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Changes in Asparagine-linked Sugar Chains of Glycoproteins in *Ricinus communis* Seeds during Callus Induction

Yoshinobu Kimura and Shigeaki Takagi
(Department of Bioresources Chemistry)

Using glycotechnology procedures, structural changes in asparagine-linked sugar chains (N-glycans) of glycoproteins in *Ricinus communis* seeds during dedifferentiation (callus induction) have been explored. N-Glycans were released from the glycoproteins in the 2,4-D derived callus tissues by hydrazinolysis, and the resulting oligosaccharides were N-acetylated and coupled with 2-aminopyridine. Structures of the purified pyridylaminated (PA-) N-glycans could be deduced by two-dimensional (2D) sugar chain mapping method. Structural analysis clearly showed that the relative amount of high-mannose type N-glycans of the endospermic glycoproteins decreased as the plant cells dedifferentiated, while that of complex type N-glycans increased. This observation suggested that enhancement of expression and/or activation of certain α-mannosidase(s) involved in N-glycan processing could occur during dedifferentiation of plant cells.

Key words : N-glycan structure, plant glycoprotein, callus induction, *Ricinus communis*

Introduction

In the last decade, it has been revealed that structural changes of N-linked sugar chains (N-glycans) of cell surface glycoproteins or cytosolic glycoproteins of animal cells do occur during cellular differentiation or dedifferentiation.[1-5] These observations have suggested that N-glycans of various glycoproteins could be good markers to indicate the differentiation or dedifferentiation stage of cells. Indeed the N-glycans of γ-glutamyltranspeptidase (γ-GTP),[6] α-fetoprotein (AFP),[7] and carcinoembryonic antigen (CEA)[8] have already been used as the tumor markers of hepatoma or colonic cancer. Since these changes in N-glycan structure of various glycoproteins should reflect the changes in expressions of some glycosyltransferases involved in the N-glycan processing, some glycosyltransferases (GlcNAc-, Fuc-, NeuAc-transferases) have been purified, characterized, and cloned in order to help figuring out the relationship between the structural changes of N-glycans and cellular dedifferentiation.[9-11]

On the other hand, little information is available so far concerning the structural changes in N-glycan conjugated to plant glycoproteins during cellular differentiation or dedifferentiation. In this report, we carried out comparative analysis of the N-glycan structures of endospermic glycoproteins from mature *Ricinus communis* seed and 2,4-D induced callus tissues. We have used hydrazinolysis to release N-glycans from

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Abbreviations
2,4-D, 2,4-dichlorophenoxyacetic acid; LS medium, Linsmaier-Skoog medium; PA-, pyridylamino; RP-, reversed phase; SF-, size-fractionation; 2D sugar chain map, two-dimensional sugar chain map; Man, D-mannose; Xyl, D-xylose; Fuc, L fucose; GlcNAc, N-acetyl-D-glucosamine

the endospermic glycoproteins, and prepared fluorescence-labeled oligosaccharides to identify their structures using a two-dimensional (2D) sugar chain map.[12-13] Structural analysis of such N-glycans in the callus tissue showed that the relative amount of high-mannose type structure decreased, while that of fucose/xylose-containing complex type structure increased during callus induction of *R. communis* seeds.

Materials and Methods

Materials— The plants *Ricinus communis* were grown during the summer of 1992 at the farm of Okayama University. 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. Hydrazine anhydrous was purchased from Pierce. Authentic pyridylaminated sugar chains were prepared as described in our previous reports.[14,15]

Callus induction from R. communis seeds— The threshed *R. communis* endosperms were sterilized in 1 % hypochlorous acid for 30 min and then exhaustively washed by distilled water (3L). The endosperms were incubated on solid (0.2 % Gellan Gum) LS medium containing 10 μM 2.4-D at 25 °C for 7, 21, 28, and 35 days.

Preparation of total glycoproteins from the seed or callus tissue— Defatted powder of *R. communis* endosperm or callus tissue was suspended in 50 mM HEPES buffer (pH 7.4), containing 0.1 % Triton X-100. After centrifugation (10,000 *g* for 30 min), to the supernatant was added 0.2 N NH_4OH to inactivate some glycosidases or proteases and to remove O-linked oligosaccharides. After exhaustive dialysis against deionized water, the dialyzate including the resulting precipitate was lyophilized.

