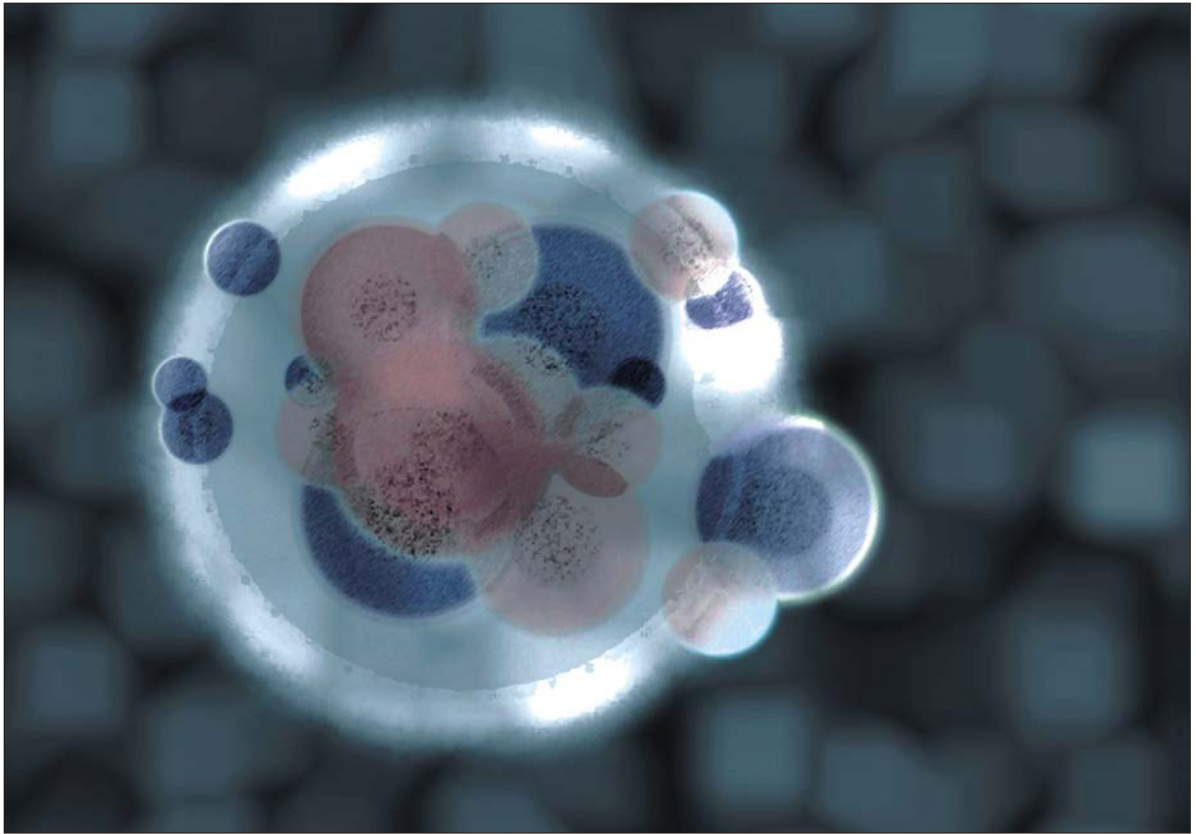

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Characterization of a Gene Encoding 3-Deoxy-D-Arabino-Heptulosonate 7-Phosphate Synthase from Rice

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ABSTRACT

3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS) is an entry enzyme of the shikimate pathway which is responsible for primary carbohydrate metabolism with the biosynthesis of most aromatic amino acids in microorganisms and plants. Enzymes of the pathway are drawing attention in the recent years because they are recognized as potential targets for design of antimicrobial drugs and herbicides. Analysis of sequences from rice revealed that the *OsDAHPS* showed a full-length open reading frame consisting of 537 amino acids, which encoded for a protein of approximately 59.0 kDa. The predicted amino acid sequence of *OsDAHPS* is highly homologous to those of DAHPS enzymes from many plants. The *OsDAHPS* expression in a aroH mutant of *Escherichia coli* showed that the gene was functionally capable of complementing the mutant. These results showed that the *OsDAHPS* encoded for a protein in 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase in rice.

