http://dx.doi.org/10.5012/bkcs.2011.32.10.3756

A Plant-specific Tau Class Glutathione *S*-transferase from *Oryza sativa* Having Significant Detoxification Activity Towards Chloroacetanilide Herbicides

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Received July 19, 2011, Accepted August 8, 2011

Key Words : Glutathione *S*-transferase, Herbicide activity, *Oryza sativa* GST, Rice, Tau class GST

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are enzymes that detoxify endobiotic and xenobiotic compounds by covalently linking glutathione (GSH) to a hydrophobic substrate, forming a less reactive and more polar glutathione *S*-conjugate.[1] In plants, GSTs detoxify herbicides, organic pollutants, and natural toxins; they also protect cells from a wide range of biotic and abiotic stresses, including pathogen attack, xenobiotic and heavy-metal toxicity, and oxidative stress.[2,3] 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.[2] The function of these plant-specific GSTs is detoxification of herbicides in both crops and weeds. Despite all available research, both the biochemical properties and functions of plant GSTs remain to be elucidated.

Rice is the most important crop for human consumption with more than a half of the world's population utilizing it as an energy source. The entire rice genomes for the japonica and indica subspecies have been sequenced.[4] From these genome sequences, a GST gene homolog (GenBank Accession No. AF309379) was identified from *Oryza sativa* by homolog searches in the NCBI database. As yet, there have been no reports concerning this gene product. Therefore, we cloned this gene and expressed it in *Escherichia coli*.

The mRNA from *Oryza sativa* L. cv. Yamahousi was isolated from the cell culture and transcribed into cDNA. Primers for PCR, using the cDNA as a template, were derived from a published cDNA sequence from *Oryza sativa* L. cv. Japonica.[5] Subsequently, a 702-bp fragment of *Oryza sativa* cDNA was amplified by PCR, subcloned into the expression vector pET-26b(+) and transformed into the *E. coli* strain BL21 (DE3). Both DNA strands were sequenced with the final amino acid sequence bearing a difference between the PCR-identified sequence and the earlier cDNA-derived sequence (GenBank Accession No. AF309379). The gene whereby guanine was substituted with thymine at position of nucleotide 166 gave an Asn 55 residue instead of Lys 55. The cloned gene was composed of 702-bp encoding for the 233 amino acids and designated *Os*GSTU3. A multiple alignment of the *Os*GSTU3 protein with other GSTs is shown in Figure 1. Sequence aliment was performed using the T-Coffee and Boxshade programe (http://www.ch.embnet.org). GST isoenzymes belonging to the same class showed more than approximately 60% identity in their primary structure, whereas enzymes belonging to phi and theta classes generally had less than 2% sequence identity.[2] Zeta class enzymes also bore less than 24% sequence identity. Based on its gene sequence, *Os*GSTU3 was presumed to belong to the plant-specific tau class GST.

The *E. coli* BL21 (pET-*Os*GSTU3) clone was tested for production of recombinant *Os*GSTU3 protein. The expressed *Os*GSTU3 protein was purified by *S*-hexylGSH affinity

