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Characterization of a Gene Encoding Acetylornithine Deacetylase from Rice

[1,2]Md. Shafiqul Islam Sikdar and [2]Jung-Sup Kim
[1]Department of Agronomy,
Hajee Mohammad Danesh Science and Technology University, 5200, Dinajpur, Bangladesh
[2]Faculty of Biotechnology, Jeju National University, 690-756, Jeju, Korea

Abstract: Acetylornithine Deacetylase (AODA) is an enzyme involved in arginine biosynthesis in bacteria and plants and is required for the release of ornithine from acetylornithine. AODA activity has been reported in many bacteria and few plants. In the consecutive study of recently identifying a gene encoding for ornithine carbamoyltransferase (OsOTC) in rice, it was characterized a gene encoding AODA from rice (OsAODA) in this study. Sequence analysis revealed that a full-length open reading frame consisted of 443 amino acids which corresponded to a protein with a molecular weight of approximately 48.7 kDa. The predicted amino acid sequence of OsAODA was highly homologous to that of the enzyme of plants and many bacteria. Expression of OsAODA in argE mutants of *Escherichia coli* showed that the gene was able to functionally complement to the mutant. These results suggest that the OsAODA gene encodes an enzyme for acetylornithine deacetylase in rice.

Key words: Acetylornithine deacetylase, argE, arginine, rice (*Oryza sativa*)

INTRODUCTION

Arginine (Arg) represents as much as 40% of the total nitrogen in seed storage proteins (VanEtten *et al*., 1963) and is highly catabolized during germination as a nitrogen source in pea cotyledons (De Ruiter and Kolloffel, 1985). Arg is serving as a precursor to synthesize the important nitrogen-containing compounds such as nitric oxide, creatine, urea and polyamines (Morris, 2006). Arg synthesis and its regulation have been well characterized in prokaryotes, fungi and animals (Caldovic and Tuchman, 2003; Cunin *et al*., 1986; Davis, 1986). In contrast, there have been few studies in this area for plant metabolism since the last major review of this topic nearly two decades ago (Shargool *et al*., 1988).

In Arg biosynthesis, two different enzymes are required for catalysis of the fifth step, where one is acetylornithine deacetylase (AODA, N2-acetyl-L-ornithine amidohydrolase; EC 3.5.1.16; the argE gene product) which catalyses deacetylation of N-acetylornithine (NAO) yielding ornithine and acetate (Vogel and Bonner, 1956). The other enzyme is glutamate N-acetyltransferase (GAT, EC 2.3.1.35) which catalyses the transfer of the acetyl group from NAO into glutamate yielding ornithine and N-acetylglutamate (Fig. 1). This linear pathway is regulated by Arg-induced feedback-inhibition of N-acetylglutamate synthase which is involved in the first step of this pathway (Leisinger and Haas, 1975). AODA converts NAO into ornithine which is used for the synthesis of citrulline and Arg in the urea cycle. Ornithine is required for the Arg synthesis as well as for polyamines which function in DNA replication and cell division in bacteria (Girodeau *et al*., 1986). After an Arg auxotrophic bacterial strain was transformed with a plasmid containing argE, AODA activity and an Arg+ phenotype was observed (Meinnel *et al*., 1992). Bacterial AODA, an enzyme in Arg synthetic pathway, is a metallohydrolase and harbors a dinuclear active site in bacteria (McGregor *et al*., 2005). The Arg biosynthetic pathway has been observed in all Gram-negative and most Gram-positive bacteria, including Enterobacteriaceae (Vogel and MacLellan, 1970), *Myxococcus* (Harris and Singer, 1998), *Vibrionaceae* (Xu *et al*., 2000), the thermophilic archaeon *Sulfelobus* (Van de Casteele *et al*., 1990) and *Bacillus thermoglucosidius* (Sakanyan *et al*., 1990). Few inhibitors have been reported for the argE product (McGregor *et al*., 2007) to date and fluoride ions were shown to be uncompetitive inhibitors, exhibiting a modest Ki of 3.4 mM. Due to the crucial role argE in prokaryotic cell growth and proliferation, the development of specific and potent inhibitors of the argE product are of key importance (Hlavacek *et al*., 2010).

