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Full Length Research Paper

Expression of a natural fusion gene for uracil phosphoribosyltransferase and uridine kinase from rice shows growth retardation by 5-fluorouridine or 5-fluorouracil in *Escherichia coli*

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Uridine kinase (UK) and uracil phosphoribosyltransferase (UPRT) are enzymes in pyrimidine salvage pathway. UK or UPRT were reported as separate genes in bacteria. Amino acid sequence of *OsUK/UPRT1* from rice shows homology to bacterial enzymes. Amino-terminal region is similar to UDK with an ATP/GTP-binding motif, whereas the carboxyl-terminal similar to UPP with two binding motifs for a uracil and a PRPP, respectively. Expression of OsUK/UPRT1 in an udk and/or upp mutant of *Escherichia coli* led to growth inhibition with 5-fluorouridine or 5-fluorouracil. These results suggest that the OsUK/UPRT1 product would be a natural fusion protein of two enzymes.

Key words: 5-Fluorouracil, 5-fluorouridine, ***Oryza sativa,*** uridine kinase, uracil phosphoribosyltransferase.

INTRODUCTION

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. UTP is synthesized from UMP by successive reactions and further metabolized to synthesis of CTP. Besides ***de novo*** synthesis of pyrimidines, uracil, uridine or cytosine, are salvaged to synthesize UMP or cytidine 5'-monophosphate by pyrimidine salvage pathway (Islam et al., 2007).

Uracil phosphoribosyltransferase (UPRT, EC 2.4.2.9 and UPP in bacteria) 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 and UDK in bacteria) 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 be upp, udk and pyrF in ***Escherichia coli*** and FUR1, URK1 and URA3 in ***Saccharomyces cerevisiae***, respectively (Andersen et al., 1992). The genes encoding UPRT have been cloned and characterized from bacteria, yeasts, protozoa (Schneider et al., 1974; Kelley, 1983; Dai et al., 1994) including ***Bacillus subtilis*** (Martinussenet al., 1995), ***Lactococcus lactis*** (Martinussen and Hammer, 1994) and ***Streptococcus salivarius*** (Gifferd et al., 1993). UK also has been characterized from ***E. coli*** (Fast and Sköld, 1977), ***S. cerevisiae*** (Kern, 1990) and currently described as a fusion gene for UK and UPRT in ***Arabidopsis thaliana*** (Islam et al., 2007).

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

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Abbreviations: UK, Uridine kinase; **UPRT,** uracil phosphoribosyltransferase; **PRPP,** 5-phosphoribosyl-1-pyrophosphate; **5-FD,** 5-fluorouridine; **5-FU,** 5-fluorouracil; **CTP,** cytidine 5'-triphosphate; **UMP,** uridine 5'-monophosphate; **ATP,** adenosine 5-triphosphate; **UTP,** uridine 5'-triphosphate; **GTP,** guanosine 5'-triphosphate; **UDK ,** uridine kinase in bacteria; **MM-Ura**, minimal medium without Ura; **ORF,** open reading frame; **PCR,** polymerase chain reaction; **RGRC,** rice genome resource center; **EMBL,** European molecular biology laboratory; **BLAST,** basic local alignment search tool; **Clustal W,** clustal weights program.

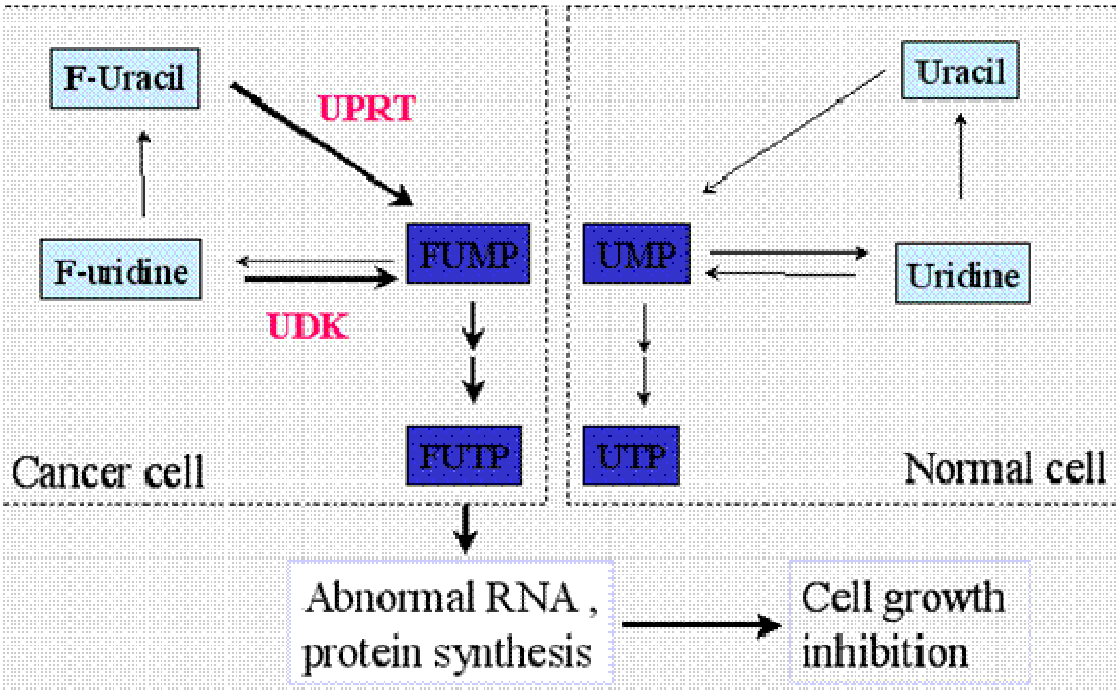

Figure 1. Cancer gene therapy (possible mechanism of cell death in cancer cells by treatment of 5-FU and 5-FD.

