

Reduced Immunogenicity of *Arabidopsis hgl1* Mutant N-Glycans Caused by Altered Accessibility of Xylose and core Fucose Epitopes^{*,§}

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Arabidopsis N-glycosylation mutants with enhanced salt sensitivity show reduced immunoreactivity of complex N-glycans. Among them, hybrid glycosylation 1 (*hgl1*) alleles lacking Golgi α -mannosidase II are unique, because their glycoprotein N-glycans are hardly labeled by anti-complex glycan antibodies, even though they carry β 1,2-xylose and α 1,3-fucose epitopes. To dissect the contribution of xylose and core fucose residues to plant stress responses and immunogenic potential, we prepared *Arabidopsis hgl1 xylT* double and *hgl1 fucTa fucTb* triple mutants by crossing previously established T-DNA insertion lines and verified them by mass spectrometry analyses. Root growth assays revealed that *hgl1 fucTa fucTb* but not *hgl1 xylT* plants are more salt-sensitive than *hgl1*, hinting at the importance of core fucose modification and masking of xylose residues. Detailed immunoblot analyses with anti- β 1,2-xylose and anti- α 1,3-fucose rabbit immunoglobulin G antibodies as well as cross-reactive carbohydrate determinant-specific human immunoglobulin E antibodies (present in sera of allergy patients) showed that xylose-specific reactivity of *hgl1* N-glycans is indeed reduced. Based on three-dimensional modeling of plant N-glycans, we propose that xylose residues are tilted by 30° because of untrimmed mannoses in *hgl1* mutants. Glycosidase treatments of protein extracts restored immunoreactivity of *hgl1* N-glycans supporting these models. Furthermore, among allergy patient sera, untrimmed mannoses persisting on the α 1,6-arm of *hgl1* N-glycans were inhibitory to immunoreaction with core fucoses to various degrees. In summary, incompletely trimmed glycoprotein N-glycans conformationally prevent xylose and, to lesser extent, core fucose accessibility. Thus, in addition to N-acetylglucosaminyltransferase I, Golgi α -mannosidase II emerges as a so far unrecognized target for lowering the immunogenic potential of plant-derived glycoproteins.

Biosynthesis and transfer of N-glycans, needed for correct folding of secreted glycoproteins in the endoplasmic reticulum (ER),² play pivotal roles in stress tolerance and plant development (staurosporin and temperature-sensitive 3a (*stt3a*) (1, 2), defective glycosylation 1 (*dgl1*) (3), asparagine-linked glycosylation 1 (*alg1*) (4), and hypersensitive to ammonium 1/vitamin c1 (*hsn1/vtc1*) (5)). In contrast, complex N-glycan modifications in the Golgi apparatus do not affect normal plant growth by itself but still influence stress tolerance of the host plants (6–8). The obligatory step for initiating complex N-glycan formation in the plant Golgi apparatus is catalyzed by N-acetylglucosaminyltransferase I (GnTI), followed by Golgi α -mannosidase II, β 1,2-xylosyltransferase (XylT), α 1,3-fucosyltransferase (FucTa, FucTb), and N-acetylglucosaminyltransferase II (GnTII), which altogether produce N-glycan structures containing β 1,2-xylose and/or core α 1,3-fucose residues (compare Fig. 1; the details of N-glycan structures appearing as acronyms in Table 1, in Figs. 1 and 3, and throughout the text are specified in supplemental Figure S3). In addition, modification of terminal GlcNAc residues can occur through β 1,3-galactosyltransferase and α 1,4-fucosyltransferase, forming a structure known as Lewis-a epitope (Ref. 9 and references cited therein). This structure was hardly detectable in leaves (10) but found in the stem tissue of *Arabidopsis* (11). Mutants defective in GnTI (such as *cgl1* (complex glycan 1) (12) entirely lack complex-type N-glycans characterized by xylose and core fucose residues (Fig. 1), whereas other glycosyltransferase mutants produce N-glycan structures lacking specific residues (6, 8). *hgl1* (*Arabidopsis* hybrid glycosylation 1) (13) mutants defective in Golgi α -mannosidase II (such as *manII*, the mannosidase II mutant, equivalent to *hgl1*) retain branched mannoses on their α 1,6-arm and produce N-glycans containing xylose as well as core fucose residues (Fig. 1).

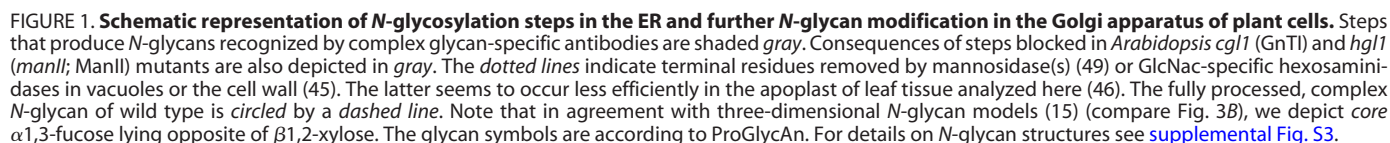
Plant-derived complex N-glycans with β 1,2-xylose and/or α 1,3-fucose are immunogenic for humans and other mammals and often responsible for IgE recognition of several glycoallergens. Together with insect- and other invertebrate-derived complex-type N-glycans, they are referred to as cross-reactive

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² The abbreviations used are: ER, endoplasmic reticulum; CCD, cross-reactive carbohydrate determinants; Ig, immunoglobulin; MII, α -mannosidase II; PHA-L, phytohemagglutinin-L; GN, N-acetylglucosaminidase; MES, 2-(4-Morpholino)ethanesulfonic acid.



