A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Characterization of Rice Genes in Amino Acid Biosynthesis and Pyrimidine Salvage Pathway by Functional Complementation

MD. SHAFIQUL ISLAM SIKDAR

Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

February, 2010

A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Characterization of Rice Genes in Amino Acid Biosynthesis and Pyrimidine Salvage Pathway by Functional Complementation

Md. Shafiqul Islam Sikdar

Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

February, 2010

Collection @ jeju

Characterization of Rice Genes in Amino Acid Biosynthesis and Pyrimidine Salvage Pathway by Functional Complementation

Md. Shafiqul Islam Sikdar

(Advised by Dr. Jung-Sup Kim)

A thesis submitted in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

This thesis has been examined and approved by

Thesis director, Dr. Young-Hwan Ko, Professor, Jeju National University

----
Dr. Young-Hoon Yang, Professor, Jeju National University

Dr. Yi Lee, Associate Professor, Chungbuk National University

----
Dr. Ho Bang Kim, Research Professor, Myongji University

Dr. Jung-Sup Kim, Assistant Professor, Jeju National University

2010.02.

…………………………

Department of Biotechnology
GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

Collection @ jeju

Collection @ jeju

CONTENTS

국문 요약………………………………………………………………… v

SUMMARY ………………………………………..…….………… vi

LIST OF FIGURES……………………………………………….…………….. viii

LIST OF TABLES …………………………………………………………….. x

ABBREVIATIONS …………………………………………………………….. xi

BACKGROUND……………………………………………………………... 1

PART I. Functional analysis of genes encoding threonine synthase

and dihydrodipicolinate synthase from rice………………… 5

ABSTRACT…………………………………………….……………………… 6

1. INTRODUCTION……………………………………….……………………….. 7

2. MATERIALS AND METHODS…………………….………………………... 11

2.1. Strains ……………….……………………………………………….. 11

2.2. DNA sequence analysis …..…………………………………….. 12

2.3. Polymerase chain reaction (PCR) and recombinant constructs 12

2.4. Functional complementation ………………..……………….. 14

2.5. Growth assay in *E. coli*……………………………………… 14

3. RESULTS……………………………………………….……….……….. 15

3.1. Sequence analysis of OsTS and OsDHDPS…………………. 15

3.2. *OsTS* and *OsDHDPS* expression in *E. coli* and *in vivo* activity 23

3.3. Expression of *OsTS* can complement the *thrC* mutant of *E. coli* 25

3.4. The Growth of the *thrC* mutant of *E. coli* was influenced by the expression

of *OsTS* in high concentration of Met……… ………. 26

Collection @ jeju

3.5. Lysine sensitivity of *E. coli* mutants was influenced by the expression of *OsDHDPS*………………………………………… 29

4. DISCUSSION………………………………………………………………. 31

PART II Analysis and characterization of genes encoding acetolactate synthase and 3-isopropylmalate dehydrogenase from rice...... 33

ABSTRACT…………………………………………………………………… 34

1. INTRODUCTION………………………………………………………….. 35

2. MATERIALS AND METHODS……………………………………….... 39

2.1. Strains ………………………………………………………………… 39

2.2. DNA sequence analysis ………………………………………………. 40

2.3. Polymerase chain reaction (PCR) and recombinant constructs 40

2.4. Functional complementation ………………………………… 42

2.5. Growth assay in *E. coli*………………………………………… 42

3. RESULTS……………………………………………………………….. 43

3.1. Sequence analysis of OsALS and OsIPMDH………………… 43

3.2 *OsALS* and *OsIPMDH* expression in *E. coli* and *in vivo* activity……………………………………………………… 51

3.3. Expression of *OsALS* can complement the *ilvH* mutants of *E. coli*……………………………………………………………. 53

3.4. Leucine sensitivity of *E. coli* mutants was influenced by the expression of *OsIPMDS*……………………………………… 56

4. DISCUSSION……………………………………………………………. 58

Collection @ jeju

PART III. A natural fusion gene encoding uridine kinase and uracil phosphoribosyltransferase from rice………………………… 60

ABSTRACT……………………………………………………………… 61

1. INTRODUCTION…………………………………………………….. 62

2. MATERIALS AND METHODS………………….……….………... 66

2.1. Strains ………….……………………………………………. 66

2.2. DNA sequence analysis …..…………………………………. 67

2.3. Expression of *OsUK/UPRT1* in *E. coli*……………………… 67

2.4. Functional complementation ………………………..………… 68

2.5. Growth inhibition assay with FU and FD in *E. coli*……… 69

3. RESULTS……………………………………………….………….….. 70

3.1. Sequence analysis of OsUK/UPRT1………………………… 70

3.2. *OsUK/UPRT1* expression in *E. coli* and *in vivo* activity……. 76

3.3. FU sensitivity of *E. coli upp-udk* mutants was influenced by the expression of *OsUK/UPRT1* ………………………………… 78

3.4. FD sensitivity of *E. coli upp-udk* mutants was influenced by the expression of *OsUK/UPRT1*…………………………………… 83

4. DISCUSSION…………………………………….………………. 88

CONCLUSIONS……………………………………………..……. 91

REFERENCES…………………………………………….……….. 92

ACKNOWLEDGEMENTS…………………………………….….… 108

BIOGRAPHY …………………… ……………………………….….. 110

Collection @ jeju

DEDICATION

This dissertation is dedicated to

The departed soul of my father,

beloved mother and daughter (Tasnia)

Collection @ jeju

국문 요약

트레오닌(Thr), 라이신(Lys), 발린(Val), 이소류신(Ile)과 류신(Leu)은 인간을 포함한 동물들에서 필수 아미노산이다. 트레오닌과 라이신은 아스파르산으로부터 유래된다. 그리고 식물에서 발린, 이소류신과 류신은 분지된 아미노산으로부터 유래된다. 이들 아미노산들은 외떡잎과 쌍떡잎 식물들에서 불균형하게 합성되고 저장된다. 분자생물학 및 생화학적 수준에서 조절기작을 밝히기 위해, 중요한 작물인 벼에서 중요조절단계에서의 유전자들에 초점을 맞추었다. 그리고 벼로부터 트레오닌 합성효소(TS), 디히드로디피콜리네이트 합성효소(DHDPS), 아세토락테이트 합성효소(ALS), 이소프로필말레이트 가수분해효소(IPMDH)를 위한 유전자들을 탐구하였다. TS, DHDPS, ALS와 IPMDH는 많은 세균과 몇 개의 식물에서 분석되고 동정되었지만 벼에서는 보고되지 않았다. 전체길이의 cDNA 가 애기장대나 세균 유전자들과의 서열비교에서 BLAST와 다른 생물정보학 프로그램에 의해 예측되었고 SALK나 NAIS로부터 주문되었다. TS, DHDPS, ALS 와 IPMDH의 연역된 효소의 분자량은 각각 대략 57.2, 41.4, 37.1 과 59.9kDa이다. 그들은 대장균의 *thrC, dapA, ilvH*와 *leuB* 돌연변이체를 각각 보상할 수 있었다. TS와 DHDPS는 아스파트산 경로 아미노산들의 합성과 분배의 기작을 연구하고 작물에서 식품 품질을 증진하기 위해 유용할 것이다. ALS와 IPMDH는 제초제 개발을 위한 타겟이다.

핵산에서 염기들은 모든 생물체에서 전사와 복제를 포함하는 여러 핵산 대사를 위해 중요하다. 우리딘 인산화효소(UK)와 우라실 포스포리보실전달효소(UPRT)는 피리미딘 구제경로에서 필수 효소들이다. UPRT 또는 UK의 기질로서 우라실과 우리딘의 독성 유사물질인 플루오로우라실(FU) 과 플루오로우리딘(FD)은 항암제로 사용되어 왔다. 암치료의 원리는 FU 또는 FD가 UMP 또는 UTP의 플루오로 유도체로 대사되어 독성을 나타내어 최종적으로 세포사망을 결과하는 것이다. 벼로부터 *OsUK/UPRT1* 은 *udk/upp* 돌연변이체 대장균을 보상할 수 있었다. 벼로부터 UK와 UPRT 효소를 위한 두 기능을 가진 단일 유전자의 분석과 동정은 식물에서 피리미딘 구제경로를 탐구하고 더 나아가 인간에서 암유전자치료에 응용될 수 있을 것이다.

Collection @ jeju

SUMMARY

Threonine (Thr), lysine (Lys) valine (Val), isoleucine (Ile) and leucine (Leu) are essential amino acids in all animals including humans. Thr and Lys are derived from aspartate (Asp). Val, Ile and Leu are derived from branched amino acids in plants. These amino acids are unequally synthesized and stored in monocot and dicot plants. To reveal the regulation mechanism in the molecular and biochemical level, the genes of key regulation steps were focused in rice, an important crop plant. Specifically, the genes were investigated for threonine synthase (TS), dihydrodipicolinate synthase (DHDPS), acetolactate synthase (ALS), 3-isopropylmalate dehydrogenase (IPMDH) from rice. They have been analyzed and characterized in many bacteria and few plants, but have not been reported in rice plant. The full length cDNAs were predicted by BLAST and other bioinformatics program in comparison with Arabidopsis or bacterial genes and ordered from SALK or NAIS. The molecular weights of the deduced enzymes for TS, DHDPS, ALS and IPMDH are approximately 57.2, 41.4, 37.1 and 59.9 kDa, respectively. Those four rice genes were treated in M9 minimal medium with IPTG, ampicillin, and 19 /18 amino acids without Thr, Lys, Ile, Val and Leu for respective rice genes. They showed functional activity in respective genes. They were able to complement in *thrC*, *dapA*, *ilvH* and *leuB* mutants of *Escherichia coli*, respectively. TS and DHDPS were functionally complemented, so its might be useful to investigate mechanisms of synthesis and distribution of Asp-pathway amino acids and to improve food qualities in crop plants. ALS and IPMDH are known to be targets for development of herbicides.

The bases in nucleic acids are also important for various nucleotide metabolisms including transcription and replication in all living things. Uridine kinase (UK) and uracil phosphoribosyltransferase (UPRT) are essential enzymes in the pyrimidine salvage pathway. 5-Fluorouracil (FU) and 5-Fluorouridine (FD), toxic analogs of uracil and uridine

Collection @ jeju

which are substrates of UPRT or UK, have been used successfully as anticancer agents. The principle of cancer therapy is that FU and/or FD are toxic to metabolize as fluoro-derivatives of UMP or UTP and finally cause cell death. The *OsUK/UPRT1* from rice was grown in M9 minimal medium with FU/FD containing ampicillin without uracil. *OsUK/UPRT1* was able to functionally complement in *udk/upp* mutants of *E. coli*. Analysis and characterization of a gene with dual functions for UK and UPRT enzymes from rice would be able to investigate the pyrimidine salvage pathway in plants and to be applied further cancer gene therapy in humans.

Collection @ jeju

LIST OF FIGURES

Fig. 1	Schematic diagram of the threonine and lysine biosynthesis pathway of aspartate family in plants………………………………………	8
Fig. 2	Amino acid sequence alignment of TSs ………………………...	16
Fig. 3	Amino acid sequence alignment of DHDPSs……………………..	18
Fig. 4	Phylogenetic analysis of OsTS related proteins using ClustalW and Mega 4.1 neighbor-joining program…………………………….	19
Fig. 5	Phylogenetic analysis of OsDHDPS related proteins using ClustalW and Mega 4.1 neighbor-joining program……………………….	21
Fig. 6	Functional complementation assay for OsTS and OsDHDPS……..	24
Fig. 7A	Growth curves of *E. coli* mutant Gif41 and sφ415 harboring *OsTS* without Met ……………………………...……………………	27
Fig. 7B	Growth curves of *E. coli* mutant Gif41 and sφ415 harboring *OsTS* supplementing Met………………………………………………	28
Fig. 8	Growth curves of *dapA* mutant *E. coli* strain *AT997* harboring *OsDHDPS* and control plasmid……………………………………	30
Fig. 9	Biosynthetic pathway of branched amino acids in plants………..	37
Fig. 10	Amino acid sequence alignment of ALSs………………………..	44
Fig. 11	Amino acid sequence alignment of IPMDHs…………………….	45
Fig. 12	Phylogenetic analysis of OsALS related proteins using ClustalW and Mega 4.1 neighbor-joining program ………………………………..	47
Fig. 13	Phylogenetic analysis of OsIPMDH related proteins using ClustalW and Mega 4.1 neighbor-joining program ……………………….	49
Fig. 14	Functional complementation assay for OsALS and OsIPMDH……..	52

Collection @ jeju

Fig. 15A Growth curves of *E. coli* mutant M1262 harboring *OsALS* and control plasmid without Ile and Val………………………………………. 54

Fig. 15B Growth curves of *E. coli* mutant FD1062 (B) harboring *OsALS* and control plasmid without Ile and Val………………………………… 55

Fig. 16 Growth curves of *E. coli* mutant M1262 harboring *OsIPMDH*…….. 57

Fig. 17 Pyrimidine salvage pathway………………………………………. 63

Fig. 18 Possible mechanism cancer gene …………………………………… 64

Fig. 19 Amino acid sequence alignment of UPRT or UK from bacteria and plants……………………………………………………………. 72

Fig. 20 Phylogenetic analysis of OsUK/UPRT1 related proteins using ClustalW……………………………………………………… 74

Fig. 21 Functional complementation of *OsUDK/UPRT1* by FU and FD...... 77

Fig. 22 Growth curves of *E. coli upp-udk* mutant X2224 expressing *OsUK/UPRT1* and control plasmid and *upp* mutant GT4 & sφ408 expressing *OsUK/UPRT1* in response to FU…………………… 80

Fig. 23 Growth curves of *E. coli upp-udk* mutant, X2224, and wild type expressing *OsUK/UPRT1*and control plasmid in response to FU… 82

Fig. 24 Growth curves of *E. coli upp-udk* mutant X2224 expressing *OsUK/UPRT1*or control and *upp* mutant GT4 & sφ408 expressing *OsUK/UPRT1* in response to FD……………………………… 85

Fig. 25 Growth curves of *E. coli upp-udk* mutant, X2224, and wild type (JM109) expressing *OsUK/UPRT1*or control in response to FD …. 87

Collection @ jeju

LIST OF TABLES

Table 1. *E. coli* strains used in part I 11

Table 2. *E. coli* strains used in part II 39

Table 3. *E. coli* strains used in part III 66

Collection @ jeju

ABBREVIATIONS

AK	aspartate kinase
ALS	acetolactate synthase
Amp	ampicillin
ASA	3-aspartic semialdyde
Asp	aspartate
ATP	adenine 5'-triphosphate
CGSC	*E. coli* Genetic Stock Center
CGS	cystathionine γ-synthase
CTP	cytidine 5’-triphosphate
DHDPS	dihydrodipicolinate synthase
EST	expressed sequence tag
FD	5-fluorouridine
FU	5-fluorouracil
GTP	guanosine 5’-triphosphate
HSD	homoserine dehydrogenase
ICDH	isocitrate dehydrogenase
Ile	**isoleucine**
IPMDH	3-isopropylmalate dehydrogenase
IPTG	isoptopyl β-D-thiogalactopyranoside
Leu	leucine
Lys	lysine
Met	methionine
MM	M9 minimal medium

Collection @ jeju

NIAS	National Institute of Agro Biological Science
OMP	orotate 5-monophosphate
OPH	*O*-phosphohomoserine
ORF	open reading frame
PCR	polymerase chain reaction
PLP	pyridoxal-5’-phosphate
PRPP	phosphoribosyl-α-1-pyrophosphate
RGRC	rice genome resource center
SAM	*S*-adenosylmethionine
TDH	threonine dehydratase
ThDP	thiamine diphosphate
Thr	threonine
TPP	thiamine pyrophosphate
TS	threonine synthase
UDP	uridine 5-diphosphate
UK	uridine kinase
UMP	uridine 5’-monophosphate
UPRT	uracil phosphoribosyltransferase
UPM	uridine 5’-monophosphate
UTP	uridine 5’-triphosphate
Val	valine

Collection @ jeju

BACKGROUND

Rice (*Oryza sativa*) is a very important crop as the staple food for more than half of the world's population. Rice is an ideal model plant among the monocot cereal crop species for genetic and molecular studies because of its genome compared to those of other cereals, in additional, its suitable for efficient genetic analysis and transformation (Rakwal and Agrawal, 2003). Rice is also an important protein source for humans especially in Asian countries. The demand for rice as a dietary protein source is expected to increase dramatically in the future since the world population will be doubled by 2030 (Mann, 1997). Furthermore, to feed the increasing population of the world, it is essential to improve yield and quality of rice (Khan and Komatsu, 2004). Similar to other cereals, rice seed protein is deficient in some essential amino acids (Sotelo *et al*., 1994).

Cereal crops represent approximately 50% of the plant protein consumed worldwide; however, cereal seeds are nutritionally deficient in important amino acids such as lysine (Lys), threonine (Thr) and tryptophan (Trp). This situation has presented researchers with the major task of improving the nutritive value of cereal seeds (Azevedo *et al*., 2006). Amino acids such as Thr and Lys are essential for human and monogastric animals. Thus the modification of amino acid profile of the rice grain to develop high nutritional varieties is one of the most important objectives in breeding of rice seed. This implies a demand for large numbers of analysis to determine the amino acid composition of rice grain (Jianguo *et al*., 2002). Amino acids that are produced by fermentation are often added to animal feed to improve its nutritional value. The world-wide cost of these supplemented amino acids is considerable, estimated at several billion dollars annually (Mueller and Huebner, 2003).

The essential amino acids Lys, methionine (Met), Thr and the branched chain amino acids Ile, Leu and Val are synthesized in plants and microorganisms by the aspartate

Collection @ jeju

(Asp)-derived and the branched-chain amino acid pathways, respectively. The two pathways are interconnected both in terms of carbon precursor filiations and allosteric interactions (Curien *et al*., 2008). The Asp derived amino acid pathway in plants leads to the biosynthesis of Lys, Met, Thr and Ile. These four amino acids are essential in the diets of humans and other animals, but are present in growth-limiting quantities in some of the world's major food crops (Jander and Joshi, 2009). Research interest in the biosynthesis of Asp derived amino acids is driven in part by their economic value. Major field crops, which either directly or indirectly (as animal feed) make up the majority of the diets of most human populations, are deficient in one or more of the Asp-derived amino acids. These deficiencies include Lys and Thr in cereals crops (Debadov, 2003).

An additional practical reason for studying amino acid biosynthesis pathways comes from their role as herbicide targets. The fundamental requirement of amino acids for plant survival, as well as the absence of essential amino acid biosynthesis in humans and other animals, makes the Asp-derived amino acid pathway an attractive target for herbicide development. For instance, acetolactate synthase, an enzyme in the biosynthetic pathway leading from Thr to Ile, is the target of several classes of economically important herbicides, including sulfonylureas, imidazolinones, triazolopyrimidines, and pyrimidinyl oxybenzoates (Ott *et al*., 1996). Although biosynthesis of Asp-derived amino acids has been studied in several plant species, much of the recent advances in this field have come from research conducted with *Arabidopsis thaliana.* The well-developed genetic resources available for this model plant have led to numerous new discoveries, including not only previously unknown biosynthetic enzymes, but also novel regulatory mechanisms for pathway enzymes (Jander and Joshi, 2009).

The branched chain amino acids Val, Leu and Ile are three among the ten essential amino acids that are not synthesized in mammals. Therefore, biosynthesis of branched chain amino acids in plants has interest due to their importance in animal diets including

Collection @ jeju

humans. In addition, in the early 1980s, two new classes of herbicides (imidazolinones and sulfonylureas) were introduced that inhibit the branched chain amino acid biosynthetic pathway (Levitt *et al*., 1981; Ray, 1984). These new herbicides were unique in that they control many weeds in different crops at low-use rates. The introduction of these two classes of herbicides initiated a new era in agricultural practices worldwide due to their unique mode of action, coupled with their low mammalian toxicity and high potency. The discovery and development of these herbicides and of crops resistant to them have led to an explosion in the scientific literature on biochemical, molecular, and genetic aspects of branched chain amino acid biosynthesis (Singh and Shaner, 1995).

Uridine kinase (UK) and uracil phosphoribosyltransferase (UPRT) have an important function in the pyrimidine salvage pathway. The cDNA encoding a bifunctional enzyme expressing both UK and UPRT have been identified in *Arabidopsis thaliana* (Islam *et al*., 2007). The UK activity was found in the N-terminal region whereas the UPRT activity was located in the C-terminal region. The molecular function of UK, i.e. catalyzing the formation of uridine 5'-monophosphate (UMP) from uridine and adenine 5'-triphosphate (ATP) and UPRT, i.e. catalyzing the formation of UMP from uracil and phosphoribosyl-alpha-1-pyrophosphate (PRPP), respectively was confirmed in mutants and double mutants of *E. coli*. These mutants were unable to express UK and/or UPRT activities and therefore unable to use either 5-fluorouracil (FU) or the 5-fluorouridine (FD) (Islam *et al*., 2007).

The FU and FD are toxic analogs of uracil and uridine which are substrates of UPRT and UK, respectively. These toxic activities have been well used as an anticancer agent since 1950's and are used medically to treat various cancers in the large intestine, stomach, pancreas, breast, prostate etc. (Miyagi *et al.*, 2003). The successive production of the fluro-derivatives of UMP and UTP by UMP kinase and UDP kinase results in RNA damage and inhibition to protein synthesis, which causes a broad range of growth retardation in bacterial cells (Koyama *et al*., 2003). The principle of cancer therapy is that FU and FD are

Collection @ jeju

\ toxic to metabolize fluro-derivatives of UMP and UTP, and finally cause mistakes of RNA base and gene expression especially in cancer cells if UK and UPRT are overexpressed and active in their catalytic activity (Koyama *et al.,* 2003).

Considering the above information, the present study was undertaken with the following specific objectives:

To analyze the functional complementation of rice genes as well as

- to improve rice qualities
- to target development of harbicides
- to investigate pyrimidine salvage pathway in plants

dCollection @ jeju

Part I

Functional analysis of genes encoding threonine synthase and dihydrodipicolinate synthase from rice

Collection @ jeju

ABSTRACT

Threonine synthase (TS) and dihydrodipicolinate synthase (DHDPS) are enzymes of the aspartate family pathway leading to biosynthesis of Thr and Lys in plants and microorganisms, respectively. Sequence analysis of the cDNA of those enzymes from rice revealed that they harbor a full-length open reading frame for OsTS encoding for 521 amino acids, corresponding to a protein of approximately 57.2 kDa and OsDHDPS also encoding for 380 amino acids, corresponding to a protein of approximately 41.4 kDa. The predicted amino acid sequence of OsTS and OsDHDPS are highly homologous to those of *Arabidopsis* and many bacterial respective sequences that are encoded for *thrC* and *dapA* gene, respectively. The OsTS protein harbors a signature binding motif for pyridoxal-5'-phosphate at the amino terminus. The *OsTS* expression was complemented by a *thrC* mutant strain of *E. coli*. The *OsTS* expression was correlated with the survival of the *thrC* mutant, which is affected by the supplementation of an Asp pathway metabolite, Met. Expression of *OsDHDPS* in *dapA* mutants of *E. coli* showed that the gene was able to functionally complement with the mutant. The result suggested that the OsTS and OsDHDPS encode a protein TS and DHDPS, respectively in rice.

Collection @ jeju

1. INTRODUCTION

Thr and Lys are two of ten essential amino acids that cannot be synthesized in animals and humans but must be provided in the diet for animals including humans. In contrast, plants and bacteria can synthesize Thr and Lys and they share similar biosynthetic pathways that use Asp as a precursor (Elena *et al*., 2008). The biosynthetic pathway of Thr and Lys are initiated from Asp, and is called the Asp family pathway in plants (Fig. 1). The Asp derived amino-acid pathway from plants is well suited for analyzing the function of the allosteric network of interactions in branched pathways (Curien *et al*., 2009). In plants and microorganisms, Thr and Lys synthesis are component of the multibranched biosynthetic pathway originating with Asp and resulting in the synthesis of Lys, Met, Thr and Ile (Curien *et al*., 1996). In order to increase Thr and Lys formation in different organisms by molecular techniques, information of Thr and Lys biosynthesis and availability of the functional genes are required.

Threonine synthase (TS: EC 4.2.99.2) is pyridoxal 5'-phosphate (PLP)-dependent enzyme and catalyses the final step of Thr formation (Curien *et al*., 2008; Mas-Droux *et al*., 2006). TS catalyzes the conversion of *O*-phosphohomoserine (OPH) into Thr and inorganic phosphate via a PLP dependent reaction in plants (Mas-Droux *et al*., 2006; Casazza *et al*., 2000). The TS activity has been identified, purified and described in a variety of microorganisms, such as *Neurospora crassa* (Flavin and Slaughter, 1960), *E. coli* (Farrington *et al*., 1993) and *Corynebacterium glutamicum* (Eikmanns *et al*., 1993), *Crytococcus neoformans* (Kingsbury and McCusker, 2008), *Streptococcus sp.* (Tang *et al*., 2007) and *Mycobacterium tuberculosis* (Covarrubias *et al*., 2008). The corresponding gene was isolated from a number of bacteria (Han *et al*., 1990; Clepet *et al*., 1992). The characterization and analysis of several plant genes have been reported, including those of *Arabidopsis thaliana* (Curien *et al*., 1998; Avraham and Amir, 2005; Lee *et al*., 2005), *Solanum tuberosum* L (Casazza *et al*., 2000) and *Sorghum bicolor* (Ferreira *et al*., 2006).

Collection @ jeju

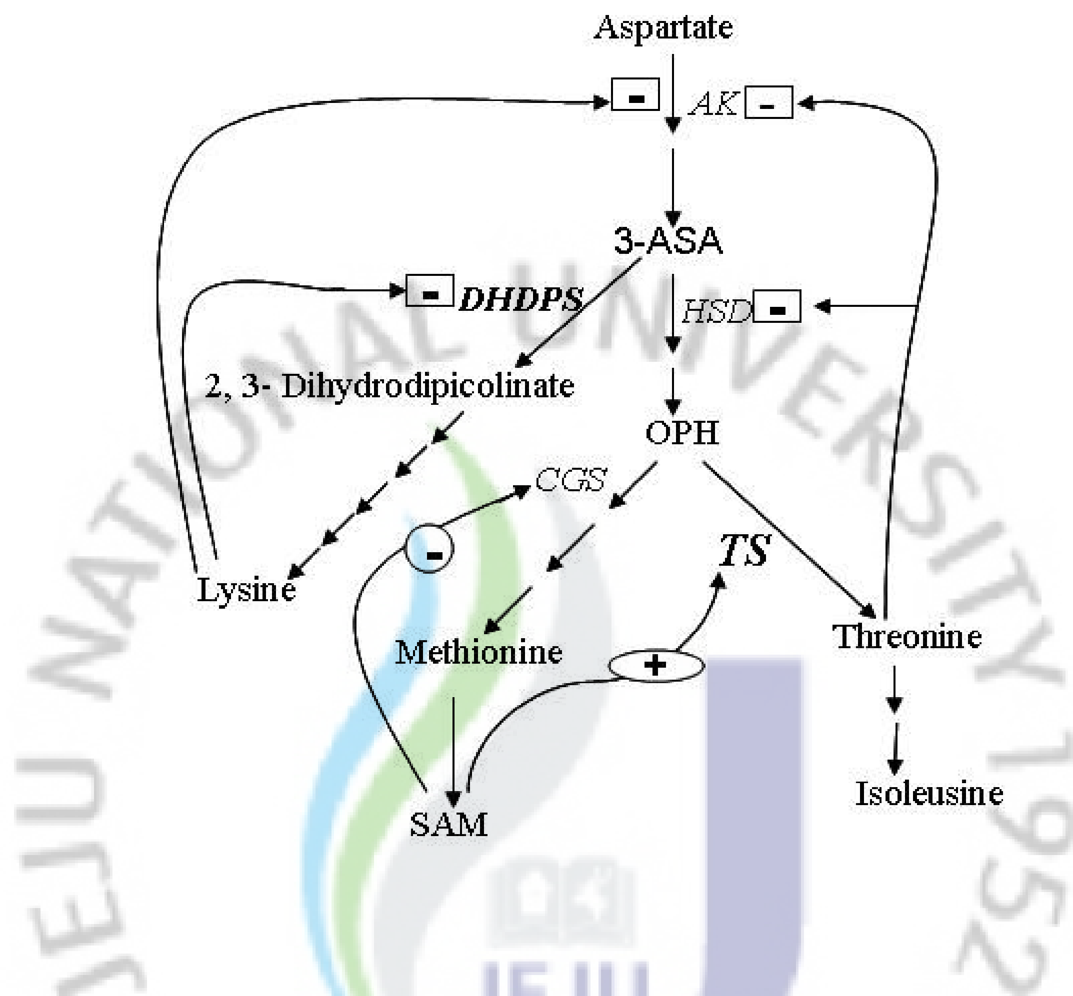


Fig. 1. Scheme of the Thr and Lys biosynthesis pathway of Asp family in plants. The abbreviations are AK, aspartate kinase; 3-ASA, 3-aspartic semialdyde; HSD, homoserine dehydrogenase; OPH, *O*-phosphohomoserine; SAM, *S*-adennosylmethionine, CGS, cystathionine γ-synthase; TS, threonine synthase; TDH, threonine dehydratase. Symbols are indicated: ⊕ ; allosteric activation, ⊖; feedback repression and ⊟; feedback inhibition.