Key words: 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase, rice, functional complementation, shikimate pathway

INTRODUCTION

The shikimate pathway is consist of the several enzymes that catalyze the first seven reactions in the synthesis of a diverse set of important aromatic compounds in multiple organisms converting primary carbon metabolites into chorismate. Chorismate is a common precursor for the synthesis of a number of aromatic metabolites, vitamin K, folic acid, ubiquinone and other secondary metabolites including the three aromatic amino acids (AAA): phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) (Bentley and Haslam, 1990; Roberts *et al*., 1998; Ma *et al*., 2012; Light and Anderson, 2013). In plants, the shikimate and AAA biosynthetic pathways provide the substrates for numerous secondary metabolites, such as alkaloids, flavonoids, lignin, coumarins, indole derivatives, para-amino benzoic acid and other phenolic compounds (Gilchrist and Kosuge, 1980). The pathway is a bridge between primary and secondary metabolism. Therefore, it is being looked upon as an attractive target for the development of herbicides and antimicrobial agents against a plethora of diseases (Bentley and Haslam, 1990). The shikimate pathway is necessary for it's an important target for the development of antibiotics and herbicides such as glyphosate in plants and microorganisms. Glyphosate is a commercially important herbicide that inhibit to 5-enolpyruvylshikimate-3-phosphate synthase, which catalyzes the sixth step in the pathway (Steinrucken and Amrhein, 1980).

The first reaction in the shikimate pathway that convert phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) to chorismate links between the primary and specialized metabolism leading to aromatic amino acids (Fig. 1), provides an unusually fertile ground to examine the role of quaternary structure in enzyme function and regulation. The first committed enzyme of the shikimate pathway, the *aroH* gene encoding enzyme is 3-deoxy-D-arabino-heptulosonate-7-

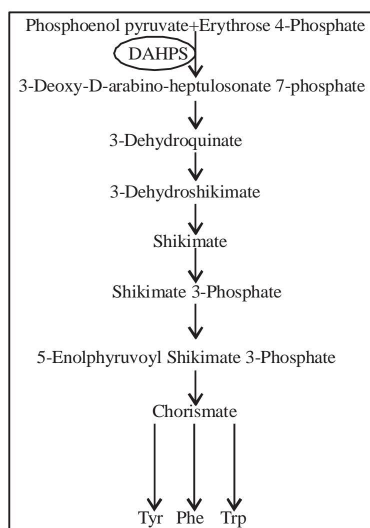

Fig. 1: Schematic diagram of the shikimate pathway in plants

phosphate synthase (DAHPS; EC 4.1.2.15), which catalyses the first committed step in the biosynthesis of aromatic compounds (Fig. 1) in bacteria, fungi, plants and some lower eukaryotes the conversion of PEP and E4P to 3-deoxy-D-arabino-heptulosonate 7-phosphate and the phosphate group of PEP, the first reaction in the process (Schoner and Herrmann, 1976; Herrmann, 1995; Nazmi *et al*., 2014). As the first enzyme in the pathway DAHPS is located at a central gateway, where pathway input can be competently controlled in response to modify in the cellular concentration of pathway outputs. Evolutionary processes have led to at least four different mechanisms of conferring DAHPSs allosterically responsive to downstream reaction products (Light *et al*., 2012). The activities of DAHPS enzyme is generally regulated by inhibition with end-product amino acids Tyr, Phe, Trp, or other intermediates of the pathway (Cross *et al*., 2013).

Although, the regulation of the synthesis of AAAs from chorismate has been studied extensively in plants (Yamada *et al*., 2008; Tzin *et al*., 2009; Maeda *et al*., 2011). The most studied enzyme of the shikimate pathway is enolpyruvylshikimate 3-phosphate synthase (EPSPS), catalyzing the formation of enolpyruvylshikimate 3-phosphate, because of its association with resistance to the herbicide glyphosate (Singer and McDaniel, 1985; Smart *et al*., 1985; Klee *et al*., 1987; Duke and Powles, 2008; Vivancos *et al*., 2011).