Pyridylamination of the sugar chains— N-Glycans were released by hydrazinolysis (100 °C, 12 hr, in 20 ml of hydrazine anhydrous) from the lyophilized total glycoproteins obtained above (250~300 mg). After *N*-acetylation of the hydrazinolysate with the saturated ammonium bicarbonate (3 ml) and acetic anhydride (300 μl), the acetylated hydrazinolysate was desalted by Dowex 50 × 2 resin. Pyridylamination of the sugar chains was done by the method of Kondo *et al.*[16] 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). On the Cosmosil 5C18-AR column, the PA-sugar chains were eluted by increasing the content of 1-butanol from 0.05 to 0.25 % in 0.1M ammonium acetate buffer, pH 4.0, at a flow rate of 1.2 ml/min. In the case of size-fractionation (SF-) HPLC using the Asahipak NH2P-50 column, the PA-oligosaccharide was eluted by increasing the water content in the water-acetonitrile mixture from 30 to 50 % linearly for 25 min at a flow rate of 0.8 ml/min.

α-Mannosidase Assay— PA-Sugar chain ($Man_9GlcNAc_2$-PA, about 100 pmol) was incubated with 36 μl (containing about 1.4 mg protein/ml) of the crude extract prepared from each sample at each different stage and 100 μl of 0.1M Na-acetate buffer (pH 5.0, containing 0.1 % Triton X-100) at 37 °C for 12 hr. The reactions were stopped by boiling the reaction mixtures for 3 min. The resulting digests were analyzed by SF-HPLC. The fragments were eluted by increasing the water content from 20 to 50 % linearly.

Results and Discussion

Purification of PA-sugar chains from the glycoproteins expressed in callus tissue— Pyridylaminated derivatives prepared from the total glycoproteins in *Ricinus communis* endosperm and the callus tissue were first separated by Sephadex G-10 column, and the thus obtained PA-sugar chains were applied to RP-HPLC using the Cosmosil 5C18-AR column. As shown in Fig. 1, four PA-sugar chain fractions (F-I, -II, -III, and -IV) were always found at each

dedifferentiation stage (7, 21, 28, 35 days); however, the relative amounts of the several fractions varied during callus induction. The relative amount of F-II increased; on the contrary, that of F-III significantly decreased (Fig. 1). This result clearly suggested that the N-glycan structures of glycoproteins in *R. communis* seeds dramatically changed with time of dedifferentiation.

To identify these N-glycan structures, the partially purified PA-sugar chains were further purified by SF-HPLC on the Asahipak NH2P-50 column, and each elution position of the purified PA-sugar chains both on RP- and SF-HPLC was compared with those of well-characterized PA-sugar chains obtained from the endospermic or microsomal glycoproteins in developing *R. communis* seeds.[15,16] One major PA-sugar chain was obtained from F-I and F-III (data not shown), respectively; on the other hand, from F-II and F-IV several PA-sugar chains were further separated and purified as shown in Fig. 2. By comparing each elution position of these purified PA-sugar chains with those of various authentic PA-sugar chains both on RP- and SF-HPLC (2D sugar chain mapping method[12,13]), several structures of N-glycans obtained from the total glycoproteins in *R. communis* callus tissue could be deduced as summarized in Table 1.

N-Glycans in F-I and F-III— The structure of the major oligosaccharides in F-I and F-III could be identified as $Man_3Fuc_1Xyl_1GlcNAc_2$ (M3FX) and $Man_6GlcNAc_2$ (M6B), respectively (Table 1). These two N-glycans have been revealed to occur in abundance in the storage and microsomal glycoproteins of *R. communis* seeds; the former (M3FX) is an antigenic plant complex type structure and the latter (M6B) is a typical high-mannose type structure which ubiquitously occurs in both plant and animal cells. As shown in Table 2, the relative amount of M3FX in F-I was almost constant (35.4～51.3 %), while that of M6B in F-III dramatically decreased from 26.5 % to 5.8 % during the cellular dedifferentiation.

