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Article

Cloning and Characterization of the UDP Glucose/Galactose Epimerases of *Oryza sativa*

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UDP-glucose/galactose-4-epimerase (UGE) catalyzes the interconversion of UDP-glucose and UDP-galactose. *Oryza sativa* contains four UGEs. Expression of *OsUGE3* was higher than other OsUGEs in all tissues except the stem. *OsUGE1* expression was enhanced after drought, salt or UV-irradiation stress. Four rice *UGE*s (*OsUGE1-4*) were cloned by polymerase chain reaction and sequenced. *OsUGE1-4* were expressed in *Escherichia coli* with glutathione *S*-transferase fusion protein and purified. All four OsUGEs could covert UDP-glucose into UDP-galactose better than vice versa. Kinetic parameters of OsUGEs showed that OsUGE1 was most efficient. These OsUGEs could be used for biosynthesis of various UDP-sugars, which serve as a cosubstrates of UDP-dependent glycosyltransferase modification reactions of antibiotics or flavonoids.

Key words: Oryza sativa, *UDP-glucose/galactose-4-epimerase, UDP-sugar*

Plants have an ability to utilize light energy to convert inorganic carbon into organic forms. Simple sugars derived from photosynthesis can serve as energy sources or as building blocks of cell components, such as cell walls, membranes, and glycoproteins. Simple sugars generally found in plants are glucose, galactose, xylose, arabinose, rhamnose, glucuronic acid galacuronic acid, mannose, and fucose, and fructose. These sugars need to be activated to serve as building blocks for cell components. Nucleotide diphosphate sugars (NDP-sugars) are activated forms which serve not only as intermediates for the intercoversion of simple sugar but also as building blocks for complex carbohydrate synthesis. Most of these simple monosaccharides are generated from UDP-glucose, however, fucose and mannose are derived from GDP-mannose [Reiter and Vanzin, 2001]. Glycosyltransferases mediate reactions in complex carbohydrate biosynthesis using NDP-sugars [Egelund *et al.,* 2004] and nucleotide interconversion enzymes are involved in the interconversion of sugar moiety of NDP-sugars [Seifert, 2004]. Enzymes catalyzing the epimerization of UDP-glucose into UDP-galactose, of UDP-xylose into UDP-arabinose, and of UDP-glucuronic acid into UDP-galacturonic acid are UDP-glucose epimerase (UGE), UDP-xylose-epimerase (UXE), and UDP-glucuronic acid 4-epimerase (UGlcAE), respectively. Because *Arabidopsis thaliana* genome has been sequenced, it was possible to conduct a genome-wide search for a particular gene. Five UGEs, four UXEs and six UGlcAEs were present in *A. thaliana* [Seifert, 2004]. Each UGE from *A. thaliana* had different catalytic properties [Barber *et al*., 2006] and UGE2 and UGE4 are involved in cell wall biosynthesis and growth of *A. thaliana* [Rösti *et al*., 2007]. All UGEs have activity for both UDP-glucose and UDP-galactose. One UXE and one UGlcAE from *A. thaliana* are characterized [Burget and Reiter, 1999; Burget *et al.,* 2003; Gu and Bar-Peled, 2004].

Rice is a model crop plant and its genome sequence is completed. Only a few rice enzymes involved in the biosynthesis of NDP-sugar were reported [Suzuki *et al.*, 2003; Liu *et al*., 2007]. Rice contains four *UGE* genes (*OsUGE 1-4*). *OsUGE1* is activated upon abiotic stress [Liu *et al.*, 2007]. However, none of *OsUGE*s have been characterized *in vitro*. In this report, we analyzed the

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Abbreviations: RT-PC, reverse transcription polymerase chain reaction; UGE, UDP-glucose epimerase; UGlcAE, UDP-glucuronic acid 4-epimerase; UXE, UDP-xylose-epimerase

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Table 1. OsUGEs primers and GeneBank accession numbers

