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Conversion of the Carbohydrate Structures of Glycoproteins in Roots of *Raphanus sativus* Using Several Glycosidase Inhibitors

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An attempt was made to convert the *N*-glycan structures in *Raphanus sativus* seeds during germination with a view to develop a method for regulating the *N*-glycan structures using glycosidase inhibitors. The *N*-glycan structures of glycoproteins in the roots of seedlings germinated for three days were analyzed by hydrazinolysis followed by *N*-acetylation, pyridylamination and HPLC. Pyridylaminated sugar chains obtained in the absence of the inhibitors had plant type structures consisting of $Man_3FucXylGlcNAc_2$(M3FX), $Man_{5-9}GlcNAc_2$(high-Man) and $GlcNAc_{1-2}Man_3FucXylGlcNAc_2$(GnM3FX and Gn2M3FX). When germinated in the presence of a glucosidase inhibitor (castanospermine or deoxynojirimycin), the amount of glucosyl high-Man-type structure increased and plant growth was inhibited. When germinated in the presence of a mannosidase inhibitor (swainsonine or deoxymannojirimycin), the amount of the high-Man-type structure increased and that of M3FX was low, and the growth was normal. In the presence of 2-acetamido 1, 2 di-deoxynojirimycin, those of GnM3FX and Gn2M3FX increased and the growth was normal. These results show that the *N*-glycan processing in both the endoplasmic reticulum (ER) and Golgi apparatus can be controlled artificially using glycosidase inhibitors, and that the glucosidase inhibitors could be useful for the study of the function of *N*-glycans in plants.

Key words: castanospermine, *N*-glycan, glycosidase inhibitor, *Raphanus sativus*, seed germination.

Abbreviations: CST, castanospermine; dFucNJ, deoxyfuconojirimycin; dGalNJ, deoxygalactonojirimycin; dGnNJ, 2-acetamido-1,2-dideoxynojirimycin; dMNJ, deoxymannojirimycin; DNJ, deoxynojirimycin; ER, endoplasmic reticulum; GNT 1, GlcNAc transferase 1; PA-, pyridylamino-; PBS, phosphate-buffered saline; SW, swainsonine. The abbreviations and structures of PA-sugar chains (G3M9, M9, M3FX, GnM3FX, Gn2M3FX, *etc.*) are shown in Fig. 4.

Most living cells contain Asn-linked oligosaccharides (*N*-glycans) and many of their structures have been elucidated; however, their functions are remain to be clarified in spite of the numerous studies on glycoproteins. In particular, we have only little information regarding plant *N*-glycan functions (*1*), although various biological activities have been proposed for *N*-glycans (*2–6*).

The biosynthesis of glycoproteins (*7*) is basically common from protozoans to animals. Briefly, the sugar chain of the precursor, Glc3Man9GlcNAc2-PP-dolichol, is transferred en bloc to a specific Asn residue of a nascent polypeptide, followed by processing by glycosidases and glycosyltransferases in the endoplasmic reticulum (ER) and Golgi apparatus. The trimming of Glc by glucosidases I and II, and the partial trimming of Man by mannosidase proceed in the ER. After the glycoproteins have been transported to the Golgi apparatus, they are further processed by mannosidase and GlcNAc transferase (GNT 1). Then in plants, Xyl and Fuc are transferred to *N*-glycans to yield plant-type structures (*1*).

To examine the functions of *N*-glycans, two approaches have been made commonly: one involves the use of glycosidase inhibitors and the other is a genetic approach involving mutants that lack a specific glycosidase or glycosyltransferase activity. A mutant *Arabidopsis* that lacks glucosidase I activity can not form seeds, indicating the importance of the glucose trimming of *N*-glycans in the ER (*8*, *9*). On the other hand, a mutant *Arabidopsis* that lacks *N*-acetylglucosaminyl transferase (GNT I) activity grows normally, and produces flowers and seeds, although the mutant lacks complex-type *N*-glycans (*10*). These findings are thought to be important clues as to the functions of *N*-glycans, which need to be reexamined by a different approach. Because of the lack of glucosidase I, seeds could not be formed, therefore further analysis of their functions is difficult.

