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The Possibility that Plant Complex Type Free *N*-Glycans Localize in Cell Wall Fraction

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In this report, we bring up the possibility that complex type free N-glycans may localize in cell wall fraction of developing seeds (*Ginkgo biloba* seeds). Several free *N*-glycans extracted by mild acid hydrolysis of cell wall fraction were coupled with 2-aminopyridine and purified by gel filtration, size-fractionation HPLC, and reversed-phase HPLC. The structures of the pyridylaminated free N-glycans were identified by two-dimensional sugar chain mapping, α-1, 2-mannosidase digestions, and ion-spray tandem mass spectrometry. The structural analyses showed that high-mannose type free N-glycans having one GlcNAc residue ($Man_{8-5}GlcNAc_1$) and plant complex type free N-glycans having the N-acetyl chitobiose unit also occur in the cell wall fraction of the developing *Ginkgo* seeds. However, quantitative analysis of such free N-glycans showed that the plant complex type free glycans found in small amounts (~3%) in the cytosolic fraction accounted for nearly 40% of total free *N*-glycans. This observation suggested that the complex type free N-glycans might occur and localize in the cell wall fraction.

Key words : free *N*-glycan, plant *N*-glycan, localization of free *N*-glycan, cell wall fraction, *Ginkgo biloba*

Introduction

In our previous reports,[1–5] we reported that free *N*-glycans ubiquitously occur in developing or growing plant cells, suggesting that such free *N*-glycans might play an important role for plant cell development or growth. During the structural and distributional analyses of such free *N*-glycans in plant cells, we noticed that high-mannose type free glycan always accounted for more than 90% of total free *N*-glycans in water soluble fraction (cytosolic fraction), although both high-mannose type and complex type free *N*-glycans were found ubiquitously. Furthermore, we recently reported that a pumpkin endo-β-*N*-acetylglucosaminidase, which is involved in the production of the high-mannose type free *N*-glycans, localizes in the cytosolic fraction of cotyledons. These results suggested that high-mannose type free *N*-glycans may be produced and accumulated in the cytosolic fraction of the developing plant cell.

On the other hand, glycoamidase (peptide: *N*-glycanase) activities, which must be involved in production of the plant complex type free *N*-glycans, have also been also found in various plant cells,[6–8] suggesting that this amidase might also play an important role in glycoprotein metabolism in plant cells. In our previous reports,[4,9,10] we revealed that free *N*-glycans (both high-mannose type (97%) and plant complex type (3%) already occur in developing *Ginkgo biloba* seeds, and the glycoproteins in the seeds carry only the plant complex type *N*-

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glycans. These results led us to postulate that the plant complex type free *N*-glycans derived by the glycoamidase may not be localized in the cytosolic fraction but in other organelle, such as the cell wall. Therefore, in this report, using the developing *Ginkgo* seeds for a model plant, we started the structural and distributional analyses of two types of free *N*-glycans occurring in cell wall fraction.

Materials and Methods

Materials — Developing seeds of *Ginkgo biloba* were collected on the campus of Okayama University in the first week of July, 1998. A Cosmosil 5C18-AR column (0.60×25 cm) was purchased from Nacalai Tesque, Inc., and an Asahipak NH2P-50 column (0.46×25 cm) from Showa Denko Co. α-1, 2-Mannosidase (*Aspergillus oryzae*) was the generous gift of Dr. T. Yoshida (Hirosaki University, Japan). Authentic PA-sugar chains were prepared as described in our previous papers.[1–4]

Preparation of oligosaccharide from cell wall fraction of Ginkgo seeds — Developing *Ginkgo biloba* seeds (119 g) were homogenized in 500 ml of 25 mM Tris-HCl buffer (pH 8.5). The suspension was exhaustively dialyzed against running tap water over night to exclude the water-extracted free *N*-glycans in cytosol fraction. After centrifugation of the resulting dialysate, the precipitate was heated in 0.01 N HCl/20% methanol at 100°C for 30 min and then dialyzed against deionized water (2 L twice). The resulting outer solution (4 L) was concentrated to about 25 ml by rotary evaporator. The concentrated outer solution was desalted by a Sephadex G-25 column (1.8×40 cm, in 50 mM NH_4OH). The eluent from the Sephadex G-25 column (elution volume: 60 ml to 120 ml) was pooled and lyophilized.

Pyridylamination of free N-glycans — Pyridylamination of free *N*-glycans was done as described in our previous papers.[3,4] Separation of PA-sugar chains was done 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 an Asahipak NH2P-50 column (0.46×25 cm) as described in our previous papers.[3,4]

α-1, 2-Mannosidase Digestions — α-1, 2-Mannosidase (*Aspergillus oryzae*) digestion and the HPLC analysis of the product were done slso as described in our previous reports.[3,4]

Ion-spray mass spectrometry — IS-MS and MS/MS analyses of PA-oligosaccharides were performed as described in our previous reports,[1–4] using a Perkin Elmer Sciex API-III, triple-quadrupole mass spectrometer with an atmospheric-pressure ionization ion source.

