Cytosolic Free N-Glycans Are Retro-Transported Into the Endoplasmic Reticulum in Plant Cells

Makoto Katsube, Natsuki Ebara, Megumi Maeda and Yoshinobu Kimura*

Department of Biofunctional Chemistry, Graduate School of Environmental and Life Science, Okayama University, Okayama, Japan

During endoplasmic reticulum (ER)-associated degradation, free N-glycans (FNGs) are produced from misfolded nascent glycoproteins via the combination of the cytosolic peptide N-glycanase (cPNGase) and endo-β-N-acetylglucosaminidase (ENGase) in the plant cytosol. The resulting high-mannose type (HMT)-FNGs, which carry one GlcNAc residue at the reducing end (GN1-FNGs), are ubiquitously found in developing plant cells. In a previous study, we found that HMT-FNGs assisted in protein folding and inhibited β-amyloid fibril formation, suggesting a possible biofunction of FNGs involved in the protein folding system. However, whether these HMT-FNGs occur in the ER, an organelle involved in protein folding, remained unclear. On the contrary, we also reported the presence of plant complex type (PCT)-GN1-FNGs, which carry the Lewis^a epitope at the non-reducing end, indicating that these FNGs had been fully processed in the Golgi apparatus. Since plant ENGase was active toward HMT-N-glycans but not PCT-N-glycans that carry β1-2xylosyl and/or α1-3 fucosyl residue(s), these PCT-GN1-FNGs did not appear to be produced from fully processed glycoproteins that harbored PCT-N-glycans via ENGase activity. Interestingly, PCT-GN1-FNGs were found in the extracellular space, suggesting that HMT-GN1-FNGs formed in the cytosol might be transported back to the ER and processed in the Golgi apparatus through the protein secretion pathway. As the first step in elucidating the production mechanism of PCT-GN1-FNGs, we analyzed the structures of free oligosaccharides in plant microsomes and proved that HMT-FNGs (Man'^9-7GlcNAc and Man'^9-8GlcNAc^2) could be found in microsomes, which almost consist of the ER compartments.

Keywords: free N-glycans, ER-associated degradation, peptide:N-glycanase, endo-β-N-acetylglucosaminidase, plant glycoproteins

INTRODUCTION

Free N-glycans (FNGs), which are related to asparagine-linked glycoproteins, are widely found in various eukaryotes, including yeast, plants, and animals. These FNGs can be classified into two types, GN1 and GN2, based on the reducing terminal structure; GN1-FNGs have one GlcNAc residue, whereas GN2-FNGs have GlcNAcβ1-4GlcNAc (the N,N-diacyetyl chitobiosyl unit). Regarding the protein quality control system in the endoplasmic reticulum (ER) in both animal
and plant cells, it has been believed that misfolded N-glycoproteins that harbor high-mannose type (HMT)-N-glycans are transported through the dislocone complex (0s9-Sel1-HRAD1 complex) into the cytosol for proteasomal degradation or ER-associated degradation (ERAD; Suzuki and Funakoshi, 2006; Hosokawa et al., 2009, 2010; Abei et al., 2010; Hütter and Strasser, 2012; Suzuki and Harada, 2014; Suzuki, 2015). Before proteolytic degradation, these misfolded N-glycoproteins are first de-N-glycosylated by cytosolic PNGase (cPNGase), and the resulting GN2-FNGs are then further processed into GN1-FNGs by endo-β-N-acetylglucosaminidase (ENGase), which is highly specific for HMT-GN2-FNGs. It has been believed that the resulting HMT-GN1-FNGs are further degraded by cytosolic α-mannosidase and finally transported to the lysosome, where the FNGs are degraded to monosaccharides, in animal cells (Suzuki et al., 2006; Kato et al., 2011; Wang and Suzuki, 2013). The fate of HMT-GN1-FNGs formed in plant cytosol, in sharp contrast to those formed in mammalian cytosol, remains to be clarified, since cytosolic α-Manase has not been found and no orthologous gene of the animal cytosolic α-Manase has been identified to date. This indicates that the HMT-FNGs produced during ERAD may be metabolized via a slightly different pathway in animal cells.

