



Structural features of *N*-glycans linked to glycoproteins expressed in three kinds of water plants: Predominant occurrence of the plant complex type *N*-glycans bearing Lewis a epitope



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ABSTRACT

The Japanese cedar pollen allergen (Cry j1) and the mountain cedar pollen allergen (Jun a1) are glycosylated with plant complex type *N*-glycans bearing Lewis a epitope(s) (Galβ1-3[Fucα1-4]GlcNAc-). The biological significance of Lewis a type plant *N*-glycans and their effects on the human immune system remain to be elucidated. Since a substantial amount of such plant specific *N*-glycans are required to evaluate immunological activity, we have searched for good plant-glycan sources to characterize Lewis a epitope-containing plant *N*-glycans. In this study, we have found that three water plants, *Elodea nuttallii*, *Egeria densa*, and *Ceratophyllum demersum*, produce glycoproteins bearing Lewis a units. Structural analysis of the *N*-glycans revealed that almost all glycoproteins expressed in these three water plants predominantly carry plant complex type *N*-glycans including the Lewis a type, suggesting that these water plants are good sources for preparation of Lewis a type plant *N*-glycans in substantial amounts.

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1. Introduction

It is well known that plant cells express plant specific complex type *N*-glycans bearing β1-2 xylosyl and/or α1-3 fucosyl residues on many glycoproteins [1–3]. The plant complex type *N*-glycans are also referred to as antigenic plant *N*-glycans, since these plant specific *N*-glycans stimulate the mammal immune system to produce antibodies [4–7]. Interestingly, it has been reported that these antigenic plant *N*-glycans occur on several pollen allergens including BG60 [8], Cup a1 [9], Jun a1 [10], Cry j1 [11], and Cha o1 [12]. Some of these pollen allergens carry the Lewis a epitope

(Galβ1-3[Fucα1-4]GlcNAcβ1-) at the non-reducing terminal of their *N*-glycans [9–11]. Furthermore, it has been reported that antigenic plant *N*-glycans serve as the carbohydrate determinants of plant allergies [4–6,13,14]. We found, however, that the core structure of antigenic plant *N*-glycan, Man₃Xyl₁Fuc₁GlcNAc₂ (M3FX), was not recognized as major IgE epitope among patients with Japanese cedar pollinosis [15]. In addition, the proliferation and the IL-4 production of Th2 cells derived from Japanese cedar pollinosis patients are inhibited by addition of M3FX, when the Th2 cells are stimulated with Japanese cedar pollen allergen Cry j1 [15]. This result suggests that M3FX in free form might have potential anti-pollinosis activity for treatment of cedar pollinosis. Therefore, we have established a new method to prepare large amounts of M3FX-Asn peptide from storage glycoproteins of *Ginkgo biloba* seeds, in which 103 mg of M3FX-Asn was able to be purified from 950 g defatted *Ginkgo* seed powder [16].

As mentioned above, the *N*-glycans of some pollen allergens and many other plant type glycoproteins are modified with the Lewis a epitope [9–11,17–19], but the immunological significance of Lewis a type plant *N*-glycans in the human immune system remains to be

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Abbreviations

RP-HPLC	reversed-phase HPLC
NP-HPLC	normal-phase HPLC
PA-	pyridylamino
Hex	hexose
HexNAc	N-acetylhexosamine
Pen	pentose
Deoxyhex	deoxyhexose
Man	D-mannose
GlcNAc	N-acetyl-D-glucosamine
Xyl	D-xylose
Fuc	L-fucose
M2FX	Man α 1-6(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA
M3FX	Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA
GN1M3FX	GlcNAc β 1-2Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA or Man α 1-6(GlcNAc β 1-2Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA
GN2M3FX	GlcNAc β 1-2Man α 1-6(GlcNAc β 1-2Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA
(Le ^a)GN1M3FX	Gal β 1-3(Fuc α 1-4)GlcNAc β 1-2Man α 1-6(GlcNAc β 1-2Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA or GlcNAc β 1-2Man α 1-6(Gal β 1-3(Fuc α 1-4)GlcNAc β 1-2Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA
(Le ^a)2M3FX	Gal β 1-3(Fuc α 1-4)GlcNAc β 1-2Man α 1-6(Gal β 1-3(Fuc α 1-4)GlcNAc β 1-2Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA

deciphered. Up to date, an effective method to prepare Lewis a type plant N-glycans in substantial amounts has not been established. Therefore, we have begun searching for a good plant-glycan source to determine the immunological activity of Lewis a type plant N-glycans. We have previously reported that various seaweeds, sea grass (*Zostera marina*), and many marine algae growing in the sea express high-mannose type N-glycans, but not plant complex type N-glycans [20]. In this study, we have found that three fresh water plants (*Elodea nuttallii*, *Egeria densa*, and *Ceratophyllum demersum*), which are readily found in rivers or lakes in Japan, produce glycoproteins bearing the Lewis a unit, suggesting these water plants are a good source of Lewis a type plant N-glycans.

2. Materials and methods

2.1. Materials

Elodea nuttallii was collected at an irrigation canal of Okayama City (Okayama Prefecture, Japan), and *Egeria densa* and *Ceratophyllum demersum* were collected at an irrigation canal of Kurashiki City (Okayama Prefecture, Japan). Sephadex® G-25 fine was purchased from GE Healthcare Japan (Tokyo), Shodex Asahipak NH2P-50 4E (0.46 × 25 cm) and Shodex Asahipak NH2P-50 10E columns (1.0 × 25 cm) from Showa Denko (Tokyo), Cosmosil 5C18-AR-II (0.6 × 25 cm) and Cosmosil 5C18-AR-II columns (1.0 × 25 cm) from Nacalai Tesque (Kyoto, Japan). M2FX, M3X, M3FX, GN1M3FX, GN2M3FX, (Le^a)GN1M3FX, and (Le^a)2M3FX were prepared as

described in our previous papers [10,11,21,22]. Pepsin from porcine gastric mucosa, recombinant β -1,3/6-galactosidase from *Xanthomonas manihotis* expressed in *E. coli*, and β -N-acetylglucosaminidase from Jack bean were purchased from Sigma-Aldrich (St. Louis, MO), and *Streptomyces* sp.142 α -1,3/4-fucosidase from Takara (Kyoto, Japan).

2.2. Preparation of glycopeptides from water plant glycoproteins

Each water plant (approximately 300 g) was washed with running water to remove mud and tiny organisms. Plant materials were homogenized in 5% formic acid with a blender and digested with 100 mg of pepsin at 37 °C for 48 h [20,23]. The peptic digests were filtered with gauze and the filtrates were centrifuged at 10,160 g for 20 min. The supernatants were concentrated to about 100 mL with a rotary evaporator and were dialyzed against 5 L of deionized water. The outer solutions were concentrated to about 50 mL with a rotary evaporator and applied to Dowex 50 × 2 resin to remove the neutral oligosaccharides. The adsorbed fractions containing glycopeptides were eluted with 0.1N NH₄OH and concentrated in a small amount of solution with a rotary evaporator. The glycopeptide-fractions were applied to a Sephadex® G-25 fine column (4.0 × 35 cm) equilibrated with 0.1N NH₄OH. The glycopeptides-fractions were detected by the Phenol-H₂SO₄ method [24], and were concentrated with a rotary evaporator and lyophilized (Fig. 1A).

2.3. Preparation of pyridylaminated N-glycans from glycoproteins in each water plant

N-Glycans were released by hydrazinolysis at 100 °C for 10 h out of the glycopeptides prepared from each water plant. The hydrazinolysate was N-acetylated with saturated sodium bicarbonate and acetic anhydride, and then the acetylated hydrazinolysate was desalted with Dowex 50 × 2 resin and a Sephadex® G-25 fine column (4.0 × 35 cm) equilibrated with 0.1N NH₄OH [16]. The oligosaccharides were pyridylaminated and then they were partially purified by the method of Natsuka and Hase [25].

