Plant complex type free *N*-glycans occur in tomato xylem sap

Yuta Tsujimori,[a] Mikako Ogura,[b] Md. Ziaur Rhaman,[a, c] Megumi Maeda,[a,b] and Yoshinobu Kimura [a,b]†

[a] *Department of Biofunctional Chemistry, Graduate School of Environmental and Life Science, Okayama University, 1-1-1 Tsushima-Naka, Okayama 700-8530, Japan*

[b] *Faculty of Agriculture, Division of Agricultural Science, Okayama University, 1-1-1 Tsushima-Naka, Okayama 700-8530, Japan*

[c] Institute of Food and Radiation Biology, Atomic Energy Research Establishment, Bangladesh Atomic Energy Commission, Dhaka-1207, Bangladesh.

Corresponding Authors†

Yoshinobu Kimura, E-mail: yosh8mar@okayama-u.ac.jp; Tel: +81-86-251-8296

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, D-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.

Abstract

Free *N*-glycans (FNGs) are ubiquitous in growing plants. Further, acidic peptide:*N*-glycanase (PNGase) is believed to be involved in the production of plant complex type FNGs (PCT-FNGs) during the degradation of dysfunctional glycoproteins. However, the distribution of PCT-FNGs in growing plants has not been analyzed. Here, we report the occurrence of PCT-FNGs in the xylem sap of the stem of the tomato plant.

Keywords: free *N*-glycan, PNGase, deglycosylation, xylem sap, *Solanum lycopersicum*

Free *N*-glycans (FNGs) ubiquitously occur in various eukaryotes, including animals and plants, and it is believed that some of these FNGs are produced from misfolded glycoproteins by cytosolic peptide:*N*-glycanase (cPNGase) and endo-β-*N*-acetylglucosaminidase (ENGase) in the ERAD system. On the other hand, in plants, an alternative acidic PNGase, (aPNGase, optimum activity at acidic pH), plays a pivotal role in the release of plant complex type *N*-glycans bearing α-1-3 fucosyl and/or β-1-2 xylosyl residue(s) from glycopeptides or glycoproteins during metabolic degradation of such proteins when they have become dysfunctional. Plant aPNGases have been purified and their molecular characterization has been reported [1–5]; in addition, the enzyme products, FNGs carrying the chitobiosyl unit at their reducing end side (GN2-type), have been found in seedlings, fruits, seeds and tissue-culture broth [6–11]. Although the high-mannose type FNGs (HMT-FNGs) with one GlcNAc residue at their reducing end side (GN1-type) occur in the cytosol, the occurrences of GN1- or GN2-type PCT-FNGs in the apoplast fluid of growing plants is still unknown. We analyzed, therefore, the occurrences and structural analysis of free oligosaccharides in the xylem sap. In this study, we found for the first time that GN2-type PCT-FNGs and several exoglycosidase-activities involved in their degradation occur in the xylem sap of tomato plants, indicating that FNGs produced by aPNGase are secreted into the apoplast space and degraded in the apoplast fluid or xylem sap.

Tomato seeds (KGM 172, Kagome Co. Tochigi, Japan) were placed on sterile vermiculite under a 16-h-light/8-h-dark photoperiod at 24℃ until germination. Nineteen-day-old seedlings were transferred to 10.5 cm pots containing sterile culture soil (Metro-Mix 350, Sun Gro Horticulture MA, USA) and incubated under a 16-h-light/8-h-dark photoperiod at 24℃. Water was supplied every two days, and a HYPONeX® solution (1,000 times diluted with water) was supplied every two weeks. Stems of 55-day-old seedlings were decapitated at approximately 5 cm above the roots with a stainless-steel razor. After discarding the first two or three drops of exudate emerging from the cut surface on the root side, we washed the cut surface with distilled water and collected the exudate as xylem sap in tubes on ice [12]. The xylem sap extruding on the top of the stems was collected by micropipette with a 200-μL tip. The sample solutions were stored at -20ºC until use. Total xylem sap (4.6 mL) was centrifuged and filtered with a VIVASPIN 15R (MWCO 10,000) (Sartorius Stedim Biotech, Goettingen, Germany) at 15,000 x *g* for 20 min. The resulting filtrate (4.0 mL) was used for the structural analysis of free glycans and the concentrated solution (0.6 mL, 700 μg protein/mL)) was used for the assay of exoglycosidase activities.

