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A plant mutase that interconverts UDP-arabinofuranose and UDP-arabinopyranose

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Plant cell walls constitute the bulk of the earth renewable source of energy and are a component in the diet of humans and herbivores. L-Arabinofuranosyl (Ara*f*) residues are a quantifiably important constituent of these walls. Plants use uridine diphosphate (UDP)-L-arabinofuranose (UDP-Ara*f*) to donate Ara*f* residues in the biosynthesis of Ara*f*-containing polysaccharides, proteoglycans, and glycoproteins. However, little is known about the formation of UDP-Ara*f*. We now describe the purification and partial characterization of a rice UDP-arabinopyranose mutase (UAM) that catalyzes the formation of UDP-Ara*f* from UDP-arabinopyranose (UDP-Ara*p*). The reaction is reversible and at thermodynamic equilibrium the pyranose form is favored over the furanose form (90 : 10). Three related proteins that are encoded by rice gene loci Os03g40270, Os04g56520, and Os07g41360 were identified from partial amino acid sequences of UAM. These proteins have >80% sequence identity with polypeptides that are reversibly glycosylated in the presence of UDP-sugars. The rice mutase and two functionally active recombinant mutases were shown to be reversibly glycosylated in the presence of UDP-Glc. The cofactor, flavin-adenine-dinucleotide (FAD), is required for the catalytic activity of UDP-galactose mutases of prokaryotes, fungi, and protozoa. The plant mutases, which do not require a cofactor, must therefore have a different catalytic mechanism. Putative UAM-encoding genes are present in the green algae *Chlamydomonas reinhardtii*, the moss *Physcomitrella patens*, the gymnosperm *Pinus taeda* (loblolly pine), and in numerous dicots and monocots, indicating that UAMs are widespread in green plants.

Key words: arabinofuranose/reversibly glycosylated polypeptide/UDP-arabinopyranose mutase

Introduction

Plant cell walls are dynamic structures that are involved in the morphology, growth, and development of cells and tissues, and in the interactions between plants and their pathogens (Johnson et al. 2003; O'Neill and York 2003). Cell walls are a source of dietary fiber for humans (Eastwood and Kritchevsky 2005), and a source of nutrition for many livestock (Jung and Allen 1996). Plant cell walls are also a renewable source of biomass for the production of fuels and other chemicals (Ragauskus et al. 2006). Generating plants with altered wall composition and structure may improve the growth characteristics of economically important plants and increase their resistance to pathogens. Manipulating cell wall composition may also improve the nutritional value of forage plants and increase the efficiency of converting plant biomass to fuel (Ragauskus et al. 2006). The development of methods to modify wall structure requires a thorough understanding of the mechanisms of polysaccharide biosynthesis.

L-Arabinofuranosyl (Ara*f*) residues are a quantifiably important constituent of plant primary and secondary cell walls (Johnson et al. 2003; O'Neill and York 2003). However, the structure of the activated form of Ara*f* required for the synthesis of plant polysaccharides, proteoglycans, and glycoproteins that contain Ara*f* residues has been a subject of debate. Nucleoside 5′-diphospho sugars are the high-energy donor molecules typically used by glycosyltransferases and glycan synthases in the formation of complex carbohydrates (Reiter and Vanzin 2001; Seifert 2004). No plant enzymes have been identified that catalyze the formation of uridine diphosphate (UDP)-Ara*f*. By contrast, UDP-L-arabinopyranose (UDP-Ara*p*) is known to be formed from UDP-D-xylopyranose by a UDP-xylopyranose 4-epimerase and from L-Ara*p*-1-phosphate and uridine triphosphate by a UDP-Ara*p*-1-phosphate pyrophosphorylase (Reiter and Vanzin 2001). The results of early studies led to the hypothesis that UDP-Ara*p* is the sugar donor and that conversion to Ara*f* occurs during the glycosyl transfer reaction (Fry and Northcote 1983). This mechanism is unlikely because UDP-Ara*f* has been shown by using plant extracts to be the sugar donor for the synthesis of Ara*f*-containing oligosaccharides in plants (Konishi et al. 2006). Several workers (Nunan and Scheller 2003; Harholt et al. 2006) have speculated that UDP-Ara*f* is formed from UDP-Ara*p* by the action of a mutase (Figure 1A). Indeed, a flavin-adenine-dinucleotide (FAD)-dependent bacterial UDP-D-galactose (UDP-Gal*p*) mutase also catalyzes the interconversion of UDP-Ara*p* and UDP-Ara*f* (Zhang and Liu 2001), albeit less efficiently than it catalyzes the interconversion of UDP-Gal*p* and UDP-Gal*f*. This mutase is present in numerous prokaryotes, fungi and protozoa (Nassau et al. 1996; Koplin et al. 1997; Sanders et al. 2001; Beverley et al. 2005), but no plant homologs have been identified (Harholt et al. 2006).

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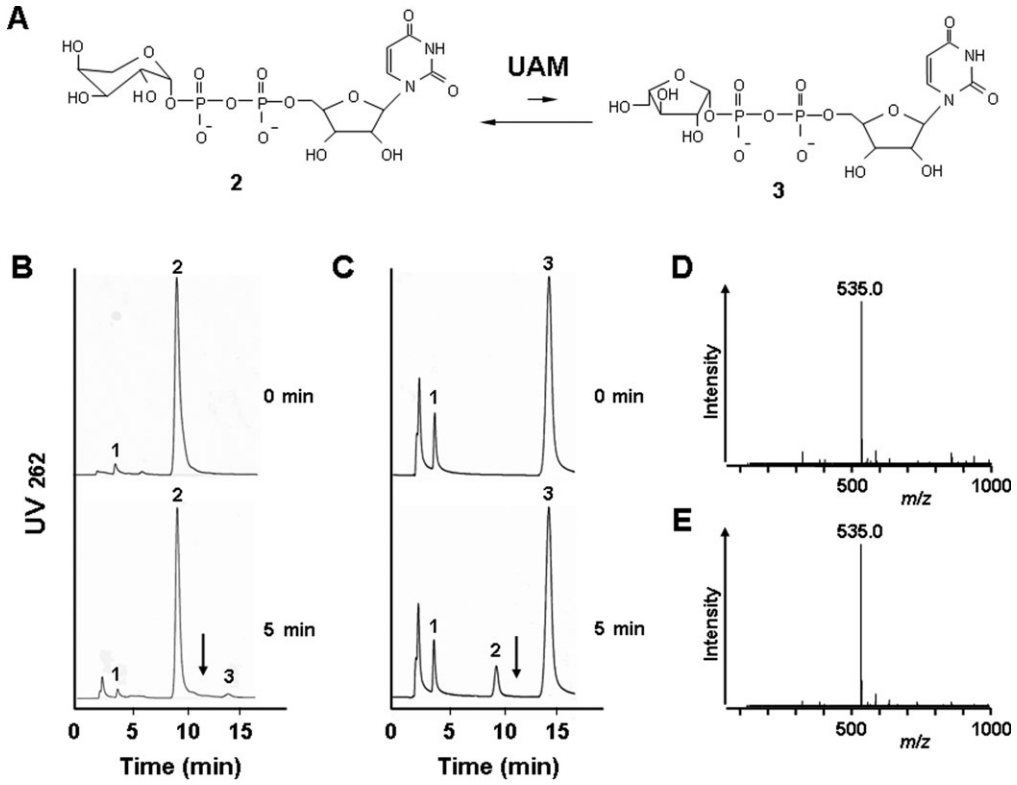