The cDNA encoding DAHPS has been identified from some micro-organism and plants including tobacco (Wang *et al*., 1991), *Arabidopsis thaliana* (Keith *et al*., 1991), potato (Dyer *et al*., 1990; Zhao and Herrmann, 1992), tomato (Gorlach *et al*., 1993; Tzin *et al*., 2012) and *Populus trichocarpa* (Tuskan *et al*., 2006), grapes (Zhang *et al*., 2011), *Thermotoga maritima* (Cross and Parker, 2013), *Corynebacterium glutamicum* (Li *et al*., 2013); *Neisseria meningitidis* (Cross *et al*., 2013) and *Hyperthermophile pyrococcus* (Nazmi *et al*., 2014). To date, there is no studies elucidation and manipulation of metabolic regulatory in the conversion of primary metabolism into aromatic metabolites of rice genes of shikimate pathway. Hence, it is necessary to study the shikimate pathway in rice so, as to better uncover the formation and regulation mechanism of diverse flavonoids in rice crop. To elucidate the impact of the shikimate pathway on the production of specialized metabolites in rice, this work expressed a bacterial Phe-feedback-insensitive *aroH* under a fruit-specific promoter in transgenic plants. The manipulation of the rice gene that encoding DAHPS enzyme controlling the conversion of primary to specialized metabolism could be an attractive tool for improving rice aroma and flavor qualities. Here, we report the analysis and characterization of a gene for the DAHPS enzyme from rice (*Oryza sativa*), an important crop plant.

MATERIALS AND METHODS

Mutant strains and plasmids: The mutant strains which were used in this study are ME8637 and JW0001. The genotypes of the mutant strains are ME8637 (aroH367, thi-1, argE3, proA2, galK2, lacY1, tsx-29) and JW0001 (thrA). Both mutant strains are obtained from National Bio-Resource Project (NBRP) Japan. The EST clone carrying the expected gene encoding for DAHP synthase, having the GenBank accession number AK069968 and clone number is J023038D13 that was collected from Rice Genome Resource Center (RGRC) Japan.

DNA sequence analysis: The sequence analysis of rice EST clone (J023038D13), which was derived from rice cDNA library (Osato *et al*., 2002) from developing seeds prepared in pBluescript that was done by using various bioinformatics tools. The DNA sequencing and sequence analysis were described previously (Sikdar and Kim, 2010). The nucleotide and amino acid sequences were compared with various sequences present in GenBank and were analyzed by using Nucleotide-BLAST (Wheeler *et al*., 2003) and CLUSTAL X a multiple sequence alignment program (Thompson *et al*., 1994). Motifs regions were searched by the Genome Net computation Service at Kyoto University (http://www.genome.ad.jp) and the phylogenic tree analysis was performed by MEGA 4.1 neighbor_joining program (Kumar *et al*., 2008).

Polymerase chain reaction and recombinant construct: Particular primers were designed from the sequence of *OsDAHPS* information surrounding the translational start and stop codons of *OsDAHPS* 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). The ORF region

of *OsDAHPS* was amplified by own designed forward and reverse primers. The cDNA of *OsDAHPS* was amplified by using the following designed forward and reverse primers from the *OsDAHPS* sequence; *Os*DAHPS-F (5'-GCATGAGGATCCAATGGCGCTCGCCACCAA-3') OsDAHPS-R (5'-GCATGAAAGCTTCTTCCCCCGTTTCA GCAGTTTA-3') both the primers contain *Bam*HI and *Hin*dIII restriction sites at 5' end. The PCR reaction was conducted using a MY Cyler TM PCR system (BioRad, U.S.A) for 35 cycles with denaturation at 95°C for 10 min, annealing at 58°C for 1 min and extension 72°C for 1 min, with 10 µM primers. The PCR products were analyzed on 1% (w/v) agarose gel (data not shown) and then subcloned into the *Bam*HI and *Hin*dIII site of pBluescript II plasmid to construct *OsDAHPS*. Restriction analysis was conducted to confirm the orientation of the contract.