2.4. Purification of pyridylaminated N-glycans

The PA-derivatives obtained by gel-filtration were evaporated to dryness. The resulting residues were dissolved in desalted water (300 μ L) and separated by HPLC on a Jasco 2080-PU HPLC apparatus equipped with a Jasco 920-FP Intelligent Spectrofluorometer (excitation 310 nm, emission 380 nm) using a Cosmosil 5C18-AR-II column (0.6 × 25 cm). The PA-sugar chains were eluted by increasing the acetonitrile concentration in 0.02% TFA linearly from 0 to 7% over 60 min at a flow rate 1.5 mL/min. The N-glycan-fractions were pooled from 35 min to 50 min and evaporated to dryness (Fig. 1B). The partially purified PA-sugar chains were further separated by NP-HPLC using a Shodex Asahipak NH2P-50 column (0.46 × 25 cm). The PA-sugar chains were eluted by increasing the water content in the water-acetonitrile mixture from 26 to 50% linearly over 40 min at a flow rate of 0.7 mL/min.

2.5. Exo-glycosidase digestion

Digestion of PA-sugar chains with α -1,3/4-fucosidase, β -1,3/6-galactosidase, and β -N-acetylglucosaminidase was performed using about 200 pmol of PA-sugar chains under the conditions described in a previous report [26]. Digestion was stopped by heating in a boiling water bath for 3 min. The resulting exo-

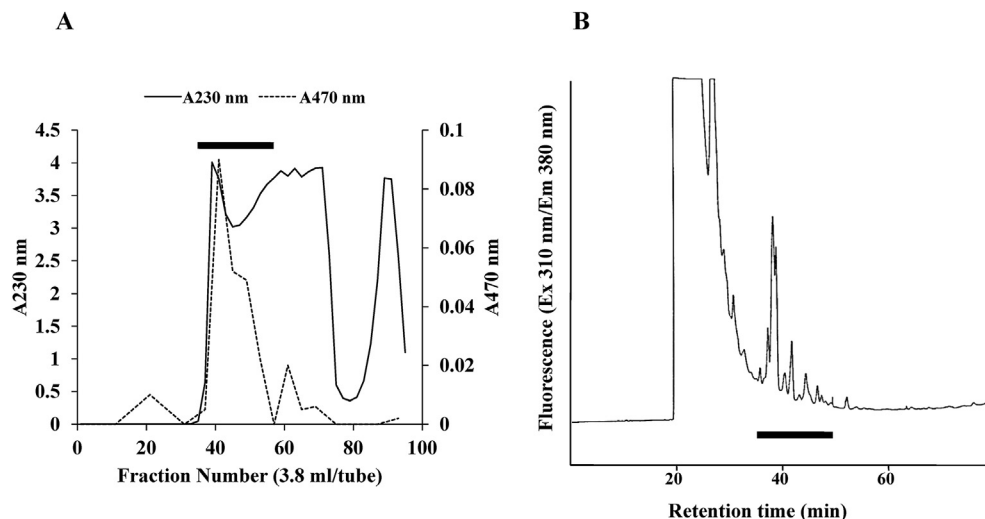


Fig. 1. Preparation of PA-Sugar Chains from Peptic Digest of Water Plant Glycoproteins. A, Gel-filtration of the peptic digest of glycoproteins using a Sephadex® G-25 fine column (4.0×35 cm) equilibrated with 0.1 N NH_4OH . The solid line indicates the elution of peptides (A230) and the dotted line indicates the elution of glycopeptides (A470). The glycopeptide-fraction was pooled, as indicated by horizontal bars, and was used for structural analysis of N-glycans. B, RP-HPLC separation of PA-labeled sugar chains. The N-glycan-fraction was pooled, as indicated by horizontal bars, and was applied to NP-HPLC.

glycosidase digests were centrifuged at 20,000 g for 5 min, and the supernatant was applied to NP-HPLC using an Asahipak NH2P-50 column. In the case of the analysis of exo-glycosidase digests, the PA sugar chains were eluted by increasing the water content in the water-acetonitrile mixture from 26 to 50% linearly over 40 min at a flow rate of 0.7 mL/min.

2.6. Electrospray ionization (ESI) mass spectrometry

LC/MS and MS/MS analyses of PA-oligosaccharides were performed using an Agilent 6500 series HPLC-Chip/QTOF-MS system equipped with a microwell-plate auto sampler (maintained at 10.0 °C), capillary sample loading pump, nanopump, HPLC-Chip interface, and an Agilent 6520 Q-TOF LC/MS, as described previously [16]. An Agilent Technologies porous

graphitized carbon (PGC)-Chip was used for separation of PA-sugar chains.

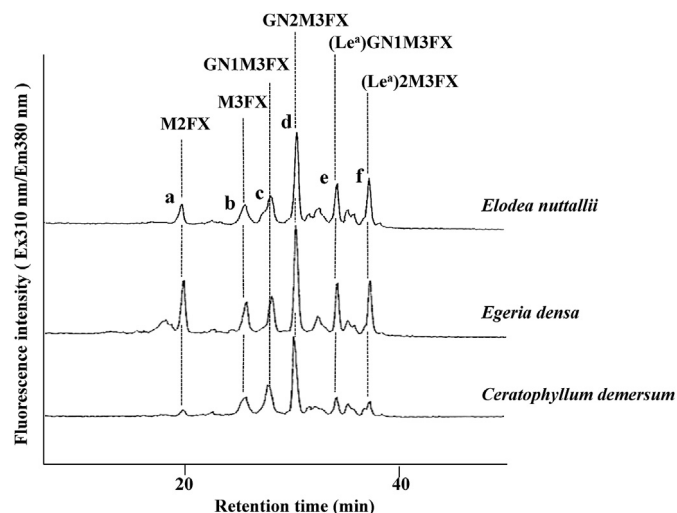


Fig. 2. NP-HPLC Profile of PA-Sugar Chains Prepared from Water Plant Glycopeptides. NP-HPLC separation of PA-sugar chains prepared from three kinds of water plants, *Elodea nuttallii*, *Egeria densa*, and *Ceratophyllum demersum*. Dotted lines indicate elution positions of authentic M2FX, M3FX, GN1M3FX, GN2M3FX, $(\text{Le}^a)\text{GN1M3FX}$, and $(\text{Le}^a)2\text{M3FX}$.

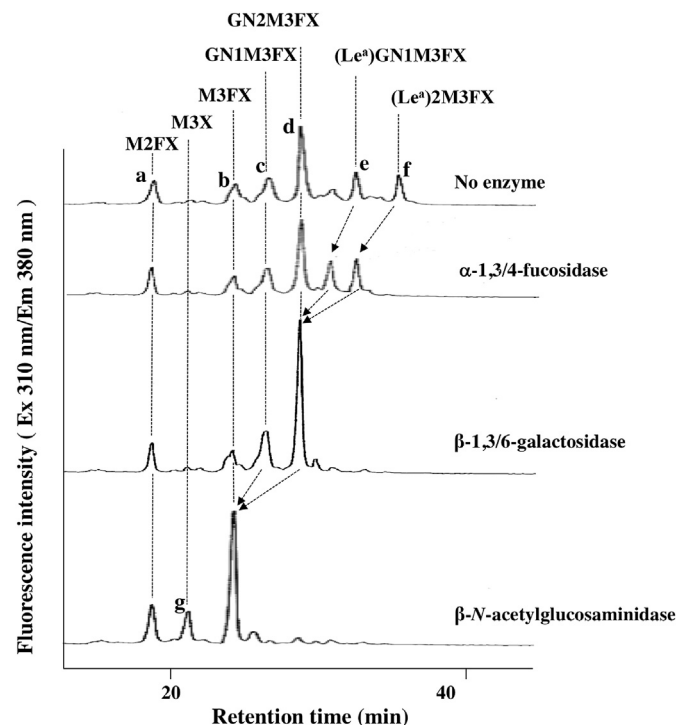


Fig. 3. NP-HPLC Profile of Exo-Glycosidase Digests of PA-Sugar Chains. PA-sugar chains, peak-e and peak-f, were successively digested with α -1,3/4-fucosidase. The α -1,3/4-fucosidase digest was digested by β -1,3/6-galactosidase. The β -1,3/6-galactosidase digest was digested by β -N-acetylglucosaminidase. Dotted lines indicate elution positions of authentic M2FX, M3FX, GN1M3FX, GN2M3FX, $(\text{Le}^a)\text{GN1M3FX}$, and $(\text{Le}^a)2\text{M3FX}$.