Glucosidase inhibitors (castanospermine and deoxynojirimycin) and mannosidase inhibitors (swainsonine and deoxymannojirimycin) are well known to inhibit glycosidases participating in the processing and changes of the *N*-glycan structures when applied to cultured cells (*11*), but plant cells are not usually used. Purified plant glucosidase I is inhibited by castanospermine (*12*). An analytical method for determining *N*-glycan structures has been developed (*13–15*). These facts prompted me to study, using plant not cultured cells, to examine the functions of *N*-glycans in the presence of glycosidase inhibitors. To my knowledge, the application of such inhibitors to seed germination has not previously been performed. In this study an attempt was made to elucidate the effects of glycosidase inhibitors on the structures of the *N*-glycans in a plant. *Raphaunus sativus* was chosen as a

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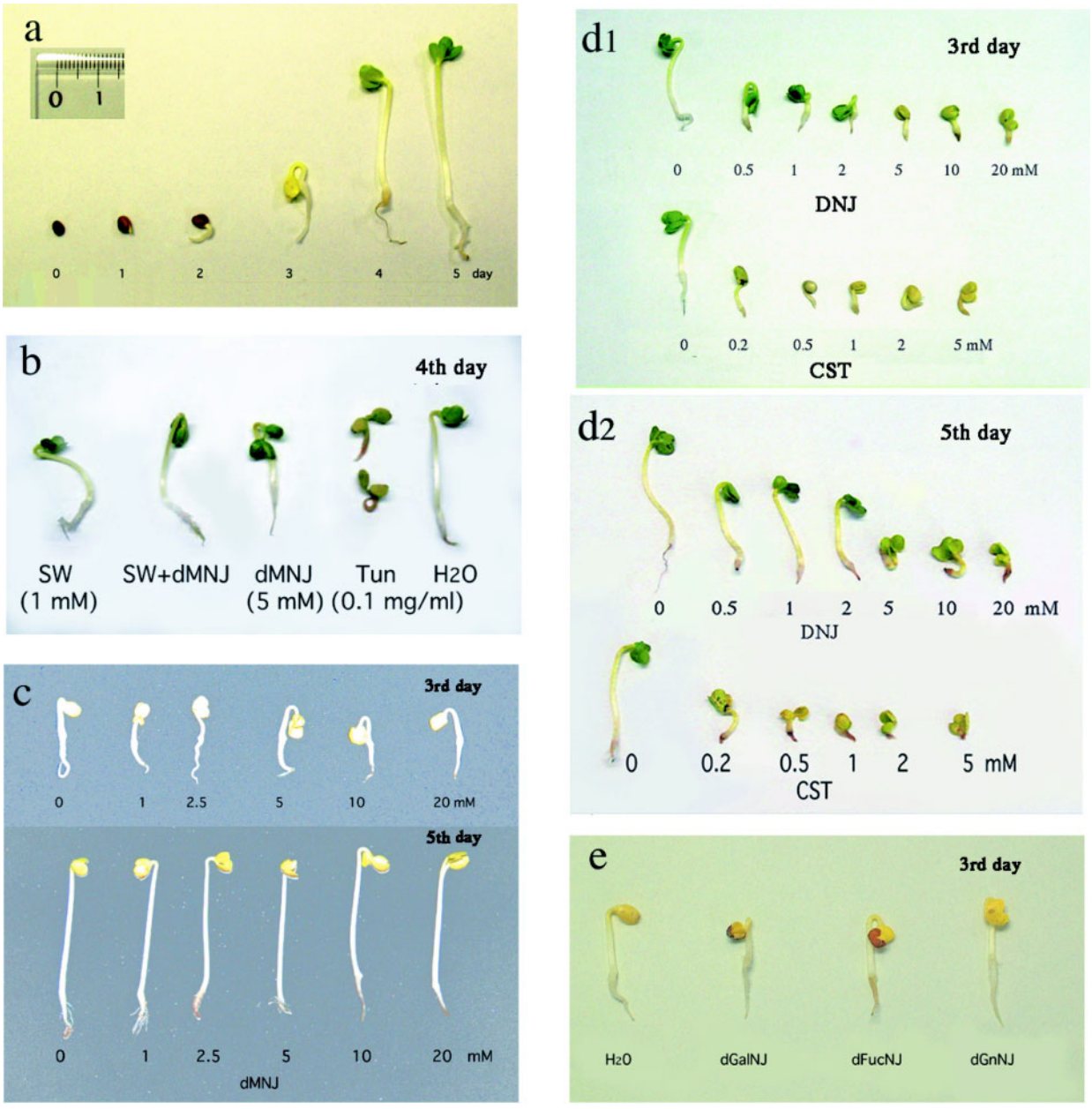


