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A Phi Class Glutathione *S*-transferase from *Oryza sativa* (*Os*GSTF5): Molecular Cloning, Expression and Biochemical Characteristics

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A glutathione *S*-transferase (GST) related to the phi (F) class of enzymes only found in plants has been cloned from the *Oryza sativa*. The GST cDNA was cloned by PCR using oligonucleotide primers based on the *Os*GSTF5 (GenBank Accession No. AF309382) sequences. The cDNA was composed of a 669-bp open reading frame encoding for 223 amino acids. The deduced peptide of this gene shared on overall identity of 75% with other known phi class GST sequences. On the other hands, the *Os*GSTF5 sequence showed only 34% identity with the sequence of the *Os*GSTF3 cloned by our previous study (Cho *et al*., 2005). This gene was expressed in *Escherichia coli* with the pET vector system and the gene product was purified to homogeneity by GSH-Sepharose affinity column chromatography. The expressed *Os*GSTF5 formed a homo-dimer composed of 28 kDa subunit and its p*I* value was approximately 7.8. The expressed *Os*GSTF5 displayed glutathione conjugation activity toward 1-chloro-2,4-dinitrobenzene and 1,2-epoxy-3-(*p*-nitrophenoxy)propane and glutathione peroxidase activity toward cumene hydroperoxide. The *Os*GSTF5 also had high activities towards the herbicides alachlor, atrazine and metolachlor. The *Os*GSTF5 was highly sensitive to inhibition by *S*-hexylGSH, benastatin A and hematin. We propose from these results that the expressed *Os*GSTF5 is a phi class GST and appears to play a role in the conjugation of herbicide and GPOX activity.

Keywords: Glutathione *S*-transferase, Herbicide activity, *Oryza sativa* GST, Phi class GST, Rice

Abbreviations: CDNB, 1-chloro-2,4-dinitrobezene; GSH, glutathione; GST, glutathione *S*-transferase; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy) propane; CP, cumene hydroperoxide; GPOX, glutathione peroxidase

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Introduction

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are a family of substrate-specific enzymes that catalyze GSH conjugation, thereby potentially alleviating toxicity (Hayes and Pulford, 1996). Plant GSTs have been identified with regard to herbicide detoxification and environmental safety (Hatton *et al*., 1996). In plants, GSTs detoxify herbicides, organic pollutants and natural toxins, in addition to less characterized role in general stress tolerance (Frova, 2003).

Plant GSTs are grouped into five classes, phi, zeta, tau, theta and lambda, based on sequence identity, gene organization and active site residues in the proteins. A further GST-like class, DHAR (proteins with dehydroascorbate reductase activity), was recently reported with members in *Arabidopsis*, rice and soybean (Frova, 2003). GST isoenzymes belonging to the same class show 40-60% identity in their primary structure, whereas enzymes belonging to different classes generally have less than 20% sequence identity (Armstrong, 1997). The plant-specific tau and phi class GSTs catalyze the detoxification of herbicides in both crops and weeds. The relative rate of herbicide detoxification in crop is a major determinant of herbicide selectivity and class specificity (Cole, 1994; Dixon *et al*., 2001; Thom *et al*., 2002).

In a previous study, we reported the cloning and characterization of *Os*GSTF3, the phi class GST from rice (Cho *et al*., 2005). As an extension of this study, we cloned and expression *Os*GSTF5 in *E. coli* and purified to electrophoretic homogeneity by affinity chromatography, and characterized with respect to the enzymatic properties. The aim of this study was to identify and characterize the phi class GST in *Oryza sativa* with selectivity towards herbicide.

Materials and Methods

Materials. pET-26b(+), expression vector used in this study was supplied from Novagen (Wisconsin). *E. coli* BL21(DE3) and XL1-blue were obtained from Pharmacia Biotech (Uppsala). Restriction

enzymes and DNA-modifying enzymes were obtained from Takara Shuzo (Otsu, Shiga). Isopropyl-β-D-thioglactopranoside (IPTG), ethacrynic acid, 1,2-epoxy-3-(*p*-nitrophenoxy)propane, indole-3-acetic acid, glutathione, kanamycin, hematin, *S*-hexylglutathione, *S*-methylglutathione, alachlor, atrazin, fluorodifen and metolachlor were purchased from Sigma (St. Louis). The synthesis of DNA primers was performed by COSMO genetech (Seoul). All the chemicals and reagents used were of the highest reagent grade commercially available.

