



Analysis of Tissue-Specific and Inhibitor Directed Plant Glycan Composition by Fluorescent Labeling with 1,5-EDANS and Combined Gel-Electrophoretic, High-Performance Liquid and Thin-Layer Chromatography Techniques.

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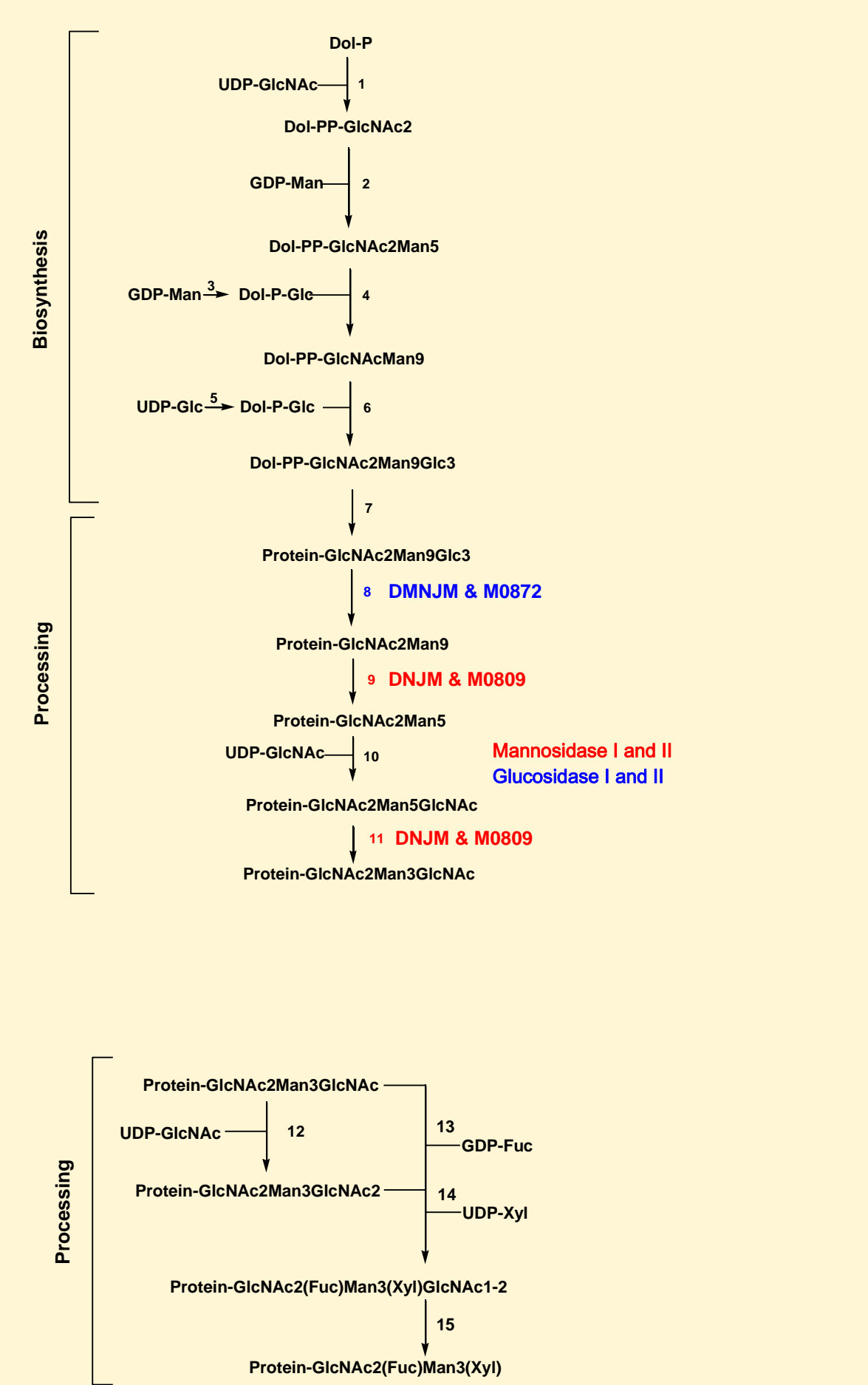


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Introduction

The processing pathway for N-glycan synthesis in plants begins with a transfer of the block oligosaccharide Glc3Man9GlcNAc2 to nascent proteins in the endoplasmic reticulum followed by processing of three glucose and four mannose residues. Inhibition of this processing in *Arabidopsis thaliana* strain DW150-307 by administration of the selective mannosidase and glucosidase inhibitors deoxymannojirimycin, deoxymannojirimycin as well as their β -D-glucuronide conjugates was evaluated by measurement of the resulting monosaccharide ratios using a new fluorescent labeling technique. Glycoproteins were isolated from leaf, stem and floral tissues of *Arabidopsis thaliana* Landsberg erecta strain DW150-307 which is homozygous for a LFY::GUS insertion containing the GUS gene under control of the full length LFY promoter that expresses the *E. coli* enzyme β -glucuronidase. Expression of GUS in this strain is developmentally regulated, showing the highest levels 9 days after germination (at the onset of meristem floral initiation under a long-day light regime), but having basal levels in vegetative apices throughout secondary growth. Acidic hydrolysis and hydrazinolysis of derived glycoproteins, released either the individual monosaccharides or glycans, which were labeled with the fluorophore 1,5-EDANS using reductive amination through the reducing end of the saccharides. The EDANS fluorophore has the advantages of (1) a lower charge due to single sulfonic acid group, allowing both PAGE, TLC and HPLC analysis, and (2) a primary amino function which provides quantitative reactivity for Schiff's base formation and reductive amination. Analysis of the resulting labeled saccharides was performed using polyacrylamide gel electrophoresis, with or without prior borate complexation. The same samples were also subjected to reversed-phase HPLC analysis as well as 2-Dimensional Thin Layer chromatographic analysis with or without precolumn sulfonic acid modification. Separation of individual monosaccharide and oligosaccharide patterns indicated changes in N-glycan processing with inhibitor application. This work was supported, in part, under grants NSF9710722 and NSF9561199 from the National Science Foundation, USA.

