the amino acid sequence of the *OsHSK* and the homologous sequences from *Zea mays*, *A. thaliana* and *E. coli* revealed high identities of 88, 64 and 33%, respectively (Figure 2).

Analysis of the OsHSK amino acid sequence revealed a GHMP kinases putative ATP-binding domain that was the position between 150 and 161 (Figure 2). The motif sequence (LPLGSGLGSSAA) was highly similar to the consensus sequence [LIVM]-[PK]-x-[GSTA]-x(0,1)-G-[LM]-[GS]-S-S-[GSA]-[GSTAC], where the underlined amino acids were well conserved. The binding motif for ATP is present in bacterial ThrBs. The other two binding motifs from the N-terminal and C-terminal domains of GHMP kinases were also present in the sequence, located at 144 - 209 and 272 - 350, respectively. The

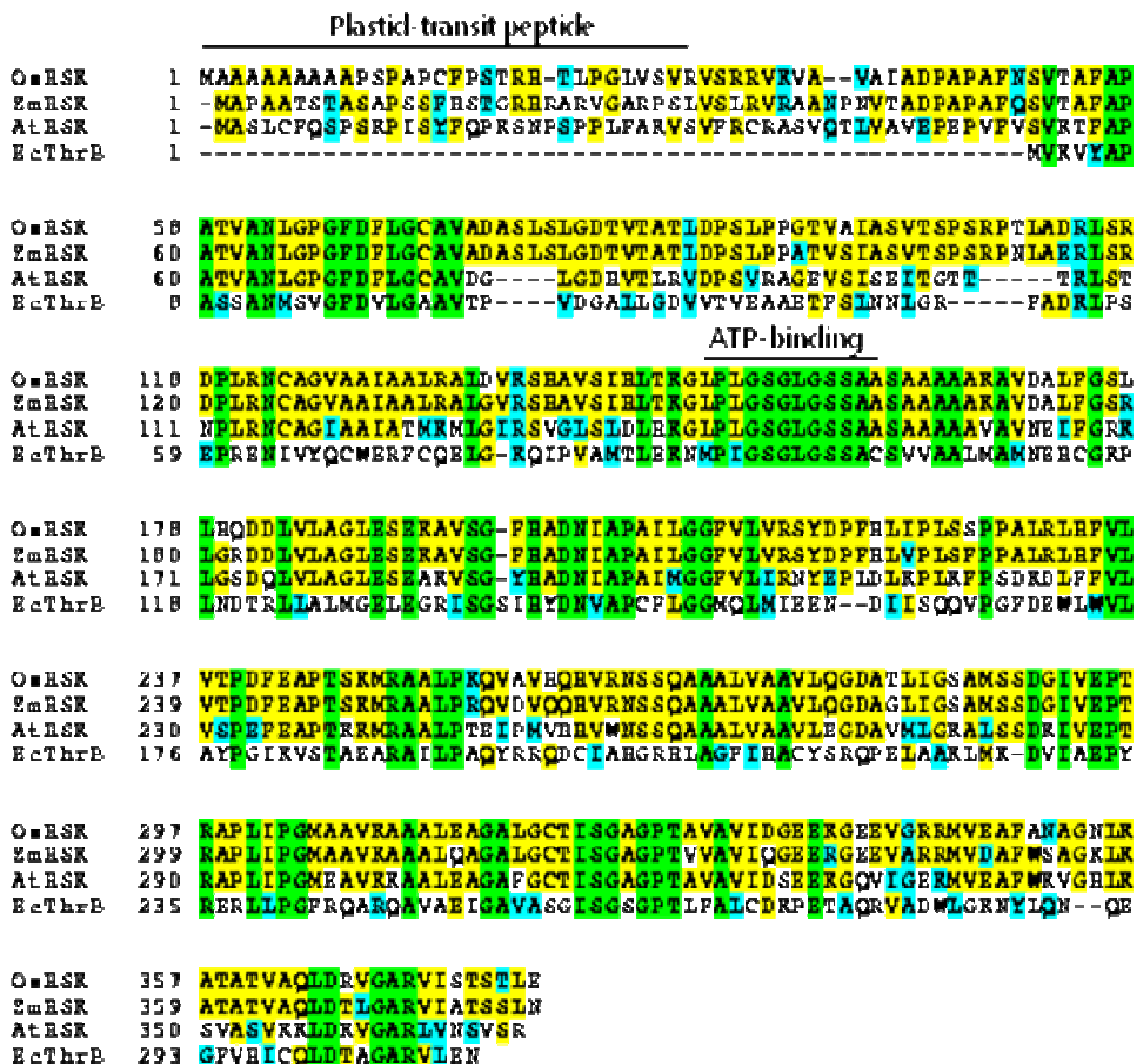

Figure 2. Amino acid sequence alignment of HSK from *Oryza sativa* (OsHSK), *Zea mays* (ZmHSK) *Arabidopsis thaliana* (AtHSK), and *Escherichia coli* (EcHSK). Amino acid residues that are completely identical or identical 3 out of 4 or similar are visually shown consequently as green, yellow and cyan, respectively. GenBank Accession Numbers; AK060519 (OsHSK from *Oryza sativa*), ACG46592 (ZmHSK from *Zea mays*), AAD33097 (AtHSK from *Arabidopsis thaliana*), YP_001742119 (EcThrB from *Escherichia coli*), respectively. The possible plastid transit peptide and an ATP-binding domain are indicated by upper-lines.

N-terminus of OsHSK contains several alanine and hydroxyl amino acids, a feature of plastid transit peptide, and its predicted localization is to chloroplast as determined by iPSORT program. Phylogenic analysis based on comparison of the related sequence further indicated that OsHSK was divergent and had evolved from ancestral bacterial HSK. The number at the nodes indicate the levels of bootstrap support based on the neighbor-joining analysis of a 1000 re-sampled data set using Mega 4.1 (Kumer et al., 2008). Numbers on the branches are the percentage of bootstrap analysis supporting the grouping of each branch (Figure 3).

OsHSK expression in *E. coli* and *in vivo* activity

The recombinant DNA, *pB::OsHSK*, was constructed using the ORF of a PCR-amplified *OsHSK* fragment. After the transformation of *E. coli* with the recombinant DNA, *OsHSK* activity was monitored *in vivo* in medium containing isopropyl-β-D-thio-galactoside (IPTG), Amp and 19 amino acids, excluding Thr. Functional complementation was performed using the *thrB* mutant of *E. coli* to confirm the enzyme activity of the gene product of *OsHSK*. To assess the viability of *E. coli* cells, the *OsHSK*-expressing cells were cultured for 16 h with