Molecular cloning and construction of an expression vector containing the *Os*GSTF5 gene. The open reading frame of the cDNA encoding *Os*GSTF5 was amplified by PCR from the cDNA library. The nucleotide sequence of the *Os*GSTF5 gene was used to design PCR primer to amplify the coding region. These primers, 5'-GAATTCCATATGAAAGTGTACGGGTGGGTGGTA-3' (the *Nde*I site is underlined; 34mer) and 5'-CGCGGATCCGCTACTATGGTATGTTCCCACTTCCGAA-3' (the *Bam*HI site is underlined; 37mer), also added restriction sites to facilitate cloning. The PCR procedure was conducted under the following conditions: 35 cycles (1 min at 94°C, 2 min at 50°C and 3 min 72°C). The resulting PCR product was digested with *Nde*I and *Bam*HI and subcloned into the plasmid expression vector pET-26b(+) (Novagene), which contains the T7 promoter, previously cut with the same restriction enzymes. The resultant plasmid, pET-*Os*GSTF5 was used to transform *E. coli* strain BL21 (DE3). Colonies containing the appropriate insert were kept and the insert was identified by sequencing.

Protein expression. Expression was achieved following a procedure similar to that of Cho and Kong (Cho and Kong, 2005). The transformed colony was cultured until the cell density reached an absorbance of 0.3-0.4 at 600 nm (A_{600}) in Luria-Bertani broth medium containing kanamycin (30 μg/ml). Expression of the recombinant enzyme was induced by the addition of 0.4 mM isopropyl-β-D-thioglactopranoside (IPTG) and incubation was continued for further 11 h at 37°C. The induced cells were harvested by centrifugation at 10,000 g for 10 min at 4°C, resuspended in 20 mM potassium phosphate buffer (pH 7.0) and subjected to sonication for 10 min with an ultrasonic processor (Sonics and Materials Inc.), then obtained the supernatant by centrifugation at 40,000 g for 30 min.

Purification of the recombinant *Os*GSTF5. The resulting solution was loaded on to a GSH-Sepharose affinity column equilibrated with 20 mM potassium phosphate buffer (pH 7.0). The column was exhaustively washed with 20mM potassium phosphate buffer (pH 7.0) containing 50 mM potassium chloride. The active *Os*GSTF5 was eluted with 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM glutathione followed by dialysis against 20 mM potassium phosphate buffer (pH 7.0). The dialyzed purified *Os*GSTF5 was used for the next experiment. Unless otherwise indicated, all purification procedures were performed either at 4°C or on ice.

Protein assay and electrophoresis. Protein concentration was determined by method of Bradford (1976), using γ-globulin as standard. Denaturing SDS-PAGE was carried out by 12.5% gels (Laemmli, 1970). The molecular-mass markers were SDS molecular weight standard markers (Bio-Rad). The gel was stained with Coomassie Blue R-250.

Enzyme activity and kinetic studies. The specific activities were determined by measuring the initial rates of the enzyme-catalysed conjugation of GSH with CDNB, DCNB, EPNP, 4-nitrophenethyl bromide and ethacrynic acid (Habig and Jacoby, 1987). GSH-dependent peroxidase activity was assayed as described by Flohe and Güzler (1985). GST activities towards herbicides were based on the quantification of the respective herbicide-glutathione conjugate by reversed-phase HPLC using the assay procedure described (Cho and Kong, 2005). Kinetic studies with GSH and electrophilic substrates were carried out at 30 (Lineweaver and Burk, 1934).

