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Free *N*-Glycans Already Occur at an Early Stage of Seed Development[1]

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As a part of our studies to elucidate the physiological significance of free *N*-glycans in differentiating or growing plant cells, we first demonstrate that two kinds of free *N*-glycans already occur at an early stage of seed development. In this report, we used the developing *Ginkgo biloba* seeds as a model plant, since we have already revealed a functional feature of the *Ginkgo* endo-β-*N*-acetylglucosaminidase and structural features of *N*-glycans linked to storage glycoproteins in the developing seeds [Kimura, Y. *et al.* (1998) *Biosci. Biotechnol. Biochem.* 62, 253–261; Kimura, Y. and Matsuo, S. (2000) *Biosci. Biotechnol. Biochem.* 64, 562–568]. The structures of free *N*-glycans, which were determined by a combination of ESI-MS, sequential α-mannosidase digestions, partial acetolysis, and two dimensional sugar chain map, fell into two categories. One dominant species is a high-mannose type structure having one GlcNAc residue at the reducing end ($Man_{9\text{-}5}GlcNAc_1$). The concentration of this type of free glycan (as the pyridylaminated derivatives) is about 2.2 nmol in 1 g fresh weight. The detailed structural analysis revealed that the high-mannose type structures have a common core unit; Manα1-6(Man1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAc. The other minor species of free *N*-glycans is the plant complex type structure having an *N*-acetylchitobiose unit at the reducing end ($Man_3Xyl_1Fuc_1GlcNAc_2$). The concentration of this type of free glycan (as the pyridylaminated derivative) was about 75 pmol in 1 g fresh weight.

Key words: free *N*-glycan, *Ginkgo biloba*, *N*-glycan metabolism, plant endo-β-*N*-acetylglucosaminidase, seed development.

Free *N*-glycans have been found in various plant cells, especially in developing or growing cells (*1–6*). As part of a study to elucidate the physiological significance of such free *N*-glycans in the growth or development of plant cells, we previously purified and characterized several endo-β-*N*-acetylglucosaminidases (endo-β-GlcNAc-ase) (*8–10*) and one peptide:*N*-glycanase (PNGase) from various plant materials (*11*), which must be involved in the production of free oligosaccharides, and revealed their detailed substrate specificities. Our detailed analysis of the substrate specificity showed that plant endoglycosidase is highly active towards the high-mannose type *N*-glycans bearing the Manα1-2Manα1-3Manβ1- structural unit (*8–10*). Furthermore, we revealed the structural features of free *N*-glycans occurring in various plant cells (*4–7*); the high-mannose-type free glycans always have only one GlcNAc residue at the reducing end, while the plant complex type free glycans have an *N*-acetylchitobiose unit. These structural features clearly suggest that the former structures must be produced by endo-β-GlcNAc-ase and PNGase must produce the latter structures, although the endogenous substrates for the two enzymes remain obscure.

Concerning the endogenous substrate(s) for the plant endo-β-GlcNAc-ase, we have revealed the following facts using the seeds of *Ginkgo biloba* as a plant model. (i) The storage glycoproteins in mature *Ginkgo* seeds bear only xylose-containing *N*-glycans, although the endoglycosidase (endo-GB) occurs in the same seeds (*10, 12*). (ii) There are no glycoproteins with high-mannose type *N*-glycans even during early stages of seed development (*13*). This circumstantial evidence suggests that *Ginkgo* endoglycosidase (endo-GB) already functions to produce the putative bioactive free glycans at the stage of seed development, but not at the stage of seed germination.

To confirm our working hypothesis, we surveyed the occurrence of free *N*-glycans in *Ginkgo* seeds at early stages of seed development, and analyzed the structures of the actually occurring free glycans in detail. In this report, we first show that the high-mannose type and plant complex type free *N*-glycans already occur during the early stages of seed development, showing that plant endo-β-*N*-acetylglucosaminidase works during seed development in addition to during shoot development (*4–7*).