Genes	Primers for cDNA (forward/reverse)	Real time PCR primers (forward/reverse)	GenBank accession number
OsUGE1	CACACGCACACAACAACAACAA/ CTGCTGCTACTGGAGGATTGGA	CTGGGCACTGGAAAGGGGAC/ GGCATCTCCAGGCCTTCGTC	NM_001063047
OsUGE2	GAGAGGCGCACACACGAA/ AGAGGCACGCATGTCTTATT	TTGGGGCTCATCCTAGCGGA/ AGCTGGCCTCCTGCCAACAG	NP_001063727.1
OsUGE3	GCACACACGAAGACGAGGC/ TGATCGCCCTAATTCTGCTC	TTGCCGCACTGGAGAAGCTC/ TCGAACGCCTTCACCACCTC	NP_001062869.1
OsUGE4	GTCAGAGAAGGGGGATCGGC/ TCGGCGTTGATCAGTGTGAA	CCTGTTGGAGCTCATCCCAGTG/ GACGGTCTCCTCCCGACAGC	NP_001061668.1

expression of four *OsUGE*s in different tissues and upon different abiotic stresses. Enzymatic characterization using the purified recombinant OsUGEs was carried out.

Materials and Methods

Cloning of *OsUGEs*. The full-length cDNA of *OsUGEs* was cloned by reverse transcription polymerase chain reaction (RT-PCR). cDNA was synthesized from total RNA from one-month-old whole rice (*Oryza sativa* Nakdong) using Omniscript reverse transcriptase (Qiagen, Hilden, Germany). Primers are listed in Table 1. PCR products were subcloned and sequenced.

Real-time PCR was used to analyze all four *OsUGE*s in different tissues and after different abiotic stresses [Kim *et al*., 2007]. The initial transcript concentration was estimated using the comparative threshold cycle method. Rice plants three-weeks after flowering were used to examine the expression of *OsUGE*s in different tissues. One-month old whole rice plants were used for abiotic stress tests. For UV-B irradiation treatments, we followed the method of Kim *et al.* [2009]. For salt treatments, plants were soaked in 200 mM NaCl solution for 24 and 48 h. For drought treatments, plant did not receive water for 1 day. Primers for real time PCR are listed in Table 1.

Induction and purification of recombinant OsUGEs. The open reading frame of each *OsUGE* was subcloned into *E. coli* expression vector pGEX 5X-3 and the resulting construct was transformed into *E. coli* BL21. The recombinant protein was induced by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The culture was incubated at 18°C for 12 h with shaking. Purification of the recombinant protein was carried out using GST affinity column (GST trap, Amersham Bioscience, Piscataway, NJ) following the manufacture's instructions.

Enzyme assay and analysis of reaction product. The reaction mixture for the UGE enzyme assay contained 0.1 to 1 μg of the purified recombinant OsUGE and 1 mM of UDP-sugars (UDP-glucose or galactose) in 200 μL of 20 mM Tris/HCl (pH 8.0). The reaction mixture was incubated at 37°C for 30 min. The reaction was stopped by heating at 95°C for 5 min. The reaction products were analyzed by high performance liquid chromatography (HPLC) equipped with a J' sphere ODS-H80 column (JH08S04-2546WT, JH-303, 250×4.6 mm, S-4 μm, 8 nm YMC, Dinslaken, Denmark) and a photodiodearray detector. The mobile phase consisted of two buffers: buffer A was 100 mM KH_2PO_4 containing 8 mM tetrabutylammonium hydrogensulfate (pH adjusted to 5.3 with KOH) and buffer B was 70 % buffer A plus 30% methanol (pH adjusted to 5.9 with KOH). The column was eluted at 1 mL/min by a 0-20%(v/v) gradient of buffer A in buffer B under the following conditions: 0-15 min, linear gradient of 100-80% buffer A concomitant with 0-20% buffer B; 15-17 min, linear gradient of 80-100% buffer A; 17-30 min 100% buffer A. UV detection was dually performed at 250 nm and 270 nm.