Fig. 1. **Germination and seedling growth of *Raphanus sativus*.** a: Daily growth of seeds in the absence of inhibitors, shown as a control. b: A picture of seeds germinated for four days in the presence of SW (1 mM), SW (0.5 mM) + dMNJ (2.5 mM), dMNJ (5 mM), and tunicamycin (0.1 mg/ml), showing that seedling growth was normal in the presence of mannosidase inhibitors but was inhibited by tunicamycin. c: A picture of seeds on the third and fifth days in the presence of dexymannojirimycin (dMNJ) at different concentrations showing no inhibition of growth. d1 and d2: Pictures of seeds on the third and fifth days in the presence of glucosidase inhibitors DNJ and CST. These glucosidase inhibitors markedly inhibited growth at concentrations higher than 5 mM DNJ and 0.5 mM CST. e: A picture of seeds on the third day in the presence of 1-deoxygalactonojirimycin (dGalNJ, 5 mM), fuconojirimycin (dFucNJ, 5 mM), and 2-acetamido-1,2-dideoxynojirimycin (dGnNJ, 2.5 mM), showing that these inhibitors had no influence on germination.

model organism due to its high germination yield, the ease of its cultivation and its suitable size for analysis. This study shows that the processing of *N*-glycans in this plant can be artificially modulated effectively with glycosidase inhibitors.

MATERIALS AND METHODS

Castanospermine (CST) and swainsonine (SW) were purchased from Wako Chemicals—Deoxynojirimycin (DNJ), deoxymannojirimycin (dMNJ), 2-acetamido-1, 2-dideoxy nojirimycin (dGnNJ), deoxygalactonojirimycin (dGalNJ), and deoxyfuconojirimycin (dFucNJ) were from Funakoshi. Jack bean α-mannosidase was from Seikagaku (Tokyo). *Diplococous pneumoniae* β-*N*-acetyl glucosaminidase was from Boehringer (Mannheim, Germany).

Germination of Raphanus sativus Seeds—One commercially available *Raphanus sativus* seed was incubated in a test tube at 25°C in the dark with 40 μl of water or a glycosidase inhibitor solution at various con-