Enzymes in plant Arg biosynthesis and their metabolic control have not been fully characterized; however, they appear to be similar to those of prokaryotes

Corresponding Author: Jung-Sup Kim, Faculty of Biotechnology, Jeju National University, 690-756, Jeju, Korea
Tel: +82-64-754-3393 Fax: +82-64-754-3393

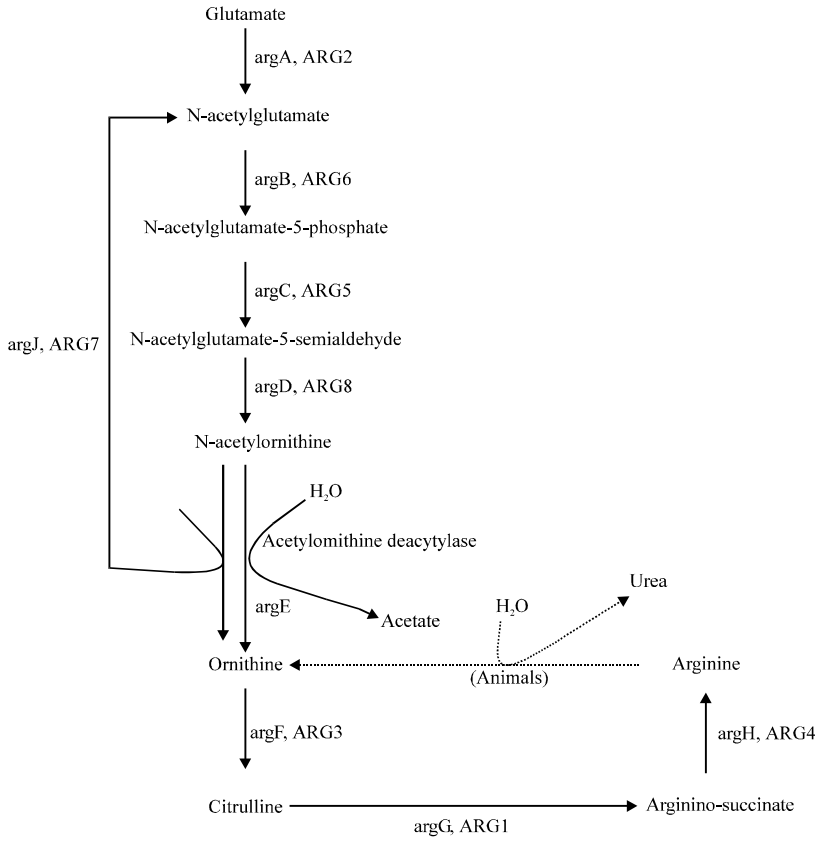

Fig. 1: Schematic diagram of the arginine biosynthesis pathway in plants. The figure was adopted and slightly modified from Sikdar and Kim (2009)

and lower eukaryotes. Subcellular compartmentation of Arg synthesis is still poorly understood. With few exceptions, plant genes encoding enzymes in Arg synthesis have not been identified and their gene products have not been characterized (Slocum, 2005). Major cereal crops such as rice, wheat, corn etc contain lower amounts of protein compared to leguminous crops. To improve the nutritional qualities of cereal crops, it is necessary to investigate the Arg biosynthesis pathway in crop plants. Therefore, in this study, a gene encoding AODA from rice is characterized.

MATERIALS AND METHODS

Strains and plasmids: Two *E. coli* strains were used in this study, 2435 and Gif41. The genotype of the strains was 2435 [recA441, sulA11, lacU169, thr1, leu6, his4, argE3, ilv (ts), galK2 rp] and Gif41 [hfr(PO1), thrC1001, λ-, e14-, relA1, spoT1, thi-1] (Theze *et al*., 1974), respectively. The 2435 strain was obtained from the Korean Collection for Type Cultures (KCTC), Korea and the Gif41 strain was provided by the *E. coli* Genetic Stock Center (CGSC) at Yale University, USA.

DNA sequence analysis: The EST clone (GenBank Accession Number AK105916, cDNA ID 001-204-H10) used in this study was obtained from the Rice Genome Resource Center (RGRC), National Institute of Aerobiological Science (NIAS), Japan. The clone was derived from a rice cDNA library (Osato *et al*., 2002) from developing seeds. The methods used for DNA sequencing and sequence analysis were described previously (Sikdar and Kim, 2010). Nucleotide sequences and amino acid sequences were compared with sequences present in the GenBank and EMBL databases and analyzed using 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).

Comparison of sequences was performed at the nucleotide and amino acid level. Motifs were searched by GenomeNet Computation Service at Kyoto University (http://www.genome.ad.jp) and a Phylogenetic tree with bootstrap value was prepared using the Mega 4.1 program (Kumar *et al*., 2008).

