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Expression of a gene encoding acetolactate synthase from rice complements two *ilvH* mutants in *Escherichia coli*

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Abstract

Acetolactate synthase (ALS) is a thiamine diphosphate-dependent enzyme in the biosynthetic pathway leading to isoleucine, valine and leucine in plants. ALS is the target of several classes of herbicides that are effective to protect a broad range of crops. In this study, we describe the functional analysis of a gene encoding for ALS from rice (*OsALS*). Sequence analysis of an EST from rice revealed that it harbors a full-length open reading frame for OsALS encoding a protein of approximately 69.4 kDa and the N-terminal of OsALS contains a feature of chloroplast transit peptide. The predicted amino acid sequence of OsALS is highly homologous to those of weed ALSs among plant ALSs. The *OsALS* expression showed that the gene was functionally capable of complementing the two *ilvH* mutant strains of *Escherichia coli*. These results indicate that the *OsALS* encodes for an enzyme in acetolactate synthase in rice.

Keywords: acetolactate synthase, rice (*Oryza sativa*), sequence analysis, functional complementation, *ilvH* mutants

Abbreviations: ALS_Acetolactate synthase; BCAAs_Branched chain amino acids; Ile_Isoleucine; Val_Valine; Leu_Leucine; TPP_Thiamine diphosphate; 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.

Introsuction

Most plants and microorganisms have full sets of biosynthetic pathways and their component enzymes; therefore they are able to synthesize all of their organic constituents from inorganic sources. In contrast, animals have complex dietary requirements due to their inability to synthesize some fatty acids, amino acids, and vitamins (Duggleby and Pang, 2000). The branched chain amino acids (BCAAs) like isoleucine (Ile), valine (Val) and leucine (Leu) are not synthesized by animals but it is essential for their life. The Ile, Val and Leu are synthesized in all plant parts as indicated by the ubiquitous presence of the mRNAs, the encoded proteins, and the activities of various enzymes of the pathway (Hattori et al., 1992). In plant and microbes, Val and Leu are synthesized by a common pathway that begins with the formation of 2-acetolactate from two molecules of pyruvate (Yoon et al., 2003). Ile is synthesized in a parallel pathway involved three more enzymes starting with the formation of 2-acetohydroxybutyrate from pyruvate and 2-ketobutyrate (Fig. 1). The acetolactate synthase (ALS, EC 4.1.3.18) is an enzyme that catalyzes the first step in the synthesis of the BCAAs leading Ile, Val and Leu. ALS belongs to a homologous family of thiamine diphosphate (TPP) dependent enzymes present in plants, algae, fungi, and bacteria (Gonzalo et al., 2010). ALS enzyme is substantially important because it is the target of several classes of herbicides, including all members of the popular sulfonylurea and imidazolinone families (Duggleby et al., 2008). Sulfonylureas and imidazolinones classes of compounds are notable for their high herbicidal potencies, their low mammalian toxicities, and, their selective toxicity for some analogs to weed species as compared to crop species (Levitt et al., 1981; Shaner et al., 1984). These compounds inhibit plant growth by inactivating an enzyme in biosynthetic pathways for essential amino acids, rather than by the alternate herbicidal mode of inactivating a component in photosynthetic pathway (Mazur et al., 1987). The ALS requires FAD, TPP and a bivalent metal ion, Mg^{2+} or Mn^{2+}, for enzyme activity (Singh et al., 1988). The enzyme uses TPP as coenzyme in the condensation reactions, and Mg^{2+} is presumed to be required for the binding of TPP to the enzyme, as it is for other TPP-dependent enzymes (Muller et al., 1993). ALS-inhibiting herbicides do not act as analogs of the substrates and cofactors, suggesting that the inhibition mechanism is complex. The most active ALS research areas are the structural studies of the herbicide binding site, as well as herbicide resistant mutations (Yoon et al., 2003). The ALS genes have been identified, purified, and sequenced in a variety of microorganisms and plant species, such as *Escherichia coli* (Eoyang and Silverman, 1984), *Saccharomyces cerevisiae*, (Falco and Dumas, 1985; Kingsbury and McCusker, 2010), *Mycobacterium tuberculosis* (Choi et al., 2005), *Triticum aestivum* (Southan and Copeland, 1996), *Arabidopsis thaliana* (Chang and Duggleby, 1997), *Nicotiana*

