Running head: Hydroxyproline O-galactosyltransferase in Arabidopsis thaliana

Corresponding author:

Yoshifumi Jigami

Research Center for Medical Glycoscience,
National Institute of Advanced Industrial Science and Technology (AIST),
AIST Central 6, 1-1-1 Higashi,
Tsukuba, Ibaraki 305-8566, Japan
Tel: +81-29-861-6160
Fax: +81-29-861-6161
E-mail: jigami.yoshi@aist.go.jp

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Characterization of ER localized UDP-D-galactose: hydroxyproline O-galactosyltransferase using synthetic peptide substrates in Arabidopsis thaliana

Takuji Oka, Fumie Saito, Yoh-ichi Shimma, Takehiko Yoko-o, Yoshiyuki Nomura, Ken Matsuoka and Yoshifumi Jigami

Institutions:

Research Institute for Cell Engineering, National Institute of Advanced Industrial Science and Technology (AIST), AIST Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan (T. O., F. S., S. Y., T. Y., Y. J.); Department of Applied Microbial Technology, Faculty of Biotechnology and Life Science, Sojo University, 4-22-1 Ikeda, Kumamoto, Japan (Y. N.); and Laboratory of Plant Nutrition, Faculty of Agriculture, Kyushu University, Higashi-ku, Fukuoka, Japan (K. M.)
Footnotes

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2 Present address: Department of Applied Microbial Technology, Faculty of Biotechnology and Life Science, Sojo University, 4-22-1 Ikeda, Kumamoto, Japan

3 Present address: Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST), AIST Tsukuba Central 6, Higashi 1-1-1, Tsukuba, Ibaraki 305-8566, Japan

*Corresponding author: Yoshifumi Jigami; jigami.yoshi@aist.go.jp
Abstract

We characterized peptidyl hydroxyproline O-galactosyltransferase (HGT), which is the initial enzyme in the arabinogalactan biosynthetic pathway. An in vitro assay of HGT activity was established using chemically synthesized fluorescent peptides as acceptor substrates and extracts from Arabidopsis thaliana T87 cells as a source of crude enzyme. The galactose residue transferred to the peptide could be detected by HPLC and MALDI-TOF-MS analyses. HGT required a divalent cation of manganese for maximal activity and consumed UDP-D-galactose as a sugar donor. HGT exhibited an optimal pH range of pH 7.0 to 8.0 and an optimal temperature of 35°C. The favorable substrates for the activity seemed peptides containing two alternating imino acid residues including at least one acceptor hydroxyproline residue, although a peptide with single hydroxyproline residue without any other imino acids also functioned as a substrate. The results of sucrose density-gradient centrifugation revealed that the cellular localization of HGT activity is identical to those of ER markers such as Sec61 and Bip, indicating that HGT is predominantly localized to the ER. This is the first characterization of HGT and the data provide evidence that arabinogalactan biosynthesis occurs in the protein transport pathway.
Introduction

O-glycosylation is the addition of a sugar to hydroxy amino acids such as threonine, serine (Ser), hydroxyproline (Hyp), hydroxylsine, or tyrosine (Lehle et al., 2006). This type of protein modification occurs in many organisms to modify a large variety of proteins. Several types of sugars can be linked to proteins via O-glycosylation, including mannose, N-acetylglucosamine, glucose, xylose, N-acetylgalactosamine, fucose, galactose (Gal), and arabinofuranose (Araf). In addition, elongation of the added sugar residues yields a large variety of oligo- and polysaccharide extensions on the substrate proteins. These modifications are known to play important roles in various phenomena, including pathways required to maintain biological systems and basic cellular functions.

Structural analysis of oligo- and polysaccharides in plant cell walls has revealed the presence of three types of O-linked structures, Gal-O-Hyp, Araf-O-Hyp, and Gal-O-Ser (Kieliszewski and Shpak, 2001; Seifert and Roberts, 2007). A part of these three structures has been found on proteins in the super family that includes arabinogalactan protein (AGP) and extensin, which are localized to the cell surface. AGPs contain O-linked arabinogalactan oligo- or polysaccharides attached to Hyp residues (Gal-O-Hyp). It is known that arabinogalactan polysaccharides mainly consist of beta 1,3 linkages of galactose polymers (Seifert and Roberts, 2007). Extensin contains short arabino-oligosaccharide chains attached to Hyp residues (Araf-O-Hyp) and single Gal residues linked to Ser residues (Gal-O-Ser). It has been suggested that these O-linked structures play an important role in many stages of growth and development in plants, including signaling, embryogenesis, and programmed cell death (Seifert and Roberts, 2007; Knox, 2006). However, our understanding on the biosynthesis of these O-linked structures is limited at present.