Fig. 1. The interconversion of UDP-L-Ara*p* and UDP-L-Ara*f*. (**A**) UDP-Ara*p* mutase catalyzes the interconversion of UDP-Ara*p* (2) and UDP-Ara*f* (3). The pyranose form is favored at equilibrium. (**B**) High-performance anion-exchange chromatograms showing the products formed when UDP-Ara*p* is reacted with the purified rice mutase. (**C**) High-performance anion-exchange chromatograms showing the products formed when UDP-Ara*f* is reacted with the purified rice mutase. UDP-Ara*f* is somewhat unstable under the experimental conditions used and is partially converted to UMP and arabinose. (**D**) ESI-MS spectrum of UDP-Ara*p*. (**E**) ESI-MS spectrum of UDP-Ara*f*. The elution positions of standard UMP (1), UDP-Ara*p* (2), UDP-Ara*f* (3), and UDP-Xyl (allow) are shown.

Here, we describe the identification, purification, and partial characterization of a rice UDP-arabinopyranose mutase (UAM) that catalyzes the interconversion of UDP-Ara*f* and UDP-Ara*p*. We show, using bioinformatics together with the functional characterization of two recombinant proteins, that UAM is identical to plant polypeptides that are reversibly glycosylated in the presence of UDP-Glc (Dhugga et al. 1991, 1997; Delgado et al. 1998; Bocca et al. 1999; Faik et al. 2000; Langeveld et al. 2002; Zhao et al. 2002). We also provide evidence that the rice and recombinant UAMs (rUAMs) do not require a cofactor and must therefore have a different catalytic mechanism than FAD-dependent UDP-Gal mutases.

Results

Identification of a UDP-arabinopyranose mutase in rice seedlings

We used UDP-Ara*f* as the substrate to detect UAM activity in rice seedling extracts (Figure 1B) because at thermodynamic equilibrium the pyranose form is favored over the furanose form (90 : 10; Zhang and Liu 2001). Indeed, only a small amount of UDP-Ara*f* was generated when UDP-Ara*p* was the substrate (Figure 1C). Enzymatically formed UDP-Ara*f* and UDP-Ara*p* were identified by liquid chromatography-mass spectrometry (LC-MS) (Figure 1D and E). The peak of UDP-xylose was not present in the reaction mixture, showing that the rice UAM has no activity of UDP-xylose 4-epimerase (Figure 1B and C). Approximately 80% of the UAM activity was detected in the cytosolic fraction with lesser amounts in a salt extract of rice cell walls (~20%). The microsomal membrane fraction of rice seedlings contained ~1% of the total mutase activity. UAM was also detected in mung bean seedling extracts (data not shown), showing that mutase activity is present in dicots as well as monocots.

Rice UAM was purified using a combination of hydrophobic interaction, size-exclusion, and anion-exchange chromatographies (Figure 2). The specific activity of UAM increased 430-fold during the purification procedure. The purified UAM gave a single broad band (~41 kDa) on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Figure 2E). However, the mutase is likely to exist as a complex composed of numerous proteins because its molecular weight was estimated by size-exclusion chromatography (SEC) to be ~460 kDa (Figure 2D). Mutase activity was stable for 3 weeks at 4 °C and for at least 3 months at −20 °C. Repeated cycles of freezing and thawing did not significantly affect mutase activity (data not shown).

General characteristics of rice UDP-arabinopyranose mutase

We determined some of the properties of rice UAM by reacting the purified enzyme at different temperatures and pH values with varying amounts of either UDP-Ara*p* or UDP-Ara*f*. The amounts of UDP-Ara*p* and UDP-Ara*f* formed are proportional to the amounts of UAM added (Figure 3A) and increased with time up to ~30 min (Figure 3B). The