Polymerase chain reaction (PCR) and construct for expression: The specific primers were designed from the sequence information around the translational start and stop codons of OsAODA to amplify the full-length Open Reading Frame (ORF) and to express the gene product in *E. coli*. The Polymerase Chain Reaction (PCR) (Sambrook and Russell, 2001) was conducted to amplify the full-length ORF. After a plasmid was purified from a pellet harvested from a liquid culture containing ampicillin (Amp), the cDNA was amplified by using designed primers from the OsAODA sequence: OsAODA-F (5'-AGGATCCGCTCAAGGAAGCGCATCAGATGG C-3') andOsAODA-R(5'-AGGATCCTGTGGCCTTAGCCTGA GGTTCCTC-3') with restriction sites underlined. The polymerase chain reaction was performed using a MY Cyler TM PCR system (BioRad, U.S.A) for 40 cycles with 95°C for 1 min, 45°C for 1 min and 72°C for 2 min, with 10 μM primers. PCR products were analyzed on 1% (w/v) agarose gel. The 1.38 kb PCR fragment was subcloned into pBluescript II KS+ (Stratagene Inc., USA) to produce pB::OsAODA. Restriction analysis was performed to confirm the construct with the right orientation.

Functional complementation and growth inhibition assay: The two strains, 2435 and Gif41, were transformed with pB::OsAODA and pBluescript II KS+ which was used as a control, by electroporation (ECM399, BTX, USA) using a cuvette with a 1 mm 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 selection of transformed cells and culture conditions were described previously (Sikdar and Kim, 2010) except excluding a specific amino acid in this case of Arg. The plates were incubated at 37°C overnight and the growing colonies were retested for growth on Arg free medium (Kim and Leustek, 1996).

The argE mutant *E. coli* (2435) harboring the pB::OsAODA construct, control plasmid and wild-type (Gif41) with control plasmid were grown at 37°C in MM containing 19 amino acids excluding Arg, IPTG (Sigma, Germany), 20% glucose (20 mL L^{-1}) and Amp (25 μg mL^{-1}). The bacterial cell growth was monitored through optical density measurements every hour using a spectrophotometer (UV1101, Biochrom, England) at 595 nm (OD_{595}) (Sikdar and Kim, 2010).

RESULTS AND DISCUSSION

Sequence analysis of OsAODA: An EST clone (GenBank accession Number AK105916, cDNA ID 001-204-H10) obtained from the Rice Genome Resource Center (RGRC) was analyzed to determine the nucleotide sequence. The cDNA (OsAODA) sequence contained a full-length open reading frame and consisted of 1332bp which encoded for a protein that had a molecular weight of approximately 48.7 kDa. The expected isoelectric point of the protein was 5.03. Data analysis indicates that the OsAODA sequence was identical to the genomic region located in chromosomes II, Os02g0690800 in rice. Alignment of the amino acid sequences with the predicted amino acid sequence for the OsAODA (Fig. 2) demonstrated that the OsAODA was highly homologous among diverse plant species, including *Brassica oleracea* (73%), *Ricinus communis* (71%) and *Arbidopsis thaliana* (63%). In addition, it was also similar to those from some bacteria, such as *E. coli* (25%) and *Bacillus subtilis* (25%) in the level of amino acid sequences.

Phylogenic analysis based on a comparison of the related sequence further indicated that OsAODA was divergent and evolved from ancestor bacterial AODA (Fig. 3). The number at the nodes indicate the bootstrap support values based on the neighbor-joining analysis of 1000 re-sampled data sets using Mega 4.1 (Kumar *et al*., 2008). The numbers on branches correspond to the percentage of bootstrap analysis supporting the grouping of each branch.

OsAODA expression in *E. coli* and *in vivo* activity: The recombinant DNA, pB::OsAODA, was constructed using ORF of the PCR-amplified OsAODA fragment. After transformation to *E. coli*, pB::OsAODA activity *in vivo* was monitored in the presence of IPTG and 19 amino acids, excluding Arg. The functional complementation was performed using the argE mutants of *E. coli* to confirm the enzyme activity by the gene product of pB::OsAODA. To check the viability of *E. coli* cells based on OsAODA activity, the cells expressing OsAODA were cultured for 12 h with shaking and a diluted portion was plated on agar medium containing the 19 amino acids and ampicillin (100 mg mL^{-1}) without Arg (Fig. 4). The argE mutants of *E. coli* expressing OsAODA restore its

