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Intracellular and extracellular free *N*-glycans produced by plant cells: occurrence of unusual plant complex-type free *N*-glycans in extracellular spaces

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As a part of the study to reveal the biological significance of de-*N*-glycosylation in plants, we analysed the structural features of free *N*-glycans (FNGs) accumulated inside cells and secreted to the extracellular space using a rice cell culture system. The structural analysis of FNGs obtained from the intracellular fraction revealed that the high-mannose type *N*-glycans with one GlcNAc residue (GN1-type) occurred at a concentration of ~10 nmol/g, while the truncated complex type *N*-glycans with a *N*, *N*′-diacetylchitobiosyl unit (GN2-type) occurred at a concentration of ~1 nmol/g. This result suggested that two kinds of glycoenzymes, cytosolic endo-β-*N*-acetylglucosaminidase (ENGase) and intracellular acidic peptide:*N*-glycanse (PNGase), are involved in the production of FNGs in rice cell as well as in other plant cells. On the other hand, in the culture medium, Lewis a epitope-containing complex and high-mannose type FNGs with the *N*, *N*′-diacetylchitobiosyl unit were found, suggesting extracellular acidic PNGase to be involved in the release of *N*-glycans from folded/processed glycoproteins in extracellular space. Furthermore, in the culture medium, we found unusual GN1-FNGs that have a biantennary complex type structure harbouring the Lewis a epitope, suggesting cytosolic ENGase and golgi *N*-glycan-processing enzymes to be involved in the production of these plant complex type FNGs.

Keywords: de-*N*-glycosylation/ENGase/*Oryza sativa*/plant free *N*-glycans/PNGase.

Abbreviations: deoxyHex, deoxy hexose; ENGase, endo-β-*N*-acetylglucosaminidase; ERAD, ER associate degradation; ERQC, ER quality control; ESI-MS, electrospray ionization mass spectrometry; FNG, free *N*-glycan; GN1-FNG, free *N*-glycan with one GlcNAc residue at the reducing end; GN2-FNG, free *N*-glycan with the chitobiosyl unit; GN2M3FX, GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA; Hex, hexose; HexNAc, *N*-acetyl hexosamine; Lewis a epitope, Galβ1-3(Fucα1-4)GlcNAc M3FX, Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA; M_n, $(Man)_nGlcNAc_2$-PA; M_n', $(Man)_nGlcNAc_1$-PA; MS/MS, tandem mass; PA-, pyridylamino; Pen, pentose; PNGase, peptide:*N*-glycanase; RP-HPLC, reversed phase HPLC; SF-HPLC, size-fractionation HPLC.

Recently it has become evident that the free *N*-glycans (FNGs) found in animal cells are released from misfolded glycoproteins by the nascent protein quality control system working in the cytosol. The high-mannose type FNGs in animal cells are thought to be formed by a combination of cytosolic endo-β-*N*-acetylglucosaminidase (ENGase), cytosolic peptide:*N*-glycanse (PNGase) and α-mannosidase (*1–4*) in the ERAD system. In plant cells, however, the mechanism of deglycosylation remains unclear, although FNGs are reported to occur ubiquitously at micromolar concentrations in various cells or tissues during cell differentiation, plant growth or fruit ripening (*5–12*) and auxin-like activity has been postulated (*13*, *14*). Recently, Meli *et al.* (*15*) have reported that suppression of the α-mannosidase and β-*N*-acetylhexosaminidase genes reduced rates of tomato fruit softening, suggesting that the degradation of *N*-glycans is closely associated with fruit softening or ripening. But it is not clear whether FNGs or conjugated *N*-glycans are the true substrates for these two glycoenzymes involved in tomato softening.

As a part of the study to elucidate the physiological significance of the deglycosylation involved in plant differentiation or development, we have analysed structural features of FNGs occurring in developing seeds, seedlings or fruits (*9–12*) and characterized some of the ENGases that produce high-mannose FNGs in developing plants (*16–19*). To date, it has been reported that two types of FNG, a high-mannose type and a plant complex type, occur in developing seeds, elongating hypocotyls and ripening fruits (*5–12*). Among FNGs, in most cases, the high-mannose type usually has a single GlcNAc residue (GN1-FNG) at the reducing end, whereas the complex type with β1-2xylose and/or α1-3fucose always has an

N-acetylchitobiosyl unit (GN2-FNG), indicating that the former structures may be produced by endo-β-*N*-acetylglucosaminidase (ENGase) and the latter structures by the plant specific acidic peptide:*N*-glycanase (PNGase). As for the complex type FNGs, the occurrence of GN2-FNG in plants indicates that the acidic PNGase is responsible for the release of fully processed *N*-glycans from secreted or vacuolar glycoproteins, since all the plant acidic PNGases purified and characterized so far have a optimum pH in the acidic region and can hydrolyse glycopeptides having complex type *N*-glycans (*20–27*). According to the processing mechanism for plant *N*-glycoproteins (*28*), in general, glycoproteins accumulated in the vacuole or protein body carry truncated complex type *N*-glycans with a β1-2 xylosyl residue and/or α1-3 fucosyl residue and secreted glycoproteins carry biantennary complex type *N*-glycans with a β1-2 xylosyl residue and/or α1-3 fucosyl residue. In several cases, Lewis a epitope unit has been observed at the non-reducing end of biantennary complex type *N*-glycans (*28–33*). Therefore, the truncated plant complex FNGs are probably produced from the vacuolar glycoproteins and the biantennary complex type FNGs from the secreted glycoproteins. By contrast, almost all high-mannose type FNGs in plants have only one GlcNAc residue at their reducing end (GN1 type structure), indicating the involvement of a cytosolic ENGase in their production, although it is unclear whether the cytosolic (or neutral) PNGase, like the cytosolic PNGase in animal cells, releases the high-mannose type *N*-glycans from misfolded glycoproteins prior to the action of cytosolic ENGase. Recently, we found that the amount of high-mannose type GN1-FNGs increases as tomato fruits mature (*11*) whereas the gene expression of cytosolic ENGase does not vary significantly with the ripening process (*19*). These observations suggest the increase in the amount of high-mannose type FNGs to be due to the increase in the amount of substrates, misfolded glycoproteins carrying the high-mannose type glycans in the cytosol, during the maturation of fruit. Although it is still unclear whether FNGs play a physiological role as signalling molecules, it appears likely that the de-*N*-glycosylation mechanisms working in plants are associated with plant development, plant growth or fruit ripening.

To clarify the physiological significance of deglycosylation and to reveal the physiological functions of FNGs involved in plant development or fruit ripening, in this study we analysed structural features of FNGs produced in the intracellular (IC) and the extracellular (EC) spaces of plants using rice cell culture system. The structural features of IC FNGs were found to be quite different from that of EC FNGs. From the cell lysate (IC fraction), high-mannose type GN1-FNGs and truncated plant complex type GN2-FNGs were obtained with the former predominating. From the culture medium (the EC space), in addition to the highmannose GN1-FNGs, high-mannose GN2-FNGs and biantennary complex type GN2-FNGs were obtained and the GN2-FNGs were predominant, indicating they were produced from secreted glycoproteins by an EC acidic PNGase. Furthermore, a biantennary complex type GN1-FNG bearing the Lewis a epitope and β1-2 xylosyl residue was obtained from the culture medium, suggesting the ENGase activity to be involved in the production, and this unusual FNG was processed in the golgi apparatus. The occurrence of the biantennary complex type GN1-FNG in the EC space leads us to postulate that some of the high-mannose type GN1-FNG produced by the cytosolic ENGase from misfolded glycoproteins could be translocated to the endoplasmic reticulum (ER) or golgi apparatus and then processed together with *N*-glycans linked to various secreted type glycoproteins in these organelle.