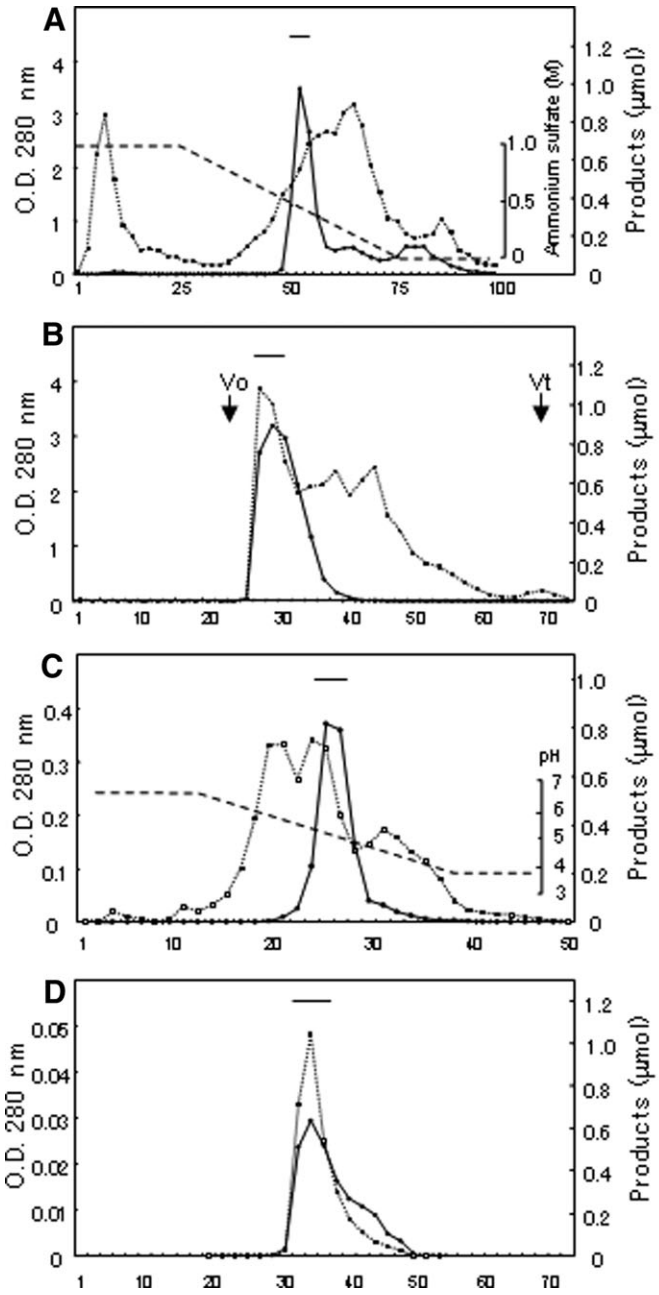

Fig. 2. Purification of UDP-Ara*p* mutase from rice seedlings. (**A**) Butyl-Toyopearl S-650 chromatography. A decreasing linear gradient (24–0%) of saturated ammonium sulfate (·). The A_{280nm} of the eluent (□). UDP-Ara*p* mutase activity (—). The horizontal bar indicates pooled fractions. (**B**) Superdex 200 SEC. V_0 and V_t indicate the void volume and total bed volume of the column, respectively. (**C**) Mono P weak anion-exchange chromatography. The dotted line represents pH. (**D**) Superdex 200 SEC. (**E**) SDS–PAGE of protein fractions obtained in the course of purifying UDP-Ara*p* mutase from rice seedlings. Proteins obtained at each purification stage were analyzed by SDS–PAGE. The gel was stained with Coomassie Brilliant Blue. Lane 1, 30–65% ammonium sulfate fraction; lane 2, Butyl-Toyopearl fraction; lane 3, Superdex 200 fraction; lane 4, Mono P fraction; and lane 5, Superdex-200 fraction. The arrow indicates the position of the purified UDP-Ara*p* mutase.

interconversion of UDP-Ara*p* and UDP-Ara*f* had an optimum temperature of 55 °C (Figure 3C and D), with maximum activity for UDP-Ara*f* formation occurring between pH 7.0 and 7.5 (Figure 3E), and for UDP-Ara*p* formation between pH 5.5 and 6.0 (Figure 3F). The reaction is reversible because UAM converts either substrate into an equilibrium mixture of ~90% of UDP-Ara*p* and ~10% of UDP-Ara*f* (Figure 3B). Kinetic values for the conversion of UDP-Ara*f* to UDP-Ara*p* ($K_m = 55$ μM and $V_{max} = 1.18$ nmol min^{-1} ng protein^{-1}), and for the conversion of UDP-Ara*p* to UDP-Ara*f* ($K_m = 531$ μM and $V_{max} = 0.55$ nmol min^{-1} ng protein^{-1}) confirm that the reaction favors pyranose formation. Rice UAM has a higher affinity for UDP-Ara*f* ($K_m = 55$ μM) than a recombinant *Escherichia coli* UDP-Gal*p* mutase ($K_m = 600$ μM) (Zhang and Liu 2001). This is the expected result, as UDP-Gal*f* ($K_m = 22$ μM) rather than UDP-Ara*f* is the likely substrate for the microbial mutase (Zhang and Liu 2001).

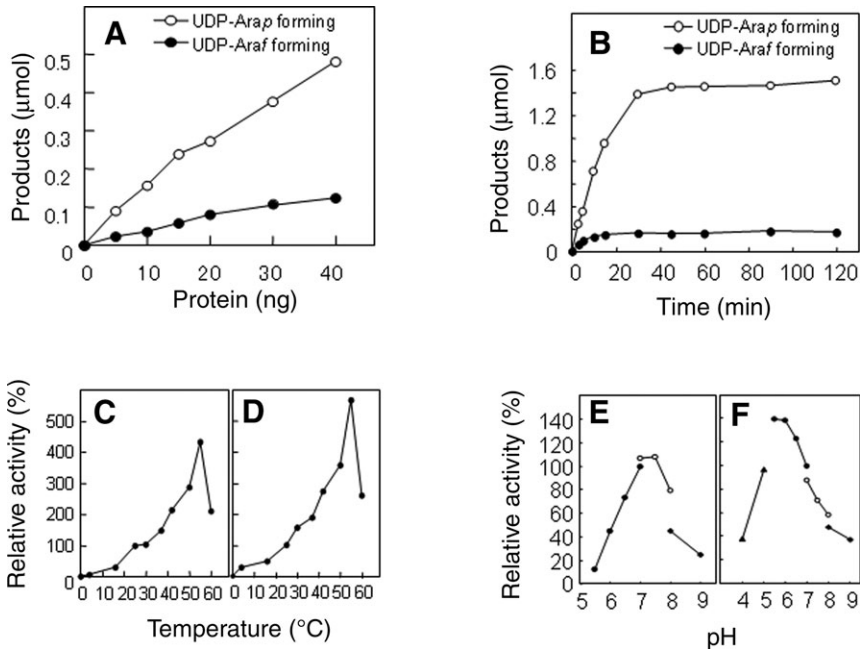

Fig. 3. Selected properties of rice UDP-Arap mutase. (**A**) The effect of protein concentration on the interconversion of UDP-Ara*p* and UDP-Ara*f*. UDP-Ara*p* or UDP-Ara*f* (2 mM) was reacted with the mutase and the products formed analyzed by HPAEC. (**B**) Time course of the interconversion of UDP-Ara*p* and UDP-Ara*f*. (**C**) The effects of temperature on the formation of UDP-Ara*f*. (**D**) The effects of the temperature on the formation of UDP-Ara*p*. (**E**) The effects of pH on the formation of UDP-Ara*f*. (**F**) The effects of the pH on the formation of UDP-Ara*p*. Enzyme activity was measured using acetate (▲), MES-KOH (○), HEPES-KOH (○), and Tris-HCl (■) buffers.

Identification of the genes that encode rice UDP-arabinopyranose mutase

To identify genes that encode rice UAM, we obtained partial amino acid sequences of the mutase proteins (Figure 4) using high-performance liquid chromatography (HPLC)-MS/MS analysis of trypsin-generated peptides. Peptide sequences were profiled against the annotated rice genome (http://rapdb.lab.nig.ac.jp and http://rice.tigr.org) and proteins identified using the Mascot search engine (www.matrixscience.com). Three related proteins (Figure 4) were identified, which correspond to coding sequences NM_194076 (LOC_Os03g40270), XM_474209 (LOC_Os04g56520), and XP_479089 (LOC_Os07g41360). We have used the system of numbering rice genes (LOC_Os0#g#####) that is described at the TIGR rice genome annotation database and resource (http://rice.tigr.org). A search of the rice massively parallel signature sequencing database (http://mpss.udel.edu/rice) indicated that all three genes are expressed at various levels in rice roots, leaves, and stems (Supplementary data, Table SI).