Results and Discussion

Purification of pyridylaminated (PA-) free N-glycans

PA-Derivatives of oligosaccharide fraction prepared from the cell wall fraction were first separated by SF-HPLC. As shown in Fig. 1-(I), eight PA-sugar chain fractions (F1～8) were separated. IS-MS analysis showed that the main PA-sugar chains contained in these fractions were hexose oligomers such as Hexose9～5Hexose-PA. Some pyridylaminated *N*-glycans, however, were found in F2 to F5 (Fig. 1-(II)). IS-MS analyses of such pyridylaminated *N*-glycans showed H-A, -B, -C, and -D were typical high-mannose type structures with one GlcNAc resi-

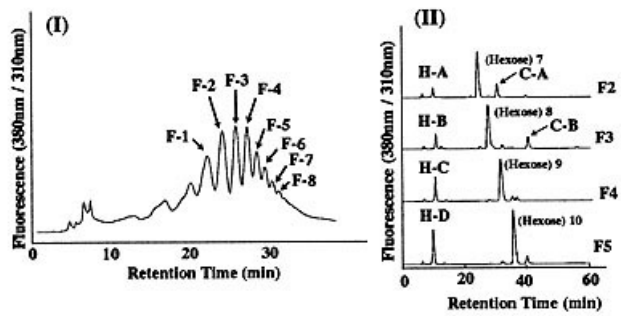

Fig. 1 HPLC of PA-derivatives prepared from the cell wall fraction of developing *Ginkgo biloba* seeds.
(I) Size-fractionation HPLC.
(II) RP-HPLC of F2, F3, F4, and F5 in (I).

due at their reducing end; $Man_5GlcNAc_1$-PA (m/z 1110.0) for H-A, $Man_6GlcNAc_1$-PA (m/z 1272.0) for H-B, $Man_7GlcNAc_1$-PA (m/z 1435.0) for H-C, and $Man_8GlcNAc_1$-PA (m/z 1596.5) for H-D. IS-MS analyses of C-A and-B had typical plant complex type with *N*-acetyl chitobiose unit; $Man_3Xyl_1Fuc_1GlcNAc_2$-PA (m/z 1267.5) for C-A, and $GlcNAc_1Man_3Xyl_1Fuc_1GlcNAc_2$-PA ($m/z$ 1470.5) for C-B.

Structural analysis of free N-glycans purified from cell wall fraction of developing Ginkgo seeds

As shown in Fig. 2, the typical high-mannose type free *N*-glycans were converted to $Man_5GlcNAc_1$-PA by *Aspergillus* α-1, 2-mannosidase digestion, suggesting these high-mannose type glycans share a common structural unit; α Man1-6 (Manα1-3) Manα1-6 (Manα1-3) Manβ1-4GlcNAc. This structural feature of high-mannose type free *N*-glycans found in cell wall fraction of *Ginkgo* seed agreed with that of the same type free glycans found in other plant cells.[1-5] The amount of the high-mannose type free *N*-glycans recovered from the cell wall fraction was 462.7 pmol/g fresh weight of seed (Table 1), and this was about 1/5 of that of high-mannose type free *N*-glycans recovered in cytosolic fraction.

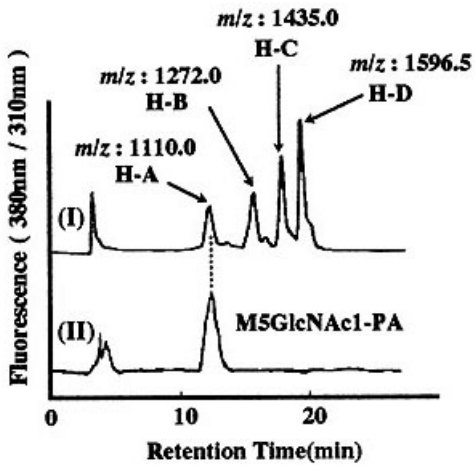

Fig. 2 α-1, 2-Mannosidase Digestion of mixture of H-A, -B, -C and -D.
(I) SF-HPLC of mixture of H-A, -B, -C, and -D.
(II) SF-HPLC of α-1, 2-mannosidase digest of I.
H-A Man5GlcNAcl-PA; H-B, Man6GlcNAcl-PA
H-A Man7GlcNAcl-PA; H-D, Man8GlcNAcl-PA

The structures of C-A and -B were analyzed by IS-MS analysis and the two dimensional PA-sugar chain map.

The elution position of C-A on the 2D sugar chain map corresponded to that for M3FX, and the molecular mass $[M+H]^+$ was 1267.5, which agreed with the calculated molecular mass (1267) for M3FX. Furthermore, as shown in Fig. 3-(I),-(II), the relevant signals observed by MS/MS analysis of C-A could be reasonably assigned as fragment ions derived from the M3FX; m/z 1121.5 ($Man_3Xyl_1GlcNAc_2$-PA), m/z 990.0 ($Man_3GlcNAc_2$-PA), m/z 959.5 ($Man_2Xyl_1GlcNAc_2$-PA), m/z 503.0 (GlcNAc-GlcNAc-PA), m/z 466.0 (Fuc1GlcNAc1-PA), m/z 300 (GlcNAc-PA). These results suggested the structure of C-A should be M3FX.