Molecular size and p*I* value determination. The purified enzyme was applied to a Superdex® 200 HR 10/30 fast protein liquid chromatography column (Pharmacia Biotech) equilibrated with potassium phosphate buffer (pH 7.0) to estimate molecular size. Molecular size standards used were blue dextran, yeast alcohol dehydrogenase, bovine serum albumin, trypsin inhibitor and cytochrome C (Sigma). Chromatofocusing was performed on FPLC system (Pharmacia Biotech) at room temperature using a Mono P HR 20/5 column equilibrated in 25 mM diethanolamine with 100 mM hydrochloride (pH 9.5). After sample application, *Os*GSTF5 was eluted at 0.5 ml/min with a decreasing pH gradient of pH 9.0 to pH 6.0 using Poly buffer 96 with 100 mM hydrochloride (pH 6.0), diluted 10-fold in water. The eluant was ministered for UV absorbance at 280 nm and fractions were assayed for GST activity with CDNB.

Inhibition studies. The inhibitory effects on the activity of the enzyme were measured by preincubating the enzyme with 1 mM GSH and the inhibitor for 2 min and initiating the reaction by addition of 1mM CDNB at 30°C. The concentration of inhibitor giving 50% inhibition (I_{50}) was determined from plot of residual activity against inhibitor concentration.

Effect of pH and temperature. Effect of pH on the *Os*GSTF5 were determined by using the following buffers (200 mM) at the indicated pH, citrate-sodium phosphate buffer, from pH 4.0 to pH 6.0; potassium phosphate buffer, from pH 6.0 to pH 8.5; Tris-HCl buffer, from pH 8.5 to pH 9.5; glycine-NaOH buffer, from pH 9.5 to pH 10.5. The effects of temperature on the enzyme activity were detected by GSH-CDNB conjugation reaction at temperatures ranging from 30°C to 80°C.

Heat inactivation assays. The enzyme was incubated at each temperature for 10 min at a protein concentration of 50 μg/ml in 20 mM potassium phosphate buffer (pH 7.0) containing 10 mM DDT and 10 mM EDTA to prevent the oxidative inactivation. The remaining activity was assayed 100 mM potassium phosphate buffer (pH 6.5) with 1 mM GSH and 1 mM CDNB at 30°C (Park *et al*., 2005).

Results and Discussion

Molecular cloning and construction of an expression vector containing the *Os*GSTF5 gene. The mRNA from *Oryza sativa* L. cv. Yamahousi was isolated from the cell culture and transcribed to cDNA. Primers for PCR, using the cDNA as template, were derived from a published cDNA sequence from *Oryza sativa* L. cv. Japonica (Soranzo *et al*., 2004). Subsequently, a 669-bp fragment of *Oryza sativa* cDNA was amplified by PCR and was subcloned into the expression vector pET-26b(+) under the control of the strong inducible bacteriophage T7 promoter. The pET-*Os*GSTF5 plasmid was transformed *E. coli* strain BL21(DE3). Colonies containing the appropriate insert were kept and the insert was identified by sequencing.

Primary structure comparison. Both DNA strands were sequenced and the deduced amino acid sequence showed 99.5% identity to the earlier cDNA-derived sequence (GenBank Accession No. **AF309382**). The gene substituted phe 217 (TTC) with leu 217 (TTA) residue. Since the 217 residue was nearly located in C-terminal, the substitution has not an effect on structure or function of *Os*GSTF5. The cloned gene was composed of 669-bp encoding for 223 amino acids and designated *Os*GSTF5 as suggested by Soranzo *et al*. (Soranzo *et al*., 2004). A multiple alignment of the *Os*GSTF5 protein with other GSTs was shown in Fig. 1. Sequence aliment was performed by T-Coffee and Boxshade programe (http://www.ch.embnet.org). The *Os*GSTF5 protein showed 77% and 74% identity to *Ta*GSTF4 and *Zm*GSTF8, respectively, while the lowest identity was found with *At*GSTZ, *Gm*GSTT25 and *Zm*GSTU6. The *Os*GSTF5 sequence showed only 34% identity with the sequence of the *Os*GSTF3 (Cho *et al*., 2005). GST isoenzymes belonging to the same class show 40-60% identity in their primary structure, whereas enzymes belonging to different classes generally have less than 20% sequence identity (Frova, 2003). Phylogenetic analysis using gene and amino acid sequences data showed that *Os*GSTF5 is in the same sister taxon of *Os*GSTF3, *Ta*GSTF4 and *Zm*GSTF8 within the glutathione *S*-transferase subfamily (Fig. 2). Based on its gene sequence, *Os*GSTF5 is presumed to belong to phi class GST.