Os03g40270, Os04g56520, and Os07g41360 are annotated in rice genomic databases as encoding putative UDP-glucose : protein transglucosylases or as encoding reversibly glycosylated polypeptides (RGP). RGPs are a family of plant-specific proteins that form reversible complexes with UDP-sugars in vitro, but until now have no known biological functions (Dhugga et al. 1991, 1997; Delgado et al. 1998; Bocca et al. 1999; Faik et al. 2000; Langeveld et al. 2002; Zhao et al. 2002). We will refer to the proteins encoded by Os03g40270 and Os07g41360 as UAMs1 and 3, respectively, as our data show that these proteins are UDP-Ara*p* mutases. We obtained no functional data for the protein encoded by Os04g56520. Nevertheless, we will refer to this protein as UAM2 as it is associated with the rice UAM complex.

UAM1 and UAM3 are 88 % identical but share only 46% identity with UAM2 (Figure 4). UAM1 and UAM3 and the family 1 RGPs have similar sequences, whereas UAM2 has sequence similarity with the family 2 RGPs (Delgado et al. 1998). The deduced molecular masses of the three UAMs (39–41 kDa) are comparable to the molecular mass (~41 kDa) of the rice mutase proteins, which are recognized by antibodies generated against rice RGP1 and RGP2 (Figure 5A). Such results when taken together suggest that UAMs1, 2, and 3 and proteins annotated as RGPs 1, 2, and 3 are encoded for by the same genes.

Recombinant UDP-arabinopyranose mutases are reversibly glycosylated in the presence of UDP-Glc

The high similarity (>80%) of the amino acid sequences of rice UAM1 and UAM3 and the RGP1 family of proteins (Figure 4) led us to suspect that UAM would be capable of autoglycosylation. We then decided to confirm that mutase and autoglycosylating activity are present in the same protein. Toward this goal, we generated recombinant versions of UAM1, UAM2, and UAM3 by separately expressing rice *Uam1*, *Uam2*, and *Uam3* in *E. coli*. rUAM1 and rUAM3 interconverted UDP-Ara*p* and UDP-Ara*f* (Figure 5D), although their specific activities (35.2 and 16.4 units mg protein^{-1}, respectively) are 10-fold lower than that of the purified rice mutase (262 units mg protein^{-1}). This was the expected result, as we had obtained evidence that the mutase most likely exists in *planta* as a complex composed of several different UAMs. However, we did not observe an increase in

Fig. 4. The predicted amino acid sequences of the proteins present in rice UDP-Ara*p* mutase. The predicted amino acid sequences of rice UAM1 (NM_194076, Os03g40270), rice UAM2 (XM_474029, Os04g56520), and rice UAM3 (XP_479089, Os07g41360) are shown. The underlined and boldface regions were identified the sequences of the peptides that were used to identify each protein. Amino acids within the box show the putative D × D motif. The residue indicated by * within the square is the conserved arginine residue that is believed to be the site of auto-glycosylation. The deduced amino acid sequences of proteins from *Arabidopsis thaliana* (At5g15650), Pine (TC65319), *Physcomitrella patens* (contig 11029), and *C. reinhardtii* (TC47939) that have high sequence homology (>80%) to Os RGP1. The alignments were generated using ClustalW (http://www.ebi.ac.uk/clustalw/index.html).

the mutase activity by mixing several combinations of the rUAMs nor were we able to demonstrate that rUAM2 alone had mutase activity, suggesting that other plant factors are required for high activity. Nevertheless, the demonstration that rUAM1 and rUAM3 are UDP-Ara mutases provides experimental evidence that these proteins participate in the synthesis of complex carbohydrates.

Rice UAM together with rUAM1 and rUAM3 were auto-glycosylated when each protein was reacted separately with UDP-[^{14}C]-Glc (Figure 5B and C). No autoglycosylation occured with rUAM2 (Figure 5C). This result is consistent with a previous study that showed a recombinant version of wheat RGP2 was also incapable of self-glycosylation in vitro (Langeveld et al. 2002). Glycosylation of rice UAM is reversible, and the radiolabeled Glc was displaced by UDP-Glc, UDP-Xyl, UDP-Gal, UDP-Ara*p*, and UDP-Ara*f* (Figure 5B, lanes 2, 3, 4, 5, and 6). Interestingly, we also found that there was a ~30% and a ~50% decrease in mutase activity when these three nucleotide sugars (UDP-Glc, UDP-Xyl, or UDP-Gal) were separately reacted for 30 min and 6 h, respectively, with rice UAM prior to the addition of UDP-Ara*f*.

Rice UDP-arabinopyranose mutase requires divalent cations but not FAD for activity

A previous study has shown that divalent cations are required for reversible glycosylation of pea RGPs (Dhugga et al. 1991). We found that divalent cations are also required for mutase activity, which almost doubled in the presence of $MnCl_2$ (5 mM), but was completely inhibited by EDTA (5 mM, data not shown). Five millimolar of $CaCl_2$, $MgCl_2$, $CuSO_4$, $ZnCl_2$, and $CoCl_2$ were not effective or inhibitory effect (Supplementary data, Figure S1).

Microbial UDP-Gal*p* mutases require reduced FAD for activity (Sanders et al. 2001; Soltero-Higgin et al. 2004). The activity of these mutases is abolished in the presence of the oxidant K_3FeCN_6 (Sanders et al. 2001). The addition of sodium dithionite to the K_3FeCN_6-oxidized enzyme restores mutase activity. No peaks characteristic of a flavin cofactor (A_{382nm} and A_{450nm}) were present in the ultraviolet-visible light spectra of rice UAM and the rUAMs, showing that these proteins contain no FAD. Moreover, the presence of K_3FeCN_6 or sodium dithionite did not have a discernible effect on rice mutase activity.