In allergy diagnosis, pineapple stem bromelain with a single complex *N*-glycan M2XF (MUXF) and HRP with at least seven complex *N*-glycan chains are broadly used as marker proteins (for *N*-glycan structures and their nomenclature, see Refs. 15 and 18–21). *N*-Glycan modification can vary, not only for different glycoproteins, but among them also at different positions within the same polypeptide (microheterogeneity) resulting in highly diverse *N*-glycan structures on protein surfaces. Notably, *N*-glycans of HRP are a mixture of M3XF (MMXF), M4XF, M3F (MMF), M3 (MM), M2X (MUX; Fig. 1), and an unusual Fuc(1,3)GlcNAc modification (21). Their recognition has been

well studied immunologically using crude antisera and antibody fractions purified thereof that bind independently to β 1,2-xylose and α 1,3-fucose epitopes (22). Such complex-glycan antisera usually result from immunization of rabbits with glycoproteins of either insect venom (only fucose specificity) (23) or plant origin, e.g. *Wistaria floribunda* mitogenic lectin WFM (24, 25); carrot cell-wall β -fructo(furano)sidase (26, 27); patatin, the major storage protein of potato tubers (28); bean lectin phytohemagglutinin (PHA-L) (29) with one *N*-glycan of M3XF (MMXF) structure; and HRP (22, 30) with the above listed mixture of *N*-glycan structures (21).

Although used synonymously during the past decades, CCD epitopes of commonly used marker glycoproteins seem to be differentially recognized by sera of varying sources. Previously, van Ree *et al.* (20) concluded that xylose-specific IgE antibodies that bound to bromelain M2XF (MUXF) *N*-glycan did not recognize closely related M3XF (MMXF) structures of PHA-L or HRP because of sterical hindrance caused by the presence of α 1,3-mannose in case of M3XF (MMXF), whereas Bencúrová *et al.* (31) concluded that bromelain is not useful for detection of xylose-specific antibodies because of an absence of α 1,3-mannose from M2X (MUX).

Previous studies identified *hgl1* as one of several salt-sensitive *Arabidopsis* mutants that are defective in producing normal complex *N*-glycans in the Golgi apparatus (6–8). Unlike other salt-sensitive *N*-glycosylation mutants lacking xylose and/or core fucose, *hgl1* mutant alleles produce hybrid *N*-glycans that still contain these residues. Because we found that hybrid *N*-glycans of *hgl1* mutants are barely recognized by complex glycan-specific antibodies, this study aimed at elucidating the basis for altered surface properties of cellular glycoproteins in *Arabidopsis hgl1* mutants. We investigated the influence of the presence *versus* absence of individual *N*-glycan residues concerning antibody binding (by rabbit IgG and human IgE) and *in planta* functionality with respect to salt sensitivity, whole glycan profiles, and surface accessibility. Altogether, the obtained data implicate that *hgl1* *N*-glycans are hypoallergenic.

EXPERIMENTAL PROCEDURES

Plant Growth and Root Growth Analyses—For leaf material *Arabidopsis thaliana* var. Columbia plants were grown in soil under short day regime (8 h of light). Root growth responses to NaCl were analyzed as described by Kang *et al.* (6). In general, seedlings were kept 5 days on normal medium and 5 days on salt medium and verified by genomic PRC using gene-specific oligonucleotide primers listed in Table S1.

***N*-Glycan Analysis of *Arabidopsis* Wild type and Mutant Lines**—*Arabidopsis* mutants have been described earlier (1, 6, 8). Preparation of pyridylaminated sugar chains from *Arabidopsis* wild type (Col-0) and T-DNA mutant lines was described previously (32). Molecular masses of pyridylaminated sugar chains as well as number and structure of their sugar moieties were estimated by liquid chromatography–tandem MS analyses using Agilent Technologies 1200 series (Agilent Technologies, Santa Clara, CA) equipped with HCT plus (Bruker Daltonics, Bremen, Germany). The structures of M7A, M7B, and of other *N*-glycan isoforms were deduced from their elution positions compared with glucose-oligomer units in reverse phase HPLC

(33). Because the intensities of product ions derived from the precursor ion detected in liquid chromatography–tandem MS were specific for each structure, we compared intensity patterns of the precursor ion with the in-house tandem MS library of authentic pyridylaminated sugar chains. These two parameters enabled us to distinguish and determine *N*-glycan isoforms, namely structures of molecules with the same *m/z* ratios.

Immunoblot Analyses—*Arabidopsis* leaf extracts were prepared with protein-extraction buffer (50 mM Hepes–NaOH, pH 7.5, 2 mM sodium bisulfite, 1 mM Pefabloc SC). Total protein contents were determined with Bradford reagent (Bio-Rad) and BSA as reference protein. Equal amounts were separated by 10–12% SDS-PAGE (reducing conditions) and blotted to nitrocellulose prior to reversible Ponceau S staining (0.3% (w/v) in 3% TCA). After blocking with 2% (w/v) nonfat dry milk in Tris-buffered saline containing Tween 20 (TBST; 20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween 20), the blots were incubated with crude polyclonal rabbit antisera raised either against PHA-L (α -PHA-L) (26, 34) or against HRP (α -HRP, purchased from Sigma) (30), diluted 1:10,000 in TBST or 1:20,000 in 2× TBST, respectively, including 2% (w/v) nonfat dry milk as described previously (8) or with affinity-selected fractions thereof. For IgE detection, blot membranes were blocked with 2% nonfat dry milk in TBST for 1 h at room temperature and incubated either with diluted patient sera (1:10 in TBST, 2% (w/v) nonfat dry milk) for 2 h or with undiluted patient sera supplemented with 2% (w/v) nonfat dry milk overnight at room temperature. After three wash steps with TBST, blots were incubated with affinity-purified peroxidase-labeled goat anti-human IgE(ϵ) antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:10,000 in TBST for 1 h, followed again by wash steps. Concanavalin A affino blotting was conducted as described by Faye and Chrispeels (35). Chemiluminescent signals were developed with the ECL advance Western blotting detection kit (GE Healthcare) and recorded digitally (GeneGnome; Syngene, Cambridge, UK).

