Running title: *N*-Glycans of bamboo shoot glycoproteins

# Structural feature of *N*-glycans of bamboo shoot glycoproteins: Useful source of plant antigenic *N*-glycans

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*Abbreviations*: RP-HPLC, reversed-phase HPLC; SF-HPLC, size-fractionation HPLC; PA-, pyridylamino; PCT, plant complex type; Hex, hexose; HexNAc, *N*-acetylhexosamine; Pen, pentose; Deoxyhex, deoxyhexose; Man, D-mannose; GlcNAc, *N*-acetyl-D-glucosamine; Xyl, s-xylose; Fuc, L-fucose; $Le^a$, Lewis a (Galβ1-3(Fucα1-4)GlcNAc); PCT, plant complex type; M3FX, Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA; GN2M3FX, GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA; ($Le^a$)1GN1M3FX, Galβ1-3(Fucα1-4)GlcNAc1-2 Manα1-6(GlcNAcβ1-2Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA or GlcNAc1-2Manα1-6(Galβ1-3(Fucα1-4)GlcNAc1-2Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA; ($Le^a$)2M3FX, Galβ1-3(Fucα1-4)GlcNAc1-2 Manα1-6(Galβ1-3(Fucα1-4)GlcNAc1-2Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA.

An effective method to prepare plant complex type *N*-glycans in large amounts has been required to evaluate their immunological activity. In this study, we found that glycoproteins in bamboo shoots predominantly carry plant complex type *N*-glycans including the Lewis a epitope-containing ones, suggesting that bamboo shoot is an excellent source for the plant antigenic glycans to synthesize immuno-active neoglycopolymers.

**Keywords**: Antigenic *N*-glycans, plant *N*-glycans, plant glycoproteins, Bamboo shoot, *Phyllostachys edulis*.

Plant complex type (PCT) *N*-glycans are well known to have antigenicity toward mammalians and are commonly found on Cupressaceae pollen allergens, such as Cup a1, Cry j1, Cha o1, and Jun a1.[1-5] We have already found that these PCT *N*-glycans suppressed the production of IL-4 from Th2 cells in patients with Japanese cedar pollinosis,[6] although the clinical significance of these PCT *N*-glycans involved in pollinosis has not been clarified. Our findings suggested a possibility that free PCT *N*-glycans (FNGs) have potential anti-pollinosis activity for treatment of cedar pollinosis. For evaluation of the putative anti-pollinosis activity or other immunological activities of PCT *N*-glycans, the effective method to prepare PCT-FNGs in large amount has been required but only a few good sources have been reported.[7,8] For preparation of Lewis a epitope-containing PCT *N*-glycans with high efficiency, we have analyzed the structures of *N*-glycans linked to plant glycoproteins expressed in various plant materials including edible seeds,[7,9] vegetables,[10] fresh-water plants,[8] and seaweeds.[11] In the course of the glycan-screening, we found that bamboo shoot, in addition to fresh-water plants, is a useful source for preparation of immuno-active plant *N*-glycans or *N*-glycopeptides containing Lewis a epitope. Hence, in this report, we describe the structural analysis of *N*-glycans of bamboo glycoproteins.

Bamboo shoots were collected at Yakage Town (Oda-Gun, Okayama Prefecture, Japan). Chopped bamboo shoots (732 g) were homogenized in 5% formic acid (2 L) with a waring blender and digested with 1.2 g of pepsin at 37°C for 72 h. After neutralization with ammonium