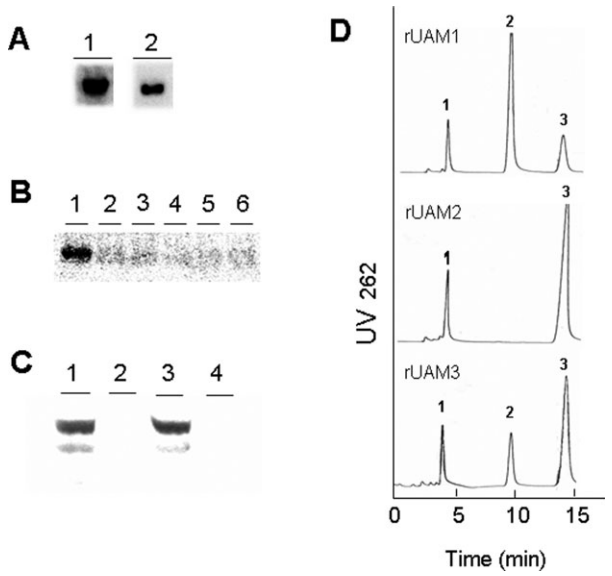

Fig. 5. Rice UDP-Ara*p* mutase is a reverisbly glycosylated protein. (**A**) Western blot analysis of the purified rice UDP-Ara*p* mutase using anti RGP1 (1) and anti RGP2 (2). (**B**) The products formed when rice UAM was reacted for 15 min with UDP-[^{14}C]-Glc (lane 1) followed by a 15 min chase with cold UDP-Glc (lane 2), UDP-Xyl (lane 3) and UDP-Gal (lane 4), UDP-Ara*p* (lane 5), and UDP-Ara*f* (lane 6). (**C**) The products formed when the recombinant proteins rUAM1 (lane 1), rUAM2 (lane 2), rUAM3 (lane 3), and vector (lane 4) were reacted for 15 min with UDP-[^{14}C]-Glc. (**D**) High-performance anion-exchange chromatograms showing the products formed when UDP-Ara*f* is reacted separately with the recombinant proteins rUAM1, rUAM2, and rUAM3. The elution positions of standard UMP (1), UDP-Ara*p* (2), and UDP-Ara*f* (3) are shown.

Putative UDP-arabinopyranose mutase-encoding genes are present in diverse green plants

Proteins with high sequence homolgy to rice UAM1 have been identified in numerous monocots and dicots (Dhugga et al. 1991, 1997; Delgado et al. 1998; Bocca et al. 1999; Faik et al. 2000; Langeveld et al. 2002; Zhao et al. 2002), in the bryophyte *Physcomitrella patens* (Wald et al. 2003), and in the green algae *Chlamydomonas reinhardtii* (Wald et al. 2003). We identified additional putative UAM1-encoding genes in two gymnosperms [*Pinus taeda* (loblolly pine) and *Picea glauca* (white spruce)], in five graminaceous monocots [barley, rye, sugar cane, sorghum, and *Brachypodium distachyon* (Purple false Brome)], in a nongraminaceous monocot (onion), and in numerous dicots including, grape, lettuce, medicago, poplar, and sunflower by BLAST searches of plant databases (http://tigrblast.tigr.org/tgi).

Discussion

In this study we have shown that rice seedlings contain a mutase that interconverts UDP-Ara*p* and UDP-Ara*f* and demonstrated that recombinant versions of this enzyme are functionally active. Rice UAM and two rUAMs are reversibly glycosylated in the presence of UDP-Glc. These results together with the fact that rice UAMs and numerous RGPs have $<$80% amino acid sequence identity lead us to conclude that plant UAMs and proteins identified as RGPs are identical. These proteins are likely to be essential for normal plant growth and development, as mutations in *Arabidopsis RGP1* (At3g02230) and *RGP2* (At5g15650) when combined together are lethal (Drakakaki et al. 2006).

The results of numerous studies (Dhugga et al. 1991, 1997; Delgado et al. 1998; Bocca et al. 1999; Faik et al. 2000; Langeveld et al. 2002; Zhao et al. 2002; Porchia et al. 2002; Drakakaki et al. 2006) have indicated that RGPs have a role, albeit poorly understood, in the synthesis of plant polysaccharides. For example, during in vitro arabinoxylan synthesis, a 41 kDa protein is transiently radiolabeled when wheat Golgi vesicles are reacted with UDP-[^{14}C]-Ara*p* and then with UDP-Xyl (Porchia et al. 2002). We hypothesize that this transient labeling resulted from the UAM catalyzed conversion of UDP-Ara*p* to UDP-Ara*f*, prior to transfer of the Ara*f* to the xylan backbone.

The mechanism for interconverting UDP-Ara*p* and UDP-Ara*f* by plant UAMs is not known. Our data show that rice UAM does not require a cofactor to catalyze ring interconversion and must therefore have a different catalytic mechanism than the FAD-dependent UDP-Gal*p* mutases. These mutase generate a flavin-galactose intermediate prior to ring interconversion (Soltero-Higgin et al. 2004). The guanidino nitrogen of the arginine residue that is believed to be the site of autoglycosylation in RGPs (Singh et al. 1995; Figure 4) may have a catalytic function that is similar to FAD. Indeed, our data suggest that ring interconversion and reversible glycosylation occur at the same site, since rice UAM activity is decreased by $\sim$50% by pretreatment with UDP-Glc, UDP-Gal, or UDP-Xyl.

UDP-Ara*p* mutase and all RGPs that have been identified to date contain a "D $\times$ D" motif (Figure 4) that is a characteristic of the GT-A family of glycosyltransferases (Breton et al. 2006). This motif is believed to be involved in the coordination of Mn^{2+} with the phosphate groups of the nucleotide donor (Breton et al. 2006). No experimental evidence has been obtained to show that RGPs are glycosyltransferases (Faik et al. 2000; Langeveld et al. 2002). Indeed, we found that rice UAM did not catalyze the transfer of Ara from UDP-Ara*p* or UDP-Ara*f* to 2-aminobenzamide labeled Ara*f*-oligosaccharide acceptors. Nevertheless, we did find that UAM requires Mn^{2+} for activity. Similar Mn^{2+}-dependent reactions would likely occur at the active sites of sugar nucleotide mutases and glycosyltransferases. For example, one of the earliest reactions catalyzed by these enzymes is the cleavage of the bond between the anomeric carbon of the glycose and the nucleoside diphosphate (Soltero-Higgin et al. 2004). The Mn^{2+} ion may counter the negative charge formed on the β-phosphate following cleavage of this linkage (Unligil and Rini 2000). The mutase reaction then proceeds via intramolecular transfer of the anomeric carbon to either the C4 or the C5 oxygen of the glycose. In contrast, a glycosyltransferase reaction involves the intermolecular transfer of the anomeric carbon to an acceptor molecule.

