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Characterization of a gene encoding for dihydrodipicolinate synthase from rice

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Abstract

Dihydrodipicolinate synthase (DHDPS, EC 4.2.1.52) is a key enzyme in lysine biosynthesis in the aspartate family pathway of plants and microorganisms. The *dapA* gene encoding for DHDPS has been reported in many bacteria and plants and was utilized to enhance the level of lysine in cereal crops. In this study, we describe the functional analysis and characterization of a gene encoding for DHDPS from rice (*OsDHDPS*). Analysis of the *OsDHDPS* sequence showed a full-length open reading frame consisting of 380 amino acids, which encoded for a protein of approximately 41.4 kDa. The predicted amino acid sequence of OsDHDPS is highly homologous to those of other DHDPS enzymes from bacteria and plants. The *OsDHDPS* expression in a *dapA* mutant of *Escherichia coli* showed that the gene was functionally capable of complementing the mutant. These results indicated that the *OsDHDPS* encoded for a protein in dihydrodipicolinate synthase in rice.

Keywords: Dihydrodipicolinate synthase, lysine, rice (*Oryza sativa*), functional complementation

Abbreviations: DHDPS_Dihydrodipicolinate synthase; Lys_Lysine; Asp_Aspartate; Met _Methionine; Ile_Isoleucine; Thr_Threonine; CGSC_E. coli Genetic Stock Center; RGRC _Rice Genome Resource Center; ORF_Open reading frame; PCR_Polymerase chain reaction; Amp_Ampicillin; MM_M9 minimal medium; IPTG_Isopropyl β-D-thiogalactopyranoside.

Introduction

Lysine (Lys) is not able to be synthesized in animals and is a mostly deficient nutrient (Sotelo et al., 1994). Therefore, it is classified as an essential amino acid and should be provided for animal diets. By contrast, plants and bacteria can synthesize Lys *de novo* and share a similar biosynthetic pathway, which utilizes aspartate (Asp) as a precursor (Bryan, 1980). The biosynthesis of Lys including methionine (Met), isoleucine (Ile), and threonine (Thr) is initiated from Asp; this is referred to as the Asp family pathway in plants (Azevedo et al., 1997). The dihydrodipicolinate synthase (DHDPS; EC.4.2.1.52) is a unique enzyme used in the conversion of 3-aspartate-ß-semialdehyde (3-ASA) and pyruvate to form dihydrodipicolinate as a branch point specific to Lys synthesis (Fig.1). In plants and bacteria, DHDPS catalyzes the first step specific for Lys synthesis in the pathway for the biosynthesis of Asp-derived amino acids including Thr, Ile, and Met. Because many, although not all, of the enzymes involved in the Lys-specific pathway have already been isolated, higher plants are thought to synthesize Lys via the diaminopimelate pathway (Bryn, 1980). The genes encoding for DHDPS have been cloned and characterized in several microbes and plants such as *Escherichia coli* (Laber et al., 1992; Emma et al., 1995; Carolyn et al., 1999; Dobson et al., 2005), *Bacillus Licheniformis* (Halling and Stahly, 1976), *Thermotoga maritima* (Pearce et al., 2006), *Coix lacryma-jobi* (Ricardo et al.,1999), *Spinacea oleracea* (Wallsgrove and Mazelis, 1981), *Triticum aestivum* (Kumpaisal et al., 1987), *Glycine max* (Silk et al., 1994), *Nicotiana tabacum* (Ghislain et al., 1990), *Zea mays* (David et al., 1991) and *Arabidopsis thaliana* (Marc et al., 1999). The amino acid sequences of the active sites of DHDPS proteins and the amino acid residues involved in the packing of dimmers are markedly conserved. Additionally, a Lys binding site is located within the cleft at the tight dimer interface, with one Lys molecule binding per monomer (Blickling et al., 1997). The enzyme is particularly sensitive to feedback inhibition by Lys (Galili, 1995). Mutant or transgenic plants expressing feedback-insensitive forms of DHDPS or ones less sensitive to Lys accumulated free Lys (Negrutiu et al., 1984). Additionally, the positive correlations detected between DHDPS activity or DHDPS protein levels and free Lys contents indicate that the amount of the enzyme may influence Lys accumulation (Perl et al., 1992; Falco et al., 1995). Many antibiotics or herbicides for the killing of microbes or plants, respectively, are targeted to a specific enzyme in amino acid biosynthesis (Girodeau et al., 1986; Kelland et al., 1986). Insensitivity to the Lys-induced inhibition of DHDPS enzyme activity from gram-positive bacteria has previously been utilized in the development of maize plants, in an effort to accumulate increased levels of free Lys in grain (Huang et al., 2005). Major cereal crops such as rice, wheat, corn, etc. harbor smaller quantities of Lys than leguminous crops. In order to improve the nutritional qualities of cereal crops, it is first necessary to gain insight into the Lys biosynthesis pathway in crop plants. Our group previously reported the gene of diaminopimelate decarboxylase for the last