solution, the peptic digests were filtered with two-layered gauze and the filtrates were centrifuged at 12,450 *g* for 20 min. The supernatants were concentrated to 415 mL with a rotary evaporator and were dialyzed (using Visking tubing UC24/32, Viskase Co. IL, USA) against deionized water (2.5 L twice) at 4°C for three days. Outer solution was concentrated to about 200 mL with a rotary evaporator and applied to Dowex 50 × 2 resin (200 ml) to remove the neutral oligosaccharides. After washing the resin with 1% formic acid, adsorbed positive-charged compounds containing glycopeptides were eluted with 3L of 0.2N $NH_4OH$ and concentrated to a small amount of solution with a rotary evaporator. The glycopeptide-fractions were applied to a Sephadex G-25 fine column (4.0 × 80 cm) equilibrated with 0.1N $NH_4OH$. The glycopeptide-fractions (elution volume, 390-700 ml) were detected by the Phenol-$H_2SO_4$ method,[12] and were concentrated with a rotary evaporator and lyophilized. The glycopeptide-fraction (483 mg) was dissolved in deionized water (25 mL) and applied to a Wako Gel 100C18 column (3.2 x 42.5 cm). Glycopeptides were eluted by a linear gradient of acetonitrile from 0 to 25% in deionized water, and the glycopeptide-fraction eluted between 7 to 20 % of acetonitrile was concentrated with a rotary evaporator and lyophilized. The partially purified peptic glycopeptides (233 mg) were further digested with actinase (20 mg) in 25 mL of 0.1 M Tris-HCl (pH 8.0) containing 5 mM $CaCl_2$ at 37°C for 72 h. After incubation, the reaction mixture was concentrated to small amount and desalted by a gel-filtration with a Sephadex G-25 fine column (4.0 × 80 cm) equilibrated with 0.1N $NH_4OH$. The glycopeptide-fraction (elution volume, 400-700 ml) was concentrated with a rotary evaporator and lyophilized. The lyophilized glycopeptides (95.1 mg) were dissolved in 50% acetonitrile/water, and Asn-glycopeptides were further purified by the hydrophilic partitioning with Shodex Asahipak NH2P-50 resin as described in our previous paper,[7] Asn-glycopeptides were predominantly eluted with 0.1% TFA/water and the final amount of Asn-glycopeptides purified in this study was 15. 7 mg.

To identify structures of *N*-glycans linked to the bamboo shoot glycopeptides, *N*-glycans were liberated by hydrazinolysis (100°C, 10 hr) from the peptides (about 1 mg). The

liberated *N*-glycans were *N*-acetylated and pyridylaminated.[13] After gel filtration to remove of excess amount of 2-aminopyridine, the PA-sugar chains were partially purified by RP-HPLC using a Cosmosil 5C18 AR column (6.0 x 250 mm, Nacalai Tesque, Kyoto),[7] and PA-sugar chains were pooled as indicated by a horizontal bar in Fig. 1-I. Structural features of the PA-sugar chains obtained by RP-HPLC were analyzed by SF-HPLC using a Asahipak NH2P-50 column (0.46 x 25 cm, Showa Denko, Tokyo).[7] As shown in Fig. 1-II, seven PA-sugar chains (peak-a, peak-b, peak-c, peak-d, peak-e, peak-f, and peak-g) were observed, and each elution position was compared with those of authentic PCT *N*-glycans purified the water-plant glycoproteins.[7] The elution position of peak-a on SF-HPLC coincided with that of $Man_2Xyl_1Fuc_1GlcNAc_2$-PA (M2FX), peak-b with that of M3FX, peak-c with that of $GlcNAc_1Man_3Xyl_1Fuc_1GlcNAc_2$-PA (GN1M3FX), peak-d with that of $GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$-PA (GN2M3FX), peak-e with that of $Gal_1GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$-PA (G1GN2M3FX), peak-f with that of $Gal_1Fuc_1GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$-PA ((Le$^a$)1GN1M3FX), and peak-g with that of $Gal_2Fuc_2GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$-PA ((Le$^a$)2M3FX). These deduced structures were confirmed by ESI-MS analyses as follows: peak-a, *m/z* 1105.4 [$(M+H)^+$]; peak-b, *m/z* 1267.5 [$(M+H)^+$]; peak-c, *m/z* 735.8 [$(M+2H)^{2+}$]; peak-d, *m/z* 837.3 [$(M+2H)^{2+}$]; peak-e, *m/z* 918.4 [$(M+2H)^{2+}$]; peak-f, *m/z* 991.4 [$(M+2H)^{2+}$]; peak-g, *m/z*1145.4 [$(M+2H)^{2+}$]. These structures were further analyzed by exoglycosidase digestions (Fig. 2). Peaks-f and -g were converted to peak-d (GN2M3FX) by the sequential digestion with α-1,3/4-fucosidase and β-1,3/6-galactosidase, suggesting that PA-sugar chains in peak-f and peak-g must be modified with Lewis a epitope(s) at their non-reducing terminals. Finally, PA-sugar chains, peaks-c and -d, and the enzyme digests obtained from peaks-f and -g, were converted to peak-b by β-*N*-acetylglucosaminidase, suggesting modification with *N*-acetylglucosamine (GlcNAc) residue(s). Since peak-h emerged after digestion of the β-1,3/6-galactosidase digest with β-*N*-acetylglucosaminidase, xylosyl and afucosyl *N*-glycans ($GlcNAc_1Man_3Xyl_1GlcNAc_2$-PA or