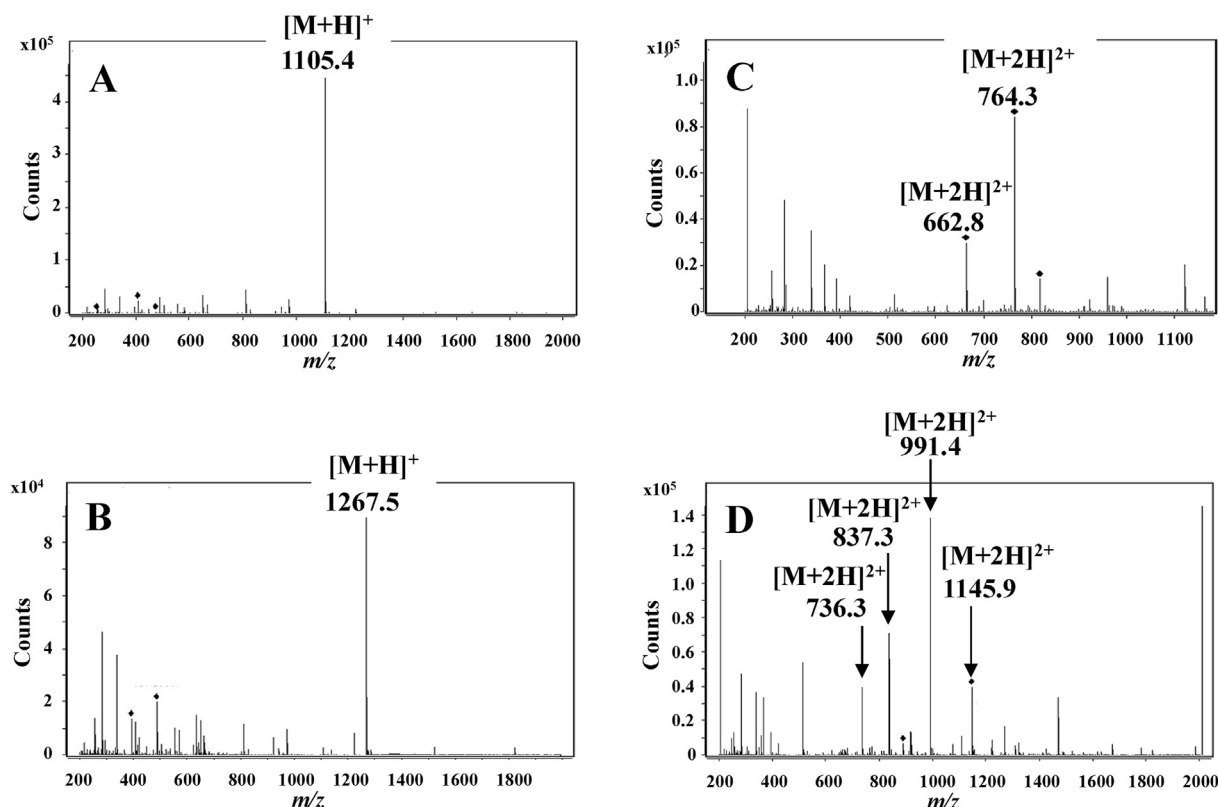


Fig. 4. LC/MS Analysis of PA-Sugar Chains. A, MS-analysis of M2FX in peak-a. B, MS-analysis of M3FX in peak-b. C, MS-analysis of GN1M3X in peak-b, and GN2M3X in peak-c, respectively. D, MS-analysis of GN1M3FX in peak-c, GN2M3FX in peak-d, (Le^a)GN1M3FX in peak-e, (Le^a)2M3FX in peak-f, respectively. The mixture of PA-sugar chains obtained by RP-HPLC were analyzed by LC/MS using a PGC-Chip.

3. Results

3.1. Plant antigenic N-glycans from three fresh water plants

The PA-sugar chains prepared from glycoproteins produced in the water plants were partially purified by RP-HPLC (Fig. 1B). The fraction indicated by a horizontal bar was pooled as the N-glycan-fraction and the PA-sugar chains were further separated by NP-HPLC (Fig. 2). The elution profiles of PA-sugar chains obtained from the three water plants (*Elodea nuttallii*, *Egeria densa*, and *Ceratophyllum demersum*) were very similar to each other, and six PA-sugar chains (peak-a, peak-b, peak-c, peak-d, peak-e and peak-f) were observed. The elution position of peak-a on NP-HPLC coincided with that of Man₂Xyl₁Fuc₁GlcNAc₂-PA (M2FX), peak-b with that of M3FX, peak-c with that of GlcNAc₁Man₃Xyl₁Fuc₁GlcNAc₂-PA (GN1M3FX), peak-d with that of GlcNAc₂Man₃Xyl₁Fuc₁GlcNAc₂-PA (GN2M3FX), peak-e with that of Gal₁Fuc₁GlcNAc₂Man₃Xyl₁Fuc₁GlcNAc₂-PA ((Le^a)GN1M3FX), and peak-f with that of Gal₂Fuc₂GlcNAc₂Man₃Xyl₁Fuc₁GlcNAc₂-PA ((Le^a)2M3FX). The structure of N-glycans and abbreviations are shown in Scheme 2. Truncated type structures, M3FX and M2FX, may have been produced from complex type structures by the action of Golgi or vacuolar β-N-acetylglucosaminidase [27]. However, it is noteworthy that plant complex type structures such as GN2M3FX, (Le^a)GN1M3FX or (Le^a)2M3FX predominantly occur on glycoproteins in these fresh water plants. The structures of these PA-sugar chains were analyzed by sequential exo-glycosidase digestions (Fig. 3). Two peaks of -e and -f were converted to peak-d by sequential digestion with α-1,3/4-fucosidase and β-1,3/6-galactosidase, suggesting that the structures of PA-sugar chains in peak-e and peak-f are modified with Lewis a epitope(s) at the non-reducing terminal. Finally, PA-sugar

chains, peaks-c and -d, and enzyme digests obtained from peaks-e and -f, were converted to peak-b by β-N-acetylglucosaminidase, suggesting modification with N-acetylglucosamine (GlcNAc) residue(s). Since peak-g appeared after digestion of the β-1,3/6-galactosidase digest with β-N-acetylglucosaminidase, xylosyl and afucosyl N-glycans (GlcNAc₁Man₃Xyl₁GlcNAc₂-PA or GlcNAc₂Man₃Xyl₁GlcNAc₂-PA), which elute similarly to M3FX and GN1M3FX, might exist in peak-b or peak-c, respectively. These results indicate that almost all glycoproteins expressed in these three water plants carry plant complex type N-glycans but not high-mannose type ones, and some of them are modified with Lewis a epitope(s).