salvage activity is a more important source of UMP than the ***de novo*** pathway for many mammalian cells (Webler et al., 1978). The 5-FU and 5-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 used medically to treat various cancers in the large intestine, stomach, pancreas, breast, prostate, etc. (Nakamura et al., 2001). The successive production of the fluro-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 principle of cancer therapy is that 5-FU and 5-FD are toxic to metabolize fluro-derivatives of UMP and UTP, and finally cause mistake of RNA base and gene expression (Figure 1) especially in cancer cells if UPRT is over expressed and active in their catalytic activity (Koyama et al., 2000). The UK activity is usually higher than that of UPRT and thus for UMP synthesis, uridine is typically more efficiently salvaged than uracil (Kanamori et al., 1981; Ashihara et al., 2000).

Here we report a key gene with dual domains for UPRT and UK enzyme from rice and the analysis of its function to investigate the pyrimidine salvage pathway in crop plants.

MATERIALS AND METHODS

Strains and plasmids

The ***E. coli*** strains (GT4, X2224, Sφ408 and JM109) are used in this study which is presented in Table 1. The source of all strains except JM109 (Strata gene) were the ***E. coli*** Genetic Stock Center (CGSC) in Yale University. The JM109 strain was used as a host for cloning purposes.

DNA sequence analysis

An EST clone harboring putative uracil phosphoribosyltransferase/ uridine kinase (Genbank accession number AK102065 and clone number J03308E21) was obtained from the Rice Genome Resource Center (RGRC). The clone 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) designed 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; University of California San Diego, USA). Comparison of sequences was performed at the nucleotide and amino acid level. The motifs were searched by GenomeNet Computation Service at Kyoto University (http://www.genome.ad.jp).

Expression of OsUK/UPRT1 in *E. coli*

The sequence analysis is revealed to be a TAA stop codon in-frame at –39 positions upstream from the possible translation-starting site. Therefore, the specific primers were designed from the sequence information around the translational start and stop codon of OsUK/UPRT1 to amplify full-length open reading frame (ORF) and to over express the gene product in ***E. coli***. The ORF of OsUK/UPRT1 was obtained by PCR from the EST clone described above as a template and the designed primers with P1 (5'-ACGGAT

Table 1. ***E.coli*** strains used in this study.

Strains	Genotype	Source
GT4	***ushA1, glnV44 (AS), λ-, thi-***1and ***upp-32.***	CGSC
Sφ408	***upp-11, relA1, rpsL254 (strR)*** and ***metB1.***	CGSC
X2224	***thr-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*** and ***upp-30.***	CGSC
JM109	***e14-(McrA-), recA1, endA1, thi-1, supE44, relA1, Δ (lac-proAB), [F"traD36 proABlacI^{q}Z ΔM15)*** and ***hsdR17 (r_k-mk+)].***	Stratagene

CCAATGCCGGAAGATT-3') and P4 (5'-CGAGCTCTACTGTC GCTCTAGT-3') using AmpliTaqGold polymerase (Perkin-Elmer, USA). The underline in the primer P1 and P4 is the designed restriction site for ***Bam***HI and ***Sac***I to facilitate subcloning, respectively. The amplified fragment (1.5 kb) was subcloned into pBluescript II KS+ (pB, Stratagene Inc., USA) to give ***pB:: OsUK/UPRT1***. The recombinant DNA for overexpression of OsUK/UPRT1 was confirmed by restriction analysis. The ***pB::OsUK/UPRT1*** construct and pBluescript II KS+ as a control were used to transform ***E. coli*** JM109, an ***upp*** mutants of GT4 and Sφ408 and a ***upp-udk*** double mutant X2224, respectively. Transformation of ***E. coli*** was performed by electroporation (ECM399, BTX, USA) at 1300 voltage using a cuvette with 0.1 cm electrode gap (Sambrook et al., 2001) and then plated onto LB agar containing 100 µg/ml ampicillin (Amp) and grown overnight at 37°C.

Functional complementation and growth inhibition assay with 5-FU and 5-FD in *E. coli*

The fully grown culture was tested in growth retardation in 100 ml M9 minimal medium without Ura (MM-Ura) containing 25 µg/ml Amp in the presence of 5-FU and 5-FD and grown at 37°C with shaking. The bacterial growth was determined spectometrically by measuring optical density at 595 nm at one-hour interval. The ***E. coli*** mutants and wild type (JM1098) harboring the ***pB::OsUK/ UPRT1*** construct and pB were grown at 37°C in MM-Ura medium containing 25 µg/ml Amp in the presence of 5-FU and 5-FD at final concentrations of 0.1, 0.01 and 0.005 µg/ml 5-FU and 5-FD, respectively. The bacterial growth was monitored by measuring optical density every hour using the spectrophotometer (UV1101, Biochrom, England) at 595 nm (OD_{595}).