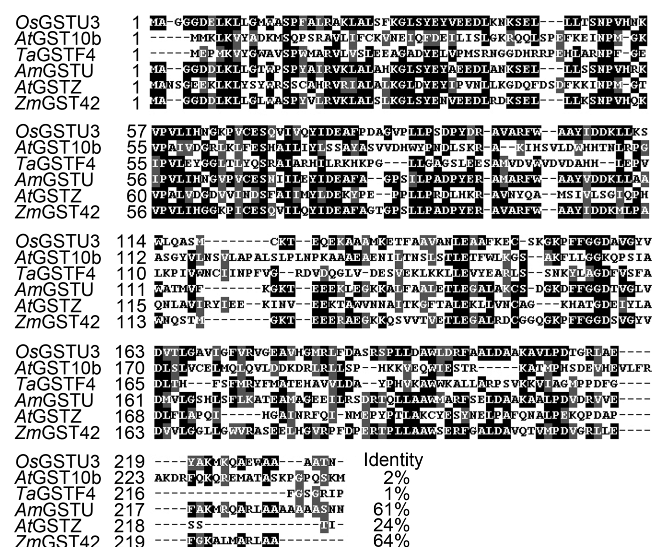

Figure 1. Comparison of the deduced amino acid sequence of the *Os*GSTU3 and other class GSTs. The sequences have been aligned with dashes indicating gaps. The regions conserved are shade in black. This sequence alignment was created using the following sequences; (Organism, Genbank accession number and class in brackets) *Os*GSTU3 (*Oryza sativa*, AAG32472); *At*GST10b (*Arabidopsis taliana*, CAA10662-theta class); *Ta*GSTF4 (*Triticum asetium*, CAD29477-phi class); *Am*GSTU (*Alopecurus myosuroides*, CAA0918861-tau class); *At*GSTZ (*Arabidopsis thaliana*, AAO60039-zeta class); ZmGST42 (*Zea may*, AAG34850-tau class).

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

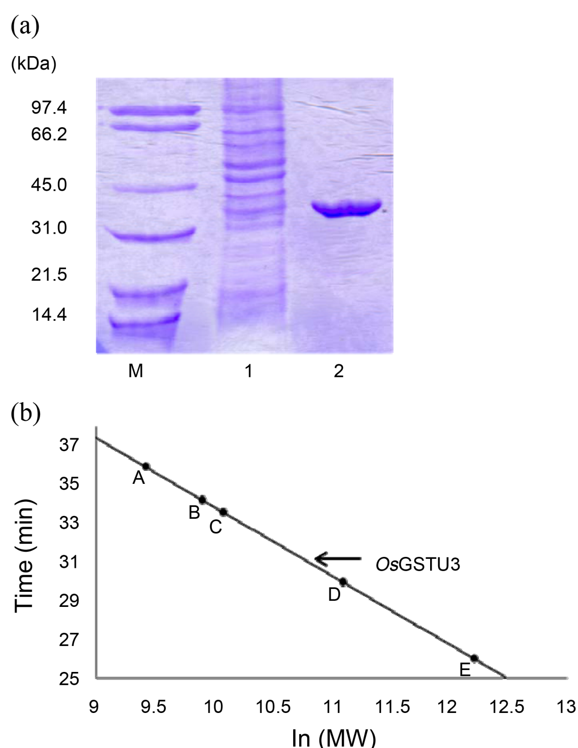

Figure 2. (a) SDS-polyacrylamide gel electrophoresis (PAGE) analysis of the *Os*GSTU3. Denaturing SDS-PAGE was carried out using the method of Laemmli (1970) in 12.5% gel. Coomassie blue R-250 was used for staining. Lane M, molecular weight standard marker; lane 1, crude cell extract (pET-*Os*GSTU3 transformants); lane 2, purified *Os*GSTU3 protein by *S*-hexylGSH-Sepharose column chromatography. (b) Determination of molecular weight of the *Os*GSTU3. The purified enzyme was applied to Superdex 200 column (H/R 10/30), equilibrated with 20 mM potassium phosphate buffer (pH 7.0). The enzyme was eluted using the same buffer at a flow rate of 0.5 mL/min. The *x*-axis enzyme elution time and the *y*-axis was plotted by calculating ln(molecular weight). A: cytochrome C (12.4 kDa); B: trypsin inhibitor (20.1 kDa); C: carbonic anhydrase (24 kDa); D: bovine serum albumin (66 kDa); E: *β*-amylase (200 kDa).

chromatography because the protein had low affinities for GSH-Sepharose. In this purification, the final product was purified approximately 52-fold to apparent homogeneity from the crude extract with a yield of 10%. Approximately 0.05 mg of a recombinant 26-kDa protein, as determined by SDS-PAGE (Fig. 2(a)), was purified from 1.0 L of the transformant culture medium. The molecular mass of the purified enzyme estimated by FPLC gel-filtration chromatography was approximately 52 kDa (Fig. 2(b)). Therefore, the *Os*GSTU3 protein was likely to exist as a homo-dimeric structure. The *Os*GSTU3 seemed to be similar to those of wheat, maize, and rice tau class GSTs, all of which were dimers with a molecular weight of 50-60 kDa.[6-8]