Shpak et al. described a novel strategy to elucidate O-glycosylation of AGPs via introduction of synthetic genes encoding a protein substrate of glycosyltransferases into plant cells (Shpak et al., 1999; Estevez et al., 2006). This strategy provided good evidence for the substrate specificities of hydroxyproline O-galactosyltransferase (HGT). Hyp galactosylation occurs on clustered non-contiguous Hyp residues such
as Xaa-Hyp-Xaa-Hyp repeats of AGPs (where Xaa is any amino acid except Hyp) (Tan et al., 2003).

However, the arabinogalactosylation site is not limited to clustered non-contiguous Hyp residues, as isolated Hyp residues with appropriate surrounding sequences can be modified with arabinogalactan (Matsuoka et al., 1995; Shimizu et al., 2005). Therefore, the mechanism of glycosylation to Hyp residues seems complex in plants, while we have little information about the glycosyltransferase(s) involved in arabinogalactan biosynthesis. To examine the enzymatic properties and to identify genes involved in arabinogalactan biosynthesis, we first attempted to establish an *in vitro* assay for HGT activity, which catalyzes the initial step in arabinogalactan biosynthesis in plants.

Here, we report a novel assay for HGT activity based on the use of ER-enriched cell lysates extracted from *Arabidopsis thaliana* T87 cells as a source of the enzyme and chemically synthesized fluorescent peptides as enzyme substrates. The method enabled us to characterize the enzymatic properties of HGT and to determine the localization of HGT in *A. thaliana* cells. Properties of the enzyme and the usefulness of our assay for various studies are discussed.
Results

**In vitro assay for hydroxyproline O-galactosyltransferase activity.** To study the biosynthesis of arabinogalactan, we first attempted to establish an *in vitro* assay for the activity of hydroxyproline O-galactosyltransferase (HGT), the initial enzyme in arabinogalactan biosynthesis, using chemically synthesized peptides and extracts from *A. thaliana* T87 cells. AtAGP14 (Arabidopsis Genome Initiative locus: At5g56540) is one of the smallest arabinogalactan proteins and contains a signal sequence and GPI anchor attachment signal sequence at its N-terminus and C-terminus, respectively (Schultz et al., 2004). We designed a peptide based on the AtAGP14 sequence (VDAOAOSOTS; M. W. 1462.6) as a substrate. The peptide lacks the signal sequences for secretion and GPI anchor attachment that are present in the full-length AtAGP14 protein. The substrate peptides were chemically synthesized with two modifications at the N-terminus; namely, addition of fluorescein isothiocyanate (FITC) to facilitate detection of the peptide and addition of gamma-amino-butyric-acid (GABA) as a spacer (Table 1). Arabinogalactan synthetic activity was measured using 100 µM AtAGP14 peptide as an acceptor, 5 mM UDP-Gal as a donor, 1 mM MnCl₂ as a co-factor, and microsomal fractions as a source of crude enzyme. The reaction mixture was incubated at 30°C for 60 min, heated at 95°C for 3 min to stop the reaction, and then subjected to HPLC using a reverse phase column. The FITC labeled peptide was detected using a fluorescence detector. Several additional peaks from 14.5 min to 18.0 min were detected (Fig. 1A, right panel). Next, we collected the product and substrate peaks (14.5 min to 18.0 min), and analyzed their molecular weights by MALDI-TOF-MS. The MALDI-TOF-MS spectrum included a peak corresponding to the mass of the AtAGP14 peptide (1461.64) and five additional peaks were detected at mass values of 1623.65, 1785.88, 1947.57, 2110.25 and 2271.38. The mass differences for each peak were 162.23, 161.69, 162.68 and 161.13, which correspond to the mass of a deoxyhexosyl group (162.2), suggesting that up to five galactose residues were attached to the hydroxyproline and/or serine residue of the AtAGP14 peptide.

Although promising, the method still has two potential problems for measuring HGT activity. First, it is
theoretically possible that galactose residues were attached to a serine residue, as the AtAGP14 acceptor peptide contains a serine residue in addition to hydroxyproline residues. Second, it is possible that the results reflect the elongation reaction step in arabinogalactan biosynthesis rather than a first-addition step. Therefore, we attempted to improve the in vitro assay for HGT activity by addressing these issues.

To do this, we designed another peptide AtAGP14-A (VDAOAOAOAA, O: Hyp; M. W. 1401.3), in which two serine and one threonine residue were replaced by alanine residues in order to exclude the potential for transfer of galactose to serine. We then prepared three different subcellular fractions from T87 cells: P10 (ER-enriched), P100 (Golgi-enriched) and S100 (cytosolic) fractions. HGT activity was assayed using 100 µM AtAGP14-A peptide as an acceptor, 5 mM UDP-Gal as a donor, 1 mM MnCl₂ as a co-factor, and the P10, P100 or S100 fraction as a source of crude enzyme (Fig. 6A). An additional peak appeared at 15.5 min only when the P10 was used for the reaction, but not when the P100 or S100 was used for the reaction (data not shown), suggesting that the observed peak is an O-galactosylated product of AGP14-A (Fig. 2A) and that HGT activity is present in the P10 fraction of A. thaliana cells.