Schematic Representation of the Glycosylation Processing in Arabidopsis

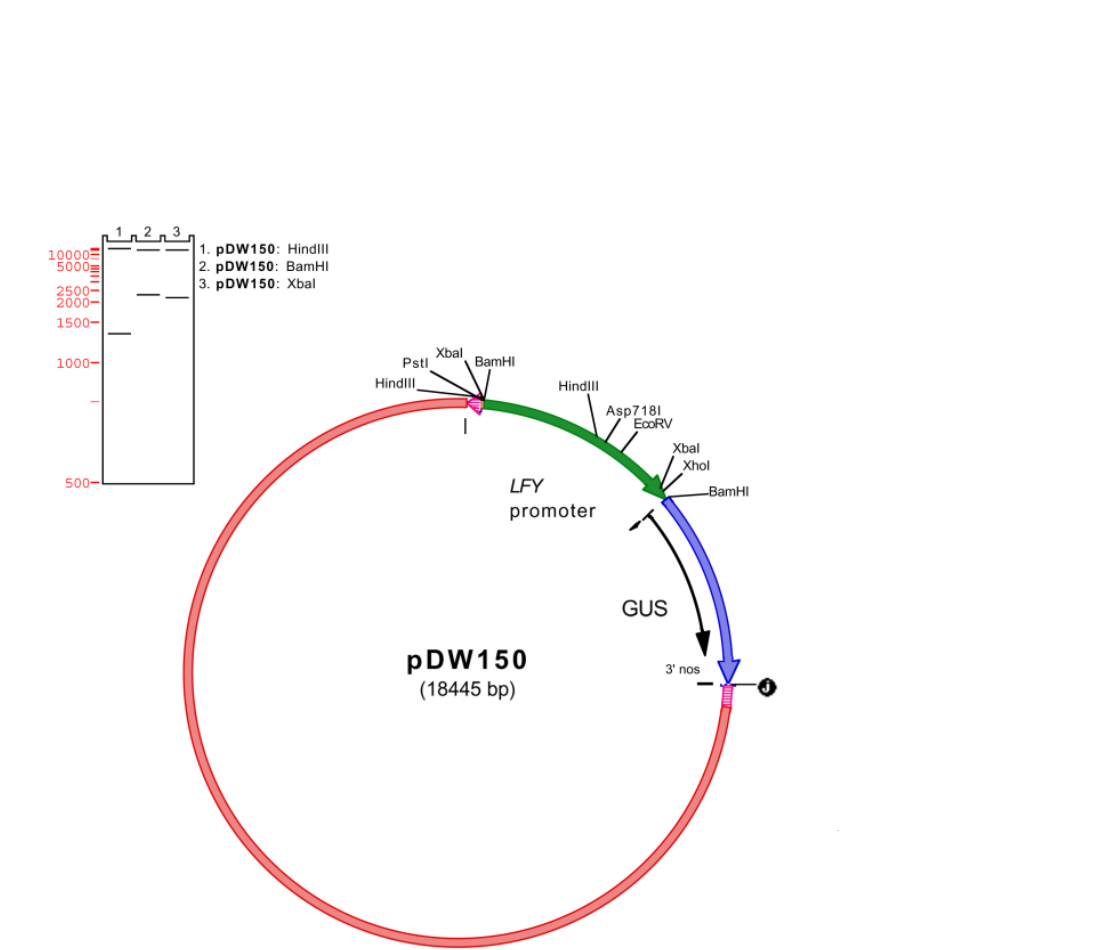


Proposed biosynthesis and processing pathway of typical N-linked Glycans from plants. The enzymes involved are GlcNAc-1-P transferase and GlcNAc-transferase (1), mannosyl-transferase (2), Dol-P-Man synthase (3), Dol-P-mannosyl-transferase (4), Dol-P-Glc synthase (5), Dol-P-glycosyl-transferase (6), oligosaccharidetransferase (7), glucosidase I and II (8), Golgi mannosidase (9), GlcNAc transferase I (10), Golgi mannosidase II (11), GlcNAc-transferase II (12), fucosyl-transferase (13), xylosyl-transferase (14), N-acetyl glucosaminidase (15). Deoxymannojirimycin (DMNJM) and Deoxynojirimycin (DNJM) will inhibit processing at steps (8), (9) and (11).

Methods

- Recombinant *Arabidopsis thaliana* Landsberg erecta plants DW150-307 homozygous for the GUS gene under control of the LFY promoter were germinated under sterile conditions.
- Plants were treated with deoxynojirimycin (DNJM), deoxymannojirimycin (DMNJM), or β -D-glucuronide conjugates of DNJM (M0809) or DMNJM (M0872), using media or spray application.
- Leaf, stem and flower samples were isolated after 35 days growth under long day (16 hour) growth conditions and glycoproteins isolated by lyophilization and extraction into PBS or H₂O.
- Glycoproteins were hydrolyzed by treatment with TFA (5h, 100°C) or by hydrazinolysis (6h, 60°C).
- Released sugars or oligosaccharides were purified and labeled with EDANS by reductive amination (NaCNBH₃).
- Carbohydrate standards were prepared from 10mM samples derivatized under identical conditions with 20 mM EDANS.
- Samples were run on PAGE gels. These gels consisted of 40% T, 5% C resolving gel and 12.5% T, 20% C stacking gel.
- HPLC analysis utilized gradient elution: Solvent A: 20mM sodium borate, 10mM tetrabutylammonium hydroxide, B: CH₃CN.
- 2D-TLC analysis was performed using 4:6:1 n-BuOH:HCOOH:H₂O in both directions.
- Seed pods from treated and control plants were removed at the end of the growth period, dried and seeds isolated.
- Second generation plants were grown from seeds under identical growth conditions as above.