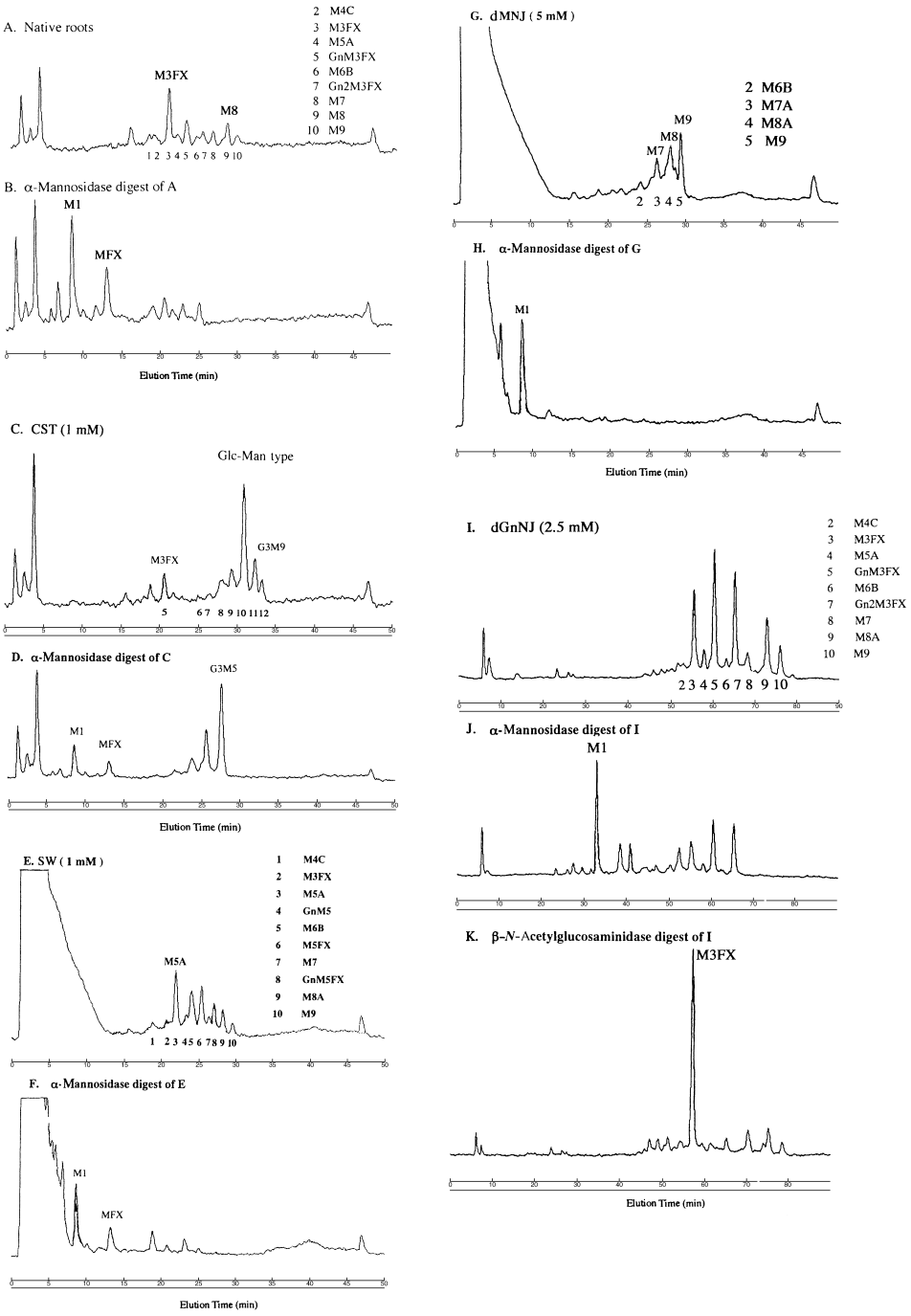


Fig. 2. **Size fractionation HPLC of PA-sugar chains of *N*-glycans obtained from third day roots in the absence of an inhibitor (A), and in the presence of 1 mM CST (C), 1 mM SW (E), 5 mM dMNJ (G), and 2.5 mM dGnNJ (I).** (B), (D), (F), (H) and (J) charts are of the α-mannosidase digests corresponding to (A)–(G). (K) shows HPLC of the β-*N*-acetylglucosaminidase digest of (I). Numbers were assigned to the peaks on size-fractionation HPLC. Most peaks were fractionated separately, but some adjacent peaks were fractionated together. These fractions were analyzed by reversed-phase HPLC. Sugar chains detected at the same elution positions on both size-fractionation and reversed-phase HPLC with the standard are shown in the figure. Reversed-phase HPLC of Control and CST is shown in Fig. 3.

centrations. When growth inhibition was observed, dose dependence was determined. When inhibition was not observed, growth was examined with higher concentrations of the inhibitor. Seed germination and root growth were observed daily for five days. Water or the inhibitor solution (0–40 μl) was added every day to ensure that the roots were immersed as the seedlings grew.

Analysis of N-Glycan Structures—The roots of *Raphanus sativus* seedlings on the third day were cut with scissors, lyophilized and powdered in a mortar. The lyophilized powder (7.5 mg) was extracted with 1 ml of PBS. The PBS solution was passed through an acrodisc and heated at 100°C for 5 min to denature the proteins in the solution, and ethanol was added to a final concentration of 60%. The precipitate was collected by centrifugation. The *N*-glycans in the precipitate were analyzed by the previously reported method (*13*, *14*). Size fractionation HPLC and reversed-phase HPLC were also carried out according to a previously described method (*15*). PA-sugar mixtures prepared from root glycoproteins were digested with Jack bean α-mannosidase in 0.01 M acetate buffer (pH 4.0) for 24 h at 37°C. The PA-sugar mixture of 2.5 mM dGnNJ roots was digested with β-*N*-acetyl glucosaminidase in 0.05 mM citrate buffer (pH 4.8) for 16 h at 37°C. PA-sugar chains were analyzed by size fractionation HPLC on a column of Shodex NH_2-P (2.0 × 250