To confirm that the product is an O-galactosylated AGP14-A, we collected the corresponding product peak (from 15 to 16 min) and the AtAGP14-A substrate peak (from 17 min to 18 min), then analyzed their molecular weights by MALDI-TOF-MS (Fig. 2B). The MALDI-TOF-MS spectrum showed that the masses of the AtAGP14-A substrate and product were 1398.4 and 1560.6, respectively. Thus, the mass difference between the AtAGP14-A substrate and the product was 162.2 Da, which corresponds to the mass of a deoxyhexosyl group, suggesting that the product is indeed an O-glycosylated peptide. Under the reaction condition that were used, no peaks of mass higher than 1560.6 were detected, indicating that only one hexose residue was attached to the AtAGP14-A peptide when the P10 fraction was used as the source for the enzyme. To identify the hexose species, we performed monosaccharide analysis using a p-aminobenzoic acid ethylester (ABEE)-labeled sugar derivative after the acid hydrolysis of the reaction product, followed by the HPLC analysis (Fig. 2C). The hydrolysis product from product peak (from 15 to
16 min) showed the ABEE-galactose peak alone. On the contrary, the ABEE-galactose peak was not detected in the hydrolysis product from the AtAGP14-A substrate peak (from 17 min to 18 min) (Fig. 2C), indicating that hexose residue that is attached to the Hyp residue is actually galactose. This shows that the method is suitable to measure HGT activity, as it does not detect the elongation activity.

**Requirement of HGT enzymatic activity for co-factors.** Most glycosyltransferases utilize nucleotide sugars or dolichyl-phosphate sugars as donors. To determine which sugar donors can be utilized in the HGT reaction, we examined if O-galactosylation can occur via consumption of UDP-D-galactose as a donor. Various UDP-sugars (UDP-D-galactose, UDP-L-arabinofuranose, UDP-L-arabinopyranose, UDP-N-acetyl-D-glucosamine, UDP-D-glucuronic acid, UDP-D-glucose, UDP-L-rhamnose and UDP-D-xylose) were added to the reaction mixture and the amount of O-galactosylated product of AtAGP14-A substrate was measured. The highest HGT activity was detected when UDP-D-galactose was used as a donor, indicating that HGT activity requires UDP-D-galactose to transfer a galactose residue to the acceptor substrate, either directly or indirectly (Fig. 3A). When UDP-D-glucose was used as a donor, the HGT activity could also be detected weakly. It is known that UGEs convert UDP-D-glucose to UDP-D-galactose (Barber, 2006). Therefore it is quite likely that HGT uses the converted UDP-D-galactose when UDP-D-glucose was used as a donor *in vitro*. The effect of dolichyl-phosphate on HGT activity was also investigated. To do this, different amounts of dolichyl-phosphates (1, 10 and 100 µg) were added to the reaction mixture. However, none of the dolichyl-phosphate concentrations we tested increased HGT activity, suggesting that dolichyl-phosphate-D-galactose is not used as a sugar donor in the HGT reaction (Fig. 3B). The effect of the presence of several divalent cations on enzyme activity was also tested (Fig. 3C). Each cation was studied at a fixed concentration (1 mM). The HGT enzyme was inactive in the absence of divalent cations (*i.e.* in the presence of 1 mM EDTA), whereas HGT activity can be detected in the presence of Mn²⁺ and Co²⁺ cations, indicating that HGT requires Mn²⁺ for maximal activity (Fig. 3C). Taken together,
the results show that UDP-D-galactose and Mn\textsuperscript{2+} are required for the optimal HGT activity.

**Effects of temperature and pH on HGT activity.** We next determined the optimal pH and temperature ranges for HGT activity. To do this, we first monitored enzyme activity over a range of pH values. Three types of pH buffer, 100 mM MES-NaOH (pH 5.5, 6.0, and 7.0), 100 mM MOPS-NaOH (pH 6.5, 7.0, 7.5, and 8.0), and 100 mM Tris-HCl (pH 7.0, 8.0, and 9.0), were tested. The P10 fraction (6 µg) was used at 30°C for 60 min and the reactions were stopped by heat treatment (95°C) for 3 min. The highest enzyme activity was observed in 100 mM MES-NaOH buffer, pH 7.0. The pH conditions permissive for HGT activity exhibited a broad optimum from pH 7.0 to pH 8.0 (Fig. 4A). Enzyme activity was also measured at different temperatures. The P10 fraction (6 µg) was incubated at a series of temperatures (20, 25, 30, 35, 40, 45, 50 and 60°C) with AtAGP14-A peptide for 60 min and the reaction was stopped by heat treatment (95°C) for 3 min. The optimal temperature for activity was 35°C and the results suggest that the enzyme is relatively active in a broad temperature range, from 20 to 40°C (Fig. 4B).