Patient Sera—The study protocol was approved by the local ethics committee, and all of the patients enrolled gave their informed consent. CCD patient sera PT-06, PT-02, and BW-69 were preselected within another study among potato/tomato (PT)-allergic and bee/wasp (BW)-allergic patients (36).

Selection of Fucose- and Xylose-specific Antibodies—Fucose- and xylose-specific antibody fractions were purified from crude complex glycan antisera. Total soluble protein was extracted from leaves of *Arabidopsis cgl1*, *xylT* (β 1,2-xylosyltransferase), and *fucTa fucTb* mutants as described above. About 1 mg of each protein extract was separated by SDS-PAGE, blotted onto nitrocellulose, and used as follows. First, HRP antiserum (Sigma) was diluted 1:2,000 in 2× TBST and incubated overnight with blotted *cgl1* extract at 4 °C (to reduce complex glycan-unrelated binding). In this way depleted antiserum was incubated with blotted *xylT* extract for 3 h at room temperature (for selection of fucose-specific antibodies) and again with blotted *fucTa fucTb* extract (for selection of xylose-specific antibodies). Blot-bound antibodies were washed extensively and eluted by pH shift as described by Frank *et al.* (8). Sodium azide (0.02% w/v) was added as preservative. Prior to use, affinity-selected antibody fractions (α -Fuc_{HRP} or α -Xyl_{HRP}) were

Arabidopsis hgl1 Mutants Are Hypoallergenic

diluted 1:5 in 2× TBST. A xylose-specific antibody fraction of the PHA-L antiserum was obtained basically as described for the HRP antiserum. Prior to incubation with blotted *fucTa fucTb* extract, the crude PHA-L serum was diluted 1:500 in TBST. The affinity-selected α -Xyl_{PHA-L} fraction was diluted 1:2.5 in TBST before use.

Bioinformatics—*N*-Glycan modeling was done as described previously (8) using GlyProt for *in silico* analyses of glycoproteins.

Glycosidase Treatments—Pure PHA-L glycoprotein (8 μ g, obtained from Sigma) was incubated with 6.4 units of recombinant α 1,3-specific mannosidase (exoenzyme, obtained from New England Biolabs) in provided buffer for 2 h at 37 °C. Plant extracts (20 or 40 μ g of protein each) were incubated with purified, recombinant *Drosophila* Golgi α -mannosidase II (Ref. 37 and references cited therein) in 40 mM MES buffer, pH 5.8, overnight at 37 °C, followed by incubation with (or without) 4–8 units recombinant β -*N*-acetylglucosaminidase (specific hexosaminidase; New England Biolabs) in provided buffer for 4 h at 37 °C. Treated glycoprotein samples and plant extracts were subjected to immunoblot analyses.

RESULTS

Missing core Fucose but Not Xylose Enhances Salt Sensitivity of Arabidopsis hgl1 Mutants—*Arabidopsis hgl1* mutants are salt-sensitive (6) despite the fact that their hybrid *N*-glycans are decorated with xylose and *core* fucose residues (13). This implied that during the stress response, xylose and/or fucose are biologically dysfunctional or inaccessible in *hgl1* *N*-glycans. We hypothesized that if both residues are functional in *hgl1*, combination of *xylT* or *fucTa fucTb* (further referred to as *fucTab*) mutations with *hgl1* should show synergistic effects during the salt stress response. Double *hgl1 xylT* and triple *hgl1 fucTa fucTb* (further referred to as *hgl1 fucTab*) mutants were prepared by crossing previously established lines (6) and selected by PCR screening of the F2 populations (supplemental Fig. S1). Root and shoot morphology of *hgl1 xylT* and *hgl1 fucTab* plants appeared unaffected under normal growth conditions (not shown). Immunoblot analyses combined with peptide:*N*-Glycosidase F digests revealed that extracts of the two newly generated *hgl1* mutant combinations are less well recognized by complex glycan-specific antibodies and confirmed that *core* fucosylation is missing in *hgl1 fucTab* (supplemental Fig. S2). Interestingly, whereas salt sensitivity of *hgl1 fucTab* was increased at 90 mM NaCl, root growth of the *hgl1 xylT* line was similar to *hgl1*. At 120 mM NaCl, which was strongly inhibitory to *hgl1* single mutants, differences between *hgl1* and the mutant combinations were less clear (Fig. 2). Together, these observations underscore that in *hgl1* mutants, xylose residues are less functional or inaccessible at the glycoprotein surface, whereas *core* fucose function is not significantly compromised *in planta*.