The substrate specificity of the *Os*GSTU3 was examined using a range of xenobiotics as substrates (Table 1). The *Os*GSTU3 enzyme displayed high activity towards the general GST substrate (CDNB) and the substrate for the epoxide ring opening reaction (EPNP). As shown in Table 1, the specific activity of the *Os*GSTU3 with EPNP was higher

Table 1. Substrate specificity of the *Os*GSTU3

Substrates	Specific activity (μmol/min/mg)
1-Chloro-2,4-dinitrobenzene	1.06 ± 0.01
Cumene hydroperoxide	ND[a]
1,2-Dichloro-4-nitrobenzene	ND[a]
1,2-Epoxy-3-(*p*-nitropheoxy)propane	2.00 ± 0.02
Ethacrynic acid	0.30 ± 0.05
4-Nitophenethyl bromide	0.15 ± 0.04

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

(2.00 μmol/min/mg) than other substrates. This value was higher than that reported for the *Os*GSTU5 (1.31 μmol/min/mg).[9] The *Os*GSTU3 showed no GSH peroxidase activity toward cumene hydroperoxide and GSH conjugation activity toward 1,2-dichloro-4-nitrobenzene. Nonetheless, the *Zm*GSTU5-5 showed no GSH conjugation activity toward EPNP, but showed GSH peroxidase activity toward cumene hydroperoxide and GSH conjugation activity toward 1,2-dichloro-4-nitrobenzene.[7] To further determine the function of the *Os*GSTU3, we investigated the kinetic parameters of the enzyme for GSH-CDNB conjugation. The observed K_m and k_{cat} parameters were 2.01 mM and 1.05 s^{-1} for CDNB and 0.37 mM and 1.02 s^{-1} for GSH, respectively. The K_m value of the *Os*GSTU3 for CDNB was higher than those of the enzyme from tobacco (*Nicotiana tabacum*, White Burley), *Zm*GSTU1, *Zm*GSTU2, and *Os*GSTU5.[9-11] Nevertheless, the K_m value of the *Os*GSTU3 for GSH was 0.37 mM, which was in general agreement with published K_m^{GSH} values of other GSTs. The I_{50} values of the various types of inhibitors for GSH-CDNB conjugating activity were determined under the standard assay conditions (Table 2). The activity of the *Os*GSTU3 was significantly inhibited by a non-substrate ligand (hematin) and a GSH derivative (*S*-methylglutathione). The high I_{50} value for ethacrynic acid and the high K_m value for CDNB indicate a lower affinity of the *Os*GSTU3 for electrophilic substrates.

We also examined the substrate specificity of the *Os*GSTU3 towards a range of herbicides (Table 3). The *Os*GSTU3 displayed high detoxification activities towards chloroacetanilide herbicides as follows: alachlor (0.82 μmol/min/mg); acetochlor (0.76 μmol/min/mg); metolachlor (0.61 μmol/min/mg); pretilachlor (0.60 μmol/min/mg) (Fig. 3). These values for the *Os*GSTU3 were higher than those reported for

Table 2. Inhibition effects of various inhibitors on GSH-CDNB conjugation of the *Os*GSTU3

Inhibitors	I_{50} (μM)
S-(2,4-dinitrophenyl)glutathione	65.8 ± 3.6
Ethacrynic acid	237 ± 12
Hematin	1.2 ± 0.08
S-Hexylglutathione	88.7 ± 6.2
S-Methylglutathione	4.52 ± 0.31

Values are Means ± S.D., generally based on $n \geq 5$.