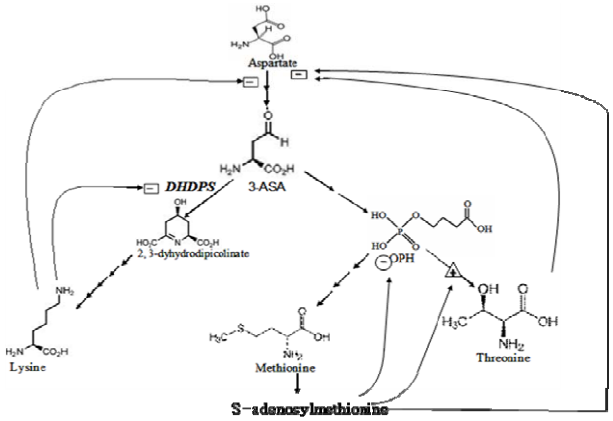

Fig 1. Schematic diagram of Lys biosynthesis and metabolic regulation in Asp family pathway in plants. The abbreviations are 3-ASA, 3-aspartate-ß-semialdehyde; OPH, *O*-phosphohomoserine and DHDPS, dihydrodipicolinate synthase. Symbols are indicated: ⊡; feedback inhibition, ⊖; feedback repression, ⚠; allosteic activation.

step in Lys synthesis from rice (Kim and Lee, 2006). These have initiated the application of molecular genetic approaches in determining gene functions and enzyme characteristics in amino acid biosynthesis of crop plants. Here, we report the functional analysis of a gene encoding for DHDPS from rice, which is an important crop plant.

Materials and methods

Strains

Two *E. coli* strains were utilized in this study-namely, AT997 and ES4, both of which were ordered from the *E. coli* Genetic Stock Center (CGSC) at Yale University, USA. The genotype of the strains was AT997 [*hfr (PO45), LAM-, e14-, dapA15, relA1, spoT1, thi*-1] and ES4 [f-, *fhuA2*, *lacY1 or lacZ4*, *tsx-1 or tsx-70*, *glnV44* (AS), *gal-6*, *LAM-*, *mtlA2*, *purA45*], respectively. The ES4 strain was utilized as a positive control and a wild-type for Lys synthesis.

DNA sequence analysis

An EST clone (GenBank accession number AK071042, clone ID 206892) was obtained from the Rice Genome Resource Center (RGRC), Japan. The clone was derived from the rice cDNA library (Osato et al., 2002) from developing seeds prepared in pBluescript SK-. The DNA sequencing and sequence analysis were described previously (Sikdar and Kim, 2010). The nucleotide and amino acid sequences were compared with sequences from the GenBank and EMBL databases, and analyzed using the BLAST (Wheeler et al., 2003) and Clustal W multiple sequence alignment programs (Thompson et al., 1994) as well as Biology WorkBench 3.2 (http://workbench.sdsc.edu; San Diego Supercomputer Center; University of California San Diego, USA). Motifs were searched by the GenomeNet Computation Service of Kyoto University (http://www.genome.ad.jp) and the phylogenetic tree with bootstrap values was prepared using the Mega 4.1 neighbor-joining program (Kumar et al., 2008).