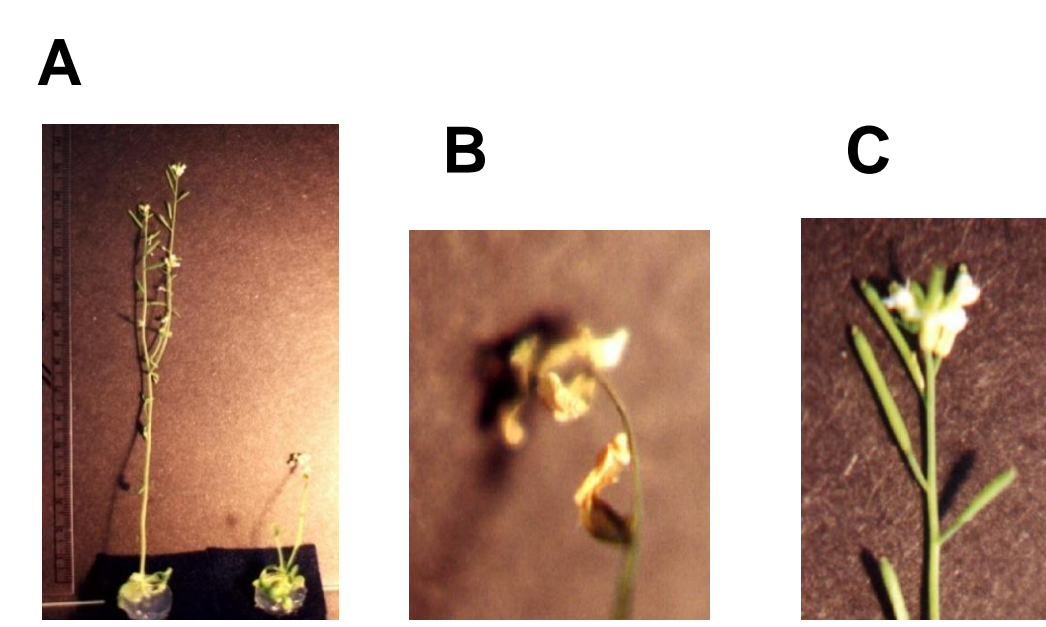
Cloning Strategies for Transgenic Plant Construction.



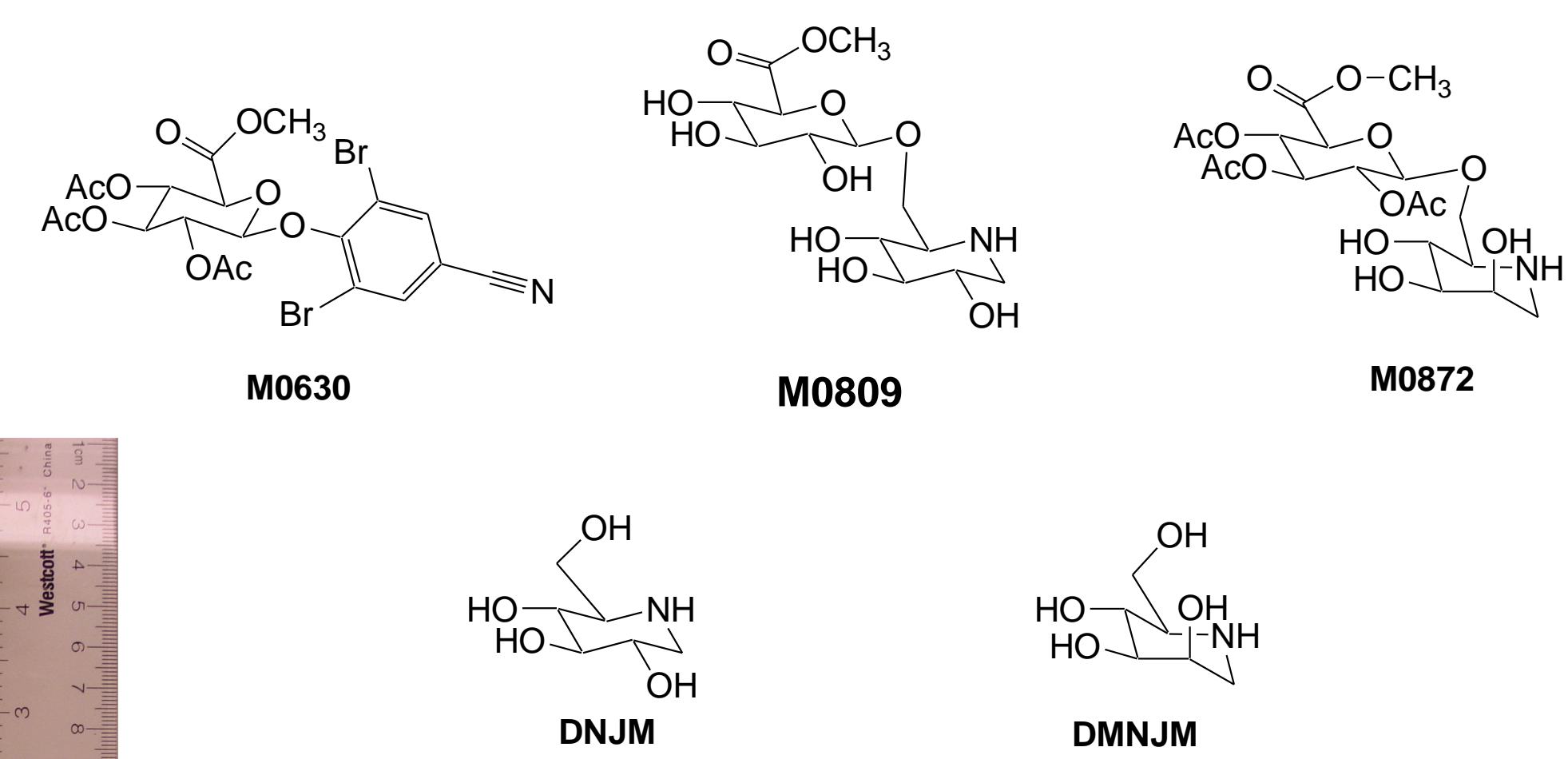
LFY::GUS vector

The homozygous *Arabidopsis thaliana* Landsberg erecta LFY::GUS line (DW150-307) was constructed using a fusion vector containing the 2.3 kb LFY promoter and the uidA gene encoding β -glucuronidase (GUS). The resulting vector pDW150 was derived from pDW137, a pCGN1547 derivative containing the HindIII-EcoRI fragment of pBI101.2, which encompasses the β -glucuronidase (GUS) coding region and the nos terminator. The 2.3 kb BamHI fragment spanning the LFY promoter was inserted in front of GUS in pDW137, such that the fusion gene uses the LFY initiation codon, which overlaps the downstream BamHI site (ATGGATCC). The authentic GUS initiation codon is seven codons downstream from the LFY initiation codon.