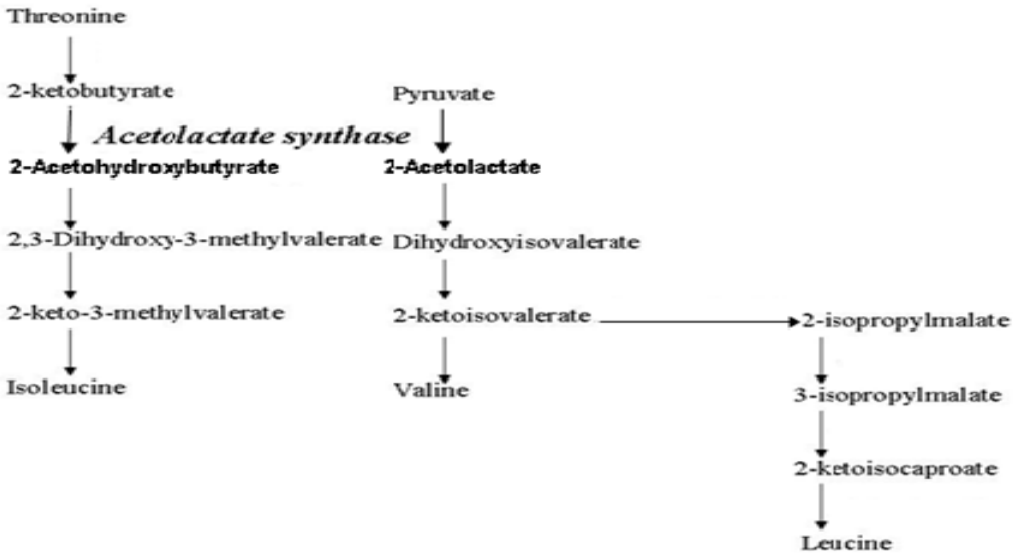

Fig 1. The biosynthetic pathway for branched chain amino acids in plants. A figure was adopted and slightly modified from Nozawa et al (2005).

tabacum (Mazur et al., 1987; Yoon et al., 2010), and *Hordeum vulgare* L (Yoon et al., 2003). The kinetic studies on inhibition of ALS enzyme by herbicides have been carried out with several bacterial ALS, which resembles the plant enzyme with regard to its sensitivity to herbicides, but is different in subunit composition and feedback regulation (Ray, 1984; Schloss et al., 1988). There are continuous demands to elucidate herbicidal mechanism and to improve nutritional qualities in rice which is a major crop in the world. These have initiated the application of molecular genetics approaches in determining gene functions and enzyme characteristics in amino acid biosynthesis of crop plants. Here, we report the analysis and characterization of a gene for ALS enzyme from rice.

Methods and materials

E. coli strains

Three *E. coli* strains were used in this study. The genotype of the strains was M1262 [hfr(PO1), *leuB6*(Am), *ilvI614*, *ilvH612*, *LAM-*, *relA1*, *spoT1*, *ilvB619*, *ilvG605*(Am), *ilvG603*(Act), *thi-1*] (Guardiola et al., 1974), FD1062 [hfr(PO1), *araC14*, *ilvI614*, *ilvH612*, *LAM-*, *glyA18*, *relA1*, *spoT1*, *ilvB619*, *bglR20*, *rbs-5::Tn5*, *ilvG468*(Act), *thi-1*] (Lawther et al., 1982), and Gif41 [hfr(PO1), *thrC1001*, *LAM-*, *e14-*, *relA1*, *spoT1*, *thi-1*] (Theze et al., 1974), respectively. The Gif41 strain was utilized as a positive control and a wild-type for BCAAs biosynthesis. The strains were obtained from the *E. coli* Genetic Stock Center (CGSC) at Yale University, USA.

DNA sequence analysis

An expressed sequence tag (EST) clone (Genbank accession number AK242817, clone name J090063G17 and clone ID 302178), derived from rice cDNA library (Osato et al., 2002), was obtained from the Rice Genome Resource Center (RGRC), Japan. DNA sequencing and sequence analysis were described previously (Sikdar and Kim, 2010). The sequences were analyzed using BLAST (Wheeler et al., 2003), 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). Comparison of sequences was performed at the nucleotide and amino acid level. Protein localization was predicted by WoLF PSORT program (http://wolfpsort.org) and phylogenetic tree with bootstrap value was prepared by using Mega 4.1- neighbor-joining program (Kumar et al., 2008).