As for complex type FNGs (CT-FNGs), both GN1-FNGs and GN2-FNGs have been found in both mammalian and plant cells or in their extracellular spaces (Priem et al., 1993; Faugerou et al., 1997a,b; Kimura et al., 1997, 2000; Ohashi et al., 1999; Ishizuka et al., 2008; Nakamura et al., 2008; Maeda et al., 2010, 2017; Iswatsukasa et al., 2013; Wang et al., 2015; Seino et al., 2016), and the structural features of these FNGs clearly suggest that these N-glycans had been modified or processed in the Golgi apparatus. It has been proposed that animal complex type (ACT)-GN2-FNGs are formed from HMT-GN2-FNGs, which are produced from dolichol-linked oligosaccharides as byproducts of the reaction involving the transfer of the glycan moiety (GlcNAcMan9GlcNAc2) to specific Asn residues in nascent polypeptides in the ER by oligosaccharyltransferase (OST) in mammalian cells (Harada et al., 2015). They have postulated that these HMT-GN2-FNGs, along with well-folded glycoproteins, are possibly transported and modified in the Golgi apparatus. Interestingly, extracellular ACT-FNGs are almost exclusively of the GN2 type, and this observation suggests that the mechanism of GN1-FNG generation may be slightly different from that of GN2-FNG generation.

In previous reports (Maeda et al., 2010, 2017), the plant complex type (PCT)-GN1-FNGs, which carry the Le' epitope [Galβ1-3(Fucα1-4)GlcNAc], were found in the culture broth of rice cells or the crude extract of a freshwater plant, Egeria densa. Since it has already been confirmed that plant ENGase is almost inactive toward typical plant-specific N-glycans, such as M3FX, GN2M3FX, and GN2M3X (Kimura et al., 1998, 2002; Maeda et al., 2017), these PCT-GN1-FNGs did not appear to be generated from N-glycopeptides or N-glycoproteins that harbored PCT-N-glycans by ENGase activity during turnover of the function-lost glycoproteins. Therefore, we propose that these PCT-GN1-FNGs might have originated from HMT-GN1-FNGs produced from misfolded glycoproteins via the combination of cPNGase and ENGase in the cytosol during ERAD (Maeda et al., 2010; Maeda and Kimura, 2014). We proposed the following hypothesis regarding the formation of PCT-GN1-FNGs from HMT-GN1-FNGs: during the first stage, HMT-GN1-FNGs produced from misfolded glycoproteins might be transported back to the ER through an unidentified transporter; then, these HMT-FNGs, along with well-formed glycoproteins, might be transported to the Golgi apparatus. During the second stage, these HMT-GN1-FNGs might be processed into PCT-GN1-FNGs via concerted reactions mediated by Golgi-glycosidases and transferases, along with the N-glycans of secreted-type glycoproteins. Finally, the resulting PCT-GN1-FNGs might be secreted into the extracellular space. The fact that these PCT-GN1-FNGs have been found in the culture broth, but not in rice cells (Maeda et al., 2010), appears to support this hypothesis. Furthermore, we found that HMT-FNGs assisted in protein folding and inhibited β-amyloid fibril formation, suggesting a possible biofunction of FNGs in the protein folding system (Tanaka et al., 2015). However, whether these HMT-FNGs occurred in the ER, an organelle involved in the folding of secreted-type glycoproteins, remained unclear.

As the first step to prove that HMT-FNGs (GN1 and/or GN2) occur in the ER, we prepared plant microsomes from pumpkin hypocotyls (Kimura et al., 2002) and analyzed the structural features of free oligosaccharides in the microsomes in this study. We found that HMT-FNGs (GN1 and GN2) occurred in the microsomes that were mainly contained in the ER compartments. These results suggested that GN1-FNGs generated from misfolded glycoproteins via the combination of cPNGase and ENGase were retro-transported from the cytosol to the ER by a putative transporter specific for FNGs.

**MATERIALS AND METHODS**

**Materials**

A Cosmols 5C18-AR column (0.60 × 25 cm) was purchased from NacalaiTesque, Inc. (Kyoto, Japan), and a Shodex Asahipak NH2P-50 column (0.46 × 25 cm) was purchased from Showa Denko Co. (Tokyo, Japan). Manα1,3GlcNAc2-PA and Manα2,3GlcNAc2-PA were prepared as described in previous reports (Kimura et al., 2000; Kimura and Matsuo, 2000). α-1,2-Manase from Aspergillus saitoi was purchased from Prozyme, Inc. (Hayward, CA, United States). Swainsonine, deoxyxymannojirimycin, deoxynojirimycin, and Jack bean β-GlcNAc'ase were purchased from Sigma-Aldrich (St. Louis, MO, United States), and Endo-H was purchased from Promega (Madison, WI, United States). Glc3Man9GlcNAc2-PA and Glc2Man9GlcNAc2-PA were purchased from Masuda Chemical Industries Co. (Kagawa, Japan).