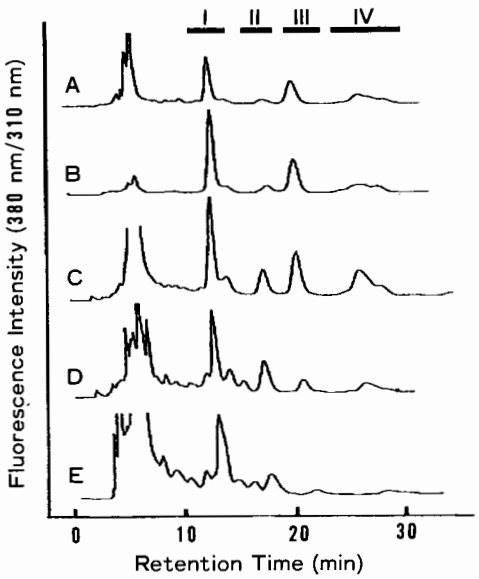


Fig. 1 RP-HPLC of PA-sugar chains prepared from glycoproteins in the callus induced from *R. communis* seeds.
A, day 0; B, day 7; C, day21; D, day 28; E, day 35

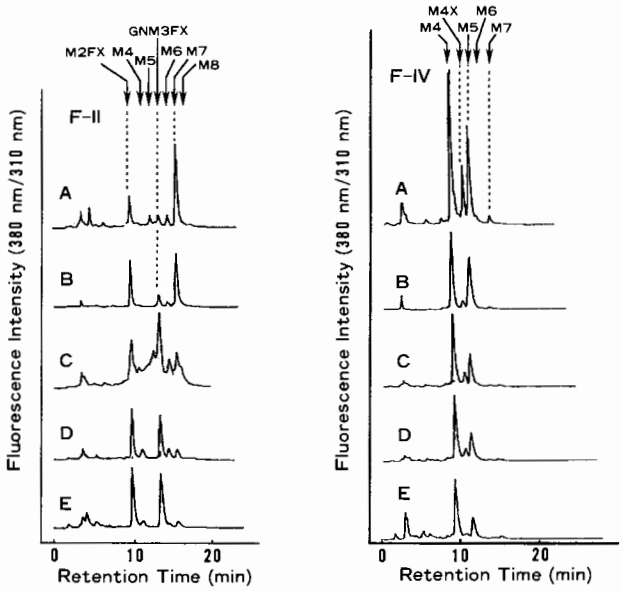


Fig. 2 SF-HPLC of Fraction-II and -IV obtained in Fig. 1.
M4～8, Man_4～$_8GlcNAc_2$-PA; M2FX, $Man_2Fuc_1Xyl_1GlcNAc_2$-PA; GNM3FX, $GlcNAc_1Man_3Fuc_1Xyl_1GlcNAc_2$-PA; M4X, $Man_4Xyl_1GlcNAc_2$-PA.
A, day 0; B, day 7; C, day21; D, day 28; E, day 35

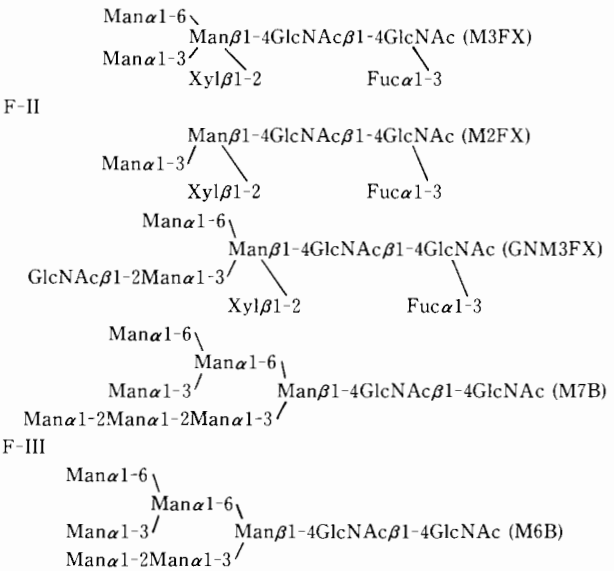
N-Glycans in F-II— As shown in Fig. 1, the

relative amount of F-II itself was very small in the early stage of dedifferentiation; however, the amount significantly increased after 21 days incubation on the 2,4-D containing LS medium. In F-II, the major structures were $Man_2Fuc1Xyl_1GlcNAc_2$ (M2FX, Table 2) and $Man_7GlcNAc_2$ (M7B, Table 2) in the early stage (0-7 days). As the cellular dedifferentiation proceeded, the relative amount of M2FX increased from 1.7 % to 14.3 %; on the other hand, that of M7B decreased from 4.5 % to 1.3 % (Fig. 2 F-II, Table 2). In addition to M2FX, the relative amount of $GlcNAc_1Man_3Fuc_1Xyl_1GlcNAc_2$ (GNM3FX, Table 2) structure, which was a minor component at the early stage, significantly increased from 0.7 % to 12.9 % (Fig. 2 F-II, Table 2).

