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Characterization of a GDP-D-mannose 3″,5″-epimerase from rice

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Abstract

The enzymatic characterization of GDP-D-mannose 3″,5″-epimerase (GME), a key enzyme in the biosynthesis of vitamin C in plants is described. The *GME* gene (Genbank Accession No. AB193582) in rice was cloned, and expressed as a fusion protein in *Escherichia coli*. Reaction products from GDP-D-mannose, as produced by GME catalysis, were separated by recycling HPLC on an ODS column, and were determined to be GDP-L-galactose and GDP-L-gulose, based on their NMR spectra and sugar analysis. The reaction catalyzed by GME was inhibited by GDP, and was strongly accelerated by NAD^+ in contrast to the case of GME from *Arabidopsis thaliana*. This difference in the effect of NAD^+ on GME activity can be attributed to the NAD binding domain which is conserved in the rice gene, but not in the *Arabidopsis thaliana* gene. The apparent K_m and k_{cat} were determined to be 1.20×10^{-5} M and 0.127 s^{-1}, respectively, in the presence of 20 μM NAD^+. The fractions of GDP-D-mannose, GDP-L-galactose and GDP-L-gulose, at equilibrium, were approximately 0.75, 0.20 and 0.05, respectively.

Keywords: *Oryza sativa*; Gramineae; Plant AsA biosynthetic pathway; GDP-D-mannose 3″,5″-epimerase; GDP-D-mannose; GDP-L-galactose; GDP-L-gulose

1. Introduction

GDP-L-galactose (**3**) is a nucleotide-sugar (Fig. 1) that serves as a biosynthetic precursor of L-galactosyl residues in polysaccharides and oligosaccharides in plants (Seifert, 2004; Reuhs et al., 2004). The L-galactosyl residue which is contained in cell wall polysaccharides, glycolipids and glycoproteins in higher plants is a minor component and little information is available concerning its biosynthesis.

It is also noteworthy that GDP-L-galactose (**3**) is also required for the synthesis of L-ascorbic acid (AsA; vitamin C) in plants. It is well-known that AsA functions as an antioxidant and an enzymatic cofactor. AsA also plays important roles in many different processes, including photosynthesis, photo-protection, stress resistance, control of cell growth and biosynthesis of hormones and cell wall constituents (Davey et al., 2000; Conklin and Barth, 2004). As shown in Fig. 1, Smirnoff and Wheeler proposed the AsA biosynthetic pathway in plants, known as the Smirnoff and Wheeler pathway, in which AsA is synthesized from D-mannose via GDP-D-mannose (**1**), GDP-L-galactose (**3**), L-galactose and L-galactono-1,4-lactone (Wheeler et al., 1998; Smirnoff and Wheeler, 2000; Smirnoff et al., 2001, 2004). GDP-D-mannose 3″,5″-epimerase (GME), a key enzyme in this pathway, catalyzes the synthesis of GDP-L-galactose (**3**) and GDP-L-gulose (**2**) from GDP-D-mannose (**1**). GME was first isolated and characterized from a type of green algae, *Chlorella pyrenoidosa* (Barber, 1975, 1979; Hebda et al., 1979; Hebda and Barber, 1982). In higher plants, GME activity has been reported in both pea and *Arabidopsis* (Wheeler et al., 1998), and was recently cloned and characterized in *Arabidopsis* (Wolucka et al., 2001b; Wolucka and Van, 2003). The reaction catalyzed by GME is possibly the rate limiting step in the

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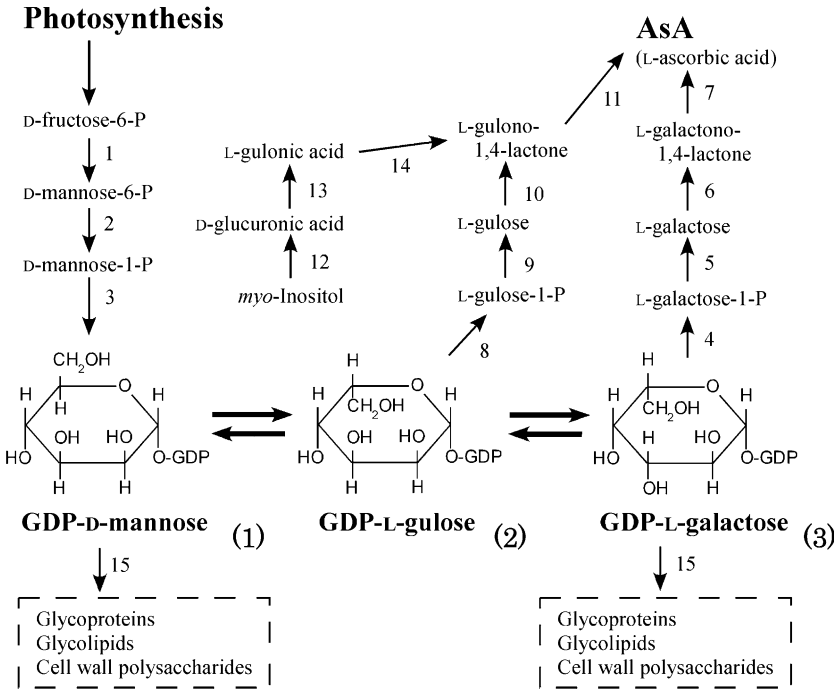