RESULTS AND DISCUSSION

Sequence analysis of *OsUK/UPRT1*

A clone (AK1020665) was determined by the nucleotide sequence using designed primers. The sequence, OsUK/ UPRT1 cDNA, contained a full-length open reading frame consisted in 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. 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. Three homologous sequences, OsUK/UPRT2, OsUK/UPRT3 and OsUK/UPRT4, are located in chromosome 8, 11 and 2 in rice genome (Os08g0530000, Os11g0265000 and Os02g0273000), respectively.

The predicted amino acid sequence of ***OsUK/UPRT1*** is homologous to the putative UK and UPRT sequences from many bacteria. The C-terminal region of OsUK/ UPRT1 sequence is similar to UPP of ***E. coli*** and FUR1 of ***S. cerevisiae*** that is encoded a functional UPRT enzyme. The deduced ***OsUK/UPRT1*** sequence is similar about 27 and 31% to that of UPP and FUR1 in amino acid sequence level, respectively. The binding motifs for a PRPP and a uracil, "DPVLATGNSA" (415-424) and "IPGLGEFGDRYFGT" (481-494), respectively, are present in rice ***OsUK/UPRT1*** and well conserved in ***E. coli*** and many bacteria (Figure 2). 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. The C-terminal region of ***OsUK/UPRT1*** is homologous to bacterial UPP sequences suggested that the region has catalytic activity for UPRT. The size of ***OsUK/UPRT1*** which consisted of 496 amino acids are about double compared to UPRT from bacteria. Amino-terminal region is similar to UDK of ***E. coli*** and many bacteria that contain an ATP/GTP-binding site motif A called P-loop. Phylogenetic analysis of the sequence indicated that rice UK/ UPRT1 is evolved from ancestral with plant UK/UPRTs as fusion proteins of UK and UPRT (Figure 3).

OsUK/UPRT1 expression in *E. coli*

The recombinant DNA, ***pB::OsUK/UPRT1***, was constructed using ORF of PCR-amplified ***OsUK/UPRT1*** fragment. After transformation to ***E. coli***, UPRT and UK activity ***in vivo*** was monitored by adding 5-FU or 5-FD.

The functional complementation was performed using the ***upp*** mutants of ***E. coli*** to confirm the enzyme activity by the gene product of ***OsUK/UPRT1***. To check the growth retardation of ***E. coli*** cells by UPRT and UK activity after treatment of 5-FU and 5-FD, the JM109 cells over-expressing ***OsUK/UPRT1*** were cultured for 14 h with and without 5-FU and 5-FD and then the diluted portion was plated. The viable colonies greatly decreased in the strain overexpressing ***OsUK/UPRT1*** treated with 5-FU and 5-FD compared to that without these toxic chemicals.