Mass Spectrometry Reveals Reduced core Fucose Levels in hgl1 xylT—*N*-Glycan analyses verified that *Arabidopsis* wild type (Col-0) contains oligomannosidic and plant-specific complex *N*-glycans (Table 1; for details on *N*-glycan structure and MS profiles, see supplemental Figs. S3 and S4). Oligomannosidic and complex *N*-glycans lacking xylose or *core* fucose were

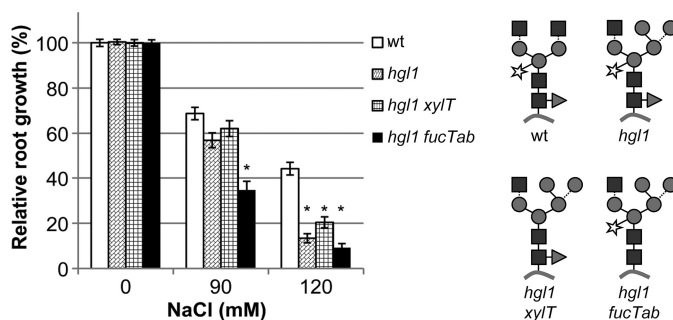


FIGURE 2. Genetic interaction between *hgl1*, *fucTab*, and *xylT* in salt sensitivity of *Arabidopsis* root growth. Five-day-old seedlings were transferred to media containing the indicated concentration of NaCl. Root growth was measured 5 days after transfer. The *hgl1 fucTab* combination shows an enhanced phenotype at 90 mM NaCl, whereas at 120 mM NaCl all of the mutants deviate similarly from wild type (wt), and differences among *hgl1* and its mutant combinations are less clear. The values are the averages of three independent experiments. Each data point corresponds to a set of more than 20 seedlings. The bars indicate the standard errors of the mean. Significant differences ($p < 0.05$; indicated by asterisks) among wild type and the mutants were determined for each NaCl concentration by one-way analysis of variance followed by a Tukey-Kramer test. The glycan symbols are according to ProGlycan.

found in parental *xylT* and *fucTab*, respectively. Although *core* fucose levels in *xylT* were slightly diminished, xylose amounts in *fucTab* were slightly elevated compared with wild type. For *hgl1-1* and *hgl1-2* alleles, similar *N*-glycan profiles with major M5XF and GnM5XF structures were found (40–45% of total *N*-glycans). Concerning *N*-glycans with both xylose and fucose residues, this fraction was similar to M3XF and Gn(2)M3XF structures of wild type (amounting to 40%). Slightly diminished *core* fucose levels in *xylT* (33% compared with 40% in wild type) already hinted at a possible dependence of fucosylation from xylosylation. This was more pronounced in *hgl1 xylT*, revealing strongly reduced fucose levels (to 18%) despite the fact that *hgl1* single mutant alleles showed *core* fucosylated *N*-glycans comparable with wild type or even higher levels (40% versus 43% for *hgl1-2* or 53% for *hgl1-1*).

Arabidopsis hgl1 Mutants Are Defective in Presenting Antigenic Xylose Epitopes—To test for xylose accessibility in *hgl1* *N*-glycans, we studied their reaction by immunoblotting of protein extracts with different complex glycan antisera and other specific mutants as references (Fig. 3A). This revealed that although crude PHA-L and HRP antisera recognized both xylose and *core* fucose residues, they reacted differently with *hgl1* extracts. Evidence for distinct xylose specificities of the two antisera became obvious when *xylT* and *fucTab* as well as their respective *hgl1* mutant combinations are compared. Much lower labeling of *xylT* by α -PHA-L (Fig. 3A, top left) confirmed that xylose-specific antibodies dominate in the PHA-L antiserum (29). Interestingly, the *N*-glycan pattern of *hgl1* resembled *xylT*, suggesting that in *hgl1* α -PHA-L recognizes only fucose epitopes. In support of this hypothesis, merely background labeling (similar to *cgl1*) was observed with α -PHA-L for the *hgl1 fucTab* combination. These results established that antibodies present in the largely xylose-specific PHA-L antiserum (29) bind to fucose but not xylose epitopes of *hgl1* *N*-glycans. Similar analyses performed with α -HRP resulted in comparable labeling of *xylT* and *fucTab* extracts, indicative of equal abundance of xylose- and fucose-specific

TABLE 1

N-Glycan composition in Arabidopsis wild type and selected N-glycosylation mutants

Relative amounts of N-glycan structures detected in *Arabidopsis* Col-0 WT and mutant lines. The ratio (%) of each structure was calculated on the basis of peak area as determined by liquid chromatography-tandem MS analysis (supplemental Fig. S4). The differences in N-glycan profiles of *hgl1-1* (exon line) and *hgl1-2* (intron line) (supplemental Fig. S1) could be the result of a different batch of cultivation (for details of listed N-glycan structures, see Fig. 1 and supplemental Fig. S3). GlcNAc-terminated xylose-linked fractions that may be unmasked by GN alone or by sequential treatment with Golgi α -MII followed by GN (Fig. 5) are in bold type. F, fucose; Gn, N-acetylglucosamine; M, mannose; X, xylose.