Materials and Methods

Materials

The rice k-1 cell line established from *Oryza sativa* L. cv. Nipponbare was kindly gift from H. Nishimura and Professor K. Kasamo (Research Institute for Bioresources, Okayama University). A Cosmosil 5C18-AR column (0.6 × 25 cm) was purchased from Nacalai Tesque (Kyoto, Japan). A Shodex Asahipak NH2P-50 column (0.6 × 25 cm) was purchased from Showa Denko (Tokyo, Japan). Authentic PA-sugar chains were prepared as described in our previous papers (*10*, *11*, *31–35*). $Glc_{3\sim1}Man_9GlcNAc_2$-PA was obtained from Masuda Chemicals Industries Co., Ltd. Jack bean α-Mannosidase (JB-Man'ase) and *Aspergillus* α-1,2-mannosidase (α-1,2-Man'ase) from Seikagaku Kogyo Co. (Tokyo), *Diplococcus pneumonia* β-*N*-Acetylglucosaminidase (β-GlcNAc'ase) from Boehringer (Mannheim, Germany), β-1,3/6-specific β-Galactosidase (β-1,3/6-Gal'ase) from Sigma (St Louis, MO, USA) and α-1,3/4--specific α-fucosidase (α-1,3/4-Fuc'ase) and Lacto-*N*-biosidase from Takara (Kyoto).

Preparation of the oligosaccharide from cultured rice cells

Rice cells in suspension cultures were filtrated and the cell pellet (33.4 g, wet weight) on filter paper was disrupted by a Teflon homogenizer (60 rpm and 10 strokes) in 50 ml of 1.0 M Tris–HCl buffer, pH 8.5 and centrifuged at 12,000*g* for 20 min. The supernatant was removed and the pellet was washed with deionized water (100 ml) and centrifuged under the same conditions. The resulting supernatant was combined with the previous supernatant and the total supernatant was used as the cell lysate (the IC fraction). The culture medium was also centrifuged at 12,000*g* for 20 min and the supernatant (770 ml) was used as the EC fraction. The culture medium was concentrated to ~200 ml by a rotary evaporator. The lysate fraction (the IC fraction) and the culture medium (the EC fraction) were separately dialyzed against deionized water (4 l twice) and the outer solution was concentrated to small amount *in vacuo*. Two samples, the IC and EC fractions, were successively desalted with Dowex 1 × 2 and Dowex 50 × 2 resins. The non-adsorbed fractions were concentrated and applied to a column (1.8 × 30 cm) of Sephadex G-25 in 0.1 M NH_4OH to remove other small molecules. Although it is well known that a strong alkaline condition often induces significant epimerization of the reducing end of oligosaccharides, in the condition (0.1 M NH_4OH) used in this study, we could not detect considerable amount of epimerized oligosaccharides. The oligosaccharide-containing fractions were detected by the phenol–sulphuric acid method (*36*) and the oligosaccharide-fraction was immediately evaporated to dryness by rotary evaporator at 35°C. After dissolving the residue in water, the sample was lyophilized and fluorescence-labelled.

Pyridylamination of free N*-glycans*

The pyridylamination of FNGs was done by the method of Natsuka and Hase (*37*). Next the excess amount of 2-aminopyridine was removed by gel filtration using a column (3.0 × 30 cm) of Sephadex G-10 in 0.1 M NH_4OH. The resulting PA-derivatives were partially purified by passage through a column (1.8 × 180 cm) of Sephadex G-50 in 0.1 M NH_4OH. The pyridylaminated oligosaccharides were monitored on a fluorescence spectrometer (excitation 310 nm, emission 400 nm, Hitachi 650 10S).

Con-A-sepharose column chromatography

The PA-derivatives obtained by gel-filtration were evaporated to dryness. The residue was dissolved in 20 mM Tris–HCl buffer, pH 7.8, containing 0.1 M NaCl, 5 mM $CaCl_2$ (5 ml) and applied to a column (2.0 × 32 cm) of Con-A-Sepharose 4B equilibrated with the same buffer. Non-adsorbed PA-sugar chains [Con-A (−)] were washed out with 200 ml of the same buffer and adsorbed PA-sugar chains [Con-A (+)] were eluted by the addition of 0.3 M methyl-α-mannoside in the same buffer (Fig. 1). The PA-sugar chains were monitored with a fluorescence spectrophotometer (excitation 310 nm, emission 400 nm, Hitachi 650 10S). Each fraction was concentrated and desalted by gel-filtration through a column (3.0 × 30 cm) of Sephadex G-10 in 0.1 M NH_4OH. The PA-derivatives obtained were further separated on a Jasco 880-PU HPLC apparatus equipped with a Jasco 821-FP Intelligent Spectrofluorometer (excitation 310 nm, emission 380 nm), using a column (0.6 × 25 cm) of Cosmosil 5C18-AR and a column (0.46 × 25 cm) of Shodex Asahipak NH2P-50 (*10–12*). For reversed-phase (RP)-HPLC using the Cosmosil 5C18-AR, the PA-sugar chains were eluted by increasing the acetonitrile concentration in 0.02% TFA linearly from 0% to 6% at a flow rate of 1.2 ml/min as described in our previous papers. For size fractionation (SF)-HPLC using the Shodex Asahipak NH2P-50, 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.

Digestion with exoglycosidase

The PA-sugar chains (~200 pmol) were digested with jack bean α-mannosidase, *Aspergillus* α-1,2-mannosidase, diplococcal β-*N*-acetylglucosaminidase, α-1,3/4-fucosidase, β-1,3/6-galactosidase and Lacto-*N*-biosidase as described in previous papers (*31–33*). The reactions were stopped on boiling the mixtures for 3 min and part of each digest was analysed by SF-HPLC. For analyses of the digests, 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.

Partial acetolysis

Partial acetolysis was done with ~10 nmol of PA-oligosaccharide essentially as described in Natsuka *et al.* (*38*) and our previous paper (*10*). Fragments having GlcNAc-PA residues were separated by SF-HPLC using the column (0.46 × 25 cm) of Shodex Asahipak NH2P-50. The PA-oligosaccharides were eluted by increasing the water content of the water–acetonitrile mixture from 10% to 38% linearly for 40 min at a flow rate of 0.7 ml/min. Fragments derived from the non-reducing end were deacetylated in the 0.2% sodium methoxide/methanol mixture at room temperature for 30 min. The deacetylated saccharides were pyridylaminated and the excess amount of 2-aminopyridine was removed by a gel filtration using a Sephadex G-25 column (1.8 × 30 cm, in 0.1 M NH_4OH). The resulting PA-oligosaccharides were analysed by SF-HPLC using the column (0.46 × 25 cm) of Shodex Asahipak NH2P-50. Authentic Man_3-PA, Man_2-PA and Man-PA were prepared from $Man_9GlcNAc_2$-PA and $Man_8GlcNAc_2$-PA by partial acetolysis (*10*). The PA-oligosaccharides bound to the column were eluted by increasing the water content of the water–acetonitrile mixture from 0% to 20% linearly for 40 min at a flow rate of 0.7 ml/min.

Electrospray ionization mass spectrometry

The mass spectrometer used was a Perkin Elmer Sciex API-III, triple-quadrupole mass spectrometer with an atmospheric pressure ionization ion source as described in our previous papers (*9–12*). The mass spectrometer was operated in the positive mode; the ion spray voltage was 4,200 V. Samples were typically dissolved in 50% acetonitrile/water (containing 0.05% formic acid) at a concentration of ~10 pmol/μl and introduced into the electrospray needle by mechanical infusion through a micro syringe at a flow rate of 5 μl/min. The collisionally activated dissociation (CAD) spectrum was measured with argon as the collision gas. The collision energy was 60–100 eV. The scanning was done with a step size of 0.5 Da and the CAD daughter ion spectrum was recorded from *m*/*z* 200.