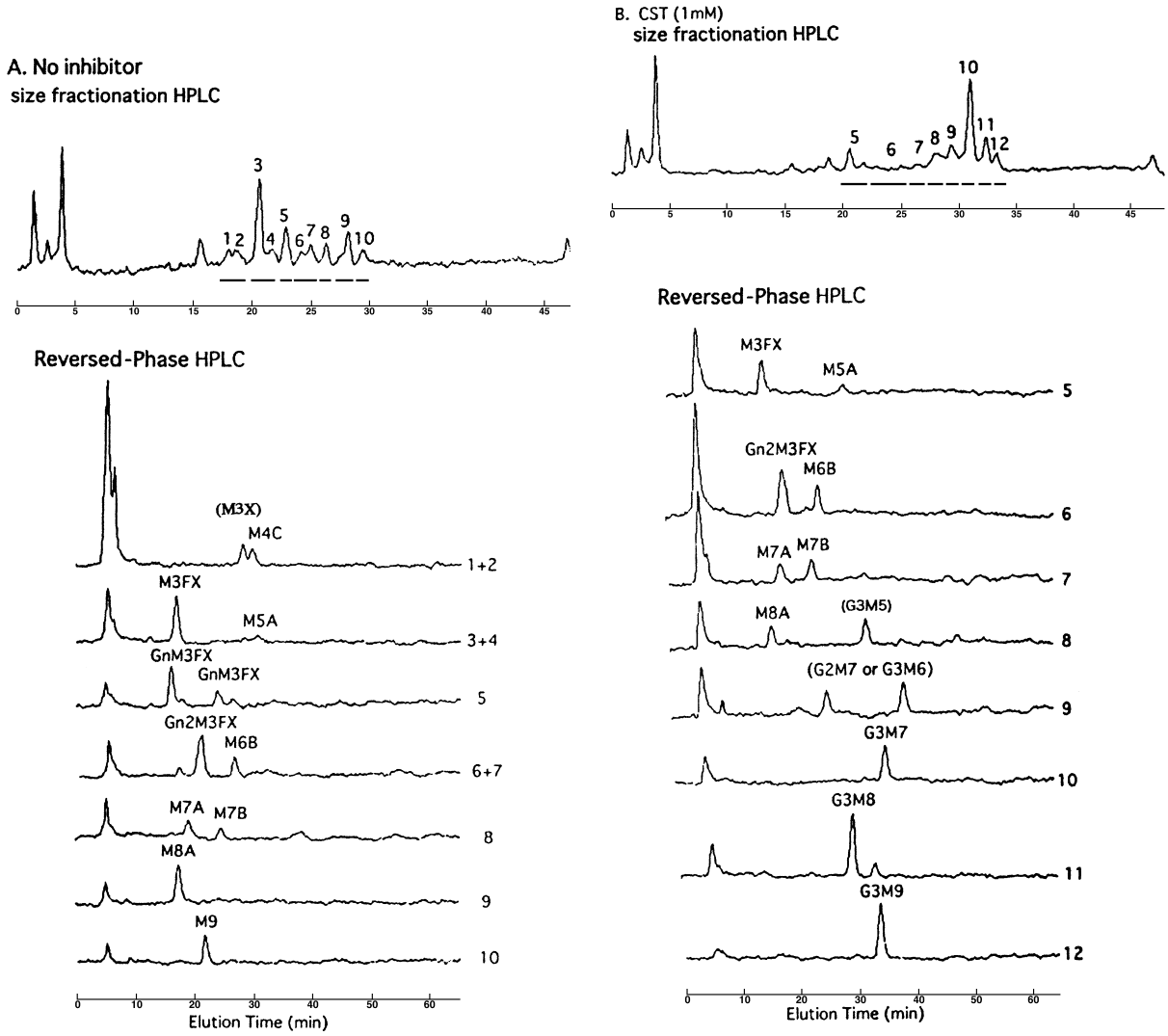


Fig. 3. **Analysis of sugar chains by both size and reversed-phase HPLC of control (A) and CST (B) root glycoproteins.** The designated peaks in Fig. 2, A and C, were fractionated as indicated by the bars and then analyzed on a reversed-phase column. The peaks obtained on HPLC were assigned based on the standard PA-oligosaccharides, but for the peaks in parentheses there was a little uncertainty.

mm) or an NH_2-P column (4.6 × 50 mm) (*14*), and by reversed-phase HPLC on a Cosmosil 5C_{18}P column (1.5 × 250 mm) (*15*). Standard sugar chains were prepared in my laboratory (*13–15*).

RESULTS

Germination and Root Growth in the Presence of Glycosidase Inhibitors—The results of germination of a *Raphanus sativus* seed and seedling growth with a small amount of water including an inhibitor are shown on Fig. 1.