MATERIALS AND METHODS

Materials—Developing seeds of *G. biloba* were collected

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Abbreviations: endo-β-GlcNAc-ase, endo-β-*N*-acetylglucosaminidase; endo-GB, endo-β-*N*-acetylglucosaminidase from *Ginkgo biloba*; PNGase, peptide:*N*-glycanase; PA-, pyridylamino; RP-HPLC, reverse-phase HPLC; SF-HPLC, size-fractionation HPLC; ESI-MS, electrospray ionization mass spectrometry; MS/MS, tandem mass; Con A, convanavalin A; Man, D-mannose; Xyl, D-xylose; Fuc, L-fucose; GlcNAc, *N*-acetyl-D-glucosamine; M3FX, Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA.

on the campus of Okayama University during the first week of July, 1997. A Cosmosil 5C18-AR column (0.60 × 25 cm) was purchased from Nacalai Tesque, and a Shodex Asahipak NH2P-50 column (0.46 × 25 cm) from Showa Denko. α-Mannosidase (jack bean) was purchased from Sigma. α-1,2-Mannosidase (*Aspergillus oryzae*) was a generous gift from Dr. T. Yoshida (Tohoku University) (*14*). M3FX was prepared from ricin D (*15*). Manβ1-4GlcNAc-PA was derived from Man6GlcNAc1-PA, which was purified from the stems of pea seedlings, by α-mannosidase digestion (*4*). ConA was purified from jack bean meal by the method of Agrawall and Goldstein (*16*). Con A Sepharose 4B was prepared from CNBr-activated Sepharose 4B according to Axèn *et al.* (*17*).

Preparation of the Oligosaccharide Fraction from Developing G. biloba Seeds—Developing *G. biloba* seeds (411.7 g) were homogenized in acetone (3 liters), and the resulting defatted powder (119.3 g) was suspended in 500 ml of 25 mM Tris-HCl buffer (pH 8.5). The suspension was dialyzed against deionized water (2 liters twice) and the resulting outer solution (4 liters) was concentrated to about 25 ml in a rotary evaporator. The concentrated outer solution was desalted by successive passages through a Dowex 50 × 2 column (3.0 × 15 cm), a Dowex 1 × 2 column (3.0 × 15 cm), and a Sephadex G-25 column (1.8 × 180 cm, in 50 mM NH_4OH). The eluent fractions from the Sephadex G-25 column (elution volume: 150 to 400 ml), which were confirmed to contain the oligosaccharides by ESI-MS analysis, were pooled. The oligosaccharide-containing fractions were also examined by the phenol–sulfuric acid method (*18*). The resulting salt-free oligosaccharide fraction was lyophilized.

Pyridylamination of Free N-Glycans—Pyridylamination of free *N*-glycans was done by the method of Kondo *et al.* (*19*). After pyridylamination, the excess 2-aminopyridine was removed by phenol/chloroform extraction as described by Tokugawa *et al.* (*20*). The resulting PA-derivatives were partially purified by passage through a Sephadex G-25 superfine column (1.8 × 180 cm) in 50 mM NH_4OH. The pyridylaminated oligosaccharide-containing fractions were monitored by a Fluorescence Spectrometer (Excitation 310 nm, Emission 400 nm, Hitachi 650 10S) and by the phenol-sulfuric acid method (*18*).

Con A–Sepharose Chromatography—PA-derivative that were partially purified by passage through a Sephadex G-25 column were evaporated to dryness. The resulting residue was dissolved in 25 mM Tris-HCl, pH 7.8, containing 0.1 M NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$ (5 ml) and applied to a Con A–Sepharose 4B column (2.2 × 33 cm) equilibrated with the same buffer. After washing the column with about 250 ml of the same buffer, the Con A–bound [Con A (+)] PA-sugar chains were eluted by the addition of 0.3 M methyl-α-mannoside (Fig. 1). The PA-sugar chains in each fraction were monitored by a fluorescence spectrophotometer (Excitation 310 nm, Emission 400 nm, Hitachi 650 10S). The Con A (+) fraction was concentrated and desalted by gel-filtration through a Sephadex G-10 column (2.5 × 40 cm) in 50 mM NH_4OH. The PA-derivatives obtained by gel-filtration were further separated by HPLC on a Jasco 880-PU HPLC apparatus equipped with a Jasco 821-FP Intelligent Spectrofluorometer, using a Cosmosil 5C18-AR column (0.6 × 25 cm) or a Shodex Asahipak NH2P-50 column (0.46 × 25 cm) as described in our previous paper (*4*).

α-Mannosidase Digestions—Jack bean α-mannosidase digestion was done using about 100 pmol of PA-sugar chains and 0.4 U of enzyme under the conditions described in our previous report (*4*). Digestions with *Aspergillus* α-1,2-mannosidase (15 μg enzyme) were done in 50 ml of 0.1 M sodium acetate buffer, pH 5.0, for 4 h at 37°C. The reaction was stopped by boiling for 2 min and a part of the digest was analyzed by SF-HPLC. For analyses of the mannosidase digests, the PA-sugar chains were eluted by linearly increasing the water content in the water–acetonitrile mixture from 20 to 47% at a flow rate of 0.8 ml/min.