DHDPS (EC.4.2.1.52) is the unique enzyme in that DHDPS catalyzes the first step

Collection @ jeju

specific to Lys synthesis in the pathway for biosynthesis of Asp derived amino acids including Thr, Met and Ile. Higher plants are supposed to synthesize Lys via the diaminopimlate pathway (Bryn, 1980). The gene encoding DHDPS has been cloned and characterized in many microbes and plants such as: *E. coli* (Emma *et al*., 1995; Dobson *et al*., 2005), *Bacillus licheniformis* (Halling and Stahly, 1976), *Thermotoga maritima* (Pearce *et al*., 2006), *Coix lacryma-jobi* (Ricardo *et al*., 1991), *Spinacia oleracea* (Wallsgrove and Mazelis, 1981), *Triticum aestivum* cell suspension culture (Kumpaisal *et al*., 1987), *Glycine max* (silk *et al.,* 1994), *Nicotiana tabacum* (Ghislain *et al.,* 1990), *Zea mays* (David *et al*., 1991), *Arabidopsis thaliana* (Marc *et al*., 1999) and *Corynebacterium glutamicum* (Elena *et al*., 2008).

The synthesis of Asp derived amino acids is subject to complex regulation. The key to pathway control of the pathway is feedback inhibition to aspartate kinase (AK) by Lys and/or Thr, or by Lys in concert with S-adenosylmethionine (SAM) (Rinder *et al*., 2008). Ak, the first enzyme in the pathway, is inhibited allosterically by Lys and Thr (Lee *et al*., 2005). TS competes with the first enzyme required for subsequent Met biosynthesis, cystathionine-γ-synthase (CGS), for their common substrate OPH (Thompson *et al*., 1982). TS enzyme activity is activated by SAM and inhibited by cysteine (Curien *et al*., 1996). SAM is, in turn, directly synthesized from Met; therefore, increasing Met levels will result in increases in the concentration of SAM and subsequently affect TS activity (Casazza *et al*., 2000).

There is also a Lys binding site in DHDPS located in the cleft at the tight dimmer interface with one Lys molecule binding per monomer (Blickling *et al*., 1997). The enzyme is particularly sensitive to Lys feedback inhibition (Galili, 1995). Transgenic plants expressing a mutant's form of DHDPS less sensitive to Lys feedback inhibition accumulate free Lys (Falco *et al*., 1995). Additionally, the positive correlations shown between DHDPS activity or DHDPS protein level and free Lys content suggest that the amount of the

enzyme may influence Lys accumulation (Falco *et al*., 1995). Many antibiotics or

Collection @ jeju

herbicides for killing microorganism or plants, respectively, are targeted to a specific enzyme in amino acid biosynthesis (Kelland *et al.,* 1986). The insensitivity of DHDPS enzyme activity from gram-positive bacteria to Lys inhibition has been used to develop maize plants that accumulate increased levels of free Lys in grain (Huang *et al*., 2005). Here, the report the analysis and characterization of two genes for the TS and DHDPS enzymes from rice, an important crop plant.

JEJU NATIONAL UNIVERSITY 1952
JEJU
1952
제주대학교

Collection @ jeju

2.1 MATERIALS AND METHODS

2. 1. Strains

Four *E. coli* strains were used in this part are presented in Table 1. The source of all strains was the *E. coli* Genetic Stock Center (CGSC) at Yale University, USA.

Table1. *E. coli* strains used in the part I

Name	Genotype	Remark
Gif41	***thrC**1001,λ14-, e14-, relA1, spoT1, th-1 thi-1*	Thr mutant
AT997	*hfr (PO45), LAM-, e14-, **dapA**15, relA1, spoT1, thi-1*	Lys mutant
Sφ415	*udk-2,upp-11,rclA1,rpsL254(strR),metB1*	wild type
ES4	*f-, fhuA2, lacY1 or lacZ4, tsx-1 or tsx-70, glnV44 (AS), gal-6, LAM-, mtlA2, purA45*	wild type

Collection @ jeju

2. 2. DNA sequence analysis

Two EST clones (Genbank accession no. AK101669 and AK071042) used were obtained from the Rice Genome Resource Center (RGRC), National Institute of Agrobiological Science (NIAS), Japan. The clones were derived from a rice cDNA library (Osato *et al*., 2002) from developing seeds prepared in pBluescript SK-. DNA sequencing was conducted using an automatic sequencer (A1Fexpress DNA sequencer, Pharmacia Biotech. Inc., UK) with synthetic oligonucleotide primers. Nucleotide sequences and amino acid sequences were compared with the sequences in the GenBank and EMBL databases and analyzed via BLAST (Wheeler *et al*., 2003) and the ClustalW 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). 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 Phylogenic tree with bootstrap value prepared by the Mega 4.1 neighbor-joining program (Kumer *et al*., 2008).

2. 3. Polymerase chain reaction (PCR) and recombinant constructs

OsTS and OsDHDPS sequence analysis showed an open reading frame. Therefore, the specific primers were designed from the sequence information around the translational start and stop codons of OsTS and OsDHDPS to amplify the full-length open reading frame (ORF) and to over express the gene product in *E. coli*. Polymerase chain reaction (PCR) (Sambrook and Russell, 2001) was conducted to amplify the full-length ORF after the ESTs were purified from a pellet harvested from a liquid culture containing Amp.

The ORF of OsTS was amplified from the EST clone as a template, and the following primers were designed from the *OsTS* sequence: OsTS-F (5'- AAA GCTTTCACTCACTCCCTAAAACCC-3') and *OsTS*-R (5' AAAGCTTCACAC TTCAGAGCTTACCCT -3') using AmpliTaqGold polymerase (Perkin-Elmer, U.S.A). The

Collection @ jeju

underlined bases in the OsTS-F and OsTS-R primers are the designed restriction sites for *Hin*dIII to facilitate subcloning, respectively. The PCR was conducted using a MYCyler™ PCR system (BioRad, U.S.A) for 35 cycles with 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with 10 μM primers. The PCR products were analyzed on 1% (w/v) agarose gel. The amplified fragment (1540 bp) was then sub cloned into pGEM-T-easy vector (Promega) and finally sub cloned into pBluescript II KS+ (Stratagene Inc., U.S.A) as a *Hin*dIII fragment, to give *pB::OsTS*.

The cDNA of OsDHDPS was amplified by using designed primers from *OsDHDPS* sequence: *OsDHDPS*-F (5'- AGGATCCAACCCTAGTCCGTTCTT TCTCCA -3') and *OsDHDPS* -R (5'- AGGATCCCATCAACGTACATGGGACTT GCA -3'). The PCR was performed using MY Cyler TM PCR system (BioRad, U.S.A) for 45 cycles with 95°C for 1 min, 45°C for 1 min, and 72°C for 1 min, with 10 μM primers. PCR products were analyzed on 1% (w/v) agarose gel. The 1140 bp PCR fragment was subcloned into a pMPM-K2 cloning vector and digested with *Bam*HI and inserted into the same site ofpBluescript II KS+ to give *pB::OsDHDPS*. Restriction analysis was conducted effort to confirm the recombinant DNA construct of *pB::OsTS* and *pB::OsDHDPS* with the right orientation for over expression.

Collection @ jeju

2. 4. Functional complementation and growth assay

The competent *thrC and dapA* mutants of the Gif41 and AT997 *E. coli* strain were transformed with *pB::OsTS* and *pB::OsDHDPS* via electroporation (ECM399, BTX, USA) using a cuvette with a 0.1 cm electrode gap, then plated on LB medium (20 g/L) with Amp (100 μg/ml). The growing culture was tested separately, for growth retardation in MM containing Amp (25 μg/ml), 20% glucose, 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and with 19 amino acids (Sigma, Germany) each at a concentration of 25 μg/ml, excluding Thr and, a second, excluding Lys. Bacterial growth was then assessed by measuring OD at 595 nm (OD_{595}) at one-hour intervals and after 12 hrs; the diluted culture was plated and incubated overnight at 37°C.

2. 5. Growth inhibition assay in *E. coli* with OsTS and OsDHDPS

The *thrC* mutant *E. coli* harboring the *pB::OsTS* construct, control vector plasmid and wild type with control vector plasmid were grown at 37°C in MM with IPTG (0.1 M), 20% glucose (20 ml/L), containing 19 amino acids and Amp (25 μg/ml), excluding Thr and the same medium was used with all the reagents kept constant, but an additional supplementation of 10-fold high Met. Similarly, *pB::OsDHDPS* construct with *dapA* mutant *E. coli* strain and control plasmid as well as wild type were grown at the same medium and 19 amino acids excluding Lys. The respective bacterial growth was monitored via OD measurements every hour using a spectrophotometer (UV1101, Biochrom, England) at 595 nm (OD_{595}).

Collection @ jeju

3. RESULTS

3. 1. Sequence analysis of OsTS and OsDHDPS

Two expressed sequence tag (EST) clones (GenBank Accession number AK101669 and AK071042) obtained from the Rice Genome Resource Center (RGRC) were analyzed to determine the nucleotide sequence using the designed primers. The cDNA (*OsTS)* sequence harbored a full-length open reading frame consisting of 1563 bp, encoding for a protein of approximately 57.2 kDa and the cDNA (*OsDSDPS*) consisting of 1140 bp, encoding 41.4 kDa The expected isoelectric point (pI) of the proteins were 6.60 and 5.99. Data analysis revealed that the OsTS and OsDHDPS sequence were identical to the genomic region located in chromosome V and IV, respectively. Comparisons of the amino acid sequence of the OsTS and the homologous sequences from maize (*Zea mays*) and arabidopsis (*A. thaliana*) revealed high identity, at 91% and 71%, respectively (Fig. 2).

Analysis of the OsTS amino acid sequence revealed a signature binding motif for PLP in the N-terminal region (189-203) (Fig. 2). The motif sequence (HCGISHTGSFKDLGM) was highly homologous to the consensus [DESH]-x (4, 5)-[STVG]-{EVKD}-[AS]-[FYI]-K-[DLIFSA]-[RLVMF]-[GA]-[LIVMGA], where the underlined amino acids were well conserved. The binding motif for PLP is present in bacterial TSs and serine/threonine dehydratases that utilize PLP as a cofactor. The exact PLP binding site seemed to be K-199, and was identified via comparison with the binding site of bacterial TS. This result indicates that the OsTS product utilizes PLP as a co-factor.

Collection @ jeju

```
OsTS     1  MAATTHAASLSFLLSHPHPTSPNPNPNPNLPLRRAPHRVRCATDAAATRH
AtTS2    1  MASFSLPHSATYFPSHS-ETSLKPHSAASFTVR-CTSASPAVPPQTPQKP
EcTS     1  ---------------------------------------------MKLYN

OsTS    51  RRAADENIREEAARHRAPNHNFSAWYAPFPPAPNGDPDERYSLDEIVYRS
AtTS2   49  RRSPDENIRDEARRRPHQLQNLSARYVPFNAPPS--STESYSLDEIVYRS
EcTS     6  LKDHNEQVSFAQAVTQGLGKNQ-GLFFPHDLP-------EFSLTEIDEML

OsTS   101  SSGGLLDVRHDMDALARFPGSYWRDLFDSRVGRTTWPFGSGVWSKNEFVL
AtTS2   97  QSGALLDVQHDFAALKRYDGEFWRNLFDSRVGKTNWPYGSGVWSKKEWVL
EcTS    48  K---LDFVTRSAKILSAFIGDEIPQEILEERVRAAFAFPAPVAN----VE

                                                PLP binding site
OsTS   151  PEIDPDHIVSLFEGNSNLFWAERLGRDHLAGMNDLWVKHCGISHTGSFKD
AtTS2  147  PEIDDDDIVSAFEGNSNLFWAERFGKQYLQ-MNDLWVKHCGISHTGSFKD
EcTS    91  SDVG---CLELFHG-----------------------------PTLAFKD

OsTS   201  LGMTVLVSQVNRLRRAPLSRPIAGVGCASTGDTSAALSAYCAAAGIPAIV
AtTS2  196  LGMSVLVSQVNRLR--KMNKPVIGVGCASTGDTSAALSAYCASAGIPSIV
EcTS   109  FGGRFMAQMLTHIAG---DKPVTILTATSGDTGAAVAHAFYGLPNVKVVI

OsTS   251  FLPANRIS--LEQLIQPIANGATVLSLDTDFDGCMRLIR------EVTAE
AtTS2  244  FLPADKIS--MAQLVQPIANGAFVLSIDTDFDGCMHLIR------EVTAE
EcTS   156  LYPRGKISPLQEKLFCTLGGNIETVAIDGDFDACQALVKQAFDDEELKVA

OsTS   293  LPIYLANSLNSLRLEGQKTAAIEILQQFDWEVPDWVIV--PGGNLGNIYA
AtTS2  286  LPIYLANSLNSLRLEGQKTAAIEILQQFNWQVPDWVIV--PGGNLGNIYA
EcTS   206  LGLNSANSINISRLLAQICYYFEAVAQLPQETRNQLVVSVPSGNFGDLTA

OsTS   341  FYKGFEMCRVLGLVDRVPRLVCAQAANANPLYRYYKSGWTE-FTPQVAEP
AtTS2  334  FYKGFHMCKELGLVDRIPRLVCAQAANANPLYLHYKSGFKEDFNPLKANT
EcTS   256  G----LLAKSLGLP--VKRFIAATNVNDTVPRFLHDG----QWSPKATQA

OsTS   390  TFASAIQIGDPVSVDRAVVALKATDGIVEEATEEELMNAMSLADRTGMFA
AtTS2  384  TFASAIQIGDPVSIDRAVYALKKSNGIVEEATEEELMDATALADSTGMFI
EcTS   296  TLSNAMDVSQPNNWPRVEELFRRKIWQLKELGYAAVDDETTQQTMRELKE

OsTS   440  CPHTGVALAALFKLRDQRIIGPNDRTVVVSTAHGLKFSQSKIDYHDSKIE
AtTS2  434  CPHTGVALTALMKLRKSGVIGANDRTVVVSTAHGLKFTQSKIDYHSKNIK
EcTS   346  LGYTSEPHAAVAYRALRDQLNPGEYGLFLGTAHPAKFKES-VEAILGETL

OsTS   490  DMACKYAN--PPVSVKADFGAVMDVLKKRLKGKL
AtTS2  484  EMACRLAN--PPVKVKAKFGSVMDVLKEYLKSNDK
EcTS   395  DLPKELAERADLPLLSHNLPADFAALRKLMMNHQ
```

Fig. 2. Amino acid sequence alignment of TS from *Oryza sativa* (OsTS), *Arabidopsis thaliana* (AtTS2), and *E. coli* (EcTS). Shaded residues represent amino acids that are identical among all sequences of the three amino acids. GenBank accession numbers; AK101669 (OsTS), Q9SSP5 (AtTS2) and NP_414545 (EcTS).

Collection @ jeju

A sequence comparison of the predicted amino acids for the OsDHDPS with the deduced sequence from maize (*Zea mays*), arabidopsis (*A. thaliana*) and *E. coli* showed high homology with an identity of 84%, 74%, and 33% respectively.

Analysis of the amino acid sequence of OsDHDPS revealed signature motifs in the N- terminal region (116-133) and middle region (209-239) positions. The DHDPS_1 motif sequence (GVIVGGTTGEGHLMSWDE) was highly homologous to the consensus [GSA]-[LIVM]-[LIVMFY]-x(2)-G-[ST]-[TG]-G-E-[GASNF]-x(6)-[EQ], where the underlined amino acids are well conserved (Fig. 3). Another signature motif exists in the middle region (YNVPSRTGQDIPPAVIEAVSSFTNLAGVKEC) of the OsDHDPS (Fig. 3). The DHDPS_2 motif sequence (*OsDHDPS*) for DAP is highly homologous to the consensus [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 motif is related to DHDPS signature 2.

Phylogenetic tree derived from the related sequence indicated that OsTS and OsDHDPS are divergent and evolved from ancestor bacterial TS and DHDPS. Branching pattern and numbers at nodes indicate levels of bootstrap value support based on neighbor-joining analysis of 1,000 re-sample data sets. Numbers on branches are percentages of bootstrap analyses supporting the grouping of each branch (Fig. 4 and Fig. 5).

Collection @ jeju

```
OsDHDPS      1  MASLL-IASTGGAHRLAWKDAAALGPAPRLARPWPAAVAAPAPLLRISRG
BsDHDPS      1  --------------------------------------------------
EcDHDPS      1  --------------------------------------------------
AtDHDPS_1    1  MSALKNYGLISIDSALHFPRSNQLQSYKRRNAKWVSPIAAVVPNF-----

OsDHDPS     50  KFALQAITLDDYLPMRSTEVKNRTSTADITSLRVITAVKTPYLPDGRFDL
BsDHDPS      1  ----------------------------MNFGNVSTAMITPFDNKGNVDF
EcDHDPS      1  -----------------------------MFTGSIVAIVTPMDEKGNVCR
AtDHDPS_1   46  -----------HLPMRSLEDKNRTNTDDIRSLRVITAIKTPYLPDGRFDL

                                DHDPS signature I
OsDHDPS    100  EAYDSLINMQIDGGAEGVIVGGTTGEGHLMSWDEHIMLIGHTVNCFGAKV
BsDHDPS     23  QKLSTLIDYLLKNGTDSLVVAGTTGESPTLSTEEKIALFEYTVKEVNGRV
EcDHDPS     22  ASLKKLIDYHVASGTSAIVSVGTTGESATLNHDEHADVVMMTLDLADGRI
AtDHDPS_1   85  QAYDDLVNTQIENGAEGVIVGGTTGEGQLMSWDEHIMLIGHTVNCFGGRI

OsDHDPS    150  KVVGNTGSNSTREAIHATEQGFAVGMHAALHINPYYGKTSIEGLISHFEA
BsDHDPS     73  PVIAGTGSNNTKDSIKLTKKAEEAGVDAVMLVTPYYNKPSQEGMYQHFKA
EcDHDPS     72  PVIAGTGANATAEAISLTQRFNDSGIVGCLTVTPYYNRPSQEGLYQHFKA
AtDHDPS_1  135  KVIGNTGSNSTREAIHATEQGFAMGMHGALHINPYYGKTSIEGMNAHFQT

                                 DHDPS signature II
OsDHDPS    200  VLPMG--PTIIYNVPSRTGQDIPPAVIEAVSS-FTNLAGVKECVG----H
BsDHDPS    123  IAAETSLPVMLYNVPGRTVASLAPETTIRLAADIPNVVAIKEASGDLEAI
EcDHDPS    122  IAEHTDLPQILYNVPSRTGCDLLPETVGRLAK-VKNIIGIKEATGNLTRV
AtDHDPS_1  185  VLHMG--PTIIYNVPGRTCQDIPPQVIFKLSQ-NPNMAGVKECVG----N

OsDHDPS    243  ERVKCYTDKGITIWSGNDDECHDSRWKYGATGVISVASNLIPGLMHDLMY
BsDHDPS    173  TKIIAETPEDFYVYSG-DDALTLPILSVGGRGVVSVASHIAGTDMQQMIK
EcDHDPS    171  NQIKELVSDDFVLLSG-DDASALDFMQLGGHGVISVTANVAARDMAQMCK
AtDHDPS_1  228  NRVEEYTEKGIVVWSGNDDQCHDSRWDHGATGVISVTSNLVPGLMRKLMF

OsDHDPS    293  EGEN------KTLNEKLFPLMKWLFCQPNPIALNTALAQLGVVRP-VFRL
BsDHDPS    222  NYTNGQTANAALIHQKLLPIMKELFKAPNPAPVKTALQLRGLDVG-SVRL
EcDHDPS    220  LAAEGHFAEARVINQRLMPLHNKLFVEPNPIPVKWACKELGLVATDTLRL
AtDHDPS_1  278  EGRN------SALNAKLLPLMDWLFQEPNPIGVNTALAQLGVARP-VFRL

OsDHDPS    336  PYVPLPLEKRVEFVRIVESIGRENFVGENEARVLDDDDFVLVSRY
BsDHDPS    271  PLVPLTEDERLSLSSTISEL
EcDHDPS    270  PMTPITDSGRETVRAALKHAGLL
AtDHDPS_1  321  PYVPLPLSKRIEFVKLVKEIGREHFVGDRDVQVLDDDDFILIGRY
```

Fig. 3. Amino acid sequence alignment of DHDPSs using Box shade program after CLUSTALW alignment. Amino acids that are identical among at least 4, 3 of the 4 amino acids residues are visually shown as yellow, green and cyan, respectively. 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*).

dCollection @ jeju

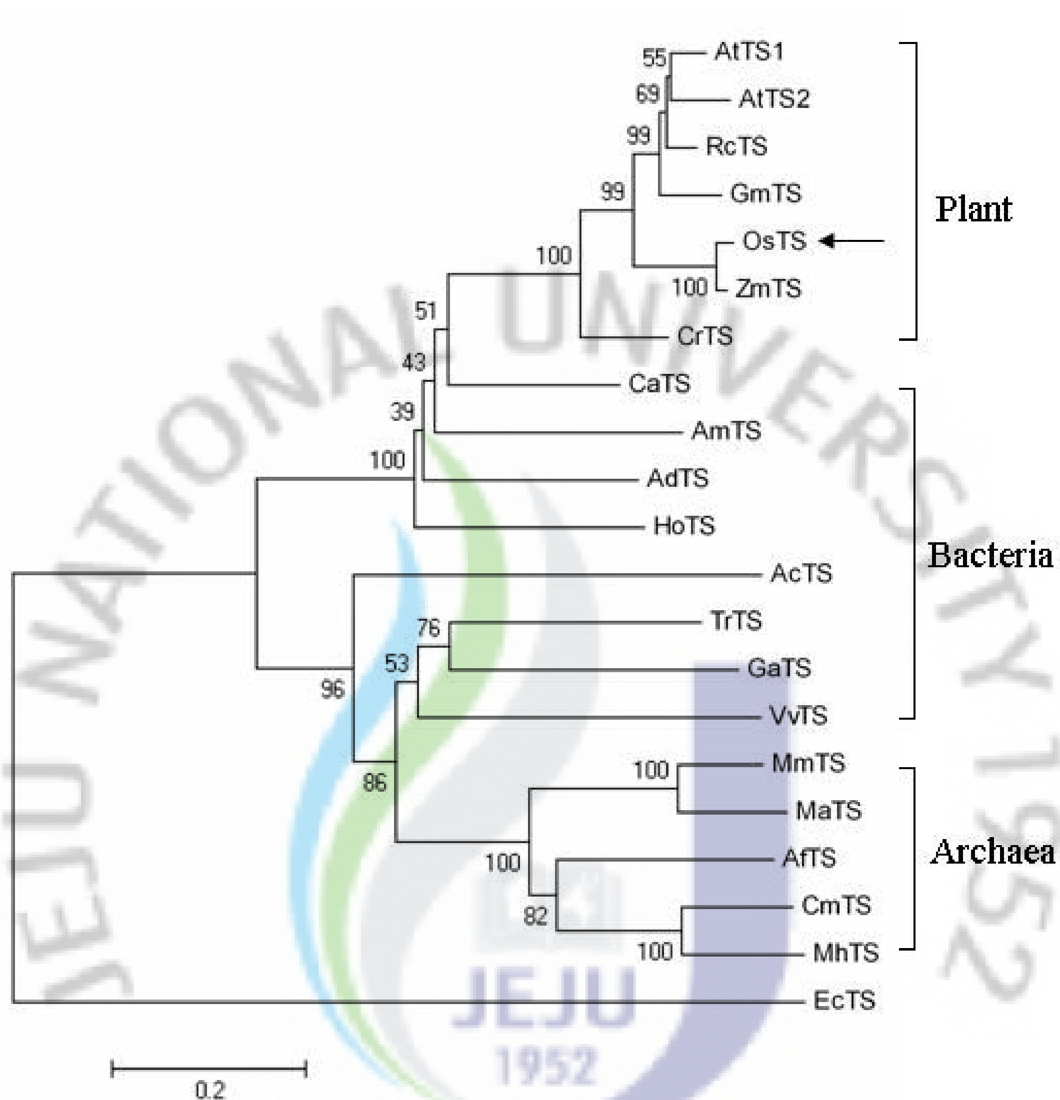


Fig. 4. Phylogenetic analysis of OsTS related proteins using ClustalW and Mega 4.1 neighbor-joining program. Accession numbers are as follows: AK101669 (OsTS from *Oryza sativa*), XP_002514088 (RcTS; *Ricinus communis*), Q9S7B5 (AtTS1; *Arabidopsis thaliana*), Q9SSP5 (AtTS2; *Arabidopsis thaliana*), ABC00741(GmTS; *Glycine max*), ACG39080 (ZmTS; *Zea mays*), XP_001698517 (CrTS; *Chlamydomonas reinhardtii*), YP_001515596 (AmTS; *Acaryochloris marina*), YP_002463167(CaTS; *Chloroflexus aurantiacus*), YP_003264969 (HoTS; *Haliangium ochraceum*), YP_002492618 (AdTS; *Anaeromyxobacter dehalogenans*), YP_002753372 (AcTS; *Acidobacterium capsulatum*),

Collection @ jeju

YP_002522459 (TrTS: *Thermomicrobium roseum*), YP_002760880 (GaTS; *Gemmatimonas aurantiaca*), ZP_01923848 (VvTS; *Victivallis vadensis*), YP_001330351 (MmTs; *Methanococcus maripaludis*), NP_070145 (AfTS; *Archaeoglobus fulgidus*), YP_002466596 (CmTS; *Candidatus Methanosphaerula*), YP_503069 (MhTS; *Methanospirillum hungatei*) and NP_414545 (*E. coli*).

Collection @ jeju

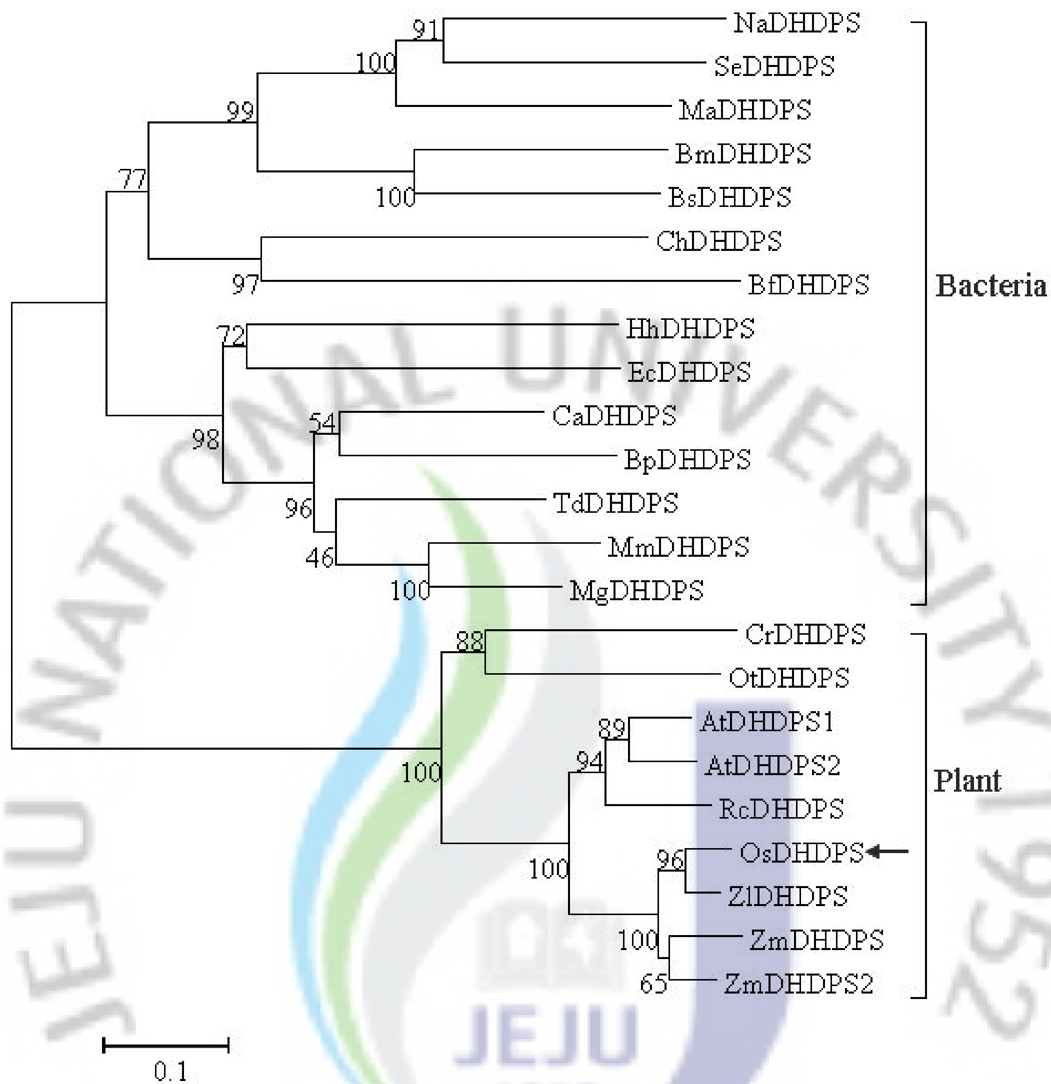


Fig. 5. Phylogenetic analysis of OsDHDPS related proteins using ClustalW and Mega4.1 neighbor-joining program. Accession numbers are as follows: AK071042 (OsDHDPS from *Oryza sativa*), ZP_03766378 (NaDHDPS from *Nostoc azollae*), YP_172957 (SeDHDPS from *Synechococcus elongatus*), YP_001661282 (MaDHDPS 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

Collection @ jeju

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 (AtDHDPS from *Arabidopsis thaliana* 1), AAG28565 (AtDHDPS from *Arabidopsis thaliana* 2), XP_002521713 (RcDHDPS from *Ricinus communis*), ABE28526 (ZlDHDPS from *Zizania latifolia*), NP_001105425 (ZmDHDPS from *Zea mays*), NP_001148623 (ZmDHDPS from *Zea mays*2)

3. 2. *OsTS* and *OsDHDPS* expression in *E. coli* and *in vivo* activity

Collection @ jeju

The recombinant DNA, *pB::OsTS* and *pB::OsDHDPS*, were constructed using respective ORF of a PCR-amplified *OsTS* and *OsDHDPS* fragment. After the transformation of *E. coli* with the recombinant DNA, OsTS activity was monitored *in vivo* in a medium containing IPTG and 19 amino acids, excluding Thr and in case OsDHDPS, excluding Lys. Functional complementation was performed separately, using the TS and DHDPS mutant of *E. coli* to confirm the enzyme activity of the gene product of *OsTS* and *OsDHDPS*. To assess the viability of *E. coli* cells by OsTS and OsDHDPS activity, the OsTS expressing cells were cultured for 12 hrs with shaking, and the diluted portion was plated on agar medium containing the 19 amino acids and Amp (25 mg/ml) without Thr and another time, the *OsDHDPS* expressing cells were cultured for 12 hrs with shaking, and the diluted solution was plated on agar medium containing the 19 amino acids and Amp (25 mg/ml) without Lys (Fig. 6). The viable colonies greatly differed among the plasmids. The *thrC* mutant of *E. coli* with OsTS could grow under conditions in which the mutant without OsTS could not. This showed that the OsTS was capable to complement as an evidence of functional TS activity. Similar results showed in case of OsDHDPS.