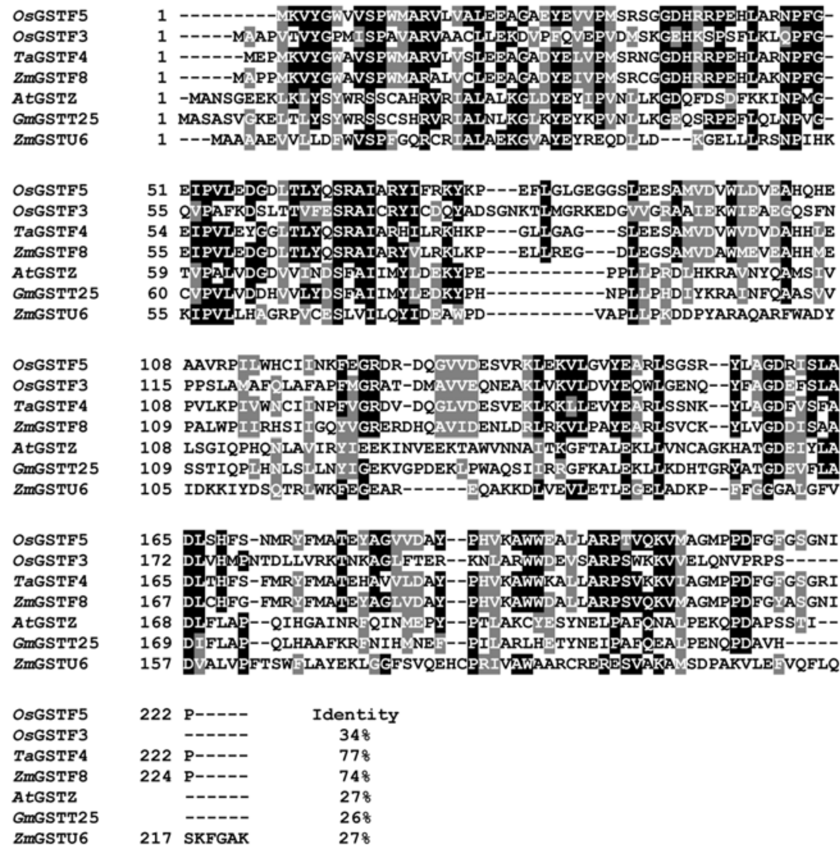

Fig. 1. Comparison of deduced amino acid sequence of the *Os*GSTF5 and other class GSTs. The sequences have been aligned with dashes indicating gaps. The regions conserved are shade in black. This sequence alignment was created using the following sequences (Organism, NCBI protein ID and class in brackets) *Os*GSTF3 (*Oryza sativa*, AAG32477-phi class); *Ta*GSTF4 (*Triticum asetium*, CAD29477-phi class); *Zm*GSTF8 (*Zea may*, AAG34816-phi class); *At*GSTZ (*Arabidopsis thaliana*, AAO60039-zeta class); *Gm*GSTT25 (*Glycine max*, AAG34815-theta class) and *Zm*GSTU6 (*Zea may*, CAB38120-tau class).

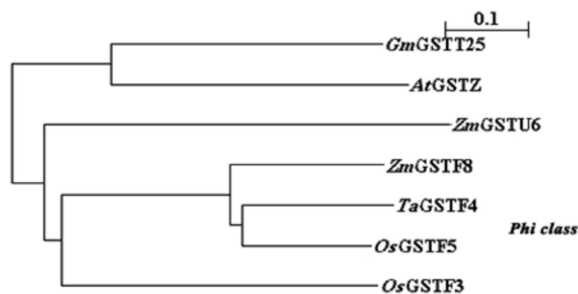

Fig. 2. Phylogenetic tree showing relationship of the *Os*GSTF5 and other class GSTs.