**Substrate specificity of HGT.** To investigate the influence of amino acids neighboring the hydroxyproline residue in the substrate peptide on HGT activity, we tested various sequence versions of the synthesized peptide as potential substrates (Table 1). The reaction products were separated and collected by HPLC and their molecular weights were analyzed by MALDI-TOF-MS. AtAGP14-A (FITC-gaba-VDAOAOAOAA), AtAGP14-1 (FITC-gaba-VDAOAPAOA), AtAGP14-2 (FITC-gaba-VDAAAAOAOAA), AtAGP14-3 (FITC-gaba-VDAOAAAIOOA) and AtAGP14-4 (FITC-gaba-VDAAAAIAOAAA) could be acceptors, suggesting that HGT can transfer a galactose residue to hydroxyproline residues located in polypeptide sequences *in vivo* (Fig. 5). On the contrary, AtAGP14-5 (FITC-gaba-VDAAAOOOAAA) was not an acceptor of HGT activity in the assay, suggesting that the contiguous Hyp residues cannot act as an acceptor of HGT (Fig. 5). Among the active substrate peptides that we found above, peptides contain two or more
alternate imino acids, such as AtAGP14-A, AtAGP14-1 and AtAGP14-2 seemed better substrates than which did not contain such sequence (AtAGP14-3, AtAGP14-4) as MS peaks with galactose showed higher in the former three peptides than the other two. This observation suggested that higher order of substrate peptide affect the preference of HGT although we cannot rule out a possibility that the difference was a result of the difference of ionization efficiency of peptides.

Subcellular localization of HGT. To determine the protein localization of HGT in A. thaliana cells, we first prepared P10, P100 and S100 fractions from T87 cells and measured their HGT activities. HGT activity was detected only in the P10 fraction (Fig. 6A), consistent with the presence of HGT in the P10 cell fraction. We next analyzed marker proteins by immunoblotting with specific antibodies that recognize TLG2a or JIM84 (for the Golgi apparatus), Bip or Sec61 (ER membrane), or PIP1 (plasma membrane). Bip, Sec61 and PIP1 were mainly detected in the P10 fraction, suggesting that HGT is localized to the ER and/or plasma membrane. To address the localization of HGT more precisely, we performed a sucrose density gradient centrifugation experiment. Total subcellular fractions were extracted from T87 cells in buffers containing either Mg2+ or EDTA. The Mg2+ cation is required for ribosome binding to ER membrane and forming the rough ER. The extracts were separated by sucrose density gradient centrifugation in the presence or absence of Mg2+ (Fig. 6B and Fig. 6C). The presence of HGT in these fractions was determined by measuring HGT activity and the distribution of marker proteins was analyzed by immunoblotting. In the absence of Mg2+, HGT activity could be detected in fractions 8 to 13. This pattern of migration was similar to what was observed for Sec61 and Bip, markers for the ER membrane (Fig. 6B). In the presence of Mg2+, HGT-containing fractions were detected in fractions 5 to 12. Under the same conditions, the other subcellular markers that were tested, TLG2a, JIM84 and PIP1, did not co-migrate with HGT activity in either the presence or absence of Mg2+ (Fig. 6B and Fig. 6C). Taken together, the results indicate that like the ER markers Bip and Sec61, HGT is predominantly localized to the ER membrane.
Discussion

In this study, we have demonstrated the hydroxyproline O-galactosyltransferase (HGT) activity involved in the biosynthesis of arabinogalactan, using chemically synthesized peptides as acceptors and UDP-D-galactose as a sugar donor, together with manganese divalent cations as a co-factor. Although Lang previously measured β-galactosyltransferase activity of a green alga via detection of incorporation of \([^{14}C]\)-galactose from UDP-[\(^{14}C\)]-galactose in glycoproteins (Lang, 1982), the method has the significant
disadvantage that it detects not only HGT activity but also elongation of arabinogalactans. By contrast, the method described here distinguishes HGT activity from other galactosylation activities.

Protein O-mannosyltransferase 1 (Pmt1p) from yeast and protein O-fucosyltransferase (POFUT1) from human have been identified as ER localized protein O-glycosyltransferases (Willer et al., 2003; Luo and Haltiwanger, 2005). However, the membrane topology and enzymatic properties of Pmt1p and POFUT1 are different. Pmt1p is a seven-transmembrane protein and requires a divalent magnesium cation for activity (Strahl-Bolsinger and Scheinost, 1999). In contrast, POFUT1 is retained in the ER via the presence of a KDEL-like sequence at its C-terminus, and requires a divalent manganese cation for activity (Luo and Haltiwanger, 2005). A manganese divalent cation is also required for most type II membrane bound protein O-glycosyltransferases. Because the characteristics of HGT are more similar to those of POFUT1, it is plausible that HGT could be a type II membrane protein rather than an integral transmembrane protein. However this speculation requires further experimental evidences to prove it.