Results and Discussion

Cloning and expression analysis of OsUGEs. Rice has four *OsUGE*s in its genome [Seifert, 2004]. *OsUGE1* is located on chromosome 5, *OsUGE2* is on chromosome 8 and *OsUGE3* and *OsUGE4* are on chromosome 9. The open reading frames of *OsUGE1-4* are 1065, 1227, 1122, and 1110-bp encoding 39.0, 44.2, 40.4, and 39.6 kDa protein, respectively. The amino acid similarity among four OsUGEs was from 74 to 93% (Table 2). All OsUGEs

Table 2. Comparison of amino acid sequence identity and similarity (%) among OsUGEs

	OsUGE1	OsUGE2	OsUGE3	OsUGE4
OsUGE1	-	87/76	77/61	86/76
OsUGE2		-	77/63	93/86
OsUGE3			-	74/58

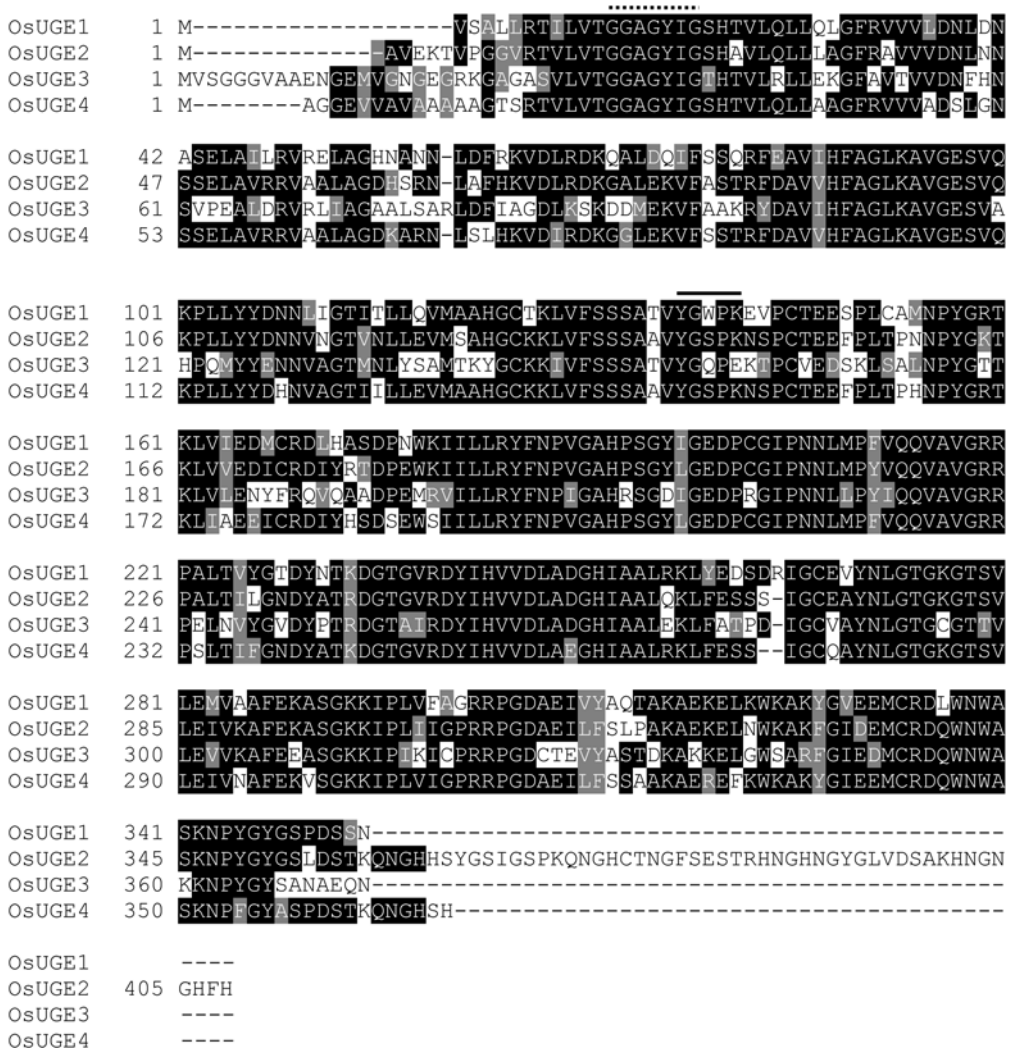

Fig. 1. Comparison of OsUGEs. The conserved NAD binding site (GXXGXXG) is indicated as a dotted line and the catalytic triad (YXXXK) is indicated as a straight line. The box indicates % of similarity and identity among OsUGEs.

contained the conserved β-NAD$^+$ binding domain (GXXGXXG, where X is any amino acid) at the N-terminal. The catalytic triad (YXXXK) conserved in UGEs [Weirenga *et al.*, 1986] was found in OsUGE1, OsUGE2, and OsUGE4. OsUGE3 had glutamate (position at 161) instead of lysine in the catalytic triad (Fig. 1) but still had comparable catalytic capacity to other OsUGEs (see below). In addition, the mutation of E161K in OsUGE3 did not change the reactivity of OsUGE3 (data not shown), indicating that glutamic acid is acceptable in the catalytic triad of UGEs.