Polymerase chain reaction and Recombinant construct

Specific primers were designed from the sequence information surrounding the translational start and stop codons of *OsDHDPS* to amplify the full-length open reading frame (ORF) and to construct a recombinant DNA to express the gene product in *E. coli*. Polymerase chain reaction (PCR) was conducted in accordance with the method described by Sambrook and Russell (2001). After a plasmid was purified from a pellet harvested from a liquid culture containing Amp, the ORF of *OsDHDPS* was amplified using the following designed primers: OsDHDPS-F (5'-AGGATCCAACCCTAG TCCGTTCTTTCTCCA-3') and OsDHDPS-R (5'-AGGATC-CCATCAACGTACATG GGACTTGCA-3'). The PCR reaction was conducted using a MY Cyler 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 approximately 1.1 kb PCR fragment was then subcloned into the *Bam*HI site of pBluescript II KS+ plasmid to construct *pB::OsDHDPS*. Restriction analysis was conducted to confirm the orientation of the contract.

Functional complementation

Two *E. coli* strains, AT997 and ES4, were transformed with *pB::OsDHDPS* and pBluescript II KS+ as a control, respectively, by electroporation (ECM399, BTX, USA) after preparing competent cells by washing with water and glycerol (Kim and Leustek, 1996) using a cuvette with a 0.1 cm electrode gap, and subsequently plated into LB (20g L^{-1}) containing ampicillin (Amp, 100 µg ml^{-1}). Amp-resistant colonies were then selected and replica plated onto M9 minimal medium (MM) [5 x M9 salts (200 ml L^{-1}), 1M $MgSO_4$ (2 ml L^{-1}), 1M $CaCl_2$ (0.1 ml L^{-1})] plates including 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), and 19 amino acids excluding Lys, each at the concentrations specified by Sigma (Germany). The plates were incubated overnight at 37°C. The growing colonies were retested for growth on Lys-free medium (Kim and Leustek, 1996).

Growth inhibition assay of OsDHDPS in E. coli

The *dapA* mutant *E. coli* strain harboring the *pB::OsDHDPS* construct, control plasmid, and wild-type strain harboring the control plasmid were grown at 37°C in MM medium, 19 amino acids excluding Lys, 1 mM IPTG (Sigma, Germany), and 20% glucose (20 ml L^{-1}), containing Amp (25 µg ml^{-1}). Bacterial cell growth was monitored via measurements of optical density using a spectrophotometer (UV1101, Biochrom, England) at 595 nm (OD_{595}) at one hour intervals (Sikdar and Kim, 2010).

Results and discussions

Sequence analysis of OsDHDPS

The EST clone (clone ID: 206892) obtained from the RGRC was analyzed to determine the nucleotide sequence using the designed primers. The cDNA sequence (*OsDHDPS)* contained a full-length open reading frame consisting of 1140 bp and encoded for a protein of approximately 41.4 kDa. The expected isoelectric point of the protein was 5.99. Data analysis shows