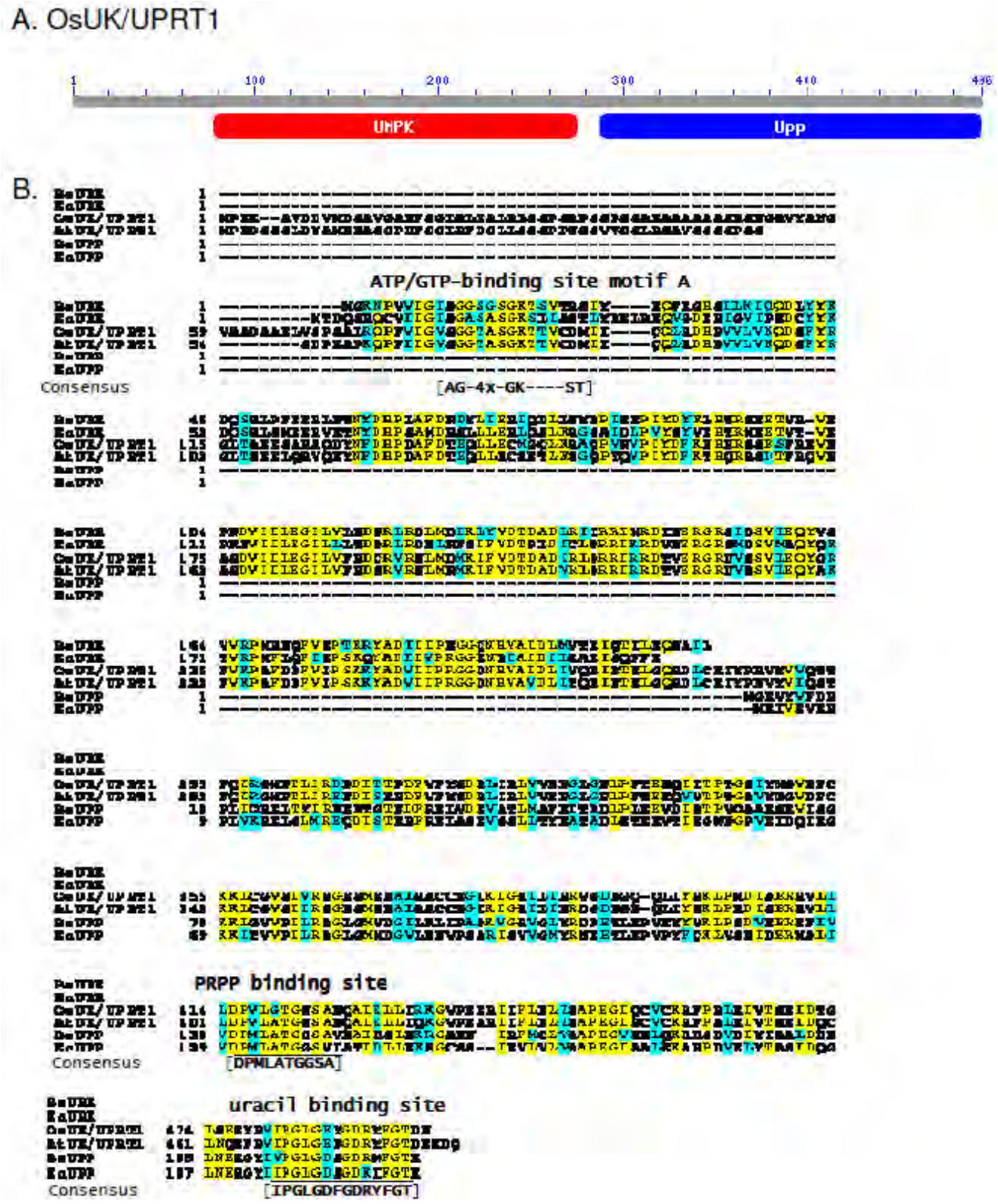

Figure 2. Amino acid sequence alignment of UPRT or UK from bacteria and plants. Program was used to generate the alignment. Shaded residues represent amino acids which are identical among at least four of the four amino acids. The GenBank accession numbers for each sequence are as follows: AK102065 (***OsUK/UPRT1***, ***Oryza sativa***), AB011477 (AtUK/UPRT1, ***Arabidopsis thaliana***), NP_834977 (BcUPP, ***Bacillus cereus***), YP_001376312 (BcURK, ***Bacillus cereus***), P25532 (EcUPP, ***Escherichia coli***) and CAA50593 (EcUDK, ***Escherichia coli***). The binding motifs for ATP/GTP, PRPP and uracil are indicated with a line including consensus sequences, respectively. Amino acid sequence alignment of UPRTs and/or UKs.