Fig. 1

Fig. 2

GlcNAc$_2$Man$_3$Xyl$_1$GlcNAc$_2$-PA), which are eluted at almost the same elution positions to M3FX and GN1M3FX respectively, must be contaminated in peak-b or peak-c. These results indicate that almost all glycoproteins expressed in bamboo shoot carry PCT *N*-glycans but not high-mannose type ones, and some of them are modified with Lewis a epitope(s). This glycoform of bamboo *N*-glycans is basically similar to that of *N*-glycans linked to glycoproteins in a fresh-water plant, *Elodea nuttallii*,[9] although the relative amount of M3FX in the bamboo PCT *N*-glycans (32.6%) was higher than that in the water plant PCT *N*-glycans (12.1%). Since we have already confirmed that Lewis a epitope-containing *N*-glycans and the truncated type *N*-glycans (M3FX and M2FX) were separated by a gel-filtration with Sephadex G-25 column used in our previous study,[7] the Lewis a epitope-containing *N*-glycans can be easily prepared from the mixture of bamboo *N*-glycans or *N*-glycopeptides. The yield of each PCT *N*-glycans is summarized in Table. The yield of the Le$^a$ epitope-containing PCT *N*-glycans as pyridylaminated derivatives was more than 300 nmol from 700 g of bamboo shoot, indicating that bamboo shoot is one of good sources for preparation of Le$^a$ epitope-containing PCT *N*-glycans to evaluate their putative immunological activities. In previous study,[9] we found that the fresh-water plants, *Elodea nuttallii, Egeria densa, Ceratophyllum demersum,* are good sources for preparation of immunogenic PCT *N*-glycans. However, large amount contamination of water soluble pigments contained in these fresh-water plants interfered the extraction and purification steps, and we had to remove these pigments by repeat gel-filtration or hydrophilic chromatographic steps. On the other hand, the bamboo shoot extract was almost uncolored and the decolorization steps to purify PCT *N*-glycans or *N*-glycopeptides were not required. Furthermore, chips of bamboo shoot can be easily homogenized with 5% formic acid using a waring blender compared with the bulky water-plants.

Table

Using bamboo shoot glycopeptides obtained by the method established in this study, we have already succeeded in synthesis of a neoglycopolymer consisting of γ-poly glutamic acid and plant antigenic *N*-glycans (Itano, S., *et al*., Proceedings of the Japanese Society for

Immunology. **45**, 166 (2016)). The immunological activities of this neoglycopolymer will be described elsewhere.

**Author contribution**

Y.K. shared responsibility for the writing of the manuscript with C.T., K.F., and M.M. All authors were responsible for the study concept and design. C.T., K.F., and M.M. carried it out. All authors contributed to the critical revision of the manuscript.

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**Legends to figures**

**Fig. 1. RP-HPLC and SF-HPLC profiles of PA-sugar chains obtained from bamboo shoot glycopeptides.**

I. RP-HPLC profile of PA-sugar chains obtained from bamboo shoot glycopeptides.

II. SF-HPLC profiles of PA-sugar chains obtained in I.

**Fig. 2. SF-HPLC profiles of exoglycosidase digests of PA-sugar chains obtained from bamboo shoot glycopeptides**

1, PA-sugar chains from bamboo shoot glycopeptides. 2, *Streptomyces* α-1,3/4-Fuc'ase digest of 1, 3, β-1,3/6-Gal'ase digest of 2; 4, Jack bean β-GlcNAc'ase digest of 3.