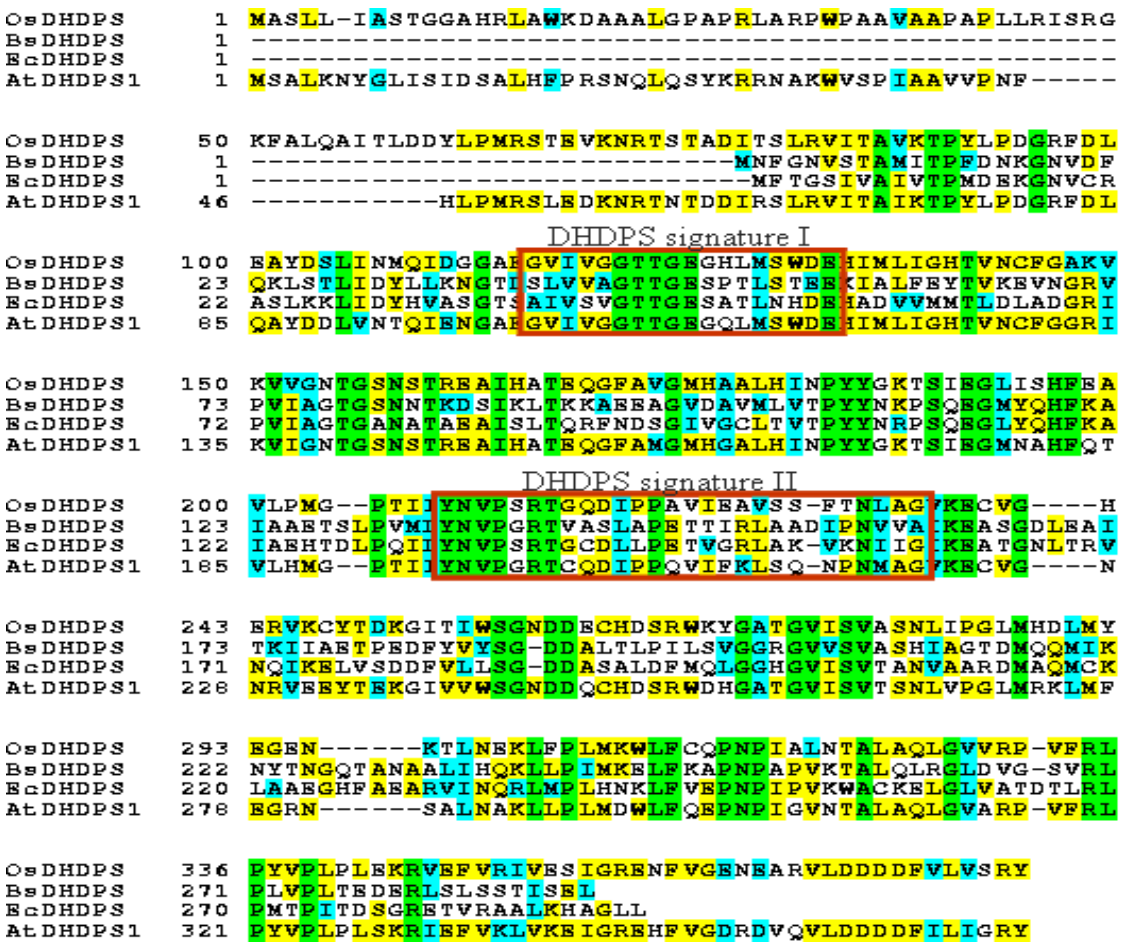

Fig 2. Amino acid sequence alignment of DHDPSs using Boxshade program after Clustal W alignment. Amino acids that are from completely identical to less similar amino acid residues are visually depicted consequently in yellow, green, and cyan, respectively. GenBank accession numbers are as follows: AK071042 (OsDHDPS from *Oryza sativa*, this study), NP_850730 (AtDHDPS1 from *Arabidopsis thaliana*), NP_416973 (EcDHDPS from *E. coli*) and NP_389559 (BsDHDPS from *Bacillus subtilis*).