UDP-Ara*p* mutase activity is reduced by $\sim$30 or $\sim$50% when the enzyme is treated with UDP-Glc*p*, UDP-Xyl*p*, and UDP-Gal*p* for 30 min or 6 h, respectively, prior to the addition of UDP-Ara*f*, which suggest that these nucleotide sugars compete with UDP-Ara for binding to UAM. A reversible reaction between UAM and UDP-Glc*p* or UAM and UDP-Xyl*p* is unlikely to result in ring contraction because the interaction between C4 and C1 that is required for furanose ring formation is sterically hindered by an equatorial

C-4 hydroxy group. When UDP-Glc*p* and UDP-Xyl*p* was treated with UAM, peaks corresponding to furanose forms were not detected (data not shown). A prolonged reaction (>3 h) between RGPs and UDP-sugars may lead to irreversible inhibition of mutase activity because of the formation of an irreversible covalent bond between the glycosyl residue and the protein (Faik et al. 2000). Thus, inhibition of UAM by nucleotide sugars may be one of the mechanisms that regulate the amounts of UDP-Ara*f* available for polysaccharide and glycoprotein synthesis.

The amino acid sequences of UAMs1, 2, and 3 (Figure 4) reveal no known membrane spanning domains or N-terminal secretory signals, which is consistent with our finding that mutase activity is present predominantly in the cytosol. Indeed, only ~1% of the mutase activity was detected in the rice seedling microsomal membrane fraction. Nevertheless, the possibility cannot be discounted that UAM is a membrane-associated protein located on the cytosolic face of the Golgi complex and released into the cytosol during tissue disruption (Dhugga et al. 1991). The mutase is likely to be associated with the Golgi complex, as this cellular organelle is the site of synthesis of complex carbohydrates (Jonson et al. 2003). Approximately 20% of the UAM activity was detected in a salt-soluble extract of rice cell walls. The plant cell wall proteome is not well defined, but is likely to include many hundreds of different structural and enzymatic proteins (Lee et al. 2004; Jamet et al. 2006). Plasmodesmata-associated proteins are often present in cell walls prepared from plant tissues (Sagi et al. 2005). Indeed, proteins with >80% sequence homology to UAM1 and UAM3 have been localized to plasmodesmata of maize and transgenic tobacco (Sagi et al. 2005). There is no evidence that Ara*f*-containing polymers are synthesized at the plasmodesmata. However, the possibility cannot be discounted that mutases, if they are indeed present in plasmodesmata, have a role in regulating the intercellular transport of UDP-Ara*p* and UDP-Ara*f*.

The presence of proteins in *C. reinhardtii*, *P. patens*, and *P. taeda* with >80% amino acid identity with rice UAM1 (Figure 4) extends the occurrence of putative UAMs to the green algae, to avascular land plants, and to nonflowering vascular land plants. *C. reinhardtii* (O'Neill and Roberts 1981), *P. patens* (Lee et al. 2005), and the gymnosperms (Thomas et al. 1987) are known to synthesize Ara*f*-containing complex carbohydrates. *C. reinhardtii* also synthesizes Gal*f*-containing glycans (O'Neill and Roberts 1981). This alga has been reported to contain a open-reading frame with sequence homolgy to FAD-dependent UDP-Gal*p* mutases, although no functionally protein has been characterized (Beverley et al. 2005). Thus, *Chlamydomonas* may provide a unique opportunity to investigate the mechanisms of biosynthesis of two structurally related glycofuranoses.

The identification and characterization of a UDP-Ara*p* mutase in rice seedlings and the production of enzymatically active recombinant proteins provide some of the tools required to generate UDP-Ara*f* more efficiently than by chemical synthesis (Zhang and Liu 2001). The identification of plant enzymes that catalyze the interconverion of UDP-Ara*p* and UDP-Ara*f* and the demonstration that genes encoding these proteins are likely to be present in land plants and in green algae provide opportunities to study the synthesis of Ara*f*- and Gal*f*-containing polysaccharides and glycoproteins.

Materials and methods

Materials and plant growth conditions

Rice (*Oryza sativa* L. cv. Koshihikari) caryopses were purchased from a local seed store (Ibaraki, Japan). Caryopses were surface sterilized for 1 day at 25 °C with aqueous 0.5% benlate (Sumitomo Chemicals, Osaka, Japan), rinsed with water and then kept in water for 2 days. The caryopses were germinated and grown in the absence of light for 7 days at 25 °C in potting soil (Kureha Chemicals, Tokyo, Japan). UDP-arabinose (UDP-Ara*p* and UDP-Ara*f*) and UDP-Xyl were purchased from the Peptide Research Institute (Osaka, Japan), and Complex Carbohydrate Research Center, The University of Georgia (Athens, GA), respectively.

Preparation of buffer soluble cytosolic protein, microsomal membrane, and cell wall fractions of rice seedlings

The coleoptiles, and the first, second, and third leaves of 7 day-old rice seedlings were cut into small pieces with a razor blade. The tissue (4 g) was suspended in ice cold 20 mM Hepes-KOH, pH 6.8 (30 mL) containing 1 mM dithiothreitol, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, and 0.4 M sucrose, and homogenized for 2 min with a Polytron. The homogenized tissue was filtered through Miracloth and the filtrate centrifuged for 5 min at 4 °C and at 1000*g*. The supernatant was collected and then centrifuged for 60 min at 4 °C at 100 000*g*. This supernatant is the buffer-soluble cytosolic fraction. The pellet that was obtained was washed twice with the homogenization buffer and is the microsomal membrane fraction.

The insoluble residue (cell wall fraction) obtained by centrifugation at 1000*g* was extracted for 3 h at 4 °C with 50 mM Hepes-KOH buffer, pH 6.8, containing 2.5 M NaCl and 3% β-mercaptoethanol. The suspension was centrifuged for 5 min at 12 000*g*. The salt-soluble fraction contains proteins released from the cell wall. The pellet was washed with 50 mM Hepes-KOH buffer, pH 6.8, and the insoluble material removed by centrifugation. The salt and buffer-soluble supernatants were combined and then desalted and concentrated by ultrafiltration (50 kDa cutoff, Millipore, Tokyo, Japan). Total protein in each fraction was determined with bovine serum albumin as a standard using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Purification of rice seedling UDP-arabinopyranose mutase