**Table. Comparison of relative amount of *N*-glycans linked to *Elodea nuttallii* and bamboo shoot glycoproteins**

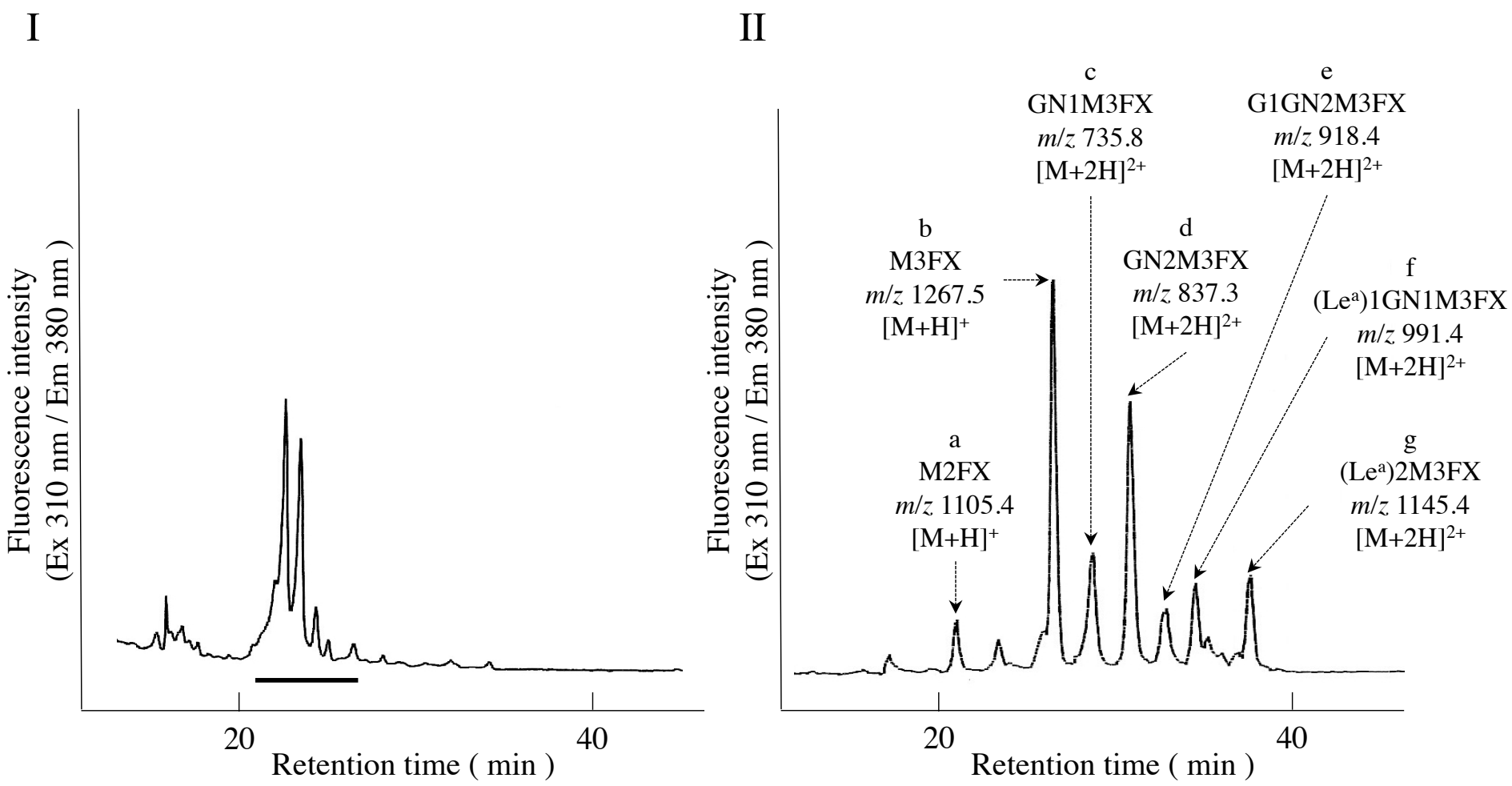