Polymerase chain reaction (PCR) and recombinant constructs

The specific primers were designed from the sequence information around the translational start and stop codons of *OsALS* to amplify the full-length open reading frame (ORF) and to express the gene product in *E. coli*. Polymerase chain reaction (PCR) 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 *OsALS* was amplified from the EST clone as a template, and the following primers were designed from the *OsALS* sequence: OsALS-F (5'-AAAGCTTATAGAACCAAGCTCTCTCCC-3') and OsALS-R (5'-AAAGCTTGGCATACCACTCTTTATGGG-3') using Taq polymerase (Perkin-Elmer, U.S.A). The underlined bases in the OsALS-F and OsALS-R primers are the designed restriction sites for *Hin*dIII to facilitate subcloning. The PCR reaction was conducted using a MYCyler^{TM} PCR system (BioRad, USA) for 35 cycles with 95°C for 1 min, 55°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 (1.6 kb) was then subcloned into pBluescript II KS+ (Stratagene Inc., U.S.A) as a *Hin*dIII fragment, to give *pB::OsALS*. Restriction analysis was conducted in an effort to confirm the recombinant DNA of *pB::OsALS* with the right orientation for expression.

Functional complementation and growth assay

Two competent *ilvH* mutant strains of *E. coli,* M1262 and FD1062, were transformed with *pB::OsALS* via electroporation (ECM399, BTX, USA) after preparing competent cells (Kim