The elution position of C-B on the 2D sugar chain map corresponded to that for ^{GN}M3FX, and the molecular mass $[M+H]^+$ was 1470.5, which

Table 1 Summary of Free N-Glycans in Developing *Ginkgo biloba* Seeds

Structure	Water soluble fraction[a)]		Cell wall fraction	
	pmol/g	(%)	pmol/g	(%)
Man3Xyl1Fuc1GlcNAc2	74.5	3.3	156.9	20.4
GlcNAc1Man3Xyl1Fuc1GlcNAc2	ND	ND	152.8	19.8
Man5GlcNAc1	101.2	4.5	68.3	8.8
Man6GlcNAc1	167.6	7.4	91.3	11.8
Man7GlcNAc1	277.0	12.3	129.7	16.8
Man8GlcNAc1	1220.8	54.1	173.4	22.4
Man9GlcNAc1	424.3	18.4	ND	ND

a), Reference 4): ND, not detected

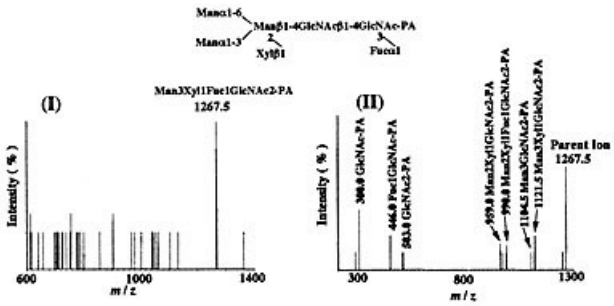

Fig. 3 IS-MS Analysis of C-B in Fig. 1.
(I) IS-MS Analysis
(II) MS/MS Analysis of $[M+H]^+=1267.5$

agreed with the calculated molecular mass (1470) for ^{GN}M3FX. Furthermore, the relevant signals observed by MS/MS analysis of C-B could be reasonably assigned as fragment ions derived from the ^{GN}M3FX; m/z 1267.5 ($Man_3Xyl_1Fuc_1GlcNAc_2$-PA) m/z 1121,5 ($Man_3Xyl_1GlcNAc_2$-PA), m/z 990.0 ($Man_3GlcNAc_2$-PA), m/z 959.5 ($Man_2Xyl_1GlcNAc_2$-PA), m/z 503.0 (GlcNAc-GlcNAc-PA), m/z 466.0 ($Fuc_1GlcNAc_1$-PA), m/z 300 (GlcNAc-PA). These results suggested the structure of C-B should be ^{GN}M3FX.

The amount of plant complex type free N-glycans recovered from the cell wall fraction was about 310 pmol/g fresh weight of seed (Table 1), and this amount was comparable with that of the high-mannose type free glycans (460 pmol/g fresh weight of seed). Comparing the amount of the plant complex type free N-glycans in cytosolic and cell wall fraction, the relative amount in the cytosolic fraction was only 3% of total free glycans, whereas the amount in cell wall fraction was more than 40% of total free N-glycans, indicating that the plant complex type free N-glycans may reside in the cell wall fraction (Table 1). Since these plant complex type free N-glycans could be recovered from the cell wall fraction by heat treatment in 0.01 N HCl/20% methanol, they may be fixed with cellulose or hemicellulose matrix through hydrogen bond networks and/or hydrophobic interactions.

Acknowledgment

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植物複合型遊離*N*-グリカンが細胞壁に局在する可能性

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登熟期銀杏種子の細胞壁画分に局在する遊離型*N*-グリカンの構造解析を行った.登熟期銀杏種子抽出物を徹底的に透析して細胞質中に存在する遊離*N*-グリカンを除去した後,細胞壁画分を遠心分離により回収した.得られた細胞壁画分を希酸処理（0.01 N HCl/20％メタノール中で100℃，30分間処理）した後，透析によりオリゴ糖鎖を透析外液に回収した．オリゴ糖鎖画分を2-アミノピリジンで蛍光標識した後，遊離*N*-グリカンをゲルろ過およびサイズフラクショネーション HPLC により精製した。遊離*N*-グリカンの構造解析は，IS-MS 分析，α-1，2-マンノシダーゼ消化，および糖鎖2次元マップ法を組み合わせることにより行った．その結果，細胞壁画分からは，ハイマンノース型および植物複合型構造を有する遊離*N*-グリカンが検出されたが，細胞質画分には74 pmol/ g fresh weight of seed（全遊離グリカンの3％相当）程度の存在量であった植物複合型遊離*N*-グリカンが，細胞壁画分では310 pmol/ g fresh weight of seed（全遊離グリカンの40％相当)程度の濃度を占めることが明らかとなった．この結果は，植物複合型構造の遊離*N*-グリカンは主に細胞壁画分に局在し，水素結合あるいは疎水結合により細胞壁マトリックスに強固に結合していることを示唆するものと考えられる．