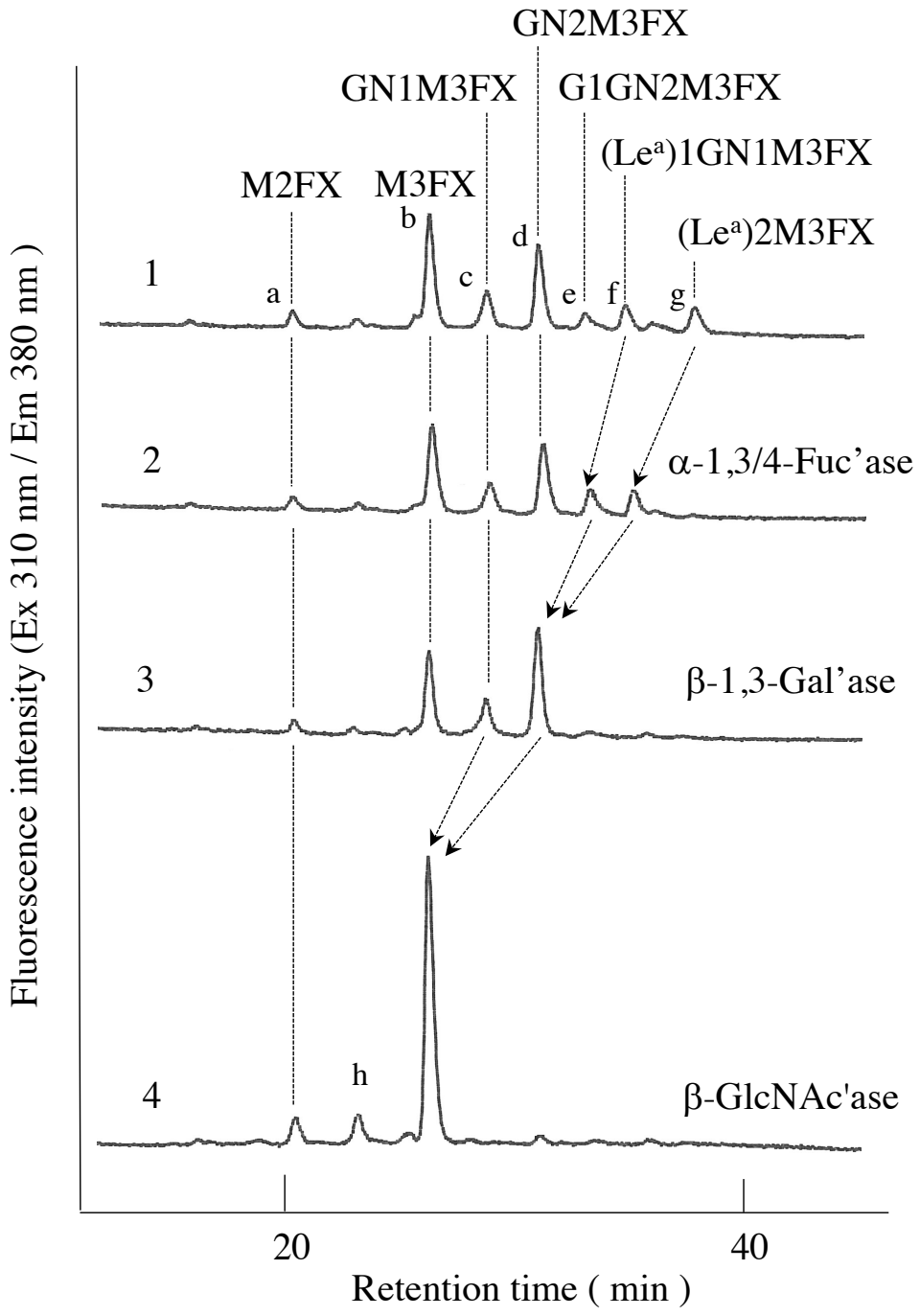
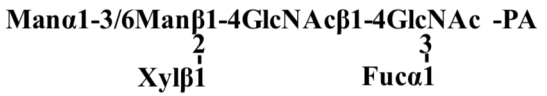


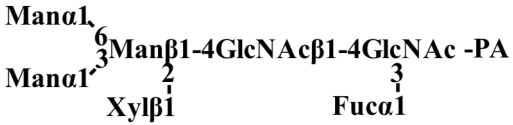

Fig. 1 Tanabe *et al*.