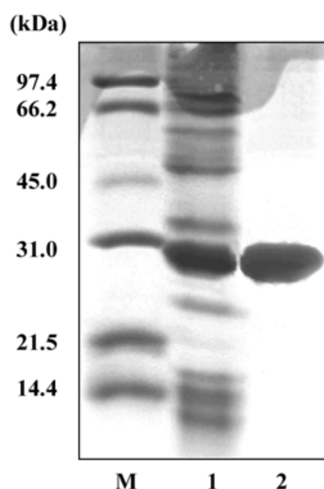

Fig. 3. SDS-polyacrylamide gel electrophoresis (PAGE) analysis of the *Os*GSTF5. Denaturing SDS-PAGE was carried out using the method of Laemmli (1970) in 12.5% gel. Coomassie blue R-250 was used for staining. Lane M, molecular weight standard marker; lane 1, crude cell extract (pET-*Os*GSTF5 transformants); lane 2, purified *Os*GSTF5 protein by GSH-Sepharose column chromatography.

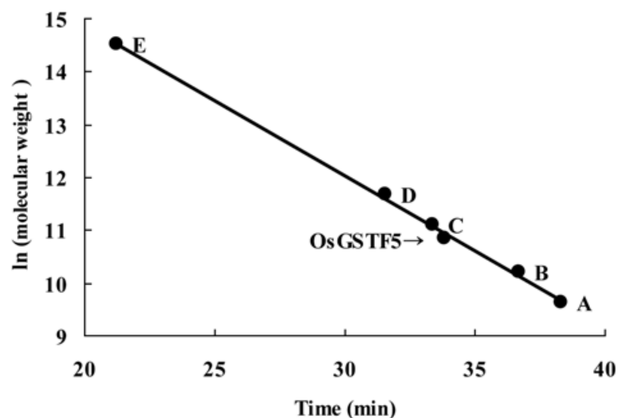

Fig. 4. Determination of molecular weight of the *Os*GSTF5. The purified enzyme was applied to Superdex 200 column (H/R 10/30) which was equilibrated with 20 mM potassium phosphate buffer (pH 6.5). The enzyme was eluted using same buffer at flow rate of 0.5 ml/min. X axis was elution time of enzymes and Y axis was plotted by calculate to ln(molecular weight). A: cytochrome c (12.4 kDa), B: trypsin inhibitor (29.8 kDa), C: bovine serum albumin (66 kDa), D: yeast alcohol dehydrogenase (150 kDa), E: blue dextran (2,000 kDa).

Expression, purification and molecular weight of *Os*GSTF5. The DNA fragment was then sub-cloned into the pET -26b(+) plasmid to generate recombinate *E. coli* BL21(DE3) expressing GST. The recombinant protein was showed optimum expression yield by cultivating *E. coli* for 11 h at 37°C following addition of 0.4 mM IPTG. Following affinity chromatography with glutathione-Separose 4B, the *Os*GSTF5 protein was selectively retained by matrix and then released in the presence of glutathione. In this purification, the final product was purified 6.5-fold with a yield of 36% to apparent homogeneity from crude extract. Approximately 13 mg of *Os*GSTF5 protein was purified from 1L of the transformant culture medium.

The *Os*GSTF5 protein was analyzed by SDS-PAGE (Fig. 3). It was appeared as a band of about 28 kDa. The molecular mass of the purified enzyme estimated by FPLC gel-filtration chromatography was approximately 55 kDa (Fig. 4). Therefore, the *Os*GSTF5 protein was likely to exist as a homo-dimeric structure. The *Os*GSTF5 seemed to be similar to those of mammalian, plant and microorganism GSTs, all of which were dimers with a molecular weight of 40-60 kDa (Armstrong, 1997; Sheehan *et al*., 2001).