Plants were transfected with pDW150 using the vacuum-infiltration method and grown at 23°C in long-day (16 hours light/8 hours dark) conditions under sterile conditions with illumination using a mixture of Cool White and Gro-Lux/W5 fluorescent lights (Osram, Sylvania). Transgenic plants were selected by histochemical analysis using 4-methylumbelliferyl glucuronide (MUG), which is converted by the GUS enzyme into the fluorescent product 4-methyl umbelliferone (4-MU).



Effect of M0630: Bromoxynil β -D-Glucuronide, methyl ester, tri-O-acetate (media application at 10ug/mL, 42 days after planting) on *Arabidopsis thaliana* Landsberg erecta plants. Panel A: Wild type plant (left) vs. recombinant DW150-307 plant (right). Panel B: Ablated floral tissues of DW150-307 plant (closeup). Panel C: Normal inflorescence with seed pods for Wild-type plant (closeup).



Structures of Conjugates and Inhibitors

Results

HPLC Analysis

TLC Analysis

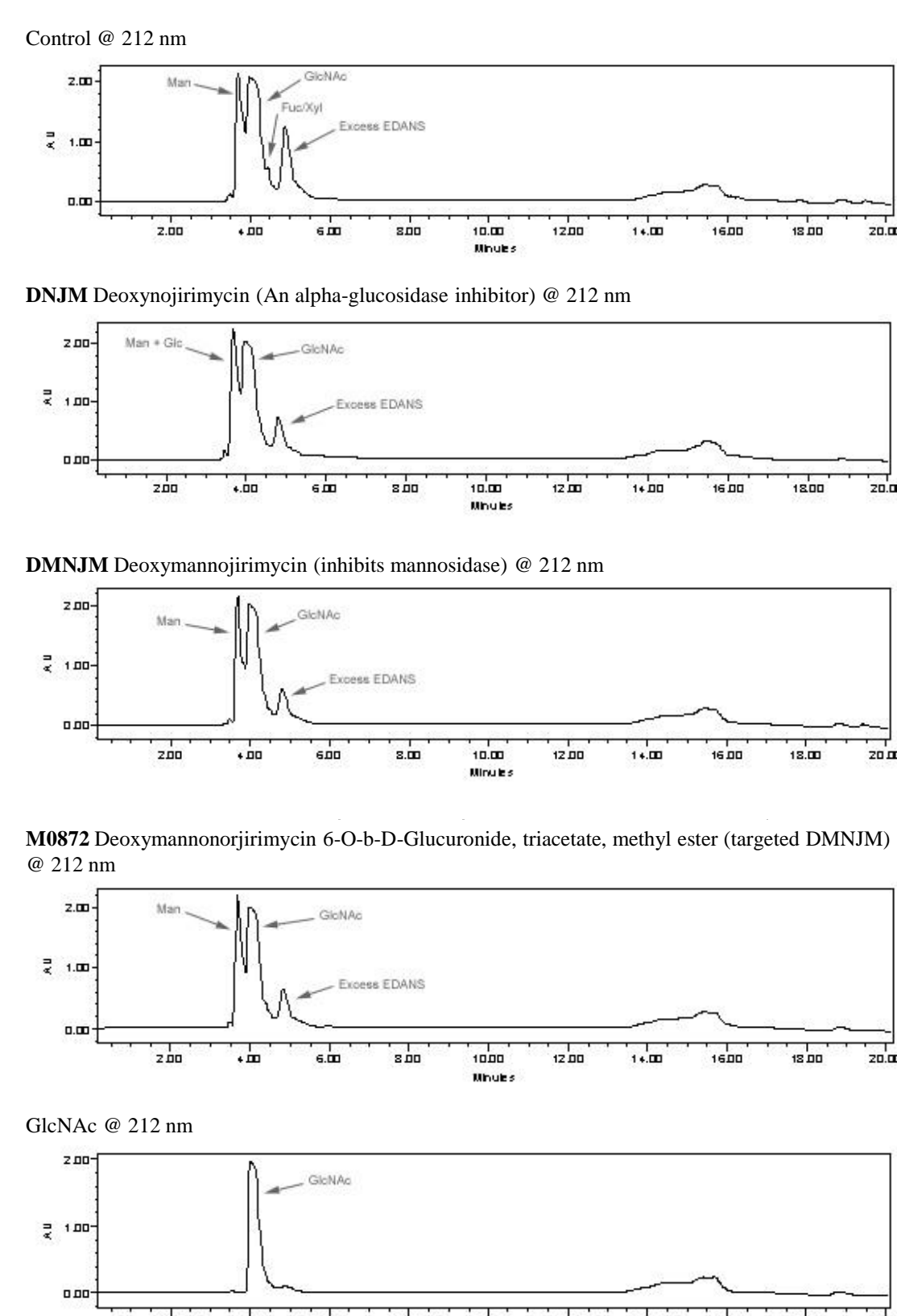


Figure 1: HPLC of Arabidopsis thaliana samples

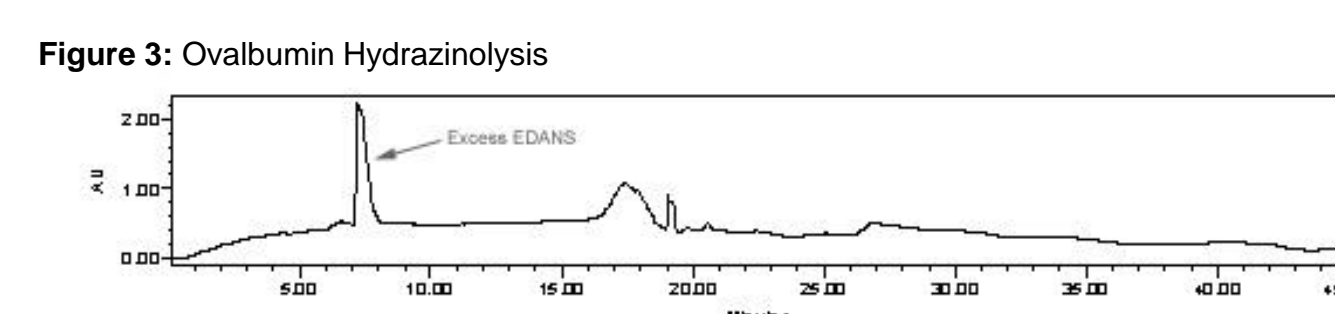


Figure 3: Ovalbumin Hydrazinolysis

Figure 3: HPLC of Ovalbumin treated by hydrazinolysis. Oligosaccharides were isolated from Ovalbumin by hydrazinolysis (hydrazine, 6 h, 60°C). Free primary amino groups were reacylated by addition of acetic anhydride. Sample was passed through 50W-X8 resin column, lyophilized, and labeled with EDANS (uL, 20 mM) and NaCNBH₃ (5 uL, 1M) in 100mM sodium bicarbonate buffer overnight. Sample was diluted in 1 mL HPLC Solvent A and run under HPLC conditions, with solvent A containing 10 mM Methanesulfonic acid instead of 20 mM Sodium Borate, pH 5.0, as shown in Figure 2