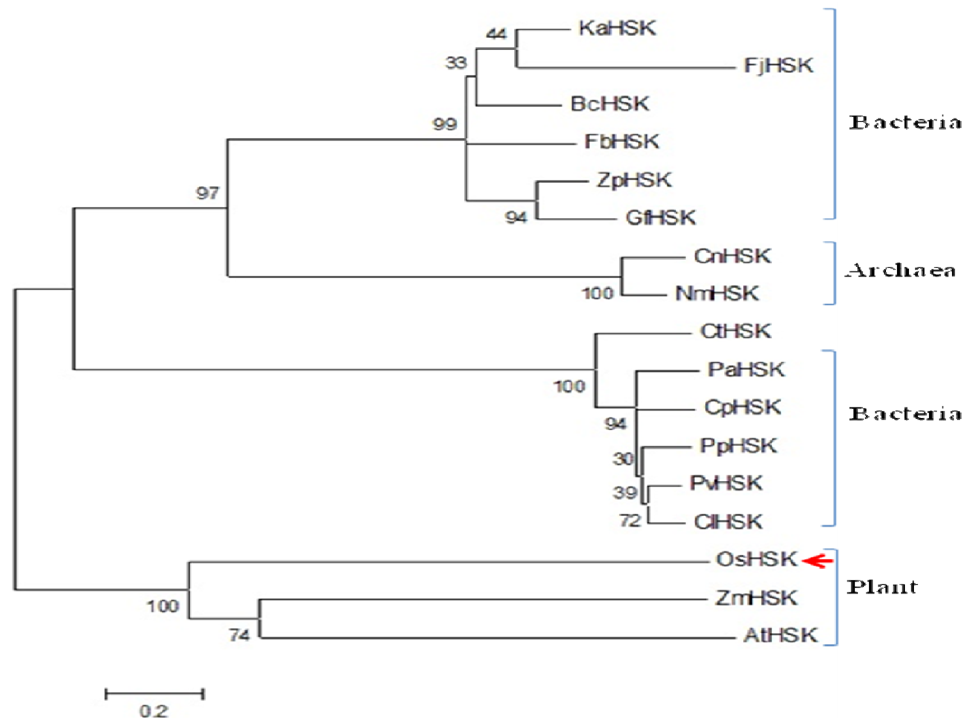

Figure 3. Phylogenetic tree: Phylogenetic analysis of OsHSK related proteins using Clustal W and Mega 4.1. Accession numbers are as follows: ZP_02163168 (KaHSK from *Kordia algicida*), YP_001194920 (FjHSK from *Flavobacterium johnsoniae*), ZP_08459439 (BcHSK from *Bacteroides coprosuis*), ZP_03701200 (FbHSK from *Flavobacteria bacterium*), YP_003584788 (ZpHSK from *Zunongwangia profunda*), YP_862001 (GfHSK from *Gramella forsetii*), ZP_08257796 (CnHSK from *Candidatus Nitrosoarchaeum*), YP_001581379 (NmHSK from *Nitrosopumilus maritimus*), YP_001995787 (CtHSK from) *Chloroherpeton thalassium*), YP_002014965 (PaHSK from *Prosthecochloris aestuarii*), YP_001999502 (CpHSK from *Chlorobaculum parvum*), YP_002019481 (PpHSK from *Pelodictyon phaeoclathratiforme*), YP_001131141 (P*rosthecochloris vibrioformis*), YP_375871 (ClHSK from *Chlorobium luteolum*), AK060519 (OsHSK from *Oryza sativa*), ACG46592 (ZmHSK from *Zea mays*), AAD33097 (AtHSK from *Arabidopsis thaliana*).

shaking, and a diluted portion was plated on agar medium containing the 19 amino acids without Thr. The viable colonies greatly differed among the plasmids. The *thrB* mutant of *E. coli* with *OsHSK* could grow under conditions in the same medium containing IPTG, Amp and 19 amino acids, excluding Thr that the mutant without *OsHSK* could not. This showed that the *OsHSK* was capable of functioning as a complement, and provided evidence for functional HSK activity.

Expression of *OsHSK* can complement the *thrB* mutant of in *E. coli*

A growth assay in liquid medium was performed to determine whether the *OsHSK* gene could increase the sensitivity of bacterial cells to Thr. The *pB::OsHSK* construct or a control plasmid was transformed into the *thrB* mutant *E. coli* strain KCTC 2435. A control plasmid was also transformed into the wild-type *E. coli* strain KCTC 2323 as a positive control. Bacterial cells were grown and the growth of bacteria was monitored in MM medium containing IPTG, Amp and the 19 amino acids excluding Thr. The *pB::OsHSK* activity was also monitored via a growth assay in the same medium in the absence of Thr. The wild-type *E. coli* strain KCTC 2323, which harbored the control plasmid grew normally and showed an S-shaped classical growth curve in the MM medium containing the 19 amino acids excluding Thr. It is a positive control for the KCTC 2323 strain to synthesize Thr itself, and thus grew normally in the medium despite the absence of Thr. The *thrB* mutant strain KCTC 2435 expressing *pB::OsHSK* also grew normally and adopted the S-shaped classical growth curve in the same medium (Fig. 4), although the KCTC 2435 strain harboring the control plasmid in the same medium without Thr showed dramatically retarded growth. In this case, it is a negative control which the *thrB* mutant *E. coli* strain KCTC 2435 was unable to produce Thr itself, and thus could not grow due to a lack of the amino acid Thr. However, the same