Expression of these *OsUGE*s was analyzed in leaves, stems, seeds and roots using real time PCR. The overall expression of *OsUGE3* was higher than the other *OsUGE*s. *OsUGE3* showed the highest expression in all the tissues investigated except the stem. In stem, *OsUGE4* had the highest expression (Fig. 2). The response of each *OsUGE* was different upon UV-B irradiation. Expression of *OsUGE1* was increased after 2 hrs but decreased until 24 h to below its initial expression level. Expression of *OsUGE2* increased about 1.6 fold until 6 hrs but decreased at 24 h. Expression of *OsUGE3* fluctuated during the times investigated. Expression of *OsUGE4* increased after 6 h (Fig. 3A). The expression of *OsUGE1* upon drought and salt stresses increased, which was also observed in microarray [Rabbani *et al.*, 2003]. However, the expressions of the other *OsUGE*s were not enhanced by abiotic stresses (Fig. 3B). The role of *OsUGE1* upon abiotic stress was shown by overexpressing it in *A. thaliana* [Liu *et al.*, 2007]. An *Arabidopsis* transformant overexpressing *OsUGE1* showed an enhanced tolerance to salt, cold, and drought stresses, which agreed with our finding of increased expression of *OsUGE1* upon salt and drought stresses.

Enzymatic characterization of OsUGEs. No UGE from rice has been shown biochemically to catalyze the

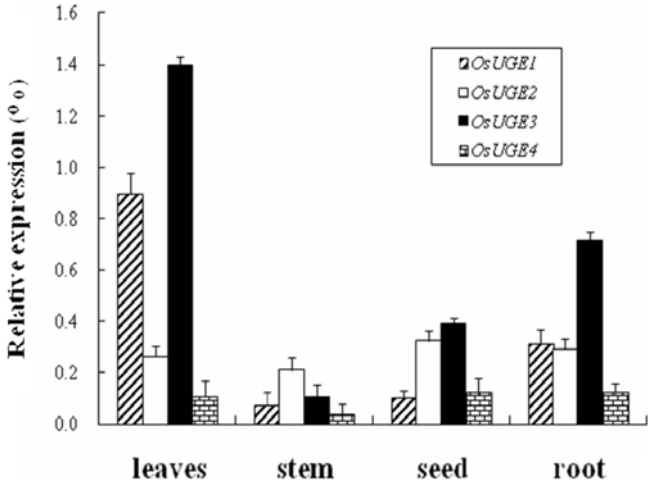

Fig. 2. Expression of *OsUGE*s in different tissues. Total RNA was isolated from leaves, stems, seeds, and roots. The relative activity indicates the ratio of expression of rice actin gene (GenBank accession number X16280) to expression of each OsUGE. All values are the means of three replicates (error bars indicate standard deviation.).

reaction from UDP-glucose to UDP-galactose. *OsUGE1* was complemented in a *E. coli* UGE mutant instead of an *in vitro* functional analysis [Liu *et al.*, 2007]. Four *OsUGE*s were expressed in *E. coli* as GST-fusion proteins. The recombinant proteins were successfully expressed and purified (Fig. 4). The molecular weight of each recombinant OsUGE was the sum of GST and the predicted molecular weight of each OsUGE.

Because OsUGEs are UDP-glucose/galactose epimerases, the purified OsUGEs reacted with either UDP-glucose or UDP-galactose. The reaction product was analyzed by HPLC. The elution profile of authentic UDP-galactose and UDP-glucose are shown in Fig. 5A and B, respectively. The UDP-galactose was eluted before UDP-glucose with our separation condition. Figure 5C and 5D illustrated the reaction product of OsUGE2. The reaction product showed two peaks: The first peak at 15.3 min had the same retention time as that of UDP-galactose and the second peak at 16.1 min had the same retention time as that of UDP-glucose (Fig. 5C).