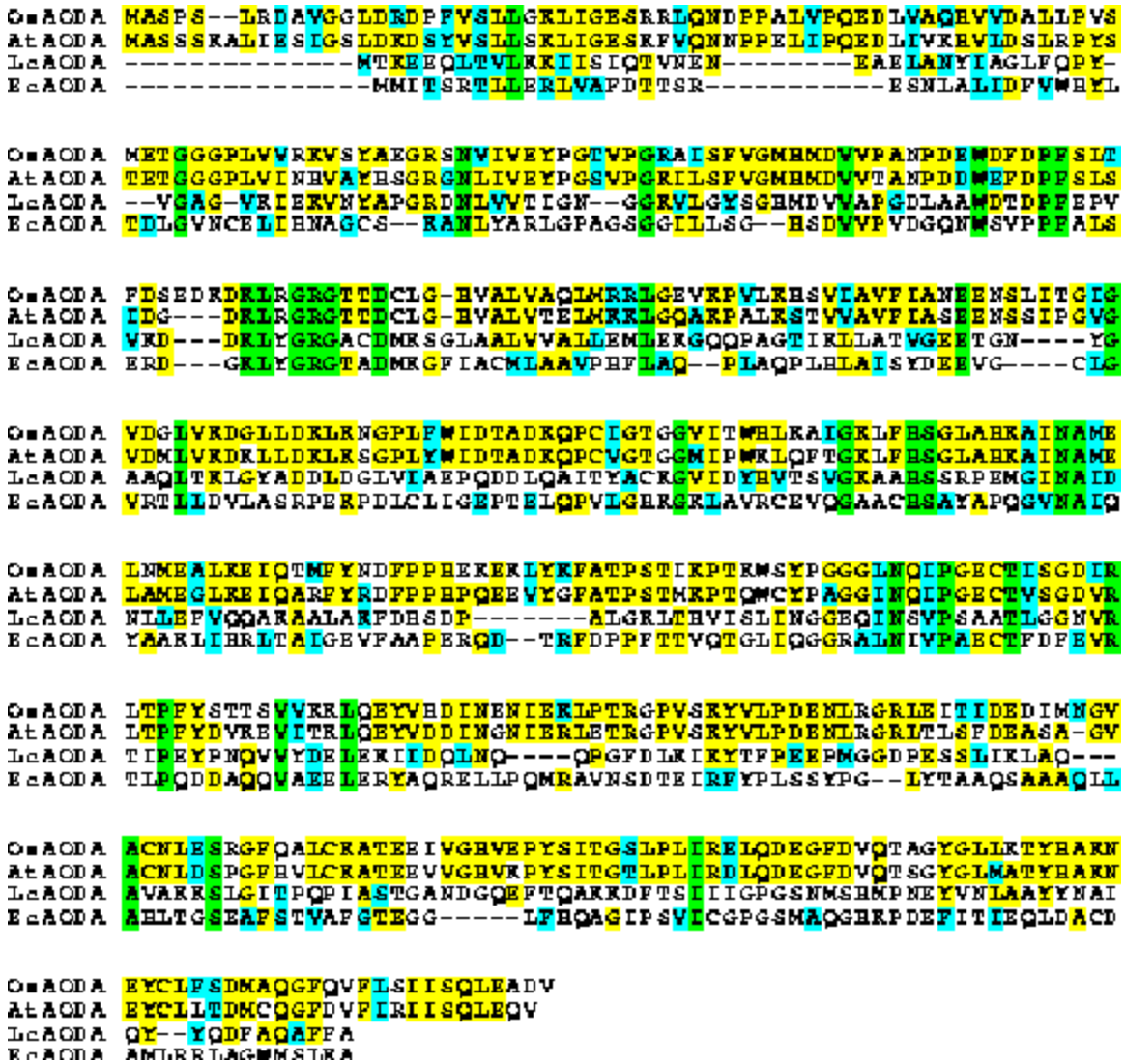

Fig. 2: Amino acid sequence alignment of AODA using the Boxshade program after CIUSTAL W alignment. Completely conserved, identical and similar residues are visually shown as green, yellow and cyan, respectively. Accession numbers are as follows: AK105916 (OsAODA from *Oryza sativa*, this study), NP_193517 (AtAODA from *Arabidopsis thaliana*), ZP_05553724 (LcAODA from *Lactobacillus coleohominis*) and ZP_07247995 (EcAODA from *Escherichia coli*)

viability like wild type. These results indicated that OsAODA growth retardation and viability of the gene product of pB::OsAODA was capable of functioning as a complement and provided evidence of functional AODA activity.