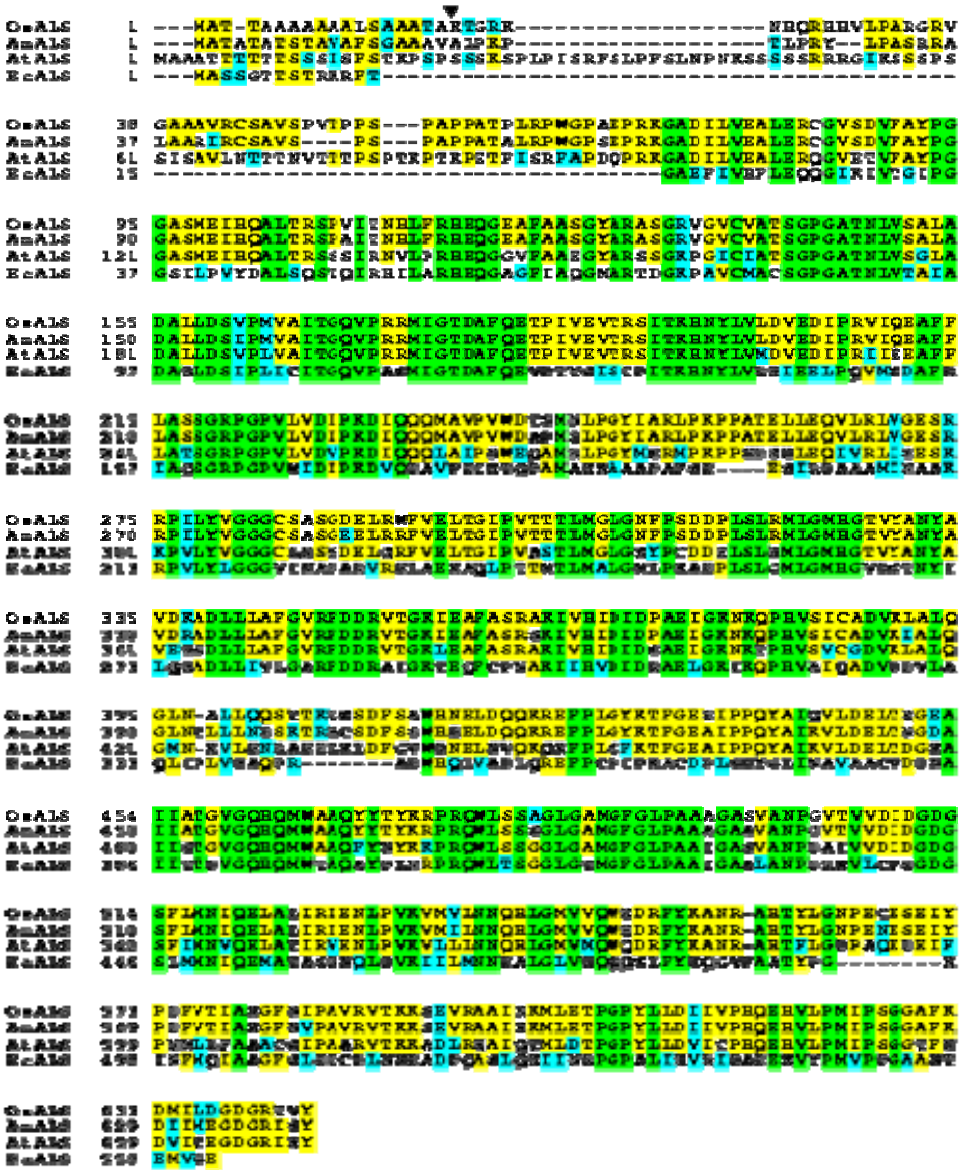

Fig 2. Amino acid sequence alignment of ALSs using Boxshade program after Clustal W alignment. Amino acids that are from completely identical to less similar amino acid residues are visually shown consequently as green, yellow and cyan, respectively. GenBank accession numbers are as follows: AK242817 (OsALS from *Oryza sativa*, this study), CAD24801 (AmALS from *Alopecurus myosuroides*), AAK68759 (AtALS from *Arabidopsis thaliana*) and NP_418127 (EcALS from *E. coli*). The possible cleavage site of chloroplast transit peptide in OsALS is indicated by an arrow.

and Leustek, 1996) using a cuvette with a 0.1 cm electrode gap, then plated on LB medium (20 g L^{-1}) with Amp (100 µg mL^{-1}). The growing culture was tested for growth retardation in M9 minimal medium (MM) [(5 x M9 salts (200 mL L^{-1}), 1 M $MgSO_4$ (2 mL L^{-1}), 1 M $CaCl_2$ (0.1 mL L^{-1})], containing Amp (25 µg mL^{-1}), 20% glucose, 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and 18 amino acids (Sigma, Germany) each at a concentration of 25 µg mL^{-1}, excluding Ile and Val. The *E. coli* mutants harboring the *pB::OsALS* construct or control plasmid, and wild type with control plasmid were grown at 37°C in MM with IPTG, 20% glucose (20 mL L^{-1}), containing 18 amino acids and Amp (25 µg mL^{-1}), excluding Ile and Val. The bacterial growth was monitored via optical density measurements at 595nm (OD_{595}) using a spectrophotometer (UV1101, Biochrom, England) at one-hour intervals (Sikdar and Kim, 2010). After 12 h, the diluted culture was plated and incubated overnight at 37°C for survival test.

Results

Sequence analysis of *OsALS*

An EST clone (GenBank accession number AK242817) obtained from the RGRC was analyzed to determine the nucleotide sequence. The cDNA (*OsALS)* sequence harbored a full-length ORF encoding for a protein of approximately 69.4 kDa. The N-terminal of OsALS contains several alanine and hydroxyl amino acids, a feature of plastid transit peptide, and is