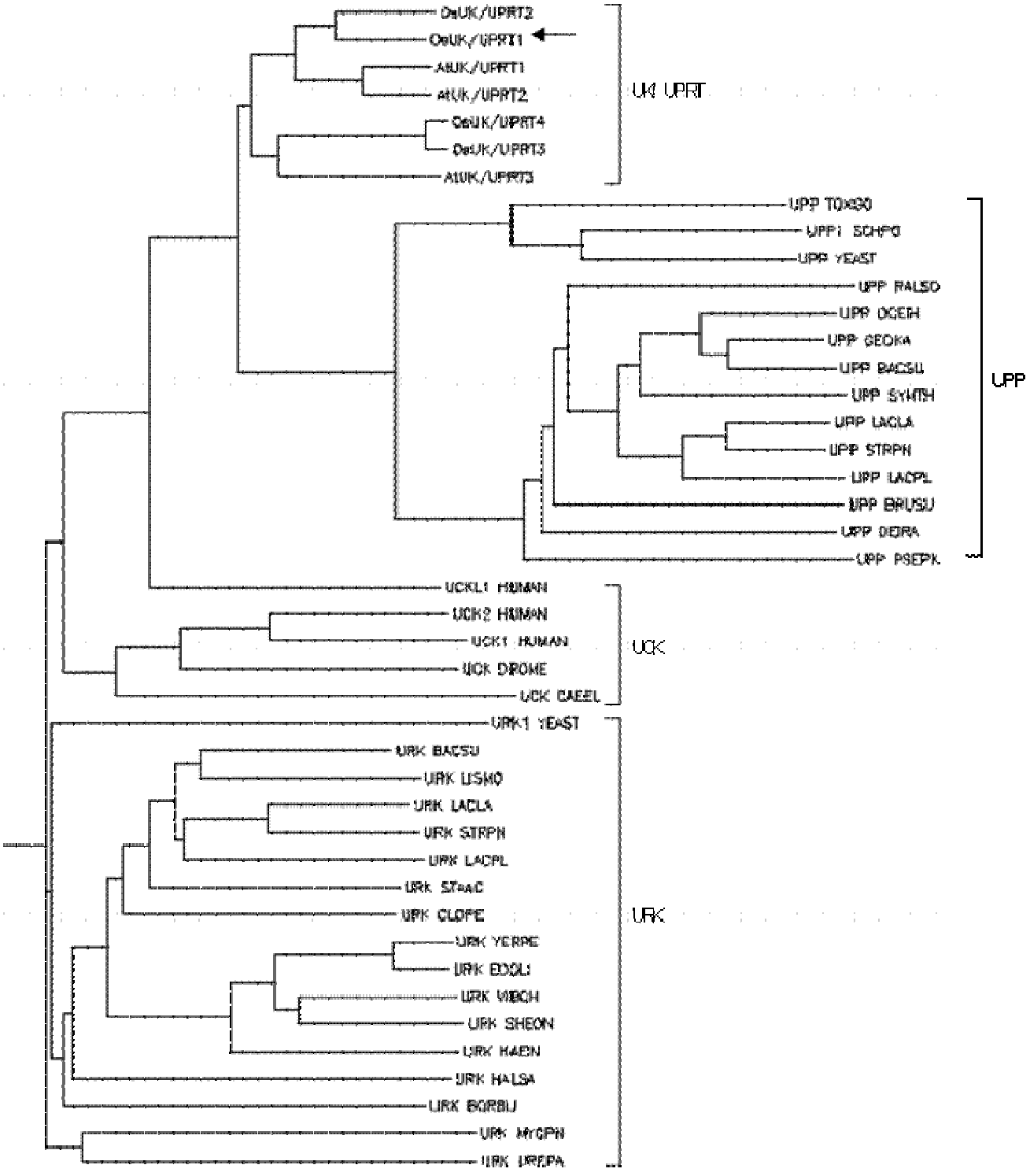

Figure 3. Phylogenetic analysis of ***OsUK/UPRT1*** related proteins using Clustal W. Accession numbers are as follows: AK102065 (OsUK/UPRT1), BAF24236 (OsUK/UPRT2), AB011477 (AtOSUK/UPRT1), AP000381 (AtUK/UPRT2), BAF28020 (OsUK/UPRT3), BAF08444 (OsUK/UPRT4), AC002304 (AtUK/UPRT3), Q26998 (UPP_TOXGO), 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) and Q9PQF9 (URK_UREPA).

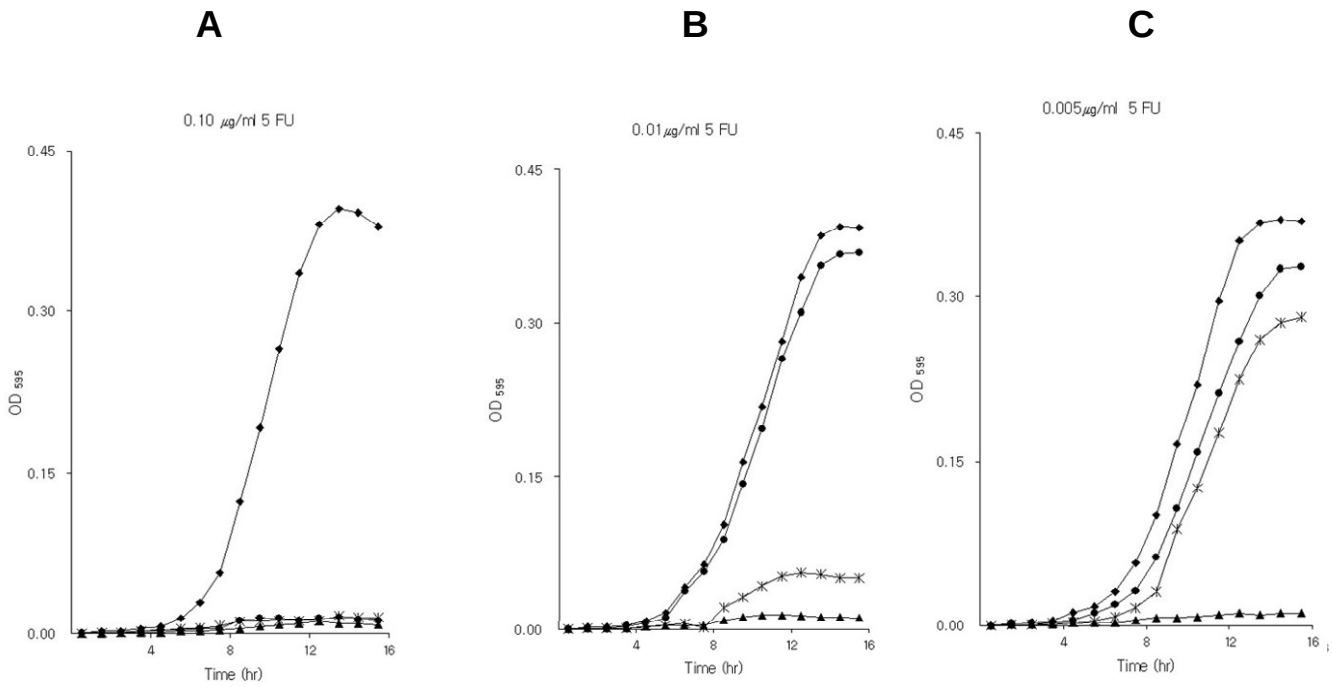

Figure 4. Growth curves of ***E. coli upp-udk*** mutant, X2224 and wild type harboring ***pB::OsUK/UPRT1*** in response to 5-FU. Bacterial cells were grown at 37°C in MM-Ura medium. Growth was monitored by optical density measurement at 595 nm (OD_{595}). Symbols: ♦, X2224 + pB, ●, wild type + pB, *, wild type (JM109) + ***pB::OsUK/UPRT1*** and ▲, X2224+ ***pB::OsUK/UPRT1***.