Growth determination in *E. coli* mutant strains: The transformed *E. coli* mutant strains ME8637 harbouring *pB:OsDAHPS*, control plasmid and wild type (JW0001) harboring control plasmid. Single colony was inoculated in 1 mL of LB broth with Amp (50 µg mL^{-1}) and incubated at 37°C for overnight with shaking. The 1 mL overnight cultured medium were grown in 100 mL of M9 minimal media containing 1 mM IPTG, 20% glucose, amp (50 µg mL^{-1}) and 19 amino acids excluding Trp and the same medium was used with all the reagents kept constant. The bacterial growth of *E. coli* was monitored for every hour by measuring the optical density measurement with a UV-spectrophotometer (UV1101, Biochrom, England) at 595 nm (OD_{595}).

RESULTS

Sequence analysis of OsDAHPS: The EST clone (clone ID: J023038D13) obtained from the RGRC was analyzed to determine the nucleotide sequence using the designed primers and bioinformatics tools. The results of data analysis with the cDNA sequence and complete rice genome indicated that an ORF was identical to that of the rice genomic region located on chromosome VII (Os07g42960) in rice sequence. The cDNA sequence (*OsDAHPS)* contained a full-length open reading frame consisting of 1614 bp and encoded for a protein with 537 amino acids and approximate molecular weight of 59.0 kDa. The expected isoelectric point of the protein was 8.266. The sequences of different species and organisms such as rice, *Arabidopsis*, potato, bacteria and fungi were analyzed. The most of the regions are highly conserved in plant species showing that they were sharing similar domains but in the case of bacteria and fungi they share very few conserved regions with plant species. A sequence comparison of the predicted amino acids for the *OsDAHPS* with the deduced sequences from maize (*Zea mays*), *Arabidopsis* (*A. thaliana*), *Ricimus communi, Saccharomyces cerevisiae* and *E. coli* evidenced a high degree of homology with identity values of 95, 97, 95, 19 and 17%, respectively (Fig. 2). Motif analysis of the amino acid sequences of DAHPS revealed that there is one motif found from 77-514 in the sequence OsDAHPS (Fig. 2). The OsDAHPS is highly conserved with other plant DAHPS and shows very less similarity with bacteria and fungi DAHPS. This evident shows that plant has two types DAHPS such as type II and type I. Type I DAHP synthases are commonly found in microorganisms. The phylogenetic tree derived from the related sequences showed that OsDAHPS is divergent and evolved from ancestor bacterial DAHPS using the MEGA 4.1 program (Kumar *et al*., 2008). The branching pattern and Numbers at the nodes indicate levels of bootstrap support based on neighbor_joining analyses of 1000 resampled data sets using the same program (Fig. 3).

Expression in *E. coli* and *in vivo* activity: The recombinant DNA, *pB::OsDAHPS* was constructed using the ORF of a PCR-amplified *OsDAHPS* fragment. After the transformation of *E. coli* with the recombinant DNA, *OsDAHPS* activity was observed *in vivo* in a medium containing IPTG and 19 amino acids, excluding Trp. Functional complementation was carried out using the *DAHPS* mutant of *E. coli* to prove the enzyme activity of the gene product of *OsDAHPS*. To evaluate the viability of *E. coli* cells by *OsDAHPS* activity, the *OsDAHPS* -expressing cells were cultured for overnight at 37°C with shaking and the diluted portion of culture was plated on agar medium containing the 19 amino acids except Trp with Amp (50 mg mL^{-1}). The viable colonies greatly differed on the agar medium among the plasmids. The *aroH* mutant of *E. coli* with *OsDAHPS* could grow under conditions in which the mutant without OsDAHPS could not. This showed that the OsDAHPS was capable of functioning as a complement and evidenced functional DAHPS activity.