Experimental conditions		
Column:	Discovery [®] C ₁₈ 5µm	
Diameter:	25cm x 4.6mm	
Mobile Phase:	A: 10 mM Tetrabutylammonium Hydroxide 20 mM Sodium Borate, pH 5.0	
Flow Rate:	1.0 mL/min	
Gradient:	Time (min)	Profile (%A, %B)
	0.0	100 0
	5.0	100 0
	7.0	75 25
	12.0	50 50
	30.0	50 50
	31.0	100 0
	45.0	100 0
Injection Volume:	10.0 µL	
Temperature:	26°C	
Detection:	UV Scan 200 - 495 nm	
Time Constant:	0.5	
Instrument:	WATERS 515 HPLC Pump with WATERS 717plus Auto Sampler and WATERS 996 Photodiode Array Detector	

Figure 2: HPLC experimental conditions

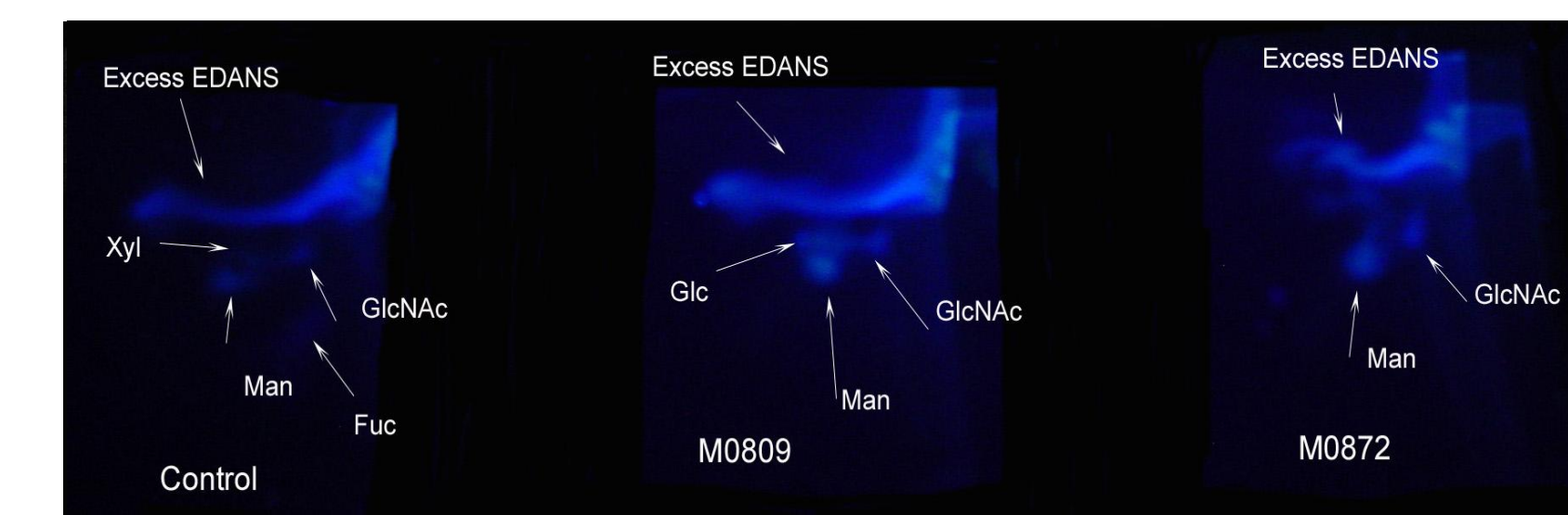


Figure 4: 2D-Thin Layer Chromatographic Analysis of monosaccharides released from control and M0809 and M0872 treated plants. Glycoproteins were isolated from lyophilized stem, floral and leaf tissues of treated *Arabidopsis thaliana* Landsberg erecta DW150-307 plants followed by acidic hydrolysis (TFA, 5h, 100°C), purification and labeling with EDANS (5 uL, 20 mM) and NaCNBH₃ (5 uL, 1M) in 100mM sodium bicarbonate buffer overnight. Samples (2uL) were spotted on 50x50 mm Kieselgel 60 - F254 aluminum plates and eluted with 4:6:1 n-butanol:formic acid:water as eluent in both directions. Detection using fluorescence emission (EX 360 nm) and Sony Cybershot Photo Documentation versus standards run under identical conditions.