Filtrates (1 mL each) were desalted by gel-filtration using a Sephadex G-25 superfine column (1.5 x 16 cm) in 0.1 N NH_4OH, and the oligosaccharide-fraction was lyophilized. The resulting oligosaccharides were pyridylaminated by the method previously described [10,11]. After gel filtration to remove of excess 2-aminopyridine, PA-sugar chains were partially purified by RP-HPLC using a Cosmosil 5C18 AR column (6.0 x 250 mm, Nacalai Tesque, Kyoto) [10,11], and the pyridylaminated *N*-glycan fraction was 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 an Asahipak NH2P-50 column (0.46 x 25 cm, Showa Denko, Tokyo) [10,11]. As shown in Fig. 1-II, five PA-sugar chains (peaks a-e) were observed, and each elution position was compared with that of authentic PCT *N*-glycans purified from water plant glycoproteins or Japanese cypress pollen allergen (Cha o3) [13,14]. The elution positions of peaks a, b, c, d, and e on SF-HPLC, coincided with those of $Man_2Xyl_1Fuc_1GlcNAc_2$-PA (M2FX), $Man_3Xyl_1Fuc_1GlcNAc_2$-PA (M3FX), $GlcNAc_1Man_3Xyl_1Fuc_1GlcNAc_2$-PA (GN1M3FX), $GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$-PA

Fig. 1

(GN2M3FX), and $Gal_1Fuc_1GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$-PA ($(Le^a)$1GN1M3FX), respectively. These structures were further analyzed by exoglycosidase digestions (Fig. 1-II-2, -3). Peak e was 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 e must be modified with one unit of Lewis a epitope at the non-reducing terminals. Finally, PA-sugar chains, peaks c and d, and the enzyme digests obtained from peak e, were converted to peak b by jack bean β-*N*-acetylglucosaminidase, suggesting modification with *N*-acetylglucosamine (GlcNAc) residue(s). These results indicated that the GN2-type PCT-FNGs including the Le^a-containing glycan, but not GN2-type HMT-FNGs, dominantly occur in tomato xylem sap, suggesting that these GN2-type FNGs must be produced by aPNGase from the glycopeptides formed by proteolysis of secreted glycoproteins that have become dysfunctional. In the previous report [11], we also found the GN2-type PCT-FNGs such as (Le^a)1GN1M3FX , GN2M3FX, and M3FX in the culture broth of rice cells and M3FX was a major component, indicating a similar structural feature to that of GN2-FNGs found in the tomato xylem sap. However, in the rice culture broth, GN1-type HMT-FNGs were also found in the extract of rice cells [11]. These GN1-type PCT-FNGs, however, could not be detected from the xylem sap in this study, suggesting that GN1-type PCT-FNGs must be rapidly degraded by several exoglycosidases after secretion into the apoplast fluid while GN2-type PCT-FNGs accumulate. In fact, the α-mannosidases were more active toward GN1-type FNGs than GN2-type FNGs [15, 16], indicating that the reducing end GlcNAc residue in GN2-type FNGs decreased the reaction rate of the glycosidase. On the occurrence of GN1-type PCT-FNGs in the cell culture broth, we have proposed a new biosynthesis mechanism responsible for the production of these GN1-type PCT-FNGs [11,17], since the plant ENGase was almost inactive toward the plant complex type *N*-glycans bearing α-fucosyl and/or β-xylosyl residue(s) [18,19]. The proposed mechanisms for the production of GN1-type PCT-FNGs are as follows. GN1-type HMT produced from the misfolded glycoproteins by PNGase and ENGase in the plant cytosol may be transported back to the ER, and then the GN1-type HMT-FNGs may be processed to the GN1-type PCT-FNGs in the Golgi apparatus, and finally the resulting processed *N*-glycans, GN1-PCT-FNGs, must be secreted into the extracellular or apoplast fluid [11,17]. In fact, we have

recently found that both GN1-type and GN2-type HMT-FNGs occur in the microsome fraction prepared pumpkin seedlings [20].