These deduced structures of these PA-sugar chains were further analyzed by LC/MS and MS/MS analysis using the Agilent 6500 Series HPLC-Chip/QTOF-MS System (Figs. 4 and 5). The LC-MS analysis of peak-a showed a N-glycan ion *m/z* 1105.4 [(M + H)⁺], corresponding to [(Pen)₁(Hex)₂(HexNAc)₁(Deoxyhex)₁(HexNAc-PA) + H]⁺ or M2FX (Fig. 4A and Table S1). Peak-b showed two N-glycan ions *m/z* 1267.5 [(M + H)⁺] and *m/z* 662.8 [(M + 2H)²⁺], corresponding to [(Pen)₁(Hex)₃(HexNAc)₁(Deoxyhex)₁(HexNAc-PA) + H]⁺ and [(Pen)₁(Hex)₃(HexNAc)₂(HexNAc-PA) + H]⁺ or M3FX and GlcNAc₁Man₃Xyl₁GlcNAc₂-PA (GN1M3X), respectively (Fig. 4B and C and Table S1). Peak-c showed two N-glycan ions *m/z* 736.3 [(M + 2H)²⁺] and *m/z* 764.3 [(M + 2H)²⁺], corresponding to [(Pen)₁(Hex)₃(HexNAc)₂(Deoxyhex)₁(HexNAc-PA) + H]⁺ and [(Pen)₁(Hex)₃(HexNAc)₃(HexNAc-PA) + H]⁺ or GN1M3FX and GlcNAc₂Man₃Xyl₁GlcNAc₂-PA (GN2M3X), respectively (Fig. 4C and D and Table S1). Peak-d showed a N-glycan ion *m/z* 837.3 [(M + 2H)²⁺], corresponding to [(Pen)₁(Hex)₃(HexNAc)₃(Deoxyhex)₁(HexNAc-PA) + H]⁺ or GN2M3FX (Fig. 4D and Table S1). Peak-e showed a N-glycan ion *m/z* 991.4 [(M + 2H)²⁺],

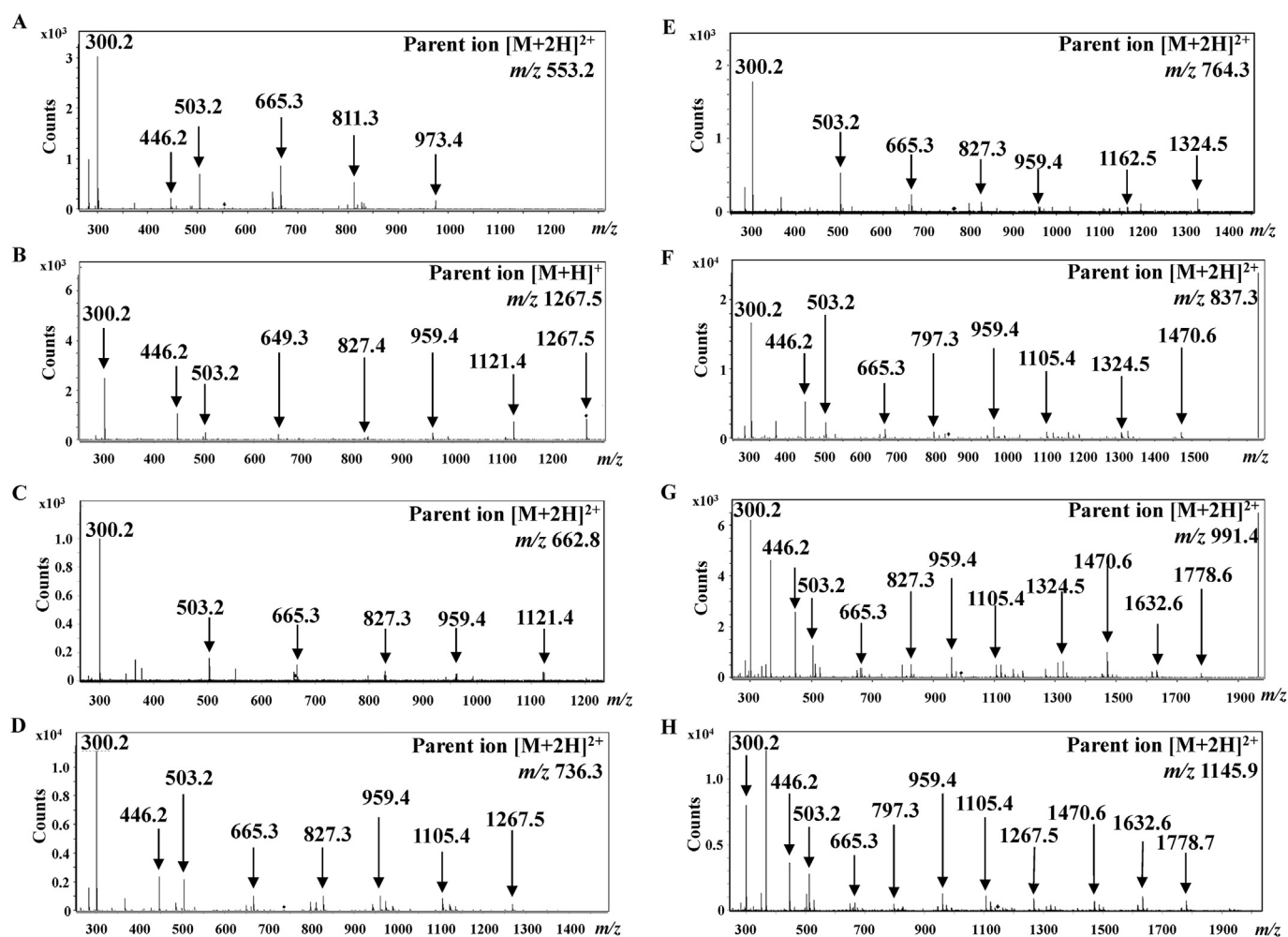


Fig. 5. MS/MS Analysis of PA-Sugar Chains. A, MS/MS analysis of *N*-glycan ions m/z 553.2 $[M+2H]^{2+}$; B, m/z 1267.5 $[M+H]^+$; C, m/z 662.8 $[M+2H]^{2+}$; D, m/z 736.3 $[M+2H]^{2+}$; E, m/z 764.3 $[M+2H]^{2+}$; F, m/z 837.3 $[M+2H]^{2+}$; G, m/z 991.4 $[M+2H]^{2+}$; H, m/z 1145.9 $[M+2H]^{2+}$.

corresponding to $[(Pen)_1(Hex)_4(HexNAc)_3(Deoxyhex)_2(HexNAc-PA) + H]^+$ or $(Le^a)GN1M3FX$ (Fig. 4D and Table S1). Peak-f showed a *N*-glycan ion m/z 1145.9 $[(M + 2H)^{2+}]$, corresponding to $[(Pen)_1(-Hex)_5(HexNAc)_3(Deoxyhex)_3(HexNAc-PA) + H]^+$ or $(Le^a)2M3FX$ (Fig. 4D and Table S1). The results of MS/MS analysis of these PA-sugar chains were shown in Fig. 5 and summarized in Table S2. The MS/MS fragment ions (m/z 973.4, m/z 811.3, m/z 665.3, m/z 503.2, m/z 446.2, and m/z 300.2) derived from the parent ion m/z 553.2 $[(M + 2H)^{2+}]$ were assigned as fragments derived from M2FX (Fig. 5A and Table S2). The MS/MS fragment ions (m/z 1121.4, m/z 959.4, m/z 827.4, m/z 649.3, m/z 503.2, m/z 446.2, and m/z 300.2) derived from parent ion m/z 1267.5 $[(M + H)^+]$ were assigned as fragments derived from M3FX (Fig. 5B and Table S2). The MS/MS fragment ions (m/z 1121.4, m/z 959.4, m/z 827.3, m/z 665.3, m/z 503.2, and m/z 300.2) derived from parent ion m/z 662.8 $[(M + 2H)^{2+}]$ were assigned as fragments derived from GN1M3X (Fig. 5C and Table S2). The MS/MS fragment ions (m/z 1267.5, m/z 1105.4, m/z 959.4, m/z 827.3, m/z 665.3, m/z 503.2, m/z 446.2, and m/z 300.2) derived from parent ion m/z 736.3 $[(M + 2H)^{2+}]$ were assigned as fragments derived from GN1M3FX (Fig. 5D and Table S2). The MS/MS fragment ions (m/z 1324.5, m/z 1162.5, m/z 959.4, m/z 827.3, m/z 665.3, m/z 503.2, and m/z 300.2) derived from parent ion m/z 764.3 $[(M + 2H)^{2+}]$ were assigned as fragments derived from GN2M3X (Fig. 5E and Table S2). The MS/MS fragment ions (m/z 1470.6, m/z 1324.5, m/z 1105.4, m/z 959.4, m/z 797.3, m/z