All OsUGEs except OsUGE3 converted UDP-galactose into UDP-glucose better than they converted UDP-glucose into UDP-galactose. The K_{cat}/K_m valuea of OsUGE1, OsUGE2, OsUGE3, and OsUGE4 for UDP-glucose was 174.9, 31.8, 121.4, and 17.4 (S^{-1} μM^{-1}), respectively. Kinetic parameters showed that OsUGE1 most efficiently converts UDP-galactose into UDP-glucose, followed by OsUGE3, OsUGE2, and OsUGE4 (Table 3). The ratio of UDP-glucose to UDP-galactose at the equilibrium was about 8:2 for all four OsUGEs. Even though some of UGEs are NAD^+-dependent, the

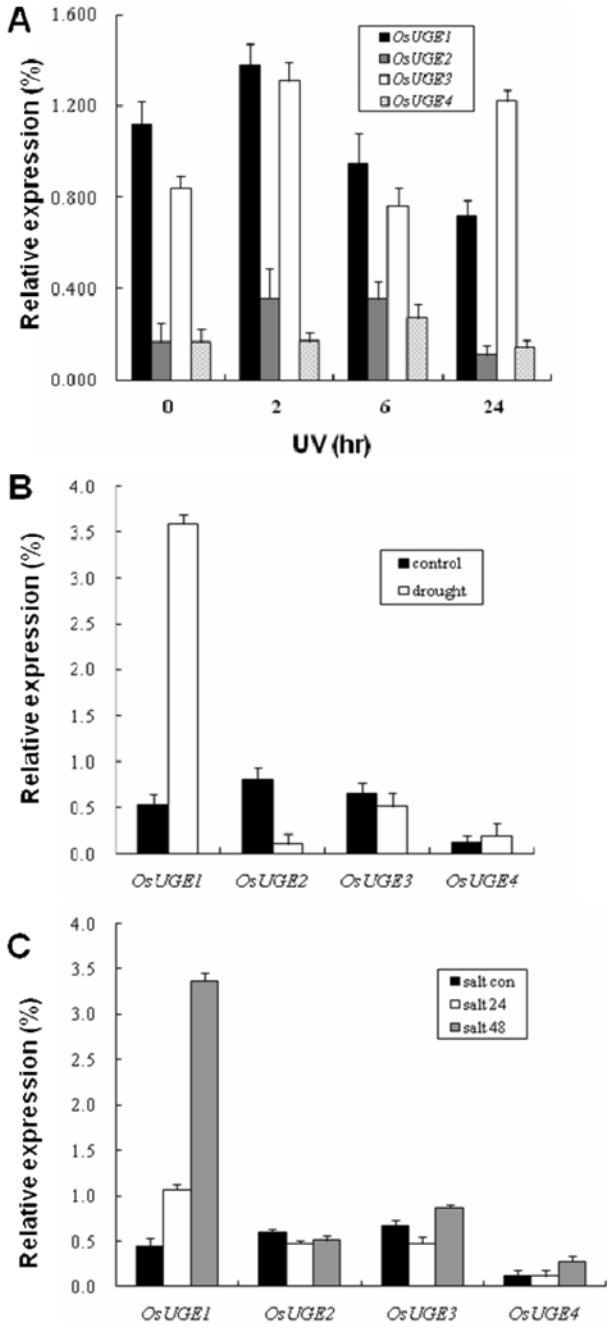

Fig. 3. Expression of *OSUGE*s upon abiotic stress. A, UV-B irradiation; B, drought; C, salt. Total RNA was isolated from one-month old whole plants after treatment. All values are the means of three replicates (error bars indicate standard deviation.).

requirement of NAD^+ for OsUGEs was not tested because NAD^+ was incorporated into UGE when UGE was expressed in *E. coli* (Zhang *et al*., 2006).