N-Glycans in F-IV— From F-IV in Fig. 1, three major N-glycans ($Man_5GlcNAc_2$ (M5A), $Man_4GlcNAc_2$ (M4C), and $Man_4Xyl_1GlcNAc_2$ (M4X)) were found in the early stage (Fig. 2 F-IV, Table 2). The relative amount of M5A, which is a core structure of high-mannose type N-glycan, gradually decreased from 7.1 % to 1.2 %. In addition to M5A, the amount of M4X also decreased from 3.2 % to 0.2 %; however, the amount of M4C did not show any dramatic change for 4 weeks. This result may suggest that (1) the M4C structure could be resistant to α-

Table 1 Possible structures of N-glycans expressed in the callus tissue induced from *R. communis* seeds

F-I



F-II

F-III

F-IV

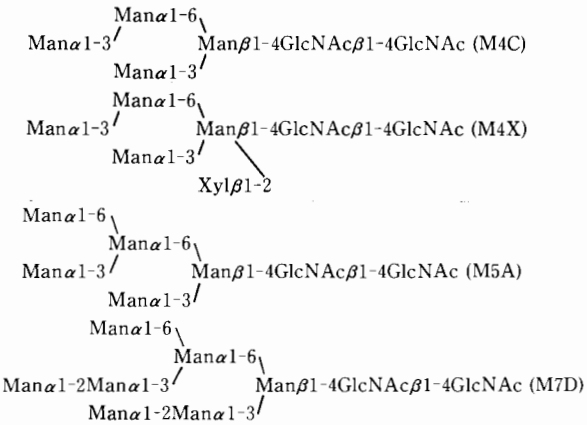


Table 2 Relative amount (%) of N-glycans linked to glycoproteins in the callus tissue induced from *R. communis* seeds

Culture Days	F-I			F-II					F-III	F-IV			
	M3FX	UN-1	UN-2	M2FX	UN-3	GNM3FX	UN-4	M7B	M6B	M4C	M4X	M5A	M7D
0	35.4	ND	1.3	1.7	0.6	0.7	0.7	4.5	26.5	15.7	3.2	7.1	2.6
7	44.6	ND	2.1	2.7	ND	0.9	0.2	3.7	28.3	9.6	1.0	6.7	0.2
21	32.7	2.7	0.8	4.9	1.7	7.1	2.4	3.3	21.8	17.3	1.5	3.8	ND
28	36.3	6.2	1.2	11.0	3.3	13.8	3.7	2.8	9.7	9.3	1.5	1.2	ND
35	51.3	5.2	1.0	14.3	1.6	12.9	0.9	1.3	5.8	4.3	0.2	1.2	ND

UN, Unknown structure; ND, not detected

mannosidase (s) in *R. communis* seed, or (2) a β-xylosidase, which is active with the M4X structure, could be activated during dedifferentiation of *R. communis* seeds. The amount of $Man_7GlcNAc_2$ (M7D), which was found as a minor component of N-glycans in the native seeds (2.6 %), also decreased and disappeared after 21 days incubation.