The Dictyostelium GnT51 gene encodes a UDP-N-acetylglucosamine: hydroxyproline polypeptide N-acetylglcosaminyltransferase (Van Der Wel et al., 2002). This enzyme is the only reported example of a glycosyltransferase that can transfer a sugar residue to the hydroxyproline of a polypeptide. BLAST analysis fails to identify any A. thaliana proteins with significant similarity to the protein encoded by GnT51, suggesting that the A. thaliana HGT protein is unrelated at the primary sequence level.

The results of subcellular separation revealed that HGT is mainly localized to the ER. Prolyl 4-hydroxylase (P4H) is reportedly a resident protein in the lumen of the ER in vertebrate and mammalian cells (Kivirikko et al., 1989; Walmsley et al., 1999; Ko and Kay, 2001), whereas P4H is detectable not only in the ER but also in the Golgi apparatus in Nicotiana tabacum (tobacco) BY-2 cells (Yuasa et al., 2005). Because the galactose transfer reaction occurs after proline hydroxylation by P4H, HGT must be located further downstream of the protein transport pathway than P4H. Because TLG2a and JIM84 antibodies are trans-Golgi and not cis-Golgi markers, it remains possible that the HGT is localized not only in the ER but
also in the cis-Golgi. Therefore, further analysis will be necessary to determine the precise sub-cellular
distribution of HGT.

When a microsomal fraction was used as an enzyme source, galactosylation occurred on up to 5 residues.
In contrast, when the P10 fraction was used as an enzyme source, only one galactose residue was
transferred to a synthetic peptide, suggesting that the elongation activity of arabinogalactans is localized to
an organelle other than the ER, probably the Golgi apparatus. A hypothetical model of arabinogalactan
synthetic pathway in plant cells is shown in Figure 7. The proteins are first modified by hydroxylation of
proline. Next, HGT transfers a galactose residue to a hydroxyproline using UDP-D-galactose as a donor.
This activity requires a divalent manganese cation. Finally, galactosylated AGPs are transported to the
Golgi apparatus, where the elongation reactions, occurs, and finally, the proteins localize to the cell surface.
The identification of genes involved in HGT reactions and the following arabinogalactan synthesis should
help improve our understanding of these important protein modification steps.

The results of substrate specificity analysis revealed that HGT can transfer a galactose residue to the
hydroxyproline residue of the unique polypeptide involving the minimal sequence of A-(O/P/A)-A-O-A
(Fig. 5). This is partly consistent with the previous indication on the consensus sequence of X-O-X-O-
repeats of AGPs for hydroxyproline O-galactosylation (Tan et al., 2003). However, it remains unclear if the
above sequence containing the A at the second amino acids instead of O or P (-A-A-A-O-A-) may have an
enough activity as compared with those containing (-A-O-A-O-A-) or (-A-P-A-O-A-), because the
observed MS intensity for the O-galactosylated product is weaker for AtAGP14-5 peptide than those for the
other substrate peptides, while the MS intensity does not correctly reflect the amount of the observed peak.
Therefore, further experiments are necessary to confirm if the alternating hydroxyproline (O) residues is
essential for the recognition by HGT.

The preferable substrates seemed to be the peptides containing at least two alternate imino acid
residues at least one of which is hydroxyproline residue. This nature fits to the previously described
hypothesis that the repeated non-contiguous hydroxyprolines, which takes polyproline II structure (van Holst and Fincher, 1984), are the site for the attachment of arabinogalactan (Kieliszewski, 2001). However, HGT could transfer galactose residue to not only peptides containing two alternate imino acids but also a peptides with only single hydroxyproline residue with no other imino acids (AtAGP14-4). This observation is consistent with the in vivo characterization of arabinogalactosylation motif (Shimizu et al., 2005) as the surrounding sequence of hydroxyproline residue in AtAGP14-4 clearly matches with the motif reported in the paper.

Materials and Methods

These results indicate that a galactose residue could be attached to a unique hydroxyproline residue of a recombinant protein, for example, human antibody, when expressed in plants. There is a possibility that the extraneous glycosylation become an antigen for human. Although several studies have been made on remodeling of N-glycosylation in various hosts for expression of recombinant glycosylated proteins, little is made on remodeling by O-glycosylation (Chiba Y and Jigami Y, 2007). In the future, it will be important to develop strategies for remodeling and repression of heterologous O-glycosylation in plants. We expect that
the in vitro assay method described here will be useful to screen for specific inhibitors of HGT activity.

Recently, several unique glycosyltransferases were characterized using a cell extract approach, and the genes encoding these activities were subsequently identified in plants (Akita et al., 2002; Konishi et al., 2006; Sterling et al., 2006; Qu et al., 2008). Our success in detecting HGT activity in the ER-enriched fraction of A. thaliana and the assay method developed in this study open the way to identification of the corresponding genes.