N-Glycan structure	Ratio						
	WT (Col-0)	<i>fucTab</i>	<i>xylT</i>	<i>hgl1-1</i> (exon)	<i>hgl1-2</i> (intron)	<i>hgl1 fucTab</i>	<i>hgl1 xylT</i>
%							
Mannose-type							
M3		6.9	7.0				
M4		1.6	2.0			4.4	
M5	22.6	15.5	26.2	13.2	21.0	14.3	37.7
M6	8.9	5.0	8.1	7.1	6.5	1.4	9.9
M7A	10.2	3.7	7.3	4.9	2.0	2.9	2.9
M7B			1.8	1.6		0.8	
M8A	5.6	3.5	6.6	6.5	6.3	1.0	5.7
M8B							3.7
M9	5.0	3.2	5.0	1.5	3.5	1.2	3.3
GlcNAc-terminated							
GnM3		2.5	2.6				
GnM5				2.0	3.2	4.9	19.2
Gn2M3		2.1					
Complex-type (fucose-linked)							
M3F			19.0				
M4F				3.5	1.9		
M5F				1.9			4.5
Complex-type (xylose-linked)							
M3X	5.7	30.5					
M4X		2.6				1.2	
M5X				4.9	7.5	35.4	
Complex-type (fucose- and xylose-linked)							
M3XF	24.9						
M4XF				2.7	1.5		
M5XF				24.0	22.3		
Complex-type (GlcNAc-terminated, fucose and/or xylose-linked)							
GnM3F			7.2				
Gn2M3F			7.2				
GnM5F							13.1 ^a
GnM3X		6.6					
GnM3X	2.1	6.1					
Gn2M3X		7.3					
GnM4X		2.9					
GnM5X				5.1	7.3	32.5	
GnM3XF	7.2						
Gn2M3XF	7.9						
GnM5XF				21.1 ^a	17.1 ^a		
Total xylose-linked	47.8	56.0	0.0	57.8	55.7	69.1	0.0
Total fucose-linked	40.0	0.0	33.4	53.2	42.8	0.0	17.6
Ratio of complex-type with/without terminal GlcNAc	0.6	0.7	0.8	0.7	0.7	0.9	2.9

^a Fucose-linked fractions that may be unmasked by GlcNAc-dependent Golgi α -mannosidase II trimming (Fig. 6B).

antibodies in this anti-complex glycan antiserum (Fig. 3A, *top right*). Recognition of *hgl1* by α -HRP was only slightly diminished (see also supplemental Fig. S2). However, compared with *hgl1* or *fucTab*, binding to *hgl1 fucTab* extracts was significantly reduced, which supports the possibility that xylose epitopes in *hgl1* mutants are not fully recognized by the HRP antiserum.

Further immunoblot analyses with xylose-specific antibodies purified from both crude anti-complex glycan antisera (α -Xyl_{PHA-L} and α -Xyl_{HRP}) substantiated this assumption: α -Xyl_{HRP} gave strong signals with wild type and *fucTab* extracts (positive controls) but only background signals with *cgl1* and *xylT* extracts (negative controls). Intensities of *hgl1* and *hgl1 fucTab* signals were intermediate (*i.e.* lay between those of wild type and *fucTab* compared with *cgl1* and *xylT*), indicating that α -Xyl_{HRP} bound only partly to xylose residues present in *hgl1* and *hgl1 fucTab* extracts. Thus, apparently in the HRP antiserum two populations of xylose-specific antibodies exist: the

majority that does not and a minority that does recognize xylose epitopes in the *hgl1* background (Fig. 3A, marked by asterisks in lower panels). We termed them A and B form antibodies of α -Xyl_{HRP}, respectively. Based on this nomenclature, A form antibodies should have similar epitope specificity as α -Xyl_{PHA-L}.

Three-dimensional Glycan Modeling Predicts Altered Xylose Position in *hgl1* Mutants—To assess the basis for differential xylose recognition in *hgl1*, complex N-glycan structures (fully versus incompletely trimmed wild type) as well as M(5)4XF N-glycans present on glycoproteins of the *hgl1* mutant were analyzed with three-dimensional model algorithms. N-Glycans of vacuolar glycoproteins usually terminate with two mannoses attached to the mannose core as M3XF (MMXF; Fig. 3B), representing a highly abundant complex glycan of *Arabidopsis* wild type plants (Refs. 6 and 13 and Table 1). In this structure, xylose residues protrude from the central mannose core (in