665.3, m/z 503.2, m/z 446.2, and m/z 300.2) derived from parent ion m/z 837.3 $[(M + 2H)^{2+}]$ were assigned as fragments derived from GN2M3FX (Fig. 5F and Table S2). The MS/MS fragment ions (m/z 1778.6, m/z 1632.6, m/z 1470.6, m/z 1324.5, m/z 1105.4, m/z 959.4, m/z 827.3, m/z 665.3, m/z 503.2, m/z 446.2, and m/z 300.2) derived from parent ion m/z 991.4 $[(M + 2H)^{2+}]$ were assigned as fragments derived from $(Le^a)GN1M3FX$ (Fig. 5G and Table S2). The MS/MS fragment ions (m/z 1778.6, m/z 1632.6, m/z 1470.6, m/z 1267.5, m/z 1105.4, m/z 959.4, m/z 797.3, m/z 665.3, m/z 503.2, m/z 446.2, and m/z 300.2) derived from parent ion m/z 1145.9 $[(M + 2H)^{2+}]$ were assigned as fragments derived from $(Le^a)2M3FX$ (Fig. 5H and Table S2).

These LC/MS analyses suggested that PA-sugar chains of peak-b and peak-c contain GN1M3X and GN2M3X in addition to M3FX and GN1M3FX, respectively. Thus, peak-g on NP-HPLC (Fig. 3) must be $Man_3Xyl_1GlcNAc_2-PA$ (M3X) derived from β -*N*-acetylglucosaminidase digestion of GN1M3X and GN2M3X.

3.2. Identification of isomeric structure of $(Le^a)GN1M3FX$ carrying one Lewis a epitope

The occurrence of two isomeric structures of $(Le^a)GN1M3FX$ was confirmed by the combination of sequential enzyme digestion and RP-HPLC analysis of the digests (Fig. 6). The peak-f, $(Le^a)GN1M3FX$, was converted to GN1M3FX by the sequential

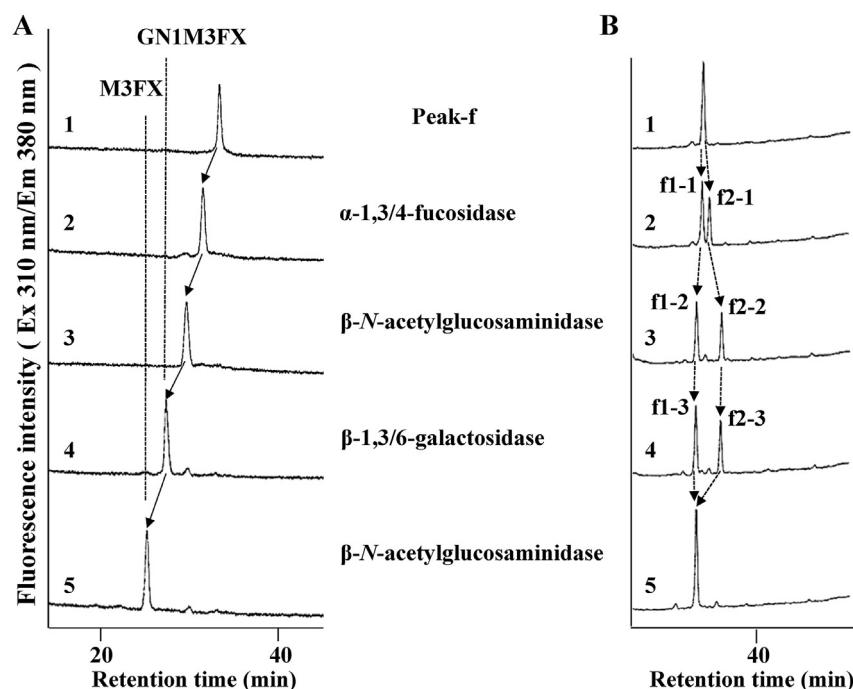
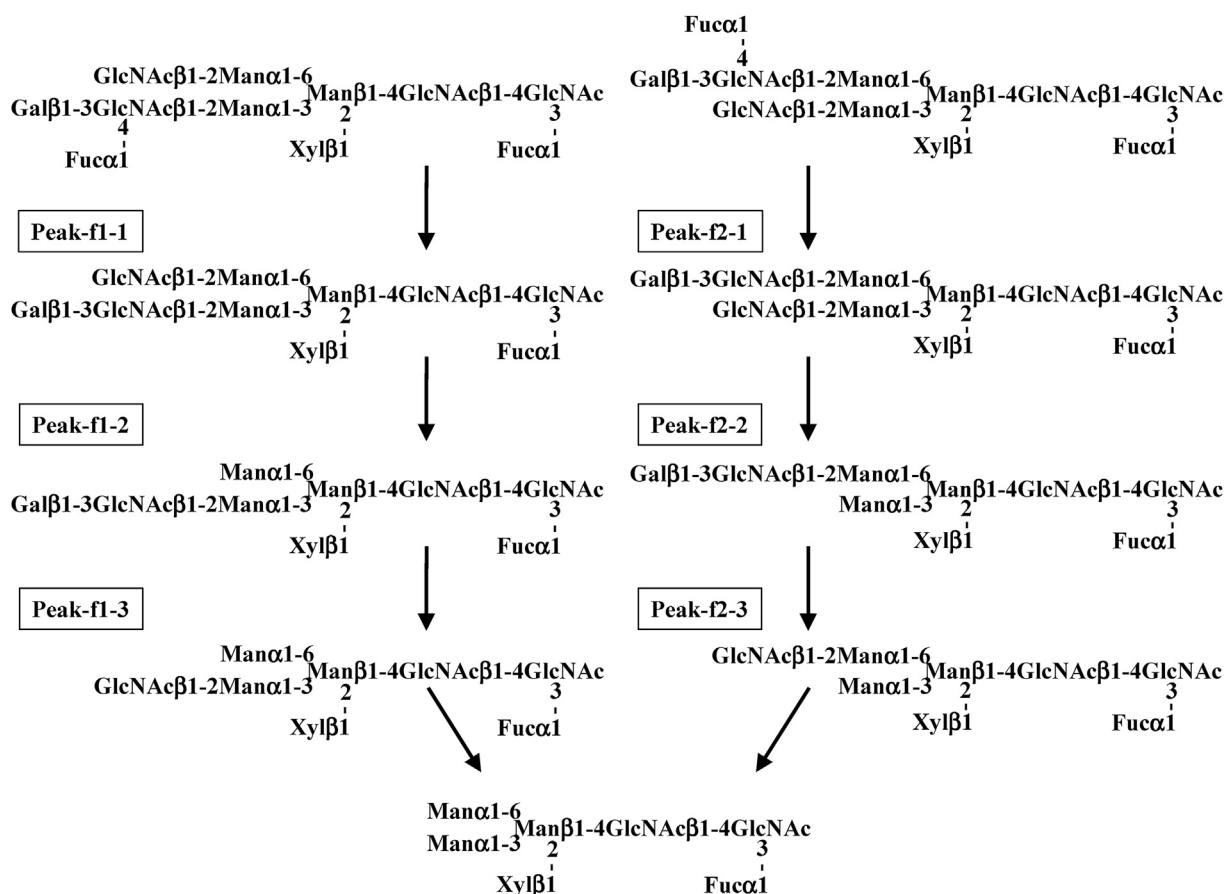
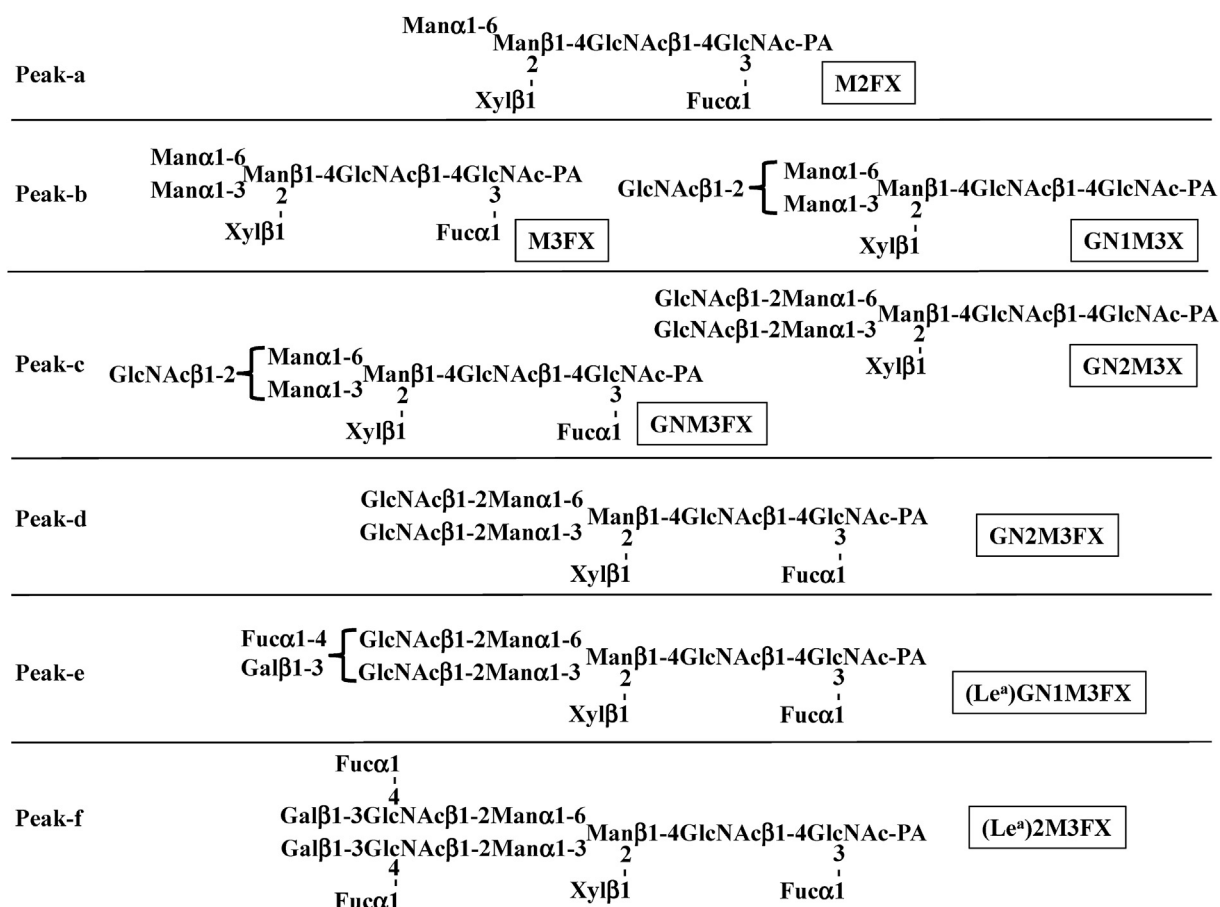