Table 3. Herbicide activity of the *Os*GSTU3

Substrates	Specific activity (nkat/mg)
Acetochlor	0.76 ± 0.04
Acifluorofen	0.03 ± 0.01
Alachlor	0.82 ± 0.11
Atrazin	ND[a]
2,4-D	ND[a]
Dicamba	ND[a]
Fluorodifen	0.22 ± 0.02
Metolachlor	0.61 ± 0.05
Pretilachlor	0.60 ± 0.03

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

the tau class *Zm*GSTU5-5, *Zm*GST7-7, and *Gm*GSTU1-1.[12,13] In addition, the *Os*GSTU3 showed low activities towards the diphenyl ether herbicides, acifluorofen and fluorodifen. However, the tau class GSTs in maize and soybean had significant activity towards photobleaching herbicides, such as diphenyl ethers.[5,14] The *Os*GSTU3 had no activities towards atrazin, 2,4-D, and dicamba. From these results, we suggest that the *Os*GSTU3 in plant possesses an unique herbicide specificity and plays an important role in the detoxification reaction of chloroacetanilide herbicides.

In conclusion, we expressed the hypothetical protein of the AF309379 gene from *Oryza sativa* and characterized the purified recombinant protein. The hypothetical protein first reported herein is a novel tau class *Os*GSTU3 displaying high specificity towards 1,2-epoxy-3-(*p*-nitrophenoxy)propane and chloroacetanilide herbicides. Further studies are underway to elucidate the structure and function of *Os*GSTU3 and provide the basis towards development of transgenic plants with improved phytoremediation capabilities for future use in the environmental cleanup of herbicides.

Experimental Section

Cloning and Construction of *Os*GSTU3 Gene. The mRNA from *Oryza sativa* L. cv. Yamahousi was isolated from the cell culture and transcribed to cDNA. The open reading frame (including the stop codon) of the gene encoding *Os*GSTU3 was amplified by PCR from the cDNA library. The nucleotide sequence of the *Os*GSTU3 gene was used to design the PCR primer to amplify the coding region. These primers, 5'-GGAATTCCATATGGCCGGTGGAGGAGATGAGCTGAAG-3' (the *Nde* I site is underlined) and 5'-CGCGGATCCGCTATCAGTTGGTGGCAGCTGCTGCCCA-3' (the *Bam*H I site is underlined), also added restriction sites to facilitate cloning. All PCR was performed in the design PCR primer and *Taq* polymerase with Thermo Hybraid PCR sprinter (Waltham, UK) in the final volume (50 μL). All PCR conditions were optimized by 35 cycles of 1 min at 94 °C, 2 min at 60 °C and 3 min 72 °C. The resulting PCR product was digested with *Nde* I and *Bam*H I and subcloned into the plasmid expression vector pET-26b(+) (Novagen, USA), which contains the T7 promoter, previously cut with the same restriction enzymes. The resultant plasmid (pET-*Os*GSTU3) was used to transform the *E. coli* strain BL21 (DE3). Colonies containing the appropriate insert were kept and the insert identified by sequencing.