These results showed the growth retardation by of the gene product of ***OsUK/UPRT1***.

5-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 5-FU. The construct of ***pB::OsUK/UPRT1*** and the pB as a control plasmid were transformed into ***upp*** mutants (GT4 and 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 5-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 and 0.005 µg/ml 5-FU. Whereas, X2224 containing ***pB::OsUK/UPRT1*** was dramatically retarded in growth phase in same medium and concentrations of 5-FU (Figures 4 and 5). The wild type JM109 expressing control plasmid was also found to express growth retardation in high (0.1 µg/ml 5-FU) concentration (Figure 4).

However, the GT4 and Sφ408 containing ***pB::OsUK/UPRT1*** were dramatically retarded in growth phase in the same medium and the same concentrations of 5-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 h (Figure 5). 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*** utilize 5-FU as a substrate that is why there was growth retardation due to toxicity of 5-FU. So these results revealed the consequence of UPRT activity of rice ***OsUK/UPRT1***.

5-FD sensitivity of *E. coli upp* mutants was influenced by the expression of *OsUK/UPRT1*

The growth pattern of ***E. coli*** mutants complemented with ***pB::OsUK/UPRT1*** was also investigated in presence of 5-FD. The X2224 harboring control plasmid grew normally and showed S-shape classical growth curve in the MM-Ura medium with different concentrations 0.1, 0.01 and 0.005 µg/ml 5-FD, respectively (Figures 6 and 7). The JM109 with control plasmid was retarded in growth by the supplementation of 5-FD. The retardation of the growth was more severe in the medium containing with high concentration 0.1µg/ml 5-FD. On the other hand, JM109 harboring ***OsUK/UPRT1*** was much more severe in the growth retardation compare with the wild type containing control plasmid and even almost not grown in the concentration of 0.01µg/ml 5-FD which was grown the

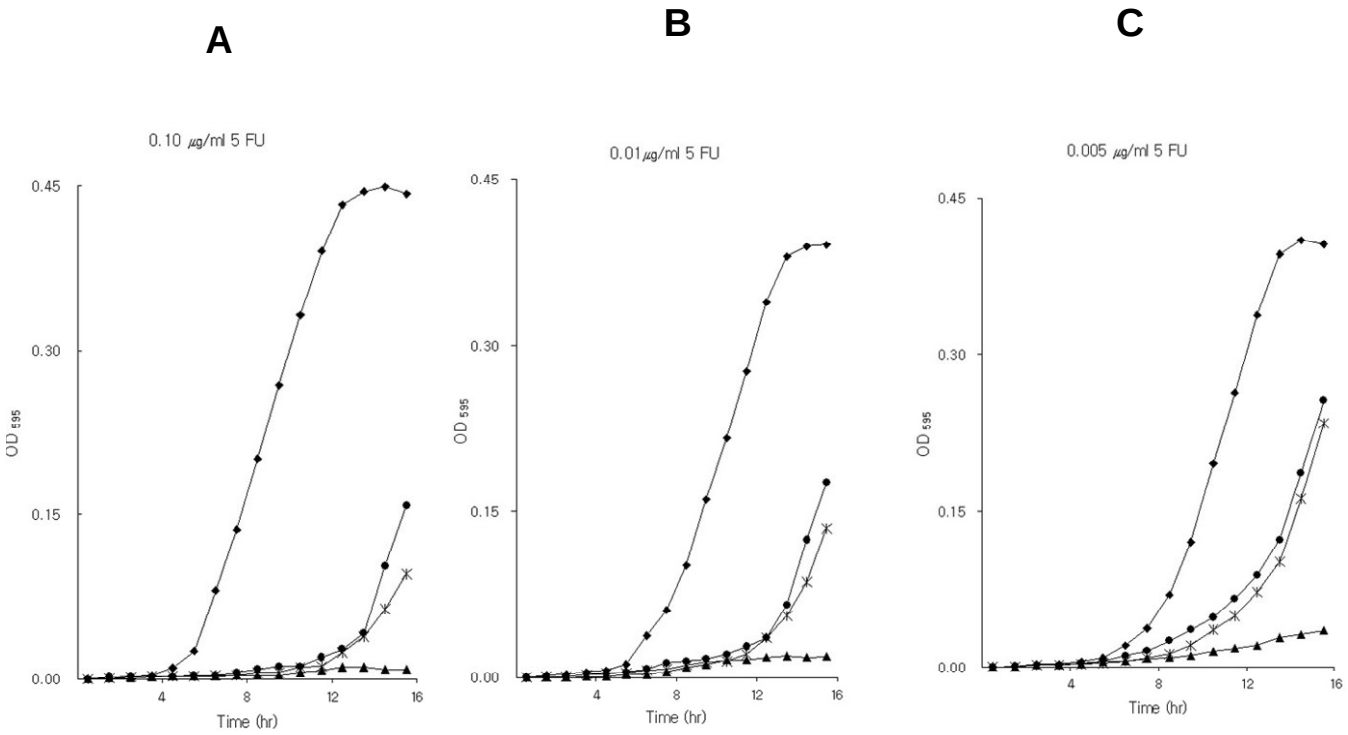

Figure 5. Growth curves of ***E. coli upp-udk*** mutant, X2224 and ***upp*** mutant GT4 and sφ408 harboring ***pB::OsUK/UPRT1*** in response to 5-FU. Bacterial cells were grown at 37°C in MM-Ura medium. Growth was monitored by optical density measurement at 595 nm (OD_{595}). Symbols: ♦, X2224+ pB, ●, GT4+ ***pB::OsUK/UPRT1***, *, Sφ408 + ***pB::OsUK/UPRT1*** and ▲, X2224+ ***pB::OsUK/UPRT1***.