Arginine sensitivity of *E. coli* mutants was influenced by the expression of OsAODA: A growth study was performed to determine whether the OsAODA gene would increase the sensitivity of bacterial cells to Arg. The pB::OsAODA construct was transformed into an argE mutant *E. coli* strain 2435. The other two pBluescript II KS+ which were used as controls, were also transformed into wild type mutant *E. coli* strain (Gif41) and argE mutant strain 2435. The pB:: OsAODA activity was monitored through a growth assay in the absence of Arg. Bacterial cells were grown in MM containing 19 amino acids, excluding Arg, IPTG and Amp. The wild type

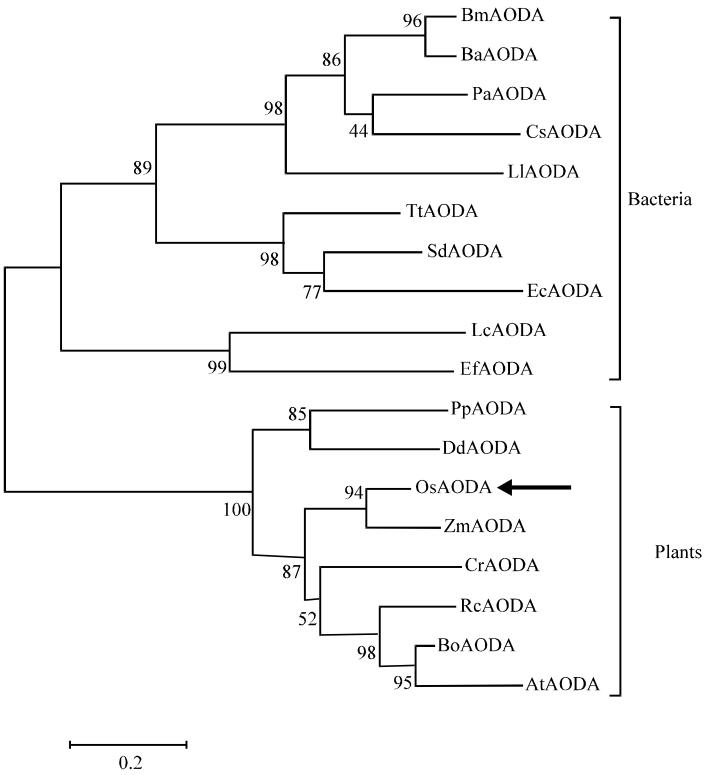

Fig. 3: Phylogenetic tree: Phylogenetic analysis of OsAODA related proteins using Clustal W. Accession numbers are as follows: ZP_03571630 (BmAODA from *Burkholderia multivorans*), ZP_02909425 (BaAODA from *Burkholderia ambifaria*), YP_001351487 (PaAODA from *Pseudomonas aeruginosa*), YP_574432(CsAODA from *Chromohalobacter salexigens*), ZP_06188127 (LlAODA from *Legionella longbeachae*), YP_003072038 (TtAODA from *Teredinibacter turnerae*), YP_525839 (SdAODA from *Saccharophagus degradans*), YP_001465454 (EcAODA from *Escherichia coli*), ZP_05553724 (LcAODA from *Lactobacillus coleohominis*), ZP_04648914 (EfAODA from *Enterococcus faecalis*), EFA86188 (PpAODA from *Polysphondylium pallidum*), XP_647163 (DdAODA from *Dictyostelium discoideum*), AK105916 (OsAODA from *Oryza sativa*, this study), NP_001149829 (ZmAODA from *Zea mays*), XP_001699756 (CrAODA from *Chlamydomonas reinhardtii*), P_002519823 (RcAODA from *Ricinus communis*), (BoAODA from *Brassica oleracea*), NP_193517 (AtAODA from *Arabidopsis thaliana*)