**Reverse-Phase-High Performance Liquid Chromatography and Size Fractionation-HPLC**

Fluorescence-labeled oligosaccharides were separated by high performance liquid chromatography (HPLC) using a Jasco performance liquid chromatography (HPLC) using a Jasco...
2080-PU HPLC system equipped with a Jasco 920-FP Intelligent Spectrofluorometer (excitation 310 nm and emission 380 nm; Jasco, Tokyo, Japan). For reverse-phase (RP)-HPLC using a Cosmosil 5C18-AR-II (4.6 × 250 mm), the PA-sugar chains were eluted by increasing the acetonitrile concentration in 0.02% trifluoroacetic acid (TFA) linearly from 0 to 7% at a flow rate of 1.2 ml/min. For size fractionation (SF)-HPLC using a Shodex Ashahipak NH2P-50 4E (4.6 × 250 mm), the PA-sugar chains were eluted by increasing the water content of the water-acetonitrile mixture from 26 to 50% linearly at a flow rate of 0.7 ml/min.

ENGase Assay
ENGase activity was assayed using M6B as a substrate and M3FX [Manα1-6(Manα1-3)(Xyl)/β1-2Manβ1-4GlcNAcβ1-4(Fucor1-3) GlcNAc-PA] as an internal standard, as described in our previous paper (Kimura et al., 2011). Briefly, an enzyme solution (100 μl) was mixed with M6B and M3FX (approximately 100 pmol) in 0.1 M MES buffer (44 μl, pH 6.5) containing 5 mM swainsonine (3 μl) and 5 mM deoxymannojirimycin (3 μl). After incubation at 37°C overnight, the reaction was stopped by heating at 100°C for 3 min. After centrifugation, an aliquot (50 μl) of the resulting supernatant was analyzed by RP-HPLC using the Cosmosil 5C18-AR-II column. The PA-sugar chains (M6B, M3FX, and PA-GlcNAc) were eluted as described above. The substrate specificity of pumpkin ENGase has been reported using various pyridylaminated N-glycans in our previous report (Kimura et al., 2002).

ER α-Glucosidase I and II Assay
Endoplasmic reticulum α-glucosidase I and II activities were assayed using GlcαManα1-3GlcNAcβ1-4PA (G3M9') and GlcαManα1-3GlcNAcβ1-4PA (G2M9') as substrates, respectively. G3M9' and G2M9' were prepared from GlcαManα1-3GlcNAcβ1-4PA and GlcαManα1-3GlcNAcβ1-4PA, respectively, by Endo-H digestion followed by pyridylamination, as described below. An enzyme solution (100 μl) was mixed with G3M9' and G2M9' (approximately 50 pmol) in 0.1 M MES buffer (44 μl, pH 6.5) containing 5 mM swainsonine (3 μl), 5 mM deoxymannojirimycin (3 μl), and 5 mM deoxynojirimycin (3 μl). After incubation at 37°C overnight, the reaction was stopped by heating at 100°C for 3 min. The digested substrates were analyzed by SF-HPLC using the Shodex NH2P-50 4E column.

α-1,2-Mannosidase Digestion
PA-sugar chains obtained from the pumpkin microsomal fraction were incubated with Aspergillus α-1,2-Mannase (100 μU) in 0.1 M Na-acetate buffer (pH 5.0) at 37°C overnight. The reactions were stopped by boiling the mixtures for 3 min, and a part of each digested substrate was analyzed by SF-HPLC using the Shodex Ashahipak NH2P-50 4E column.

Preparation of Microsomes From Pumpkin Hypocotyls
Pumpkin (Cucurbita sp. cv. Kurokawa Amakuri) seeds (17.7 g) were soaked overnight, planted in moist rock fiber, and allowed to germinate at 25°C in the dark. The seedlings were grown for 6 days in the dark, and the etiolated hypocotyls were used for subcellular fractionation. Hypocotyls (41.5 g) were chopped using a razor and 0.15 M tricine buffer (50 ml, pH 7.5) containing 13% sucrose and 2 mM MgCl₂ (buffer A). The chopped materials were squeezed through a nylon mesh. The resulting filtrate (10 ml each from 60 ml) was layered on the same tricine buffer, which consisted of two layers, a 20% sucrose layer (15 ml) and a 60% sucrose layer (10 ml), and centrifuged at 100,000 × g for 3 h (HITACHI 55P-72, Tokyo, Japan). After centrifugation, four fractions, from top to bottom (F-1, 10 ml; F-2, 10 ml; F-3, 10 ml; and F-IV, 5 ml), were collected, as shown in Supplementary Figure 1-I. Significant ENGase activity was detected in F-1 and F-2, indicating that these fractions contained cytosolic components (Supplementary Figure 1-II). On the contrary, α-Glc'ase I activities were detected in F-3 (Supplementary Figure 1-III); therefore, we used F-3 for further preparation of microsomes, which contained mainly ER. F-3 was diluted with Buffer A (35 ml) and centrifuged at 100,000 × g for 3 h. After centrifugation, the resulting precipitates were resuspended and washed in the 13% sucrose-containing buffer (25 ml), and the suspended sample was centrifuged again at 100,000 × g for 3 h. A part of the resulting precipitates were solubilized with 25 mM HEPES-NaOH buffer (1 ml, pH 7.5) containing 0.1% Triton X-100 by ultrasonication. As shown in Figure 1, the solubilized F-3 precipitates contained α-Glc'ase I and II but not ENGase, suggesting that F-3 contained the ER compartment.