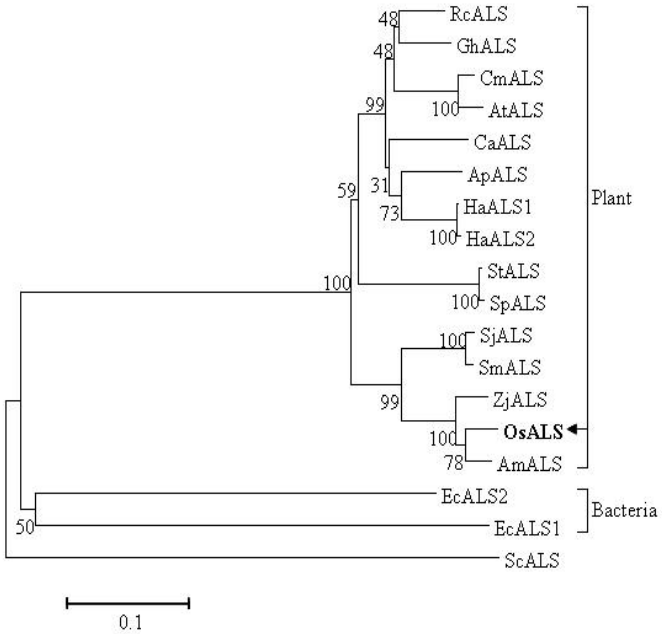

Fig 3. Phylogenetic analysis of OsALS-related proteins using Clustal W and Mega4.1 neighbor-joining program. GenBank accession numbers are as follows: AK242817 (OsALS from *Oryza sativa*), XP_002511176 (RcALS from *Ricinus communis*), CAA87084 (GhALS from *Gossypium hirsutum*), AAR06607 (CmALS from *Camelina microcarpa*), AAK68759 (AtALS from *Arabidopsis thaliana*), ACF17639 (CaALS from *Capsicum annuum*), AAK50821 (ApALS from *Amaranthus powellii*), AAT07325 (HaALS1 from *Helianthus annuus*), AAT07327 (HaALS2 from *Helianthus annuus*), BAF57909 (StSLS from *Sagittaria trifolia*), BAH60833 (SpALS from *Sagittaria pygmaea*), BAE97677 (SjALS from *Schoenoplectus juncoides*), ACD93201 (SmALS from *Schoenoplectus mucronatus*), BAI44129 (ZjALS from *Zoysia japonica*), CAD24801 (AmALS from *Alopecurus myosuroides*), NP_418127 (EcALS1 from *E. coli),* YP_543276 (EcALS2 from *E. coli)*, EDV11602 (ScALS from *Saccharomyces cerevisiae).* Numbers on branches are percentages of bootstrap analyses supporting the grouping of each branch.

predicted localization to chloroplast with a possible cleavage site between 18 and 19 amino acid by WoLF PSORT program. The expected isoelectric point of the protein was 6.5. Data analysis revealed that the *OsALS* sequence was identical to the genomic region located in chromosome II (Os02g0510200) and existed as a single copy gene in rice. Comparisons of the amino acid sequences using BLOSUM50 score of the OsALS and the homologous sequences from *A. myosuroides, A. thaliana* and *E. coli* revealed identity at 89.8%, 68.1% and 39.2%, respectively (Fig. 2). Analysis of the OsALS amino acid sequence revealed three signature motifs for N-terminal (71-241), central (263-396), and C-terminal (458-558) TPP binding domains characteristic in TPP-dependent enzymes. To determine the relationship between plant and bacterial ALS enzymes, phylogenetic analysis was performed with the Mega 4.1 neighbor-joining program (Kumar et al., 2008). Phylogenetic tree derived from the related sequences further indicated that OsALS is divergent and grouped interestingly with ALSs from *Zoysia* grass and *Alopecurus myosuroides*, major weeds in cereal crops, among several plant groups and far from bacterial and yeast ALS (Fig. 3). Branching pattern and numbers at nodes indicate levels of bootstrap support based on neighbor-joining analysis of 1000 re-sample data sets.

Functional complementation of *OsALS* in *E. coli*

The recombinant DNA, *pB::OsALS*, was constructed using the ORF of a PCR-amplified *OsALS* fragment. After the transformation of *E. coli* with the recombinant DNA, *OsALS* activity was monitored *in vivo* in a medium containing IPTG and 18 amino acids, excluding Ile and Val. Functional complementation was performed using the *ALS* mutants of *E. coli* to confirm the enzyme activity of the gene product of *OsALS*. To assess the viability of *E. coli* cells by *OsALS* activity, the *OsALS*-expressing cells were cultured for 12 h with shaking, and the diluted portion was plated on agar medium containing Amp (25 mg mL^{-1}) and the 18 amino acids without Ile and Val (Fig. 4). The viable colonies greatly differed among the plasmids. The *ilvH* mutants of *E. coli* with *pB::OsALS* could grow under conditions in which the mutant with control plasmid could not. A growth study was performed further to determine whether the *OsALS* gene would increase survival of bacterial cells of the *ilvH* mutants. The *pB::OsALS* construct was transformed into the two *ilvH* mutant strains of *E. coli,* M1262 and FD1062. A control plasmid was also transformed into wild type (Gif41) and the *ilvH* mutants. The *OsALS* activity was monitored via a growth assay of the transformed *E. coli* strains in MM with 18 amino acids excluding Ile and Val, containing IPTG and Amp. The wild type *E. coli* strain Gif41 harboring the control plasmid grew normally and showed an S-shaped classical growth curve in the medium without Ile and Val. The Gif41 strain could synthesize Ile and Val itself, and thus grew normally in the medium. Both of the *ilvH* mutant strains, M1262 and FD1062, expressing *pB::OsALS* also grew normally and evidenced an S-shaped classical growth curve in the same medium, similar to the wild type strain containing the control plasmid (Fig. 5A & 5B), although the M1262 and FD1062 strains harboring the control plasmid in the same medium without Ile and Val evidenced dramatically retarded growth. In this case, the *ilvH* mutant strains of *E. coli,* M1262 and FD1062, could not synthesize Ile and Val itself, and thus could not grow in the medium; however, the same *ilvH* mutant strains harboring *pB::OsALS* grew well because the *ilvH* mutants were able to synthesize Ile and Val using rice ALS expressed by the *pB::OsALS* plasmid (Fig. 5A & 5B). These results led us to the conclusion that *OsALS* confers the ability to functionally complement the inability of the *ilvH* mutants to produce Ile and Val and the rice gene of *OsALS* encodes an enzyme for functional acetolactate synthase.