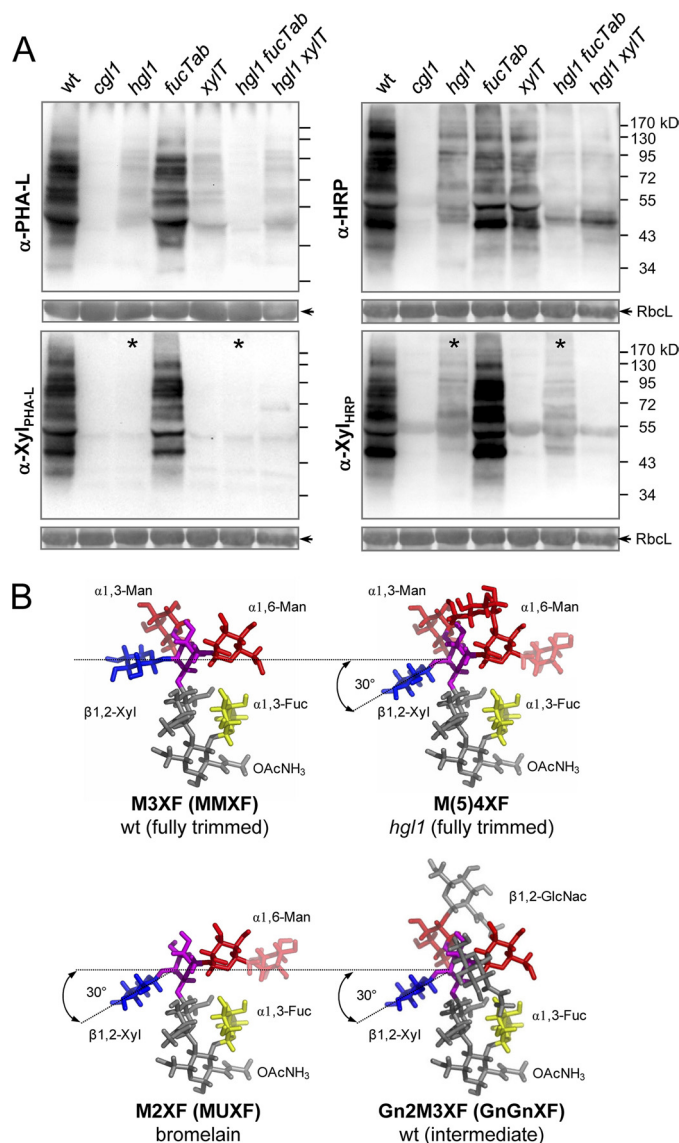


FIGURE 3. Xylose-specific binding of two complex glycan antisera differs as revealed by comparison of *hgl1 xy1T* and *hgl1 fucTab* extracts. A, protein extracts (20 μ g/lane) of *Arabidopsis* wild type and the indicated *N*-glycosylation mutants were separated by 10% SDS-PAGE and blotted onto nitrocellulose. The blots were developed either with crude phytohemagglutinin antiserum (α -PHA-L) (34), horseradish peroxidase antiserum (α -HRP; Sigma), or affinity-selected antibody fractions thereof (α -Xyl_{PHA-L} or α -Xyl_{HRP}). Prominent differences between α -Xyl_{PHA-L} and α -Xyl_{HRP} in binding xylose are highlighted by asterisks. B, three-dimensional models of fully trimmed *N*-glycan structures prevailing in wild type (*wt*) and *hgl1* compared with that of bromelain and a prominent GlcNAc-terminated intermediate structure Gn2M3XF (GnGnXF) of wild type. Note that PHA-L and HRP are both vacuolar glycoproteins that carry M3XF (MMXF; Table 1) structures. Thus, α -PHA-L and α -HRP should preferentially bind to fully trimmed wild type proteins (for microheterogeneity of HRP *N*-glycan structures, see detailed descriptions in the text). *N*-Glycan nomenclature according to ProGlycAn. The central mannose (pink) is often not mentioned (MMXF) unless unsubstituted (U in MUXF).

pink, compare Figs. 3B and 4) at 90° relative to the GlcNAc2 stem region attached to the amino group of asparagine (A form). By contrast, GlcNAc-terminated Gn2M3XF (GnGnXF) glycans (also present in wild type; Table 1), M(5)4XF glycans that dominate in *hgl1* (Table 1) and the unusual M2XF (MUXF) glycan of bromelain (18) are characterized by distinct xylose conformation: xyloses tilt 30° downward (B form) compared with M3XF (MMXF) (A form; Fig. 3B). Similar B form confor-

mation was also predicted for singly GlcNAc-terminated glycans (GnM3XF(F); Table 1). These three-dimensional models suggested that β 1,2-xylose residues can exist in at least two different conformations, which is consistent with the reported structures: HRP carries minimal complex *N*-glycans of both A (M3XF) and B form (M4XF, M2X; 21), whereas PHA-L carries only a single M3XF *N*-glycan in addition to M8, a high mannose ER-type *N*-glycan lacking fucose and xylose residues (Refs. 29 and 38 and Fig. 1).

Mannosidase Treatment of PHA-L Alters Xylose-Specific Recognition—According to the three-dimensional modeling of plant *N*-glycans, presentation of β 1,2-xylose epitopes can vary, depending on the presence or absence of terminal GlcNAc and/or mannose residues on α 1,3- and α 1,6-antennae, respectively. To check the validity of these predictions, we investigated the immunoreactivity of enzymatically modified *N*-glycans. At first, pure glycoprotein PHA-L was subjected to α 1,3-mannosidase treatment. To favor glycan-specific (and to avoid peptide-based reactions), affinity-selected antibodies of PHA-L and HRP antisera (α -Xyl_{PHA-L} and α -Xyl_{HRP}, respectively) served as probes. A fucose-specific HRP fraction (α -Fuc_{HRP}) and concanavalin A, a lectin preferentially binding to terminal mannoses of high mannose-type and bisected hybrid-type *N*-glycans (compared with the central tri-mannosyl region of complex-type *N*-glycans) (39), served as controls for *N*-glycan integrity and protein contents. Basically, the single complex glycan of PHA-L differs from that of bromelain only by the presence of α 1,3-mannose (Fig. 3B). Thus, α 1,3-mannosidase treatment of PHA-L should convert M3XF (MMXF, with A form xylose) to bromelain-like M2XF structure (MUXF, with B form xylose; Fig. 4, top panel). As predicted, α 1,3-mannosidase treatment of PHA-L decreased xylose-specific binding (Fig. 4, bottom panel). Noteworthy, fucose-specific antibody binding (α -Fuc_{HRP}) was not affected by the treatment, and concanavalin A affinity blotting verified intactness of the high mannose-type *N*-glycan of PHA-L. Importantly, these results showed that recognition of xylose residues depends on the structural environment within complex-type *N*-glycans.