Preparation and Pyridylation of FNGs
The microsome fraction was heated in boiling water for 3 min and then solubilized with 25 mM Tris-HCl buffer (pH 7.5) containing 0.2% SDS by ultrasonication for 15 min. The solubilized samples were desalted using Dowex 1 × 2 resins, and the run-through fraction was pooled and concentrated to approximately 10 ml. The run-thought fraction was applied onto a Sephacryl G-25 superfine column (2.7 × 33 cm) equilibrated with 0.1 N ammonium water. The oligosaccharide fractions (elution volumes from 61 to 115 ml) were pooled and concentrated to dryness using a rotary evaporator. The residue was suspended in distilled water (approximately 1 ml) and lyophilized. The lyophilized oligosaccharides were pyridylaminated (Natsuka and Hase, 1998). An excess amount of 2-aminopyridine was removed by gel filtration using a Sephadex G-25 Fine (1.8 × 40 cm) in 0.1 M NH₄OH. The pyridylaminated oligosaccharides were monitored using a JASCO FP-8200 Fluorescence Spectrometer.

Electrospray Ionization Mass Spectrometry
LC/MS and MS/MS analyses of PA-oligosaccharides were performed using an Agilent 6,500 series HPLC-Chip/QTOF-MS system equipped with a microwell-plate auto sampler (maintained at 10°C), capillary sample loading pump, nanopump, HPLC-Chip interface, and an Agilent 6,520 Q-TOF LC/MS, as described in our previous report (Maeda et al., 2017).
A porous graphitized carbon (PGC)-Chip (Agilent Technologies) was used for separation of the PA-sugar chains.

RESULTS AND DISCUSSION

Structural Analysis of FNGs in the Pumpkin Microsomes

Since we confirmed that the microsomal fraction obtained from the pumpkin hypocotyls showed α-Glc’ase I and II activities, but not ENGase activity, as shown in Figure 1, we prepared pyridylaminated oligosaccharides from the 0.1% SDS-extract of the microsomes (mainly ER). First, the pyridylaminated oligosaccharides obtained from the microsomes were partially purified by RP-HPLC, as shown in Figure 2-I. As described in our previous reports (Kimura and Matsuo, 2000; Maeda et al., 2010, 2017), GN1-FNGs were eluted in the run-through or slightly retained fraction (F1, in this RP-HPLC system before 20 min), and GN2-FNGs were eluted in the bound fraction (F2, after 20 min). The PA-oligosaccharides in these two fractions were further analyzed by SF-HPLC. As shown in Figure 2-II, the elution positions of peaks a, b, and c obtained from F1 coincided with those of authentic M7', M8', and M9', respectively, whereas the elution positions of peaks d and e from F2 coincided with those of authentic M8 and M9, respectively. Peaks c and e could be analyzed by electrospray ionization mass spectrometry (ESI-MS), as shown in Supplementary Figures 2, 3. For peak c, a parent ion was observed at m/z 879.8 as a double-charged ion, indicating that this signal was obtained from M9' (Man9GlcNAc2-PA). All signals obtained from peak c by MS/MS analysis could be assigned to fragment ions from Man9GlcNAc2-PA. For peak e, a parent ion was observed at m/z 981.38 as a double-charged ion, indicating that this signal was obtained from M9 (Man9GlcNAc2-PA). All signals obtained from peak e by MS/MS analysis could be assigned to fragment ions from Man9GlcNAc2-PA. The structures of GN1- and GN2-FNGs in the pumpkin microsomes were further analyzed by exoglycosidase digestion. As shown in Figure 3-I, when the PA-oligosaccharides obtained from F1 and F2, as shown in Figure 2-II, were treated with Endo-H, peaks a, b, and c were not digested, but peaks d and e were digested as shown in Figure 3-II, indicating that peaks d and e represented HMT-GN2-FNGs. However, peaks a, b, and c were converted to M5' (Man5GlcNAc2-PA) upon α-1,2-Man'ase digestion, indicating that peaks a, b, and c represent Man9GlcNAc2-PA, Man9GlcNAc2-PA, and Man9GlcNAc2-PA, respectively. The positions of peaks d and e were converted to M5 (Man5GlcNAc2-PA) upon α-1,2-Man'ase digestion, indicating that peaks d and e represented Ma8GlcNAc2-PA and Ma9GlcNAc2-PA, respectively. These results indicated that in addition to HMT-GN2-FNGs, HMT-GN1-FNGs (cytosolic ENGase products) occurred in the ER compartments. The yield of these pyridylaminated GN1-FNGs