Collection @ jeju

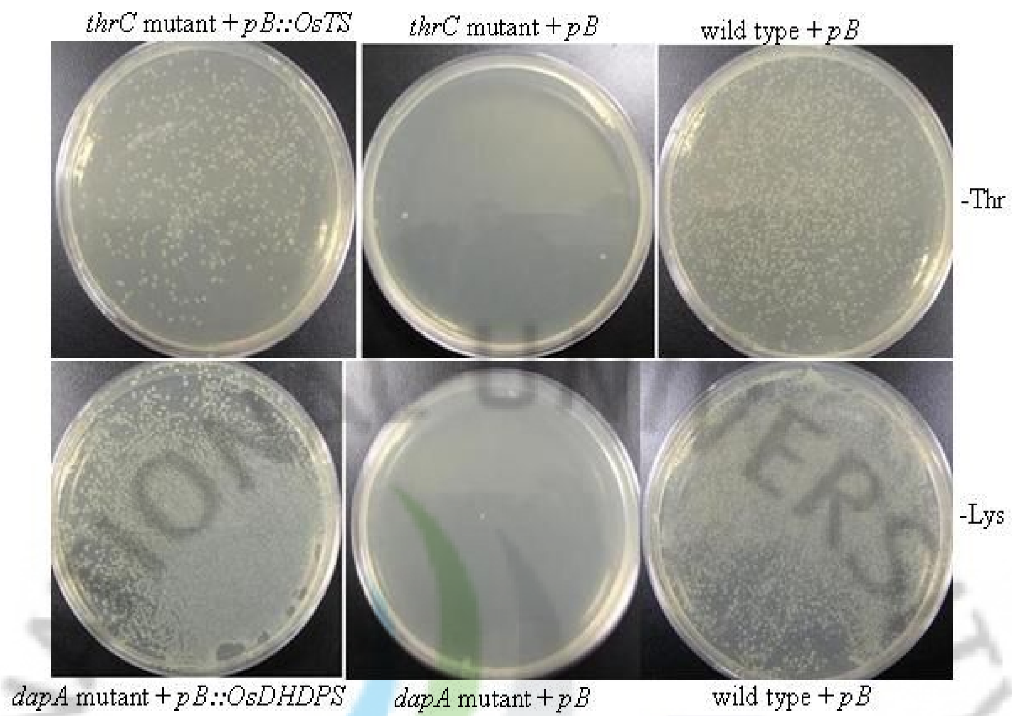


Fig. 6. Functional complementation assay for OsTS and OsDHDPS. The *thrC* mutant *E. coli* strain Gif41 containing *pB::OsTS* and control plasmid and Sφ415 wild type *E. coli* containing control plasmid (upper line). The *dapA* mutant *E. coli* strain AT997 containing *pB::OsDHDPS* and control and wild type *E. coli* containing control plasmid (lower line).

Collection @ jeju

3. 3. Expression of *OsTS* can complement the *thrC* mutant of *E. coli*

A growth study was performed to determine whether the *OsTS* gene would increase the sensitivity of bacterial cells to Thr. The *pB::OsTS* construct was transformed into the *thrC* mutant *E. coli* strain Gif41. A control plasmid was also transformed into wild type strain (Sφ415) and the *thrC* mutant Gif41. The *pB::OsTS* activity was monitored via a growth assay in the absence of Thr. Bacterial cells were grown in MM with 19 amino acids excluding Thr, containing IPTG and Amp. The wild type *E. coli* strain Sφ415 harboring the control plasmid grew normally and evidenced an S-shaped classical growth curve in the medium without Thr (Fig. 7A). The Sφ415 strain could synthesize Thr itself, and thus grew normally in the medium. The *thrC* mutant strain Gif41 expressing *pB::OsTS* also grew normally and evidenced an S-shaped classical growth curve in the same medium, but grew slightly more slowly than the wild type strain containing the control plasmid (Fig. 7A). The Gif41 strain harboring the control plasmid in the same medium without Thr evidenced dramatically retarded growth. In this case, the *thrC* mutant *E. coli* strain Gif41 could not synthesize Thr itself, and thus it was not grow; however, the same *E. coli* strain Gif41 containing *pB::OsTS* grew well because the *thrC* mutant *E. coli* strain was able to synthesize Thr using TS expressed by the *pB::OsTS* plasmid (Fig. 7A). This is a consequence of *pB::OsTS* activity. From the above finding, it was concluded that *OsTS* expression can functionally complement the *thrC* mutant *E. coli*.

Collection @ jeju

3. 4. The Growth of the *thrC* mutant of *E. coli* was influenced by the expression of *OsTS* in high levels of Met

The growth pattern of the *thrC* mutant of *E. coli* complemented with *pB::OsTS* was also assessed in the presence of high Met levels. The wildtype *E. coli* strain Sφ415 harboring the control plasmid grew normally and evidenced an S-shaped classical growth curve in MM with 19 amino acids (excluding Thr, containing 1 mM IPTG and supplemented with additional 10-fold high Met). The *E. coli* strain Gif41 grew and evidenced an S-shaped classical growth curve in the same medium, but the growth pattern was much more vigorous than in the medium without Met (Fig. 7B). In this case, when a high level of Met was added, the Met was converted to SAM and the SAM allosterically activated TS activity--this is why the *thrC* mutant of *E. coli* grew so vigorously. This result is consistent with previously reported results in studies of bacteria and plants (Curien *et al*., 1996; Casazza *et al*., 2000; Ferreira *et al*., 2006). The principal feature of plant TS, in contrast to its bacterial counterpart, may be allosteric regulation by SAM, which induces a dramatic stimulation of TS activity (Hesse *et al*., 2004). However, the Gif41 strain harboring the control plasmid also evidenced dramatically retarded growth in the same medium owing to a lack of Thr, even when 10-fold high Met was added (Fig. 7B). This finding indicates that Met has a marked influence on *OsTS* activity in rice plants.

Collection @ jeju

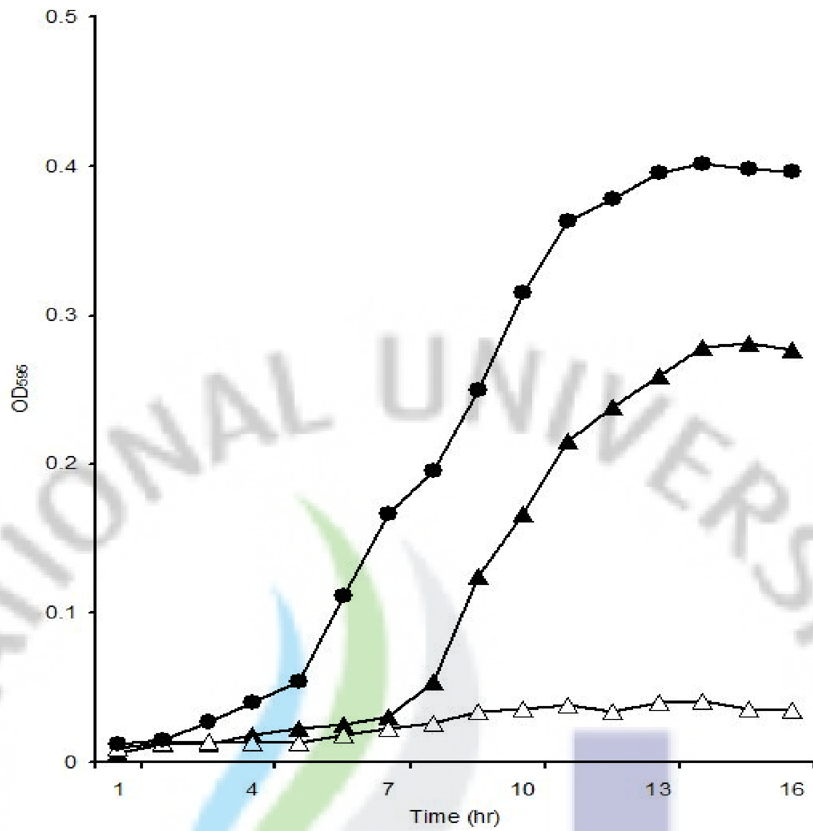


Fig. 7A. Growth curves of *E. coli* mutant Gif41 and sφ415 harboring *OsTS* without Met (A). Bacterial cells were grown at 37°C in MM containing 19 amino acids except Thr. Growth was monitored via optical density measurements at 595 nm (OD_{595}). Symbols: ▲, Gif41+ *pB::OsTS*; ●, Sφ415+ control; Δ, Gif41+ control

dCollection @ jeju

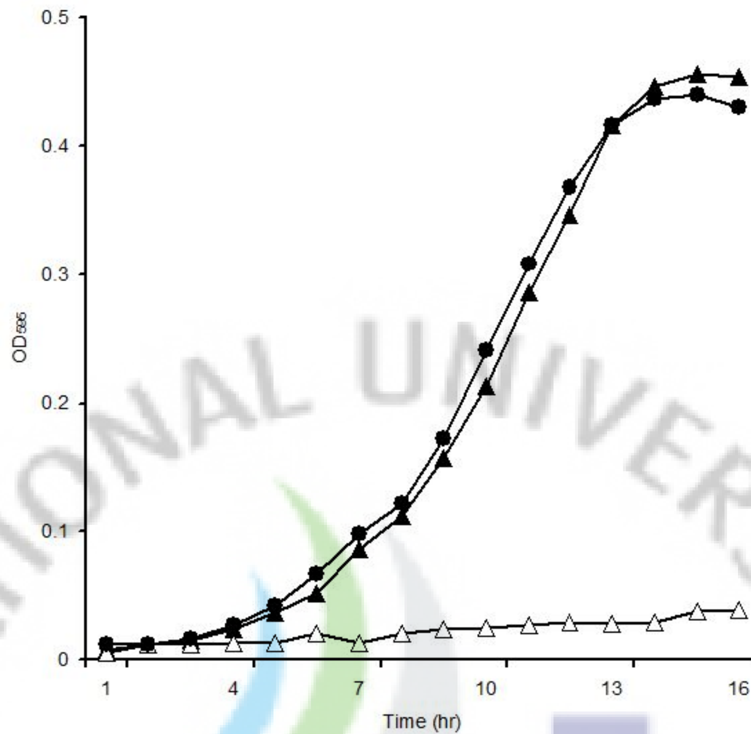


Fig. 7B. Growth curves of *E. coli* mutant Gif41 and sφ415 harboring *OsTS* and supplementing Met. Bacterial cells were grown at 37°C in MM containing 19 amino acids except Thr in which the same medium was supplemented with an additional 10 times high Met. Growth was monitored via optical density measurements at 595 nm (OD_{595}). Symbols: ▲, Gif41+ *pB::OsTS*; ●, Sφ415+ control; Δ, Gif41+ control

dCollection @ jeju

3. 5. Lysine sensitivity of *E. coli* mutants was influenced by the expression of *OsDHDPS*

A growth study was performed to determine whether the *OsDHPDS* gene would increase the sensitivity of bacterial cells to Lys. The *pB::OsDHDPS* construct was transformed into *dapA* mutant *E. coli* strains AT997. The others two control plasmids were also transformed into wild type strain (ES4) and *dapA* mutant strain AT997. The *pB::OsDHDPS* activity was monitored by the growth assay in the absence of Lys. Bacterial cells were grown in MM with 19 amino acids excluding Lys, containing IPTG and Amp. The wild type *E. coli* strain *ES4* harboring control plasmid grew normally and showed S-shape classical growth curve in the medium in spite of lack of Lys amino acid (Fig. 8). The *ES4* strain had produced Lys itself; that's why it grew normally in the medium. However, the *dapA* mutant strain AT997 expressing *pB::OsDHDPS* also grew normally and showed S-shape classical growth curve in the same medium (Fig. 8). When the mutant strain AT997 harboring control then it was dramatically retarded in growth phase in the same medium due to lack of the essential amino acid Lys. In this case, the *dapA* mutant *E. coli* strain AT997 could not produce Lys itself, so it showed dramatically retarded in growth phase in the same medium but when the same mutant strain containing *OSDHDPS* then grew well because *dapA* mutant strain got Lys from rice plasmid and rapid growth resulted (Fig. 8). This is an outcome of *OsDHDPS* activity. From the above situation, it was concluded that the expression of the *OsDHDPS* is able to functionally complement and it has the function of Lys.

Collection @ jeju

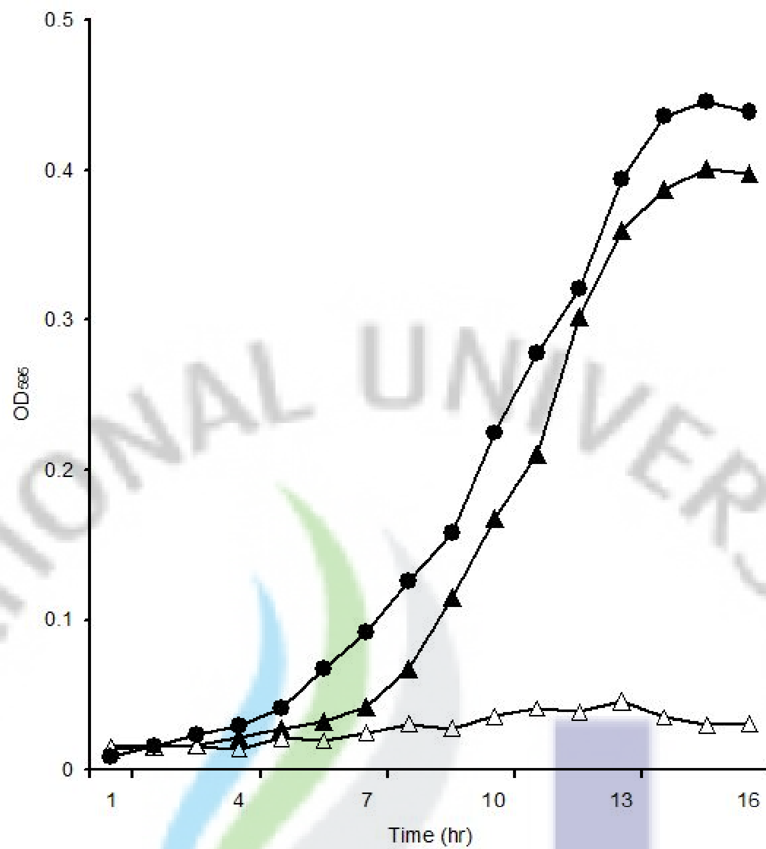


Fig. 8. Growth curves of *dapA* mutant *E. coli* strain AT997 harboring *pB::OsDHDPS* and control plasmid. Wild type strain was harboring control plasmid. Bacterial cells were grown at 37°C in MM containing all amino acids except Lys. Growth was monitored by optical density measurement at 595 nm (OD_{595}). Symbols: ▲, AT997 + *pB::OsDHDPS*; ●, wild type + control; Δ, AT997 + control.

Collection @ jeju

4. DISCUSSION

Cereal crops such as rice, corn, wheat, etc are considered to be of major nutritional importance in human foods and animal feedstuffs. Among cereal crops, rice is the primary source of carbohydrates for millions of people world-wide (particularly in South Asia). However, the amount of protein in these crops is comparatively low as compared to leguminous crops such as the soybean, chickpea, etc. Amino acids are important nitrogen containing compounds and protein constituents, and amino acid metabolism is an essential process in plant growth and development (Andrews *et al*., 2004; Raven *et al*., 2005). To improve the protein contents of cereal crops, then, it is clearly necessary to gain a greater understanding of the amino acid biosynthetic pathways in crop plants.

TS and DHDPS are the essential enzymes for the Thr and Lys biosynthetic pathway in plants. TS and DHDPS genes have been previously cloned and characterized from many bacteria and a few plant species. We identified and characterized two genes encoding TS and DHDPS from rice. The deduced amino acid sequence of *OsTS* evidenced profound similarity to its counterparts in other plant species and bacterial TSs and similar to OsDHDPS that deduced amino acid sequence of *OsDHDPS.* We noted a severe retardation in the growth of the *thrC* mutant *E. coli* containing the control plasmid, as the mutant itself was not able to synthesize the amino acid Thr. However, the same *E. coli* containing the *pB::OsTS* plasmid grew and evidenced an S-shaped classical growth curve. It also noted the growth retardation of the *dapA* mutant *E. coli* harboring the control plasmid whereas when the same mutant *E. coli* containing pB*::OsDHDPS* then it showed S-shape growth curve, in this case the mutant strain got Lys from pB*::OsDHDPS;* thus, it grew well. Attempts are currently underway to obtain some important information about the substrate specificity of the enzymes by purifying recombinant *OsTS* and *OsDHDPS* in *E. coli* and to assess the physiological functions of these novel enzymes for Thr and Lys metabolism by screening T-DNA insertion mutants in which the *OsTS* and *OsDHDPS* genes are knocked out in rice. The

Collection @ jeju

report regarding the cloning and characterization of the cDNAs encoding for TS and DHDPS from rice have generated bioinformatics predictions, as well as motifs and complementation, in a *thrC* and *dapA* mutants of *E. coli*. These results may constitute a starting point for investigations at the molecular level to investigate Thr and Lys biosynthesis in rice, which might eventually be applied to modify the nutritional compositions of crop plants.

Collection @ jeju

Part II

Analysis and characterization of genes

encoding acetolactate synthase and

3-isopropylmalate dehydrogenase from rice

Collection @ jeju

ABSTRACT

Acetolactate synthase (ALS) and 3-isopropylmalate dehydrogenase (IPMDH) are key enzymes in the biosynthetic pathway leading to Ile, Val and Leu in plants and microorganisms. ALS and IPMDH are the target site of several classes' of herbicides including sulfonylureas, imidazolinones and trizolopyrimidines that are effective to protect a broad range of crops from different weeds. The ALS and IPMDH enzymes have been characterized and sequenced in many bacteria and few plants. Sequence analysis of the cDNA from rice revealed that it harbors a full-length open reading frame for OsALS encoding for 558 amino acids and approximately 59.9 kDa protein. The cDNA for OsIPMDH encoded of 348 amino acids and approximately 37.1 kDa protein. The predicted amino acid sequence of OsALS is highly homologous to that of many bacterial ALS encoded by the *ilvH* gene and *OsIPMDH* which is also highly homologous to the enzyme for IPMDH encoded by *leuB*. *OsALS* or *OsIPMDH* expression was complemented by an *ilvH* or a *leuB* mutant strain of *E. coli,* respectively. OsALS and OsIPMDH expression were correlated with the survival of respective mutants.

Collection @ jeju

1. INTRODUCTION

Most plant and microorganism have a full complement of biosynthetic pathways and component enzymes of the pathway are able to synthesize all of their organic constituents from CO_2. In contrast, animals have complex dietary requirements due to their inability to make fats, amino acids, vitamins and so on. The branched amino acids (Val, Leu and Ile) are not produced by animals but it is essential for diet of animals. In plant and many microorganism, Val and Leu are synthesized by a common pathway that begins with the formation of 2-acetolactate from two molecules of pyruvate. Ile is synthesized in a parallel pathway starting with the formation of 2-aceto-2-hydroxy-butyrate from pyruvate and 2-ketobutyrate. The parallel step involved four enzymes; anabolic acetolactate synthase is one of four enzymes (Fig. 9).

Acetolactate synthase (ALS, EC 4.1.3.18) is an enzyme that catalyzes the first step in the synthesis of the branched-chain amino acids. ALS enzyme is of substantial importance because it is the target of several classes of herbicides, including all members of the popular sulfonylurea and imidazolinome families (Duggleby *et al.*, 2008). ALS requires FAD, thiamine diphosphate (ThDP) and a bivalent metal ion, Mg^{2+} or Mn^{2+}, for its activity (Singh *et al.*, 1988). The enzyme used the ThDP as coenzyme in the condensation reactions, and Mg^{2+} is presumed to be required for the binding of ThDP to the enzyme, as it is for other ThDP-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 metabolic enzyme 3-isopropylmalate dehydrogenase (IPMDH: EC 1.1.1.85), a product of the *leuB* gene is a bifunctional dimeric enzyme that catalyzes dehydrogenation and decarboxylation reactions in the presence of NAD^+ and a divalent cation, such as Mg^{2+} or Mn^{2+}, which is involved in Leu biosynthesis. The reaction involves a

dCollection @ jeju

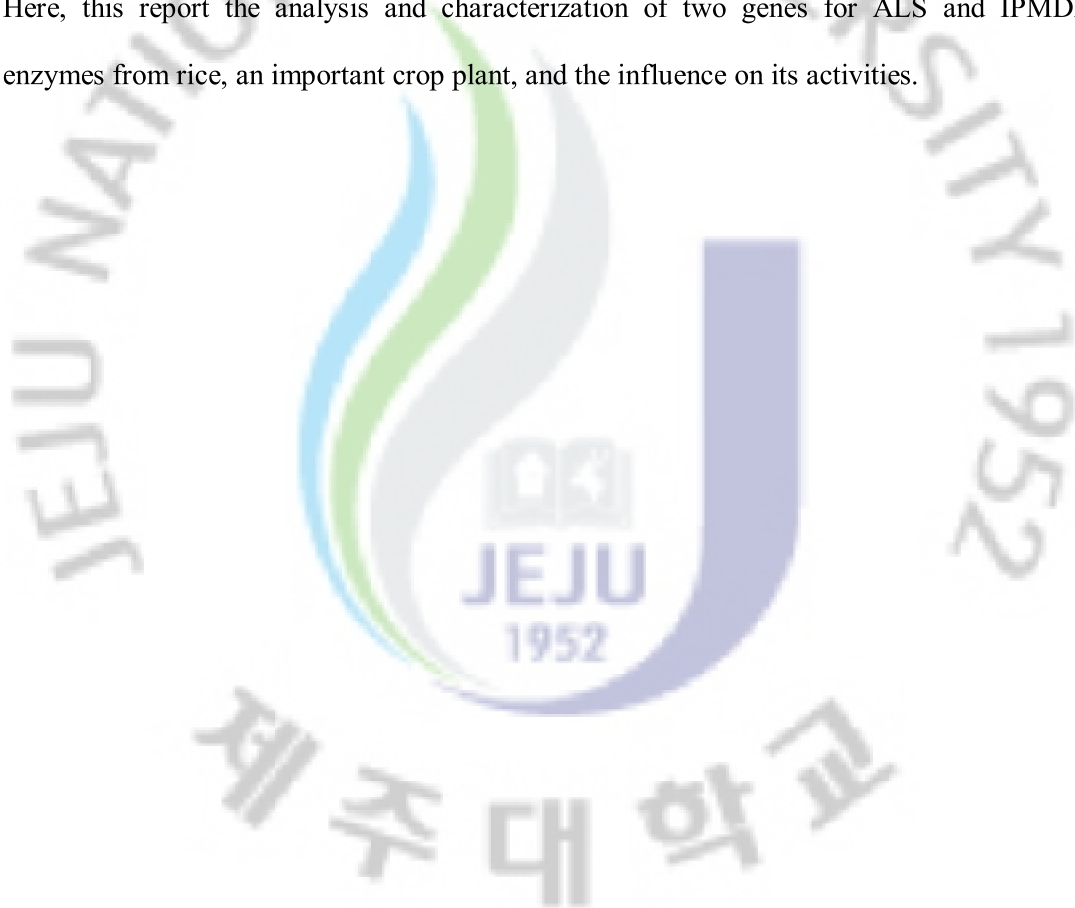
dehydrogenation and subsequent decarboxylation of threo-D-3-isopropylmalate to 2-oxoisocaprorate with the concomitant reduction of NAD^{+}. This dimeric enzyme belongs to a new family of dehydrogenases which comparies only two members: isocitrate dehydrogenase (ICDH) and isopropylmalate dehydrogenase (IPMDH). IPMDH oxidizes 3-isopropylmalate with NAD^{+} to generate 2-isopropyl-3-oxosuccinate and NADH (Wallon *et al.,* 1997). The ALS gene activity has been identified, purified, and sequenced in a variety of microorganisms and plant species, such as *Saccharomyces cerevisiae* (Falco and Dumas., 1985), *Mycobacterium tuberculosis* (Choia *et al*., 2005), *Escherichia coli* (Eoyang and Silverman, 1984; Grimminger and Umbarger, 1979), *Salmonella typhimurium* (Schloss *et al*., 1985) *Arabidopsis thaliana* (Chang and Duggleby, 1997), *Nicotiana tabacum* (Mazur *et al*.,1987), *Hordeum vulgare* L (Durner and Boger, 1988 and Yoon *et al*., 2003), *Triticum eastivum* leaves (Southan and Copeland, 1996) and *Zea mays* (Muhitch *et al*., 1987). The enzymological and kinetic studies on inhibition of ALS by herbicides have been carried out with the 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, 1988). The IPMDH has been purified and characterized in many bacteria and few plants such as *E. coli* (Umbarger, 1996), *Thiobacillus ferrooxidans* (Matsunami *et al*.,1998), *Bacillus subtillis* (Nagahari and Sakaguchi, 1978), *Candida utilis* (Hamasawa *et al.,* 1987), *Sulfobus* sp. (Suzuki *et al*., 1997), *Salmonella typhimurium* (Umbarger, 1996; Wittenbach *et al*., 1994), *Pseudomonas aeruginosa* (Hoang and Schweizer, 1997), *Arabidopsis thaliana* (Nozawa *et al*., 2005), *Brassica napus* (Ellerstrom *et al.,* 1992), *Solanum tuberosum* (Jackson *et al*., 1993).

Collection @ jeju

Fig. 9. Schematic diagram of branched amino acids pathway in plants.

Collection @ jeju

The IPMDH has herbicidal activity and the sensitivity of this enzyme to O-Isobutenyl oxalyhydroxamate (O-IBOHA) and that only *LeuB* and 2-ketoisocaproate were able to protect the roots from inhibition of the enzyme (Wittenbach *et al.,* 1994). Several enzymes in this pathway are inhibited by commercial and experimental herbicides (Singh and Shaner, 1995). Sulfonylureas and imidazolinones classes of compounds are notable for their high herbicidal potencies, their low mammalian toxicities, and, for some analogs, their selective toxicity to weed species as compared to crop species (Levitt *et al*., 1981; Shaner *et al*., 1984). Here, this report the analysis and characterization of two genes for ALS and IPMDH enzymes from rice, an important crop plant, and the influence on its activities.


JEJU NATIONAL UNIVERSITY 1952
JEJU
1952
제주대학교

Collection @ jeju

2. MATERIALS AND METHODS

2.1 Strains

Three *E. coli* strains were used in the part II that is presented in Table 2. The source of all strains was the *E. coli* Genetic stock Center (CGSC) at Yale University, USA.