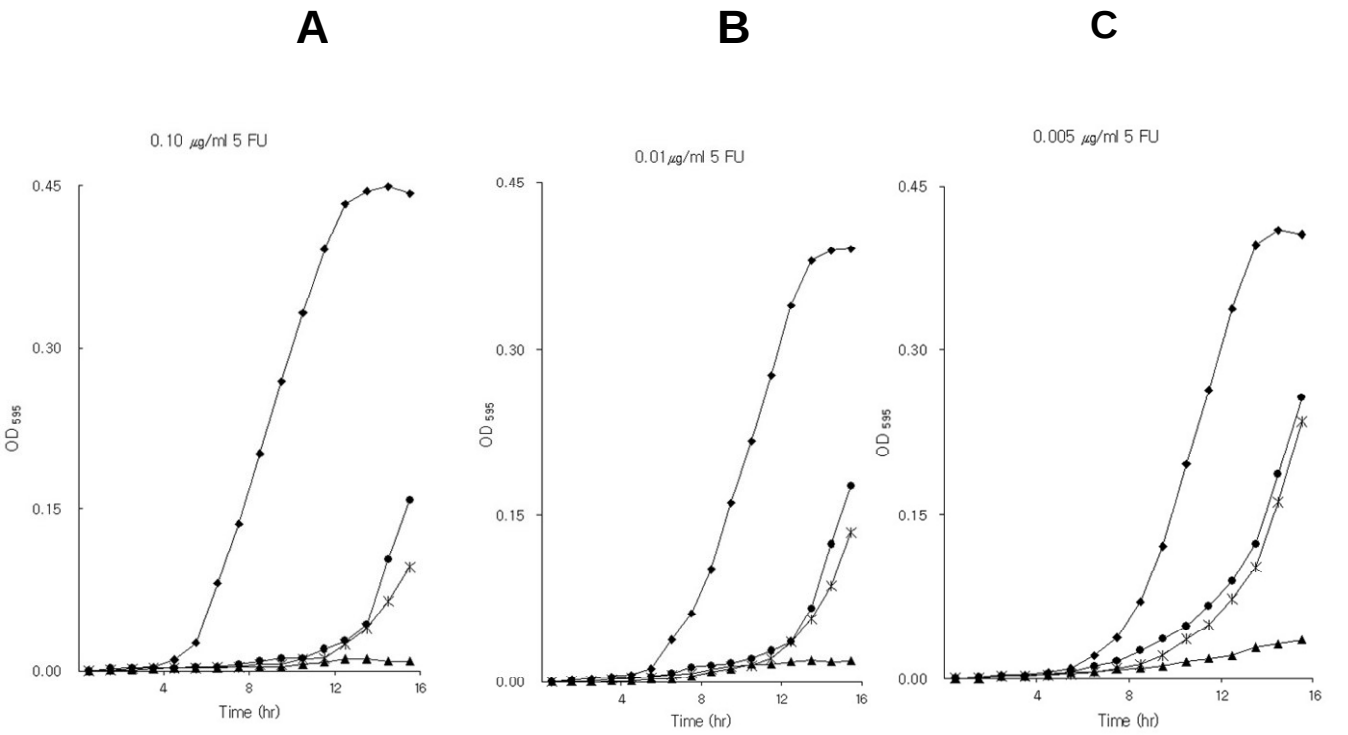

Figure 6. Growth curves of ***E. coli upp-udk*** mutant, X2224 and wild type harboring ***pB::OsUK/UPRT1*** in response to 5-FD. Bacterial cells were grown at 37°C in MM-Ura. Growth was monitored by optical density measurement at 595 nm (OD_{595}). Symbols: ♦, X2224 + pB, ●, wild type + pB, *, wild type (JM109) + ***pB::OsUK/UPRT1*** and ▲, X2224***+ pB::OsUK/UPRT1***.