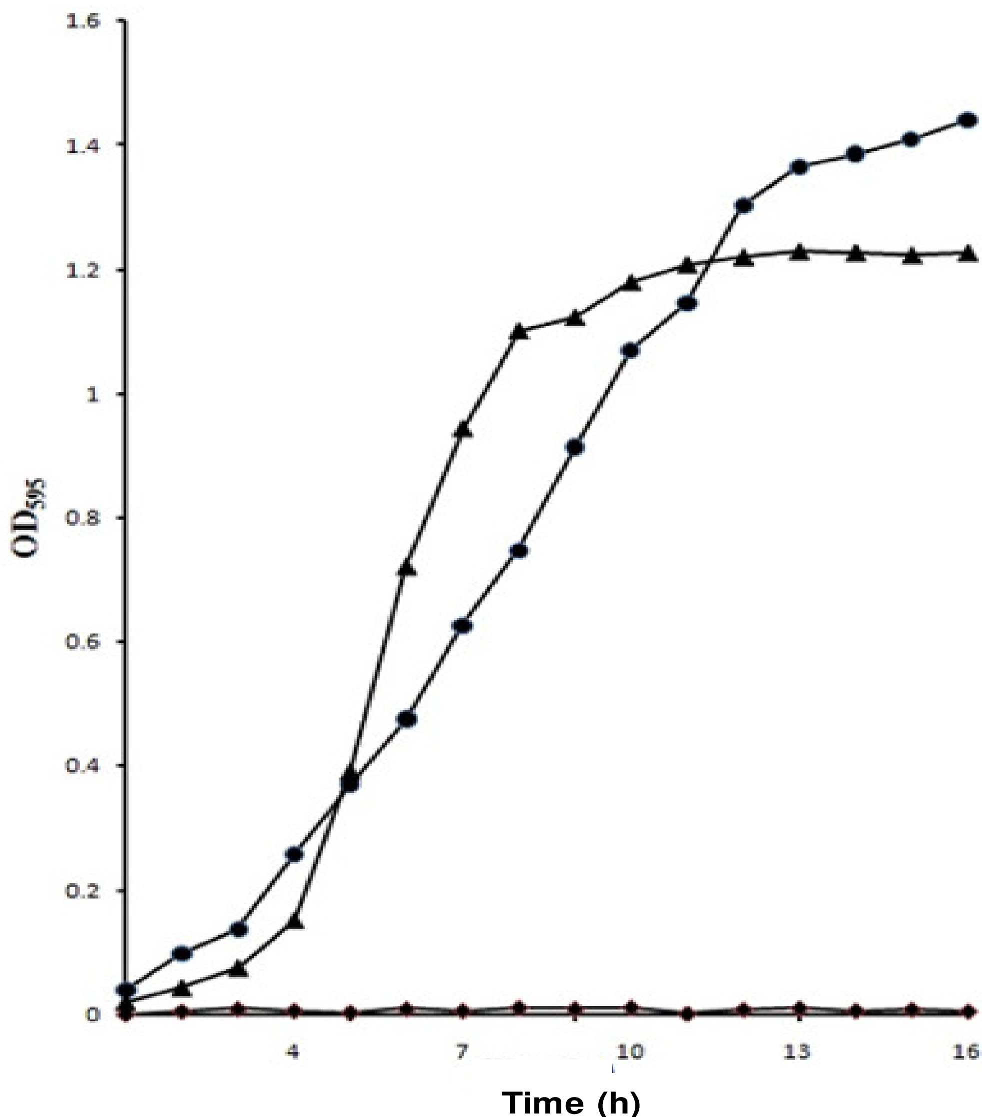

Figure 4. Growth curves of *E. coli* mutant KCTC 2435 harboring *pB::OsHSK* and control plasmid or KCTC 2323 containing control plasmid. Bacterial cells were grown at 30°C in MM containing 19 amino acids excluding Thr. Growth was monitored via optical density measurements at 595 nm (OD_{595}). Symbols: ●, KCTC 2435 + *pB::OsHSK*; ▲, KCTC 2323 + control; ♦, KCTC 2435 + control.

thrB mutant *E. coli* strain KCTC 2435 containing *pB::OsHSK* exhibited normal growth almost similar to the wild type strain KCTC 2323 because the *thrB* mutant *E. coli* KCTC 2435 could synthesize Thr using *pB::OsHSK* (Figure 4). These results demonstrated that *pB::OsHSK* expression functionally complemented the *thrB* mutant of *E. coli* and OsHSK encoded for a functional HSK enzyme. We are currently attempting to obtain more information on the enzyme activity by purifying recombinant OsHSK. These activities are expected to provide important information on the substrate specificity and the physiological functions of this enzyme for the synthesis of *thrB* and GHMP kinase in rice plants, which could be used to improve the nutritious value of rice through the development of transgenic rice plants.

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