Table 2. *E. coli* strains used in part II

Name	Genotype	Remark
M1262	*hfr(PO1),* ***leuB****6(Am), ilvI614,* ***ilvH****612, LAM-, relA1, spoT1, ilvB619, ilvG605(Am), ilvG603(Act), thi-1*	Leu, Ile mutant
FD1062	*hfr(PO1), araC14, ilvI614,* ***ilvH****612, LAM-, glyA18, relA1, spoT1, ilvB619, bglR20, rbs-5::Tn5, ilvG468(Act), thi-1*	Ile mutant
Gif41	*hfr(PO1), thrC1001, LAM-, e14-, relA1, spoT1, thi-1*	wild type

Collection @ jeju

2. 2. DNA sequence analysis

Two EST clones (GenBank accession aumber AK242817 and AK120254, Clone ID 206892 and 214973) were obtained from the Rice Genome Resource Center (RGRC). The clones were derived from the rice cDNA library (Osato *et al.,* 2002) from developing seeds prepared in pBluescript SK-. DNA sequencing was performed by an automatic sequencer (A1Fexpress DNA sequencer, Pharmacia Biotech. Inc., UK) with synthetic oligonucleotide primers. 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 CLUSTALW 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 Genome Net Computation Service at Kyoto University (http://www.genome.ad.jp) and phylogenetic tree with bootstrap value was prepared by using Mega 4.1- neighbor-joining program (Kumar *et al*., 2008).

2. 3. Polymerase chain reaction (PCR) and recombinant constructs

The two sequences analysis showed the presence of an ATG start codon located in-frame at -2540 and -53 positions upstream from the translation-starting site. Therefore, the specific primers were designed from the sequence information around the translational start and stop codons of OsALS and OsIPMDH to amplify the full-length open reading frame (ORF) and to over express the gene products in *E. coli*. Polymerase chain reaction (PCR) (Sambrook and Russell, 2001) was conducted to amplify the full-length ORF.

After the EST was purified from a pellet harvested from a liquid culture containing 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’-

Collection @ jeju

AGTCGACTGGGACAGCTTAAAAGTGGGCTA-3') and *OsALS*-R (5' AGTCGACCCCTGATTAGTCTGTACCGAAGT-3') using AmpliTaqGold polymerase (Perkin-Elmer, U.S.A). The underlined bases in the OsALS-F and OsALS-R primers are the designed restriction sites GTCGAC of for *Sal*I to facilitate subcloning, respectively. The PCR was conducted using a MYCyler^{TM} PCR system (BioRad, U.S.A) for 35 cycles with 95°C for 1 min, 45°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 sub cloned into pEZZ18 vector and finally sub cloned into pBluescript II KS+ (Stratagene Inc., U.S.A) as a *Sal*I fragment, to give *pB::OsALS*.

In case of IPMDH, also using designed primers were also used from OsIPMDH sequence: *OsIPMDH* -F (5'-AGGATCCTACTACTCCTCTTTCCCACTCCT-3') and *OsIPMDH* -R (5'- AGGATCCGGTGCATGGGGACCTGATTTTTA-3'). The polymerase chain reaction was performed sing MY Cyler TM PCR system (BioRad, U.S.A) for 45 cycles with 95°Cfor 1 min, 45°C for 1 min, and 72°C for 1 min, with 10 μM primers. PCR products were analyzed on 1% (w/v) agarose gel. The 1030 bp PCR fragment was sub cloned into a pMPM-K2 cloning vector and digested with *Bam*HI and inserted into the same site of GGATCC plasmid to contrast OsIPMDH. Restriction analysis was performed to confirm the constract of both enzymes. Restriction analysis was conducted in an effort to confirm the recombinant DNA constructs of *pB::OsALS* and *pB::OsIPMDH* with the right orientation for overexpression.

Collection @ jeju

2. 4. Functional complementation and growth assay

The competent *ilvH* mutants of the M1262 and FD1062 *E. coli* strains were transformed with *pB::OsALS* and *LeuB* mutants of the M1262 which were also transformed with *pB::OsIPMDH* via electroporation (ECM399, BTX, USA) using a cuvette with a 0.1 cm electrode gap, then plated on LB medium (20 g/L) with Amp (100 µg/ml). The growing culture was tested for growth retardation in MM medium containing Amp (25 µg/ml), 20% glucose, IPTG, Amp (25 µg/ml) and 18 amino acids excluding Ile and Val (For OsALS) and/or 19 amino acids excluding Leu (For OsIPMDH). Bacterial growth was then assessed by measuring OD at 595 nm (OD_{595}) at one-hour intervals. After 12 hrs, the diluted culture was plated and incubated overnight at 37°C.

2. 5. Growth inhibition assay of ***OsALS*** and ***OsIPMDH*** in ***E. coli***

The respective *E. coli* mutant strains harboring the *pB::OsALS* and *pB::OsIPMDH* construct, control plasmid, and wild-type with control plasmid were grown at 37°C in MM with IPTG (0.1 M), 20% glucose (20 ml/L) and Amp (25 µg/ml) and In the case of, ALS, containing 18 amino acids excluding Ile and Val; in the case of IPMDH, 19 amino acids excluding Leu. The bacterial growth was monitored via OD measurements every hour using a spectrophotometer (UV1101, Biochrom, England) at 595 nm (OD_{595}).

Collection @ jeju

3. RESULTS

3. 1. Sequence analysis of OsALS and OsIPMDH

Two expressed sequence tag (EST) clones (GenBank accession number AK242817 and AK120254) cDNA (OsALS and OsIPMDH*)* sequences harbored a full-length open reading frame consisting of 1677 bp and 1047 bp encoding for a protein of approximately 59.9 kDa and 37.1 kDa, respectively. The expected pI of the proteins was 6.54 and 5.87. Data analysis revealed that the OsALS and OsIPMDH sequence was identical to the genomic region located in chromosome V and III, respectively. Comparisons of the amino acid sequence of the OsALS and the homologous sequences from maize (*Zea mays*) and arabidopsis (*A. thaliana*) revealed high identity, at 91% and 71%, respectively.

Analysis amino acid sequence of OsALS in Pfam database revealed that there are three thiamine pyrophosphate enzyme (TPP_enzyme) site found in the sequence such as N-terminal TPP binding domain (71-241), central TPP binding domain (263-396) and C-terminal TPP binding domain (458-558) (Fig. 10). The predicted amino acids for the OsIPMDS with the deduced sequence from maize (*Zea mays*), arabidopsis (*A. thaliana*), bacteria (*Bacillus subtilis*) and *E. coli* showed conserved sequence exaggerates with an identity 76.4%, 68.4%, 50.3% and 43.9%, respectively. Analysis of the amino acid sequence of OsIPMDS revealed a signature binding motif for PLP in the N- terminal region (266-285). The motif sequence (NIFGDILSDEASMLTGSIGM) was highly homologous to the consensus [[NSK]-[LIMYTV]-[FYDNH]-[GEA]-[DNGSTY]-[IMVYL]-x-[STGDN]-[DN]-x(1, 2)-[SGAP]-x(3, 4)-[GE]-[STG]-[LIVMPA]-[GA]-[LIVMF] where the that underlined amino acids are well conserved (Fig. 11). The binding motif for NAD^+ is present and well in bacterial IPMDH in which the underlined amino acids are well conserved (Hofmann *et al.,* 1999). The motif is related to isocitrate and isopropylmalate dehydrogenases signature.

Collection @ jeju

```
OsALS      1 ----MATTAAAAAAALSAAATAKTGRK-------------------NHQR
AtALS      1 MAAATTTTTTSSSISFSTKPSPSSSKSPLPISRFSLPFSLNPNKSSSSSR
EcALS_I    1 ---------------MASSGTTSTRKR-----------------------

OsALS     28 HHVLPARGRVGAAAVRCSAVSPVTPPSPAPPATP---LRPWGPAEPRKGA
AtALS     51 RRGIKSSSPSSISAVLNTTTNVTTTPSPTKPTKPETFISRFAPDQPRKGA
EcALS_I   13 ----------------------------------------------FTGA

OsALS     75 DILVEALERCGVSDVFAYPGGASMEIHQALTRSPVITNHLFRHEQGEAFA
AtALS    101 DILVEALERQGVETVFAYPGGASMEIHQALTRSSSIRNVLPRHEQGGVFA
EcALS_I   17 EFIVHFLEQQGIKIVTGIPGGSILPVYDALSQSTQIRHILARHEQGAGFI
                   N-terminal TPP binding domain
OsALS    125 ASGYARASGRVGVCVATSGPGATNLVSALADALLDSVPMVAITGQVPRRM
AtALS    151 AEGYARSSGKPGICIATSGPGATNLVSGLADALLDSVPLVAITGQVPRRM
EcALS_I   67 AQGMARTDGKPAVCMACSGPGATNLVTAIADARLDSIPLICITGQVPASM

OsALS    175 IGTDAFQETPIVEVTRSITKHNYLVLDVEDIPRVIQEAFFLASSGRPGPV
AtALS    201 IGTDAFQETPIVEVTRSITKHNYLVMDVEDIPRIIEEAFFLATSGRPGPV
EcALS_I  117 IGTDVFQEVDTYGISIPITKHNYLVRHIEELPQVMSDAFRIAQSGRPGPV

OsALS    225 LVDIPKDIQQQMAVPVWDTSMNLPGYIARLPKPPATELLEQVLRLVGESR
AtALS    251 LVDVPKDIQQQLAIPNWEQAMRLPGYMSRMPKPPEDSHLEQIVRLISESK
EcALS_I  167 WIDIPKDVQTAVFEIETQPAMAEKAAAPAFSE----ESIRDAAAMINAAK

OsALS    275 RPILYVGGGCSASGDELRWFVELTGIPVTTTLMGLGNFPSDDPLSLRMLG
AtALS    301 KPVLYVGGGCLNSSDELGRFVELTGIPVASTLMGLGSYPCDDELSLHMLG
EcALS_I  213 RPVLYLGGGVINAPARVRELAEKAQLPTTMTLMALGMLPKAHPLSLGMLG
              Central-terminal TPP binding domain
OsALS    325 MHGTVYANYAVDKADLLLAFGVRFDDRVTGKIEAFASRAKIVHIDIDPAE
AtALS    351 MHGTVYANYAVEHSDLLLAFGVRFDDRVTGKLEAFASRAKIVHIDIDSAE
EcALS_I  263 MHGVRSTNYILQEADLLIVLGARFDDRAIGKTEQFCPNAKIIHVDIDRAE

OsALS    375 IGKNKQPHVSICADVKLALQGLNALLQQSTTKTSSDFSAWHNELDQQKRE
AtALS    401 IGKNKTPHVSVCGDVKLALQGMNKVLENRAEELKLDFGVWRNELNVQKQK
EcALS_I  313 LGKIKQPHVAIQADVDDVLAQLIPQVEAQPR------AEWHQLVADLQRE

OsALS    425 FPLGYKTFGEEIPPQYAIQVLDELTKGEAIIATGVGQHQMWAAQYYTYKR
AtALS    451 FPLSFKTFGEAIPPQYAIKVLDELTDGKAIISTGVGQHQMWAAQFYNYKK
EcALS_I  357 FPCRIPKACDPLSHYGLINAVAACVDDNAIITTDVGQHQMWTAQAYPLNR
             C-terminal TPP binding domain
OsALS    475 PRQWLSSAGLGAMGFGLPAAAGASVANPGVTVVDIDGDGSFLMNIQELAL
AtALS    501 PRQWLSSGGLGAMGFGLPAAIGASVANPDAIVVDIDGDGSFIMNVQELAT
EcALS_I  407 PRQWLTSGGLGTMGFGLPAAIGAALANPDRKVLCFSGDGSLMMNIQEMAT

OsALS    525 IRIENLPVKVMVLNNQHLGMVVQWEDRFYKAAYV
AtALS    551 IRVENLPVKVLLLNNQHLGMVMQWQDRFYKANRAHTFLGDPAQEDEIFPN
EcALS_I  457 ASENQLDVKIILMNNEALGLVHQQQSLFYEQGVFAATYPG-------KIN

OsALS
AtALS    601 MLLFAAACGIPAARVTKKADLREAIQTMLDTPGPYLLDVICPHQEHVLPM
EcALS_I  500 FMQIAAGFGLETCDLNNEADPQAALQEIINRPGPALIHVRIDAEEKVYPM

OsALS
AtALS    651 IPSGGTFNDVITEGDGRIKY
EcALS_I  550 VPPGAANTEMVGE
```

Fig. 10. Amino acid sequence alignment of ALSs using Boxshade program after CLUSTALW alignment. Completely conserved, identical and similar residues are visually shown as yellow, green and cyan, respectively. Accession numbers are as: AK242817 (OsALS from *Oryza sativa*, this study), AAK68759 (AtALS from *Arabidopsis thaliana*) and NP_418127 (EcALS from *E. coli*).

dCollection @ jeju

```
OsIPMDH     1 MAPPAP-------------ATARRPMA--AVRCSAA---KRSYNVTLLPG
AtIPMDH     1 MAAALQTNIRTVKVPATFRAVSKQSLAPFRVRCAVASPGKKRYTITLLPG
EcIPMDH     1 ------------------------MMS-------------KNYHIAVLPG

OsIPMDH    33 DGIGPEVVAVAKDVLSLAGALEGVEFRFQEKLMGGAAVDAYGVPLPEETL
AtIPMDH    51 DGIGPEVVSIAKNVLQQAGSLEGVEFNFREMPIGGAALDLVGVPLPEETI
EcIPMDH    14 DGIGPEVMTQALKVLDAVRNRFAMRITTSHYDVGGAAIDNHGQPLPPATV

OsIPMDH    83 AAAQASDAVLLGAIGGYKWDNNEKHLKPETG-LLQIRAGLGVFANLRPAA
AtIPMDH   101 SAAKESDAVLLGAIGGYKWDNNEKHLRPEKG-LLQIRAALKVFANLRPAT
EcIPMDH    64 EGCEQADAVLFGSVGGPKWEHLPPDQQPERGALLPLRKHFKLFSNLRPAK

OsIPMDH   132 VLPQLVDASTLKKEVAEG-VDIMVVRELTGGIYFGQPRGFGKNDKGEDTG
AtIPMDH   150 VLPQLVDASTLKREVAEG-VDLMVVRELTGGIYFGEPRGIKTNENGEEVG
EcIPMDH   114 LYQGLEAFCPLRADIAANGFDILCVRELTGGIYFGQPKGREGSGQ-YEKA

OsIPMDH   181 FNTEVYSASEIDRITRVAFEVARKRRGKLCSVDKANVLEASMLWRKRVTS
AtIPMDH   199 FNTEVYAAHEIDRIARVAFETARKRRGKLCSVDKANVLEASILWRKRVTA
EcIPMDH   163 FDTEVYHRFEIERIARIAFESARKRRHKVTSIDKANVLQSSILWREIVNE

                                                   IPMDH signature
OsIPMDH   231 LASEFPDIELSHMYVDNAAMQLIRNPKQFDTIVTNNIFGDILSDEASMLT
AtIPMDH   249 LASEYPDVELSHMYVDNAAMQLVRDPKQFDTIVTNNIFGDILSDEASMIT
EcIPMDH   213 IATEYPDIELAHMYIDNATMQLIKDPSQFDVLLCSNLFGDILSDECAMIT

OsIPMDH   281 GSIGMLPSASVGESGPGLFEPIHGSAPDIAGQDKANPLATILSAAMLLRY
AtIPMDH   299 GSIGMLPSASLSDSGPGLFEPIHGSAPDIAGQDKANPLATILSAAMLLKY
EcIPMDH   263 GSMGMLPSASLNEQGFGLYEPAGGSAPDIAGKNIANPIAQILSLALLLRY

OsIPMDH   331 GLGEENAAKRLKPQLQRH
AtIPMDH   349 GLGEEKAAKRIEDAVLVALNNGFRTGDIYSAGTKLVGCKEMGEEVLKSVD
EcIPMDH   313 SLDADDAASAIERAINRALEEGIRTGDLARG-AAAVSTDEMGDIIARYVA

OsIPMDH
AtIPMDH   399 SQVPASV
EcIPMDH   362 EGV
```

Fig. 11. Amino acid sequence alignment of IPMDHs using Boxshade program after CIUSTALW alignment. Completely conserved, identical and similar residues are visually shown as yellow, green and cyan, respectively. Accession numbers are as: AK120254 (OsIPMDH from *Oryza sativa*), NP_178171 (AtIPMDH from *Arabidopsis thaliana*) and AAG54377 (EcIPMDH from *E. coli*).

Collection @ jeju

To determine the relationship between plant and bacterial ALS and IPMDH enzymes, we performed phylogenetic analysis with the Mega 4.1 neighbor-joining program (Kumar *et al*., 2008). Phylogenetic tree derived from the related sequence indicated further that OsALS and OsIPMDH are divergent and evolved from ancestor bacterial ALS and IPMDH, respectively. Branching pattern and numbers at nodes indicate levels of bootstrap support based on neighbor-joining analysis of 1,000 re-sample data sets. Numbers on branches are percentages of bootstrap analyses supporting the grouping of each branch (Fig. 12 & Fig. 13).

dCollection @ jeju

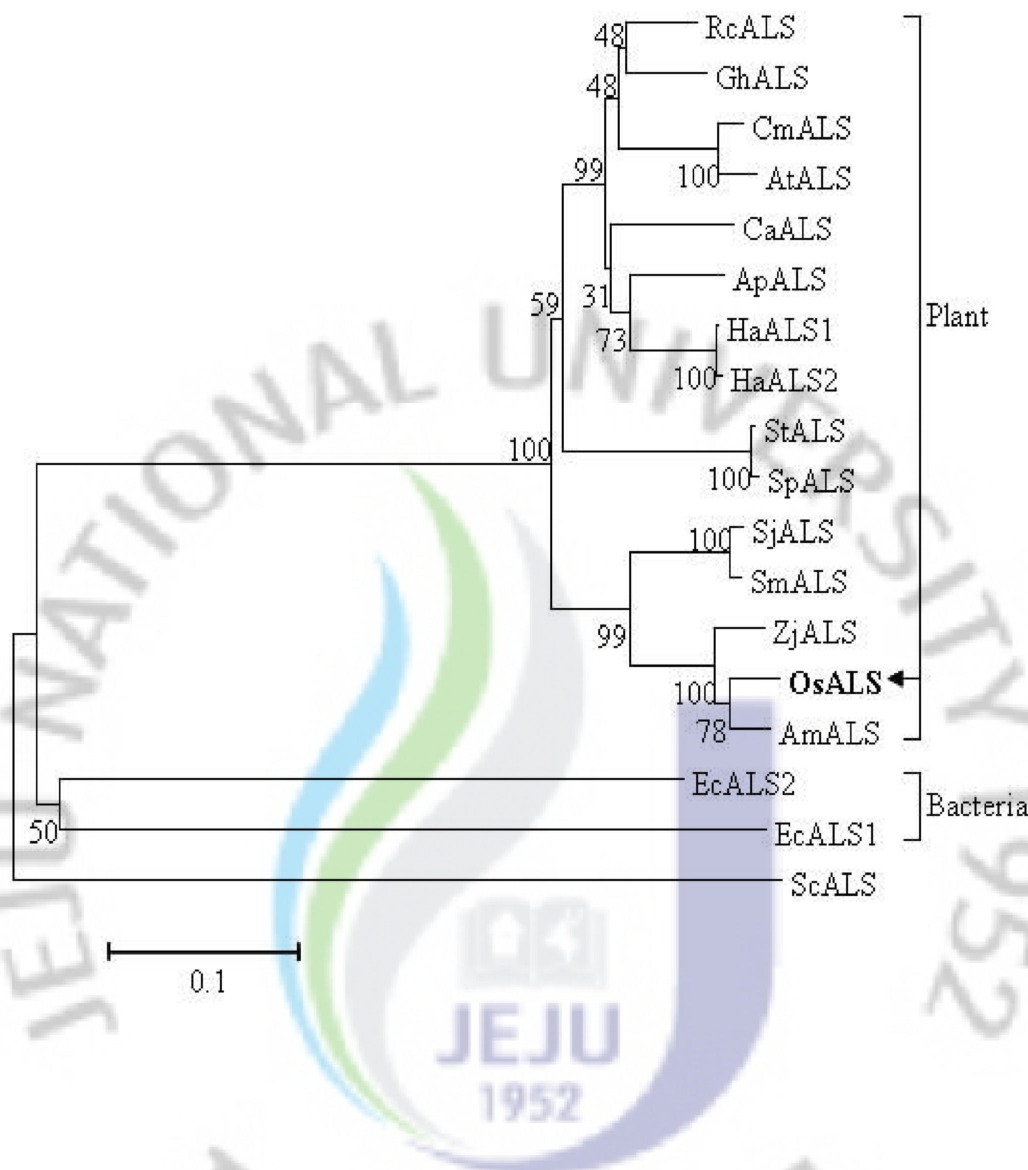


Fig. 12. Phylogenetic analysis of OsALS related proteins using ClustalW and Mega4.1 neighbor-joining program. Accession numbers are as follows: AK242817 (OsALS from *Oryza sativa*), XP_002511176 (RcALS from *Ricinus communis*), CAA87084 (GhALS from *Gossypium hirsutum*), AAR06607 (CmALS from *Camelina 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

Collection @ jeju

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)*

JEJU NATIONAL UNIVERSITY 1952
JEJU
1952
제주대학교

Collection @ jeju

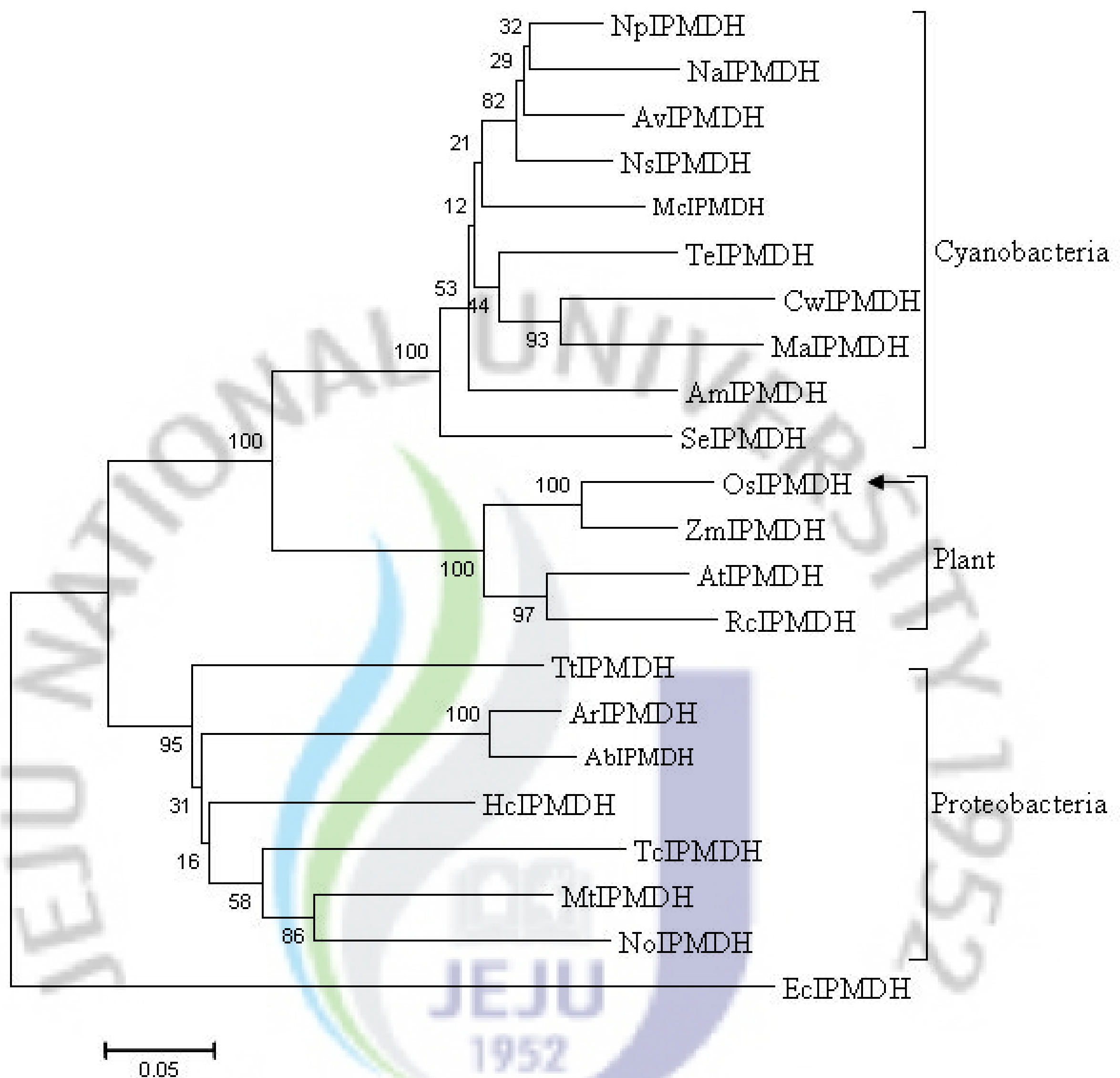


Fig. 13. Phylogenetic analysis of OsIPMDH related proteins using ClustalW and Mega4.1 neighbor-joining program. Accession numbers are as follows: AK120254 (OsIPMDH from *Oryza sativa*), YP_001866278 (NpIPMDH, from *Nostoc punctiforme*), ZP_03766655 (NaIPMDH from *Nostoc azollae*), YP_323492 (AvIPMDH from *Anabaena variabilis*), ZP_01632298 (NsIPMDH from *Nodularia spumigena*), ZP_05030606 (McIPMDH from *Microcoleus chthonoplastes*), YP_723864 (TeIPMDH from *Trichodesmium erythraeum*) ZP_00514246 (CwIPMDH from *Crocosphaera watsonii*), YP_958834 (MaIPMDH from

Collection @ jeju

Marinobacter aquaeolei), ZP_03272601 (AmIPMDH from *Arthrospira maxima*), YP_173200 (SeIPMDH from *Synechococcus elongates*), YP_001515254 (AmIPMDH from Acaryochloris marina), NP_001150956 (ZmIPMDH from *Zea mays*), NP_178171 (AtIPMDH from *Arabidopsis thaliana*), XP_002530452 (RcIPMDH from *Ricinus communis*), YP_003073929 (TtIPMDH from *Teredinibacter turnerae*), ZP_05362385 (ArIMPDH from *Acinetobacter radioresistens*),YP_001708216 (AbIPMDH from *Acinetobacter baumannii*),P_433663 (HcIPMDH from *Hahella chejuensis*), YP_391067 (TcIPMDH from *Thiomicrospira crunogena*), ZP_05105225 (MtIPMDH from *Methylophaga thiooxidans*), YP_343052 (NoIPMDH from *Nitrosococcus oceani*) AAG54377 (EcIPMDH from *E. coli*).

Collection @ jeju

3. 2. ***OsALS*** **and** ***OsIPMDH*** **expression in** ***E. coli*** **and** ***in vivo*** **activity**

The recombinant DNAs, *pB::OsALS and*, *pB::OsIPMDH*, were constructed using the ORF of a PCR-amplified *OsALS and OsIPMDH* fragment, respectively. After the transformation of *E. coli* with the recombinant DNA, OsALS and OsIPMDH activity were monitored *in vivo* in a medium containing IPTG and 18 amino acids, excluding Ile and Val (OsALS) and 19 amino acids excluding Leu (OsIPMDH). Functional complementation was performed using the ALS mutant of *E. coli* to confirm the enzyme activity of the gene product of *OsALS,* and similarly the *leuB* mutants of *E. coli* to be sure the enzyme activity by the gene product of *pB:: OsIPMDS*. To assess the viability of *E. coli* cells by OsALS and OsIPMDH activity, the OsALS *and* OsIPMDH expressing cells were cultured for 12 hrs with shaking, and the diluted portion was plated on agar medium containing the 18 amino acids and Amp (25 mg/ml) without Ile and Val and 19 amino acids excluding Leu (Fig. 14). The viable colonies greatly differed among the plasmids. These could grow under conditions in which the mutant without OsALS/OsIPMDH could not. This showed that the OsALS and OsIPMDH were capable to complement, as an evidenced functional ALS and IPMDH activity, respectively.

Collection @ jeju

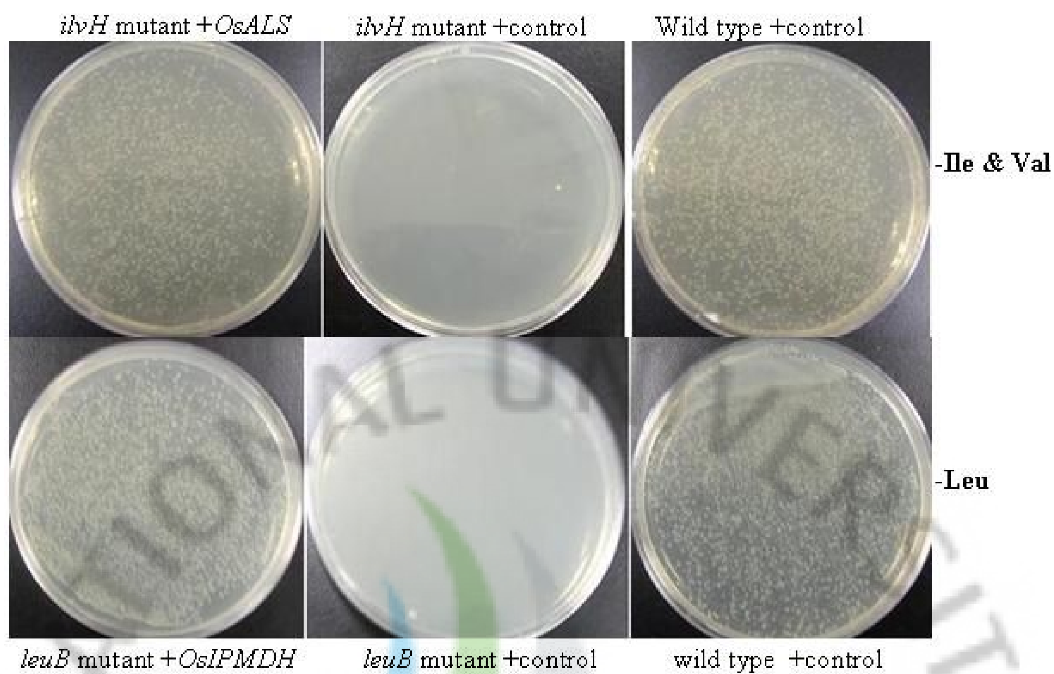


Fig. 14. Functional complementation assay for OsALS and OsIPMDH. The *ilvH* mutant *E. coli* strain M1262 containing *pB::OsALS* and control and wild-type *E. coli* strain Gif41containing control plasmid (upper line). The *leuB* mutant *E. coli* strain M1262 containing *pB::OsIPMDH* and control; and Gif41, wild-type *E. coli* containing control plasmid (lower line).