In addition, the p*I* value of the recombinant protein was approximately 7.8 by chromatofocusing using a Mono P column. This value was higher than the predicted p*I* value (5.6). The difference between the predicted p*I* value and the actual value seems to be due to dimmer form. The predicted p*I* value means the value of monomer. On the other hand, the actual p*I* value means the value of GST dimer. The p*I* value of the *Os*GSTF5 also was higher than p*I* values (pH 4.3-5.2) of GSTs from maize, sugarcane leaves and wheat (Edwards and Owen, 1987; Singhal *et al*., 1991; Pascal and Scalla, 1999).

Biochemical properties of *Os*GSTF5. The biochemical properties of the recombinant *Os*GSTF5 were investigated with GSH-conjugation reaction towards CDNB as an eletrophilic substrate. The optimum activity of the *Os*GSTF5 on GSH-CDNB conjugation reaction was observed at pH 9.0. The *Os*GSTF5 showed less than 20% of its maximum activity below pH 6.0. The optimum temperature of the *Os*GSTF5 was 45. The activity of *Os*GSTF5 toward CDNB was significantly inactivation more than 55°C.

The *Os*GSTF5 was characterized with respect to enzyme activities towards a range of xenobiotics substrates. The model GST substrate CDNB was used, together with the structurally diverse herbicides metolachlor, alachlor, atrazine and fluorodifen. The *Os*GSTF5 had a very similar range of activities toward xenobiotics. The *Os*GSTF5 activity toward CDNB was much lower than *Os*GSTF3 (7.6 μmol/min/mg) and *Os*GSTU5 (4.48 μmol/min/mg) (Cho and Kong, 2005; Cho *et al*., 2006). However, *Os*GSTF5 did differ from other

Table 1. Substrate specificity of the *Os*GSTF5

Substrates	Specific activity (μmol/min/mg)
Alachlor	2.32 ± 0.22
Atrazin	2.51 ± 0.03
1-Chloro-2,4-dinitrobenzene	0.91 ± 0.04
Cumene hydroperoxide	0.15 ± 0.01
1,2-Dichloro-4-nitrobenzene	ND[a]
1,2-Epoxy-3-(*p*-nitropheoxy)propane	0.45 ± 0.12
Ethacrynic acid	0.04 ± 0.01
Fluorodifen	0.21 ± 0.01
Metolachlor	1.18 ± 0.16
4-Nitrophenethyl bromide	ND

Values are means ± S.D., generally based on n ≥ 5.
[a]ND, not detectable activity.

*Os*GSTs in having measurable GPOX activity (Table 1). The *Os*GSTF5 showed higher activity than phi class *Ta*GSTF toward herbicide (Cummins *et al*., 2003). On the other hand, The CDNB activity of *Os*GSTF5 was lower than *Ta*GSTF. Rice phi class *Os*GSTF3 and *Os*GSTF5 have not activity toward DCNB and 4-NPB. Only *Os*GSTF5 showed GPOX activity. The *Os*GSTF3 showed higher activity than *Os*GSTF5 toward CDNB, ETA, alachlor and metoalachlor (Cho *et al*., 2005). Although the *Os*GSTF5 showed low sequence identity with the *Os*GSTF3, *Os*GSTF5 and *Os*GSTF3 were showed the similar specificity towards substrates and herbicides. We propose from these results that the phi *Os*GSTF5 enzymes show herbicide specificities, and they play an important role in the detoxification reaction of rice.

The effects of substrate concentration toward CDNB, cumene hydroperoxide and 1,2-epoxy-3-(*p*-nitrophenoxy)propane and were investigated at 30°C and kinetic parameters are summarized in Table 2. The K_m values of the *Os*GSTF5 for GSH were approximately 0.34-0.84 mM, which were in general agreement with published K_m^{GSH} values of other GSTs (Bartling *et al*., 1993; Hahn and Strittmatter, 1994; Hong *et al*., 1999; Cummins *et al*., 2003). In contrast, the K_m values of the *Os*GSTF5 for CDNB and cumene hydroperoxide were 0.55 mM and 0.16 mM, respectively. These values were one order of magnitude lower than K_m values reported for the *Triticum aestivum* GSTF1-1 and the *Zea mays* GST1-1 (Dixon *et al*., 1997; Cummins *et al*., 2003). Particularly, the K_m value of the *Os*GSTF5 for 1,2-epoxy-3-(*p*-nitrophenoxy) propane was 0.05 mM, which indicated a higher affinity of the *Os*GSTF5 for 1,2-epoxy-3-(*p*-nitrophenoxy)propane.