Expression of OsDAHPS can complement the *aroH* mutant of *E. coli*: A growth study of the *pB::OsDAHPS* activity was monitored to determine by functional complementing the *aroH* mutant *E. coli* strain. The *pB::OsDAHPS* construct was transformed into *E. coli* mutant *aroH* strain ME8637. The was used for the transformation in this experiment, the *pB:OsDAHPS* construct was transformed into ME8637 *E. coli* mutant strain. A control plasmid was also transformed into wild type (JW0001) and the *aroH* strain ME8637 to differentiate the activity of gene in minimal media without Trp. All of these transformed mutant strains were monitored via a growth assay for functional complementation in MM media with 1mM IPTG, AMP (50 µg mL^{-1}) containing 19 amino acids excluding Trp. The *E. coli* strain harboring *pB::OsDAHPS* which was encoded by *aroH* showed they grew normally under tryptophan deficit condition in the MM media, this result indicated that the *aroH* strain was able to synthesize tryptophan, which grew normally in the medium and thus produced S-shaped growth curve. The wild type *E. coli* strain JW0001 harboring the control plasmid also grew normally and

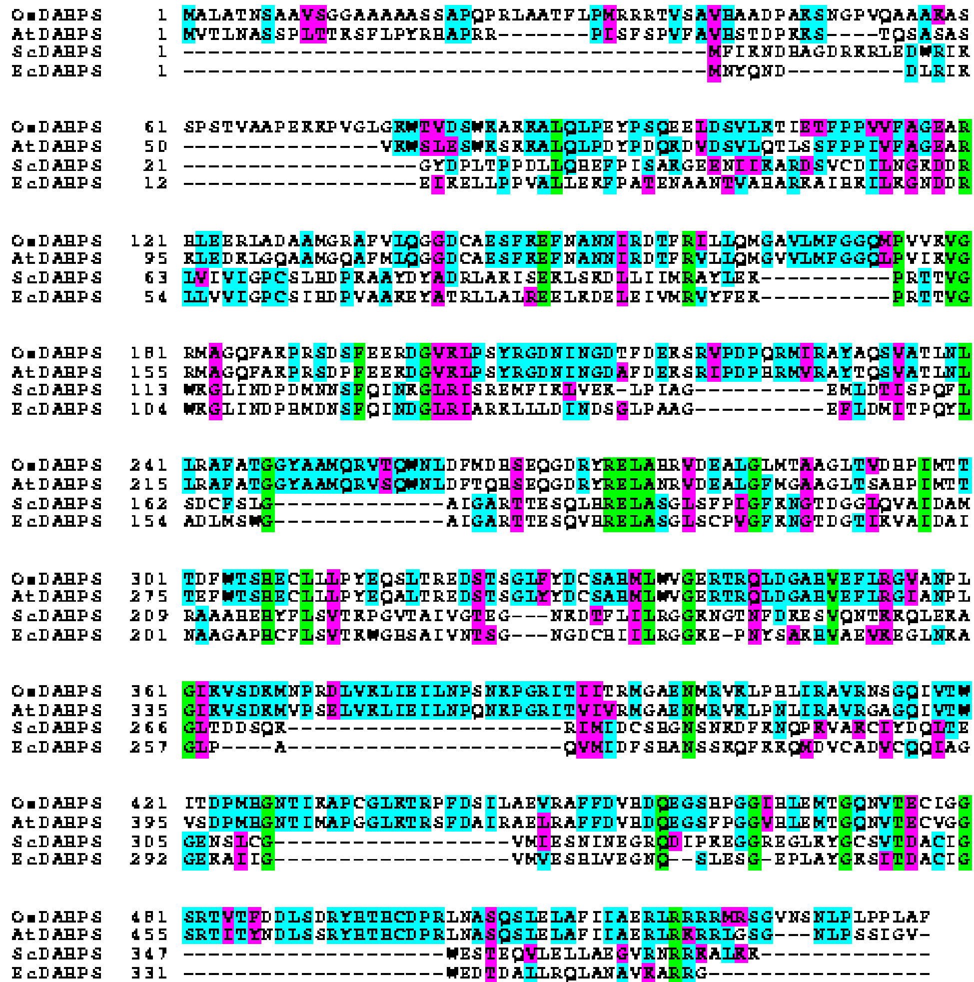

Fig. 2: Multiple sequence alignment of DAHPS was analyzed by using Boxshade program. The amino acid sequences of DAHPS from different organisms that were obtained from NCBI database such as *Oryza sativa* (OsDAHPS; accession number: AK069968), *Arabidopsis thaliana* (AtDAHPS; accession number: NP_195077), *Saccharomyces cerevisiae* (ScDAHPS; accession number: NP_010320) and *Escherichia coli* (EcDAHPS; accession number:NP_415275), (In figure, Characters shows different color like: Completely Conserved Residues, Identical Residues, Similar Residues and Different Residues are green, cyan, magenta and white color, respectively)