Gel Electrophoresis (PAGE) Analysis of Carbohydrate Structures.

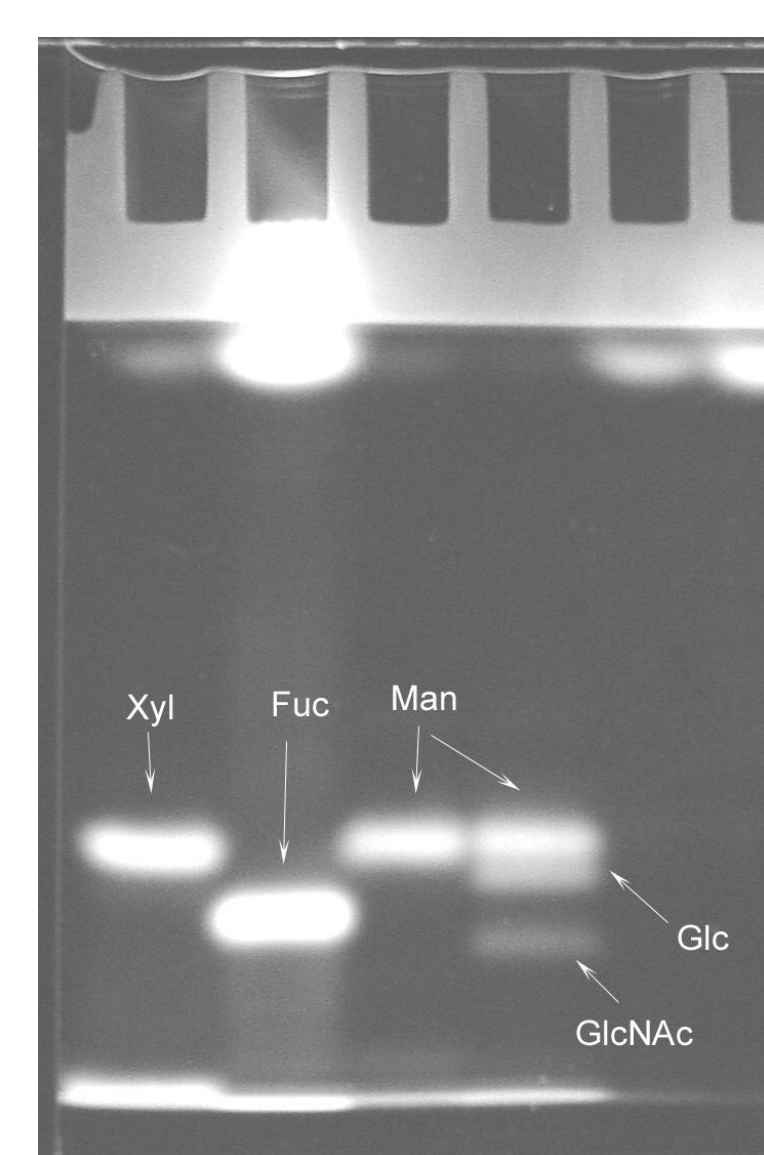


Figure 6: PAGE analysis of EDANS labeled monosaccharides.