Fig. 1. Schematic diagram of a possible pathway for the biosynthesis of GDP-L-galactose and AsA (L-ascorbic acid). Enzymes catalyzing the numbered reactions are: (1) mannose-6-phosphate isomerase (EC 5.3.1.8); (2) phosphomannomutase (EC 5.4.2.8); (3) GDP-D-mannose pyrophosphorylase (EC 2.7.7.22); (4) GDP-L-galactose pyrophosphatase (Barber, 1971); (5) L-galactose-L-phospate phosphatase (Laing et al., 2004); (6) L-galactose dehydrogenase (Gatzek et al., 2002); (7) L-galactono-1,4-lactone dehydrogenase (EC 1.3.2.3); (8) GDP-L-gulose pyrophosphatase; (9) L-gulose-1-phosphate phosphatase; (10) L-gulose dehydrogenase; (11) L-gulono-1,4-lactone oxidase/dehydrogenase; (12) *myo*-inositol oxygenase (Lorence et al., 2004); (13) D-glucuronic acid reductase (EC 1.1.1.19); (14) aldono lactonase; (15) glycosyl transferase. When EC numbers are not shown in parenthesis, they have not yet been identified. (Baig et al., 1970; Conklin, 2001; Reiter and Vanzin, 2001; Valpuesta and Botella, 2004).

AsA biosynthetic pathway in plants. Experiments with an *Arabidopsis* cell suspension culture indicate that the rate limiting step in AsA biosynthesis occurs between D-mannose and L-galactose (Davey et al., 1999). Moreover, a strong correlation between GME reaction and accumulation of AsA has been reported in the colorless microalga, *Prototheca moriformis* (Running et al., 2003).

In this study, we report on the cloning, expression and characterization of GME from rice (*Oryza sativa* L.). There appears to be a large difference in the activity of this enzyme between rice and *Arabidopsis*, which may involve a difference in amino acid sequence. We also attempted to establish a method for the efficient separation and recovery of rare nucleotide-sugars, GDP-L-galactose (**3**) and GDP-L-gulose (**2**), which represent key compounds in the plant AsA biosynthesis pathway (the Smirnoff and Wheeler pathway).

2. Results and discussion

2.1. Characterization of the OsGME cDNA clone

The cloned cDNA, *OsGME* (*Oryza sativa* GDP-D-mannose 3″,5″-epimerase), appeared to encode a polypeptide of 378 amino acids. The peptide sequence, as deduced from *OsGME* cDNA is assumed to contain neither a transmembrane domain nor a signal peptide sequence, from the prediction using the SOSUI program (Mitaku et al., 2002) and SignalP version 3.0 (Bendtsen et al., 2004). An analysis by means of iPSORT (Bannai et al., 2002) predicted that the product of *OsGME* was localized in the cytoplasm. The calculated molecular mass and isoelectric point for the enzyme was 42,778 Da and 5.75, respectively.

Using the clustal W program (Thompson et al., 1994), the amino acid sequence deduced from *OsGME* indicated a 91% identity with that from *Arabidopsis thaliana* (Fig. 2A), and the sequence of OsGME shared high similarities with putative GME from *Zea mays* (97%), *Sorghum bicolor* (97%), *Hordeum vulgare* (93%), *Triticum aestivum* (92%), *Lycopersicon esculentum* (92% and 91%), *Medicago truncatula* (91%), *Lotus corniculatus* var. *japonicus* (91%), *Solanum tuberosum* (91%), *Mesembryanthemum crystallinum* (90%), *Glycine max* (90%), and *Nicotiana tabacum* (89%) at the amino acid level. Fig. 2B shows a phylogenetic tree constructed from the multiple amino acid sequence alignment obtained for these plants. Such a high similarity between monocots and dicots is very unique and a characteristic of the GME enzyme. In addition, the *GME* gene is a single gene, as no homologous gene has been found in the same species except for *Lycopersicon esculentum*.

The OsGME enzyme contains consensus motifs for the epimerase/dehydratase family, GxxGxxG and YxxxK (Fig. 2A and C). The motifs, GxxGxxG and YxxxK have