Plant Material. Suspension-cultured A. thaliana T87 cells were grown on Murashige and Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid and 3% sucrose and cultured for 2 weeks. The flasks were shaken at 120 rpm at 25°C.

Preparation of microsomal fractions. T87 cells were harvested on filter paper and lysed using a mortar and pestle under liquid nitrogen. The lysed cells were suspended in Buffer A, 100 mM MOPS-NaOH (pH 7.0), 1 mM MnCl₂ with the EDTA-free protease inhibitor (1 tablet of Complete per 50 ml; Roche Diagnostics GmbH, Mannheim, Germany). The suspension was centrifuged at 3,000 x g for 10 min at 4°C to remove cell debris. The resultant supernatants were centrifuged at 160,000 x g for 60 min at 4°C. The pellets were resuspended in Buffer A to generate a microsomal fraction. The fraction was dialyzed in 10 mM ammonium-acetate buffer (pH 7.0) overnight at 4°C. The protein concentration was determined according to the manufacturer’s protocol using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL).

Preparation of P10, P100 and S100 enzyme fractions. Cells lysed as described above were suspended in Buffer A. The suspension was centrifuged at 3,000 x g for 10 min at 4°C to remove cell debris. The resultant supernatants were centrifuged at 14,000 x g for 10 min at 4°C. The ER-enriched pellets were resuspended in Buffer A to generate a P10 fraction. The supernatant was further centrifuged at 160,000 x g.
for 60 min at 4°C to generate a Golgi-enriched pellet fraction (P100) and a cytosolic supernatant fraction (S100). Each fraction was dialyzed in 10 mM ammonium-acetate buffer (pH 7.0) overnight at 4°C. The protein concentration was determined according to the manufacturer’s protocol protocol using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL).

**Assay for hydroxyproline O-galactosyltransferase (HGT) activity.** Assay mixtures contained the following components in a total volume of 50 µl: 1 mM UDP-galactose, 100 µM substrate peptide acceptor, 100 mM MOPS-NaOH buffer, pH 7.0, containing (final concentrations) 0.2% (w/v) Triton X-100, 1 mM MnCl₂ and 30 µg of crude enzyme fractions (i.e. P10, P100, or S100 fractions). After the addition of the enzyme preparation, the mixtures were incubated at 30°C for 60 min. The reactions were stopped by heating at 95°C for 3 min. Products (10 µl) were separated by reverse phase chromatography and detected using a fluorescent detector (model RF-10A XL; Shimadzu, Kyoto, Japan). FITC-labeled peptides were purchased from Operon Co., Ltd. (Tokyo, Japan) or AnyGen (Gwang-ju, Korea), and used as an acceptor substrate. FITC-labeled glycopeptides were detected at a fluorescence intensity of 530 nm (excitation at 488 nm). The FITC-labeled peptides were synthesized using Fmoc/tBu technique (Coin et al., 2007).

**Sucrose gradient.** T87 cells were grown for 14 to 20 days at 25°C. All manipulations were done on ice or at 4°C. T87 cells (5 g fresh weight) were ground with a mortar and pestle under liquid nitrogen. Lysed cells were suspended at 0.5 ml/g in Buffer B containing 50 mM MOPS-NaOH, pH 7.0, 45% (w/v) sucrose, EDTA-free complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany), and either 5 mM EDTA or 5 mM MgCl₂. The suspension was centrifuged at 3,000 x g for 10 min to remove cell debris. The supernatant was loaded onto a 50% sucrose solution. A discontinuous gradient was formed by adding 0.5 ml of 55% sucrose, 0.5 ml of 50% sucrose, and 1.2 ml of 45% sucrose (including the cell sample), then adding 0.5 ml of 40%, 35%, 30%, 25% 20% sucrose solutions sequentially on the surface of
the supernatant. The gradient was centrifuged at 100,000 x g for 18 h and collected in 280 µl increment fractions. Specific membrane fractions were identified by immunoblotting using antibodies against organelle-specific markers.

**Immunoblotting and antibodies.** The following antibodies were used for immunoblotting. Anti-PIP1 (Ohshima et al., 2001) and anti-Sec61 (Yuasa et al., 2005) antibodies were described previously. Anti-Bip and JIM84 antibodies were purchased from Santa Cruz Biotechnology or CarboSource Services (Athens, GA USA), respectively. Anti-AtTLG2a (Bassham et al., 2000) was kindly provided by Dr. N.V. Raikhel. Each primary antibody was used at a dilution of 1: 1000. We then used anti-rabbit IgG conjugate HRP (Cell Signaling Technology, Beverly, MA) to detect anti-Sec61, anti-AtTLG2a and anti-PIP1; anti-rat IgM conjugate HRP to detect JIM84; or anti-goat IgG conjugate HRP (Santa Cruz Biotechnology) to detect anti-Bip. In each cases, secondary antibodies were used at a dilution of 1: 5000. An ECL Plus kit (Amersham Biosciences) was used to visualize the immunoreactive proteins. Chemical fluorescent signals on a PVDF membrane were recorded using a LAS-3000 imaging system (FUJIFILM corporation, Tokyo, Japan).