Fig. 6. Identification of Branching of (Le^a)GN1M3FX. PA-sugar chain of peak-f, (Le^a)GN1M3FX, was successively digested with α -1,3/4-fucosidase. The α -1,3/4-fucosidase digest was digested by β -N-acetylglucosaminidase. The β -N-acetylglucosaminidase digest was digested by β -1,3/6-galactosidase. The β -1,3/6-galactosidase digest was digested by β -N-acetylglucosaminidase. A, NP-HPLC separation of PA-sugar chains of peak-f and digests. Dotted lines indicate elution positions of authentic M3FX and GN1M3FX. B, RP-HPLC of PA-sugar chains of peak-f and digests.



Scheme 1. Proposed Analytical Digestion Pathway for (Le^a)GN1M3FX.



Scheme 2. Structural Features of *N*-Glycans Linked to Glycoproteins in Water Plants.

Table 1
Summary of *N*-Glycans linked to glycoproteins in water plants.

Peaks	Structure	<i>Elodea nuttallii</i> (nmol/g) (%)	<i>Egeria densa</i> (nmol/g) (%)	<i>Ceratophyllum demersum</i> (nmol/g) (%)
Peak-a	M2FX	0.09	3.70	0.40
Peak-b	M3FX	0.29	12.1	0.21
	GN1M3X			12.5
Peak-c	GN1M3FX	0.28	11.7	0.22
	GN2M3X			13.3
Peak-d	GN2M3FX	0.94	39.8	0.44
Peak-e	(Le ^a)GN1M3FX	0.38	16.0	0.17
Peak-f	(Le ^a)2M3FX	0.39	16.7	0.23
				14.0
				0.69
				44.8
				0.14
				8.90
				0.16
				10.5

digestion with α -1,3/4-fucosidase, β -*N*-acetylglucosaminidase, and β -1,3/6-galactosidase (Fig. 6B-2, -3, -4). The product by the β -1,3/6-galactosidase digestion, GN1M3FX, was further converted to M3FX by β -*N*-acetylglucosaminidase digestion (Fig. 6B-5). Each product obtained at each digestion stage was found as a single peak on NP-HPLC (Fig. 6A). The product obtained by α -1,3/4-fucosidase digestion, however, was separated to two components on RP-HPLC (peak-f1-1 and peak f2-1, Fig. 6-2), indicating that two isomeric structures occurred in the product. When these two isomeric structures were sequentially digested by β -*N*-acetylglucosaminidase and β -1,3/6-galactosidase, each product was eluted at the different retention time on the RP-HPLC (Fig. 6B-3, -4). Finally, the two isomeric products obtained by β -1,3/6-galactosidase digestion (Fig. 6B-4) fully converged as a single product, M3FX, by the second digestion by β -*N*-acetylglucosaminidase (Fig. 6B-5). Since it has been reported that the

RP-HPLC elution time of Man α 1-6(GlcNAc β 1-2Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA is earlier than that of GlcNAc β 1-2Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA [28,29], peak-f1-3 should contain the former structure and peak-f2-3 the latter structure. These results indicated that (Le^a)GN1M3FX (peak-f) contained two isomeric structures; one has the Lewis a epitope at the Man α 1-6 arm, the other one at the Man α 1-3 arm, of the tri-mannosyl core, respectively (Scheme 1).

4. Discussion

We found that almost all *N*-glycans linked to glycoproteins in the water plants, *Elodea nuttallii*, *Ceratophyllum demersum*, and *Egeria densa*, have plant complex type *N*-glycans bearing β 1-2 xylosyl and/or α 1-3 fucosyl residues but not high-mannose type

structures. Eight types of plant complex type *N*-glycans, M2FX, M3FX, GN1M3X, GN2M3X, GN1M3FX, GN2M3FX, (Le^a)GN1M3FX, and (Le^a)2M3FX, were commonly obtained from glycoproteins expressed in the three water plants (Scheme 2 and Table 1).