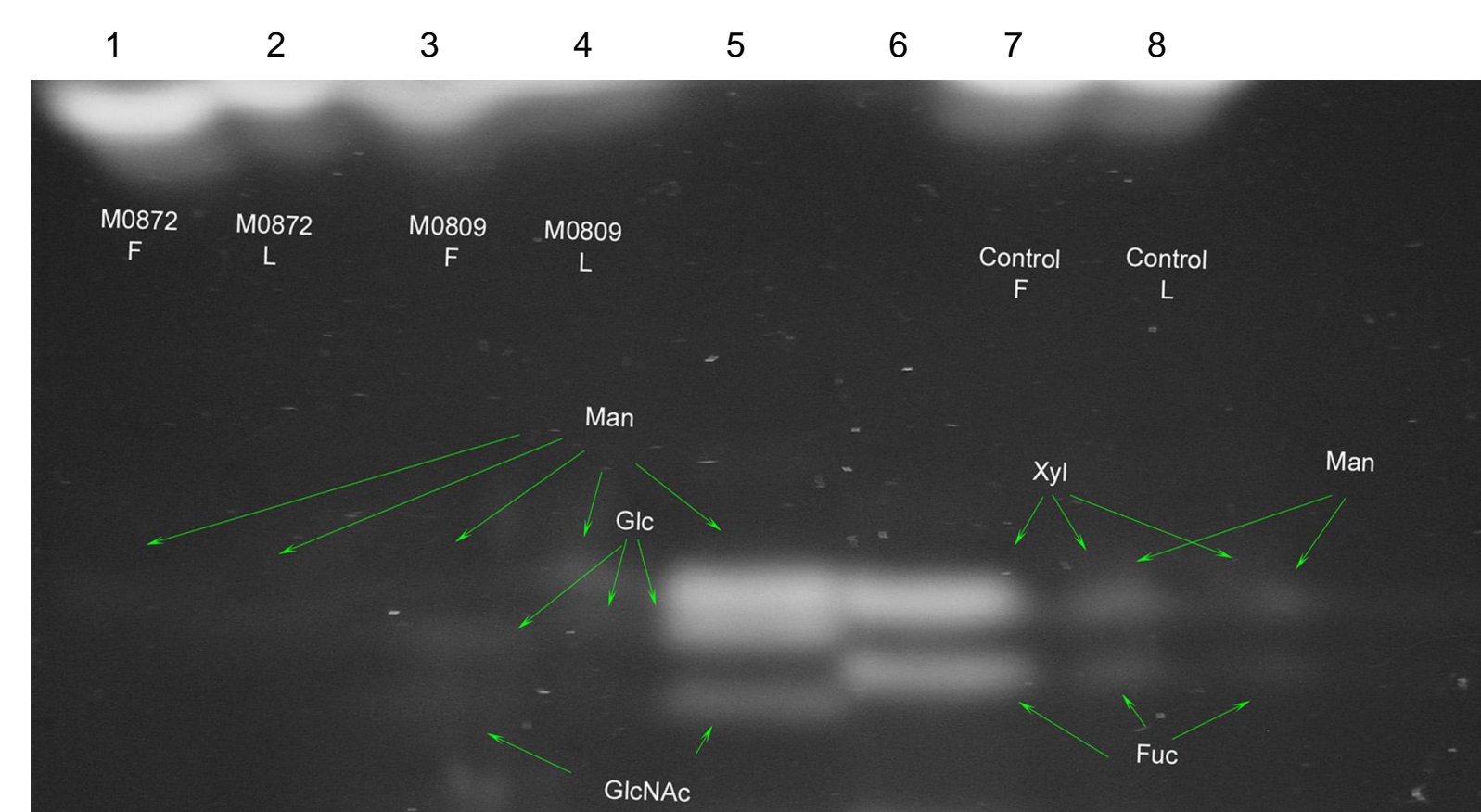


Figure 7: PAGE analysis of flower and leaf protein glycans from M0809 and M0872 treated *Arabidopsis thaliana* DW150-307 plants after complete hydrolysis (TFA, 5h, 100°C) and labeling with EDANS. Lane 5 and 6 = standards. Lane 5: Man, Glc and GlcNAc; Lane 6: Xyl and Fuc.

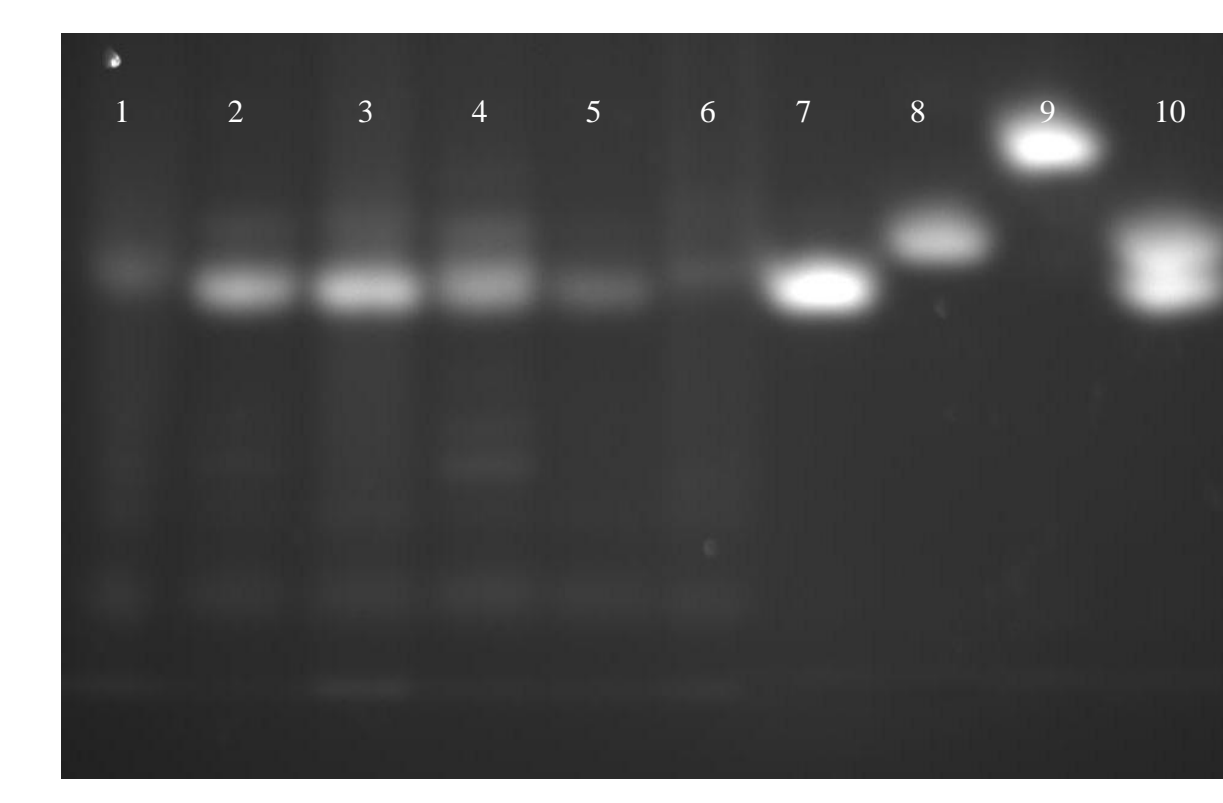


Figure 8: PAGE analysis of protein glycosylation from after complete hydrolysis (TFA, 5h, 100°C) and labeling with AMAC. Lane 1-5 for proteins isolated from *Arabidopsis thaliana* DW150-307 floral tissues with the following treatments: Lane 1: DNJM; Lane 2: DMNJM; Lane 3: M0872; Lane 4: M0809; Lane 5: Control; Lane 6: Ovalbumin; Lane 7: Glc; Lane 8: Man; Lane 9: GlcNAc; Lane 10: GlcNAcMan.

Note: Ovalbumin hydrolysis should produce GlcNAc:Man (-8.3). Control (GlcNAc:Fuc:Man:Xyl 2:1:3:1). DNJM and M0809: GlcNAc:Man:Glc 2:9:3. DMNJM and M0872: GlcNAc:Man 2:9.

Second Generation Seed Propagation and Viability Analysis.