In vivo substrates of OsUGEs remain elusive. Generally, UGEs are thought to be responsible for the biosynthesis of the cell wall and for detoxification of high levels of galactose [Dörmann and Benning, 1996; 1998]. Recent studies showed that UGEs are involved in

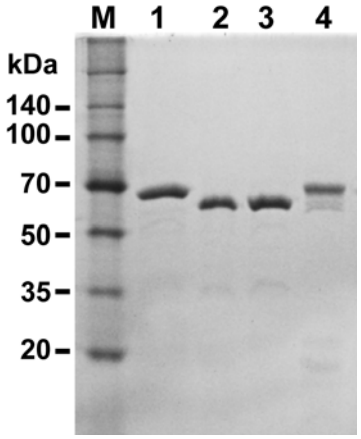

Fig. 4. Purification of the recombinant OsUGEs. 1.5 μg of the purified protein was loaded on the gel. M, protein standard size marker; 1, OsUGE3; 2, OsUGE1; 3, OsUGE4; 4, OsUGE2.

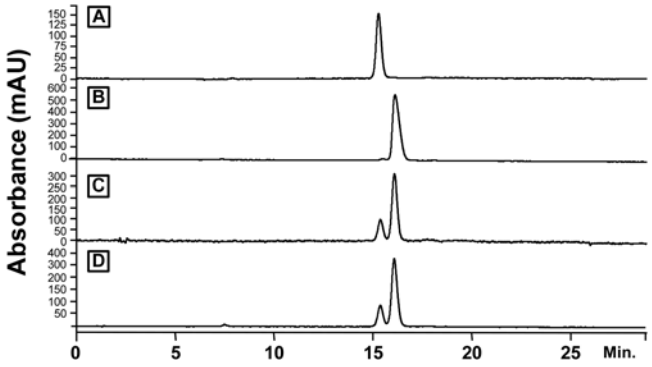

Fig. 5. Analysis of OsUGE reaction product using HPLC. A, authentic UDP-glactose; B, authentic UDP-glucose; C, reaction product of OsUGE2 with UDP-galatcose as a substrate, D: reaction product of OsUGE2 with UDP-glucose as a substrate.

tolerance against abiotic stresses. Overexpression of *OsUGE1* in *A. thaliana* increased the content of raffinose (a trisaccharide composed of galactose, fructose and glucose), which resulted in tolerance against abiotic stresses [Liu *et al.,* 2007]. The increased level of UDP-galactose resulted in an increased level of raffinose because raffinose is synthesized from UDP-galactose and sucrose [Pridham and Hassid, 1965]. Transgenic rice overexpressing a putative UDP-galactose epimerase from *Paspalum vaginatum* also showed tolerance against salt [Endo *et al.,* 2005]. The analysis of expression in different tissues and after different stresses can provide clues about the *in vivo* functions of OsUGEs. *OsUGE3* is likely to have a role in cell wall biosynthesis rather than in the stress response because its expression in all the tissues was much higher than other OsUGEs and abiotic stress had little effect on its expression.

UDP-sugars are sugar donors for various UDP-dependent glycosyltransferase (UGTs). UGTs from plants are highly specific for UDP-sugars, but microbial UGTs have broad UDP-sugar ranges [Vogt and Jones, 2005]. In addition, depending on the sugar, the physical and biological activities of some antibiotics and flavonoids are changed [Hollman *et al.,* 1999]. Thus, the OsUGEs reported in this study may provide different UDP-sugars for the use in the biosynthesis of valuable compounds.

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Table 3. Kinetic parameters of OsUGEs

Genes	UDP- galactose				UDP- glucose			
	Km (μM)	Vmax (μKcat/mg)	Kcat (S^{-1})	Kcat/Km (S^{-1} μM^{-1})	Km (μM)	Vmax (μKcat/mg)	Kcat (S^{-1})	Kcat/Km (S^{-1} μM^{-1})
OsUGE1	495.4	1209.7	86.7	174.9	6.1	945.7	67.7	11.1
OsUGE2	97.3	45.6	3.1	31.8	4.0	511.4	29.9	7.4
OsUGE3	423.3	778.1	51.4	121.4	7.6	926.1	61.2	8.1
OsUGE4	298.8	77.5	5.2	17.4	2.1	564.5	37.9	18.2

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