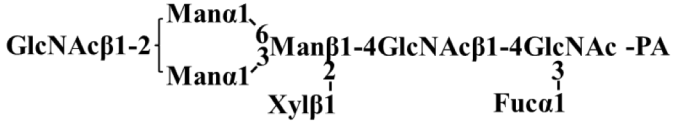

M2FX



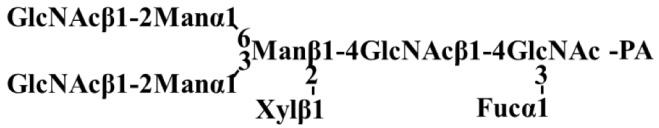

M3FX



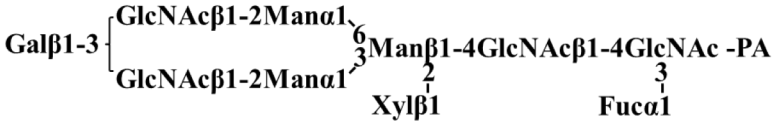

GN1M3FX



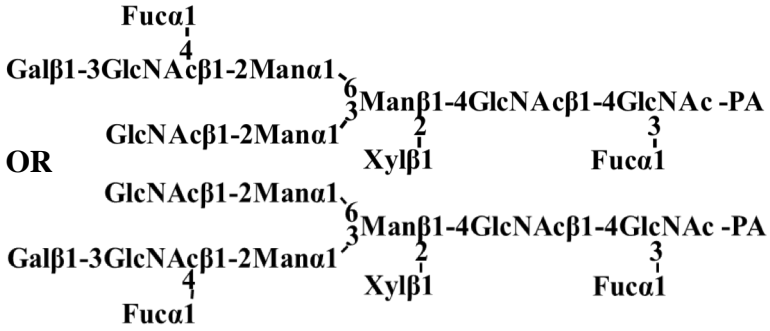

GN2M3FX




G1GN2M3FX




(Le^a)1GN1M3FX




(Le^a)2M3FX

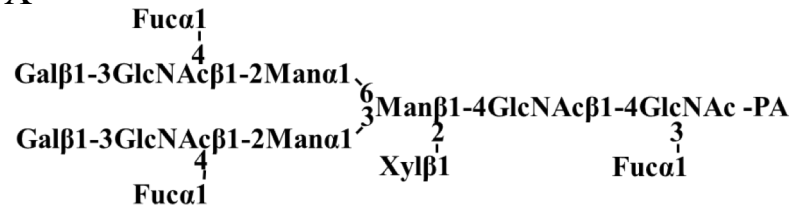



Fig. 2 Tanabe *et al*.

| Structure | *Elodea nuttallii* a) | | bamboo shoot | | |
|---|---|---|---|---|---|
| | (nmol/g) | (%) | (nmol/g) | (%) | Peaks |
| M2FX | 0.09 | 3.70 | 0.11 | 4.60 | Peak-a |
| M3FX | 0.29 | 12.1 | 0.79 | 32.6 | Peak-b |
| GN1M3X | | | | | |
| GN1M3FX | 0.28 | 11.7 | 0.26 | 10.6 | Peak-c |
| GN2M3X | | | | | |
| GN2M3FX | 0.94 | 39.8 | 0.63 | 26.1 | Peak-d |
| G1GN2M3FX | ND | | 0.17 | 7.10 | Peak-e |
| ($Le^a$)1GN1M3FX | 0.38 | 16.0 | 0.22 | 9.00 | Peak-f |
| ($Le^a$)2M3FX | 0.39 | 16.7 | 0.24 | 10.0 | Peak-g |

a) Reference [8]