Carbohydrate analysis

Carbohydrate composition was analysed by gas–liquid chromatography (GLC) of trimethylsilyl derivatives after methanolysis (1.5 N methanolic HCl) (*39*). For GLC analysis, a Hitachi G-3000 gas

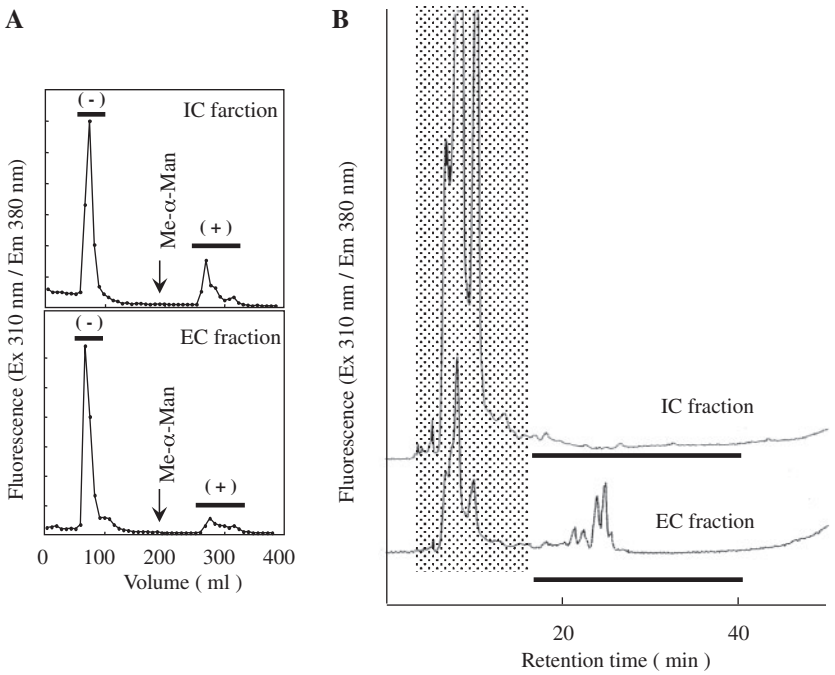


Fig. 1 Con A affinity chromatography and RP-HPLC of pyridylaminated FNGs. (A) Con A affinity chromatography of PA-FNGs. IC, PA-derivatives from the IC fraction (inside cell); EC, PA-derivatives from the EC fraction (culture medium). The non-adsorbed fraction was designated Con A (−) and the adsorbed fraction was designated Con A (+). (B) RP-HPLC profiles of PA-sugar chains of the Con A (+) fractions obtained in A. IC, PA-sugar chains from the soluble fraction; EC, the pyridylaminated derivatives from EC fraction (culture medium). Shaded fractions were pooled as FNGs having one GlcNAc residue at the reducing end side (GN1-FNGs), and fractions indicated by horizontal bars were pooled as FNGs having an *N*, *N*′-diacetylchitobiosyl unit (GN2-FNGs).

chromatograph with a DB-1 capillary column (30 m × 0.25 cm, J & W Scientific) was used.

Results

Structural analysis of high-mannose type FNGs

The Con-A (+) fraction was subjected to RP-HPLC to separate FNGs having one GlcNAc (GN1 type) residue at the reducing end side from those having the *N*-acetylchitobiosyl unit (GN2 type). As shown in Fig. 1B, GN1-FNGs were obtained in the run-through fraction (shaded fraction) and GN2-FNGs were obtained in the fraction indicated by horizontal bars. The FNGs obtained by RP-HPLC were further purified by SF-HPLC. As shown in Fig. 2A, five PA-sugar chains having GN1 (peak-a, -b, -c, -d and -e) were obtained from the two fractions (the IC fraction and the EC fraction) by SF-HPLC. The structures of each of these PA-sugar chains (a, b, c, d and e) was first analysed by α-mannosidase digestion. Since the glycosidase digestions of PA-sugar chains obtained from the two fractions gave similar results, the analysis on sugar chains from the IC fraction are described below. As shown in Fig. 2A, almost all the PA-sugar chains in peaks-b, -c, -d and -e were converted to peak-a ($Man_5GlcNAc_1$-PA, M5′) by α1-2 mannosidase and the product was further converted to $Hex_1GlcNAc_1$-PA (M1′) by jack bean α-mannosidase, indicating that these sugar chains had a typical high-mannose type structure. But some minor peaks (peak-f and peak-g) were observed on the chromatograms of the α-1,2-mannosidase digest or jack bean α-mannosidase digest, suggesting that some glucosylated high-mannose type *N*-glycans occur in the Con-A bound FNGs.

To confirm the occurrence of glucose residue(s) in peaks-d and -e, we purified the peaks by SF-HPLC and analysed the molecular mass with electrospray ionization mass spectrometry (ESI-MS) and sugar composition using α-mannosidase and GLC. On ESI-MS, Peak-d gave a signal at *m*/*z* 1596.5, indicating that these *N*-glycans in peak-d had the $Hex_8GlcNAc_1$-PA structure (Fig. 3A). Some fragment ions obtained by tandem mass (MS/MS) also supported that these *N*-glycans consisted of eight hexoses and GlcNAc-PA, although two fragments ($Hex_6GlcNAc_1$ and $Hex_7GlcNAc_1$) were not detected in this analysis (Fig. 3B). GLC revealed that peak-d contained glucosyl residues in addition to mannosyl and GlcNAc residues, indicating that peak-d was glucosylated (data not shown). As shown in Fig. 2B, ∼50% of peak-d was converted to M5′ but the rest was not digested by α-1,2-mannosidase, indicating that peak-d consisted of two types of $Hex_8GlcNAc_1$-PA. Therefore, it could be deduced that ∼50% of the $Hex_8GlcNAc_1$-PA must be glucosylated *N*-glycans and resistant to α-1,2-mannosidase, indicating the absence of exposed an α1-2 mannosyl residue. On digestion with jack bean α-mannosidase, the resulting M5′ was converted to M1′, whereas the α-1,2-mannosidase-resistant oligosaccharide was digested and the product was eluted near where M5′ was eluted, releasing three mannose residues. Furthermore, partial acetolysis of peak-d gave two major fragments having the reducing

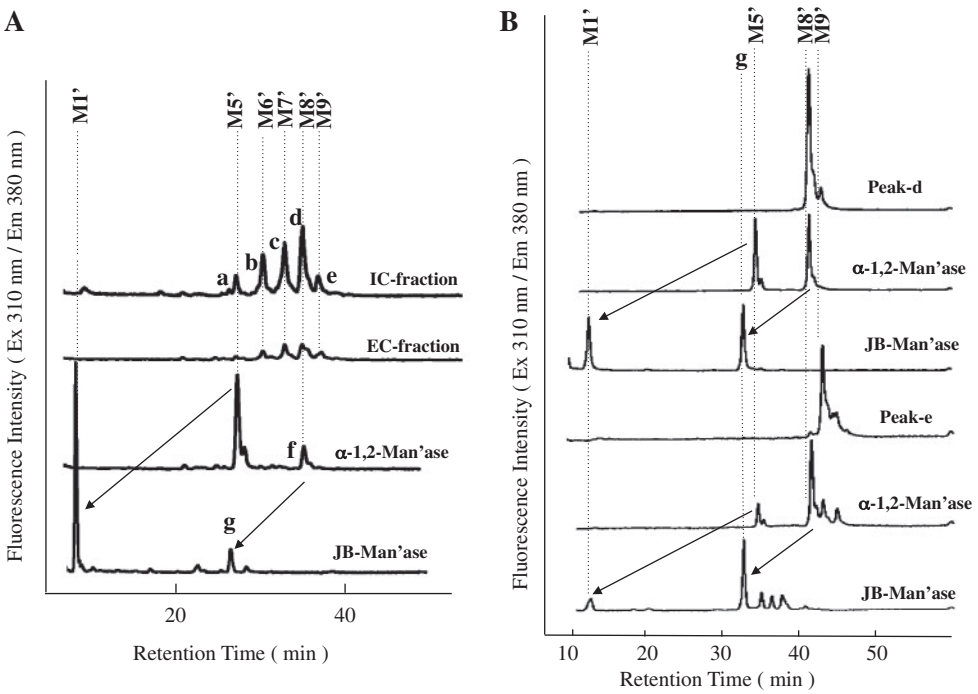


Fig. 2 SF-HPLC of high-mannose type GN1-FNGs and their α-mannosidase digests. (A) SF-HPLC of total high-mannose type GN1-FNGs obtained by Con A affinity chromatography in Fig. 1I and their α-mannosidase digests. IC fraction; FNGs from the the IC fraction (cell lysate); EC fraction; FNGs from the EC fraction (culture medium); α-1,2-Man'ase, *Aspergillus* α-1,2-mannosidase digest; JB-Man'ase, jack bean α-mannosidase digest. (B) SF-HPLC of peaks-d, -e, and their α-mannosidase digests. Dotted lines indicate the elution positions of authentic PA-sugar chains: M9′, $Man_9GlcNAc_1$-PA; M8′, $Man_8GlcNAc_1$-PA; M7′, $Man_7GlcNAc_1$-PA; M6′, $Man_6GlcNAc_1$-PA; M5′, $Man_5GlcNAc_1$-PA; M1′, $Man_1GlcNAc_1$-PA.