Electrospray Ionization (ESI-) Mass Spectrometry—The mass spectrometer used was a Perkin Elmer Sciex API-III, triple-quadrupole mass spectrometer with an atmospheric-pressure ionization ion source (*21*). 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 approximately 10 pmol/μl and introduced into the electrospray needle by mechanical infusion through a micro syringe at a flow rate of 5 μl/min (*21*). The collisionally activated dissociation (CAD) spectrum was measured with argon as the collision gas at a collision energy of 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.

Partial Acetolysis—Partial acetolysis was done with about 10 nmol of PA-oligosaccharide essentially according to Natsuka *et al.* (*22*). Fragments with GlcNAc-PA residues were separated by SF-HPLC using the Shodex Asahipak NH2P-50 column (0.46 × 25 cm). The pyridylaminated oligosaccharides were eluted by linearly increasing the water content in the water–acetonitrile mixture from 20 to 47% at a flow rate of 0.8 ml/min. Each PA-oligosaccharide with the GlcNAc-PA residue was analyzed by ESI-MS spectrometry. For the analysis of fragments derived from the non-reducing end, we used a minor modification of this method as described below. The resulting fragments were deacetylated in the 0.2% sodium methoxide/methanol mixture at room temperature for 30 min. The deacetylated saccharides were pyridylaminated by the method of Kondo *et al.* (*19*) and the excess reagents were removed by the method of Tokugawa *et al.* (*20*). The resulting PA-oligosaccharides (Man_3-PA, Man_2-PA, Man-PA) were separated from the pyridylamination reagents by HPLC using a Superdex Peptide HR 10/30 column (Pharmacia Biotech). The PA-oligosaccharides were eluted in 3% acetic acid at a flow rate of 0.25 ml/min. The partially purified PA-oligosaccharides were further purified by SF-HPLC using the Shodex Asahipak NH2P-50 column (0.46 × 25 cm). The pyridylaminated oligosaccharides were eluted by linearly increasing the water content in the water–acetonitrile mixture from 0 to 20% at a flow rate of 0.8 ml/min. The PA-derivatives were analyzed by ESI-MS spectrometry.

RESULTS

Purification of PA-Oligosaccharides from Developing Ginkgo Seeds—PA-Derivatives partially purified by Sephadex G-25 superfine column chromatography were further fractionated by Con A affinity chromatography (Fig. 1). Since it has been revealed that the plant complex type free *N*-glycans can be purified from the non-absorbed fraction [Con A (−)] and the high-mannose type free *N*-glycans from the absorbed fraction [Con A (+)], we further purified the

two kinds of free glycans from the both fractions by RP-HPLC or SF-HPLC. The Con A (+) fractions were pooled as indicated by the horizontal bar.

Structural Analysis of Xylose-Containing Free N-Glycan—On the RP-HPLC profile of Con A (−) fraction (Fig. 2A), three PA-derivatives were obtained, F-I, F-II, and F-III. Each fraction was analyzed by ESI-MS and MS/MS spectrometry to confirm which fraction contained *N*-glycans. The MS/MS analysis of F-I and F-III showed that these PA-derivatives were not *N*-glycans (data not shown), since these PA-derivatives did not give a specific fragment ion at *m/z* 300.0 corresponding to GlcNAc-PA. Furthermore, the major signal found at *m/z* 1,045.5 in F-II (Fig. 2B) was also not *N*-glycan, since MS/MS analysis of this signal gave no daughter ion at *m/z* 300.0. However, a relatively small signal at *m/z* 1,268.0 in F-II (Fig. 2B) was confirmed to contain typical plant complex type *N*-glycans ($Man_3Xyl_1Fuc_1GlcNAc$-PA; M3FX) by MS/MS analysis (Fig. 2C). Every daughter signal was well assigned to fragment ions derived from M3FX as shown in Fig. 2C; *m/z* 1,121.5 for $[Man_3Xyl_1GlcNAc_2\text{-PA}+H]^+$, *m/z* 1,105.5 for $[Man_2Xyl_1Fuc_1GlcNAc_2\text{-PA}+H]^+$, *m/z* 990.0 for $[Man_3GlcNAc_2\text{-PA}+H]^+$, *m/z* 959.5 for $[Man_2Xyl_1GlcNAc_2\text{-PA}+H]^+$, *m/z* 827.0 for $[Man_2\text{-}GlcNAc_2\text{-PA}+H]^+$, *m/z* 665.0 for $[Man_1GlcNAc_2\text{-PA}+H]^+$, *m/z* 503.0 for $[GlcNAc_2\text{-PA}+H]^+$, *m/z* 446.0 for $[Fuc_1GlcNAc_2\text{-PA}+H]^+$, and *m/z* 300.0 for $[GlcNAc\text{-PA}+H]^+$.