Overview on structural changes of conjugated N-glycans during dedifferentiation— As described above, N-glycan pattern of the endospermic glycoproteins expressed in *R. communis* seeds showed a dramatic change during cellular dedifferentiation. To our knowledge, this is the first report showing the structural changes of the conjugated N-glycans during dedifferentiation (callus induction) of plant cells. Our finding clearly indicated that N-glycosylation of plant glycoproteins should be profoundly associated with plant cell dedifferentiation. Since the relative amount of various high-mannose type structures (M7B, M7D, M6B, M5A) significantly decreased, and on the other hand, that of some complex type structures (GNM3FX, M2FX) increased during callus induction, we assumed that transcriptional level enhancement or enzyme-level activation of α-mannosidase(s) and/or N-acetylglucosaminyl transferase (GT-I) could occur during plant cell dedifferentiation. We first checked the α-mannosidase activity, using crude extract from the callus tissue in each stage of dedifferentiation. Although no α-mannosidase activity was detected in the starting sample (Fig. 3, day 0), this is probably due to an enzyme inactivation caused by hypochlorous acid, which was used for sterilization of endosperm. From the other four samples (day 7, day 14, day 21, and day 28), α-mannosidase activities were detected using $Man_9GlcNAc_2$-PA as a substrate (Fig. 3). Especially, the crude extract prepared from the day-21 sample efficiently converted $Man_9GlcNAc_2$-PA to $Man_{8\sim5}GlcNAc_2$-PA by hydrolyzing some α-1,2-mannosyl residues, suggesting an elevation of α-1,2-mannosidase activity[17] during callus induction. This observation indicated that the callus tissue derived from *R. communis* seeds could be a good source for isolation of α-1,2-mannosidase, which is a useful restriction enzyme in the field of "glycobiology" or "glycotechnology".

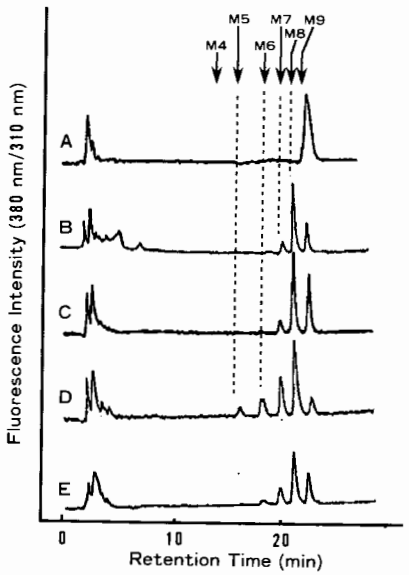


Fig. 3 SF-HPLC of $Man_9GlcNAc_2$-PA incubated with the crude extract prepared from *R. communis* callus.
A, day 0; B, day 7; C, day 14; D, day 21; E, day 28

The detailed structural analysis of the N-glycans purified in this report will be described elsewhere.

Acknowledgment

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References

1) Kobata, A : Structures and functions of the sugar chains of glycoproteins. *Eur. J. Biochem.*, **209**, 483-

501 (1992)

2) Rademacher, T. W., R. B. Parekh, and R. A. Dwek : Glycobiology. *Ann. Rev. Biochem.*, **57**, 785-838 (1988)

3) Ogier-Denis, E., P. Codogno, I. Chantret, and G. Trugnan : The processing of asparagine-linked oligosaccharides in HT-29 cells is a function of their state of enterocytic differentiation. *J. Biol. Chem.*, **263**, 6031-6037 (1988)

4) Dennis, J. W., S. Laferté C. Waghorne, M. L. Breitman, and R. S. Kerbel : β1-6Branching of Asn-linked oligosaccharides is directly associated with metastasis. *Science*, **236**, 582-585 (1987)

5) Mizoguchi, A., S. Takasaki, S. Maeda, and A. Kobata : Changes in asparagine-linked sugar chains of human promyelocytic leukemic cells (HL-60) during monocytoid differentiation and myeloid differentiation. *J. Biol. Chem.*, **259**, 11943-11957 (1984)

6) Yamashita, K., K. Totani, Y. Iwaki, I. Takamizawa, N. Tateishi, T. Higashi, Y. Sakamoto, and A. Kobata : Comparative study of sugar chains of γ-glutamyltranspeptidases purified from human hepatocellular carcinoma and from human liver. *J. Biochem.*, **105**, 728-735 (1989)

7) Yamashita, K., K. Taketa, S. Nishi, K. Fukushima, and T. Ohkura : Sugar chains of human cord serum α-fetoprotein : characteristics of N-linked sugar chains of glycoproteins produced in human liver and hepatocellular carcinomas. *Cancer Res.*, **53**, 2970-2975 (1993)

8) Yamashita, K., K. Fukushima, T. Sakiyama, F. Murata, M. Furuki, and Y. Matsuoka : Expression of Siaα2→6Galβ1→4GlcNAc residues on sugar chains of glycoproteins including carcinoembryonic antigens in human colon adenocarcinoma : application of *Trichosanthes japonica* agglutinin I for early diagnosis. *Cancer Res.*, **55**, 1675-1679 (1995)