![Figure 1](https://example.com/figure1.png)

**FIGURE 1** | High performance liquid chromatography (HPLC) analyses of α-glucosidase I (I) and II (II) and endo-β-N-acetylglucosaminidase (ENGase; III) activities of the microsomes obtained from pumpkin hypocotyls α-Glc’ase I and II activities were assayed using Glc3Man9GlcNAc2-PA (G3M9') and Glc2Man9GlcNAc1-PA (G2M9'), respectively, as substrates. The reaction mixtures were analyzed by size fractionation (SF)-HPLC, using a Shodex Asahipak NH2P-50 4E column. The ENGase activity was assayed using Man9GlcNAc2-PA (M6B) as a substrate and Man9Xyl1Fuc1GlcNAc2-PA (M3FX) as an internal standard. The reaction mixture was analyzed by reversed-phase (RP)-HPLC, using a Cosmosil 5C18-AR-II column.
(M7', M8', and M9') was approximately 40 pmol/g hypocotyls, whereas that of GN2-FNGs (M8 and M9) was approximately 30 pmol/g hypocotyls. The total amount of the HMT-GN1/GN2-FNGs obtained by the Con-A affinity chromatography from the soluble fraction (F-1 and F-2) was about 2.7 nmol/g hypocotyl, indicating that the amount of HMT-FNGs obtained from the microsomes was 1/40 of those in the soluble fraction. However, it seems that the amount maybe not reflect the real amount of FNGs occurring in the ER, since it is unlikely that total ERs have been collected during the fractionation process (some part of ERs maybe have been broken). At this moment, therefore, the real amount of FNGs occurring in the ER is obscure.

Since the microsomes prepared from the pumpkin hypocotyls showed α-Glc'ase I (a soluble enzyme) and α-Glc'ase II (a membrane-bound enzyme) activities, the microsomes probably contained intact ER compartments. The presence of HMT-GN1-FNGs (Manα6GlcNAc1) in the ER compartments suggested that these HMT-GN1-FNGs produced in the cytosol were retro-transported to the ER from the cytosol through unidentified transporter(s) specific for these free oligosaccharides. If this was true, these HMT-GN1-FNGs, together with well-folded N-glycoproteins, might have been transported to the Golgi apparatus and processed into PCT-GN1-FNGs as observed in previous reports (Maeda et al., 2010, 2017). Finally, PCT-GN1-FNGs were secreted into the extracellular space, as shown in Figure 4. It appeared that this hypothetical scheme could explain why PCT-GN1-FNGs, which contained the Lewisα epitope [Galβ1-3(Fucα1-4)GlcNAcβ1-], were found in the culture broth, but not in the rice cells (Maeda et al., 2010). Additionally, it is possible that the microsomes prepared in this study contained the Golgi apparatus as a minor component and the HMT-FNGs were obtained from the Golgi apparatus. However, since the Golgi apparatus contains several kinds of α-Man'ases (Liebminger et al., 2009; Kajiura et al., 2010), the HMT-FNGs that occurred in the Golgi apparatus might have been trimmed into smaller N-glycans, such as Manα6GlcNAc1, but not Manα6GlcNAc2. In this study, such smaller size HMT-FNGs (Manα6GlcNAc2) were not found, but in our previous study (Kimura et al., 2002), the very small amount of M5' and M6' in the ER-rich microsome fraction were found, which probably corresponded to F-3 in this study, and contamination of Golgi apparatus in F-3 cannot be completely excluded. Therefore, it seems to be necessary to assay the activities of Golgi-marker enzyme(s) to prove the complete absence or negligible amount of the Golgi apparatus in F3. This result will provide more solid evidence that the predominant occurrences of HMT-GN1-FNGs in the ER. In this study, we focused on HMT-FNGs in the ER, and at this moment it is obscure whether PCT-GN1-FNGs occur in the microsome fraction (F-3), although the amount might be very small if any. The structural analysis of FNGs in F-1 and F-2 is necessary for the next step to confirm whether the Golgi apparatus were mainly fractionated in F-2 but not F-3.