After further purification of the plant complex type *N*-glycans by SF-HPLC, it was confirmed that the elution positions of the PA-sugar chain on both RP-HPLC and SF-HPLC corresponded exactly to that of M3FX [Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA]. These results indicate that the structure of the free *N*-glycan could be M3FX as shown in Scheme 1. The amount of plant complex type free glycan (as PA-derivative) was 75 pmol/g fresh weight of developing *Ginkgo* seeds (Table I).

Structural Analysis of High-Mannose Type Free N-glycans—Several high-mannose type free *N*-glycans were purified from the Con A (+) fraction by SF-HPLC using a Shodex Asahipak NH2P-50 column. As shown in Fig. 3-[A]

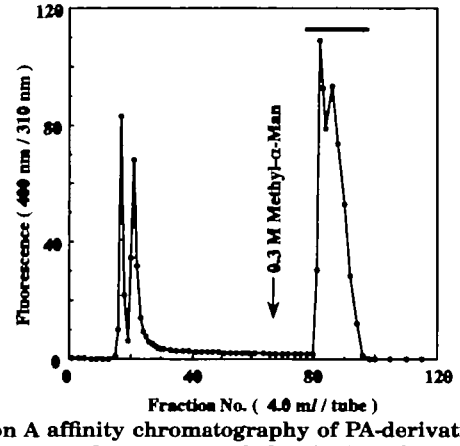


Fig. 1. **Con A affinity chromatography of PA-derivatives prepared from crude extracts of developing *Ginkgo biloba* seeds.** After desalting on Sephadex G-10, the mixture of pyridylaminated oligosaccharides was fractionated on a Con A Sepharose 4B column (2.2 × 33 cm). After washing the column with 20 mM Tris-HCl, pH 7.8, containing 0.1 M NaCl, 5 mM $CaCl_2$, and 5 mM $MgCl_2$, the adsorbed PA-sugar chains were eluted with 0.3 M methyl-α-mannoside. The non-adsorbed fraction was designated Con A (−) and the adsorbed fraction was designated Con A (+).

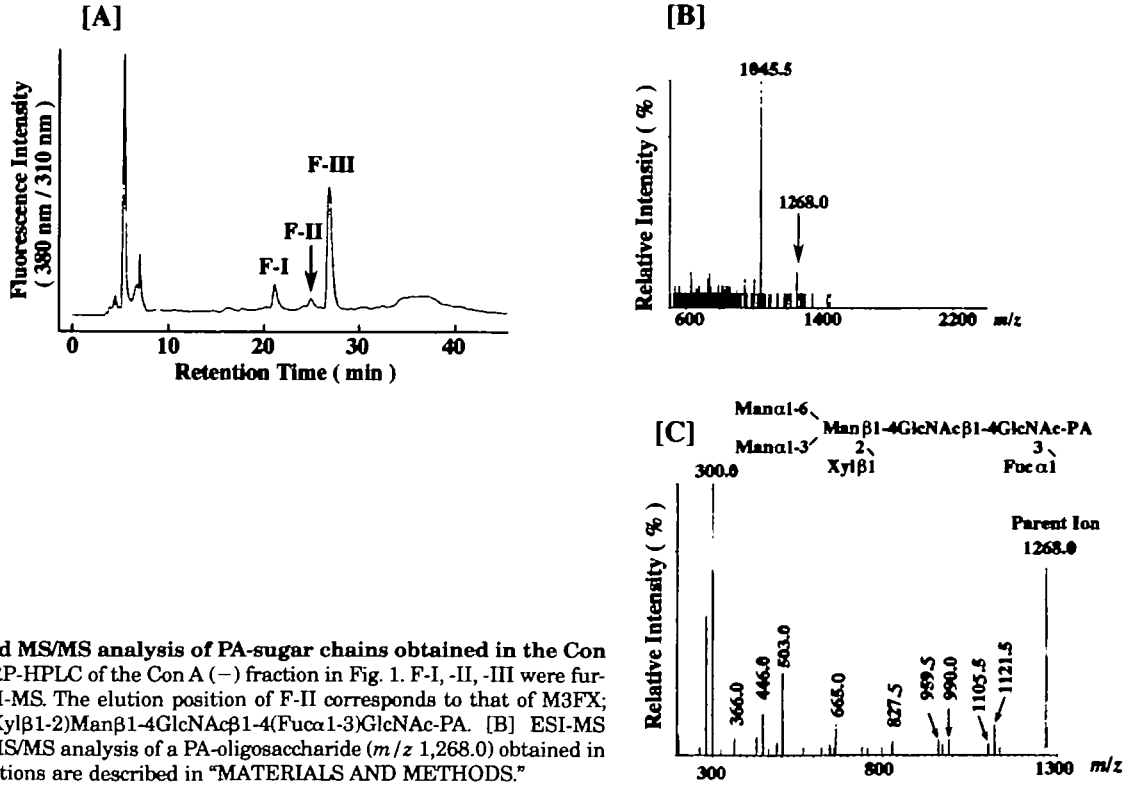
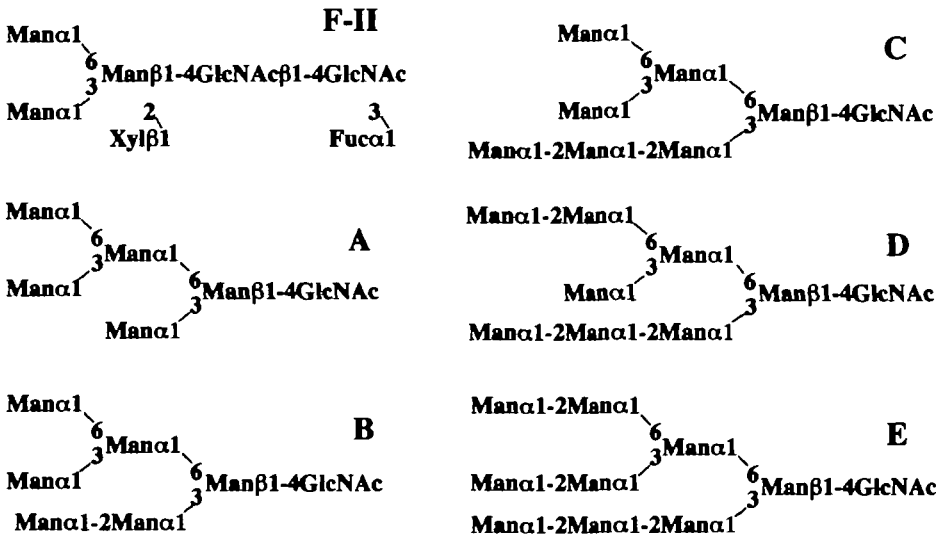