Expression and Purification of the Recombinant

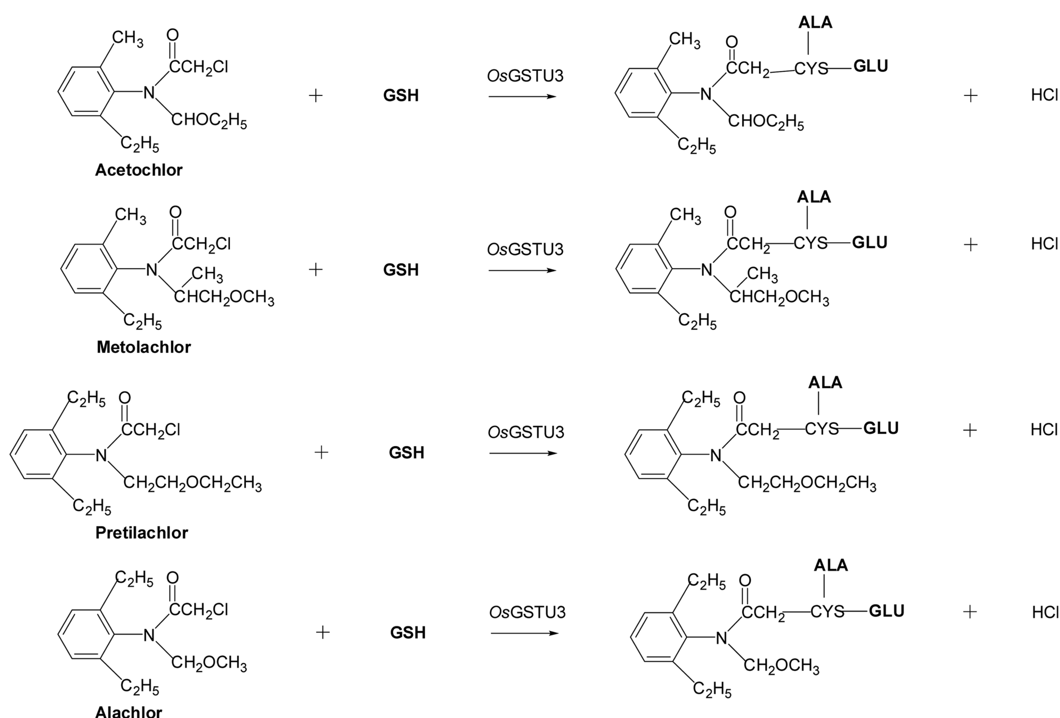

Figure 3. The scheme of the GSH-conjugation reactions towards chloroacetanilide herbicides by *Os*GSTU3.

***Os*GSTU3.** The transformed colony was cultured in Luria-Bertani broth medium containing kanamycin (30 μg/mL) until the OD_{600} reached 0.3-0.4. Expression of the recombinant enzyme was induced by the addition of 0.1-1.0 mM isopropyl-β-D-thioglactopranoside (IPTG); incubation was continued for 2-24 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), subjected to sonication for 10 min with an ultrasonic processor (Sonics and Materials Inc, USA), and obtained from the supernatant by centrifugation at 40,000 g for 30 min. The resulting solution was loaded onto a *S*-hexylGSH affinity column equilibrated with 20 mM potassium phosphate buffer (pH 7.0). The active *Os*GSTU3 was eluted with 20 mM potassium phosphate buffer (pH 7.0) containing 200 mM potassium chloride and 10 mM glutathione followed by dialysis against 20 mM potassium phosphate buffer (pH 7.0). Unless otherwise indicated, all purification procedures were performed either at 4 °C or on ice.

Protein Assay, Electrophoresis, and Molecular Size Determination. Protein concentration was determined by the Bradford method, using γ-globulin as the standard. Denaturing SDS-PAGE was carried out in 12.5% gels. The molecular-mass markers were SDS molecular weight standard markers (Bio-Rad, USA). The gel was stained with Coomassie Blue R-250. To estimate molecular size, the purified enzyme was applied to a Superdex® 200 HR 10/30 fast protein liquid chromatography column (Pharmacia Biotech, Sweden) equilibrated with potassium phosphate buffer (pH 7.0).

Enzyme Activity and Kinetic Studies. The specific activities of *Os*GSTU3 were determined by measuring the initial rates of the enzyme-catalysed conjugation of GSH with CDNB, DCNB, EPNP, 4-nitrophenethyl bromide and ethacrynic acid.[17] GSH-dependent peroxidase activity was assayed as described by Flohe and Güzler (1985).[18] All GST activities towards herbicides were based on the quantification of the respective herbicide-glutathione conjugate by reversed-phase HPLC using the assay procedure described.[19] Kinetic studies with GSH and electrophilic substrates were carried out at 30 °C.[20] The inhibitory effects on the activity of the enzyme were measured by preincubating the enzyme with 1.0 mM GSH and the inhibitor for 2 min and initiating the reaction by addition of 1.0 mM CDNB at 30 °C. The concentration of inhibitor giving 50% inhibition (I_{50}) was determined from a plot of residual activity against inhibitor concentration.

Acknowledgments. This work was supported by the National Research Foundation of Korea (NRF) Grant (NRF-2010--C00065).

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