Plant complex type *N*-glycans are usually classified into two groups based on a structural feature at the non-reducing end side, the secreted type and the vacuolar type [1–3]. Since many secreted type glycoproteins are membrane proteins that carry the Lewis a epitope or biantennary type *N*-glycans (GlcNAc₂Man₃Xyl₁Fuc₁GlcNAc₂), this type of glycan structures has been classified as secreted type *N*-glycans [1–3]. The secreted type glycoproteins are transported to the extracellular space after *N*-glycan processing by the Golgi-localized β 1,3-galactosyltransferase and α 1,4-fucosyltransferase [1,3,30]. Some pollen allergens, Cup 1 [9], Cry j1 [10], Jun a1 [11], and Cha o1 [12], are typical examples. On the other hand, the glycoproteins bearing vacuolar-type or paucimannose type *N*-glycans such as M2FX and M3FX accumulated in the vacuole or in protein bodies. This type of glycan structures has been considered as the trimmed products of the secreted type *N*-glycans by the actions of some Golgi or vacuolar exo-glycosidases [1,31]. However, it is unclear at this time whether the *N*-glycan structure bears significant information for protein sorting to the extracellular space or the vacuole.

In the case of the three water plants used in this study, we found that the secreted type *N*-glycans predominantly occur on glycoproteins, indicating many glycoproteins in water plants may localize in the extracellular space. Moreover, the Lewis a type plant *N*-glycans were commonly expressed in a substantial amount on the glycoproteins produced in these three water plants: 32.7% of *Elodea nuttallii*, 24.0% of *Egeria densa*, and 19.4% of *Ceratophyllum demersum* glycoproteins. Approximately 7.6 mg of non-labeled Asn-glycopeptides bearing Lewis a type *N*-glycans were purified from *Elodea nuttallii* (300 g wet weight), indicating that these abundant water plants are excellent sources of Lewis a type plant *N*-glycans for studying their immunological activities in mammals.

In our previous paper [20], we have reported that almost all glycoproteins expressed in various seaweeds, sea grass (*Zostera marina*) or many marine algae, exclusively carry high-mannose type *N*-glycans, but not plant complex type *N*-glycans. On the other hand, it is well known that secreted type glycoproteins expressed in land plants ubiquitously carry plant complex type *N*-glycans, which often contain the Lewis a epitope. At this time, it is unclear why sea grass (eel grass), which evolved as flowering land plants in ancient time and had returned to sea environments during the Cretaceous period [32], hardly expresses glycoproteins bearing the plant complex type *N*-glycans. However, this fact suggests that the plant complex type *N*-glycans or Lewis a type plant *N*-glycans might provide physicochemical or biological function(s) to glycoproteins expressed in plants grown in a fresh-water environments. Genetic analysis to investigate whether glycosyltransferase genes involved in the biosynthesis of plant complex type *N*-glycans occur in the genome of *Zostera marina* is in progress.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.carres.2016.09.008>.

References

- [1] P. Lerouge, M. Cabanes-Macheteau, C. Rayon, A.C. Fischette-Laine, V. Gomord, L. Faye, *Plant Mol. Biol.* 38 (1998) 31–48.
- [2] M. Maeda, Y. Kimura, *Front. Plant Sci.* 5 (2014) 429.
- [3] R. Strasser, *Glycobiology* (2016) 1–14, <http://dx.doi.org/10.1093/glycob/cww023>.
- [4] G. Garcia-Casado, R. Sanchez-Monge, M.J. Chrispeels, A. Armentia, G. Salcedo, L. Gomez, *Glycobiology* 6 (1996) 471–477.
- [5] R. van Ree, M. Cabanes-Macheteau, J. Akkerdaas, J.P. Milazzo, C. Loutelier-Bourhis, C. Rayon, M. Villalba, S. Koppelman, R. Aalberse, R. Rodriguez, L. Faye, P. Lerouge, *J. Biol. Chem.* 275 (2000) 11451–11458.
- [6] M. Bardor, C. Faveeuw, A.C. Fitchette, D. Gilbert, L. Galas, F. Trottein, L. Faye, P. Lerouge, *Glycobiology* 13 (2003) 427–434.
- [7] C. Jin, F. Altmann, R. Strasser, L. Mach, M. Schahs, R. Kunert, T. Rademacher, J. Glossl, H. Steinkellner, *Glycobiology* 18 (2008) 235–241.
- [8] H. Ohsuga, S.N. Su, N. Takahashi, S.Y. Yang, H. Nakagawa, I. Shimada, Y. Arata, Y.C. Lee, *J. Biol. Chem.* 271 (1996) 26653–26658.
- [9] C. Alisi, C. Afferni, P. Iacovacci, B. Barletta, R. Tinghino, C. Butteroni, E.M. Puggioni, I.B. Wilson, R. Federico, M.E. Schinina, R. Ariano, G. Di Felice, C. Pini, *Allergy* 56 (2001) 978–984.
- [10] Y. Kimura, M. Kamamoto, M. Maeda, M. Okano, M. Yokoyama, K. Kino, *Biosci. Biotechnol. Biochem.* 69 (2005) 137–144.
- [11] M. Maeda, M. Kamamoto, K. Hino, S. Yamamoto, M. Kimura, M. Okano, Y. Kimura, *Biosci. Biotechnol. Biochem.* 69 (2005) 1700–1705.
- [12] Y. Kimura, M. Kuroki, M. Maeda, M. Okano, M. Yokoyama, K. Kino, *Biosci. Biotechnol. Biochem.* 72 (2008) 485–491.
- [13] A. Mari, *Int. Arch. Allergy Immunol.* 129 (2002) 286–295.
- [14] F. Altmann, *Int. Arch. Allergy Immunol.* 142 (2007) 99–115.
- [15] M. Okano, Y. Kimura, K. Kino, Y. Michigami, S. Sakamoto, Y. Sugata, M. Maeda, F. Matsuda, M. Kimura, T. Ogawa, K. Nishizaki, *Clin. Exp. Allergy* 34 (2004) 770–778.
- [16] M. Maeda, N. Takeda, A. Mano, M. Yamanishi, M. Kimura, Y. Kimura, *Biosci. Biotechnol. Biochem.* 77 (2013) 1269–1274.
- [17] A.C. Fitchette-Laine, V. Gomord, M. Cabanes, J.C. Michalski, M. Saint Macary, B. Foucher, B. Cavellier, C. Hawes, P. Lerouge, L. Faye, *Plant J.* 12 (1997) 1411–1417.
- [18] I.B. Wilson, R. Zeleny, D. Kolarich, E. Staudacher, C.J. Stroop, J.P. Kamerling, F. Altmann, *Glycobiology* 11 (2001) 261–274.
- [19] T. Mega, *Biosci. Biotechnol. Biochem.* 71 (2007) 2893–2904.
- [20] T. Yoshiie, M. Maeda, M. Kimura, Y. Hama, M. Uchida, Y. Kimura, *Biosci. Biotechnol. Biochem.* 76 (2012) 1996–1998.
- [21] Y. Kimura, S. Hase, Y. Kobayashi, Y. Kyogoku, T. Ikenaka, G. Funatsu, *J. Biochem.* 103 (1988) 944–949.
- [22] M. Maeda, Y. Kimura, *Biosci. Biotechnol. Biochem.* 70 (2006) 1356–1363.
- [23] M. Kimura, T. Hara, Y. Kimura, *Biosci. Biotechnol. Biochem.* 75 (2011) 155–158.
- [24] M. DuBois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, *Anal. Chem.* 28 (1956) 350–356.
- [25] S. Natsuka, S. Hase, *Methods Mol. Biol.* 76 (1998) 101–113.
- [26] M. Maeda, M. Kimura, Y. Kimura, *J. Biochem.* 148 (2010) 681–692.
- [27] R. Strasser, J.S. Bondili, J. Schoberer, B. Svoboda, E. Liebinger, J. Glössl, F. Altmann, H. Steinkellner, L. Mach, *Plant Physiol.* 145 (2007) 5–16.
- [28] K. Yanagida, H. Ogawa, K. Omichi, S. Hase, *J. Chromatogr. A* 800 (1998) 187–198.
- [29] N. Tomiya, M. Kurono, H. Ishihara, S. Tejima, S. Endo, Y. Arata, N. Takahashi, *Anal. Biochem.* 163 (1987) 489–499.
- [30] R. Strasser, J.S. Bondili, U. Vavra, J. Schoberer, B. Svoboda, J. Glossl, R. Leonard, J. Stadlmann, F. Altmann, H. Steinkellner, L. Mach, *Plant Cell* 19 (2007) 2278–2292.
- [31] A. Vitale, M.J. Chrispeels, *J. Cell Biol.* 99 (1984) 133–140.
- [32] M. Waycott, G. Procaccini, D.H. Les, T.B.H. Reusch, in: A.W.D. Larkum, R.J. Orth, C.M. Duarte (Eds.), *Seagrasses: Biology, Ecology and Conservation*, Springer, Dordrecht, The Netherlands, 2006, pp. 25–50.