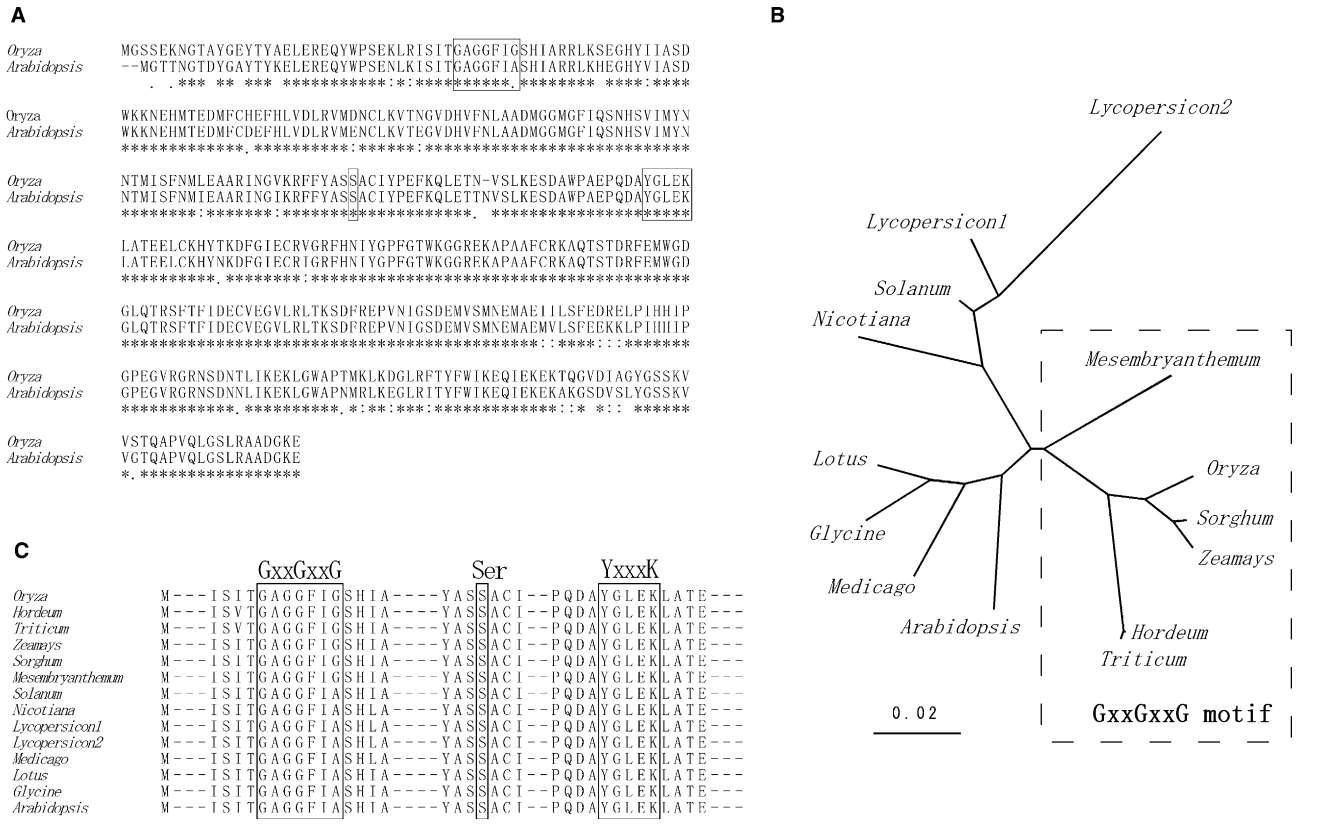

Fig. 2. Amino acid sequence alignment and phylogenetic analysis of GME. (A) Amino acid sequence alignments of GME proteins were aligned with *Oryza sativa* AB193582 and *Arabidopsis thaliana* AX647945 by the pairwise method using the clustalW program (Thompson et al., 1994). Gaps (-) were introduced to achieve maximum similarity. Identical amino acid residues among the sequences are highlighted by an asterisk (*) under the residue. The putative nicotinamide-adenine dinucleotide binding motif, GxxGxxG (Bellamacina, 1996), and a putative catalytic domain, Ser and YxxxK motif (Jornvall et al., 1995) are highlighted in each box. (B) A Neighbor-Joining tree derived from the amino acid sequences of GME from various plants. The phylogenetic analysis was performed using the TreeView program. The amino acid sequences shown in these diagrams are listed in the DDBJ database under the following GenBank Accession Nos. *Oryza*, AB193582; *Zea mays*, AX647973; *Sorghum*, AX647983; *Hordeum*, AX647987; *Triticum*, AX647985; *Arabidopsis*, AX647945; *Mesembryanthemum*, AX647975; *Medicago*, AX647981; *Lotus*, AX647989; *Glycine*, AX647977; *Solanum*, AX647979; *Lycopersicon* 1, AX647971; *Lycopersicon* 2, AX647997; *Nicotiana*, CQ809292. The bar indicates substitutions per site. Plant species which have the GxxGxxG motif conserved (see Fig. 1C) are highlighted in dashed box. (C) Two motif sequence alignments of GME protein among higher plants was highlighted. *Oryza* (rice), *Hordeum* (barley), *Triticum* (wheat), *Zea mays* (maize), and *Sorghum* (millet) are monocots. *Mesembryanthemum* (iceplant), *Solanum* (potato), *Nicotiana* (tobacco), *Lycopersicon* (tomato), *Medicago* (alfalfa), *Lotus*, *Glycine* (soybean), and *Arabidopsis* are dicots.

been reported to be the NAD-binding domain (Bellamacina, 1996) and a catalytic domain having short-chain dehydrogenase activity forms a catalytic triad with Tyr, Lys and Ser upstream (Jornvall et al., 1995). These two motifs may contribute to the first step in the dehydratase and epimerase enzyme mechanisms which occurs via abstraction of the 4-hydroxyl proton and hydride transfer from the C4 position of the sugar to the NAD^+ bound at the GxxGxxG motif, i.e. to yield the nucleotide-4-keto sugar intermediate (Liu et al., 1997). In the GME reaction mechanism proposed by Barber (1979), using tritium-labeled water and GME from *Chlorella pyrenoidosa*, the mannosyl residue of GDP-D-mannose was also converted to a 4-keto sugar intermediate, this suggests that these motifs function as for other epimerase enzymes as well. It should also be noted that the GxxGxxG motif in *Arabidopsis* is not conserved. In addition, the GxxGxxG motif in GME from dicot plants is replaced to GxxGxxA, except for *Mesembryanthemum crystallinum* (Fig. 2C).

2.2. Identification of products of OsGME

The OsGME expressed in *Escherichia coli* was successfully purified to apparent homogeneity as evidenced by SDS–PAGE (Fig. 3A). Two products, GDP-L-galactose (**3**) and GDP-L-gulose (**2**), generated from GDP-D-mannose (**1**) by the action of the purified OsGME were also isolated and characterized. The maximum yield of these products from the recombinant OsGME was 20–25% (Fig. 3B). As shown in Fig. 4, these products were separated by reversed-phase recycle HPLC. The GDP-D-mannose (**1**) was removed completely after three recycles. The reaction products were divided into three fractions, products 1, 2 and 3, respectively. These fractions were collected after the 10th, 12th and 9th cycles, respectively.