Collection @ jeju

3. 3. Expression of *OsALS* can complement the *ilvH* mutants of *E. coli*

A growth study was performed to determine whether the *OsALS* gene would increase the sensitivity of bacterial cells to *ilvH*. The *pB::OsALS* construct was transformed into the *ilvH* mutant *E. coli* strains M1262 and FD1062. A control plasmid was also transformed into wildtype (Gif41) and the *ilvH* mutants M1262 and FD1062. The *pB::OsALS* activity was monitored via a growth assay in the absence of Ile and Val. Bacterial cells were grown in MM with 18 amino acids excluding Ile and Val, containing IPTG and Amp. The wildtype *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 (Fig. 15A) The Gif41 strain could synthesize Ile and Val itself, and thus grew normally in the medium. The *ilvH* mutant strains M1262 and FD1062 expressing *pB::OsALS* also grew normally and showed an S-shaped classical growth curve in the same medium, but grew slightly more slowly than the wild type strain containing the control plasmid (Fig. 15A & B), 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 *E. coli* strains M1262 and FD1062 could not synthesize Ile and Val itself, and thus it was not grow; however, the same *E. coli* strains M1262 and FD1062 containing *pB::OsALS* grew well because the *ilvH* mutants *E. coli* was able to synthesize Ile and Val using ALS expressed by the *pB::OsALS* plasmid (Fig. 15A & B). This is a consequence of *pB::OsALS* activity. From the above finding, it was concluded that *OsALS* expression can functionally complement the *ilvH* mutants *E. coli*.

Collection @ jeju

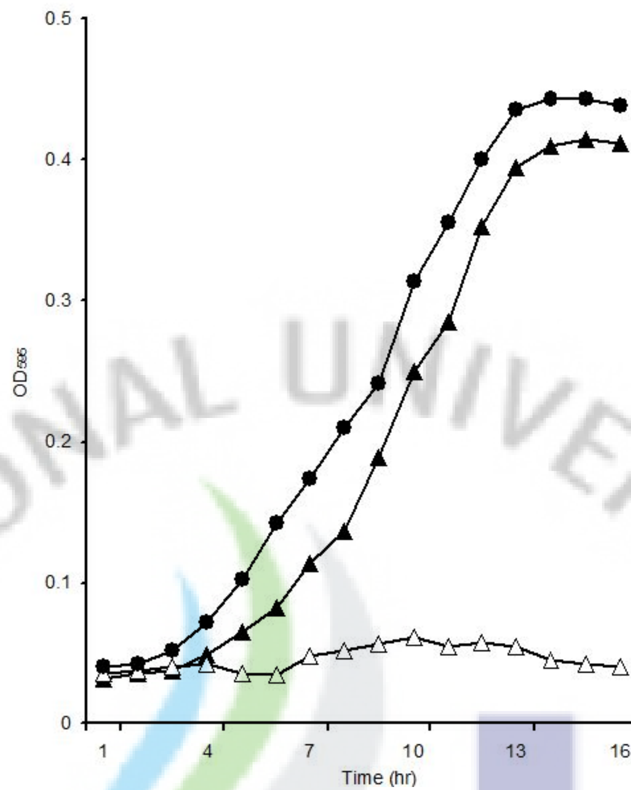


Fig. 15 A. Growth curves of *E. coli* mutant M1262 harboring *pB::OsALS* and control plasmid without Ile and Val and wild type strain containing control Plasmid. Bacterial cells were grown at 37°C in MM containing 18 amino acids except Ile and Val. Growth was monitored via OD measurements at 595 nm (OD_{595}). Symbols: A) ▲, M1262 + *pB::OsALS*; ●, wild type + control; Δ, M1262 + control.

Collection @ jeju

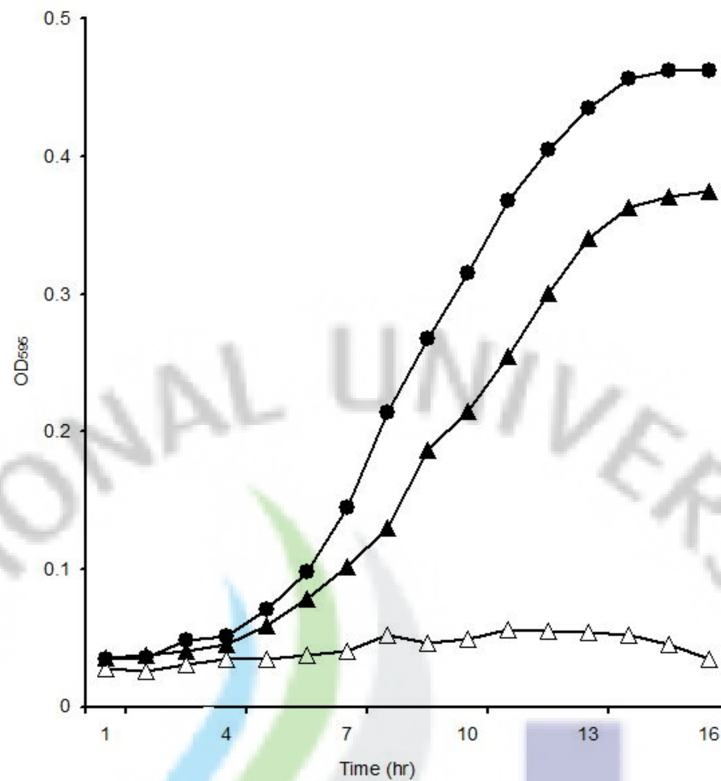


Fig.15B. Growth curves of *E. coli* mutant and FD1062 harboring *pB::OsALS* and control plasmid without Ile and Val and wild type strain Gif41containing control Plasmid. Bacterial cells were grown at 37°C in MM containing 18 amino acids except Ile and Val. Growth was monitored via optical density measurements at 595 nm (OD_{595}). Symbols: ▲, FD1062 + *pB::OsALS*; ●, wild type + control; Δ, FD1062 + control.

Collection @ jeju

3. 4. Leucine sensitivity of *E. coli* mutants was influenced by the expression of *pB:: OsIPMDS*

A growth study was performed to determine whether the *pB:: OsIPMDS,* gene would increase the sensitivity of bacterial cells to Leu. The *pB:: OsIPMDS,* construct was transformed into *leuB* mutant *E. coli* strain M1262. The control plasmid was transformed into *leuB* mutant *E. coli* strain M1262 and a wild type *E. coli* strain. The *pB::OsIPMDS,* activity was monitored by a growth assay in the absence of Leu. Bacterial cells were grown in MM with 19 amino acids excluding Leu, containing IPTG, 20% glucose and Amp. The wild type *E. coli* strain harboring a control plasmid grew normally and showed S-shape classical growth curve in the medium in spite of lack of Leu amino acid (Fig. 16). The wild type strain produced in Leu of itself resluting in normal growth in the medium. However, the *leuB* mutant strain M1262 expressing *pB:: OsIPMDS* also grew normally and showed S-shape classical growth curve in the same medium (Fig. 16). The *leuB* mutant strain M1262 harboring a control plasmid was dramatically retarded in the growth phase in the same medium due to lack of the essential amino acid Leu. In this case, the *leuB* mutant *E. coli* strain *pB:: OsIPMDS* could not produce Leu itself, thus it showed dramatically retarded growth in the same medium but when the same *E. coli* containing *pB:: OsIPMDS* it then grew well because *leuB* mutant *E. coli* got Leu from *pB:: OsIPMDS* as a result well growth (Fig. 16). This result indicated the activity of *pB:: OsIPMDS* . From the above situation, it was concluded that the expression of the *pB:: OsIPMDS* is able to functionally complement and it has the function of Leu.

Collection @ jeju

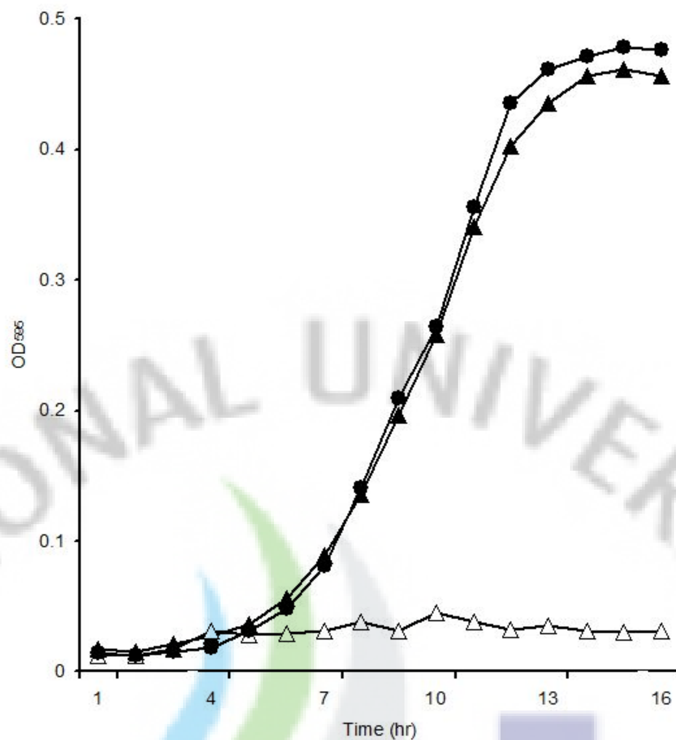


Fig. 16. Growth curves of *E. coli* mutant M1262 was harboring *pB::OsIPMDH*. M1262 and Gif41 were containing respective control plasmid. Bacterial cells were grown at 37°C in MM containing 19 amino acids except Leu. Growth was monitored by optical density measurement at 595nm (OD_{595}). Symbols: ▲, M1262 + *pB::OsIPMDH*; ●, wild type + control; Δ, M1262 + control.

Collection @ jeju

4. DISCUSSION

The breached chain amino acids Val, Leu and Ile are three among ten essentials amino acids that are not synthesized in mammals; however there is more interest due to their importance in animal diets. Leu, Ile and Val, 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). The branched chain amino acid biosynthetic pathway feeds carbon into three different amino acids. The flow of carbon must therefore be tightly regulated so that no one of these amino acids becomes limiting for plant growth (Singh and Shaner, 1995). Amino acids such as Leu, Val and Ile are essential for monogastric animal but can not synthesize it. Thus the modification of the amino acid profile of the rice grain to enhance high nutritional varieties is one of the most important objectives in breeding for seed quality.

This implies a demand for large numbers of analyses to determine the amino acid composition of the rice grain (Jianguo *et al.*, 2002). ALS is the first enzyme in the branched chain amino acids and IPMDH is third enzyme of Leu biosynthesis in higher plants. These enzymes are targeted in herbicidal activity. Here we report characterization of two genes encoded by OsALS and OsIMPDH from rice. The deduced amino acid sequence of OsALS and OsIMPDH showed extensive similarity to its counterparts in other plant species and respective bacterial ALS and IMPDH. The full-length cDNA of ALS and OsIMPDH encoded 558 and 348 amino acids, and approximately 59.9 and 37.1 kDa protein, respectively.

After transformation of both genes, we treated then separately with 18 amino acids excluding Ile and Val, with 19 amino acids without Leu in MM medium containing Amp and IPTG. We observed that *ilvH* mutants *E. coli* strains showed growth retardation when harboring control. In this case, the mutant strain could not produce Ile and Val

Collection @ jeju

thus it failed to grow. The same mutant *E. coli* strain expreesing *pB::OsALS* then grew well and the wild type strain normally grew well. Similar circumstances showed in the case of *leuB* mutant *E, coli* strain and *pB::OsIMPDH* and control. Another wild type *E. coli* strain *Gif41* containing control plasmid that grew normally also showed S-shaped classical curve, in this case, wild type strain produced Leu itself and grew normally.

Here we are investigating to find out some important clues about substrate specificity of the enzyme by purifying recombinant *pB::OsALS* and *pB::OsIMPDH* in *E. coli* and physiological functions of this novel enzyme for Ile , Val and Leu metabolism by screening T-DNA insertion mutants in which the expression of rice *pB::OsALS* and *pB::OsIMPDH* gene is knockout this would provide important clues into the substrate specificity and physiological function of this noble enzyme for nucleotide metabolism in rice plants. The ALS & IPMDH genes were cloned by functional complementation. The ALS & IPMDH genes could be used as a powerful tool for future application to develop herbicide resistant crops in selectable markers. ALS & IPMDH genes could be reintroduced into crop plants for improvement of rice qualities.

Collection @ jeju

Part III

A natural fusion gene encoding uridine kinase and uracil phosphoribosyltransferase from rice

Collection @ jeju

ABATRACT

UK and UPRT are enzymes catalyzing the formation of uridine 5'-monophosphate from urindine and adenosine 5'-triphosphate or uracil and 5-phosphoribosyl-1-pyrophosphate (PRPP) in the pyrimidine salvage pathway, respectively. The genes for UK or UPRT were reported as separate genes in bacteria or yeasts. Here we report the analysis of a gene with dual domains for UK and UPRT from rice (*OsUK/UPRT1*). Sequence analysis was revealed that it contains a full-length open reading frame for *OsUK/UPRT1* which is encoded as 496 amino acids, approximately 55.2 kDa protein. The predicted amino acid sequence of OsUK/UPRT1 is similar to the two proteins for UK and UPRT. Amino-terminal region is similar to UDK of *Escherichia coli* and many bacteria containing an ATP/GTP-binding site motif called a P-loop whereas the carboxyl-terminal is similar to UPP of *E. coli* containing signature-binding motif for a uracil and a PRPP. Expression of OsUK/UPRT1 in an *upp* and *udk* mutant of *E. coli* led to growth inhibition effect with 5-fluorouracil (FU) or 5-fluorouridine (FD). These results suggest that the OsUK/UPRT1 product can use both uracil and uridine as substrates and would be a natural fusion protein of UK and UPRT enzyme.

Collection @ jeju

1. INTRODUCTION

The diverse phenotype among different organisms is originated from the expression of nucleic acids that are composed of purine and pyrimidine bases such as adenine, guanine, thymine, cytosine and uracil. Various DNA metabolisms including replication and repair are needed for such bases as raw materials, which should be synthesized *de novo* or digested from nucleic acids in foods. The biosynthetic pathway of pyrimidines including uridine 5'-triphosphate (UTP) and cytidine 5'-triphosphate (CTP) is initiated by carbamoyl phosphate synthetase with glutamine and CO_2 (Islam *et al*., 2007). Through three more following steps, orotate is synthesized and then converted to uridine 5'-monophosphate (UMP) via orotidine 5'-phosphate. UTP is synthesized from UMP by successive reactions and further metabolized to synthesis of CTP. Besides biosynthesis of pyrimidines, uracil, uridine, or cytosine are salvaged to synthesize UMP or cytidine 5'-monophosphate by the pyrimidine salvage pathway (Fig. 17) (Islam *et al*., 2007).

Uracil phosphoribosyltransferase (UPRT, EC 2.4.2.9) is an enzyme catalyzing the formation of UMP from uracil and 5-phosphoribosyl-1-pyrophosphate (PRPP) in the pyrimidine salvage pathway. Uridine kinase (UK, EC 2.7.1.48) is also an enzyme involved in the formation of UMP from uridine and adenosine 5-triphosphate (ATP). The related genes for UPRT, UK, and orotidine 5' -phosphate decarboxylase were reported to *upp, udk, pyrF* in *E. coli* and *FUR1, URK1, URA3* in *Saccharomyces cerevisiae*, respectively (Andersen *et al*., 1992). The UPRT is well characterized in bacteria, yeasts, and protozoa. It is active in various forms such as monomer in *Toxoplasma gondii* (Carter *et al*., 1997), heterodimer in *S. cerevisiae* (Natalini *et al*., 1979), homodimer in *Crithidia luciliae* (Asai *et al*., 1990), and homotrimer in *E. coli* (Rasmussen *et al*., 1986). The UPRT from *T. gondii* is needed Ca^{2+} and Co^{2+} for enzyme activity and denatured with no activity at 50°C (Carter *et al*., 1997). It has been reported that UPRT from *T. gondii, E. coli,* and *Sulfolobus shibatae* is activated by guanosine 5'-triphosphate (GTP) (Schumacher *et al*., 2002).

Collection @ jeju

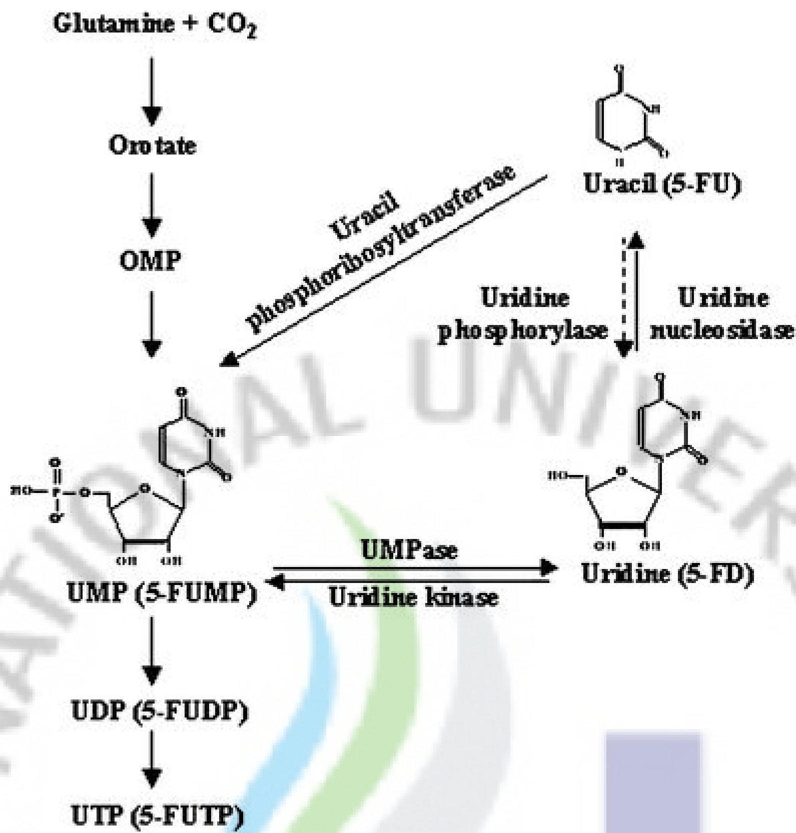


Fig. 17. The Pathway of pyrimidine biosynthesis as adapted from Moffatt and Ashihara (2002). The reaction by uridine phosphorylase was found in *E. coli* or yeast but not in plant (Anderson *et al*., 1992). The pathway of *E. coli* including FU and FD metabolism is marked by parenthesis. OMP, orotate 5-monophosphate; UMP, uridine 5'-monophosphate; UDP, uridine 5-diphosphate; UTP, uridine 5-triphoasphate (Islam *et al*., 2007).

Collection @ jeju

The genes encoding UPRT have been cloned and characterized from bacteria, yeasts and protozoa (Carter *et al*., 1997), *Bacillus subtilis* (Martinussen *et al*., 1995), *Lactococcus lactis* (Martinussen and Hammer, 1994), *Arabidopsis thaliana* (Islam *et al*., 2007) and humans (Li *et al.,* 2007). UK also has been characterized to occur from *E. coli* (Fast and Sköld, 1977), *S. cerevisiae* (Kern 1990) and as a fusion gene for UK and UPRT in *Arabidopsis thaliana* (Islam *et al*. 2007). The UPRT protein is composed of ranging from 208 to 251 amino acids in microbes such as *E. coli, S. cerevisiae*, and *T. gonddi* (Kern *et al*., 1989) and has relatively small molecular weight ranging from 23 kDa to 36 kDa. The binding motifs for the two substrates of UPRT which are PRPP and uracil are well conserved as a sequence "DPMLATGGSA" and YI(F)VPGLGDA(F)GDRL(Y,M)F(Y)G(C)T(V)K, respectively in many bacteria including *B. subtilis* and *L. lactis* (Schumacher *et al*. 1998). UPRT and UK have been found in all organisms, and the salvage activity is a more important source of UMP than the *de novo* pathway for many mammalian cells (Webler *et al*., 1978). The FU and FD are toxic analogs of uracil and uridine which are substrates of UPRT and UK, respectively.

These toxic substances have been well used as an anticancer agent since the 1950's and used medically to treat various cancers in the large intestine, stomach, pancreas, breast, prostate etc. (Miyagi *et al*., 2003). The successive production of the fluro-derivatives of UMP and UTP by UMP kinase and UDP kinase results in RNA damage and inhibition to protein synthesis, which causes broad range of growth retardation in bacterial cells (Koyama *et al*., 2003). The principle of cancer therapy is that FU and FD are toxic to metabolize fluro-derivatives of UMP and UTP, and finally cause a mistake of RNA base and also in gene expression (Fig. 18). Here this report the analysis of a gene with dual domains for UPRT and UK enzyme from rice to investigate the pyrimidine salvage pathway in crop plants.

Collection @ jeju

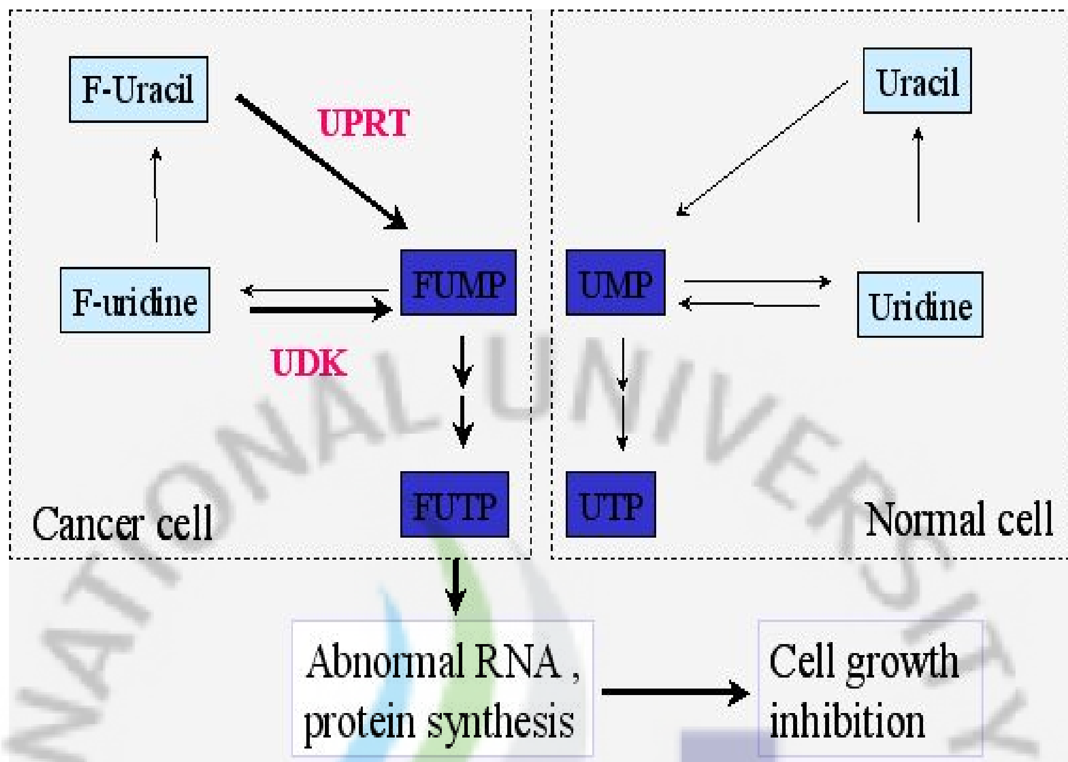


Fig. 18. Cancer gene therapy (Possible mechanism of cell death in cancer cells by treatment of FU & FD).

dCollection @ jeju

2. 1. Strains

Four *E. coli* strains were used in this study. The source of all strains except JM109 (Stratagene) was the *E. coli* Genetic Stock Center (CGSC) in Yale University, USA. *E. coli* strain JM109 was frequently used as a host for cloning purposes.

Table. 3. *E. coli* strains used in the part III

Strains	Genotype	Remark
GT4	*ushA1, glnV44, (AS)λ-, thi-1, upp-32*	*upp* mutant
Sφ408	*upp-11, relA1, rpsL254 (strR), MetB1*	*udk, upp* mutant
X2224	*thur-1, leuB6 (Am), secA208, fhuA2, lacY1, glnV44, (AS), galK2 (Oc), minB-2, rpsL109 (strR), malT1 (λR), Xyl-7, mtlA2, thi-1, tdk-2, udk-30, upp-30*	*udk, upp* mutant
JM109	*e14-* (*McrA-*), *recA1, endA1, thi-1, supE44, relA1,* Δ *(lac-proAB),* (*F'traD36 proAB* $lacI^{q}Z\Delta M15$), *hsdR17* (r_k-mk+)	wild type

Collection @ jeju

2. 2. DNA sequence analysis

The clone (Accession Number AK102065, cDNA ID 212912) was derived from rice cDNA library (Osato *et al*., 2002) from developing seeds prepared in pBluescript SK-. DNA sequencing was performed by an automatic sequencer (A1Fexpress DNA sequencer, Pharmacia Biotech. Inc., UK) with synthetic oligonucleotide primers. 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 ClustalW 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).

2. 3. Expression of *OsUK/UPRT1* in *E. coli*

It is revealed from the sequence analysis that there is a TAA stop codon in-frame at –39 position upstream from the translation-starting site. Therefore; the specific primers were designed from the sequence information around the translational start and stop codons of OsUK/UPRT1 to amplify ORF and to overexpress the gene product in *E. coli*.

The open reading frame (ORF) of *OsUK/UPRT1* was obtained by PCR with the EST clone as a template and the primers with P1 (5'-ACGGATCCAATGCCGGAAGATT-3') and P4 (5'-CGAGCTCTACTGTCGCTCTAGT-3') using AmpliTaqGold polymerase (Perkin-Elmer, U.S.A). The underline in the primer P1 and P4 is the designed restriction sites for *Bam*HI and *Sac*I to facilitate subcloning, respectively. The amplified fragment 1.500 bp was subcloned into pGEM-T-easy vector (Promega) and then finally subcloned into pBluescript II KS+ (Stratagene Inc., U.S.A) as a *Bam*HI-*Sac*I fragment, to give *pB::OsUK/UPRT1*. The recombinant DNA for overexpression of OSUK/UPRT1 was

Collection @ jeju

confirmed by restriction analysis with *Bam*HI and *Sac*I digestion and nucleotide sequencing with T3 and T7 primers. The *pB::OsUK/UPRT1* construct and control were used to transform *E. coli* JM109 an *upp* mutants of GT4 and *upp-udk* double mutant, respectively.

2. 4. Functional complementation and enzymatic activity

Transformation of *E. coli upp-udk* mutant X2224 was done with *pB::OsUK/UPRT1* construct and control as well as *upp* mutantas GT4 and Sφ408 which were also done with *pB::OsUK/UPRT1* construct by electroporation (ECM399, BTX, USA) respectfully, at 1300 voltage after producing competent cells of *E. coli* mutants and double mutant strains (Sambrook and Rusesel, 2001) by washing with water and glycerol (Kim and Leustek, 1996) using a cuvette with 0.10 cm electrode gap. Following electroporation, it was plated onto LB+Agar medium containing Amp [LB (20 g/L), Agar (15 g/L) and Amp (100 μg/ml)] and incubated overnight at 37°C.

One milliliter LB with Amp liquid medium was inoculated with a single colony of respective strain and grown over night at 37°C with shaking (140 rpm). The culture was poured into 100 ml MM with 20% glucose (20 ml/L), Drop-out medium supplement without uracil (-ura DO) (Sigma, 0.77 g/L) containing Amp (25 μg/ml) in the presence of FU and FD (Sigma, Germany) and was then grown at 37°C with shaking (140 rpm). The enzymatic activity was determined spectometrically by measuring OD at 595 nm at one-hour interval. After 12 hrs, the diluted culture was spread out on LB+Agar medium containing Amp and incubated at 37°C overnight.