The growth of seeds in the absence of inhibitors is shown as a control (Fig. 1a). The germination yield was nearly 100%, and the growth rate mainly depended on the amount of water and temperature. For normal growth, 20–40 μl of water or an inhibitor solution was added every day to immerse the roots. After three days, growth of the hypocotyls was eminent. The growth in the presence of mannosidase inhibitors, swainsonine (SW), deoxymannojirimycin (dMNJ) and tunicamycin (Tun) on the third day is shown in Fig. 1b. SW(1 mM) and dMNJ (5 mM) had no influence on the morphology of seedlings, although tunicamycin at 0.1 mg/ml inhibited their growth (Fig. 1b). dMNJ did not inhibit the germination or seedling growth up to 20 mM (Fig. 1c). The glucosidase inhibitors, castanospermine (CST) at 0.5 mM and deoxynojirimycin (DNJ) at 5 mM, inhibited the growth. The concentration dependence of germination was examined (Fig. 1, d1 and d2). Both inhibitors strongly suppressed the growth after 3 days. The seedlings could not grow further after three days with concentrations of higher than 0.2 mM CST and 5 mM DNJ, but seedlings exposed to a concentration of lower than 2 mM DNJ continued to grow after 3 days (Fig. 1, d2). Other glycosidase inhibitors, such as 2-acetamido-1,2-dideoxynojirimycin (dGnNJ), deoxygalactonojirimycin (dGalNJ), and deoxyfuconojirimycin (dFucNJ), did not have any specific influence on the morphology of seedlings (Fig. 1e). No influence on

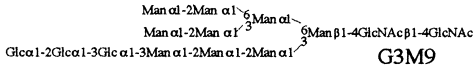
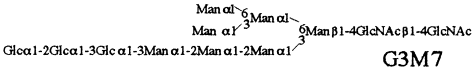
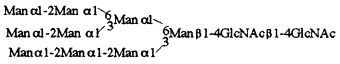
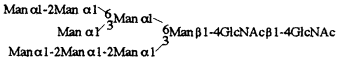
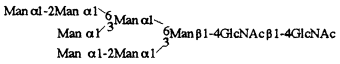
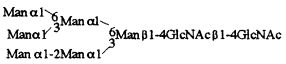
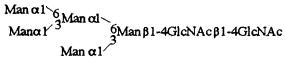
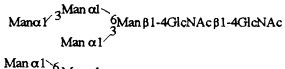
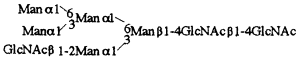
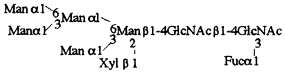
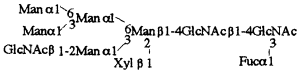
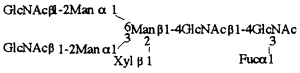
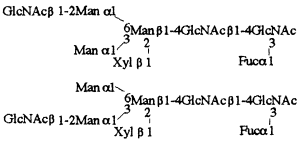
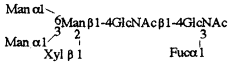
Oligosaccharide	Abbreviation	Control		CST 1 mM		dMNJ 5 mM	SW 1 mM	dGnNJ 2.5 mM
		S	T	S	T	S	S	S
	G3M9			6.9	7.6			
	(G3M8)			15	17			
	G3M7			36	31			
	(G3M6) (G2M7)			18	22			
	M9	6.3	6.8			41	4.0	5.8
	M8A+M8B	18	15	4	7	37	8.3	13
	M7A+ (M7B)	7.0	3.1	0.7	3.3	16	4.0	7.1
	M6B	+	2.1	0.6		6	20	5.4
	M5A	+	1.7	0.3			23	9.2
	M4C	9.8	9.9	4.2			4.6	5.8
	GnM5			2			3.4	
	M5FX						19	
	GnM5FX						9.2	
	Gn2M3FX	9.2	6.8					25
	GnM3FX*	11	15					18
	M3FX	37	32	7.4	2.3	+	4.6	11
Sum of the detected oligosaccharides[a)]		52	290	96	300	49	108	88

Fig. 4. **Oligosaccharides detected in glycoproteins of roots germinated with glycosidase inhibitors for three days.** (Values are the percent ratios of detected sugar chains.) S, PBS-extracted glycoproteins T, direct hydrazinolysis; +, a corresponding peak was observed but there was only a trace amount; ( ), the structure was not elucidated; *, both structures were detected; [a]pmol/mg dry root.

growth was observed up to 4 mM SW and 10 mM dGnNJ (data not shown).