**Figure 2** | HPLC profiles of PA-oligosaccharides obtained from pumpkin microsomes. (I) The RP-HPLC profile of crude PA-oligosaccharides obtained from pumpkin microsomes is shown. The PA-oligosaccharides were separated by RP-HPLC using a Cosmosil 5C18-AR-II column. F1 contained GN1-FNGs, whereas F2 contained GN2-FNGs. (II) The SF-HPLC profiles of F1 and F2 obtained in (I). M9', M8', and M7' indicate the elution positions of authentic PA-oligosaccharides (Manα6GlcNAc1-PA, Manα6GlcNAc2-PA, and Manα6GlcNAc3-PA, respectively). M9 and M8 indicate the elution positions of authentic PA-oligosaccharides (Manα6GlcNAc2-PA and Manα6GlcNAc3-PA, respectively).
The HMT-GN2-FNGs, Man$_9$GlcNAc$_2$, were also found in the pumpkin microsomes (or the ER compartment) in this study, suggesting that two putative mechanisms could be considered. One is that these GN2-FNGs were formed as byproducts during the transfer of Glc$_9$Man$_9$GlcNAc$_2$ from the dolichol-oligosaccharide intermediates to the nascent polypeptides by
OST (Harada et al., 2015), and the other is that, along with HMT-GN1-FNGs (ENGase products), the HMT-GN2-FNGs produced by cPNGase from misfolded glycoproteins were transported back to the ER. However, considering that the reaction rate of ENGase for HMT-GN2-FNG generation is very fast and the GN1-FNG concentration is greater than the GN2-FNG concentration (Kimura et al., 2002), the former mechanism appears to be more likely in plant cells, as shown in Figure 5. Furthermore, we recently confirmed that HMT-FNGs and PCT-FNGs occurred in a mutant line of Arabidopsis thaliana, in which one cPNGase and two ENGase genes were completely knocked out, indicating that HMT-GN2-FNGs were generated without cPNGase activity and converted into PCT-FNGs through the Golgi apparatus via a certain pathway (Shirai et al., 2019).

The putative transporter(s) responsible for the retro-transportation of HMT-GN1-FNGs from the cytosol to the ER have not yet been identified, and the identification of such glycan-specific transporter(s) is a prerequisite to evaluate our hypothesis or reveal the degradation mechanism of GN1-FNGs formed during plant ERAD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material and further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

YK, MK, NE, and MM wrote the manuscript. All authors contributed to the study concept and design and performed the study. All authors contributed to the critical revision of the manuscript.

FUNDING

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Basic Research C, nos. 15K07841 and 18K05559 to MM and nos. 17K08197 and 20K05959 to YK).

ACKNOWLEDGMENTS

We are grateful to the Department of Instrumental Analysis, Advanced Science Research Center, Okayama University, for their assistance in performing the ESI-MS analysis. We would also like to thank Editage (www.editage.com) for English language editing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.610124/full#supplementary-material

Supplementary Figure 1 | Preparation of microsomes mainly containing the ER compartments from pumpkin hypocotyls. (I) Composition of the sucrose-containing separation buffer used for microsome preparation by ultracentrifugation is shown. After ultracentrifugation (100,000 × g for 3 h), the samples were fractionated, as shown in the right figure. (II) HPLC analyses of the ENGase and α-Glc’ase I activities of the pumpkin microsomes obtained by the first ultracentrifugation were performed. α-Glc’ase I activity was assayed using Glc₃Man₉GlcNAc₂–PA (G3M9) as a substrate. The reaction mixtures were analyzed by SF-HPLC using a Shodex Asahipak NH2P-50 4E column. The ENGase activity was assayed using Man₆GlcNAc₂–PA (M6B) as a substrate and ManXyl-Fuc_GlcNAc₂–PA (MSFX) as an internal standard. The reaction mixture was analyzed by RP-HPLC using a Cosmosil 5C18-AR-II column. Significant ENGase activity was found in F-1 in I, whereas significant α-Glc’ase I activity was found in F-3 in I.
REFERENCES

Abei, M., Bernasconi, R., Clerc, S., and Molinari, M. (2010). N-glycan structure; recognition and processing in the ER. Trends Biochem. Sci. 35, 74–82. doi: 10.1016/j.tibs.2009.10.001

Faucher, C., Lernould, S., Lemoine, J., Costa, G., and Morvan, H. (1997b). Identification of un conjugated N-glycans in strawberry plants. Plant Physiol. Biochem. 35, 89–95.

Faucher, C., Lernould, S., Maes, E., Lerouge, P., Strecke, G., and Morvan, H. (1997a). Tomato plant leaves also contain un conjugated N-glycans. Plant Physiol. Biochem. 35, 73–79.

Harada, Y., Masahara-Negishi, Y., and Suzuki, (2015). Cytosolic-free oligosaccharides are predominately generated by the degradation of dolichol-linked oligosaccharides in mammalian cells. Glycobiology 25, 1196–1205. doi: 10.1093/glycob/cw055

Hosokawa, N., Kamiya, Y., Kamiya, D., Kato, K., and Nagata, K. (2009). Human O-9, a lectin required for glycoprotein endoplasmic reticulum-associated degradation, recognizes mannose-trimmed N-glycans. J. Biol. Chem. 284, 17061–17068. doi: 10.1074/jbc.M907725200.