Collection @ jeju

2. 5. Growth inhibition assay with FU and FD in *E. coli*

The *E. coli* mutants harboring the *pB::OsUK/UPRT1* construct, and well as wild type (JM109) were grown at 37°C in MM with –Ura DO (0.77 gm/L) (used to eliminate uracil), 20% glucose (20 ml/L) containing Amp (25 μg/ml) in the presence of FU and FD (Sigma, Germany) at final concentrations of 0.1 μg/ml, 0.01 μg/ml and 0.005 μg/ml, respectively. Growth of bacteria was monitored by measuring OD every hour using the spectrophotometer at 595 nm (OD_{595}) (UV1101, Biochrom, England).

dCollection @ jeju

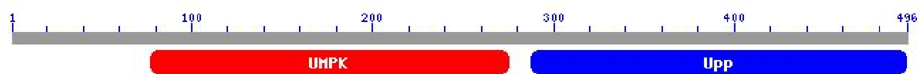
3. RESULTS

3.1. Sequence analysis of OsUK/UPRT1

The clone (AK1020665) was determined by the nucleotide sequence using designed primers. The sequence, *OsUK/UPRT1* cDNA, contained a full-length open reading frame consisting of 1850 bp and encoded a protein of approximately 55.2 kDa. The predicted amino acid sequence of OsUK/UPRT1 is highly homologous to similar sequences from rice and Arabidopsis. Three sequences from Arabidopsis found by database search with OsUK/UPRT1, AtUK/UPRT1 (GenBank accession number AB011477), AtUK/UPRT2 (GenBank accession number 5672506) and AtUK/UPRT3 (GenBank accession number BAF28020, Os11g0265000), have high homology with the identity of 96% 95% and 92% to OsUK/UPRT1 amino acid sequence, respectively. Database analysis shows that *OsUK/UPRT1* sequence is identical to a genomic region located in chromosome 9 in rice (Os09g0505800) and consisted of 14 exons (Fig. 19). Three homologous sequences, *OsUK/UPRT2, OsUK/UPRT3, OsUK/UPRT4*, are located in chromosome 8, 11, and 2 in rice genome (Os08g0530000, Os11g0265000, Os02g0273000), respectively. Three similar sequences from rice were found by database search with OsUK/UPRT1, OsUK/UPRT2 (GenBank accession number BAF24236, Os08g0530000)

A. OsUK/UPRT1

dCollection @ jeju



B.

```
BsURK          1 ---------------------------------------------------------------
EcURK          1 ---------------------------------------------------------------
OsUR/UPRT1     1 MPER--AVDDVMDSAVGAHFSGLRLEALRLSSPSAPSSPSSARAAAAAAAHSNGAVYANG
AtUR/UPRT1     1 MPEDSSSLDYAMERASGPHFSGLRFDGLLSSSPPNSSVVSSLRSAVSSSSPSS-------
BsUPP          1 ---------------------------------------------------------------
EcUPP          1 ---------------------------------------------------------------

                           ATP/GTP-binding site motif A
BsURK          1 ------------MGRNPVVIGIAGGSGSGRTSVTRSIY----EQFRGHSILMIQQDLYYR
EcURK          1 ----------MTDQSHQCVIIGIAGASASGRSLIASTLYRELREQVGDEHIGVIPEDCYYR
OsUR/UPRT1    59 VAADAAELVSPSALRQPFVIGVSGGTASGRTTVCDMII----QQLHDHRVVLVNQDSFYR
AtUR/UPRT1    54 ---------SDPEAPRQPFIIGVSGGTASGRTTVCDMII----QQLHDHRVVLVNQDSFYR
BsUPP          1 ---------------------------------------------------------------
EcUPP          1 ---------------------------------------------------------------
Consensus                                   [AG-4x-GK----ST]

BsURK         45 DQSHLPFEERLNTNYDHPLAFDNDYLIEHIQDLLNYRPIERPIYDYRLHTRSEETVH-VE
EcURK         52 DQSHLSMEERVRTNYDHPSAMDHSLLLEHLQALRRGSAIDLPVYSYVEHTRMRETVT-VE
OsUR/UPRT1   115 GLTAEESAHAQDYNFDHPDAFDTEQLLECMGQLRRAQPVNVPIYDFRNHRRSSESFRRVN
AtUR/UPRT1   102 GLTSEELQRVQEYNFDHPDAFDTEQLLHCAETLRSGQPYQVPIYDFRTHQRRSDTFRQVN
BsUPP          1 ---------------------------------------------------------------
EcUPP          1 ---------------------------------------------------------------

BsURK        104 PRDVIILEGILVLEDRRLRDLMDIRLYVDTDADLRIIRRIMRDINERGRSIDSVIEQYVS
EcURK        111 PRRVIILEGILLLTDARLRDELNFSIFVDTPLDICLMRRIRRDVNERGRSMDSVMAQYQR
OsUR/UPRT1   175 ASDVIILEGILVFHDQRVRNLMDMRIFVDTDADIRLARRIRRDTVERGRDVSSVLEQYGR
AtUR/UPRT1   162 ASDVIILEGILVFHDSRVRNLMNMRIFVDTDADVRLARRIRRDTVERGRDVNSVLEQYAR
BsUPP          1 ---------------------------------------------------------------
EcUPP          1 ---------------------------------------------------------------

BsURK        164 VVRPMHNQFVEPTRRYADIIIPEGGQNHVAIDLMVTRIQTILEQNAIL
EcURK        171 TVRPMFLQFIEPSRQYADIIVPRGGRNRIAIDILRARISQFFE
OsUR/UPRT1   235 FVRPAFDDFVLPSRRYADVIIPRGGDNHVAIDLIVQHIRTRLGQHDLCRIYPHVYVVQTT
AtUR/UPRT1   222 FVRPAFDDFVLPSRRYADVIIPRGGDNHVAVDLITQHIHTRLGQHDLCRIYPNVYVIQST
BsUPP          1 ---------------------------------------------------MGRVYVFDH
EcUPP          1 ----------------------------------------------------MRIVEVRH
```

Fig 19. contd.

Collection @ jeju

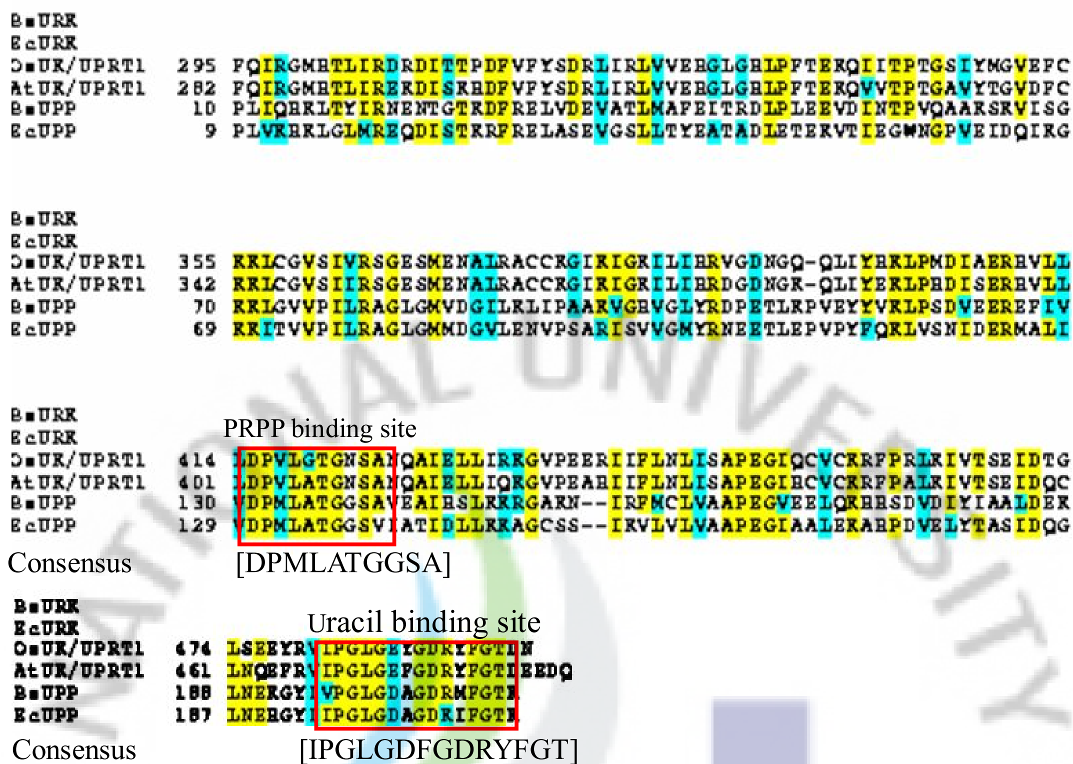


Fig. 19. Structure and amino acids sequence alignment of OsUK/UPRT1.

(A). Structure of OsUK/UPRT1showing UK and UPRT domains and separate genes. **(B)**. Deduced amino acid sequence alignment of UPRT or UK from bacteria and plants.These is *Oryza sativa* (*OsUK/UPRT1*), *Arabidopsis thaliana* (*AtUK/UPRT1*), *Bacillus cereus* (BcUPP, BcURK), and *Escherichia coli* (EcUPP, EcURK). Shaded residues represent amino acids which are identical among at all four of the four amino acids that are GenBank accession numbers as follow AK102065 (OsUK/UPRT1), AB011477 (AtUK/UPRT1), P25532 (EcUPP), and CAA50593 (EcUDK).

Collection @ jeju

The size of OsUK/UPRT1 consisted of 496 amino acids are about double compared to UPRTs from bacteria and protozoa. The C-terminal region of OsUK/UPRT1 is homologous to bacterial UPP sequences suggested that the region has catalytic activity for UPRT. By the analysis of motif with amino acid sequence of OsUK/UPRT1, it was revealed that there are signature-binding motifs for a PRPP and a uracil in the N-terminal region of the OSUK/UPRT1 (Fig. 19). The binding motifs for a PRPP and a uracil, “DPVLATGNSA” (398-407) and “IPGLGEFGDRYFGT” (468-481), respectively, are present in rice OsUK/UPRT1 and well conserved in *E. coli* and many bacteria. The motif sequences for a PRPP and an uracil are highly homologous to the consensus “DPMLATGGSA” and “YI(F)VPGLGDA(F)GDRL(Y,M)F(Y)G(C) T(V)K”, respectively. The finding suggests that the OsUK/UPRT1 product may use uracil and PRPP as substrates to synthesize UMP. Through the analysis of amino acid homology in database, it is suggested that there are functional UPRT homologs in animals and plants. The phylogenic analysis of the deduced sequence is revealed that rice UPRT and UK are evolved from ancestral with *AtUK/UPRT1* and *AtUK/UPRT2* (Fig. 20). The *OsUK/UPRT1* consisted of 496 amino acids and is about double compared to UPRT and UK from bacteria and protozoa.

Collection @ jeju

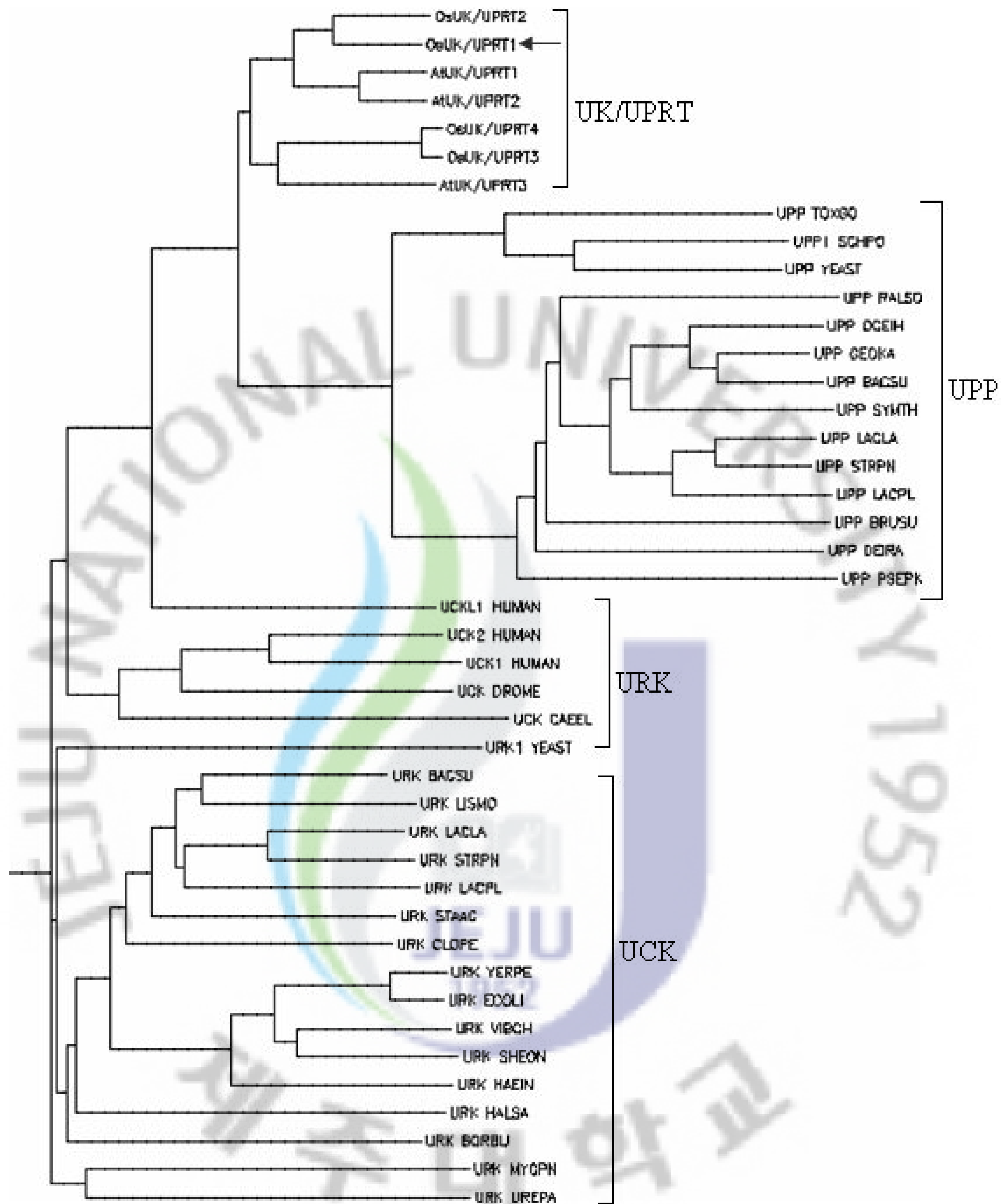


Fig. 20. Phylogenetic analysis of OsUK/UPRT1 related proteins using ClustalW. Accession numbers are as follows: AK102065 (*OsUK/UPRT1*), BAF24236 (*OsUK/UPRT2*), AB011477 (*AtOSUK/UPRT1*), AP000381 (*AtUK/UPRT2*), BAF28020 (*OsUK/UPRT3*),

dCollection @ jeju

BAF08444 (*OsUK/UPRT4*), AC002304 (*AtUK/UPRT3*), Q26998 (*UPP_TOXGO*), O13867 (*UPP_SCHPO*), P18562 (*UPP_YEAST*), Q8XXC7 (*UPP_RALSO*), Q8EM74 (*UPP_OCEIH*), Q5KUI3 (*UPP_GEOKA*), P39149 (*UPP_BACSU*), Q67TC9 (*UPP_SYMTH)*, Q9CEC9 (*UPP_LACLA*), Q97RQ3 (*UPP_STRPN*), Q9CEC9 (*UPP_LACPL*), Q8FUZ2 (*UPP_BRUSU*), Q9RU32 (*UPP_DEIRA*), Q88PV2 (*UPP_PSEPK*), Q9NWZ5 (*UCKL1 _HUMAN),* Q9BZX2 (*UCK2_HUMAN*), Q9HA47 (*UCK1_HUMAN*), Q9VC99 (UCK_DROME), Q17413 (*UCK_CAEEL*), P27515 (*URK1_YEAST*), O32033 (*URK_BACSU*), Q8Y727 (*URK_LISMO*), Q9CF21 (*URK_LACLA*), P67413 (URK_STRPN), Q88WR0 (*URK_LACPL*), Q5HFF1 (*URK_STAAC*), Q8XJI6 (*URK_CLOPE*), Q8ZFZ9 (*URK_YERPE*), Q8EDX4 (*URK_SHEON*), P44533 (*URK_HAEIN*), Q9HQC9 (*URK_HALSA*), Q59190 (*URK_BORBU*), P75217 (*URK_MYCPN*), Q9PQF9 (URK_UREPA),NP060329 (HsURKL1), NP011996 (ScFUR1), (ScURK1), P25532 (EcUPP) and CAA50593 (EcUDK).

dCollection @ jeju

3. 2. ***OsUK/UPRT1* expression in *E. coli* and *in vivo* activity**

The recombinant DNA, *pB::OsUK/UPRT1*, was constructed using ORF of PCR-amplified *OsUK/UPRT1* fragment. After transformation *E. coli*, UPRT and UK activity *in vivo* was monitored by adding FU or FD. The successive production of the fluoro-derivatives of UMP and UTP by UMP kinase and UDP kinase results to RNA damage and inhibition to protein synthesis, which causes broad range of growth retardation in bacterial cells (Koyama *et al*., 2000).

The functional complementation was performed using the *upp* and *upp-udk* mutants of *E. coli* to confirm the enzyme activity by the gene product of *OsUK/UPRT1*. To check viability of *E. coli* cells by UK/UPRT protein activity after treatment of FU and FD, the JM109 cells overexpressing OsUK/UPRT1 were cultured with agitation for 14 hrs with and without FU & FD. And then the diluted portion was plated on LB agar medium containing Amp (Fig. 21). The viable colonies greatly decreased in the strain overexpressing OsUK/UPRT1 treated with FU & FD compared to that without FU & FD. These results in growth retardation and viability suggest that the gene product of OsUK/UPRT1.

Collection @ jeju

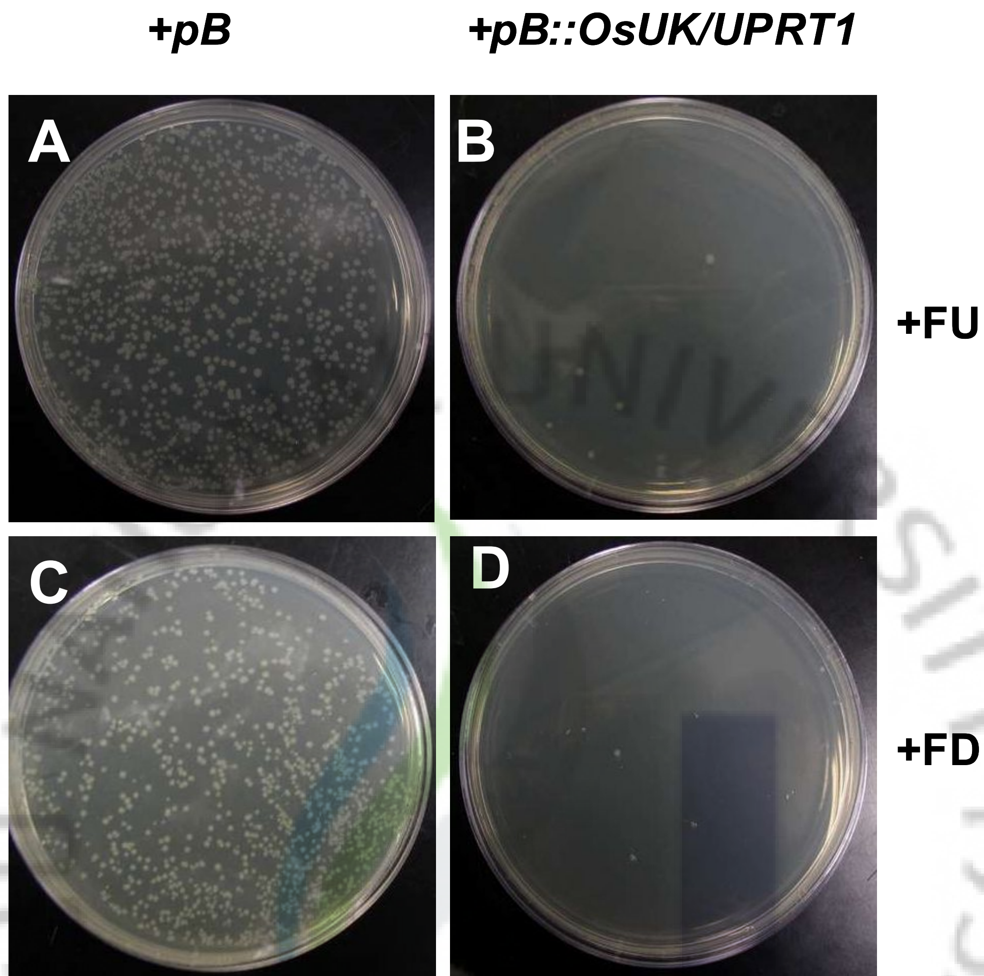


Fig. 21. Functional complementation (*In vivo* assay of OsUK/UPRT1 and *OsUDK/UPRT1* by FU and FD); A) *pB* + FU, B) *pB*::*UK/UPRT1* + FU, C) *pB* + FD, D) *pB*::*UK/UPRT1* + FD.

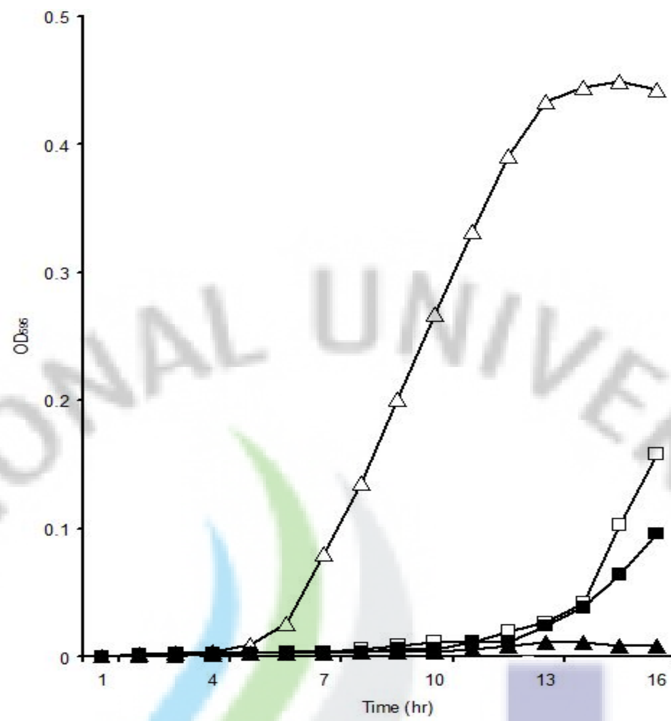
Collection @ jeju

3. 3. FU sensitivity of *E. coli upp* mutants was influenced by the expression of *OsUK/UPRT1*

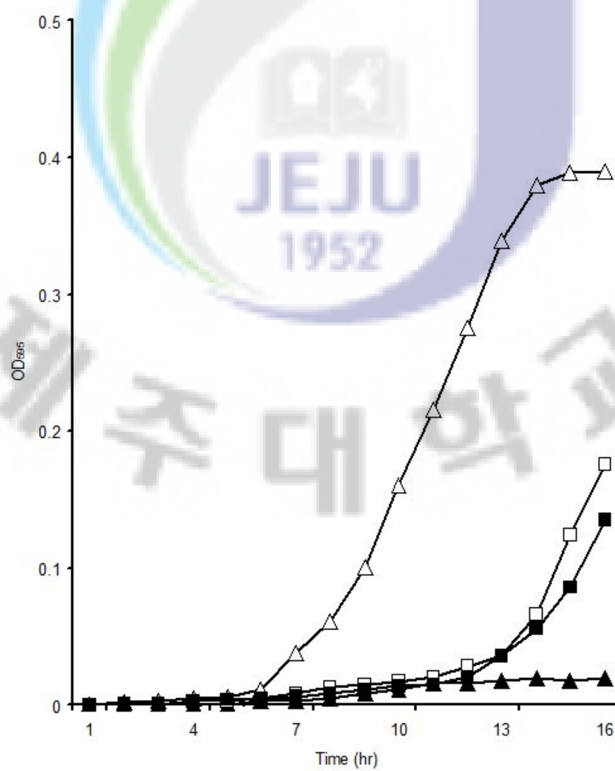
A growth study was performed to determine whether the *OsUK/UPRT1* gene would increase the sensitivity of bacterial cells to FU. The construct of *pB::OsUK/UPRT1* and the pB as a control plasmid were transformed into *upp* mutants (GT4 & Sφ408) and *upp-udk* double mutant (X2224) as well as a wild type (JM109) *E. coli*. Bacterial cells were grown in MM–Ura and the *pB::OsUK/UPRT1* activity in the presence of FU was monitored by a growth assay. The X2224 harboring control plasmid grew normally and showed S-shape classical growth curve in the MM-Ura medium in different concentrations 0.1, 0.01 & 0.005 μg/ml FU. Whereas, X2224 containing *pB::OsUK/UPRT1* was dramatically retarded in the growth phase in the same medium and concentrations of FU (Fig. 22 & Fig. 23). The wild type JM109 expressing control plasmid was also found to have growth retardation in high (0.1 μg/ml FU) concentration (Fig. 22). However, the GT4 & Sφ408 containing *pB::OsUK/UPRT1* were dramatically retarded in the growth phase in the same medium and the same concentrations of FU. The trend of growth retardation of all *upp* mutants expressing OsUK/UPRT1 is almost similar. The growth inhibition of *upp* mutants harboring *pB::OsUK/UPRT1* were even observed at lower concentrations until 12 hrs. So the bacterial growth inhibition was clearly observed in GT4, Sφ408 and X2224 expressing OsUK/UPRT1 as well as JM109 with control plasmid. These results indicated that mutants expressing OsUK/UPRT1 utilizes FU as a substrate that's why growth retardation resulted due to toxicity of FU. So these results indicated the consequence of UPRT activity of rice OsUK/UPRT1.