Analysis of the N-Glycan Structures of Root Glycoproteins—To determine whether glycosidase inhibitors in the germination solution can block the *N*-glycan processing in the ER or Golgi, the *N*-glycan structures of glycoproteins in roots were analyzed. The three-day-old roots of a plant were lyophilized and powdered. The *N*-glycans of glycoproteins in the powder were released by direct hydrazinolysis or hydrazinolysis after the extraction of glycoprotein with phosphate-buffered saline (PBS). The *N*-glycans released on hydrazinolysis were *N*-acetylated, followed by pyridylamination (*13*, *14*). The pyridylaminated sugar chains were analyzed by size fractionation

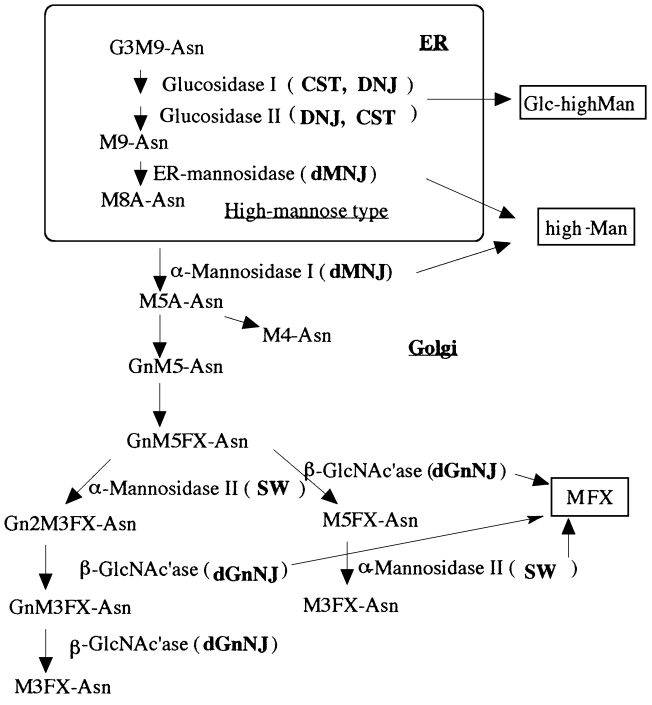


Fig. 5. **Glycosidases and their inhibitors participating in the processing of *Raphanus sativus* *N*-glycans.** Oligosaccharides boxed are expected to increase in root *N*-glycans with the processing inhibition by the glycosidase inhibitors.

HPLC (*15*). The HPLC pattern of PA-sugar chains obtained on direct hydrazinolysis was almost the same as that obtained on PBS extraction. This was confirmed with control and CST-treated seedlings. Figure 2 shows the HPLC pattern of PA-sugar chains on PBS extraction.

Size fractionation HPLC of *N*-linked PA-oligosaccharides obtained from roots grown with pure water (control), and HPLC of their α-mannosidase digest are shown in Fig. 2, A and B, respectively. The elution pattern of the PA-sugar chains shows that the oligomannose-type chain is the main sugar chain, since ManGlcNAc2-PA (M1) was the main product in the α-mannosidase digest. Most of the peaks eluted after 18 min except for peak 7, as shown in Fig. 2, A and B, decreased in amount after α-mannosidase digestion. The relatively large peaks in chromatograms A, C, E, G and I were collected separately and analyzed by reversed-phase HPLC (Fig. 3A) for comparison with standard PA-oligosaccharides.

These analyses revealed the presence of a typical plant-type oligosaccharides, with $Man_3FucXylGlcNAc_2$ (M3FX) as the main sugar chain and ten other sugar chains, as summarized in Fig. 4. The plant roots contained high-Man, M3FX and GnM3FX-type sugar chains.

The structures of *N*-glycans in the glycoproteins of roots of seedlings grown in the presence of CST and DNJ were analyzed using small roots on the third day, since the seeds germinated for 2 days at a slow rate. Size fractionation HPLC of the PA-sugar chains in the roots of seedlings grown in the presence of 1 mM CST and that of an α-mannosidase digest of the PA-sugar chains are shown in Fig. 2, C and D. Most of the root sugar chains were larger than M9. The elution positions of the peaks at around 31, 32, and 33 min corresponded to the positions of G1M9(G2M8, G3M7), G2M9(G3M8), and G3M9, respectively. These peaks were found to be G3M9, G3M8, and G3M7 on the basis of the elution positions on reversed-phase HPLC (Fig. 3B) compared with those of the standards. α-Mannosidase released 2–4 mannose residues per sugar chain and produced G3M5 as the main peak. The peak corresponding to M3FX was markedly smaller than that corresponding to the control (Fig. 2A). These results show that CST inhibited glucosidase I activity. The *N*-glycans of glycoproteins were estimated to contain mainly three Glc residues, because the peaks corresponding to G3M9, G3M8, and G3M7 those were detected around 33, 32, and 31 min before α-mannosidase digestion gave the peak corresponding to G3M5 around 28 min as the main peak after α-mannosidase digestion (Fig. 2D).