terminal GlcNAc-PA; one was eluted near the position of $Man_5GlcNAc_1$-PA and the other at $Man_4GlcNAc_1$-PA (Fig. 4A). On the other hand, from the non-reducing terminal fraction, Man_2-PA (Manα1-2Man-PA or Manα1-3Man-PA) and Man-PA were obtained as major products and Man_3-PA (Manα1-2Manα1-3Man-PA) was obtained as a minor product (Fig. 4B). Although the amount of Man_3-PA was small compared with that of Man_2-PA or Man-PA, the detection of a tri-mannosyl fragment among the products of partial acetolysis indicated clearly that at least two isomers of M8′, Manα1-2Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc (major component) and Manα1-6(Manα1-2Manα1-3) Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc(minor component), occurred. From these results, we concluded that peak-d contained $Glc_1Man_7GlcNAc_1$-PA, Manα1-6(Manα1-3)Manα1-6(Glcα1-3Manα1-2Manα1-2) Manα1-3Manβ1-4GlcNAc-PA and $Man_8GlcNAc_1$-PA (M8′).

In the case of peak-e (Fig. 2B), digestion with α-1,2-mannosidase gave a major peak that eluted at the same position as peak-d, indicating that one α1-2 mannose residue was released (Fig. 2B). With jack bean α-mannosidase, this major product was converted to a smaller oligosaccharide and its position was the same as that of the jack bean α-mannosidase digest of peak-d, which eluted near M5′ (Fig. 3B). This result indicated the major component in peak-e to be Glc1Man8GlcNAc1-PA containing the Manα1-2Manα1-6(Manα1-3)Manα1- or Manα1-6(Manα1-2Manα1-3)Manα1-unit. Since the minor products were eluted at the position of M5′ by α-1,2-mannosidase (Fig. 2B) and of M1′ by jack bean α-mannosidase (Fig. 3B), $Man_9GlcNAc_1$-PA (M9′) must be a minor component in peak-e. As for other some minor component found in the α-mannosidase digests, detailed structural analyses could not be done due to the small amounts of sample but high-mannose type FNGs containing two or three glucose residues could be expected. Furthermore, partial acetolysis of peak-e gave one major fragment having the reducing terminal GlcNAc-PA that was eluted near the position of $Man_5GlcNAc_1$-PA (Fig. 4A), indicating the major component of peak-e contained the Glcα1-3Manα1-2Manα1-2Manα1-3Manβ1-4GlcNAc-PA unit like one component in peak-d. Interestingly, two minor fragments were obtained near where $Man_6GlcNAc_1$-PA and $Man_7GlcNAc_1$-PA eluted, suggesting that these fragments contained two or three glucose residues ($Glc_2Man_4GlcNAc$-PA or $Glc_3Man_4GlcNAc_1$-PA). From the non-reducing terminal fraction, Man_3-PA, Man_2-PA and Man-PA were obtained (Fig. 4B). In the case of peak-e, the amount of Man3-PA

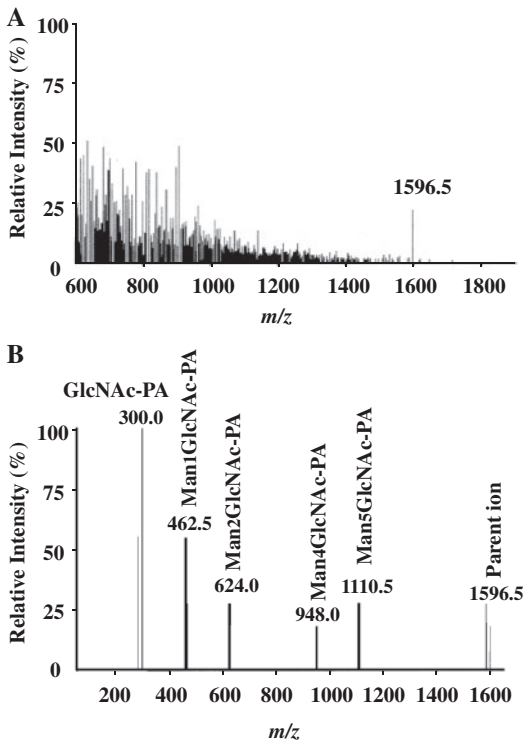


Fig. 3 ESI-MS of peak-d. (A) ESI-MS of peak-d. (B) MS/MS of a signal at *m*/*z* 1596.5.

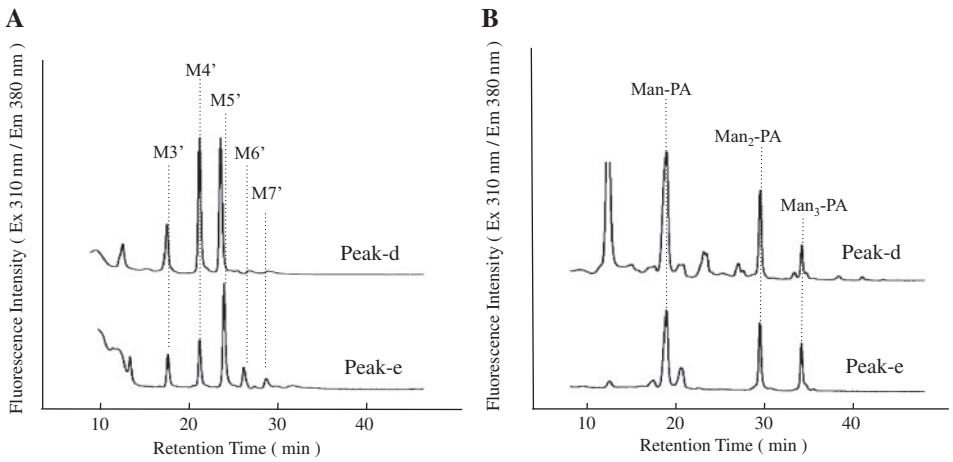


Fig. 4 SF-HPLC of PA-oligosaccharide fragments obtained from peaks-b and -d in Fig. 2 by partial acetolysis. (A) PA-fragments containing the reducing terminal GlcNAc-PA. (B) PA-fragments derived from the non-reducing ends. Dotted lines indicate the elution positions of authentic PA-sugar chains (Mn' = $Man_nGlcNAc_1$-PA).

(Manα1-2Manα1-3Man-PA) was almost equal to that of Man_2-PA (Man_3-PA:Man_2-PA=1:1.2), therefore, peak-e should contain $Glc_1Man_8GlcNAc_1$-PA, Manα1-6(Manα1-2Manα1-3)Manα1-6(Glcα1-3Man-1-2Manα1-2)Manα1-3Manβ1-4GlcNAc-PA and Manα1-2Manα1-6(Manα1-3)Manα1-6(Glcα1-3Manα1-2Manα1-2)Manα1-3Manβ1-4GlcNAc-PA and a small amount of $Man_9GlcNAc_2$-PA, as major components.

These deduced structures of the high-mannose type GN1-FNGs were further confirmed by ESI-MS. The ESI-MS analysis of peak-a, -b, -c, -d and -e showed a signal at *m/z* 1113.0 ($[Man_5GlcNAc_1\text{-}PA+H]^+$), *m/z* 1272.0 ($[Man_6GlcNAc_1\text{-}PA+H]^+$), *m/z* 1434.0 ($[Man_7GlcNAc_1\text{-}PA+H]^+$), *m/z* 1596.5 ($[Man_8GlcNAc_1$-PA or $Glc_2Man_6GlcNAc_1]+H)^+$) and *m/z* 1760.5 ($[Man_9GlcNAc_1\text{-}PA+H]^+$). The results of MS/MS of these PA-sugar chains are summarized in Table I.