**HPLC analysis.** The products were analyzed by HPLC with a reverse phase column cosmosil 5C18-AR-II (250 x 4.6 mm; Nacalai Tesque, Kyoto, Japan). The column was equilibrated with 20% acetonitrile containing 0.1% trifluoroacetic acid (TFA). The FITC labeled glycopeptides were eluted using a linear gradient of 20 to 40% acetonitrile over a period of 30 min at a flow rate of 1 ml/min. The FITC labeled glycopeptides were detected by the fluorescence intensity at 530 nm (excitation, 488 nm).

**Mass spectrometry.** The enzymatic products were collected and lyophilized and suspended with deionized water. The matrix used was CHCA (a-Cyano-4-hydroxycinnamic Acid, Sigma) dissolved at 10
mg/ml in 0.1% TFA: acetonitrile (5:5, v/v). Equal volumes (1 µl each) of the sample and the matrix solution were mixed and dried on the target plate. The fractions were analyzed by Matrix Assisted Laser Desorption/Ionization time of flight mass spectrometry (MALDI-TOF-MS). Mass spectra were obtained on an Ettan MALDI-ToF MS (GE Healthcare UK Ltd., UK) in the positive-ion mode.

**Monosaccharide analysis.** Monosaccharides from the O-glycosylated peptides were analyzed by the method described previously (Chigira et al., 2008). The HPLC-purified peptide sample was incubated in 4M TFA at 100˚C for 4 h and dried. Next, the hydrolysates were labeled with fluorescent ABEE using an ABEE labeling kit (Seikagaku Corporation, Tokyo, Japan) according to the manufacturer’s protocol. The ABEE-labeled monosaccharides were analyzed by HPLC using a cosmostil 5C18-AR-II (250 x 4.6 mm; Nacalai Tesque, Kyoto, Japan) at a flow rate of 1 mL/min with 0.1% TFA buffer containing 10% acetonitrile at 45˚C. The ABEE-labeled monosaccharides were detected by the UV intensity at 305 nm.

**Source of sugar nucleotides.** UDP-D-glucose, UDP-D-galactose, UDP-N-acetyl-D-glucosamine and UDP-D-glucuronic acid were purchased from Sigma. UDP-L-arabinopyranose and UDP-D-xylose were from CarboSource Services (Athens, GA). UDP-L-rhamnose was synthesized using a cytoplasmic fraction from yeast cells expressing *RHM2/MUM4*, which encodes UDP-L-rhamnose synthase, and purified by HPLC using a Develosil RPAQUEOUS column (250 x 4.6 mm, Nomura Chemical Co, Ltd, Seto, Japan) (Oka et al., 2007). Chemically synthesized UDP-L-arabinofuranose was obtained from the Peptide Institute (Osaka, Japan).

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References


Bassham DC, Raikhel NV (2000) AtVPS45 complex formation at the trans-Golgi network. Mol Biol Cell


Walmsley AR, Batten MR, Lad U, Bulleid NJ (1999) Intracellular retention of procollagen within the endoplasmic reticulum is mediated by prolyl 4-hydroxylase. J Biol Chem 274: 14884-14892


### Table 1 Synthetic peptides used in this study

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<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Theoretical M. W.</th>
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<td>AtAGP14</td>
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</table>
Figure Legends

Figure 1. *In vitro* assay for arabinogalactan synthetic activity of plant cells. **A.** HPLC analysis of reaction products using AtAGP14 peptide as an acceptor. Reaction mixtures were incubated with microsomal fractions for 0 min (*left panel*) or 60 min (*right panel*). **B.** MALDI-TOF-MS analysis of reaction products using AtAGP14 peptide as an acceptor. Peptides collected from 17 to 18 min (AtAGP14; *left panel*), or from 14.5 to 17 min (products generated; *right panel*) as shown in Fig. 1A, as analyzed here by MALDI-TOF-MS. Asterisks indicate no O-hexosylated peaks.

Figure 2. *In vitro* assay for HGT activity of plant cells. **A.** HPLC analysis of reaction products using AtAGP14-A peptide as an acceptor. Reaction mixtures were incubated with the P10 fraction for 0 min...
**B. MALDI-TOF-MS analysis of reaction products using AtAGP14-A peptide as an acceptor.** Peptides collected from 15-16 min (products, +O-Gal), or from 17-18 min (AtAGP14-A) as shown in Fig. 2A, were analyzed here by MALDI-TOF-MS. **C. Monosaccharide analysis of reaction products using AtAGP14-A peptide as an acceptor.** Peptides collected from 15-16 min (products, +O-Gal), or from 17-18 min (AtAGP14-A) in Fig. 2B were purified and approximately 300 pmol each was hydrolyzed by 4M TFA. The acid hydrolyzed products were labeled with ABEE and were analyzed by HPLC using a C18 column. The ABEE-galactose and ABEE-glucose eluted at 33.9 min and 35.6 min under the conditions, respectively. Asterisks indicate a nonspecific peak that eluted at 35.3 min in both samples from 15-16 min (products, +O-Gal), or from 17-18 min (AtAGP14-A).