Products 1–3 were identified by NMR spectroscopic analysis. Published NMR data for GDP-D-rhamnose (Kneidinger et al., 2001) and GDP-L-fucose (Albermann

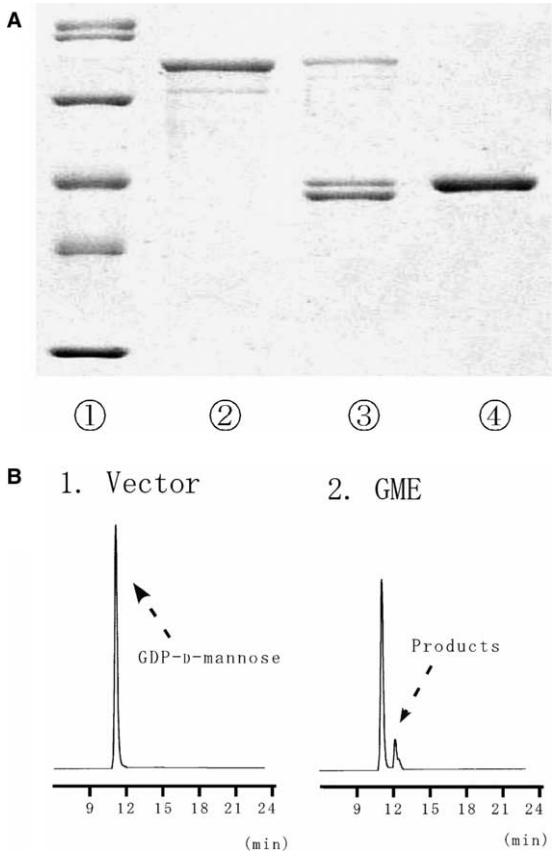

Fig. 3. SDS–PAGE analysis of GME and the HPLC products of GME reaction. (A) Aliquots of the fractions were loaded onto a 10% SDS–PAGE gel and subjected to electrophoresis. The gel was stained with Coomassie brilliant blue R-250. Lane M, size markers; lane 1, MBP-GME fusion proteins purified by amylose resin; lane 2, GME and MBP proteins digested by Factor Xa; lane 3, GME protein purified by anion exchange chromatography. (B) Elution profiles of the products of the GME reaction with 0.1 mM GDP-D-mannose for 5 min at 37 °C (pH 7.5) detected by HPLC analysis on the ODS column. Purified protein (1 μM) derived from *E. coli* expressing a vector inserted the GME gene and an empty vector are shown, respectively.

et al., 2000) were used in the identification of the products. Table 1 indicates the assignments of the NMR spectra for products 2 and 3; GDP-L-galactose (**3**) and GDP-L-gulose (**2**). When GDP-L-galactose (**3**) was analyzed by ^{1}H and ^{13}C NMR spectroscopy, the assignments of the proton and carbon signals were obtained from ^{1}H–^{1}H correlation spectroscopy (COSY; Fig. 5B), ^{13}C–^{1}H heteronuclear multiple quantum coherence (HMQC; Fig. 5A), ^{13}C–^{1}H heteronuclear multiple-bond correlation (HMBC; not shown), and Homonuclear Hartmann–Hahn (HOHAHA; not shown). Concerning GDP-L-gulose (**2**), the assignments of its proton resonances were determined by ^{1}H NMR spectroscopy and ^{1}H–^{1}H correlation spectroscopy (COSY; Fig. 5C). However, the ^{1}H NMR spectra of the product 1 showed only guanine and ribose, suggesting that the product 1 was an artifact derived from GDP sugars during the preparation for HPLC. The proton–proton coupling constants $^{3}J_{H,H+1}$ for GDP-L-galactose (**3**) and GDP-L-gulose (**2**) are summarized in Table 1.

Data for sugar analysis by gas–liquid chromatography is shown in Fig. 6. The retention times of the alditol acetate derivatives for authentic D-mannose, D-altrose, L-galactose, and L-gulose were 44.5, 45.5, 48.5, and 52.5 min, respectively. D-Altrose is produced from D-mannose by epimerization at C-3, which suggests that GDP-D-altrose is one of the products produced by OsGME. The nucleotide sugars in the reaction mixture of OsGME were hydrolyzed and the monosaccharides produced were converted to alditol acetates, with the latter separated by GLC. As a result, we confirmed the presence of D-mannose, L-galactose and L-gulose; however, no derivative of D-altrose was detected. This suggests that the epimerization step is during the conversion of GDP-D-mannose (**1**) to GDP-L-gulose (**2**) (C5 epimerization) and then GDP-L-galactose (**3**) (C3 epimerization).