Untrimmed Mannoses Interfere with Xylose and Fucose Detection—Next we tested whether xylose detection of *hgl1* *N*-glycans can be restored *in vitro*. According to the three-dimensional models (Fig. 3B), not only untrimmed mannoses on the α 1,6-arm but also terminal GlcNAc residues on either antenna should keep xylose residues in tilted positions. We used recombinant *Drosophila* Golgi α -mannosidase II (37) and commercial *N*-acetylglucosaminidase to modify glycoproteins in leaf extracts of *Arabidopsis* wild type and mutant plants prior to immunoblot analyses. Compared with buffer-incubated controls, already *N*-acetylglucosaminidase (GN) treatment of wild type extracts increased xylose recognition by both α -Xyl_{PHA-L} and α -Xyl_{HRP} antibodies (Fig. 5), indicative of converting Gn(2)M3XF to fully processed M3XF structures that amount to 36% (17.2% of 47.8%; Table 1) of all xylose-containing *N*-glycans in wild type (for conformational switch of B to A form xylose; see Fig. 3B). GN treatment of *hgl1* extracts, however, did not improve recognition by xylose-specific antibodies, presumably because untrimmed mannoses are still present on the α 1,6-arm. Importantly, Golgi α -mannosidase II (MII) can

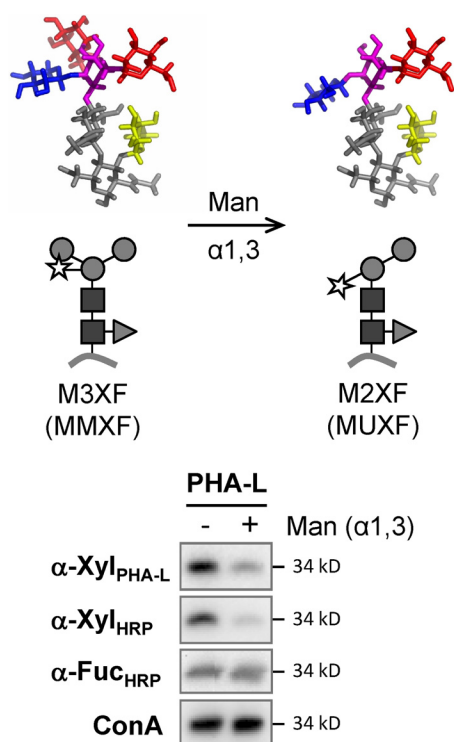


FIGURE 4. Modulation of xylose conformation by α 1,3-mannosidase treatment of PHA-L. Top panel, schematic representation of altered xylose position expected to result from the treatment. Bottom panel, PHA-L was incubated without (–) or with (+) a commercial α 1,3-mannosidase (exoenzyme; New England Biolabs) prior to 12% SDS-PAGE (1 μ g of PHA-L loaded per lane) and immunoblot development of separate blot strips. Xylose-specific and fucose-specific antibody fractions (α -Xyl_{PHA-L}, α -Xyl_{HRP}, and α -Fuc_{HRP}) were selected from the crude antisera using blotted extracts of *Arabidopsis* mutants (see “Experimental Procedures”). Binding of Concanavalin A (ConA) verified the presence of M8, a high mannose ER-type N-glycan (Fig. 1) on PHA-L. The glycan symbols below the three-dimensional models are according to ProGlycan.

only release those mannoses when the α 1,3-antenna is decorated with β 1,2-GlcNAc (37, 40). Thus, 45% (26.2% of 57.8%) of all xylose-containing N-glycans in *hgl1* may switch from the B form to the A form after sequential digestion with MII and GN, mirroring the amount of A form N-glycans present in wild type before treatment (30.6% of 47.8%). This was in good agreement with our observations: *hgl1* and *hgl1 fucTab* extracts treated with MII alone did not result in appreciable unmasking of xylose residues compared with buffer-incubated controls, but when MII was followed by GN treatment, xylose detection increased for *hgl1* (and similarly for the *hgl1 fucTab* combination) reaching almost the levels of buffer-incubated wild type controls (Fig. 5, asterisks). Remarkably, independent of GN treatment, fucose recognition increased upon MII treatment (Fig. 5, lanes labeled with bars). This revealed that core fucose residues, too, are influenced by persistence of untrimmed mannoses on the α 1,6-arm but not by that of terminal GlcNAc residues.

Glycoproteins of *Arabidopsis hgl1* Mutants Are Hypoallergenic—The potential clinical impact of altered N-glycan conformation in *hgl1* mutants on the presentation of CCD epitopes was assessed by immunoblot analyses. Blotted extracts of *Arabidopsis* wild type and investigated N-glycosylation mutants were challenged with CCD-reactive sera (obtained