that the *OsDHDPS* sequence was identical to the genomic region located in chromosome IV (Os04g0254000) in rice. A sequence comparison of the predicted amino acids for the *OsDHDPS* with the deduced sequences from maize (*Zea mays*), Arabidopsis (*A. thaliana*), and *E. coli* evidenced a high degree of homology with identity values of 84%, 74%, and 33%, respectively. Analysis of the amino acid sequence of *OsDHDPS* revealed signature motifs for DHDPS. The DHDPS signature I sequence (GVIVGGTTGEGHLMSWDE) was highly homologous to the consensus sequence [GSA]-[LIVM]-[LIVMFY]-x(2)-G-[ST]-[TG]-G-E-[GASNF]-x(6)-[EQ], where the underlined amino acids are well-conserved (Fig. 2). Another signature, substrate binding motif, exists within the central region (YNVPSRTGQDIPPAVIEAVSSFTNLAGVKEC) of the OsDHDPS (Fig 2). The DHDPS signature II in OsDHDPS is highly homologous with the consensus sequence [Y-[DNSAH]-[LIVMFAN]-P-x(2)-[STAV]-x(2,3)-[LIVMFT]-x(13,14)-[LIVMCF]-x-[SGA]-[LIVMFNS]-K-[DEQAFYH]-[STACI], in which the underlined amino acids are well-conserved (Hofmann et al., 1999). The phylogenetic tree derived from the related sequence showed that OsDHDPS is divergent and evolved from ancestor bacterial DHDPS. The branching pattern and the numbers at nodes indicate the levels of bootstrap value support based on neighbor-joining analysis of 1,000 re-sampled datasets (Fig. 3).

OsDHDPS expression in *E. coli*

The recombinant DNA, *pB::OsDHDPS*, was constructed using the ORF of the PCR-amplified *OsDHDPS* fragment. After transformation into *E. coli*, *OsDHDPS* activity *in vivo* was monitored in the presence of IPTG and 19 amino acids excluding Lys. Functional complementation was conducted using the *dapA* mutant of the *E. coli* strain, AT997, to verify the enzyme activity of the gene product of *OsDHDPS*. In order to assess the viability of *E. coli* cells by OsDHDPS protein activity in the Lys-free medium, the *OsDHDPS*-expressing cells were cultured for 12 h with shaking, and the diluted portion was plated on Amp-containing MM medium without Lys. The viable colonies appearing on the plate contained the *dapA* mutant expressing *OsDHDPS* (Fig. 4). These results demonstrate that the gene product of *OsDHDPS* is capable of