The high-mannose type FNGs having the *N*,*N*′-acetylchitobiosyl unit (GN2-FNGs), which might be produced by the acidic PNGase, were obtained from only the EC fraction (the culture medium) as shown in Fig. 1B. As shown in Fig. 5, five PA-sugar chains (peak-h, -i, -j, -k and -l) were obtained by SF-HPLC. The elution position of peak-h coincided with that of $Man_5GlcNAc_2$-PA (M5), peak-i with that of $Man_6GlcNAc_2$-PA (M6), peak-j with that of $Man_7GlcNAc_2$-PA (M7), peak-k with that of $Man_8GlcNAc_2$-PA (M8) and peak-l with that of $Man_9GlcNAc_2$-PA (M9) on SF-HPLC. Peaks -i, -j, -k and -l were converted to peak-h ($Man_5GlcNAc_2$-PA) by α-1, 2-mannosidase and the product was further converted to $Man_1GlcNAc_2$-PA (M1) by jack bean α-mannosidase, suggesting that these *N*-glycans had a typical high-mannose type structure and might be produced by the plant specific acidic PNGase (*20–27*) residing in the cell wall or some other EC region, such as the apoplast, but not in the cytosol.

Structural analysis of plant complex-type FNGs

The Con-A (−) oligosaccharides obtained from the IC (inside cells) and EC (culture medium) fractions in Fig. 1 were further separated by the SF-HPLC (Fig. 6). The elution positions of peak-m coincided with that of authentic $Man_3Xyl_1Fuc_1GlcNAc_2$-PA (M3FX), peak-o with that of $GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$-PA (GN2M3FX) and peak-q with that of $Gal_1Fuc_1GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$-PA (the Lewis a epitope-containing type structure) (*28*, *33*). As shown in Fig. 7A, ESI-MS of peak-m gave two main signals at *m/z* 1040.0 and 1268.0, suggesting that the signal at *m/z* 1268.0 $(M+H)^+$ corresponded to $Man_3Xyl_1Fuc_1GlcNAc_2$-PA. On the other hand, the signal at *m/z* 1040.0 seemed not to be *N*-glycan, since MS/MS gave no daughter ion at *m/z* 300.0 (GlcNAc-PA) (data not shown). As shown in Fig. 7B, peak-o gave a single signal at *m/z* 837.5 $[M+2H]^2$ corresponding with $HexNAc_2Hex_3Pen_1DeoxyHex_1HexNAc_2$-PA or $GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$-PA, which is a major *N*-glycans linked to glycoproteins expressed in cultured rice cells (*33*). The structure of peak-q was analysed by digestion with glycosidases as shown in Fig. 8. Peak-q was converted to $Man_3Xyl_1Fuc_1GlcNAc_2$-PA by successive digestion with diplococcal β-GlcNAc-ase, α-1, 3/4-specific fucosidase, β-1, 3/4-specific galactosidase and diplococcal β-GlcNAc-ase (second digestion). Furthermore, the α-fucosidase digest was converted to M3FX by lacto *N*-biosidase, indicating the occurrence of the Galβ1-3GlcNAc unit instead of the Galβ1-4GlcNAc unit. The deduced structures of peaks-m, -o and -q are summarized in Scheme 1. Although many unidentified peaks were obtained from the IC fraction by SF-HPLC (upper chromatogram in Fig. 6), these peaks seemed not to be *N*-glycan but might be cell wall fragments, since MS/MS analysis of main peaks gave no daughter ion at *m/z* 300.

Table I. MS/MS analysis of high mannose type GN1-FNGs obtained from IC fraction.

	Peak name				
Fragment ions $[M+H]^+$	**a**	**b**	**c**	**d**	**e**
$GlcNAc_1$-PA (300.0)	+++	+	++++	++++	++
$Man_1GlcNAc_1$-PA (462.0)	++	+	+++	+++	ND
$Man_2GlcNAc_1$-PA (624.0)	++	+	+	++	ND
$Man_3GlcNAc_1$-PA (786.0)	ND	+	+	ND	+
$Man_4GlcNAc_1$-PA (948.0)	ND	+	+	+	ND
$Man_5GlcNAc_1$-PA (1110.5)	++++	+	+	++	+
$Man_6GlcNAc_1$-PA (1272.5)	ND	++++	+	ND	ND
$Man_7GlcNAc_1$-PA (1435.0)	ND	ND	+	ND	ND
$Man_8GlcNAc_1$-PA (1596.5)	ND	ND	ND	+	++
$Man_9GlcNAc_1$-PA (1758.5)	ND	ND	ND	ND	++++

+, 10–25% relative intensity; ++, 26–50% relative intensity; +++, 51–75% relative intensity; ++++, 76–100% relative intensity; ND, not detected.

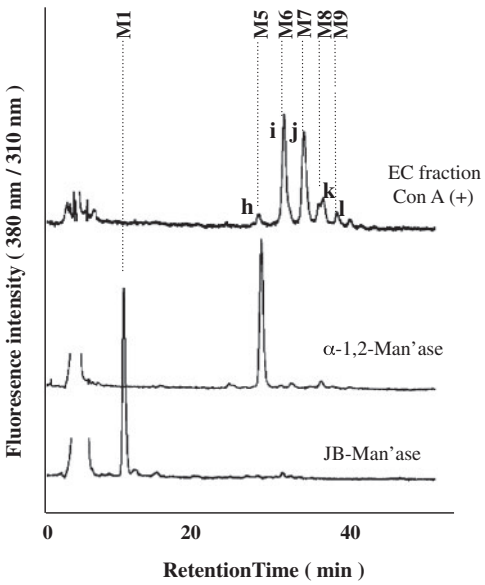


Fig. 5 SF-HPLC of high-mannose type GN2-FNGs obtained in Fig. 1B and their successive α-mannosidase digests. EC fraction Con A (+), PA-oligosaccharides obtained from the EC fraction in Fig. 1B; α-1,2-Man'ase; α-1,2-mannosidase digest; JB-Man'ase, jack bean α-mannosidase digest. Dotted lines indicate the elution positions of authentic PA-sugar chains: M9, $Man_9GlcNAc_2$-PA; M8, $Man_8GlcNAc_2$-PA; M7, $Man_7GlcNAc_2$-PA; M6, $Man_6GlcNAc_2$-PA; M5, $Man_5GlcNAc_2$-PA; M1, $Man_1GlcNAc_2$-PA.

The elution positions of peaks-n, -p and -r did not coincide with those of any authentic *N*-glycans. In the case of peak-n, this PA-sugar chain gave a single signal at *m*/*z* 1324.0 $(M+H)^+$, suggesting that the signal corresponded to $HexNAc_1Hex_3Pen_1HexNAc_2$-PA or $GlcNAc_1Man_3Xyl_1GlcNAc_2$-PA. As shown in Fig. 7D, two informative signals [GlcNAc-PA (*m*/*z* 300.5) and $Man_1GlcNAc_1$-PA (*m*/*z* 462.5)] were observed but the *N*-acetylchitobiosyl signal [$GlcANc_2$-PA (*m*/*z* 503)] was not. This MS/MS analysis indicated that the PA-sugar chain was of the GN1-FNG species, suggesting the ENGase to be involved in the production of this FNG. Other fragment ions (*m*/*z* 1121.5, *m*/*z* 959.5, *m*/*z*

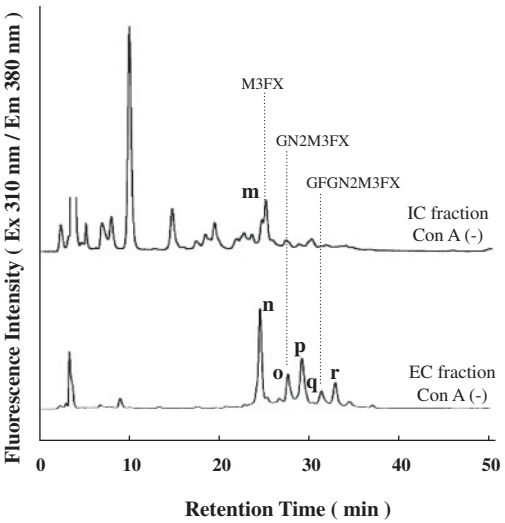


Fig. 6 SF-HPLC of Con A (−) fraction obtained in Fig. 1A. Dotted lines indicate the elution positions of authentic PA-sugar chains: M3FX, Man_3Xyl_1 $Fuc_1GlcNAc_2$-PA; GN2M3FX, $GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$-PA; GFGN2M3FX, $Gal_1Fuc_1GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$-PA.