M3FX was a major FNGs in the xylem sap (196 pmol/mL as a pyridylaminated glycan), although other PCT-FNGs also occur as minor components (GN1M3FX; 61 pmol/mL, GN2M3FX; 45 pmol/mL, M2FX; 65 pmol/mL, (Le^a)1GN1M3FX; 8.5pmol/mL). These results suggested that several kinds of exoglycosidases involved in the degradation of FNGs might occur in the xylem sap, and we analyzed α-mannosidase (α-Man'ase), β-*N*-acetylglucosaminidase (β-GlcNAc'ase), β-galactosidase (β-Gal'ase), and α-fucosidase (α-Fuc'ase) enzyme activities in the same xylem sap. Each exoglycosidase activity was assayed at pH 4.5 using various pyridylaminated *N*-glycans and the concentrated xylem sap (80 μL containing about 56 μg protein). The reaction mixtures were incubated at 37°C overnight. After stopping the enzyme reactions in boiling water, the enzyme products were analyzed by SF-HPLC using an Asahipak NH2P-50 column (0.46 x 25 cm) as described previously [21-23]. As shown in Fig. 2, activity of all four exoglycosidases was detected in xylem sap. Significant activities were measured for α-Man'ase and β-GlcNAc'ase, while that of α-Fuc'ase was moderate. These results suggested that the GN2 PTC-FNGs produced by aPNGase are degraded in the xylem sap by a combination of several exoglycosidases, although the degradation rate of M3FX by α-Man'ase seems to be slow, which explains the predominance of this structure in the xylem sap. The accumulation of free M3FX in the tomato xylem sap seems to suggest that the occurrence of the β1-2 xylosyl and α1-3-fucosyl resides on the core penta saccharide structure ($Man_3GlcNAc_2$) should decrease the reaction rate of the tomato α-mannosidase as described in the previous report [21].

Fig. 2

Given that xylem sap flows from the roots to the leaves and fruits unidirectionally, it follows that the GN2 PCT-FNGs found in the xylem sap collected from the stem near the root must be transferred or distributed to the upper parts of the plant such as the leaves, fruits, and other developing tissues. Although the physiological function(s) of these free *N*-glycans is still unknown, our study has confirmed, for the first time, the occurrence of GN2 PCT-FNGs and several secreted exoglycosidases involved in their degradation, in the xylem sap.

Author contributions

YK shared responsibility for writing the manuscript with YT, MO, ZMR, MM. All authors were equally responsible for the study concept and design, and all contributed equally to the critical revision of the manuscript.

Acknowledgments

We are grateful to Research Institute, Kagome Co. Ltd. for the supply of the tomato seeds.

Disclosure statement

No potential conflict of interests is reported by the authors

Funding

This work was partially supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Basic Research C No. 18K05559 to M.M., and No. 17K0819709 to Y.K.).

References

[1] Takahashi N, Nishibe H. Some characteristics of a new glycopeptidase acting on aspartylglycosylamine linkages. J. Biochem. 1978;84:1467–1473.

[2] Altmann F, Paschinger K, Dalik T, et al. Characterisation of peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase A and its N-glycans. Eur. J. Biochem. 1998;252:118–123.

[3] Lhernould S, Karamanos Y, Lerouge P, et al. Characterisation of the peptide-N^4-(N-acetylglucosaminyl) asparagine amidase (PNGase Se) from Silene alba Cells. Glycoconjugate J. 1995;12:94–98.

[4] Chang T, Kuo M-C, Khoo K-H, et al. Developmentally regulated expression of a peptide:*N*-glycanase during germination of rice seeds (*Oryza sativa*) and its purification and characterization. J. Biol. Chem. 2000;275:129–134.

[5] Hossain Md A, Nakano R, Nakamura K, et al. Molecular identification and characterization of an acidic peptide:N-glycanase from tomato (*Lycopersicum esculentum*) fruits. J. Biochem. 2010;147:157–165.

[6] Priem B, Gitti R, Bush C A, et al. Structure of ten free *N*-glycans in ripening tomato fruit. Plant. Physiol. 1993;102:445–458.

[7] Faugeron C, Lhernould S, Lemoine J, et al. Identification of unconjugated *N*-glycans in strawberry plants. Plant Physiol. Biochem. 1997;35:891–895.

[8] Priem B, Solokwan J, Wieruszeski J M, et al. Isolation and characterization of free glycans of the oligomannoside type from the extracellular medium of a plant cell suspension. Glycoconjugate J. 1990;7:121–132.

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

[10] Kimura Y, Matsuo S. Free *N*-glycans already occur at an early stage of seed development. J. Biochem. 2000;127(6):1013–1019.