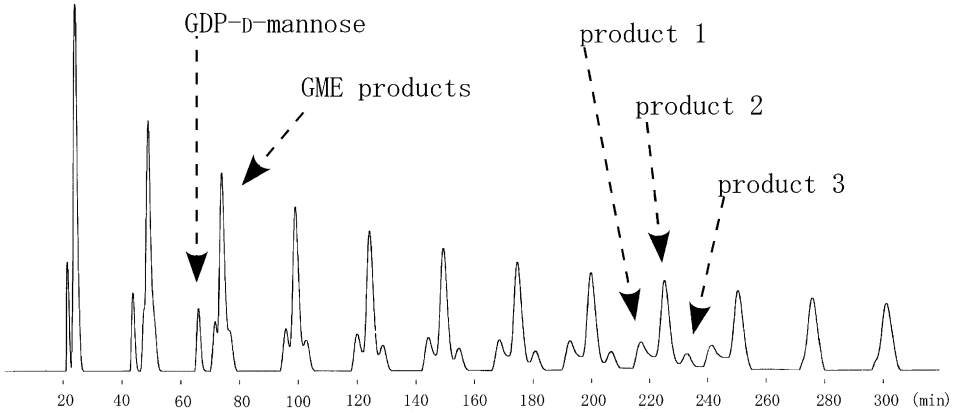

Fig. 4. Recycle HPLC of the products by GME reaction. After partial purification of the GME reaction mixture, the sample was injected into the recycling HPLC system using a reversed phase column with monitoring and detection by UV absorbance at 254 nm. Six millimolar phosphoric acid and 2 mM potassium chloride were used as the mobile phase (Wolucka et al., 2001a). As the peaks were separated, the purified product was discharged by operating a valve. As a result, three products were purified and are denoted as products 1, 2 and 3, respectively.

Table 1
^{1}H and ^{13}C NMR assignments for GDP-L-galactose (**3**) and GDP-L-gulose (**2**)

Sugar residue		1	2	3	4	5	6
β-D-ribose	^{1}H	5.95[a]	4.77	4.54	4.37	4.23 (2×)	
	^{13}C	87.8	74.8	71.4	84.7	66.4	
	$^{3}J_{H,H+1}$	6.1[b]	5.8	3.7	2.6	4.9	
β-L-galactose	^{1}H	4.97	3.62	3.69	3.93	3.75	3.75, 3.82
	^{13}C	99.5	72.2	73.3	69.6	76.9	62.2
	$^{3}J_{H,H+1}$	7.9	10	3.4	3.4	3.4	
β-L-gulose	^{1}H	5.30	3.76	4.11	3.84	4.08	3.74, 3.81
	$^{3}J_{H,H+1}$	8.1	3.2	3.4	2.7	4.1	
Nucleotide residue		2	4	5	6	8	
Guanine	^{1}H					8.13	
	^{13}C	155.0	152.7	117.2	160.0	138.5	

[a] Chemical shifts (parts per million) of ^{1}H and ^{13}C NMR signals.
[b] $J_{H,H+1}$ (Hz) value of ^{1}H NMR signal for nucleotide sugars.

2.3. Biochemical characterization of OsGME

The enzymatic properties of the purified OsGME were examined by measuring the amount of products that contained both GDP-L-galactose (**3**) and GDP-L-gulose (**2**). The maximum activity of the enzyme was observed at pH 7.5–8.0 in the absence of NAD^+; however, the optimal pH shifted to 8.0–8.5 when NAD^+ was present. Similarly, the optimal temperature for the OsGME shifted to 25 °C (addition of NAD^+) from 20 °C (no addition of NAD^+), suggesting that NAD^+ contributes to the stability of OsGME.

The effects of the substrate concentration on activity were examined by measuring enzyme activities with varying concentrations of GDP-D-mannose. When no NAD^+ was added, the apparent K_m, k_{cat}, and k_{cat}/K_m was 7.12×10^{-6} M, $3.03 \times 10^{-2}\ s^{-1}$, and $4.26 \times 10^{3}\ s^{-1}\ M^{-1}$, respectively. These values were changed to 1.20×10^{-5} M, $0.127\ s^{-1}$, and $1.06 \times 10^{4}\ s^{-1}\ M^{-1}$, respectively, when 20 μM NAD^+ was added. These kinetics data indicate that the OsGME enzyme has a high affinity for GDP-D-mannose (**1**) and that the rate of the catalytic reaction from GDP-D-mannose (**1**) to GDP-L-gulose (**2**) and GDP-L-galactose (**3**) is low. By the addition of NAD, the affinity for the substrate decreases slightly and rate of the catalytic reaction increases, indicating that NAD^+ contributes to the turnover in the OsGME reaction.

The effects of additives on OsGME activity were examined by measuring enzyme activities with various additives of nucleotides and nucleotide sugars (Table 2). It is remarkable that $NAD(P)^+$ and NAD(P)H activated the enzyme reaction by more than twice and that GDP inhibited it by more than 80%. Inhibition of the enzyme by GDP was reported in GME from *Arabidopsis*, which suggests that the configuration of GDP is significant for substrate recognition. In contrast to NAD^+, when almost all other nucleotides or nucleotide pentose were added, the inhibition of the OsGME reaction was limited from 44% to 110%. It is clear that the activity of the OsGME reaction is stimulated by exogenously added nicotinamide-adenine dinucleotides, especially the oxidized forms NAD^+ and $NADP^+$. However, it should be noted that the GME from *Arabidopsis* (Wolucka and Van, 2003) is inactivated by the reduced forms, NADH and NADPH. Moreover, the degrees with which NAD^+ and $NADP^+$ contribute to the increase in activity differ greatly in rice (805% and 531% of control, respectively) and *Arabidopsis* (145% and 110% of control, respectively). These differences in the effect of nicotinamide-adenine dinucleotides between the *Arabidopsis* GME and OsGME can be attributed to differences in their affinities for NAD^+. As mentioned above, a NAD-binding motif is conserved in rice, but not in *Arabidopsis*.