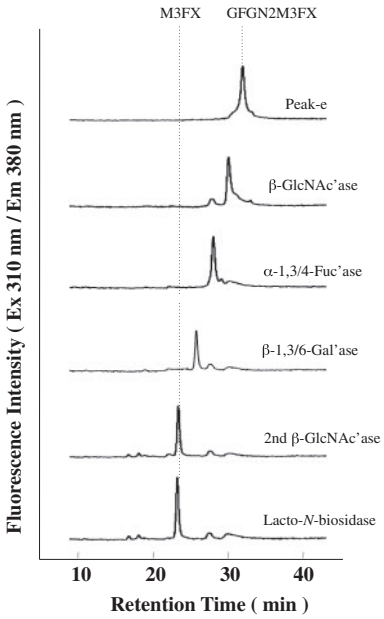


Fig. 8 SF-HPLC of exo- and *endo*-glycosidase digests of the PA-sugar chain in peak-q. Peak-q was successively digested with diplococcal β-*N*-acetylglucosaminidase (β-GlcNAc'ase), α-1, 3/4-specific fucosidase (α1-3,4-Fuc'ase), β-1, 3/4-specific galactosidase (β-13/6-Gal'ase) and diplococcal β-GlcNAc'ase (second digestion). The α-fucosidase digest was digested by lacto *N*-biosidase. Dotted lines indicate the elution positions of authentic PA-sugar chains ($Man_3Xyl_1Fuc_1GlcNAc_2$-PA and $Gal_1Fuc_1GlcNA_2Man_3Xyl_1Fuc_1GlcNAc_2$-PA).

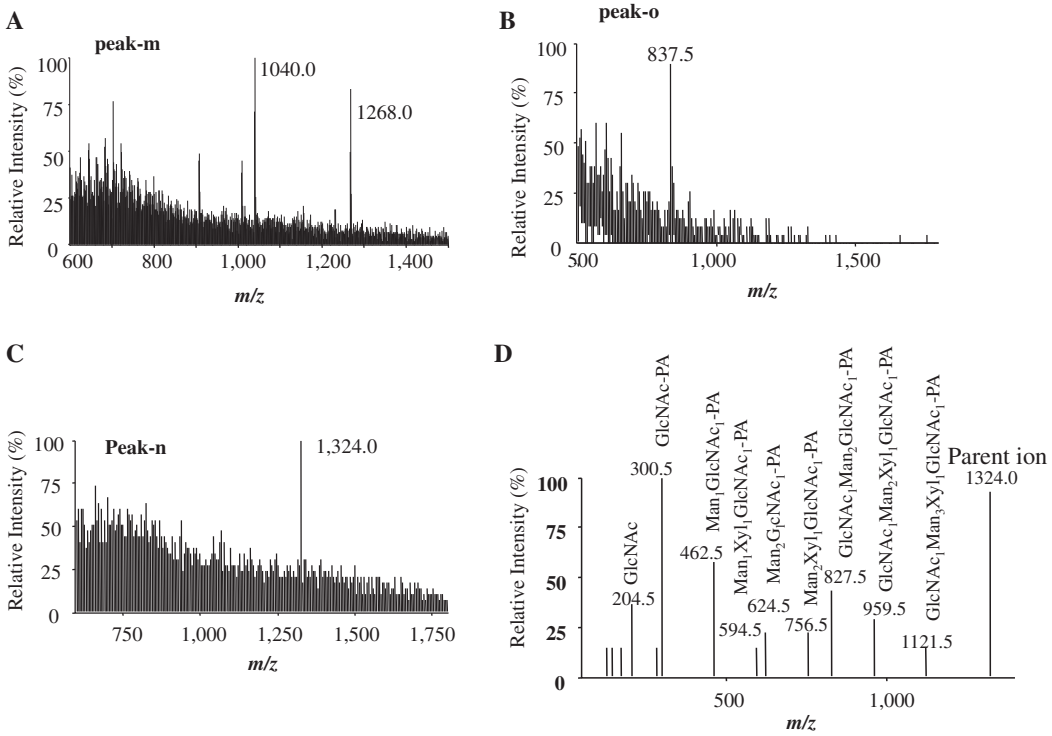
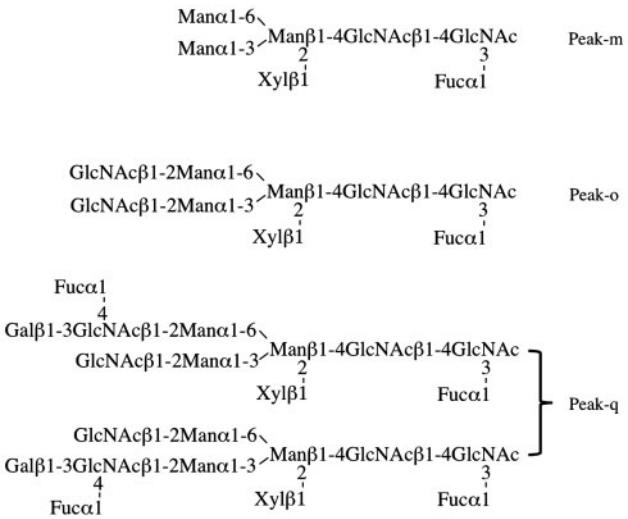


Fig. 7 ESI-MS of peaks-m, -n and -o. (A) peak-m; (B) peak-o; (C) peak-n; (D) MS/MS spectrum of peak-n (*m*/*z* 1324.0).



Scheme 1 Proposed structure of complex type GN2-FNGs (peaks-m, -o, and -q) produced by rice cells.

827.5, *m*/*z* 756,5, *m*/*z* 756.5, *m*/*z* 624.5 and *m*/*z* 594.5) could be assigned as daughter ions derived from $HexNAc_2Hex_3Pen_1HexNAc_1$-PA or $GlcNAc_2Man_3Xyl_1GlcNAc_1$-PA as shown in Fig. 7D. This PA-sugar chain was converted to $Man_3Xyl_1GlcNAc_1$-PA by diplococcal β-GlcNAc-ase (data not shown). Since peaks-p and -r could not be analysed by ESI-MS due to the small amounts of samples, these PA-sugar chains were analysed by digestion with glycosidases. In the case of peaks-p and -r, the sequential digestion of these PA-sugar chains gave similar results, although the molecular size of peak-r was larger than that of peak-p, as shown in Fig. 9A and B. As shown in Fig. 9, peak-p and -r were converted to $Man_3Xyl_1GlcNAc_1$-PA by successive glycosidase digestions with diplococcal β-GlcNAc-ase, α1-4 specific fucosidase, β1-3 specific galactosidase and diplococcal β-GlcNAc-ase (second digestion). Furthermore, the α-fucosidase digests (from both peaks-p and -r) were converted to $Man_3Xyl_1GlcNAc_1$-PA by lacto *N*-biosidase, indicating the occurrence of the Galβ1-3GlcNAc unit instead of the Galβ1-4GlcNAc unit (Fig. 9A and B). In the case of peak-p, some glycosidase digests gave two peaks on the chromatograms, suggesting that two isomers bearing the Galβ1-3(Fucα1-4)GlcNAcβ1-2Manα1-6Man unit or the Galβ1-3(Fucα1-4)GlcNAcβ1-2Manα1-3Man unit occurred in peak-p and these two isomer were slightly separated on the Asahipak NH2-50 column. Considering all these data together, we proposed the structures of peaks-n, -p and -r, shown in Scheme 2. The results clearly indicated that there are two different pathways for the production of plant complex type FNGs in rice cells; one is acidic PNGase-associated pathway and the other one is a new ENGase-associated pathway.