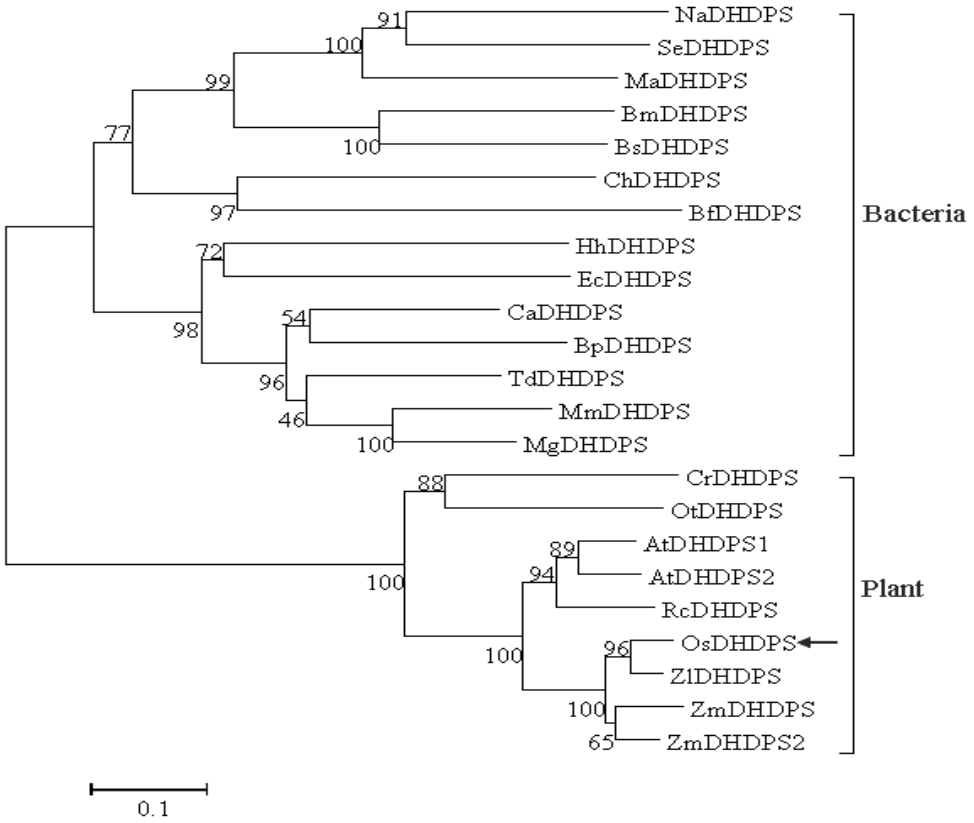

Fig 3. Phylogenetic analysis of OsDHDPS-related proteins using the Clustal W and Mega4.1 neighbor-joining programs. GenBank accession numbers are as follows: AK071042 (OsDHDPS from *Oryza sativa*), ZP_03766378 (NaDHDPS from *Nostoc azollae*), YP_172957 (SeDHDPS from *Synechococcus elongatus*), YP_001661282 (MaDHDPS from *Microcystis aeruginosa*), ACL13295 (BmDHDPS from *Bacillus methanolicus*), NP_389559 (BsDHDPS from *Bacillus subtilis*), ZP_06116634 (ChDHDPS from *Clostridium hathewayi*), ZP_05346547 (BfDHDPS from *Bryantella formatexigens*), YP_001003764 (HhDHDPS from *Halorhodospira halophila*), NP_416973 (EcDHDPS from *Escherichia coli*), YP_003166625 (CaDHDPS from *Candidatus Accumulibacter*), YP_001632085 (BpDHDPS from *Bordetella petrii*), YP_314856 (TdDHDPS from *Thiobacillus denitrificans*), YP_003049383 (MmDHDPS from *Methylotenera mobilis*), BAB61104 (MgDHDPS from *Methylobacillus glycogenes*), XP_001699738 (CrDHDPS from *Chlamydomonas reinhardtii*), CAL53889 (OtDHDPS from *Ostreococcus tauri*), NP_850730 (AtDHDPS1 from *Arabidopsis thaliana*), AAG28565 (AtDHDPS2 from *Arabidopsis thaliana*), XP_002521713 (RcDHDPS from *Ricinus communis*), ABE28526 (ZlDHDPS from *Zizania latifolia*), NP_001105425 (ZmDHDPS from *Zea mays*), NP_001148623 (ZmDHDPS2 from *Zea mays*). The numbers on branches are percentages of bootstrap analyses supporting the grouping of each branch.

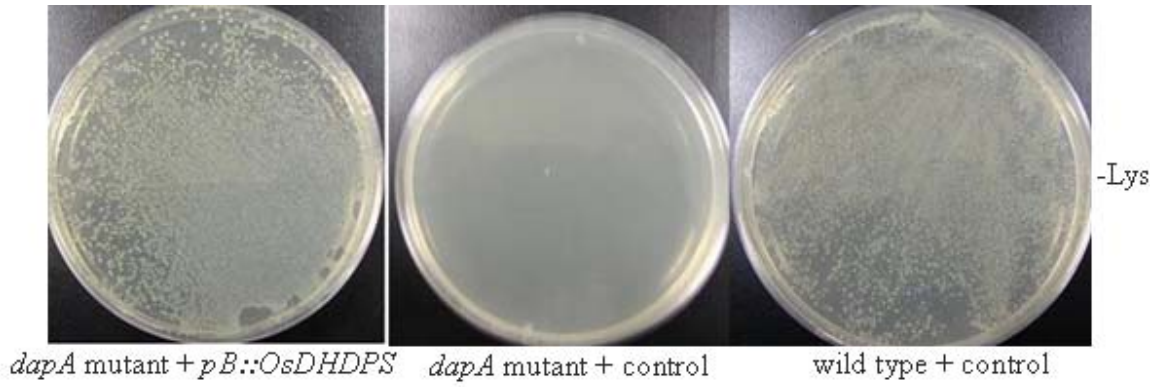

Fig 4. Functional complementation assay. Pictures were taken the bacterial cells which were grown for 2-days at 37°C in MM agar medium containing 19 amino acids excluding Lys after inoculation of overnight culture grown LB medium with Amp. The *E. coli* strains harboring plasmid were designated as *dapA* mutant + *pB::OsDHDPS, dapA* mutant + control, wild type + control.