Table S1. MS Analysis of *N*-Glycans in Water Plant Glycoproteins

Structure	<i>m/z</i>
M2FX	1105.4 [M+H] ⁺
M3FX	1267.5 [M+H] ⁺
GN1M3X	662.8 [M+2H] ²⁺
GN1M3FX	736.3 [M+2H] ²⁺
GN2M3X	764.3 [M+2H] ²⁺
GN2M3FX	837.3 [M+2H] ²⁺
(Le ^a)GN1M3FX	991.4 [M+2H] ²⁺
(Le ^a)2M3FX	1145.9 [M+2H] ²⁺

Table S2. MS/MS Analysis of *N*-Glycans in Water Plant Glycoproteins

M2FX (peak-a)	
Fragment ions [M+H] ⁺	<i>m/z</i>
Man ₂ Fuc ₁ GlcNAc ₂ -PA	973.4
Man ₁ Fuc ₁ GlcNAc ₂ -PA	811.3
Man ₁ GlcNAc ₂ -PA	665.3
GlcNAc ₂ -PA	503.2
Fuc ₁ GlcNAc ₁ -PA	446.2
GlcNAc ₁ -PA	300.2

M3FX (peak-b)	
Fragment ions [M+H] ⁺	<i>m/z</i>
Man ₃ Xyl ₁ GlcNAc ₂ -PA	1121.4
Man ₂ Xyl ₁ GlcNAc ₂ -PA	959.4
Man ₂ GlcNAc ₂ -PA	827.4
Fuc ₁ GlcNAc ₂ -PA	649.3
GlcNAc ₂ -PA	503.2
Fuc ₁ GlcNAc ₁ -PA	446.2
GlcNAc ₁ -PA	300.2

GN1M3X (peak-b)	
Fragment ions [M+H] ⁺	<i>m/z</i>
Man ₃ Xyl ₁ GlcNAc ₂ -PA	1121.4
Man ₂ Xyl ₁ GlcNAc ₂ -PA	959.4
Man ₂ GlcNAc ₂ -PA	827.3
Man ₁ GlcNAc ₂ -PA	665.3
GlcNAc ₂ -PA	503.2
GlcNAc ₁ -PA	300.2

GN1M3FX (peak-c)	
Fragment ions [M+H] ⁺	<i>m/z</i>
Man ₃ Xyl ₁ Fuc ₁ GlcNAc ₂ -PA	1267.5
Man ₂ Xyl ₁ Fuc ₁ GlcNAc ₂ -PA	1105.4
Man ₂ Xyl ₁ GlcNAc ₂ -PA	959.4
Man ₂ GlcNAc ₂ -PA	827.3
Man ₁ GlcNAc ₂ -PA	665.3
GlcNAc ₂ -PA	503.2
Fuc ₁ GlcNAc ₁ -PA	446.2
GlcNAc ₁ -PA	300.2

GN2M3X (peak-c)	
Fragment ions [M+H] ⁺	<i>m/z</i>
GlcNAc ₁ Man ₃ Xyl ₁ GlcNAc ₂ -PA	1324.5
GlcNAc ₁ Man ₂ Xyl ₁ GlcNAc ₂ -PA	1162.5
Man ₂ Xyl ₁ GlcNAc ₂ -PA	959.4
Man ₂ GlcNAc ₂ -PA	827.3
Man ₁ GlcNAc ₂ -PA	665.3
GlcNAc ₂ -PA	503.2
GlcNAc ₁ -PA	300.2

Table S2. MS/MS Analysis of *N*-Glycans in Water Plant Glycoproteins (continued)

GN2M3FX (peak-d)		(Le^a)2M3FX (peak-f)	
Fragment ions [M+H] ⁺	<i>m/z</i>	Fragment ions [M+H] ⁺	<i>m/z</i>
GlcNAc ₁ Man ₃ Xyl ₁ Fuc ₁ GlcNAc ₂ -PA	1470.6	Gal ₁ Fuc ₁ GlcNAc ₁ Man ₃ Xyl ₁ Fuc ₁ GlcNAc ₂ -PA	1778.6
GlcNAc ₁ Man ₃ Xyl ₁ GlcNAc ₂ -PA	1324.5	Gal ₁ GlcNAc ₁ Man ₃ Xyl ₁ Fuc ₁ GlcNAc ₂ -PA	1632.6
Man ₂ Xyl ₁ Fuc ₁ GlcNAc ₂ -PA	1105.4	GlcNAc ₁ Man ₃ Xyl ₁ Fuc ₁ GlcNAc ₂ -PA	1470.6
Man ₂ Xyl ₁ GlcNAc ₂ -PA	959.4	Man ₃ Xyl ₁ Fuc ₁ GlcNAc ₂ -PA	1267.5
Man ₁ Xyl ₁ GlcNAc ₂ -PA	797.3	Man ₂ Xyl ₁ Fuc ₁ GlcNAc ₂ -PA	1105.4
Man ₁ GlcNAc ₂ -PA	665.3	Man ₂ Xyl ₁ GlcNAc ₂ -PA	959.4
GlcNAc ₂ -PA	503.2	Man ₁ Xyl ₁ GlcNAc ₂ -PA	797.3
Fuc ₁ GlcNAc ₁ -PA	446.2	Man ₁ GlcNAc ₂ -PA	665.3
GlcNAc ₁ -PA	300.2	GlcNAc ₂ -PA	503.2
		Fuc ₁ GlcNAc ₁ -PA	446.2
		GlcNAc ₁ -PA	300.2
(Le^a)GN1M3FX (peak-e)			
Fragment ions [M+H] ⁺	<i>m/z</i>		
Gal ₁ Fuc ₁ GlcNAc ₁ Man ₃ Xyl ₁ Fuc ₁ GlcNAc ₂ -PA	1778.6		
Gal ₁ GlcNAc ₁ Man ₃ Xyl ₁ Fuc ₁ GlcNAc ₂ -PA	1632.6		
GlcNAc ₁ Man ₃ Xyl ₁ Fuc ₁ GlcNAc ₂ -PA	1470.6		
GlcNAc ₁ Man ₃ Xyl ₁ GlcNAc ₂ -PA	1324.5		
Man ₂ Xyl ₁ Fuc ₁ GlcNAc ₂ -PA	1105.4		
Man ₂ Xyl ₁ GlcNAc ₂ -PA	959.4		
Man ₂ GlcNAc ₂ -PA	827.3		
Man ₁ GlcNAc ₂ -PA	665.3		
GlcNAc ₂ -PA	503.2		
Fuc ₁ GlcNAc ₁ -PA	446.2		
GlcNAc ₁ -PA	300.2		