Fig. 2. **RP-HPLC and MS/MS analysis of PA-sugar chains obtained in the Con A (−) fraction.** [A] RP-HPLC of the Con A (−) fraction in Fig. 1. F-I, -II, -III were further analyzed by ESI-MS. The elution position of F-II corresponds to that of M3FX; Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA. [B] ESI-MS analysis of F-II. [C] MS/MS analysis of a PA-oligosaccharide (*m/z* 1,268.0) obtained in F-II. Analytical conditions are described in "MATERIALS AND METHODS."

and -[B]-I, main five PA-sugar chains were purified; PA-sugar chain A, m/z 1,110.5 ($[Man_5GlcNAc_1\text{-}PA+H]^+$); PA-sugar chain B, m/z 1,272.5 ($[Man_6GlcNAc_1\text{-}PA+H]^+$); PA-sugar chain C, m/z 1,435.0 ($[Man_7GlcNAc_1\text{-}PA+H]^+$) and m/z 718.0 ($[Man_7GlcNAc_1\text{-}PA+2H]^{2+}$); PA-sugar chain D, m/z 1,597.0 ($[Man_8GlcNAc_1\text{-}PA+H]^+$) and m/z 799.0 ($[Man_8GlcNAc_1\text{-}PA+2H]^{2+}$); PA-sugar chain E, m/z 880.5 ($[Man_9GlcNAc_1\text{-}PA+2H]^{2+}$). The two minor peaks appeared just after PA-sugar chains D and E with the same molecular masses as D and E. The results of MS/MS analysis of these pyridylaminated free *N*-glycans are summarized in Table II. Every fragment ion could be reasonably assigned as daughter ions derived from each parent ion. The four PA-sugar chains (B, C, D, and E) were converted to PA-sugar chain A [m/z 1,110.5 ($[Man_5GlcNAc_1\text{-}PA+H]^+$)] by α-1,2-mannosidase digestion, whereas A was resistant to digestion (Fig. 3-[B]-II). By successive α-mannosidase (jack bean) digestion, the product was converted completely to $Man_1GlcNAc_1$-PA (m/z 462.0 $[M+H]^+$) (Fig. 3-[B]-III).