Table 3. Inhibition effect of various inhibitors on GSH-CDNB conjugation of the *Os*GSTF5

Inhibitors	I_{50} (iM)
Benastatin A	1.36 ± 0.05
S-(2,4-Dinitrophenyl)glutathione	18.92 ± 0.02
Hematin	3.49 ± 0.03
S-Hexylglutathione	1.25 ± 0.03
Indole-3-acetic acid	$1.25 \times 10^3 \pm 2.30$

Values are Means ± S.D., generally based on n > 5.

Several compounds were tested for their inhibitory action on the *Os*GSTF5 (Table 3). The *Os*GSTF5 was notably inhibited by *S*-hexyl-GSH, a derivative of GSH and benastatin A, an electrophilic substrate-like compound (Aoyagi *et al*., 1992) and their I_{50} values were 1.25 μM and 1.36 μM, respectively. The I_{50} value of *S*-hexyl-GSH for the enzyme was lower than those for *Brushtail possum* GST (10.5 μM), *Mycobacterium tuberculosis* GST (15 μM) and *Solanum tuberosum* GST (150 μM) (Antonio *et al*., 1996). On the other hand, the I_{50} value of benastatin A for the enzyme was similar to those for *Brushtail possum* GST (1.52 μM), *Mycobacterium tuberculosis* GST (1.16 μM) and *Solanum tuberosum* GST (1.29 μM). Hematin, a non-substrate ligand for the enzyme strongly inhibited the enzymatic activity and its I_{50} value for the enzyme was 3.49 μM. This value for the enzyme was lower than those reported for *Mycobacterium tuberculosis* GST (5 μM) and *Solanum tuberosum* GST (15 μM), but it was higher than that for *Brushtail possum* GST (0.5 μM). The I_{50} value for the enzyme of *S*-(2,4-dinitrophenyl)glutathione, a conjugation product of GSH with CDNB was 18.92 μM. On the other hand, indole-3-acetic acid was the least potent inhibitor among the substances tested.

In conclusion, we cloned a novel glutathione *S*-transferase gene from *Oryza sativa*, expressed in *E. coli* and characterized the expressed protein. From our results on amino acid sequence comparison and detoxifying activity toward xenobiotics and herbicides, the expressed *Os*GSTF5 is a phi class GST and appears to play a role in the conjugation of herbicide and

Table 2. Kinetic parameters of 1-chloro-2,4-dinitrobenzen(CDNB)-GSH, cumene hydroperoxide(CP)-GSH and 1,2-epoxy-3-(*p*-nitrophenoxy)propane(EPNP)-GSH, on Michaelis-Menten constant(K_m) and Lineweaver-Burk plot(V_{max}) of the *Os*GSTF5

Substrates	GSH		Substrates	
	K_m (mM)	V_{max} (μmol/mg)	K_m (mM)	V_{max} (μmol/mg)
CDNB	0.35 ± 0.01	0.18 ± 0.01	0.55 ± 0.02	0.20 ± 0.02
EPNP	0.84 ± 0.03	0.26 ± 0.04	0.05 ± 0.00	0.22 ± 0.01
CP	0.34 ± 0.01	0.19 ± 0.01	0.16 ± 0.02	0.20 ± 0.02

Values are means ± S.D., generally based on n > 5.

GPOX activity. To elucidate the difference in the molecular structures and functions between the *Os*GSTF5 and GSTs from other plant sources, we are now in a process to study the detailed catalytic mechanism and the structure-function relationship of this enzyme by site-directed mutagenesis and structural analysis.

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