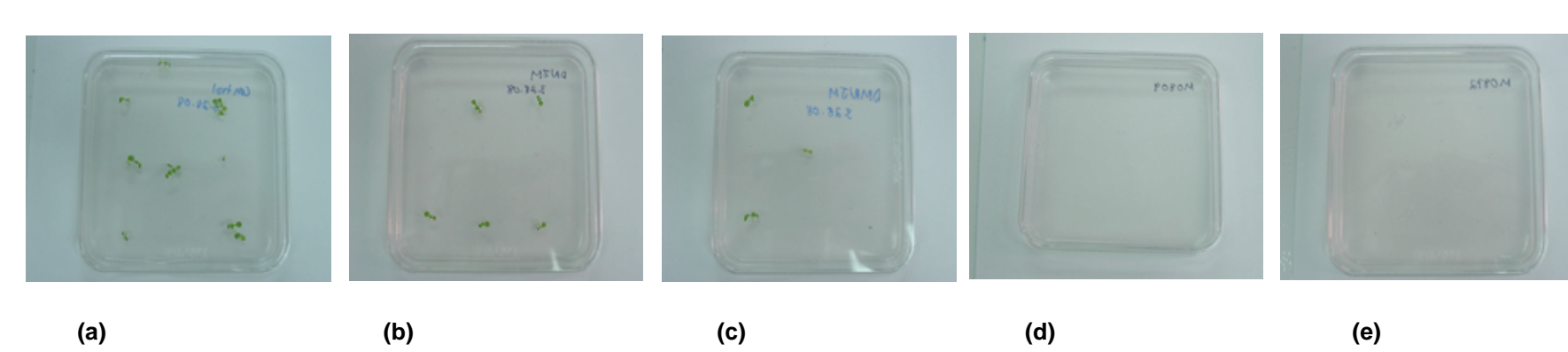
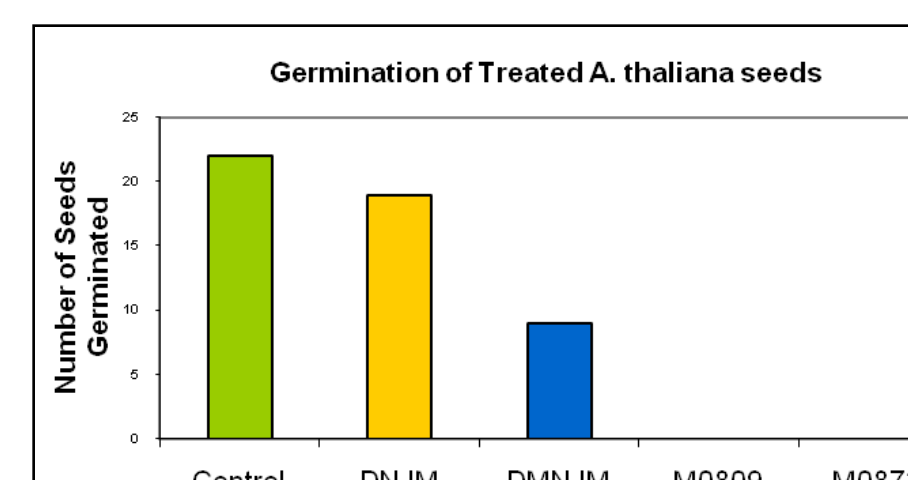


Figure 9: Growth of *Arabidopsis thaliana* seeds from treated plants. (14 days after planting) (a) Control; (b) DNJM; (c) DMNJM; (d) M0809; (e) M0872. Seeds were sterilized (1 ml 20% sodium hypochlorite, 0.5% tween-20), vortexed and incubated at room temperature for 5 minutes, centrifuged at 1700 rpm, resuspended in 1 ml 70% ethanol, vortexed, centrifuged and then washed 10 times with sterile filtered water. Approximately 9-12 seeds were transferred to sterile Murashige/Skoog plates, refrigerated for 3 days, and then incubated at 24°C under a 16h day/8h night regime to germinate 10 days. After germinating, plants were transferred into various treated media contained in 250 x 25 mm culture tubes. Base media was prepared as above. Treated media was prepared by adding 250 uL of a 10mg/mL solution of desired compound into 250 mL base media for a final concentration of 10 ug/mL. Compounds used were Deoxynojirimycin (DNJM), Deoxymannojirimycin 6-O- β -D-Glucuronide, triacetate, methyl ester (M0872), and Deoxymannojirimycin (DMNJM). Plants were grown in treated media until seedpods matured. After approximately 14 weeks (we harvested flowers twice before letting seedpods develop), seedpods were removed from plants and dried in a sterile environment. Seeds from these pods were washed and sterilized as above and grown on untreated Murashige/Skoog plates. After 14 days, 67% of the control seeds germinated (a), 56% of DNJM treated seeds (b) and 21% of DMNJM treated seeds (c) germinated but none of the M0809 and M0872 (d) and (e) treated seeds germinated (Right panel, tabulated results of sterility).



Discussion and Conclusions

In an effort to influence glycosylation patterns in recombinant plants, we developed a system utilizing transgenic *Arabidopsis thaliana* DW150-307 plants which have the *E. coli* β -glucuronidase (GUS) gene expressed in their floral tissues under control of a floral initiation promoter (LFY). Plants were grown under long-day (16h) conditions to initiate flowering.

Application of glucosidase inhibitors deoxynojirimycin (DNJM) and deoxymannojirimycin (DMNJM) should inhibit glycosylation during glycoprotein processing in the Golgi. Treatment with glucuronide conjugates of these inhibitors (M0809 or M0872) will target their effect only to the floral tissues in recombinant plants.

With media (at seed germination) or spray application (after flowering) with conjugates or inhibitors, plants grew normally, except DNJM and DMNJM treated plants exhibited reduced stems/leaf growth versus controls.

Analysis of carbohydrates isolated from glycoproteins from stem, leaf or floral tissues was performed by either complete hydrolysis (TFA) or hydrazinolysis (hydrazine) treatment and labeling with the new fluorophore EDANS using reductive amination. After labeling, HPLC, PAGE and TLC analyses were performed to analyze changes in glycosylation patterns.

Comparison of glycosylation patterns for leaf, stem, and floral tissues versus control plants (untreated) indicated truncated processing for DNJM, DMNJM, M0809 and M0872 treated plants.

Isolation of seeds from treated plants, and growth of second generation plants exhibited sterility of M0809 and M0872 plants versus controls or DNJM or DMNJM treated plants.

The combined system is being investigated as a method of influencing glycosylation of recombinant proteins expressed in transgenic plants for use in mammalian systems with reduced immunogenicity.