evidenced an S-shaped classical growth curve almost similar the *pB::OsDAHPS* which was encoded by *aroH*. The wild type train contain *aroH* as it mutant of *thrB,* so it was able to encode for DAHPS, therefore showed the activity in the medium by synthesizing the trp, as evident of S-shaped growth curve. The *E. coli* strain which was encoded by *aroH* harboring control plasmid was incubated in the same MM medium without Trp that evidenced dramatically retarded growth. In this case, the *aroH* mutant *E. coli* strains ME8637 could not synthesize Trp itself, that's why was not able to grow normally. However, the same *E. coli* strains ME8637 containing *pB::OsDAHPS* grew well because the *aroH* mutants *E. coli* was able to synthesize Trp using DAHPS expressed by the *pB::OsDAHPS* plasmid (Fig. 4). This is a consequence of *pB::OsDAHPS* activity. From the above finding, it was concluded that *OsDAHPS* expression could be functionally complement the *aroH* mutants *E. coli*.

DISCUSSION

It was reported that there is the transit signal for chloroplasts in some plants including tobacco (Wang *et al*., 1991). The classical transit peptide characteristic with many hydroxyl amino acids such as Ser and Thr is also found in the N-terminal of OsDAHPS which might be active for the biosynthesis of aromatic amino acids and other secondary metabolites in chloroplasts.

There are three DAHPS isoenzymes which are regulated by Tyr, Phe, Trp, respectively, in *E. coli* (Kikuchi *et al*., 1997). It seems that there are more than four genes (Os07g0622200,