TABLE I. **Amount of free *N*-glycans in developing *Ginkgo* seeds.**

Structure	pmol/g fresh weight	(%)
$Man_5GlcNAc_1$	102	5
$Man_6GlcNAc_1$	168	7
$Man_7GlcNAc_1$	277	12
$Man_8GlcNAc_1$	1,221	54
$Man_9GlcNAc_1$	415	19
$Man_3Fuc_1Xyl_1GlcNAc_2$	75	3



Scheme 1. **Proposed structures of free *N*-glycans occurring in developing *Ginkgo biloba* seeds.**

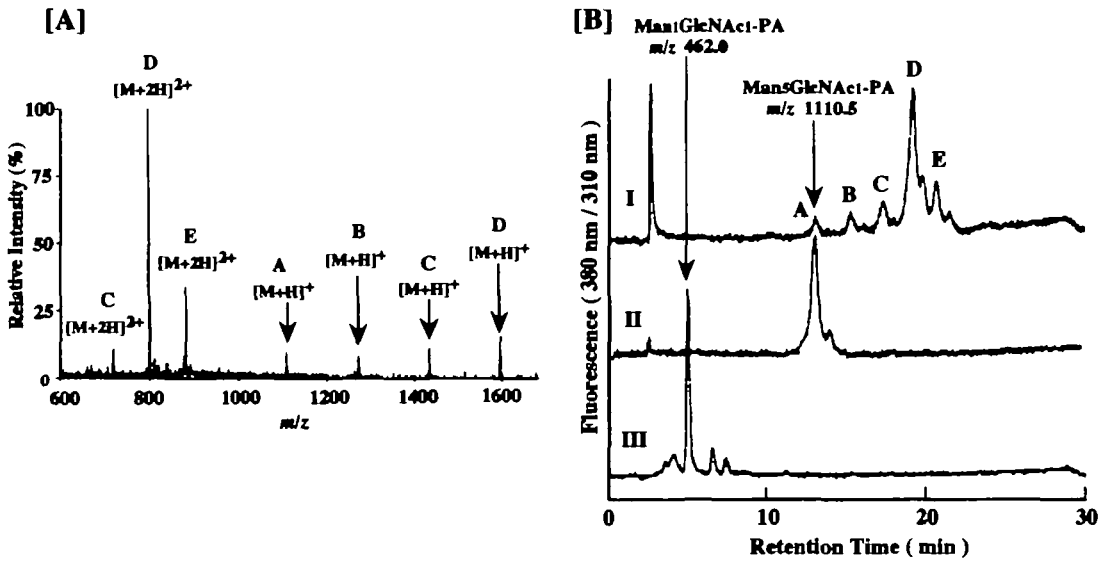


Fig. 3. **ESI-MS analysis and sequential α-mannosidase digestions of PA-sugar chains in the Con A (+) fraction in Fig. 1.** [A] ESI-MS analysis of PA-sugar chains obtained in the Con A (+) fraction. PA-sugar chains C, D, and E were observed as double charged ions $[M+H]^{2+}$. [B] α-1,2-Mannosidase (*Aspergillus oryzae*) and α-mannosidase (jack bean) of PA-sugar chains obtained in the Con A (+) fraction. I, Con A (+) fraction; II, α-1,2-mannosidase digest of I; III, α-mannosidase digest of II.

TABLE II. **MS/MS analysis of pyridylaminated free *N*-glycans in developing *Ginkgo* seeds.**

Fragment ions $[M+H]^+$	Peak name on SF-HPLC				
	A	B	C	D	E
$GlcNAc_1$-PA (300.0)	++++	++++	++	+++	+++
$Man_1GlcNAc_1$-PA (462.0)	+++	+++	++	++	++
$Man_2GlcNAc_1$-PA (624.0)	++	++	+	+	+
$Man_3GlcNAc_1$-PA (786.0)	++	+++	++	+	+
$Man_4GlcNAc_1$-PA (948.0)	+	++	++	++	++
$Man_5GlcNAc_1$-PA (1,110.5)	++	++	++	+	+
$Man_6GlcNAc_1$-PA (1,272.5)	ND	+++	++	+	++
$Man_7GlcNAc_1$-PA (1,435.0)	ND	ND	++++	+	+
$Man_8GlcNAc_1$-PA (1,597.0)	ND	ND	ND	++++	+
$Man_9GlcNAc_1$-PA (1,758.5)	ND	ND	ND	ND	++++