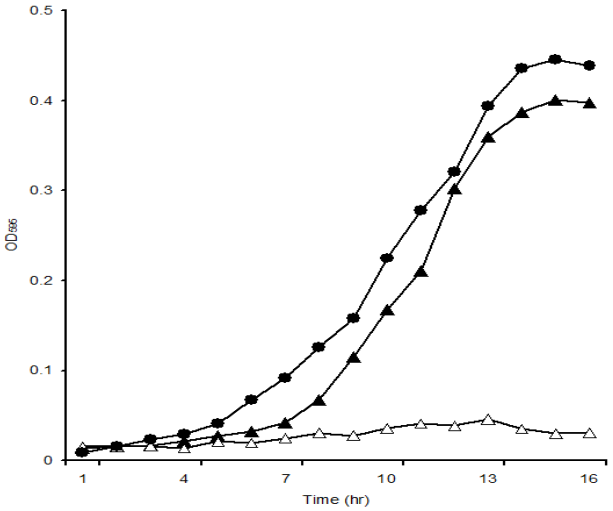

Fig 5. Growth curves of the *dapA* mutant strain AT997 harboring *pB::OsDHDPS*. Bacterial cells were grown at 37°C in MM containing 19 amino acids excluding Lys. Growth was monitored via optical density measurements at 595 nm (OD_{595}). Symbols: ●, wild type + control; Δ, *dapA* mutant + control; ▲, *dapA* mutant + *pB::OsDHDPS*.

complementing the *dapA* mutant.

Lysine sensitivity of E. coli mutant was influenced by the expression of OsDHDPS

A growth study was conducted to determine whether the *OsDHPDS* gene would increase the sensitivity of bacterial cells to Lys. The *pB::OsDHDPS* construct was transformed into the AT997 strain. The control plasmid was also transformed into the wild-type (ES4) and the *dapA* mutant (AT997) strains of *E. coli*. The *OsDHDPS* activity was monitored by the growth assay in the absence of Lys. Bacterial cells were grown in MM containing IPTG and Amp and 19 amino acids excluding Lys. The wild-type *E. coli* strain ES4 harboring the control plasmid exhibited normal growth and evidenced a classic S-shaped growth curve in the medium absent of Lys (Fig. 5). The ES4 strain synthesizes Lys itself, and so grows normally in the Lys-free medium. However, the *dapA* mutant strain AT997 harboring the control plasmid was retarded in the same medium due to the lack of Lys. The complementation experiment showed that the same *dapA* mutant strain AT997 expressing *OsDHDPS* surprisingly grew normally and evidenced an S-shaped classical growth curve, as did the wild-type strain (Fig. 5). In this case, the *dapA* mutant strain showed the retarded growth due to inability to synthesize Lys, but the same mutant strain expressing *OsDHDPS* then grew well because the mutant could overcome the mutation of *dapA* gene by using rice DHDPS (Fig. 5). These results led us to the conclusion that *OsDHDPS* expression confers the ability to functionally complement the inability of the *dapA* mutant to produce Lys and the rice gene of *OsDHDPS* encode DHDPS enzyme. We are currently attempting to purify a recombinant OsDHDPS in *E. coli* and to investigate the physiological functions relevant to Lys metabolism. Those approaches may provide us with important clues as to the substrate specificity and physiological functions of this novel enzyme for Lys synthesis in rice plants, and also provide us some insights into the use of transgenic crops to foster better nutrition.

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