Ammonium sulfate was added (to 30% saturation) to the 20 mM Hepes-KOH, pH 6.8, soluble cytosolic fraction (40 mL) and the resulting precipitate discarded. The supernatant was brought to 65% saturation by further addition of ammonium sulfate. The precipitate that formed was collected by centrifugation and then dissolved in 20% saturated ammonium sulfate (40 mL) in 20 mM potassium phosphate, pH 6.8 and applied to a Butyl-Toyopearl 650-S column (2 × 20 cm, Tosoh, Tokyo, Japan) equilibrated with the same buffer (Figure 2). The column was washed with 200 mL of the same buffer and then eluted at 4 mL min^{-1}, with a linear gradient from 24% saturated ammonium sulfate (200 mL) to 0% ammonium sulfate (200 mL). Fractions (8 mL) were collected and a portion (4 μL) analyzed for UDP-Ara*p* mutase activity by high-performance anion-exchange

chromatography (HPAEC). Fractions containing mutase activity were pooled and the protein then precipitated by the addition of ammonium sulfate to 70% saturation. The precipitate that formed was collected by centrifugation and then suspended in 20 mM potassium phosphate, pH 6.8 (4 mL), containing 0.15 M NaCl and applied to a Superdex 200 column (1.6 × 60 cm, GE Healthcare Life Sciences, Piscataway) pre-equilibrated with the same buffer. The column was eluted at 0.8 mL min^{-1} and 1.6 mL fractions were collected. Fractions containing mutase activity were pooled and precipitated with 70% saturated ammonium sulfate and centrifuged. The precipitate was dissolved in 25 mM Bis-Tris, pH 6.3 (10 mL) and dialyzed overnight at 4 °C against 2 L of the same buffer. The dialyzed solution was applied to a Mono P column (1 × 20 cm, GE Healthcare Bio-Science), pre-equilibrated 25 mM Bis-Tris, pH 6.3. The column was eluted at 0.5 mL min^{-1} with Polybuffer 74 (pH 4.0) (GE Healthcare Life Sciences, Piscataway, NJ) and 1.0 mL fractions were collected. Mutase-containg fractions were pooled and precipitated with 70% saturated ammonium sulfate and centrifuged. The precipitate was dissolved in 20 mM potassium phosphate, pH 6.8 (4 mL) containing 0.15 M NaCl and applied to a Superdex 200 column and eluted with the same buffer. The column was eluted at 0.8 mL min^{-1} and 1.6 mL fractions were collected. Mutase-containing fractions were pooled and stored at −20 °C.

UDP-arabinopyranose mutase assay

Rice mutase activity was determined at 25 °C for 5 min, unless otherwise specified in a standard reaction mixture (20 mM Hepes-KOH, pH 6.8, 5 mM $MnCl_2$, and total volume 5 μL) containing the enzyme (30 ng of rice UAM) and 2 mM UDP-Ara*p* or UDP-Ara*f*. Ethanol (20 μL) was added and the mixture then heated for 2 min at 100 °C to terminate the reaction. Water (20 μL) was added and the mixture then filtered through a 0.2 μm nylon membrane prior to analysis of the products by HPAEC. UDP-Ara*p* and UDP-Ara*f* were separated using a CarboPac PA1 column (4 × 250 mm, Dionex, Tokyo, Japan), essentially as described (Koplin et al. 1997). The column was eluted at 0.7 mL min^{-1} and 30 °C with 250 mM ammonium acetate, pH 4.4. The A_{262nm} of the eluant was monitored and the amounts of UDP-Ara*p* or UDP-Ara*f* then estimated from their peak areas using the response curve of UDP-Ara*p* as standard. One unit of enzyme activity is defined as 1 μmol of UDP-Ara*p* or UDP-Ara*f* formed per minute at 25 °C. The mutase assay of the rUAMs were determined using the chimeric form of glutathion S-transferase (GST) and standard assay conditions. Enzyme assays were performed, at least, duplicate.

Characterization of the products formed by the action of rice UDP-arabinopyranose mutase

The enzymic formation of UDP-Ara*p* and UDP-Ara*f* was confirmed by HPLC-MS. Liquid chromatography was performed using a Zorbax SB-C18 (3.0 mm × 150 mm) column. The column was eluted at 0.1 mL min^{-1} at 40 °C with a linear gradient of 1% (v/v) methanol in aqueous 0.1% formic acid to 90% (v/v) methanol in aqueous 0.1% formic acid in 50 min. The outlet of the column was connected to a LCQ electrospray mass spectrometer (Thermo Electron, Waltham, MA) operating in the negative ion mode. Electrospray ionization mass spectra were obtained using a spray voltage of 5 kV, a capillary voltage of 5 V, and a capillary temperature of 200 °C. Spectra were obtained between *m/z* 260 and 2000.

The effects of UDP-sugars, divalent cations, and ethylelendiamine tetraacetic acid on UDP-arabinopyranose mutase activity

Rice UAM (20 ng of protein) in 20 mM Hepes-KOH, pH 6.8 (5 μL) containing 5 mM $MnCl_2$ was reacted with UDP-Glc, UDP-Gal, or UDP-Xyl (400 μM) for 30 min and 6 h at 25 °C. UDP-Ara*f* (400 μM) was then added and the reaction allowed to proceed for a further 30 min at 25 °C. Ethanol (20 μL) was added and the mixture heated for 2 min at 100 °C to terminate the reaction. Water (20 μL) was added and the mixture then filtered through a 0.2 μm nylon membrane, prior to determination of the amounts of product formed by HPAEC. Mutase activity in 20 mM Hepes-KOH, pH 6.8 (5 μL) was also determined in the presence or absence of added $MnCl_2$, $CaCl_2$, $MgCl_2$, $CuSO_4$, $ZnCl_2$, and $CoCl_2$ (5 mM) and EDTA (5 mM).

The effects of oxidizing and reducing reagents on rice UDP-arabinopyranose mutase activity

Rice UAM (20 ng of protein) in 20 mM Hepes-KOH, pH 6.8 (5 μL) containing 5 mM $MnCl_2$ was reacted with K_3FeCN_6 (5 mM) or sodium dithionite (5 mM) together with UDP-Ara*f* (2 mM) for 30 min at 25 °C. Ethanol (20 μL) was added and the mixture heated for 2 min at 100 °C to terminate the reaction. Water (20 μL) was added and the mixture then filtered through a 0.2 μm nylon membrane, prior to determination of the amounts of product formed by HPAEC.

Assay for glycosyltransferase activity of rice UDP-arabinopyranose mutase

α-Arabinosyltransferase activity was determined by reacting rice UAM (20 ng of protein) for 30 min at 25 °C with 2 mM UDP-Ara*f* or 2 mM UDP-Ara*p* in 50 mM Mes/KOH, pH 6.5 (10 μL), containing 5 mM $MnCl_2$ and 10 μM 2-aminobenzamide labeled arabino-heptasaccharide. The reaction was terminated by heating for 5 min at 100 °C. The mixture was centrifuged and the supernatant then analyzed by normal-phase HPLC using fluorescence ($\lambda_{ex} = 33$ mm, $\lambda_{em} = 420$ mm) detection as described (Konishi et al. 2006).