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

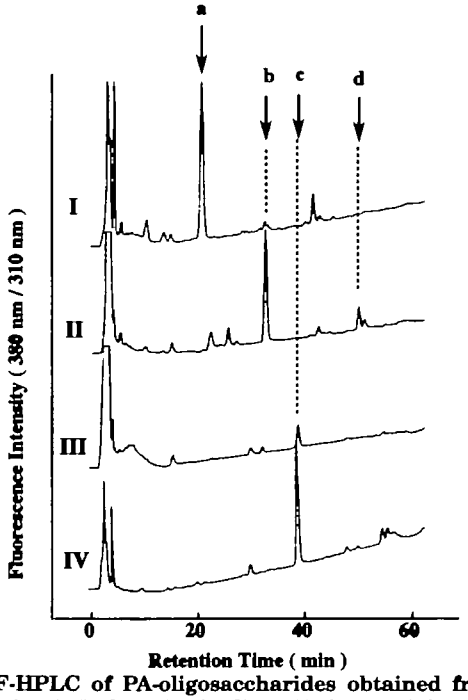


Fig. 4. **SF-HPLC of PA-oligosaccharides obtained from PA-sugar chains A, B, C, and D in Fig. 3-[A] by partial acetolysis.** Fragments containing GlcNAc-PA were analyzed on a Shodex Asahipak NH2P-50 column. I, PA-sugar chain A; II, PA-sugar chain B; III, PA-sugar chain C; IV, PA-sugar chain D. The arrows indicate the elution positions of: a, $Man_2GlcNAc_1$-PA (m/z 624.0); b, $Man_3GlcNAc_1$-PA (m/z 786.0); c, $Man_4GlcNAc_1$-PA (m/z 948.5); d, $Man_6GlcNAc_1$-PA (m/z 1,272.5). ESI-MS and MS/MS analyses of all other minor peaks showed that these peaks were not *N*-glycans.

These results clearly indicate that four PA-sugar chains (PA-sugar chains B, C, D, and E) are typical high-mannose type *N*-glycans with α-1,2-mannosyl residue(s). Since PA-sugar chain A was not digested by α-1,2-mannosidase, this PA-sugar chain contains no α-1,2-mannosyl residue. From these results (ESI-MS, MS/MS, and the sequential α-mannosidase digestions), PA-sugar chain A is assumed to be Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAc, and PA-sugar chain E could be non-trimmed high-mannose type *N*-glycan [Manα1-2Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc] (Scheme 1).

To clarify the branching structures of PA-sugar chains A,

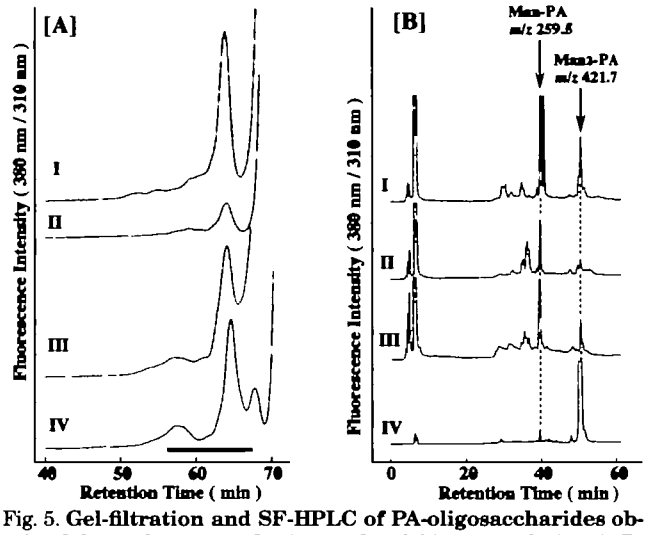


Fig. 5. **Gel-filtration and SF-HPLC of PA-oligosaccharides obtained from the non-reducing ends of PA-sugar chains A, B, C, and D by partial acetolysis.** I, PA-sugar chain A; II, PA-sugar chain B; III, PA-sugar chain C; IV, PA-sugar chain D. [A] PA-oligosaccharides containing GlcNAc-PA obtained by partial acetolysis were analyzed on a Superdex Peptide HR 10/30 column eluted with 3% acetic acid. The pyridylaminated fragments were pooled as indicated by the horizontal bars. [B] The partially purified fragments were further analyzed by SF-HPLC. Every peak observed on the chromatograms was further analyzed by ESI-MS and MS/MS (with a step size of 0.1 Da). The ESI-MS and MS/MS analyses of a peak eluted at just after Man-PA in I showed that this peak was not oligosaccharide.

B, C, D, and E, the PA-sugar chains were subjected to the partial acetolysis. As shown in Fig. 4, PA-sugar chain A gave a fragment corresponding to $Man_2GlcNAc_1$-PA ($[M+H]^+$: m/z 624.0); PA-sugar chain B gave a fragment corresponding to $Man_3GlcNAc_1$-PA ($[M+H]^+$: m/z 786.0); and PA-sugar chains C and D gave a fragment corresponding to $Man_4GlcNAc_2$-PA ($[M+H]^+$: m/z 948.5).