Kato, A., Wang, L., Ishii, K., Seino, J., Asano, N., and Suzuki, T. (2011). Occurrence of free sialyl oligosaccharides related to N-glycosylation enzymes, ENGase, and PNGase: the presence of unusual plant complex type N-glycans. Front. Plant Sci. 5:429. doi: 10.3389/fpls.2014.00429

Liebninger, E., Hütten, S., Vavra, U., Fischl, R., Schoberer, J., Grass, J., et al. (2009). Class I α-mannosidases are required for N-glycan processing and root development in Arabidopsis thaliana. Plant Cell 21, 3850–3867. doi: 10.1105/tpc.109.072363

Maeda, M., Ebara, N., Tani, M., Yavricka, C. J., and Kimura, Y. (2017). Occurrence of complex type free N-glycans with a single GlcNAc residue at the reducing termini in the fresh-water plant, Egeria densa. Glycocon. J. 34, 229–240. doi: 10.1016/j.jgb.2017.01.016

Maeda, M., and Kimura, Y. (2014). Structural features of free N-glycans occurring in plants and functional features of de-N-glycosylation enzymes, ENGase, and PNGase: the presence of unusual plant complex type N-glycans. Front. Plant Sci. 5:429. doi: 10.3389/fpls.2014.00429

Malenc, K., and Szél, A. (2012). Endoplasmic reticulum-associated degradation of glycoproteins in plants. Front. Plant Sci. 3:67. doi: 10.3389/fpls.2012.00067

Ishizuka, A., Hashimoto, Y., Naka, R., Kinoshita, M., Kakehi, K., Seino, J., et al. (2008). Accumulation of free complex-type N-glycans in MKN7 and MKN45 stomach cancer cells. Biochem. J. 413, 227–237. doi: 10.1042/BJ20071562

Iwatsuka, K., Watanabe, S., Kinoshita, M., Kamisue, K., Yamada, H., Hayakawa, T., et al. (2011). Free N-glycans derived from glycoproteins pre-sent in human sera. Glycobiology 21, 972–976. doi: 10.1093/glycob/cwpq13

Küttner, S., and Strasser, R. (2012). Endoplasmic reticulum-associated degradation of glycoproteins in plants. Front. Plant Sci. 3:67. doi: 10.3389/fpls.2012.00067

Kato, A., Wang, L., Ishii, K., Seino, J., Asano, N., and Suzuki, T. (2011). Calystegine B3 as a specific inhibitor for cytoplasmic α-mannosidase, ENGase, and high-mannose type free N-glycans in plant cells. Biosci. Biotechnol. Biochem. 75, 1013–1019. doi: 10.1002/ibmb.2009725200.

Kato, A., Wang, L., Ishii, K., Seino, J., Asano, N., and Suzuki, T. (2011). Calystegine B3 as a specific inhibitor for cytoplasmic α-mannosidase, ENGase, and high-mannose type free N-glycans in plant cells. Biosci. Biotechnol. Biochem. 75, 1013–1019. doi: 10.1002/ibmb.2009725200.

Kimura, Y., and Matsuo, S. (2000). Free N-glycans already occur at an early stage of seed development. J. Biochem. 127, 1013–1019. doi: 10.1093/oxfordjournals.jbchem.a022692

Kimura, Y., Matsuoka, S., Tsursakii, S., Kimura, M., Hara-Nishimura, I., and Nishimura, M. (2002). Subcellular localization of endo-β-N-acetylglucosaminidase and high-mannose type free N-glycans in plant cells. Biochem. Biophys. Res. Commun. 1570, 38–46. doi: 10.1016/s0006-2915(02)00149-6

Kimura, Y., Miyagi, C., Kimura, M., Nitta, T., Kawai, N., and Sugimoto, H. (2000). Structural features of N-glycans linked to royal jelly glycoproteins: structures of high-mannose type, hybrid type, and biantennary type glycans. Biosci. Biotechnol. Biochem. 64, 1847–1855. doi: 10.1271/bbb.64.1847

Kimura, Y., and Matsuoka, S. (2000). Free N-glycans already occur at an early stage of seed development. J. Biochem. 127, 1013–1019. doi: 10.1093/oxfordjournals.jbchem.a022692

Kato, A., Wang, L., Ishii, K., Seino, J., Asano, N., and Suzuki, T. (2011). Calystegine B3 as a specific inhibitor for cytoplasmic α-mannosidase, ENGase, and high-mannose type free N-glycans in plant cells. Biosci. Biotechnol. Biochem. 75, 1013–1019. doi: 10.1002/ibmb.2009725200.