Discussion

The BCAAs, Val, Leu and Ile, are three among 10 essential amino acids and important in animals and human diets. A biosynthetic enzyme ALS for Ile and Val is an attractive target for antimicrobial drugs and herbicides, since the biosynthetic pathway for Ile and Val is not present in mammals (Kingsbury and McCusker, 2010). The biosynthetic pathway of BCAAs feeds carbon into the three different amino acids, therefore the flow of carbon must be tightly regulated so that no one of these amino acids becomes limiting for plant growth (Singh. and Shaner, 1995). In plants and microbes, ALS is a key enzyme for

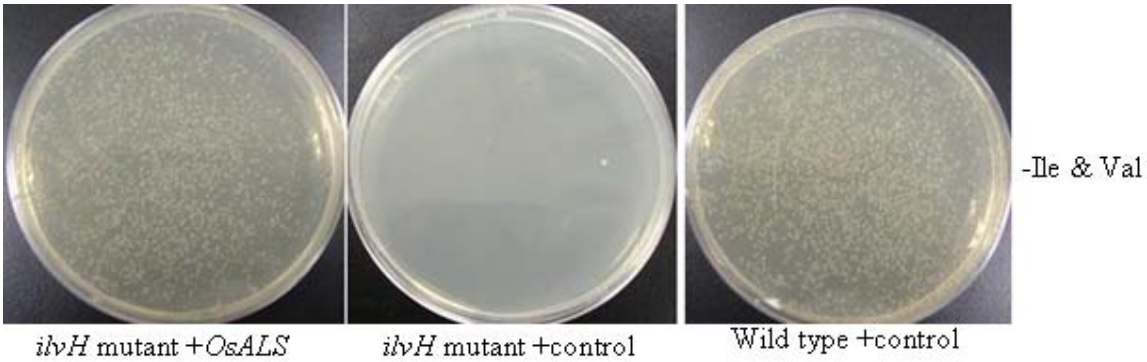

Fig 4. Functional complementation assay for OsALS. The *ilvH* mutant strain of *E. coli*, M1262, containing *pB::OsALS* or control plasmid and wild-type strain of *E. coli,* Gif41, containing control plasmid.

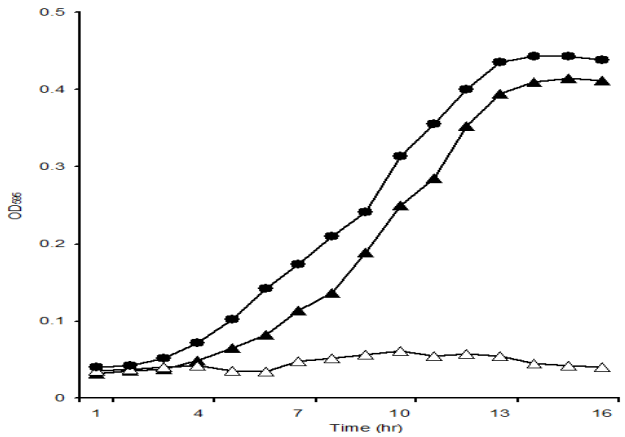

Fig 5A. Growth curves of an *E. coli* mutant M1262 harboring *pB::OsALS* or control plasmid and wild type strain Gif41 with control plasmid. Bacterial cells were grown at 37°C in MM containing 18 amino acids except Ile and Val. Bacterial growth was monitored via optical density measurements at 595 nm (OD_{595}). Symbols: ▲, M1262 + *pB::OsALS*; ●, wild type + control; Δ, M1262 + control.