Collection @ jeju

(A) 0.1 μg/ml FU



(B) 0.01 μg/ml FU



Collection @ jeju

(C) 0.005 μg/ml FU

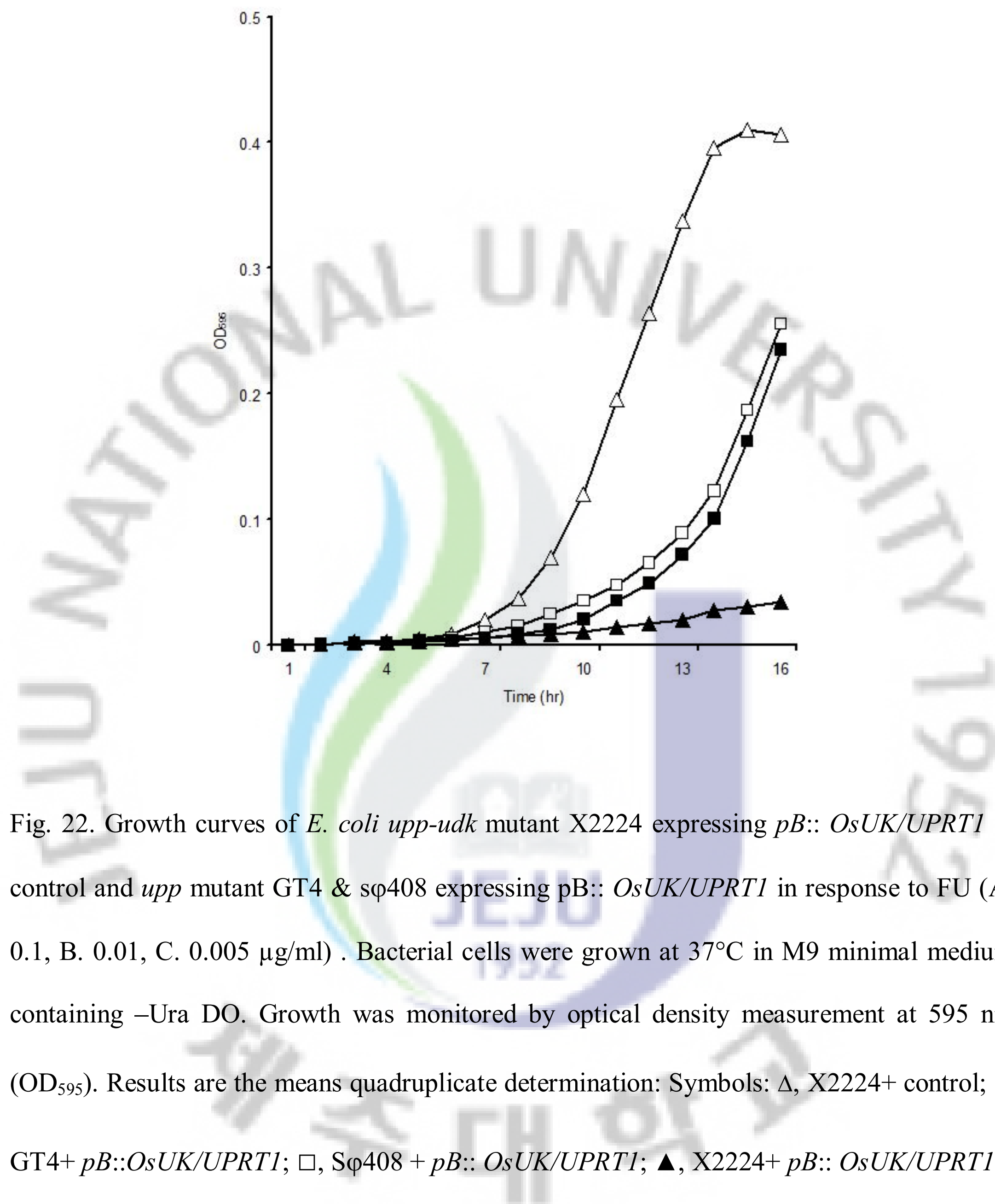
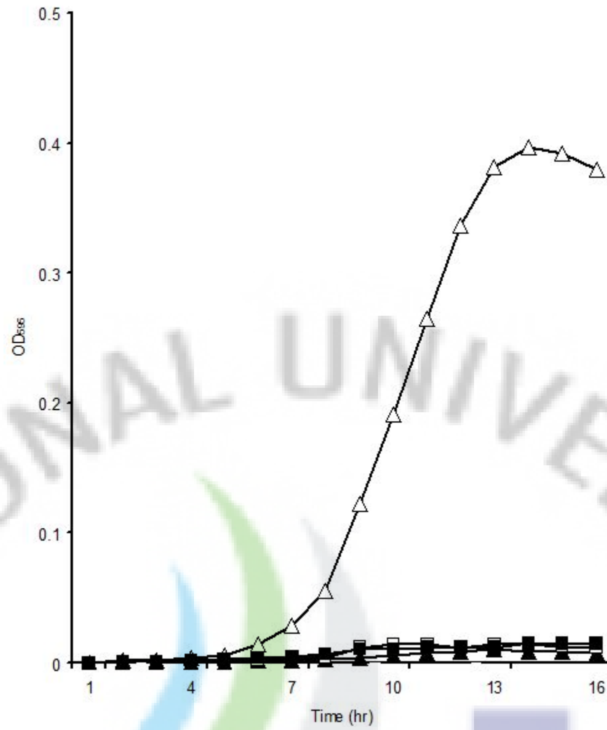


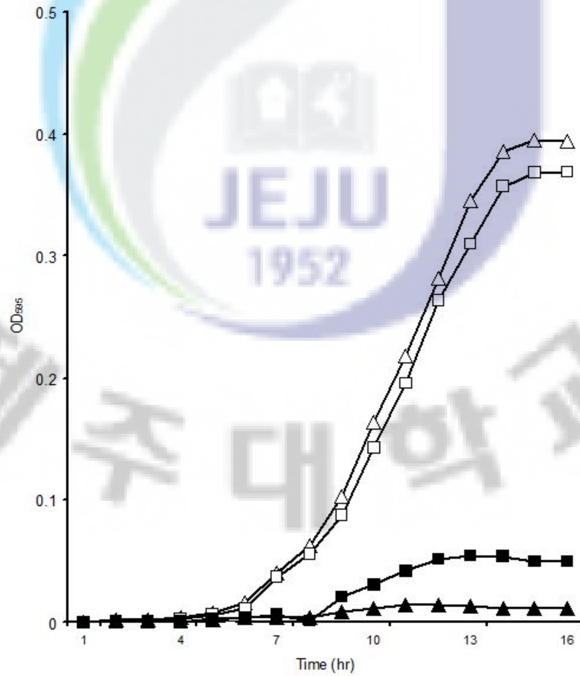
Fig. 22. Growth curves of *E. coli upp-udk* mutant X2224 expressing *pB*:: *OsUK/UPRT1* & control and *upp* mutant GT4 & sφ408 expressing pB:: *OsUK/UPRT1* in response to FU (A. 0.1, B. 0.01, C. 0.005 μg/ml) . Bacterial cells were grown at 37°C in M9 minimal medium containing –Ura DO. Growth was monitored by optical density measurement at 595 nm (OD_{595}). Results are the means quadruplicate determination: Symbols: Δ, X2224+ control; ■, GT4+ *pB*::*OsUK/UPRT1*; □, Sφ408 + *pB*:: *OsUK/UPRT1*; ▲, X2224+ *pB*:: *OsUK/UPRT1*.

(A) 0.1 μg/ml FU

Collection @ jeju



(B) 0.01 μg/ml FU



(C) 0.005 μg/ml FU

Collection @ jeju

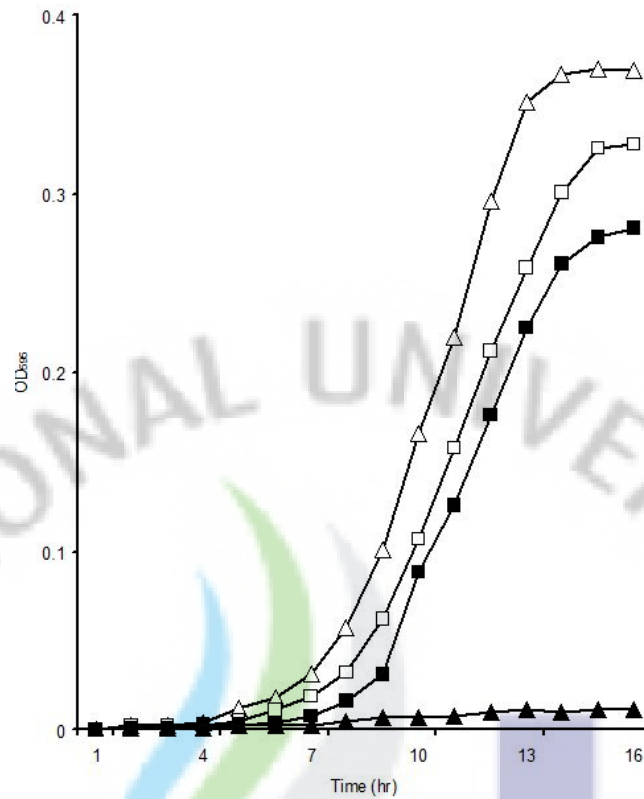


Fig. 23. Growth curves of *E. coli upp-udk* mutant, X2224, and wild type (JM109) expressing *pB:: OsUK/UPRT1*& control in response to FU. Becterial cells were grown at 37°C in MM containing –Ura DO. Growth was monitored by optical density measurement at 595 nm (OD_{595}). Results are the means quadruplicate determination: Symbols: Δ, X2224 + control; □, wild type + control, ■, wild type + *pB::OsUK/UPRT1*; ▲, X2224+ *pB::OsUK/UPRT1.*

3. 4. FD sensitivity of *E. coli upp* mutants was influenced by the expression of

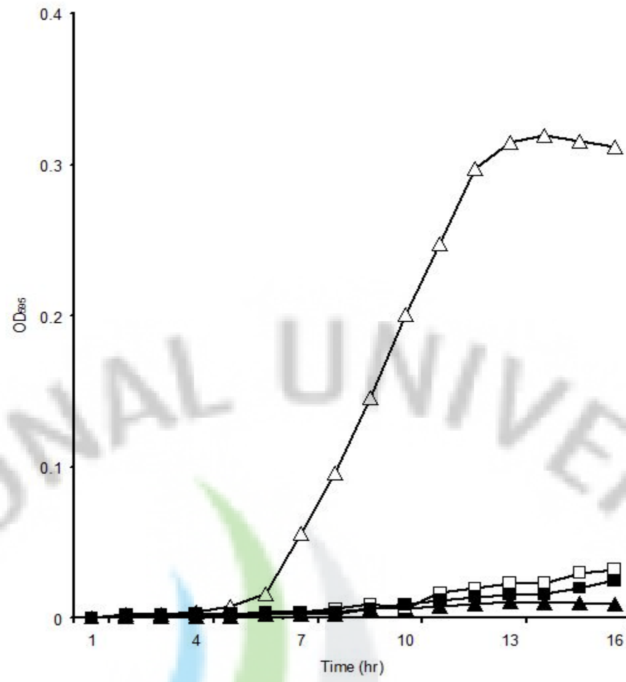
Collection @ jeju

OsUK/UPRT1

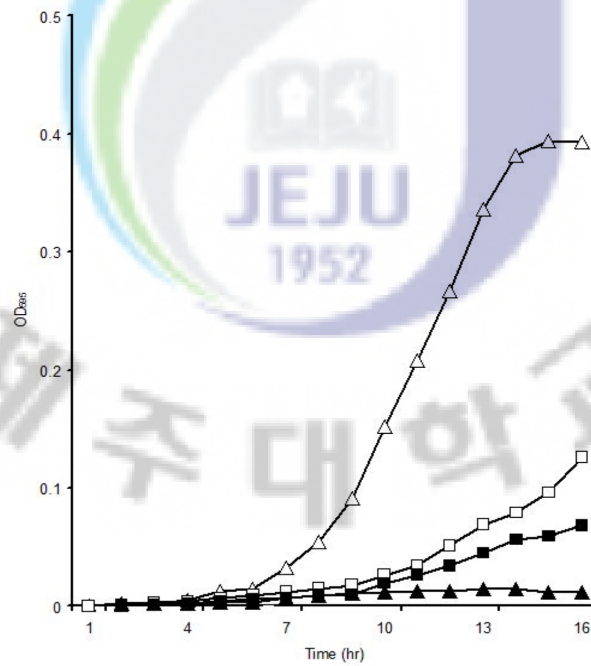
The growth pattern of *E. coli* mutants complemented with pB:: *OsUK/UPRT1* was also investigated in presence of FD. The X2224 harboring control plasmid grew normally and showed S-shape classical growth curve in the MM with –Ura with different concentrations 0.1, 0.01 & 0.005 μg/ml FD, respectively (Fig. 24 & Fig. 25). The JM109 with control plasmid was retarded in growth by the supplementation of FD. The retardation of the growth was more sever in the medium containing a high concentration 0.1 μg/ml FD. On the other hand, JM109 harboring *OsUK/UPRT1* was much more severe in the growth retardation compared with the wild type containing control plasmid and even almost didn't grow in the concentration of 0.01 μg/ml FD in which was grown the wild type was grown containing control plasmid and slight growth in lower concentration 0.005 μg/ml FD (Fig. 24). However, the GT4, Sφ408 and X2224 expressing *pB::OsUK/UPRT1* were dramatically retarded in the growth phase in the same medium and same concentrations of FD. The trends of growth retardation of all *upp* mutants and control plasmid are almost similar. Slight growth was observed in GT4 & Sφ408 expressing *pB::OsUK/UPRT1* at lower concentrations 0.01 & 0.005 μg/ml FD after 10 hrs but X2224 expressing *OsUK/UPRT1*1 continued retardation until 16 hrs (Fig. 25). One explanation in the growth of mutants expressing OsUK/UPRT1 after 10 hrs is that the growth inhibition could be due to re-directing UPRT activity using FU from FD degradation not FD as a substrate directly. The growth of X2224 with control plasmid was unaffected (Fig. 24 & Fig. 25) because there is no toxic effect of FD without UK activity. These results showed the UK activity of OsUK/UPRT1.

(A) 0.1 μg/ml FD

Collection @ jeju



(B) 0.01 μg/ml FD



(C) 0.005 μg/ml FD

dCollection @ jeju

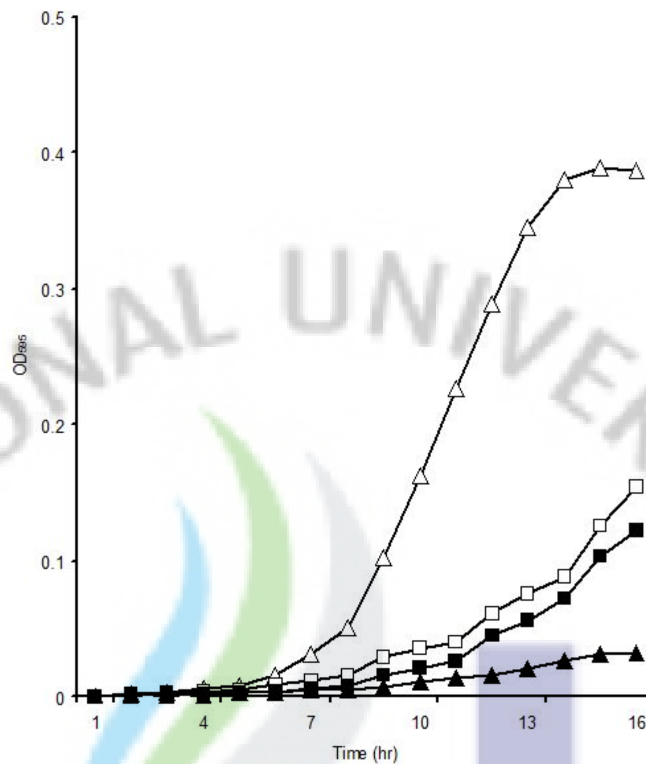
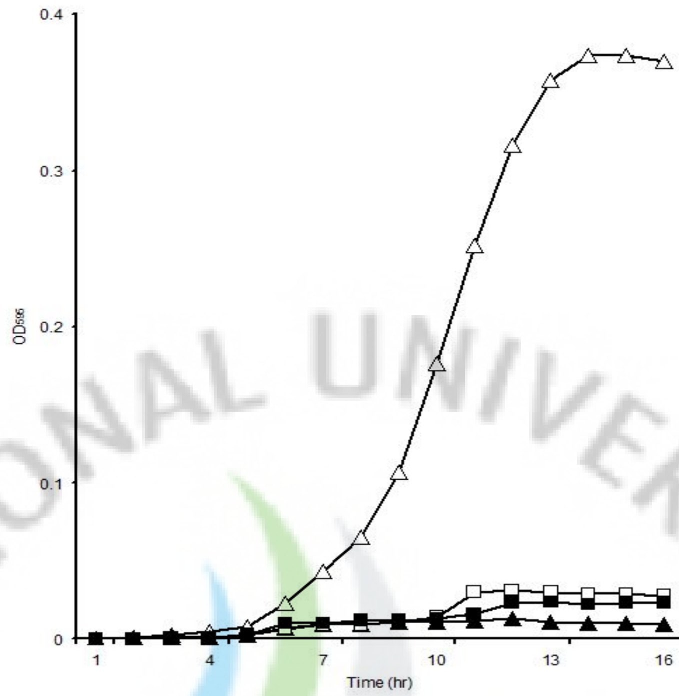


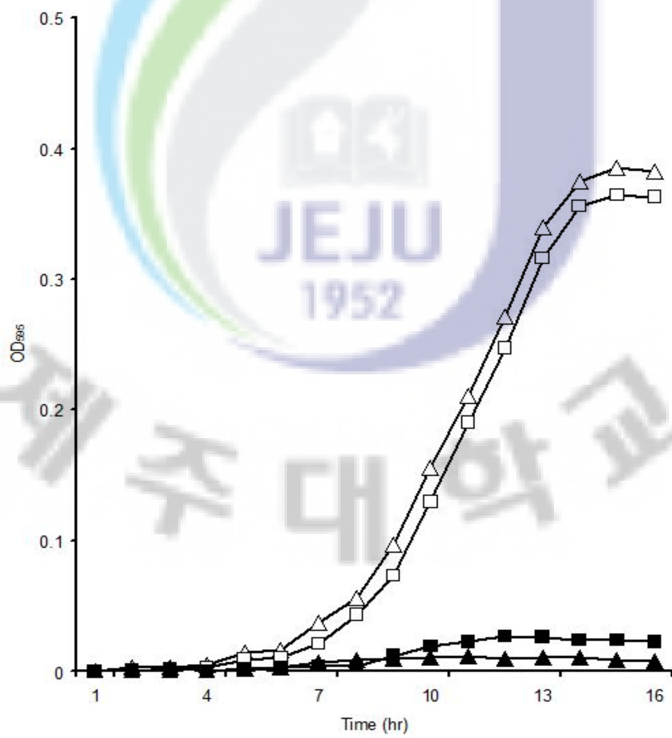
Fig. 24. Growth curves of *E. coli upp-udk* mutant X2224 expressing pB:: *OsUK/UPRT1*& control and *upp* mutant GT4 & sφ408 expressing pB:: *OsUK/UPRT1* in response to FD. Bacterial cells were grown at 37°C in MM containing –Ura DO. Growth was monitored by optical density measurement at 595 nm (OD_{595}). Results are the means quadruplicate determination: Symbols: ∆, X2224+ control; □, GT4+ pB::*OsUK/UPRT1*; ■, Sφ408 + pB:: *OsUK/UPRT1*; ▲, X2224+ pB:: *OsUK/UPRT1*.

(A) 0.1 μg/ml FD

Collection @ jeju



(B) 0.01 μg/ml FD



(C) 0.005 μg/ml FD

Collection @ jeju

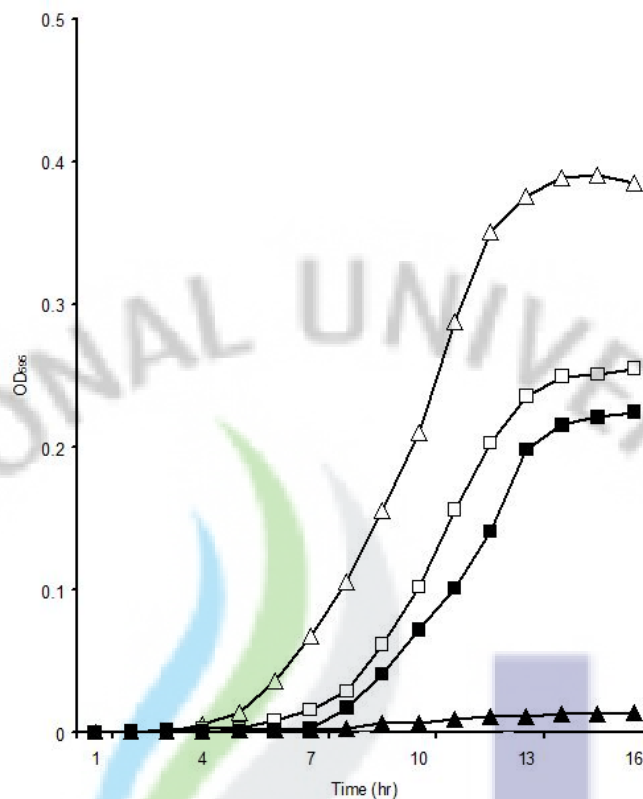


Fig. 25. Growth curves of *E.coli upp-udk* mutant, X2224, and wild type (JM109) expressing *pB*:: *OsUK/UPRT1*& control in response to FD. Becterial cells were grown at 37°C in MM containing –Ura DO. Growth was monitored by optical density measurement at 595 nm (OD_{595}). Results are the means quadruplicate determination: Symbols: ∆, X2224 + control; □, wild type + control; ■, wild type + *pB*::*OsUK/UPRT1*; ▲, X2224+ *pB*:: *OsUK/UPRT1*.

Collection @ jeju

The roles of UK and UPRT have important roles in the pyrimidine salvage pathway. This gene belongs to a class of enzymes catalyzing reactions involving 5-phosphoribosyl 1-pyrophosphate (PRPP). These enzymes are involved in different biosynthetic pathways including the synthesis of histidine, tryptopan, purines, pyrimidines and NAD (Jesen, 1983) there is no specific report on UPRT and UK in higher plants. Only a few studies have been conducted on the uridine salvage pathway of actively growing potato tubers (Katahira and Ashihara, 2002). The functional characterization of uracil phosphoribosyltransferase (UPRT) is in bacteria, yeasts, and protozoa, but not in other animals and plants (Carter *et al*., 1997). Here we report the characterization of a natural fusion gene encoding rice UK and UPRT (*OsUK/UPRT1*) and the gene product by over expression of wild type and *upp* mutant of *E. coli* followed by growth inhibition with FU. Following determination of the nucleotide sequences, the encoded protein was analyzed by a putative novel enzyme for UK and UPRT in plants. The full-length cDNA encodes a 496-amino acid protein that is highly homologous to putative UK and UPRT from arabidopsis and homologous to *FUR1* and URK1 of yeasts and *upp* and *udk* of bacteria and also homologous to upp and udk of *E. coli* (Bismuth *et al*., 1982). There were three homologous genes, in chromosome III and I of arabidopsis which has identity with 90%, 96% and 90% in nucleotide and amino acid sequence between them, respectively, which they assume to perform similar functions in plants.

The *OsUK/UPRT1* from rice is homologous to that from animals such as *C. elegans*, *M. musculus*, and *H. sapiens* compared to that from bacteria or yeasts. The identity between putative UK and UPRT from mammals, *M. musculus* and *H. sapiens*, has increased to 95% in amino acid sequence level. These findings suggest that *OsUK/UPRT1* from rice is more closely related evolutionally to higher organisms such as humans than to lower ones such as bacteria and protozoa. The findings also suggest that there is a protein performing similar function as *OsUK/UPRT1* in animals such as *C. elegans*, *M. musculus*,

and *H. sapiens*. The *OsUK/UPRT1* protein consists of 496 amino acids, which is about

Collection @ jeju

double in size compared to that from bacteria, yeasts, or protozoa, which suggests there are diversity and additional function in rice *OsUK/UPRT1* rather than the counterpart from microbes.

There is another reaction catalyzed by uridine kinase (UK) to synthesize UMP from uridine in the pyrimidine salvage pathway. The role of UPRT in the salvage of endogenously formed uracil and the utilization of exogenous uracil and cytocine has been demonstrated in several microorganisms including *E. coli* (Fast and Sköld, 1977). *E. coli upp* and *udk* are pyrimidine salvage enzymes, which can convert FU and FD into FUMP as the internal step of FU and FD activation (Andersen *et al*., 1992). To test whether *OsUK/UPRT1* from rice has the UK activity, we treated FD with *E. coli* strains of *upp* mutant over expressing *OsUK/UPRT1*. The FD would metabolize to FUMP and have a toxic effect if *OsUK/UPRT1* has UK activity. To minimize the toxic effect of FU from degradation of FD and maximize the reaction for UK by FD, uracil was added with FD. The bacterial strains harboring control plastid showed the classical S-shape growth curve in case of the addition of FD. However, the bacterial lines over expressing OsUK/UPRT1 slowed down growth in log phase after 7 hrs of initial inoculation followed by gradual decrease of the growth until 13 hrs. The strains showed gradual increase of their growth after 13 hrs of initial inoculation, which suggest the substrate (FD) was depleted at this time, and then the growth was reached to the maximum stationary phase with OD_{595} to 1.5, similar to control lines, which is clearly different to the results supplemented with FU as a substrate. In addition to the upp-dependent formation of UMP, uracil may be metabolized (Martinussen and Hammer, 1994). The effect of growth inhibition by FD lasted 6 hrs and was less efficient than that by FU. The results assume that the growth inhibition could be due to re-directing UPRT activity using FU from FD degradation not FD as a substrate directly. Some approaches by over expressing of recombinant OsUK/*UPRT1* in *E. coli* and screening T-DNA insertion mutants in

which the expression of each rice *OsUK/UPRT* gene is knockout would provide important

Collection @ jeju

clues into the substrate specificity and physiological function of this noble enzyme for nucleotide metabolism in plants. The results clearly indicate the importance of the *upp* encoded UPRT activity.

So far, none of UPRT genes from crop plants have been reported. Our report about the characterization of a gene encoding *OsUK/UPRT1* from rice would be a starting point at molecular level to investigate pyrimidine nucleotide metabolisms in rice and to apply a new selection marker in plants and further in gene therapy in humans.

Collection @ jeju

Analysis and characterization of the cDNAs encoding for TS, DHDPS, ALS, IPMDH and UK/UPRT1 from rice have generated bioinformatic predictions, as well as motifs and complementation, in the respective mutant of *E. coli*. We concluded as follow:

- ❖ The OsTS and OsDHDPS are highly homologous to plants and many bacterial TS and DHDPS, respectively. Assessment the physiological functions of novel enzymes for Thr and Lys metabolism were determinated by bioinformatics and functional complementation in a *thrC* and a *dapA* mutant of *E. coli.* Those genes would be able to modify the nutritional composition of crop plants.
- ❖ The ALS & IPMDH genes were cloned by functional complementation. The ALS & IPMDH genes could be used as a powerful tool for future application to development herbicide resistant crops in selectable markers. ALS & IPMDH genes could be reintroduced into crop plants for improvement of rice qualities.
- ❖ The OsUK/UPRT1 protein has a PRPP and Uracil binding sites. in vivo assay to FU and FD, it suggests that *OsUK/UPRT1* is encoded in UK and UPRT as a natural fusion protein in rice. The gene encoding *OsUK/UPRT1* from rice could be an important clue to investigate pyrimidine nucleotide metabolisms in rice and it could also be applied to cancer gene therapy in humans.

]

REFERENCES

Collection @ jeju

Andersen PS, Smith JM, Mygind B (1992). Characterization of the *upp* gene encoding uracil phosphoribosyltransferase of *Escherichia coli* K12. Eur. J. Biochem. 204: 51-56.

Andrews M, Lea PJ, Raven JA, Lindsey K (2004). Can genetic manipulation of plant nitrogen assimilation enzymes result in increased crop yield and greater N-use efficiency? An assessment. Annals of Appli. Biol. 145: 25–40.

Asai T, Lee CS, Chandler A, O'Sullivan WJ (1990). Purification and characterization of uracil phosphoribosyltransferase from *Crithidia luciliae*. Comp. Biochem. Physiol. 95: 159-163.

Avraham T, Amir R (2005). The expression level of threonine synthase and cystathionine-γ-synthase is influenced by level of both threonine and metheonine in Arabidopsis plants.Trans. Gen. Res. 14: 299-311.

Azevedo RA, Arruda P, Turner WL, Lea PJ (1997). The biosynthesis and metabolism of the aspartate derived amino acids in higher plants. Phytochem. 46: 395-419.

Azevedo RA, Lancien M, Lea JP (2006). The aspartic acid metabolic pathway, an exciting and essential pathway in plants. Amino acids, 30: 143-162.

Bismuth G, Thuillier L, Perignon JL, Carter PH (1982). Uridine as the only alternative to pyrimidine de novo synthesis in rate T-lyphocytes. FEBS Lett. 148: 135-139.

Blickling S, Beisel HG, Bozic D, Knäblein J, Laber B, Huber R (1997). Structure of dihydrodipicolinate synthase of *Nicotiana sylve*. Mol. Biol. 274: 608–621.

Brendel V, Bucher P, Nourbakhsh IR, Blaisdell BE, Karlin S (1992). Methods and algorithms for statistical analysis of protein sequences. Proc. Natl. Acad. Sci. 89:

Collection @ jeju

2002-2006.

Bryan A (1980). The aspartate family and Branched-Chain Amino Acids.In the Biochemistry of plants, Vol. 5. Amino acids and Derivatives (Miflin, J. ed.). New York: Academic press Inc. 403-452.

Calvo JM (1983). Leu biosynthesis in prokaryotes. In: Herrmann KM, Somerville RL (eds.) Amino acids biosynthesis and genetic regulation. Addison-Wesley, Reading, Mass. 267-284.

Carter D, Robert GK, Donald Roos D, Ullman B1997. Expression, purification, and characterization of uracil phosphoribosyltransferse from *Toxoplasma gondii*. Mol. Biochem. Parasit. 87: 137-144.

Caszza AP, Basner A, Höfgen R, Hesse H (2000). Expression of threonine synthase from *Solanum tuberosum* L. is not metaolically regulated by photosynthesis-relatate signals or by nitrogenous compounds. Plant Sci. 157: 43-50.

Chang AK, Duggleby RG (1997). Expression, purification and characterization of *Arabidopsis thaliana* acetohydroxyacid synthase. Biochem. J. 327: 161-169.

Choia K-J, Yub YG, Hahnc HG, Choid J-D, Yoona M-Y (2005). Characterization of acetohydroxyacid synthase from *Mycobacterium tuberculosis* and the identification of its new inhibitor from the screening of a chemical library. FEBS Lett. 579: 4903–4910.

Clepet C, Borne V, Krishnapillai C, Baird C, Patte JC, Cami B (1992). Organization and expression of the Pseudomonas aeruginosa threonine genes. Mol. Microbiol. 6: 3109-3119.

Covarrubias AS, Högbom M, Bergfors T, Carroll P, Mannerstedt K, Oscarson S,

Parish T, Jones TA, Mowbray SL (2008). Structural, biochemical, and *in vivo*

Collection @ jeju

investigations of the threonine synthase from *Mycobacterium tuberculosis*. J. Mol. Biol. 381(3): 622-633.

Curien G, Bastien O, Robert-Genthon M, Cornish-Bowden A, Cardenas ML, Dumas R (2009). Understanding the regulation of aspartate metabolism using a model based on measured kinetic parameters. Mol. Syst. Biol. 5(271): 1-14.

Curien G, Biou V, Mas-Droux C, Robert-Genthon M, Ferrer JL, Dumas R (2008). Amino acid biosynthesis: new architectures in allosteric enzymes. Plant Physiol. Biochem. 46(3): 325-339

Curien G, Dumas R, Ravanel S, Douce R (1996). Characterization of an *Araidopsis thaliana* cDNA encoding an S-adenosylmethionine-sensitive threonine synthase. FEBS Lett. 390: 85-90.

Curien G, Job D, Douce R, Dumas R (1998). Allosteric activation of Arabidopsis threonine synthase by S-adenosymetheonine. Biochem. 31: 13212-1322.