Discussion

The predominant FNGs obtained from rice cell lysate (IC FNGs) and from culture medium (EC FNGs) differed in structural features, suggesting that at least two types of de-*N*-glycosylation machinery function at different locations in plant cells. The predominant FNGs inside the cells are typical high-mannose type structures with only one GlcNAc residue at the reducing end (GN1-FNGs), whereas those outside the cells (in the culture medium) are two kinds of plant complex type structures, GN1-FNGs and GN2-FNGs types. Furthermore, the complex type FNGs secreted into the EC spaces had a biantennary structure (some of them harboured the Lewis a epitope), whereas the complex type GN2-FNG in the IC fraction had a typical truncated type structure (M3FX). These results indicated that the complex type GN2-FNGs found in the EC fraction (culture medium) were released from secreted type glycoproteins that had already been processed in the golgi apparatus once and secreted into the EC space but not transported into the vacuole. In the case of animal cells, it is commonly believed that FNGs formed in the cytosol are sorted into the lysosome and degraded to monosaccharides after some trimming of mannosyl residues by the cytosolic α-mannosidase (*1–4*). The occurrence of the plant complex type GN1-FNGs in the EC spaces, however, suggests that another machinery for the secretion of FNGs formed in the cytosol to EC spaces may function in plant cells.

As shown in Table II, in the IC fraction (inside cells), the amount of high-mannose type GN1-FNGs ($Man_{9\text{-}5}GlcNAc_1$ and $Glc_1Man_{7\text{-}8}GlcNAc1$) was 10.15 nmol/g (339.0 nmol/33.4 g cells) and that of plant complex type GN2-FNGs (M3FX) was 0.94 nmol/g (31.4 nmol/33.4 g cells). As for the high-mannose type glycans, the cytosolic ENGase must be involved in their production from misfolded nascent glycoproteins. However, it is not clear whether the animal type cytosolic PNGase acts prior to the hydrolysis of FGNGs-GN2 by the ENGase. In contrast, the truncated plant complex type GN2-FNG (Man3XylFuc1GlcNAc2) that accounts for only ~10% of all IC FNGs must be released from vacuole-residing glycoproteins by the plant acid PNGase that can hydrolyse the truncated complex type glycans from glycopeptides or glycoproteins. The structural features and amounts of the IC FNGs of rice culture cells are comparable with those obtained from several other plant materials (*5–12*).

In the culture medium (EC fraction), various FNGs were found; two kinds of GN1-FNGs (high-mannose type and complex type) and two kinds of GN2-FNGs (a high-mannose type and a complex type) are shown in Schemes 1 and 2. Compared with the amount of complex type FNGs, the amount of high-mannose type FNGs in the culture medium was about one-twentieth as much. Although it is not clear whether the high-mannose type GN1-FNGs were secreted through the secretion pathway or artificially-recovered in the EC fraction, the presence of the high-mannose type GN2-FNGs that are not the cytosolic ENGase-products indicates that EC de-*N*-glycosylation machinery using the EC acidic PNGase works in the outside of plant cells. On the other hand, the biantennary complex type FNGs occurred at

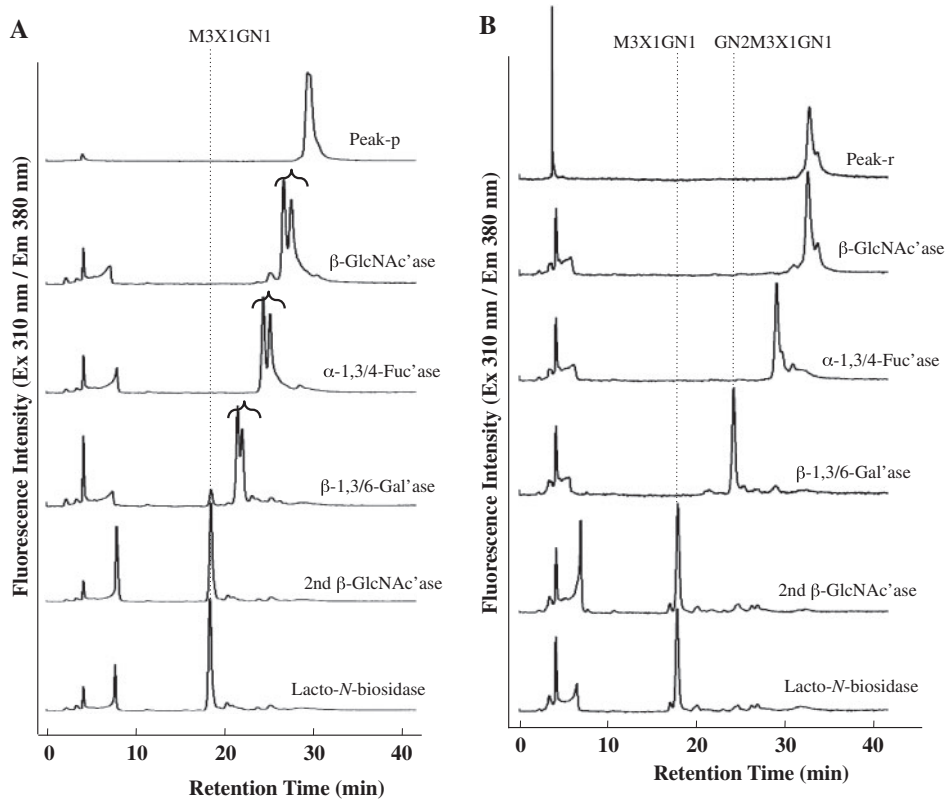
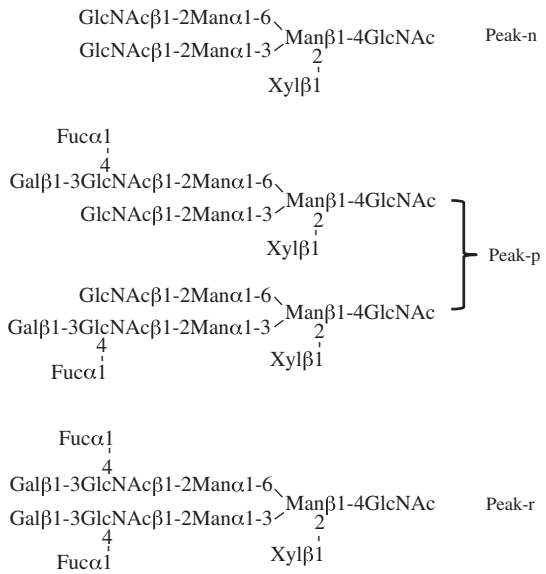


Fig. 9 SF-HPLC of *exo*- and *endo*-glycosidase digests of peaks-p and -r. (A) HPLC profile of glycosidase digests of peak-p. Peak-p was successively digested with diplococcal β-GlcNAc'ase, α-1, 3/4-specific fucosidase, β-1, 3/4-specific galactosidase and diplococcal β-GlcNAc'ase (second digestion). The α-fucosidase digest was digested by lacto *N*-biosidase. (B) HPLC profile of glycosidase digests of peak-r. Peak-r was successively digested with diplococcal β-GlcNAc'ase, α-1, 3/4-specific fucosidase, β-1, 3/6-specific galactosidase and diplococcal β-GlcNAc'ase (second digestion). The α-fucosidase digest was digested by lacto *N*-biosidase. Dotted lines indicate the elution positions of authentic PA-sugar chains ($Man_3Xyl_1GlcNAc_1$-PA(M3X1GN1) and $GlcNAc_2Man_3Xyl_1GlcNAc_1$-PA(GN2M3X1GN1).



Scheme 2 Proposed structure of complex type GN1-FNGs (peaks-n, -p, and -r) produced by rice cells.

comparable amounts to the IC high-mannose type FNGs, and one of them contained the Lewis a epitope, suggesting that these complex type GN2-FNGs were released from EC or secreted plant glycoproteins, such as pollen allergens or peroxidase (*29–32*), but not from vacuolar glycoproteins. The occurrence of two different kinds of complex type FNGs, the truncated type and biantennary type, indicates that the acidic PNGases may be constantly expressed in the different space of plant tissues; one is expressed in IC acidic organelle (vacuole) to release the truncated type glycans and the other is expressed in EC space (cell wall or apoplast) to release the biantennary complex type glycans (including the Lewis a epitope-containing ones).