When the roots grew in the presence of 5 mM dMNJ, the *N*-glycans detected were almost all of the high-Man type, as expected, and were hydrolyzed by α-mannosidase to M1, as shown in Fig. 2, G and H. The presence of M3FX was confirmed by reversed-phase HPLC, but there was only a trace amount. The high M9 content shows that dMNJ inhibited most of the α-mannosidases participating in the processing.

The elution pattern of PA-sugar chains from the roots of seedlings grown in the presence of 1 mM SW (Fig. 2E) was different from that in the case of dMNJ. Reversed-phase HPLC revealed the presence of M5A, GnM5, M6B, M5FX, GnM5FX, M8A, and M9 (Fig. 4). The contents of M5FX and GnM5FX were relatively high, showing that the trimming of Man residues linked to the α-1,6 branch was inhibited.

In the presence of 2.5 mM *N*-acetylglucosaminidase inhibitor, the PA-sugar chains of *N*-glycans in the roots, and also their digests with α-mannosidase (Fig. 2J) and β-*N*-acetylglucosaninidase (Fig. 2K) were analyzed in the same manner as for size fractionation HPLC (Fig. 2I). The increase in the M3FX content in the β-*N*-acetylglucosaninidase digest shows the high contents of Gn1M3FX and Gn2M3FX in *N*-glycans. The fractions corresponding to the peaks shown in Fig. 2I were analyzed by reversed-phase HPLC, and the sugars detected are shown in Figs. 2I and 4.

The amounts of the sugar chains detected on HPLC are summarized in Fig. 4. The values in Fig. 4 are in the percent ratios of sugar chains detected. The actual amounts were at the pico mole level, and the differences in quantity between roots of seedlings grown in the presence of different inhibitors or under different conditions were slight. The total amount of PA-sugar chains detected on size-fractionation HPLC was approximately 300 pmol per dried root powder when oligosaccharides were released by direct hydrazinolysis, and the amount was 50–108 pmol per mg root powder when PBS extraction was performed. The overall yield in the above analysis was estimated to be 10–30%.

DISCUSSION

The analysis of *N*-glycans showed that the root glycoproteins in *Raphanus sativus* contained highMan, M3FX, and GnM3FX-type sugar chains on normal germination of control seedlings, as shown in Fig. 4. These structures

are typical plant types, therefore, the biosynthesis route is considered to be the same as other typical plant routes. The sugar chains detected in the present study are shown in Fig. 5 according to the step of plant *N*-glycan processing (*1*), together with the trimming glycosidases and their inhibitors that might inhibit the enzymes.

Our sugar analyses demonstrated that glycosidase inhibitors added externally inhibited the processing in the ER and Golgi. It is clear from Figs. 2 and 3 that CST inhibited glucosidase I and produced the sugar structure of the Glc-high-Man type. dMNJ probably inhibited both ER-mannosidase and α-mannosidase I, and thus the high-mannose structures of *N*-glycans were retained. Since the content of M9 was 41% in the presence of 5 mM dMNJ (Fig. 4), the plants grown in the presence of 20 mM (Fig. 1C) were estimated to contain M9 at a much higher level, showing that the ceasation of mannosidase processing has no critical effect on germination or seedling growth. SW inhibited α-mannosidase II, which hydrolyzes the mannose residues linked to the α-1,6 branch, and therefore more GnM5FX and M5FX were produced in the roots. The increase in the content of *N*-glycans with these structures had no effect on germination or seedling growth. GnNJ inhibited β-GlcNAc'ase, as shown in Fig. 2I, and produced Gn2M3FX sugar chains. An increase in the M4-M6 content was also observed. These shifts in the proportions of *N*-glycan structures also had no effect on germination or seedling growth.

The fact that seedling growth was inhibited by glucosidase inhibitors (Fig. 1d) is in accordance with the results of studies on *Arabidopsis* glucosidase I mutants that are unable to produce seeds (*8*, *9*). It has also been reported that the content of cellulose in mutant seeds decreased (*9*). The real reason for the growth inhibition is unknown. The absence of growth inhibition by mannosidase and β-GlcNAc'ase inhibitors, particularly dMNJ, is comparable to that an *Arabidopsis* GNT-I mutant was able to grow, and produce flowers and seeds normally, although *N*-glycans of the mutant were of the oligomannose-type and there were no complex types (*10*).

These results clarified that glycosidase inhibitors inhibited the processing of glycoprotein biosynthesis during plant seed germination. This provides a method for artificially regulating the structures of whole glycoproteins in plants using glycosidase inhibitors.

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