**Figure 3. Enzymatic properties of HGT. A. Sugar nucleotide requirement of HGT.** Reaction mixtures were incubated with or without various UDP-sugars. 100% corresponds to incorporation of 1.33 x 10^1 unit (pmol/min/µg) with UDP-D-galactose. Results are plotted as mean ± SD from three independent experiments. **B. Influence of dolichyl phosphate on HGT activity.** Reaction mixtures were incubated with or without dolichyl-phosphate (Dol-P). 100% corresponds to incorporation of 6.83 x 10^2 unit (pmol/min/µg) with UDP-D-galactose and dolichyl-phosphate (1 µg). Results are plotted as mean ± SD from three independent experiments. **C. Metal cation requirement of HGT.** Reaction mixtures were incubated with EDTA or various divalent metals. 100% corresponds to incorporation of 1.98 x 10^1 unit (pmol/min/µg) with manganese. Results are plotted as mean ± SD from three independent experiments.

**Figure 4. Optimal temperature and pH ranges for HGT activity. A. Effects of temperature on HGT enzymatic activity.** The buffer used was 100 mM MOPS-NaOH (pH 7.0). 100% corresponds to incorporation of 1.65 x 10^1 unit (pmol/min/µg) at 35°C. Results are plotted as mean ± SD from three
independent experiments. **B. Effects of pH on HGT enzymatic activity.** The buffers used were 100 mM MES-NaOH (circle), 100 mM MOPS-NaOH (triangle), or 100 mM Tris-HCl (square). 100% corresponds to incorporation of \(5.63 \times 10^{-2}\) unit (pmol/min/µg) at pH 8.0 of 100 mM MOPS-NaOH. Results are plotted as mean ± SD from three independent experiments.

**Figure 5. Effects of amino acids neighboring hydroxyproline acceptor residues on HGT activity.** Effects of amino acids near hydroxyproline residues on HGT activity were tested in the standard assay (see Materials and Methods) at a concentration of 100 µM peptide. Peptide products of the reaction were separated by HPLC and their molecular weights were analyzed by MALDI-TOF-MS.

**Figure 6. Subcellular localization of *A. thaliana* HGT activity. A. Subcellular fractionation of HGT.** Total protein extract (excluding cell debris) was prepared from T87 cells as described in the Materials and Methods. **Sucrose density gradient centrifugation in the presence (B) or absence (C) of Mg\(^{2+}\).** After centrifugation, each resulting gradient was separated into 16 fractions. Anti-AtTLG2a and JIM84 antibodies were used to detect markers for the Golgi apparatus, anti-Sec61 and Bip were used to detect markers for the ER, and anti-PIP1 was used to detect a marker for the plasma membrane.

**Figure 7. Schematic of the HGT reaction.** AGPs, arabinogalactan proteins. PRO, proline residue of AGPs. HYP, hydroxyproline residue of AGPs. HGT, hydroxyproline O-galactosyltransferase. P4H, proline 4-hydroxylase. Mn\(^{2+}\), divalent cation of manganese. Arrows indicate the protein transport pathway in *A. thaliana* cells.
Figure 1. In vitro assay for arabinogalactan synthetic activity of plant cells.

A. HPLC analysis of reaction products using AtAGP14 peptide as an acceptor.

Reaction mixtures were incubated with microsomal fractions for 0 min (left panel) or 60 min (right panel).

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Peptides collected from 17 to 18 min (AtAGP14; left panel), or from 14.5 to 17 min (products generated; right panel) as shown in Fig. 1A, as analyzed here by MALDI-TOF-MS.

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A. **HPLC analysis of reaction products using AtAGP14-A peptide as an acceptor.**

A reaction mixture incubated with P10 fraction for 0 min (upper panel) or 60 min (bottom panel).

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Peptides collected from 15-16 min (products, +O-Gal), or from 17-18 min (AtAGP14-A) as shown in Fig. 2A, were analyzed here by MALDI-TOF-MS.

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Figure 3. Enzymatic properties of HGT.

A. Sugar nucleotide requirement of HGT.
Reaction mixtures were incubated with or without various UDP-sugars. 100% corresponds to incorporation of 1.33 x 10⁻¹ unit (pmol/min/µg) with UDP-D-galactose. Results are plotted as mean ± SD from three independent experiments.

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After centrifugation, each resulting gradient was separated into 16 fractions.
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