Equilibrium ratios of GDP-D-mannose (**1**), GDP-L-galactose (**3**) and GDP-L-gulose (**2**) in the reaction catalyzed by OsGME were determined by monitoring the amount of these substances produced or consumed in the reaction (Fig. 7). Using a reversed-phase recycle HPLC, we were able to separate and collect the enzymatic products, GDP-L-galactose (**3**) and GDP-L-gulose (**2**). Thus, we also performed enzymatic reactions using GDP-L-galactose (**3**) and GDP-L-gulose (**2**) as substrates. No differences in the equilibrium ratios of the reaction catalyzed by OsGME were observed. The equilibrium ratios of GDP-D-mannose (**1**), GDP-L-galactose (**3**) and GDP-L-gulose (**2**) were approximately 75%, 20% and 5%, respectively. These equilibrium ratios were not changed by the additives shown in Table 2. Interestingly, only when GDP-L-gulose (**2**) was used as a substrate (Fig. 7C), the ratio of GDP-L-galactose (**3**) temporarily increased to more than the equilibrium ratio of GDP-L-galactose (**3**) before equilibrium.

It should be finally noted that although this enzyme is considered to be involved in ascorbic acid synthesis via L-galactose; however, the genetic evidence for this using transgenic plants will be required to clarify its role in rice.

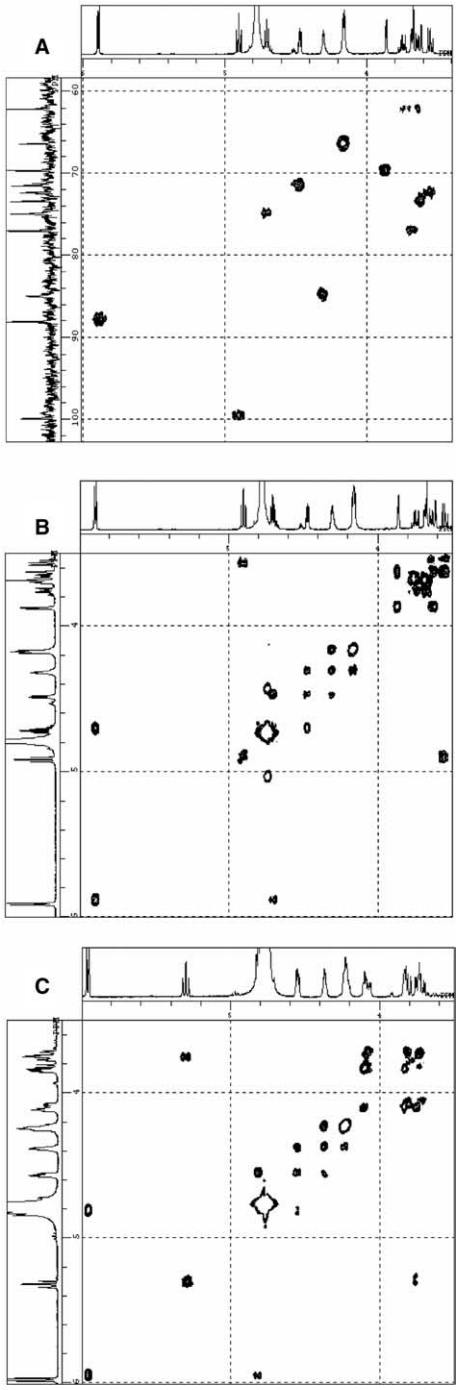

Fig. 5. Two-dimensional HMQC and H-H cosy. Panel A, a partial two-dimensional HMQC spectrum of GDP-L-galactose in D_2O shows the single bond ^{13}C–^{1}H correlations arising from the ribose protons and the pyranose protons to their respective carbons. Panels B and C show the connectivities in the ribose and pyranose moieties of GDP-L-galactose and GDP-L-gulose, respectively. The ^{1}H and ^{13}C spectroscopic assignments are listed in Table 1.

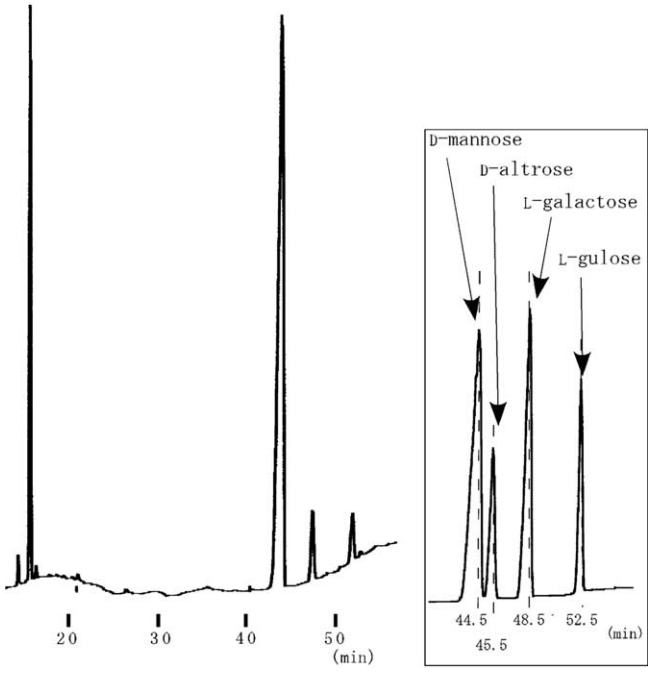

Fig. 6. Sugar analysis by GLC. A GLC of alditol acetates of L-galactose and L-gulose. The GME reaction mixture was hydrolyzed and the monosaccharides converted to alditol acetates. The peaks at 15.1 min, 44.1 min, 47.7 min, and 52.4 min correspond to alditol acetates of ribose, D-mannose, L-galactose, and L-gulose, respectively. Within the framework, references of alditol acetates of D-mannose, D-altorose, L-galactose, and L-gulose are shown.