Kato, A., Wang, L., Ishii, K., Seino, J., Asano, N., and Suzuki, T. (2011). Calystegine B3 as a specific inhibitor for cytoplasmic α-mannosidase, ENGase, and high-mannose type free N-glycans in plant cells. Biosci. Biotechnol. Biochem. 75, 1013–1019. doi: 10.1002/ibmb.2009725200.

Kimura, Y., Matsuoka, S., Tsursakii, S., Kimura, M., Hara-Nishimura, I., and Nishimura, M. (2002). Subcellular localization of endo-β-N-acetylglucosaminidase and high-mannose type free N-glycans in plant cells. Biochem. Biophys. Res. Commun. 1570, 38–46. doi: 10.1016/s0006-2915(02)00149-6

Kimura, Y., Miyagi, C., Kimura, M., Nitta, T., Kawai, N., and Sugimoto, H. (2000). Structural features of N-glycans linked to royal jelly glycoproteins: structures of high-mannose type, hybrid type, and biantennary type glycans. Biosci. Biotechnol. Biochem. 64, 2109–2120. doi: 10.1271/bbb.64.2109

Kimura, Y., Takagi, S., and Shiraishi, T. (1997). Occurrence of free N-glycans in pea (Pisum sativum L.) seedlings. Biosci. Biotechnol. Biochem. 61, 924–926.

Kimura, Y., Takeoka, Y., Inoue, M., Maeda, M., and Fujiyama, K. (2011). Double-Knockout of putative endo-β-N-acetylglucosaminidase (ENGase) genes in Arabidopsis thaliana: Loss of ENGase activity induced accumulation of high-mannose type free N-glycans bearing N,N′-acetylichitobiosyl unit. Biosci. Biotechnol. Biochem. 75, 1019–1021. doi: 10.1271/bbb.110148

Kimura, Y., Tokuda, T., Ohno, A., Tanak, H., and Ishiguro, Y. (1998). Enzymatic properties of endo-β-N-acetylglucosaminidase from developing tomato fruits and soybean seeds: substrate specificity of plant origin endoglycosidase. Biochim. Biophys. Acta 1381, 27–36.
## GLOSSARY

| TERM      | Definition                                                                 |
|-----------|-----------------------------------------------------------------------------|
| ER        | Endoplasmic reticulum                                                       |
| ERAD      | ER-associated degradation                                                   |
| ENGase    | Endo-β-N-acetylglucosaminidase                                               |
| cPNGase   | Cytosolic peptide:N-glycanase                                                |
| OST       | Oligosaccharidyltransferase                                                  |
| α-Glc'ase I | α-Glucosidase I (ER α-1,2-glucosidase)                                      |
| α-Glc'ase II | α-Glucosidase II (ER α-1,3-glucosidase)                                     |
| α-1,2-Man'ase | α-1,2-Mannosidase                                                      |
| FNG       | Free N-glycan                                                               |
| GN1-FNG   | Free N-glycan bearing one GlcNAc residue at the reducing end                |
| GN2-FNG   | Free N-glycan bearing two GlcNAc residues at the reducing end                |
| HMT-FNG   | High-mannose type FNG                                                       |
| PCT-FNG   | Plant complex type FNG                                                      |
| Le'       | Lewis a                                                                    |
| PA        | Pyridylmimo                                                                |
| RP-HPLC   | Reversed-phase HPLC                                                        |
| SF-HPLC   | Size-fractionation HPLC                                                     |
| ESI-MS    | Electrospray ionization mass spectrometry                                   |
| Glc       | D-Glucose                                                                  |
| Man       | D-Mannose                                                                  |
| GlcNAc    | N-acetyl-D-glucosamine                                                      |
| Xyl       | D-Xylose                                                                   |
| Fuc       | L-Fucose                                                                   |
| M6B       | Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-3)Manα1-4GlcNAcβ1-4GlcNAc-Pa          |
| M3FX      | Manα1-6(Manα1-3)(Xylβ1-2)Manγ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-Pa                 |
| G3M0'     | Manα1-2Manα1-6(Manα1-2Manα1-3)Manα1-6(Glcα1-2Glcα1-3Glcα1-3Manα1-2Manα1-3)Manγ1-4GlcNAc-Pa |
| G2M0'     | Manα1-2Manα1-6(Manα1-2Manα1-3)Manα1-6(Glcα1-3Glcα1-3Manα1-2Manα1-3)Manγ1-4GlcNAc-Pa |
| Mn'       | Manα,ManγGlcNAcβ1-4GlcNAc-Pa                                                 |
| Mn        | Manα,ManγGlcNAcβ1-4GlcNAc-Pa                                                 |