Peptide sequencing

Purified rice UAM (5 μg protein) was electrophoresised on a 12% SDS–PAGE gel (Laemmli 1970). The gel was stained with Coomassie Brilliant Blue and the single band corresponding to UDP-Ara*p* mutase excised using a razor blade. The excised gel was treated with trypsin and the resulting peptides were analyzed by HPLC-MS using a Paradigm MS-4 liquid chromatograph (Michrom BioResources, Inc., Auburn, CA) interfaced to a LCQ Advantage ion-trap mass spectrometer (Thermo Electron, Waltham, MA). Proteomics analysis was performed at the Kankyo-Kenkyu Center (Tsukuba, Japan). Peptide sequences were profiled against the rice genome and proteins identified using the Mascot search engine (www.matrixscience.com).

BLAST searches were performed using the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST), the TIGR plant gene

indices (http://tigrblast.tigr.org/tgi), the rice annotation database (http://rapdb.lab.nig.ac.jp), the TIGR rice genome annotation (http://rice.tigr.org), and the *Physcomitrella patens* DNA database (http://moss.nibb.ac.jp). Amino acid sequence alignments were generated using ClustalW (http://www.ebi.ac.uk/clustalw/index.html). Gene expression profiles were obtained using the rice massively parallel signature sequencing database (http://mpss.udel.edu/rice).

Generation of recombinant UDP-arabinopyranose mutase

Full-length complementary DNA clones of rice *Uam1* (Clone AK098933), *Uam2* (Clone AK071012), and *Uam3* (Clone AK061294) were obtained from the Rice Genome Resource Center (Tsukuba, Ibaraki, Japan). Truncated version of *Uam1*, *Uam 2*, and *Uam 3* were generated by polymerase chain reaction (PCR) using the forward primers 5′-ACGCCCGGGAATGGCGGGGACGGTGACGG-3′, 5′-ACGCCCGGGAATGTCTTTGGAAATTCAGGACAGTGAG-3′, and 5′-ACGCCCGGGAATGGCTTCCTCCGACGCCG-3′ for UAM1, UAM2, and UAM3, respectively, and the reverse primers 5′-**CGGAATTC**CTACTTGGCCTTGCCGTTCTCG-3′, 5′-**CGGAATTC**CTAAGCGCTTTGAGCTCCGTGAGATT-3′, and 5′-**CGGAATTC**TCACTTGCTCTTGGCAGTGCCG-3′ for UAM1, UAM2, and UAM3, respectively. These primers contain restriction sites for *Sma*I (underlined) and *Eco*RI (bold).

Constructs were generated to express the recombinant enzymes fused to the C-terminus of GST. PCR fragments of *Uam1*, *Uam2*, and *Uam3* were treated with *Sma*I and *Eco*RI and inserted into the pGEX-2TK vector (GE Healthcare Life Sciences, Piscataway, NJ) between the *Sma*I and *Eco*RI sites. The DNA sequences of each insert were determined and were consistent with the sequences of the cDNA clones from which the inserts were derived.

Expression and purification of recombinant UDP-arabinopyranose mutase

The pGEX-2TK vector constructs were introduced into *E. coli* strain BL21 (DE3) (Studier and Moffort 1986). The *E. coli* cells were then grown at 37 °C to an OD_{600nm} of 0.4. Isopropylthio β-D-galactopyranoside (0.1 mM) was added to induce the production of recombinant proteins and the cells grown for an additional 3 h at 25 °C. The *E. coli* cells were harvested, suspended in cold 20 mM potassium phosphate, pH 7.2, containing complete proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, German), and sonicated (Sonifier cell distruptor 200, Branson, Danbury, CT) 10 times for 5 min to rupture the cells. The released GST-fused UAM proteins were purified using Glutathione Sepharose 4 Fast Flow beads (GE Healthcare Life Sciences, Piscataway, NJ) according to the manufacturer's instructions. SDS–PAGE showed that the molecular weights of rUAM1 and rUAM3 were ∼69 kDa and that of rUAM2 was ∼67 kDa with GST.

Autoglycosylating activity of the rice and the recombinant UDP-arabinopyranose mutases

Autoglycosylating activity was measured as described (Delgado et al. 1998) with some modifications. Separate solutions of purified rice UAM and the rUAMs (1 μg each) in 50 mM Hepes-KOH, pH 6.8 (20 mL) containing 5 mM $MnCl_2$ and 0.15 μCi of UDP-[^{14}C]-Glc [specific activity 327μCi/mmol (GE Healthcare Life Sciences, Piscataway, NJ)] were kept for 15 min at 30 °C. An equal volume of SDS–PAGE buffer (120 mM Tris–HCl, pH 6.8, 200 mM 1,4-Dithiothreitol, 4% SDS, 0.02% bromophenol blue, and 20% glycerol) was added and the mixture then heated for 2 min at 100 °C to stop the reaction. After separation of the mixtures by SDS–PAGE, the gels were dried, and exposed to Phosphorimager screens (Fujifilm, Tokyo, Japan). Chase experiments were performed by reacting the purified rice mutase and the rUAMs first for 15 min with 0.15 μCi of UDP-[^{14}C]-Glc and then for an additional 15 min with 1 mM UDP-Glc, UDP-Xyl, UDP-Gal, UDP-Ara*p*, or UDP-Ara*f*.

Western blot analysis

The purified rice mutase was analyzed by SDS–PAGE followed by immunoblotting using RGP1 and RGP2 antibodies (Langeveld et al. 2002).

Supplementary data

Supplementary data are available at *Glycobiology* online (http://glycob.oxfordjournals.org).

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Conflict of interest statement

None declared.

Abbreviations

Ara*f*, arabinofuranose; Ara*p*, arabinopyranose; FAD, flavin-adenine-dinucleotide; Gal*f*, galactofuranose; Gal*p*, galactopyranose; GST, glutathion S-transferase; HPAEC, high performance anion-exchange chromatography; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; PCR, polymerase chain reaction; RGP, reversibly glycosylated polypeptide; rUAMs, recombinant UAMs; SEC, size-exclusion chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UAM, UDP-arabinopyranose mutase; UDP-Ara*f*, UDP-arabinofuranose; UDP-Ara*p*, UDP-arabinopyranose; UDP-Gal*f*, UDP-Galactofuranose; UDP-Gal*p*, UDP-Galactopyranose; UDP-Glc, UDP-glucose, UDP-Xyl, UDP-xylose; UDP, uridine diphosphate.

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