3. Experimental

3.1. Cloning of the OsGME gene

An annotation of the coding sequences of the *Oryza sativa* GDP-D-mannose 3,5-epimerase (*OsGME*) gene was performed, comparing the rice genome sequences with the *GME* gene sequence of *Arabidopsis thaliana* (Genbank Accession No. AX647945) using the BLAST program (Altschul et al., 1990). One candidate for the *OsGME* gene (Genbank Accession No. AB193582) was found, with a homology to the *Arabidopsis* gene sequence of 80%. To obtain cDNA of the *OsGME* gene, total RNA was isolated 14 days postanthesis from the japonica rice (*Oryza sativa* L.) variety Nipponbare, that had been grown in a greenhouse from May to September, 2001 (Suzuki et al., 2004). First-strand cDNA synthesis was carried out using 5 μg of the total RNA with the *OsGME*-specific primer (5-GAGGAGCTGATTGATCTTCATGGTG-3) according to the protocol of the SuperScript First-Strand Synthesis System (Invitrogen, Tokyo, Japan). The reaction mixture was used as a template for PCR in a iCycler (Bio Rad Laboratories, Hercules, CA). The candidate gene was amplified with a set of specific primers, S-1 (5′-GATCCCTCTCCGACCGACCAAG-3) and A-1 (5-GAGGAGCTGATTGATCTTCATGGTG-3) in a volume of 50 μl containing the cDNA template and LA *Taq* polymerase (Takara-Bio, Shiga, Japan). The PCR program was 35 cycles at 95 °C for 1 min and 62 °C for 2 min, followed by incubation at 72 °C for 4 min. Five microliters of PCR product was analyzed by electrophoresis on a 1% agarose gel with ethidium bromide staining. The nucleotide sequence was then determined.

Table 2
Effects of additives on GME activities

Additives	Reaction rate (%)
Water	100
NAD	805[0.11][a]
NADH	240[14][a]
NADP	531[0.51][a]
NADPH	297[2.0][a]
GMP	94
GDP	19[1.0][b]
GTP	74
AMP	73
ADP	64
ATP	68
CMP	51
CDP	54
CTP	52
TMP	44
TDP	55
TTP	57
UMP	44
UDP	58
UTP	64
D-mannose	67
UDP-glucose	104
UDP-galactose	110
UDP-xylose	79
UDP-arabinose	83
GDP-fucose	73

[a] Concentration (μM) of additive required for 200% activation of the enzyme reaction.
[b] Concentration (μM) of additive required for 50% inhibition of the enzyme reaction.

3.2. Expression and purification of recombinant enzyme

The coding region of the cloned cDNA of GME was amplified with the following set of specific primers, compS-1 (5′-AACAACCTCGGGATCGAGGGAAGGA-TGGGGAGCTCGGAGAAGAAC-3′) and compA-1 (5′-CGCGGATCCTTACTCCTTGCCATCGGCAGC-3′), which contain restriction sites for BsoBI and BamHI, respectively. This product was inserted between the BsoBI and BamHI sites of pMAL-C2X (New England Biolabs, Beverly, MA). The construct that subcloned *OsGME* gene in pMAL-C2X and insert-free pMAL-C2X (negative control) was transformed to *E. coli* JM109. The *E. coli* cells were grown at 37 °C, and the production of recombinant protein was induced by treatment with 1 mM isopropyl *β*-D-thiogalactopyranoside for 6 h. The *E. coli* cells were harvested and sonicated in a buffer containing 50 mM Tris–HCl buffer (pH 8.0), 50 mM NaCl, 1 mM EDTA, and 1 mM DTT. The crude soluble extract was placed on an amylose resin column (New England Biolabs), with the latter washed with 10 mM Tris–HCl buffer, pH 7.4, containing 0.8% NaCl and 0.02% KCl; the bound protein was then eluted with the same buffer containing 10 mM maltose. The purified recombinant enzyme (1 mg) was digested with 10 μg of Factor Xa (Haematologic Technologies, Vermont, USA) at 4 °C for 24 h in order to split off the fused maltose binding protein. The sample was applied onto a TOYOPEARL SuperQ-650M (Tosoh, Tokyo, Japan) column with 10 mM Tris–HCl buffer (pH 7.5), and eluted with a linear gradient of 10–500 mM Tris–HCl. Active fractions were collected, with the purified recombinant enzyme examined for purity, specific activity, and substrate specificity. The concentration of GME obtained was determined by the Bradford method (Bradford, 1976).