David AF, Burle GG, Andrew MT, Janita MS, David AS, Dorothea EM (1991). Isolation and Characterization of Dihydrodipicolinate Synthase from Maize. Plant Physiol. 96: 444–452.

Debadov VG (2003). The threonine story. *In* T Scheper, ed, Advances in Biochem. Eng. Biotechnol. 79: 113-136.

Dobson RCJ, Griffin MDW, Jameson GB, Gerrard JA (2005). The crystal structures of native and (*S*)-lysine-bound dihydrodipicolinate synthase from *Escherichia coli* with improved resolution show new features of biological significance. Acta Cryst. 61: 1116-1124.

Duggleby RG, McCourt JA, Guddat LW (2008). Structure and mechanism of inhibition of plant acetohydroxyacid synthase. Plant Physiol. Biochem. 46: 309-324.

Durner J, Boger P (1988). Acetolactate synthase from barley (*Hordeum vulgare*.):

Collection @ jeju

purification and partial characterization. Z. Naturforsch. 43: 850-856

Eikmanns BJ, Eggeling L, Sahm H (1993). Molecular aspects of lysine, threonine, and Ile biosynthesis in *Corynebacterium glutamicum*. Antonie Van. Leeuwenhoek 64: 145-163.

Elena AR, Gary AB, Kevin CG, Soon SJ, Eric JS, Timothy JR (2008). Characterization and crystal structure of lysine insensitive *Corynebacterium glutamicum* dihydrodipicolinate synthase (cDHDPS) protein. Arch. Biochem. Biophys. 480: 111–121.

Ellerström M, Josefsson LG, Rask L, Ronne H (1992). Cloning of a cDNA for rape chloroplast 3-isopropylmalate dehydrogenase by genetic complementation in yeast. Plant Mol. Biol. 18: 557–566.

Emma BB, Susanne JC, David WT, David JR, Andrea S, Chris A, John RC (1995). *Escherichia coli* dihydrodipicolinate synthase: characterization of the imine intermediate and the product of bromopyruvate treatment by electrospray mass spectrometry. Biochem. J. 305: 521-524.

Eoyang L, Silverman PM (1984). Purification and subunit composition of acetohydroxyacid synthase I from *Escherichia coli* K-12. J. Bacteriol 157: 184-189.

Falco SC, Guida T, Locke M, Mauvais J, Sanders C, Ward RT, Webber P (1995). Transgenic canola and soybean seeds with increased lysine. Biotechnol. 13: 577–582.

Falco SC, Dumas KS (1985). Genetic analysis of mutants of *Saccharomyces cerevisiae* resistant to the herbicide sulfometuron methyl. Genetics. 109(1): 21–35.

Farrington GK, Kumar A, Shames SL, Ewaskiewicz JI, Ash DE, Wedler FC

(1993). Threonine synthase of *Escherichia coli*: inhibition by classical and slow-

Collection @ jeju

binding analogues of homoserine phosphate. Arch. Biochem. Biophys. 307: 165-174.

Fast R, Sköld O (1977). Biochemical mechanism of uracil uptake regulation in *Escherichia coli*. B. J. Biol. chem. 252: 7620-7624.

Ferreia RR, Meinhardt LW, Azevedo RA (2006). Lysine and Threonine biosynthesis in sorghum seeds: Characterization of aspartate Kinase & homoserine dehydrogenase isoenzymes. Ann. Appl. boil. 149: 77-86.

Flavin M, Slaughter C (1960). Purification and properties of threonine synthase of *Neurospora*. J. Biol. Chem. 235: 1103-1108.

Galili G (1995). Regulation of lysine and threonine synthesis. Plant Cell 7: 899–906.

Ghislain M, Frankard V, Jacobs M (1990). Dihydrodipicolinate synthase of *Nicotiana sylvestris*, a chloroplast-localized enzyme of the lysine pathway. Planta 180: 480–486

Grimminger H, Umbarger HE (1979). Acetohydroxy acid synthase I of *Escherichia coli*: purification and properties. J. Bacteriol. 137: 846-853.

Guardiola J, Felice MD, Iaccarino M (1974). Mutant of *Escherichia coli* K-12 missing acetolactate synthase activity. J. Bacteriol. 120(1): 536-538.

Halling SM, Stahly DP (1976). Dihydrodipicolinic acid synthase of *Bacillus licheniformis*. Quaternary structure, kinetics, and stability in the presence of sodium chloride and substrates. Biochim. Biophys. Acta. 452: 580–596.

Hamasawa K, Kobayashi Y, Harada S, Yoda K, Yamasaki M, Tamura G (1987). Molecular Cloning and Nucleotide Sequence of the 3- Isopropylmalate dehydrogenase Gene of *Candida utilis.* J. Gen. Microbiol. 133: 1089-1097.

Hammer-Jespersen K, Munch-Petersen A (1973). Mutants of *Eschericia coli* unable to metabolize cytidine: isolation and chararization. Mol. Gen. Genet. 126: 177-186.

Collection @ jeju

Han KS, Archer AJC, Sinskey Al (1990). The molecular structure of the *Corynebacterium glutamicum* threonine synthase gene. Mol. Microbiol. 4: 1693-1702.

Hattori H, Rutledge RG, Miki BL, Baum BR (1992). DNA sequence relationships andorigins of acetohydroxy acid synthase genes of *Brassica napus*. Can. J. Bot. 70: 1957-1963.

Hesse H, Hoefgen R (2003). Molecular aspects of methionine biosynthesis.Trends plant Sci. 8: 259-262.

Hesse H., Kreft O, Maimann S, Zeh M, Hoefgen R (2004). Current understanding of the regulation of methionine biosynthesisin plants. J. Exp Bot. 55: 1799-1808.

Higgins DG, Bleasby AJ, Fuchs R (1992). Clustal V: improved software for multiple sequence alignment. Comput. Appl. Biosci. 8: 189-191.

Hoang TT, Schweizer HP (1997). Identification and genetic characterization of the *Seudomonas aeruginosa leuB* gene encoding 3-isopropylmalate dehydrogenase. Mol. Gen. Genet. 254: 166-170.

Hofmann K, Bucher P, Falquet L, Bairoch A (1999). The PROSITE database, its status in 1999. Nucleic Acids Res. 27: 215-219.

Huang S, Kruger DE, Frizzi A, D'Ordine RL, Florida CA, Adams WR, Brown WE Luethy MH (2005). High-lysine corn produced by the combination of enhanced lysine biosynthesis and reduced zein accumulation. Plant Biotechnol. J. 3: 555–569.

Islam MR, Kim H, Kang SW, Kim JS, Jeong YM, Hwang HJ, Lee SY, Woo JC,

Kim SG. (2007). Functional characterization of a gene encoding a dual domain for uridine kinase and uracil phosphoribosyltransferase in *Arabidopsis thaliana*. Plant

Collection @ jeju

Mol. Biol. 63: 465-477.

Jackson SD, Sonnewald U, Willmitzer L (1993). Cloning and expression analysis of beta isopropylmalate dehydrogenase from potato. Mol. Gen. Genet. 238: 309–314.

Jandera G, Joshia V (2009). Aspartate-derived Amino Acid Biosynthesis in *Arabidopsis thaliana.* The Arabidopsis Book. American Society of Plant Biologists. 1-15.

Jesen KF (1983). Metabolism of 5-phosphoribosyl 1-pyrophosphate (PRPP) in *Escherichia coli* and *Salmonella typhimurium*, p.1-25. In A. Munch-Petersen (ed.), Metabolisms of nucleotides, nucleosides, nucleosides and nucleobases in microorganisms. Academic press, London

Jensen KF, Mygind B (1996). Different oligomeric states are involved in the allosteric behavior of uracil phosphoribosyltransferase from *Escherichia coli*. Eur. J. Biochem. 240: 638-645.

Jianguo GW, Chunhai S, Xiaoming Z (2002). Estimating the amino acid composition in milled rice by near-infrared reflectance spectroscopy. Field Crops Res. 75 (1):1-7.

Katahira R, Ashihara H (2002). Profiles of pyrimidine biosynthesis, salvage and degradation in disks of potato (*Solanum tuberosum* L.) tubers. Planta, 215: 821-828.

Kawaguchi H, Inagaki K, Kuwata Y, Tanaka H, Tano T (1993). 3-Isopropylmalate dehydrogenase from chemolithoautotroph *Thiobacillus ferrooxidans*: DNA sequence, enzyme purification, and characterization. J. Biochem. 114**:** 370–377.

Kelland JG, Arnold LD, Palcic MM, Pickard MA, Vederas JC (1986). Analogs of

diaminopimelic acid as inhibitors of mesodiaminopimelate decarboxylase from *Bacillus sphaericus* and wheat germ. J Biol Chem 261: 13216-13223.

Collection @ jeju

Kern L, Montigny de, Jund R, Lacroute F (1989). The FUR1 gene of *Saccharomyces cerevisiae*: cloning, structure and expression of wild-type and mutant alleles. Gene 88: 149-157.

Khan MMK, Komatsu S (2004). Rice proteomics: recent developments and analysis of nuclear proteins. Phytochemistry. 65:1671–1681.

Kim J, Lee M, Chalam R, Martin MN, Leustek T, Boerjan, W (2002). constitutive overexpression of cystathionine-γ-synthase in Arabidopsis leads to accumulation of soluble methionine and S-methylmethionine. Plant physiol. 128: 95-107.

Kim J, Leustek T (2000). Repression of cystathionine-γ-synthase in *Arabidopsis thaliana* produces partial methionine auxotrophy and developmental abnormalities. Plant Sci. 151: 9-18.

Kim J, Leustek T (1996). Cloning and analysis of the gene for cystathionine g synthase from *Arabidopsis thaliana*. Plant Mol. Biol. 32: 1117-1124.

Kingsbury JM, McCusker JH (2008). Threonine biosynthetic genes are essential in *Cryptococcus neoformans*. Microbiol. 154 (9): 2767-2775.

Koyama F, Sawada H, Fujii H, Hamada H, Hirao T, Ueno M, Nakano H (2003). Adenoviral-mediated transfer of *Escherichia coli* uracil phosphoribosyltransferse (UPRT) gene to modulate the sensitivity of the human colon cancer cells to 5-fluorouracil. Eur. J. Cancer. 36: 2403-2410.

Kumar S, Nei M, Dudley J, Tamura K (2008). MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. Briefings Bioinform. 9(4): 299-306.

Kumpaisal R, Hashimoto T, Yamada Y (1987). Purification and characterization of dihydrodipicolinate synthase from wheat suspension cultures. Plant Physiol. 85: 145–

Collection @ jeju

151.

Lawther RP, Calhoun DH, Gray JE, Adams CW, Hauser CA, Hatfield GW (1982). DNA sequence fine-structure analysis of *ilvG* (IlvG+) mutations of *Escherichia coli* K-12. J. Bacteriol. 149: 294-298.

Lee M, Martin MN, Hudson AO, Lee J, Muhitch MJ, Leustek T (2005). Methionine and threonine synthesis are limited by homoserine availability and not the activity of homoserine kinase in *Arabidopsis thaliana*. Plant J. 41: 685-696.

Levitt G, Ploeg HL, Weigel RC, Filtzgerald DJ (1981). 2-Chloro-N [(4- methoxy-6- methyl- 1, 3, 5-triazin-2-yl) aminocarbonyl] benzene sulfonamide, a new herbicide. J Agric Food Chem. 29: 416-418.

Li J, Huang S, Chen J, Yang Z, Fei X, Zheng M, Ji C, Xie Y, Mao Y (2007). Identification and characterization of human uracil phosphoribosyltransferase (UPRTase). J. Hum. Genet. 52: 415-422.

Madison JT, Thompson JF (1976). Threonine synthetase from higher plants: stimulationby s-adenosylmethionine and inhibition by cysteine. Biochem. Biophys Res. Commun.71: 684-691.

Mann C (1997). Reseeding the green revolution. Science 277: 1038–1043.

Marc V, Val´erie F, Michel J (1999).The *Arabidopsis thaliana dhdps* gene encoding dihydrodipicolinate synthase, key enzyme of lysine biosynthesis, is expressed in a cell-specific manner. Plant Mol. Biol. 39: 695–708.

Martinussen J, Glaser P, Andersen PS, Saxild HH (1995). Two genes encoding uracil phosphoribosyltransferase are present in *Bacillus subtilis*. J. Bacteriol. 177: 271-274.

Martinussen J, Hammer K (1994). Cloning and Characterization of upp, a Gene Encoding Uracil Phosphosribosyltransferase from *Lactococcus lactis*. J.

dCollection @ jeju

Bacteriol. 176 (21): 6457-6463.

Mas-Droux C, Biou V, Dumas R (2006). Allosteric threonine synthase: Reorganization of the pyridoxal phosphate site upon asymmetric activation through S-adenosylmethionine binding to a novel site. J Biol. Chem. 281(8): 5188-5196.

Matsunami H, Kawaguchi K, Inagaki K, Eguchi T, Kakinuma K, Tanaka H (1998). Overproduction and substrate Specificity of 3-Isopropylmalate dehydrogenase fron *Thiobacillus ferrooxidans*. Biosci. Biotechnol. Biochem. 62(2): 372-373.

Maturin L, Curtiss J (1981). Role of ribonucleic acid synthesis in conjugational transfer of chromosal and plasmid deoxyribonucleic acids. J. Bacteriol. 146: 552-563.

Mazur BJ, Chui C-F, Smith JK (1987). Isolation and Characterization of Plant Genes Coding for Acetolactate Synthase, the Target Enzyme for Two Classes of herbicides. Plant Physiol. 85: 1110-1117.

Miyagi T, Koshida K, Hori O, Konaka H, Katoh H, Kitagawa Y, Mizokami A, Egawa M, Ogawa S, Hamada H, Namiki M (2003). Gene therapy for prostate cancer using the cytosine deaminase/uracil phosphoribosyltransferase suicide system. J. Gene Med. 5: 30-37.

Mueller U, Huebner S (2003). Economic aspects of amino acids production. *In* T Scheper, ed, Advances in Biochemical Engineering/Biotechnology. 79: 137- 170.

Moffatt BA, Ashihara H (2002) Purine and pyrimidine nucleotide synthesis and metabolism. In: Somerville CR, Meyerowitz EM (Eds) The arabidopsis book, American society of plant biologists, Rockville, MD. doi: 10.1199/tab.0018, http:// www.aspb.org/publications/arabidopsis

Muhitch MJ, Shaner DL, Stidham MA (1987). Imidazolinones and Acetohydroxyacid Synthase from Higher Plants: Properties of the Enzyme

Collection @ jeju

from Maize Suspension Culture Cells and Evidence for the Binding of Imazapyr to Acetohydroxyacid Synthase in Vivo. Plant Physiol. 83(2): 451-456.

Muller YA, Lindqvist Y, Furey W, Schulz GE, Jordan F, Schneider G (1993). A thiamin diphosphate binding fold revealed by comparison of the crystal structures of transketolase, pyruvate oxidase and pyruvate decarboxylase. Structure. 1(2): 95–103.

Nagahari K, Sakaguchi K (1978). Cloning of *Bacillus subrilis* leucina A, B and C genes with *Escherichia coli* plasmids and expression of the *leuC* gene in *E. coli.* Mol Gen Genet 158: 263-270.

Natalini P, Ruggieri S, Santarelli I, Vita A, Magni G (1979). Baker's yeast UMP: pyrophosphate phosphoribosyl transferase. Purification, enzymatic and kinetic properties. J. Biol. Chem. 254: 1558-1563.

Nozawa A, Takano J, Miwa K, Nakagawa Y, Fujiwara T (2005). Cloning of cDNAs Encoding Isopropylmalate Dehydrogenase from *Arabidopsis thaliana* and Accumulation Patterns of Their Transcripts. Biosci. Biotechnol. Biochem. 69 (4): 806-810.

Orwick PL, Marc RA, Umeda K, Shaner DL, Los M, Clarlante DR (1983). AC 252, 214-A new broad spectrum herbicide for soybeans: Greenhouse studies. Proc. South. Weed Sci. Soc. 36: 90.

Osato N, Itoh M, Konno H, Kondo S, Shibata K, Carninci P, Shiraki T, Shinagawa A, Kikuchi S, Sato K, Kawai J, Hayashizaki Y (2002). A Computer-based method of selecting clones for a full-length cDNA project: simultaneous collection of negligibly

Collection @ jeju

redundant and variant cDNAs. Genome Res. 12: 1127-1134.

Ott KH, Kwagh JG, Stockton GW, Sidorov V, Kakefuda G (1996). Rational molecular design and genetic engineering of herbicide resistant crops by structure modeling and site-directed mutagenesis of acetohydroxyacid synthase. J. Mol. Biol. 263: 359- 368.

Pearce FG, Perugini MA, McKerchar HJ, Gerrard JA (2006). Dihydrodipicolinate synthase from *Thermotoga maritime*. Gerrard, Biochem. J. 400: 359–366.

Rakwal R and Agrawal GK (2003). Rice proteomics: Current status and future perspectives. Electrophoresis. 24: 3378–3389.

Rasmussen UB, Mygind B, Nygaard P (1986). Purification and some properties of uracil phosphoribosyltransferase from *Escherichia coli* K12. Biochim. Biophys. Acta. 881: 268-267.

Raven JA, Andrews M, Quigg A (2005). The evolution of oligotrophy: implications for the breeding of crop plants for low input agricultural systems. Annals of App. Bio. 146: 261–280.

Ray TB (1984). Site of action of chlorsulfuron: inhibition of Val and Leu biosynthesis in plants. Plant Physiol. 75: 827-831.

Ricardo AD, Germano CN, Ad´ılson L, Jos´e AY, Paulo A (1999). The *DapA* gene encoding the lysine biosynthetic enzyme dihydrodipicolinate synthase from *Coix lacryma-jobi*: cloning, characterization, and expression analysis. Plant Mol. Biol. 41: 551–561.

Rinder J, Casazza AP, Hoefgen R, Hesse H (2008). Regulation of aspartate-derived amino acid homeostasis in potato plants (*Solanum tuberosum* L.) by expression of *E. coli* homoserine kinase. Amino Acids. 34(2): 213-222.

Collection @ jeju

Sambrook J, Russell DW (2001). Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press. Cold Spring Harbor.

Schloss JV, Ciskanik LM, VanDyk DE (1988). Origin of the herbicide binding site of acetolactate synthase. Nature, 331: 360-362.

Schloss JV, VanDyk DE, Vasta JF, Kutny RM (1985). Purification and properties of *Salmonella typhimurium* acetolactate synthase II from *E. coli* HB 101/pDU9. Biochem. 24: 4952-4959.

Schumacher MA, Bashor CJ, Song MH, Otsu K, Zhu S, Parry RJ, Ullman B, Brennan RG. (2002). The structural mechanism of GTP stabilized oligomerization and catalytic activation of the *Toxoplasma gondii* uracil phosphoribosyltransferase. Proc. Natl. Acad. Sci. 99: 78-83.

Schumacher MA, Carter D, Scott DM, Roos BU, Brennan RG. (1998). Crystal structures of *Toxoplasma gonddii* uracil phosphoribosyltransferase reveal the atomic basis of pyrimidine discrimination and prodrug binding. EMBO. J. 12: 3219-3232.

Singh BK, Shaner DL (1995). Biosynthesis of branched chain amino acids: from test tube to field. Plant Cell. **7**: 935–944.

Singh BK, Stidham MA, Shaner DL (1988). Assay of acetohydroxyacid synthase. Anal Biochem. 171(1): 173–179.

Shaner DL, Anderson PC, Stidham MA (1984). Imidazolinones (potent inhibitors of acetohydroxyacid synthase). Plant Physiol. 76: 545-546.

Shimamoto K (1995). The molecular biology of rice. Sci. 270: 1772–1773.

Silk GW, Matthews BF, Somers DA, Gegenbach BG (1994). Cloning and expression of the soybean *DapA* gene encoding dihydrodipicolinate synthase. Plant Mol. Biol. 26: 989–993.

Collection @ jeju

Sotelo A, Hernandez M, Montalvo I, Sousa V (1994). Amino acid content and protein biological evaluation of 12 Mexican varieties of rice. Nutrition 71: 605–609.

Southan MD, Copeland L (1996). Physical and kinetic properties of acetohydroxyacid synthase from wheat leaves. Physiologia Plantarum. 98(4): 824 – 832.

Suzuki T, Inoki Y, Yamagishi A, Iwasaki T, Wakagi T, Oshima T (1997). Molecular and Phylogentic Characterization of Isopropylmalate Dehydrogenase of a Thermoacidophilic Archaeon, *Sulfolobus sp* Strain 7. J. Bacteriol. 1174-1179.

Tang DW, Li LF, Yu YM, Liu XY, Su XD, Zhao X, Liang YH (2007). Preparation, crystallization and preliminary X-ray analysis of threonine synthase from *Streptococcus* mutans. Protein Pept Lett. 14(8): 836-838.

Theze JD, Margarita G.N, Cohen F, Borne J, Patte C (1974). Mapping of the Structural genes of the three aspartokinases and of the two homoserine dehydrogenases of *Escherichia coli* K-12. J. Bacteriol. 117: 133-140.

Thompson GA, Datko AH, Mudd SH, Giovanelli J (1982). Methionine biosynthesis in *Lemna*: Studies on the regulation of cyststhionine gamma-synthase, *O*-phosphohomoserine Sulfhydrylase and *O*-acetylserine sulhydrylase. Plant Physio. 69: 1077-1083.

Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting position-specific gap penalties and weight matrix choice.Nucleic Acids

Collection @ jeju

Res. 22: 4673-4680.

Umbarger HE (1996). Biosynthesis of the branched-chain amino acids. In: Neidhardt F, Ingraham JL, Low KB, Magasanik B, Schaechter M, Umbarger HE (eds) *Escherichia coli* and *Salmonella*. ASM Press, Washington DC, 352-367.

Wallon G, Yamamoto K, Kirino H, Yamagishi A, Lovett ST, Petsko GA, Oshima T (1997). Purification, Catalytic properties and thermostability of 3-isopropylmalate dehydrogenase from *Escherichia coli*. Biochimica et Biophysica Acta. 1337: 105- 112.

Wallsgrove RM, Mazelis M (1981). Spinach leaf dihydrodipicolinate synthase: Partial purification and characterization. Biochem. 20: 2651–2655.

Ward JB, Zahler SA (1973). Regulation of Leu biosynthesis in *Bacillus subtilis*. J. Bacteriol. 116: 727-735.

Webler G, Shiotani T, Kizaki H, Tzeng D, Williams JC, Gladstone N (1978). Biochemical strategy of the genome as expressed in regulation of pyrimidine metabolism. Adv. Enzyme Regul. 16: 3–19.

Wheeler DL, Church DM, Federhen S, Lash AE, Madden TL, Pontius JU, Schuler GD, Schriml LM, Sequeira E, Tatusova TA, Wagner L (2003). Database resources of the National Center for Biotechnology. Nucleic Acids Res. 31(1): 28-33.

Wiersma PA, Schmiemann MG, Condie JA, Crosby WL, Moloney MM (1989). Isolation, expression and phylogenetic inheritance of an acetolactate synthase gene from *Brassica napus*. Gen. Genet. 219: 413-420.

Wittenbach VA, Teaney PW, Hanna WS, Rayner DR, Schloss JV (1994).

Herbicidal Activity of an Isopropylmalate Dehydrogenase Inhibitor. Plant Physiol. 106(1): 321–328.

Collection @ jeju

Yoon J-M, Yoon M-Y, Kim YT, Choi J-D (2003). Characterization of Two Forms of Acetolactate Synthase from Barley. J. Biochem. Mol. Biol. 36(5): 456-461.

Collection @ jeju

ACKNOWLEDGEMENTS

At first, I deeply express my gratefulness to almighty Allah who gave me the capability to finish my higher study abroad.

I would like to express my deepest sense of gratitude and heartfelt thanks to my respected academic advisor, Dr. Jung-Sup Kim, Jeju National University for his scholastic guidance, sincere appreciation and constructive criticism in pursuing my research, course work and subsequent write-up of this dissertation

It's a great pleasure for me to extend sincere appreciation and heartfelt thanks to Dr. Young-Hwan Ko, Dr. Young-Hoon Yang, Jeju National University, Dr. Yi Lee, Chungbuk National University and Dr. Ho Bang Kim, Myongji University for their valuable suggestions and cooperation. I wish to thanks Mi-Sun Kim, Cheju Halla College for her suggestions and Dr. So-Mi Kim, Jeju National University for her suggestions and encouragement during course work. Also thanks to my lab members (Bo-Hwa Kim, Eun-Sung Moon, Dae-Kun Kim, Yun-Ju Lee, Seung-Won Lee, Su-Mi Son, Seong-A Kang, Ji-Won Cha and Min-Ju Kim) for their co-operation.

I wish to thanks Biogreen 21, RDA and Korea research foundation (KRF) for funding. I also thanks to Rice Genome Resource Center (RGRC), National Institute of Agro biological Science (NIAS), Japan and *E. coli* Genetic Stock Center (CGSC) at Yale University, USA for providing an EST clones and *E. coli* strains, respectively. I would like to acknowledge my employer of Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh for providing legal support to pursue my higher study at Jeju National University.

dCollection @ jeju

I also express my sincere thanks to Mary Branson Visiting Professor, Jeju National University for her valuable advice and co-operation and thanks to Jeju National University' foreign students especially Bangladeshi (Dr. Jasim, Abhijit, Adnan, Mostafiz, Al-Amin and Dr. Ashik) for their co-operation.

I am delighted to express thanks to Dr. Md. Rafiqul Islam, Associate Professor, Department of Soil Science, Bangladesh Agricultural University and my colleagues at Hajee Mohammad Danesh Sciences and Technology University, Dinajpur, Bangladesh for their help.

I am deeply indebted to my beloved mother (Sufia Khatun), younger brothers (Rafiqul, Shahidul, Shaidul and Jahangir), sister (Hasina) and maternal uncle (Mostofa Sikdar) for their sacrifices, encouragement and constant support during my stay abroad. Finally, I would like to give my special thanks to my wife (Jahanara Hydar) for her patience and love during my time at Jeju National University in the Republic of Korea.

Collection @ jeju

BIOGRAPHY

Personal Information

Name : Md. Shafiqul Islam Sikdar

Father’s name : Mohammad Ali Sikdar

Nationality: Bangladeshi

Date of birth: 1 February, 1970

Telephone : +88-01713-163322

e-mail : shafiq_agn@yahoo.com

Address:

Present address	Permanent address
Department of Agronomy	Village: Arjunpur
Faculty of Agriculture	P.O.: Rajapur hat
Hajee Mohammad Danesh Science	Upazilla: Baraigram
and Technology university	District: Natore
Dinajpur-5200	Bangladesh
Bangladesh	

Collection @ jeju

Scientific Publications:

1. **MSI Sikdar**, J-S Kim (2009). Functional analysis of a gene encoding threonine synthase from rice (Submitted). Afn J Biotechnol.
2. **MSI Sikdar**, J-S Kim (2009). Analysis and characterization of a gene encoding ornithine carbamoyltransferase from Rice. J. Plant Biotechnol. 36: 397–402.
3. **MSI Sikdar**, J-S Kim (2009). A natural fusion gene encoding uracil phosphoribosyltransferase and uridine kinase from rice (Submitted). Afn J Biotechnol.
4. **MSI Sikdar**, MM Rahman, MS Islam, MS Yeasmin, M. M.Akhter (2008). Effect of Nitrogen level on Aromatic Rice varieties and soil fertility status. Intl. J. Sustain Crop Prod. 3(3): 49-54.
5. MS Islam, **MSI Sikdar**, MM Rahman, MM Akhter, AK Azad (2008). Performance of Aromatic rice varieties as influenced by spacing. J. Innov. Dev. Stategy. 2 (1): 43-46.
6. **MSI Sikdar**, M Ahmed, MS Islam, MM Rahman, MRA Sarker (2006) Effect of Nitrogen level on the yield, yield contributing characters and protein content of some varieties of aromatic rice. Bangladesh J. Crop Sci. 17 (2): 437-442.
7. MF Hossan, MA Kabir, MA Majumder, **MSI Sikdar**, MMA Chowdury (2005). Influence of Irrigation and nitrogen level on the yield of wheat. Pakistan J. Biol.Sci. 8(1): 152-155.
8. MF Hossan, MS Akhter, MA Majumder, **MSI Sikdar**, MMAA Chowdury (2004). Effect of tillage practices and nitrogen levels on the physical properties of soil. Pakistan J. Biol. Sci.7 (1): 1876-1879.
9. MS Akter, MF Hossain, UK Majumder, **MSI Sikdar**, MAA Chowdhury (2003).

Collection @ jeju

Effect of tillage practices and nitrogen rates on the organic matter (%) and N (%) content in soil. Pakistan J. Biol. Sci. 4(4): 1202-1204.

10. TM Islam, MA Salam, Z Pervez, **MSI Sikdar**, M.Eunus (2002) A Comparative study of yield and return of late Jute seed cultivation at normal and puddle soil. Pakistan J. Aron. 1(4): 139-141.

Papers Presented in International Conference

1. MSI Sikdar, J-S Kim (2008). A natural fusion gene encoding uracil phosphoribosyltransferase and uridine kinase from rice for human gene therapy. Proceedings of the 2008, BIT's 6th Annual Congress of International Drug Discovery Science and Technology (IDDST), Beijing, China, p. 486.

Collection @ jeju