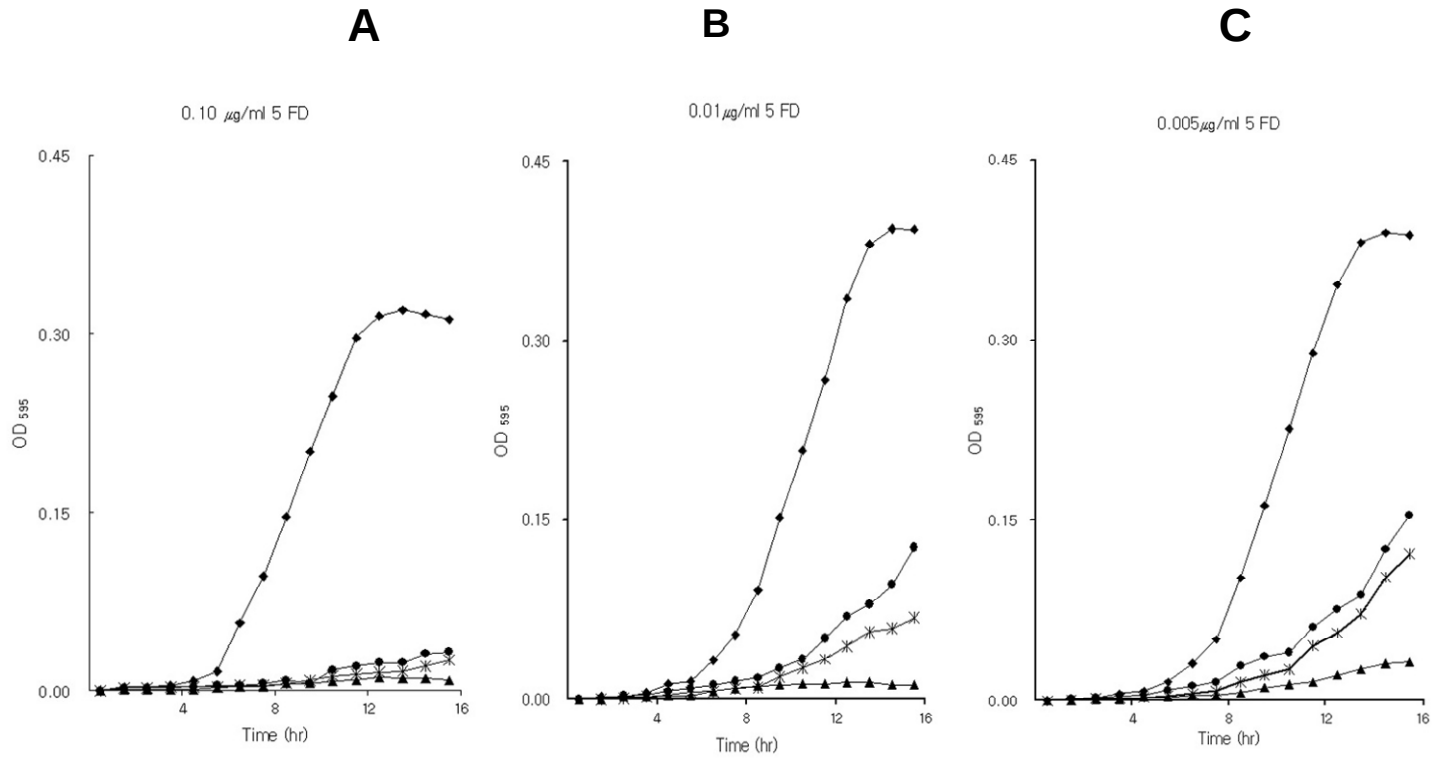

Figure 7. Growth curves of ***E. coli*** upp-udk mutant, X2224 and ***upp*** mutant GT4 and sφ408 harboring ***pB::OsUK/UPRT1*** in response to 5-FD. Bacterial cells were grown at 37°C in MM-Ura. Growth was monitored by optical density measurement at 595 nm (OD_{595}). Symbols: ♦, X2224+ pB, ●, GT4+ ***pB::OsUK/UPRT1***, *, Sφ408 + ***pB::OsUK/UPRT1*** and ▲, X2224+ ***pB::OsUK/UPRT1***.

wild type containing control plasmid and slightly growth in lower concentration 0.005 µg/ml 5-FD (Figure 6).

However, the GT4, Sφ408 and X2224 expressing ***pB::OsUK/UPRT1*** were dramatically retarded in growth phase in the same medium and same concentrations of 5-FD. The trends of growth retardation of all ***upp*** mutants and control plasmid are almost similar. Slight growth was observed in GT4 and Sφ408 expressing ***pB::OsUK/UPRT1*** at lower concentrations 0.01 and 0.005 µg/ml 5-FD after 10 h but X2224 expressing ***OsUK/UPRT1*** continued retardation until 16 h (Figure 7). One explanation in the growth of mutants expressing OsUK/ UPRT1 after 10 h is that the growth inhibition could be due to re-directing UPRT activity using 5-FU from 5-FD degradation not 5-FD as a substrate directly. The growth of X2224 with control plasmid was unaffected (Figures 6 and 7) because there is no toxic effect of 5-FD without UK activity. These results showed the UK activity of ***OsUK/UPRT1***.

Some approaches by purification of recombinant ***OsUK/UPRT1*** protein from overexpressed ***E. coli*** and screening T-DNA insertion mutants in which the expression of each rice OsUK/UPRT gene is knockout would provide important clues into the substrate specificity and physiological function of this noble enzyme for nucleotide metabolism in plants.

Our report about the characterization of a gene encoding ***OsUK/UPRT1*** from rice would be a starting point at a molecular level to investigate pyrimidine nucleotide metabolisms in rice and to apply a new selection marker in plants and further to gene therapy in humans.

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