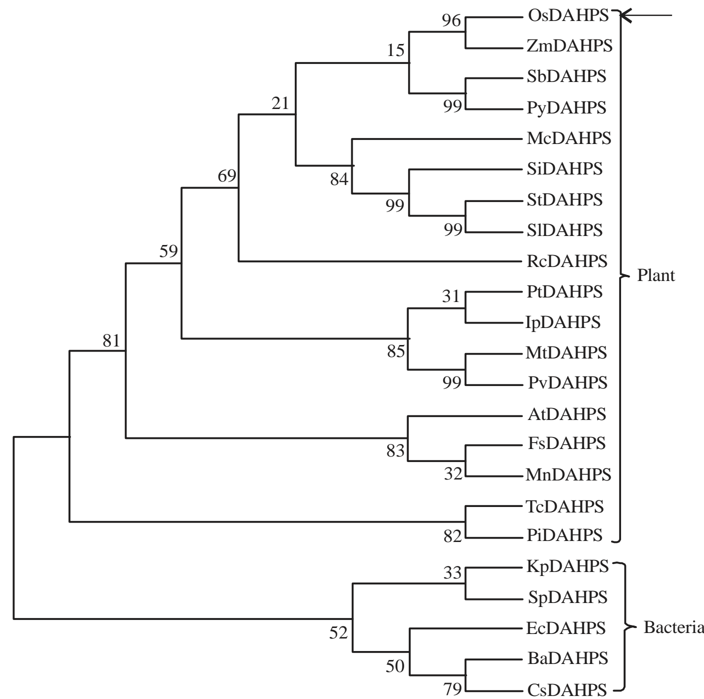

Fig. 3: Phylogenetic tree: Phylogenetic analysis of OsTS related proteins using Clustal W and Mega 4.1 program. GenBank accession numbers of above protein sequences are as follows: AK069968 (OsDAHPS from *Oryza Sativa*), NM 001157394 (ZmDAHPS; *Zea mays*), XP_002463199 (SbDAHPS; *Sorghum bicolor*), AFL02467 (PyDAHPS; *Petunia hybrid*), CAA7509223 (McDAHPS; *Morinda citrifolia*), XP_004984156 (SiDAHPS; *Setaria italic*), NP_001275361 (StDAHPS; *Solanum tuberosum*), NP_001234418 (SlDAHPS; *Solanum lycopersicum*), XM_002513905 (RcDAHPS; *Ricinus communis*), XP_002307364 (PtDAHPS; *Populus trichocarpa*), AFD62807 (IpDAHPS; *Ipomoea purpurea*), XP_003615152 (MtDAHPS; *Medicago truncatula*), AGV54666 (PvDAHPS; *Phaseolus vulgaris*), NP_195077 (AtDAHPS; *Arabidopsis thaliana*), ABA54865 (FsDAHPS; *Fagus sylvatica*), EXB75364 (MnDAHPS; *Morus notabilis*), XP_007042911 (TcDAHPS; *Theobroma cacao*), XP_002908379 (PiDAHPS; *Phytophthora infestans*), YP_005955985 (KpDAHPS; *Klebsiella pneumonia*, YP_003877294 (SpDAHPS; *Streptococcus pneumonia*), NP_415275 (EcDAHPS; *Escherichia coli*), YP_005580695 (BaDAHPS; *Bifidobacterium animalis*) and YP_003543340 (CsDAHPS; *Candidatus sulcia*)

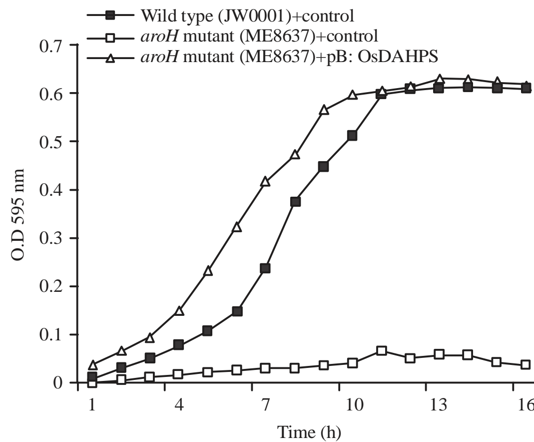

Fig. 4: Growth curves of the *aroH* mutant strain ME8637 harboring *pB::OsDAHPS.* Bacterial cells were grown at 37°C in MM containing 19 amino acids excluding Trp. Growth was monitored via optical density measurements at 595 nm (OD_{595})

Os03g0389700, Os08g0484500, Os10g0564400) in rice chromosome III, VII, VIII and X by searching in rice genome with the *OsDAHPS* sequence, even two homologous genes were reported in Arabidopsis, potato, tomato and grapes (Keith *et al*., 1991; Zhao and Herrmann, 1992; Gorlach *et al*., 1993; Zhang *et al*., 2011). These isoenzymes might be different in the sensitivity of feedback regulations by Tyr or other final products. Further research about these isoenzymes would be elucidated the overall regulation of shikimate pathway including the biosynthesis of aromatic amino acids and other secondary metabolites.

Current research elucidated that DAHPS is inhibited by Phe and would use a target for therapeutic agent against *Neisseria meningitides* (Cross *et al*., 2013).

Next step would be find out some important clues about the substrate specificity of the enzyme by purifying recombinant *pB::OsDAHPS S* in *E. coli* and physiological functions of this novel enzyme by screening T-DNA insertion mutants in which the *OsDAHPS* gene is knocked out in rice. Our reports about cloning and characterization of the cDNA encoding DAHPS from rice confirm the predictions and extend to motifs and expression in *E. coli*. The DAHPS gene could be used as a powerful tool for future application to improve the aromatic amino acids as well as others secondary metabolic compounds in rice plants.

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