In this study, we found new type of plant complex typeGN1-FNGs, indicating an involvement of ENGase activity. The occurrence of complex type GN1-FNG species in the culture medium (the EC space) suggested that new machinery involved in the production of FNGs might work in plant tissues. One possible explanation is that a quite new ENGase that can hydrolyse the chitobiosyl linkage of plant complex type *N*-glycans with β1-2 xylose and/or α1-3 fucose residues were involved in the release of *N*-glycans. But in preliminary experiments using the plant complex type *N*-glycans and a crude extract prepared from rice cells, we could not detect any ENGase activity towards the plant complex type *N*-glycans containing β1-2 xylosyl and α1-3 fucosyl residues, indicating that such new ENGase with unique substrate specificity does not occur in plants. At this moment, however, we cannot exclude a possibility of occurrence of

Table II. Summary of FNGs produced by rice culture cells.

HPLC-Peaks	Structure		IC fraction nmol (33.4 g total cell)	(%)	EC fraction nmol/770 ml medium	(%)
Peak-a	$Man_5GlcNAc_1$	(M5′)	26.05	7.2	0.36	0.2
Peak-b	$Man_6GlcNAc1$	(M6′)	70.47	19.4	0.90	0.4
Peak-c	$Man_7GlcNAc_1$	(M7′)	95.86	26.4	1.61	0.8
Peak-d	$Man_8GlcNAc_1$	(M8')	55.44	15.3	1.76	0.9
	$Glc_1Man_7GlcNAc_1$	(Glc1M7′)	69.47	19.2	0.76	4.0
Peak-e	$Glc_1Man_8GlcNAc_1$	(Glc1M8′)	10.69	2.9	Trace	Trace
	$Man_9GlcNAc_1$	(M9′)	3.34	0.9	0.68	0.3
	High mannose type (GN1-type)		331.32	91.3	6.18	3.0
Peak-h	$Man_5GlcNAc_2$	(M5)	ND	–	0.09	Trace
Peak-i	$Man_6GlcNAc_2$	(M6)	ND	–	2.27	1.1
Peak-j	$Man_7GlcNAc_2$	(M7)	ND	–	1.93	1.0
Peak-k	$Man_8GlcNAc_2$	(M8)	ND	–	0.69	0.3
Peak-l	$Man_9GlcNAc_2$	(M9)	ND	–	0.14	0.1
	High mannose type (GN2-type)		–	–	5.12	2.5
Peak-n	$GlcNAc_2Man_3Xyl_1GlcNAc_1$		ND	–	71.54	35.9
Peak-p	$Gal_1Fuc_1GlcNAc_2Man_3Xyl_1GlcNAc_1$		ND	–	49.30	24.7
Peak-r	$Gal_2Fuc_2GlcNAc_2Man_3Xyl_1GlcNAc_1$		ND	–	20.71	10.4
	Plant complex type (GN1-type)		–	–	141.55	71.0
Peak-m	$Man_3Fuc_1Xyl_1GlcNAc_2$		31.40	8.7	ND	–
Peak-o	$GlcNAc_2Man_3Fuc_1Xyl_1GlcNAc_2$		ND	–	29.63	15.0
Peak-q	$Gal_1Fuc_1GlcNAc_2Man_3Fuc_1Xyl_1GlcNAc_2$		ND	–	16.98	8.5
	Plant complex type (GN2-type)		31.40	8.7	46.61	23.5

ND, not detected.

plant specific chitobiase-like activity, which can hydrolyse the chitobiosyl linkage in the GlcNAcβ1-4(Fucα1-3)GlcNA unit and requires the hemiacetal linkage of reducing end GlcNAc residue in *N*-glycans. Another possible explanation is that the high-mannose type FNGs (GN1-FNGs) produced by the cytosolic ENGase were translocated into the ER and then transported to the golgi apparatus together with well-folded glycoproteins and processed into complex type *N*-glycans *en route* to the EC space. In this case, we can postulate a new retrograde transportation mechanism for the high-mannose type FNGs from the cytosol back into the ER, assuming that not all the FNGs produced in the cytosol are degraded into monosaccharides in the vacuole or other digestive organelle similar to the lysosome in animal cells. In previous papers (*5–12*), however, such complex type GN1-FNGs have not been found in fruits, seeds, leaves or hypocotyls, suggesting that the biantennary complex type GN1-FNGs would be degraded rapidly by a combination of several glycosidases in the EC spaces.

As for complex type FNGs formed in animal cells, Ishizuka *et al.* (*40*) found sialylated complex type GN1-FNGs (NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAc) in stomach cancer cells (MKN7 and MKN45) and postulated that they were formed in the lysosome and leaked out into the cytosol due to the low integrity of the lysosomal membrane in these cancer cells. In animal cells, lysosome contains chitobiase that hydrolyses the chitobiosyl unit in animal complex type *N*-glycans having no core α1-6 fucosyl residue (*41*), therefore, the sialylated complex type GN1-FNGs might be formed from the animal complex type GN2-FNGs lacking the core α1-6 fucosyl residue by the lysosomal chitobiase. But plant biantennary complex type *N*-glycans usually contains the core α1-3 fucosyl residue and are resistant to the acidic chitobiase. Furthermore, the plant biantennary complex type *N*-glycans bearing the Lewis a epitope are always linked to the secreted glycoproteins but not to the vacuolar proteins (*28*). Therefore, it can be assumed that the biantennary plant complex type GN1-FNGs might not be leaked out from the vacuole. To confirm our hypothesis that biantennary plant complex type GN1-FNGs may be formed from high-mannose type GN1-FNGs via the retrotranslocation from the cytosol to the ER and the *N*-glycan processing in the golgi apparatus, it is necessary to check if the generation of the complex type GN1 glycans is suppressed by inhibition of the cytosolic PNGase and/or ENGase activities.

Even if the putative ER/golgi-mediated processing of high-mannose type FNGs is the case, it is unclear whether the translocation of high-mannose type GN1-FNGs formed in the cytosol to the ER or golgi apparatus is of physiological significance. But it is worthwhile to consider the possibility of chaperone-like functions of *N*-glycans themselves as reported earlier (*42–46*). It has been suggested that the high-mannose type *N*-glycans linked to some glycoproteins [ribonuclease (*42*), soybean agglutinin (*44*) or Jack bean α-mannosidase (*45*)] could stimulate protein folding or assembly to form functional oligomeric structures after their denaturation. Furthermore, Asn-glycopeptides (*43*, *46*) or high-mannose type FNGs (*47*) could also stimulate the folding of non-glycosylated proteins (such as lysozyme and α-lactalbumine) and the functional assembly of oligomeric structures of deglycosylated α-mannosidase,

indicating that free oligosaccharides can play a chaperone-like role. These results provide a hypothetical physiological function of GN1-FNGs that impinges upon protein folding or assembly in the ER quality control (ERQC) system. If the high-mannose type GN1-FNGs ($Man_{9-5}GlcNAc_1$) are translocated and concentrated in the ER, these free oligosaccharides can stimulate the self-reliant folding or assembly of glycosylated or non-glycosylated proteins even if the chaperone proteins are absent. The high-mannose type GN1-FNGs in the ER may not compete against partially folded nascent glycoproteins for the ER-lectins (such as calnexin, calreticulin, OS9), since these sensor lectins usually recognize both *N*-glycans and unfolded polypeptide moieties at the same time (*48*). After the protein folding or assembly are completed with the aid of chaperone proteins, when GN1-FNGs are transported to the golgi apparatus together with well-folded proteins, they are processed into the plant complex type structures as well as the conjugated *N*-glycans on secreted type glycoproteins. To clarify the physiological significance of GN1-FNGs involved in ERQC, we are preparing double knock-out *Arabidopsis* plants, in which the genes of ENGase and PNGase are impaired.

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Conflict of interest
None declared.

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