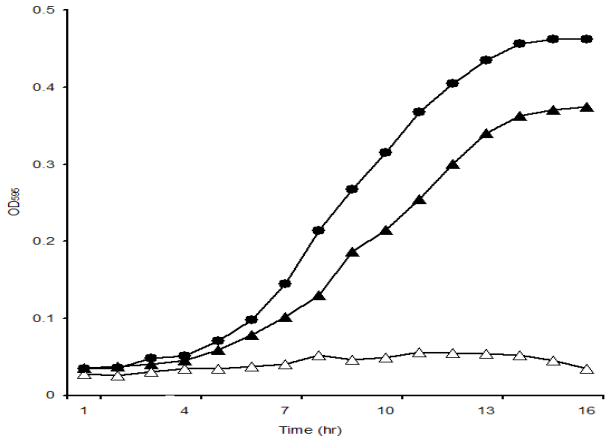

Fig 5B. Growth curves of an *E. coli* mutant FD1062 harboring *pB::OsALS* or control plasmid and wild type strain Gif41 with control plasmid. Bacterial cells were grown at 37°C in MM containing 18 amino acids except Ile and Val. Bacterial growth was monitored via optical density measurements at 595 nm (OD_{595}). Symbols: ▲, FD1062 + *pB::OsALS*; ●, wild type + control; Δ, FD1062 + control.

the BCAAs pathway, and specific inhibitors of ALS can be used as antibiotics or herbicides (Chaleff and Mauvais, 1984; Shaner et al., 1984). The well-known class of herbicides sulfonylureas inhibits enzyme activity of ALS in the biosynthesis of BCAAs in plants. The role of ALS in BCAAs pathway has been demonstrated in several bacteria and plants including *E. coli* and *A. thaliana* (Eoyang and Silverman, 1984; Chang and Duggleby. 1997). Recently, the functional relationships between ALS and rice mutants were analyzed by using gene analysis and transgenic rice plants (Okuzaki et al., 2007; Tougou et al., 2009). The recent research surrounding ALS is more focused to herbicide resistance and selection marker than the role in amino acid synthesis and nutritional improvement in plants. Okuzaki and Toriyama (2004) showed that a predicted *ALS* gene after complete genome sequencing of rice was targeted to modify a specific base using chimeric RNA/DNA oligonucleotide to be resulted in resistance to herbicides. The research group was further reported a mutated *ALS* gene from rice conferring specific resistance to pyrimidinylcarboxy herbicides (Okuzaki et al., 2007). Transgenic soybeans were produced recently using the mutated *ALS* gene from rice as the selectable marker (Tougou et al., 2009). Therefore, an attempt was made to characterize a gene for ALS from rice. Based on the results of a database search, there is an ALS gene (OsALS) in rice genome. The full-length OsALS cDNA encodes a 69.4 kDa protein which is highly homologous to ALSs from plants. The OsALS is more homologous to those from weeds than those from dicotyledon plants. These findings suggest that OsALS is more closed related evolutionally to weeds such as *A. myosuroides* or *Z. japonica* than to dicotyledon plants or bacteria. Our group previously reported several genes encoding cystathionine γ-synthase from *Arabidopsis thaliana* (Kim and Leustek, 1996), diaminopimelate decarboxylase from rice (Kim and Lee, 2006), and threonine synthase from rice (Sikdar and Kim, 2010) by functional complementation using *E. coli* mutants. The molecular genetics approach using *E. coli* mutants to characterize plant metabolic genes are proven as a powerful tool.In this study, an *ALS* gene of rice was analyzed using bioinformatics tools and expressed in two *ilvH* mutant strains of *E. coli.* Our report on the functional characterization of a gene encoding OsALS in rice confirms again the role of OsALS along with the previous reports with *in vivo* gene targeting, mutated gene analysis and transgenic approach in rice (Okuzaki and Toriyama, 2004; Okuzaki et al., 2007; Tougou et al., 2009). The analysis showed that *OsALS* is able to complement the *ilvH* mutants of *E. coli* whose system would be applied easily to screening of herbicides. Our report of the functional characterization of a

cDNA encoding ALS from rice demonstrates indirectly that *OsALS* functions as ALS enzyme. The *ALS* gene could be used as a selection marker for future application to improve or develop the herbicide-resistant plants and also reintroduced into crop plants to improve nutritional qualities in cereal foods.

Acknowledgements

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