[11] Maeda M, Kimura M, Kimura Y. Intracellular and extracellular free *N*-glycans produced by plant cells: Occurrence of unusual plant complex-type free *N*-glycans in extracellular spaces. J. Biochem. 2010;148:681–692.

[12] Masuda S, Sakuta C, Satoh S. cDNA Cloning of a Novel Lectin-Like Xylem Sap Protein and Its Root-Specific Expression in Cucumber. Plant Cell Physiol. 1998;39:1330–1336.

[13] Maeda M, Tani M, Yoshiie T. et al. 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. Carbohydr. Res. 2016;435:50–57.

[14] Osada T, Maeda M, Tanabe C. et al. Glycoform of a newly identified pollen allergen, Cha o 3, from *Chamaecyparis obtusa* (Japanese cypress, Hinoki). Carbohydr. Res. 2017;448:18–23.

[15] Oku H, Hase S. Studies of the substrate specificity of neutral a-mannosidase purified from Japanese quail oviduct by using sugar chains from glycopeptides. J.Biochem. 1991; 110:982-989.

[16] Woo K K, Miyazaki M, Hara S, Kimura M, Kimura Y. Purification and characterization of a Co (II)-sensitive a-mannosidase from *Ginkgo biloba* seeds. Biosci. Biotechnol. Biochem. 2004;68:2547-2556.

[17] Maeda M. and Kimura Y. Structural features of free *N*-glycans occurring in plants and functional features of de-*N*-glycosylation enzymes, ENGase, and PNGase: the presence of unusual plant complex type *N*-glycans. Front. Plant Sci. 2014: 04 September doi.org/10.3389/fpls.2014.00429

[18] Kimura Y, Tokuda T, Ohno A, et al. Subcellular localization of endo-β-N-acetylglucosaminidase and high-mannose type free *N*-glycans in plant cells. *Biochim. Biophys. Acta*. 1998;1381: 27-36.

[19] Nakamura K, Inoue M, Maeda M et al. Molecular cloning and gene expression analysis of tomato endo-β-*N*-acetylglucosaminidase, an endoglycosidase involved in the production of high-mannose type free *N*-glycans during tomato fruit ripening. Biosci. Biotechnol. Biochem. 2009;73:461-464.

[20] Katsube M, Abe Y, Tanaka T, et al. Protein-folding promoting and amyloid-formation inhibitory activity of HMT free *N*-glycans. The 36th Annual Meeting of the Japanese Society of Carbohydrate Research: 2017 Jul; Asahikawa, Japan, p. 94.

[21] Hossain Md A, Nakano R, Nakamura K, et al. Molecular characterization of plant acidic α-mannosidase, a member of glycosylhydrolase family 38, involved in the turnover of N-glycans during tomato fruit ripening. J. Biochem. 2010;148:603–616.

[22] Rahman Md Z, Maeda M, Kimura Y. β-Galactosidase from *Ginkgo biloba* seeds active against β-galactose-containing N-glycans: purification and characterization. Biosci. Biotechnol. Biochem. 2015;79:1464–1472.

[23] Rahman Md Z, Tsujimori Y, Hossain Md A, et al. Molecular characterization of second tomato α1,3/4-fucosidase (α-Fuc'ase Sl-2), a member of glycosyl hydrolase family 29 active toward the core α1,3-fucosyl residue in plant *N*-glycans. J. Biochem. 2018;164:53–63.

Figure Legends

Figure 1. HPLC-profiles of PA-sugar chains obtained from tomato xylem sap and their glycosidase digests.

I, RP-HPLC profile of PA-sugar chain prepared from xylem sap.

GN1 FNGs, elution range for GN1-type free N-glycans; GN2 FNGs, elution range for GN1-type free N-glycans.

II, SF-HPLC profiles of exoglycosidase digests of GN2 type FNGs obtained in I. 1, SF-HPLC profiles of PA-sugar chain obtained in I; 2, α-Fuc'ase digest of 1; 3, β-Gal'ase digest of 2; 4, β-GlcNAC'ase digest of 3. R means GlcNAcβ1-4(Fucα1-3)GlcNAc-PA.

Figure 2. Analyses of exoglycosidase activities (α-Fuc'ase, β-Gal'ase, β-GlcNAC'ase, and α-Man'ase) by SF-HPLC.