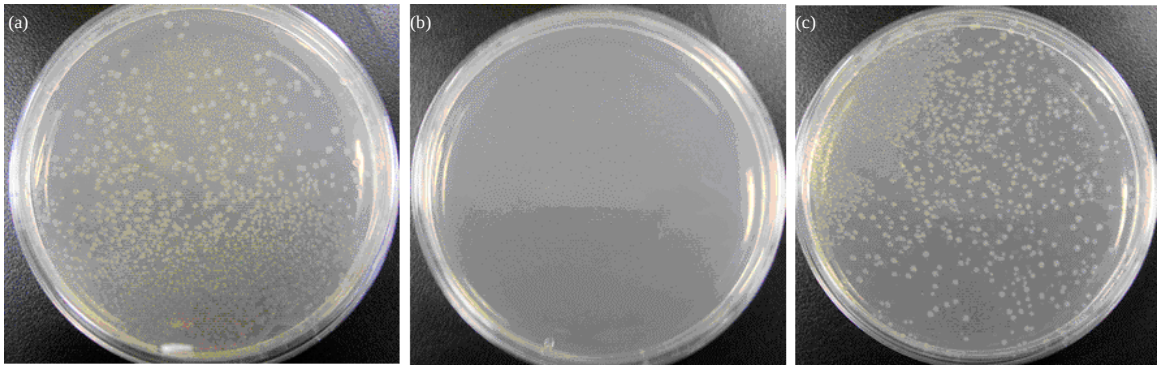

Fig. 4(a-c): Functional complementation assay. The argE mutant *E. coli* strain (2435) containing pB::OsAODA or control and wild-type *E. coli* (Gif41) containing the control plasmid which designated as (a) 2435+OsAODA, (b) 2435+control and (c) Gif41+control

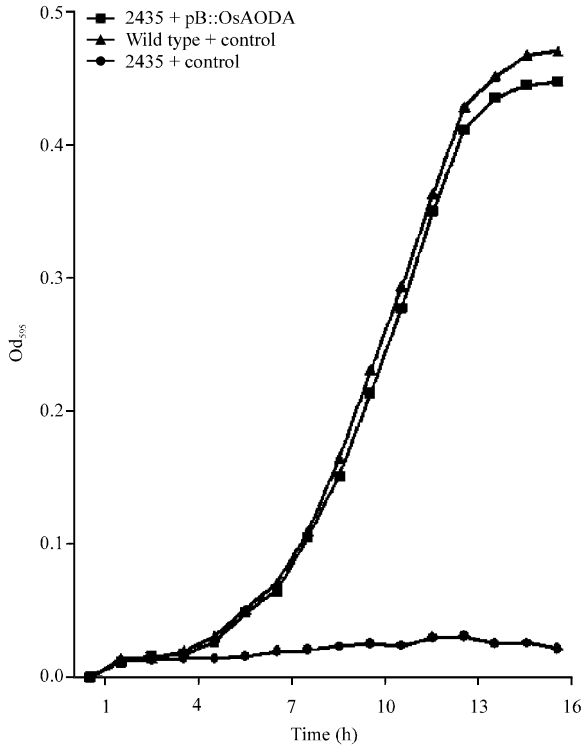

Fig. 5: Growth curves of *E. coli* mutant 2435 harboring pB::OsAODA and control plasmid and the Gif41 containing control plasmid. Bacterial cells were grown at 37°C in MM containing all amino acids, except Arg. Growth was monitored via optical density measurements at 595 nm (OD_{595})

E. coli strain Gif41 harboring the control plasmid grew normally and showed the classical S-shape growth curve in the same medium despite the lack of Arg (Fig. 5). The wild type *E. coli* strain produced Arg itself and supplied Arg to the control; thus, it grew normally in the same medium. However, the argE mutant strain 2435 expressing pB::OsAODA also grew normally and displayed the classical S-shape growth curve in the same medium (Fig. 5). Again, this occurred because the pB::OsAODA produced Arg and provided it to the mutant strain.

In contrast, the mutant 2435 which contained the control plasmid, showed dramatic retardation in growth in the medium due to a lack of the essential amino acid Arg. In this case, the argE mutant *E. coli* strain 2435 could not produce Arg (Fig. 5) which was due to the OsAODA activity. The expression of the OsAODA was functionally complementary and was shown to have functional Arg activity in the rice plant which was a consequence of the pB::OsAODA activity. Based on the above finding, it was concluded that OsAODA expression can functionally complement the argE mutant *E. coli*.

Additional studies are currently underway to examine the substrate specificity of recombinant OsAODA purified from *E. coli*. In addition, we are also examining the physiological functions of this novel enzyme for Arg metabolism by screening T-DNA insertion mutants, in which the OsAODA gene is knocked out in rice. The cloning and characterization of the cDNA encoding for AODA from rice are important to generating bioinformatic predictions, as well as motifs and complementation, in a argE mutant of *E. coli*. These results may constitute a starting point for investigations at the molecular level of Arg biosynthesis in rice which might eventually be used to modify the nutritional compositions of crop plants.

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