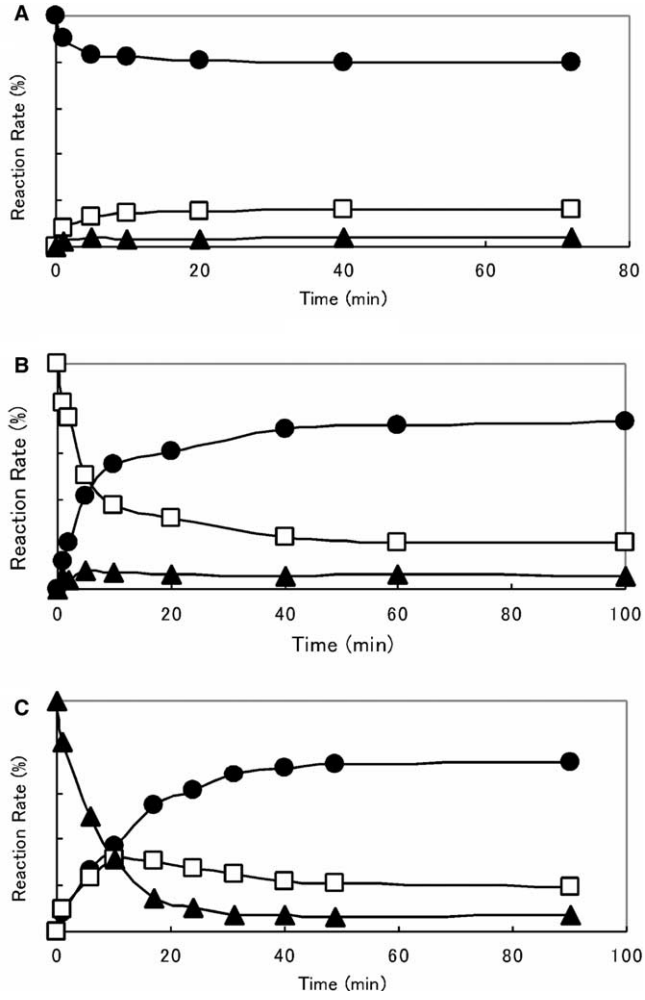

Fig. 7. Time courses of the GME reaction. Transition of GDP-D-mannose (**1**) (●), GDP-L-galactose (**3**) (□) and GDP-L-gulose (**2**) (▲) in the GME enzyme reaction. The enzyme reaction was performed in 40 mM Tris–HCl buffer (pH 8) at 20 °C using 0.1 mM substrate. Panels A–C show moment-to-moment change using GDP-D-mannose (**1**) (●), GDP-L-galactose (**3**) (□) and GDP-L-gulose (**2**) (▲) as a substrate, respectively.

3.3. Enzymatic reaction and NMR spectroscopic analysis of products

In order to identify products formed by the action of the recombinant enzyme, a relatively large scale reaction was employed. A reaction mixture consisting of 40 mM Tris–HCl buffer, pH 7.5, 40 mg of GDP-D-mannose (Calbiochem, Darmstadt, Germany), and 31 mg of the recombinant GME in a final volume of 15 ml was incubated at

37 °C for 30 min. To terminate the enzyme reaction, the enzymes were removed using Centriprep filter devices with a molecular weight cut-off of 10,000 (Millipore, Bedford, MA), and the synthesized products were confirmed by HPLC analysis on an ODS column (UK-C_{18}; 20 mm i.d. × 250 mm; Imtakt, Kyoto, Japan). Concerning the purification of the synthesized products, they were roughly fractionated by HPLC and the absorbance of the eluate was monitored at 254 nm. The roughly purified products were separated again by a recycle HPLC system using a UK-C_{18} column. A solution of 6 mM phosphoric acid and 2 mM potassium chloride was used for the mobile phase (Wolucka et al., 2001a) in both the standard HPLC system and the recycling HPLC system. Each of the three types of products were absorbed on a SuperQ Toyopearl 650M column in H_2O and eluted with 0.3 M NaCl. Finally, each product was desalted on a Sephadex G-10 gel filtration chromatography column (Pharmacia, Freiburg, Germany) and lyophilized.

Lyophilized samples were dissolved in D_2O and re-lyophilized, and then dissolved in D_2O. Spectra were recorded at 25 °C at 400 MHz for ^1H and at 100 MHz for ^{13}C NMR using JNM-AL400 and JNM-A500 spectrometers (JEOL, Tokyo, Japan). The analysis of the spectra obtained by measurement of ^1H–^1H correlated spectroscopy (COSY), ^{13}C–^1H heteronuclear multiple quantum coherence (HMQC), ^{13}C–^1H heteronuclear multiple-bond correlation (HMBC), and Homonuclear Hartmann–Hahn (HOHAHA) were performed using the ALICE software program (JEOL, Tokyo, Japan).

3.4. Gas–liquid chromatography for sugar analysis

Nucleotide sugars were hydrolyzed by treatment with trifluoroacetic acid at 100 °C for 6 h, and the monosaccharides were converted to alditol acetates by reduction, followed by treatment with acetic anhydride in an equal volume of pyridine at 120 °C for 2 h. Separation of the alditol acetates was carried out on a chromatographic device GC-17A (Shimadzu, Kyoto, Japan) on a DB-225 column (J&W scientific, Folsom, CA). One microliter of the sample was injected under split conditions at an oven temperature of 180 °C. After 45 min at 180 °C, the oven temperature was raised to 200 °C at 2 °C/min, and held at 200 °C for 15 min. The injector and detector temperatures were 230 °C.

3.5. Enzyme assay

The activity of the GME enzyme was determined by monitoring the formation of both GDP-L-galactose (**3**) and GDP-L-gulose (**2**) from GDP-D-mannose (**1**). In a typical run, the reaction mixture contained 40 mM Tris–HCl buffer (pH 8.0), 10 μM GDP-D-mannose, 10 μM NAD^+, and enzyme. In a study of the effect of nucleotide and nucleotide sugars on GME activity, the GME protein was separately mixed with each additive for 10 min at 20 °C, and the reaction was then started by the addition of GDP-D-mannose. After incubation at 20 °C for one to 15 min, the reaction was terminated by dipping the mixture into a heated block at 100 °C for 1 min. The reaction products were subjected to reversed phase HPLC on an ODS column (4.6 mm i.d. × 250 mm; Imtakt). The mobile phase solution was a mixture of H_2O–Et_3N–AcOH (100:0.2:0.1, v:v:v) that had been filtered and degassed under reduced pressure, prior to use. Separation was carried out isocratically at a flow rate of 0.8 ml/min at 37 °C, and the respective samples were detected by their absorption at a wavelength of 254 nm. In the kinetic studies, the trials were performed in triplicate, at a minimum and the standard errors were less than 10%. Primary initial velocity data were fitted to the Michaelis–Menten equation by non-linear regression (Origin, version 5.0).

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