I, α-Fuc'ase activity. G2F2GN2M3FX-PA was used as a substrate and incubated with the xylem sap (80 μL containing about 56 μg protein) at pH 4.5, 37ºC overnight. The reaction mixture was analyzed by SF-HPLC using a Shodex NH2P-50 column (4.6 x 250 mm) and the elution program as described previously [11,13,14]. 1, substrate; 2, treated with xylem sap. II, β-Gal'ase activity. G2GN2M3-PA was used as a substrate. 1, substrate; 2, treated with xylem sap. III, β-GlcNAc'ase activity. GN2M3FX-PA was used as a substrate. 1, substrate; 2, treated with xylem sap. IV, α-Man'ase activity. $Man_9GlcANc_2$-PA was used as a substrate. 1, substrate; 2, treated with xylem sap.

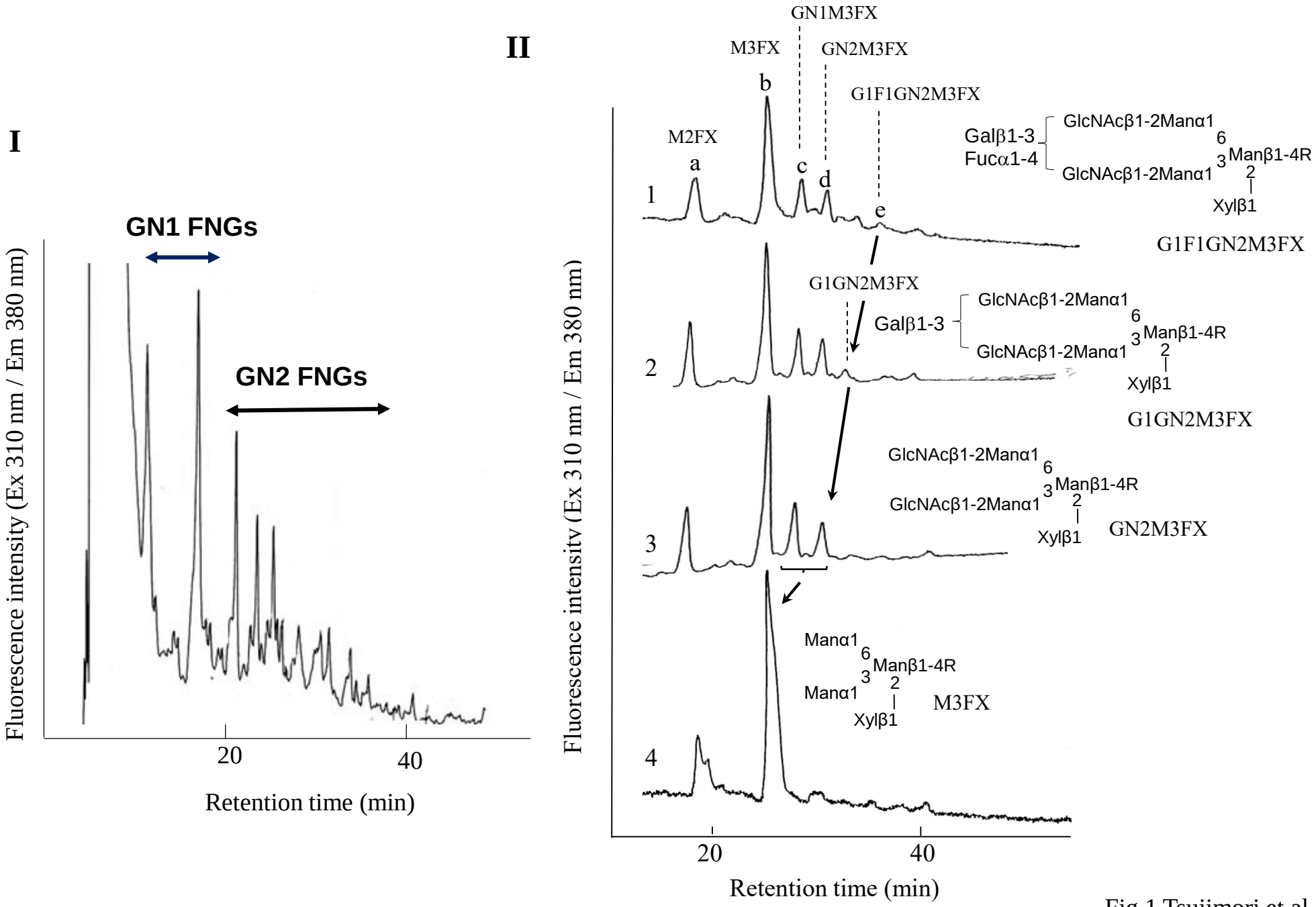

Fig.1 Tsujimori et al

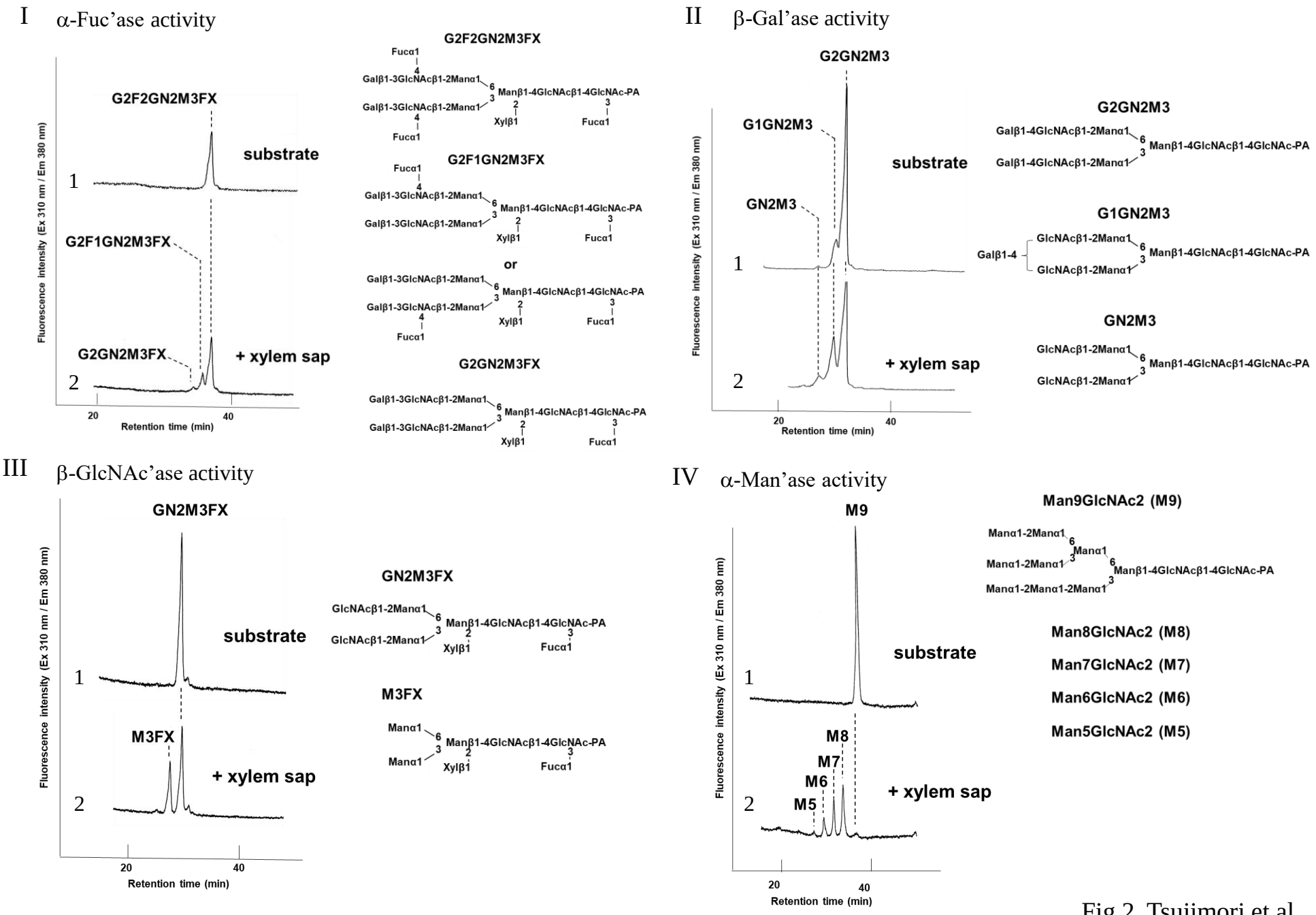

Fig.2. Tsujimori et al