9) Nishikawa, A., Y. Ihara, M. Hatakeyama, K. Kanagawa, and N. Taniguchi : Purification, cDNA cloning, and expression of UDP-N- acetylglucosamine : β-D-mannoside β-1,4 N-acetylglucosaminyl transferase III from rat kidney. *J. Biol. Chem.*, **267**, 18199-18204 (1992)

10) Kurosawa, N., T. Hamamoto, Y.-C. Lee, T. Nakaoka, N. Kojima, and S. Tsuji : Molecular cloning and expression of GalNAc α2,6-sialyltransferase. *J. Biol. Chem.*, **269**, 1402-1409 (1994)

11) Natsuka, S., K. M. Gersten, K. Zenita, Kannagi, R, and J. B. Lowe : Molecular cloning of a cDNA encoding a novel human leukocyte α-1,3-fucosyltransferase capable of synthesizing the sialyl Lewis x determinant. *J. Biol. Chem.*, **269**, 16789-16794 (1994)

12) Hase. S, and T, Ikenaka : Estimation of elution times on reversed-phase high-performance liquid chromatography of pyridylamino derivatives of sugar chains from glycoproteins. *Anal. Biochem.*, **184**, 135-138 (1990)

13) Tomiya, N., Y.-C. Lee, T. Yoshida, Y. Wada, M. Kurono, and N, Takahashi : Calculated two-dimensional sugar map of pyridylaminated oligosaccharides : elucidation of the jack bean α-mannosidase digestion pathway of $Man_9GlcNAc_2$. *Anal. Biochem.*, **193**, 90-100 (1991)

14) Kimura, Y., H. Suehisa, O. Yamaguchi, S. Nakajima, and S. Takagi : Structures of sugar chains of water-soluble glycoproteins in developing castor bean cotyledons. *Agric. Biol. Chem.*, **54**, 3259-3267 (1990)

15) Kimura, Y., Y. Nakagawa, T. Tokuda, M. Yamai, S. Nakajima, E. Higashide, and S. Takagi : Structures of N-linked oligosaccharides of microsomal glycoproteins from developing castor bean endosperms. *Biosci. Biotech. Biochem.*, **56**, 215-222 (1992)

16) Kondo. A., J. Suzuki, N. Kuraya, S. Hase, I. Kato, and T. Ikenaka : Improved method for fluorescence labeling of sugar chains with sialic acid residues. *Agric. Biol. Chem.*, **54**, 2169-2170 (1990)

17) Kimura, Y., O. Yamaguchi, H. Suehisa, and S. Takagi : In vitro hydrolysis of oligomannose-type sugar chains by an α-1,2-mannosidase from microsomes of developing castor bean cotyledons. *Biochim. Biophys. Acta.* **1075**, 6-11 (1991)

ヒマ種子カルス化に伴うアスパラギン結合型糖鎖の構造変化

木村吉伸・高木茂明
(生物資源開発学講座)

植物細胞の脱分化と糖蛋白質糖鎖（アスパラギン結合型糖鎖）の発現機構との相関を明らかにすることを目的とし，ヒマ種子から誘導したカルス中に発現されるアスパラギン結合型糖鎖（N-グリカン）の構造解析を行った．2,4-D処理によるカルス化誘導を行い，経時的に採取した組織からTriton X-100を含むHEPES緩衝液（pH 7.4）で全糖蛋白質を抽出した．得られた糖蛋白質からヒドラジン分解によりN-グリカンを遊離させた後，N-アセチル化，ピリジルアミノ(PA)化により蛍光標識糖鎖を調製した．逆相およびサイズフラクショネーションHPLCによりPA-糖鎖を単一に精製後，糖鎖2次元マップ法により構造の同定を行った．その結果，カルス化に伴い，ハイマンノース型糖鎖(Man7-4GlcNAc2)の相対量が顕著に減少するのに対して，キシロース/フコース含有型糖鎖（GlcNAc1Man3Fuc1Xyl1GlcNAc2）の相対量が増加することが明らかとなった．この現象は，植物細胞の脱分化に伴い，糖鎖プロセシングに関与するα-マンノシダーゼあるいはN-アセチルグルコサミン転移酵素の活性化が起こることを示唆するものと考えられる．