Furthermore, the fragments derived from the non-reducing end were analyzed as shown in Fig. 5. The pyridylaminated oligosaccharides were partially purified by gel-filtration using the Superdex Peptide HR column (Fig. 5-[A]). The PA-oligosaccharide fractions indicated by the horizontal bars in Fig. 5-[A] were further purified by SF-HPLC and each purified peak was analyzed by ESI-MS (Fig. 5-[B]). From PA-sugar chains A, B, C, both Man-PA ($[M+H]^+$: m/z 259.5) and Man_2-PA ($[M+H]^+$: m/z 421.7) were obtained, while PA-sugar chain D gave mostly Man_2-PA ($[M+H]^+$: m/z 421.7). The results of partial acetolysis on PA-sugar chain A further support the deduced structure described above.

Considering these results (ESI-MS, MS/MS, α-mannosidase digestions, and partial acetolysis), the structures of PA-sugar chains B, C, D can be proposed as follows: Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAc for B, Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc for C, Manα1-2Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc for D (Scheme 1).

The amounts of these high-mannose type free *N*-glycans occurring in developing *Ginkgo* seeds are summarized in Table I.

DISCUSSION

In conclusion, we have first shown using developing *Ginkgo* seeds as a model plant that free *N*-glycans (both the plant complex type and high-mannose type) are already present during the early stages of seed development. This result suggests that plant endo-β-*N*-acetylglucosaminidase already works to release the potent bioactive oligosaccharide (*23*) during seed development in addition to during shoot development (*4–7*) and fruit ripening (*1*).

Comparing the amounts of high-mannose type free glycans ($Man_{9\text{-}5}GlcNAc_1$) and plant complex type free glycans ($Man_3Xyl_1Fuc_1GlcNAc_2$) in developing seeds, the amount of the former type structure was about 2.2 nmol/g, while the latter type was present at only 75 pmol/g (Table I). It is noteworthy that, at least using the extraction method described in this report, the amount of high-mannose type free *N*-glycans ($Man_{9\text{-}5}GlcNAc_1$) always overwhelms that of the plant complex type free glycans ($GlcNAc_{2\text{-}0}Man_3Xyl_{1\text{-}0}Fuc_{1\text{-}0}GlcNAc_2$) (*4–7*). However, at present, it is not clear whether this difference in the concentrations of the two kinds of free *N*-glycans reflects a difference in the potencies of their biological activities.

In our previous papers (*10*, *12*), we have already reported that *Ginkgo* endo-β-*N*-acetylglucosaminidase (endo-GB), which reacts preferentially with the high-mannose type *N*-glycans bearing the Manα1-2Manα1-3Manβ1- structural unit, is present in mature seeds, although the storage glycoproteins carry the complex type *N*-glycans but not the high-mannose type. Furthermore, we proved that *Ginkgo* seeds even during early developmental stages carry only the plant complex type glycans (*13*), suggesting that the endogenous substrate(s) for endo-GB may not be glycoproteins containing high-mannose type glycans. Considering this circumstantial evidence, we assume that the endogenous substrate(s) for plant endo-β-GlcNAc-ase may be small glycoconjugates such as dolichol-oligosaccharide intermediates or glycopeptides with high-mannose type glycans derived from misfolded glycoproteins rather than folded and intact glycoproteins with high-mannose type glycans.

As shown in Scheme 1, the high-mannose type free *N*-glycans in developing *Ginkgo* seeds have a common structural core unit, Manα1-6(Man1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAc, which is the same unit observed in high-mannose type *N*-glycans linked to numerous glycoproteins in both animal and plant cells. It is noteworthy that the high-mannose type free *N*-glycans purified from animal cells carry a common structural core unit, Manα1-6 (Manα1-2Man1-2Manα1-3)Manβ1-4GlcNAc (*24*, *25*), which is the same unit observed in high-mannose type *N*-glycans linked to dolichol-pyrophosphate that occur on the ER during *N*-glycan biosynthesis. At present, however, it is unclear whether the remarkable difference in the free *N*-glycan structures between animals and plants indicates a difference in some physiological functions.

As the next step to elucidate the physiological significance of plant endo-β-GlcNAc-ase and free *N*-glycans in plant cell differentiation or growth, it will be necessary to identify the endogenous substrate for endoglycosidase and to analyze the distribution of free glycans in plant cells.

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