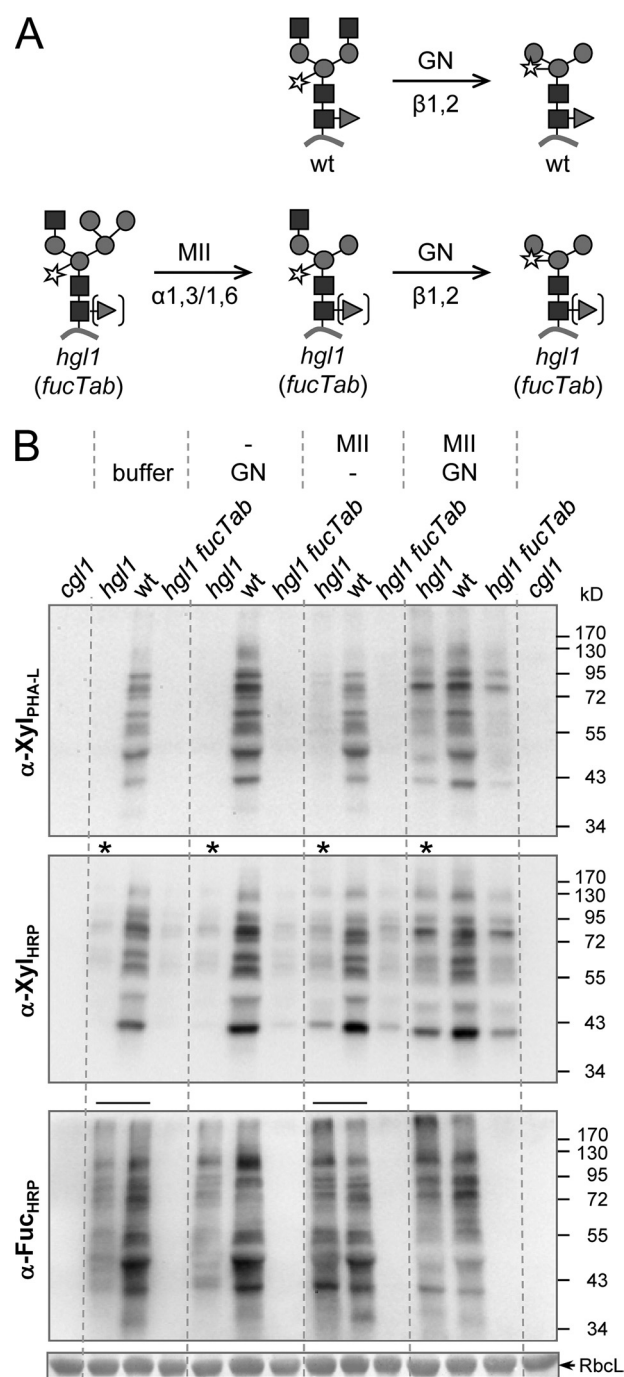


FIGURE 5. Sequential treatment of wild type and mutant extracts with Golgi α -MII and GN enhances xylose and core fucose detection. A, MII can only act on precursors with GlcNAc-decorated α 1,3-antennae (37, 40). B, extracts of buffer-incubated controls, MII-treated and/or GN-treated *Arabidopsis* wild type (wt), and the indicated mutant extracts (20 μ g of protein each) were separated by 10% SDS-PAGE, blotted to nitrocellulose, and developed with the indicated antibody fractions (after sequential stripping of the blot). Note that unmasking of xylose residues in *hgl1* extracts depends on treatment with MII followed by GN (indicated by asterisks), whereas unmasking of fucose residues in *hgl1* extracts occurs by MII treatment alone (indicated by bars). RbcL, ribulose-1,5-bisphosphate carboxylase/oxygenase, large subunit (loading reference). The glycan symbols are according to ProGlycan.

from preselected allergic patients) (36) as the first antibody and anti-human IgE-HRP conjugate as the second antibody. Compared with wild type, *hgl1* mutants displayed clearly reduced reactivity with patient IgE, revealing a hypoallergenic nature of

Arabidopsis hgl1 Mutants Are Hypoallergenic

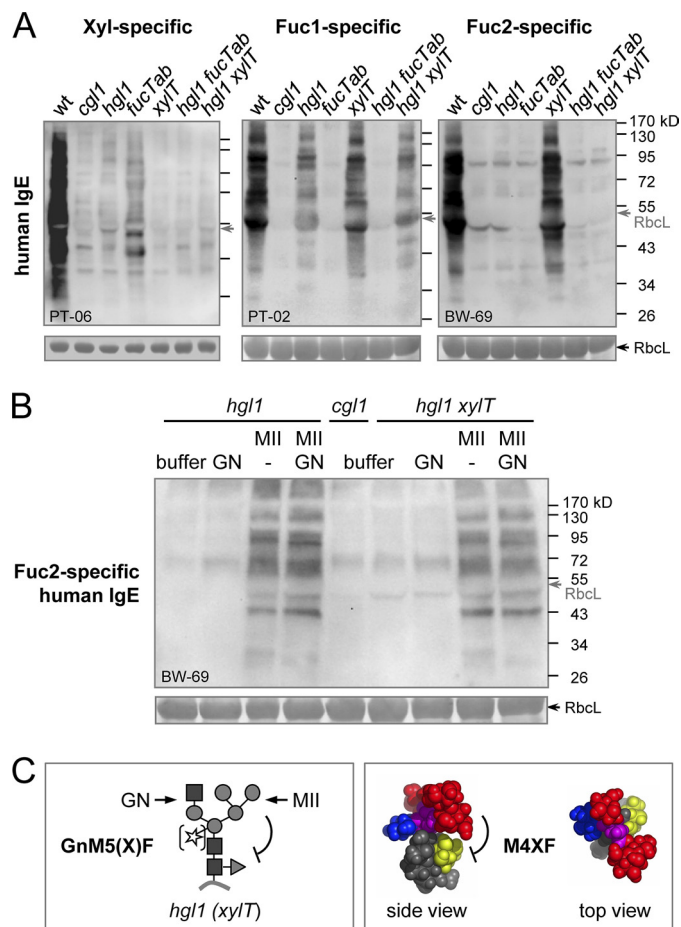


FIGURE 6. Immunodetection of human CCD-specific IgE bound to blotted Arabidopsis extracts. *A*, untreated extracts of Arabidopsis wild type (wt) and selected *N*-glycosylation mutants (20 μ g each, compare Fig. 3*A*) were developed for IgE detection with one xylose-recognizing serum (Xyl-specific) and two representative fucose-recognizing sera (Fuc1-specific and Fuc2-specific) that were preselected in another study among potato/tomato (PT)- and bee/wasp (BW)-allergic patients (36). Note that the Xyl-specific and Fuc2-specific IgE antibodies barely recognize *hgl1* and that compared with wild type, Fuc1-specific binding is also decreased. *B*, pretreatment of *hgl1* and *hgl1 xylT* extracts (40 μ g of protein each) with Golgi MII enables binding of Fuc2-specific antibodies present in this patient serum. Note that incubation with buffer or GN alone has no effect on fucose-specific recognition (compare Fig. 5). *C*, schematic depiction of the glycosidase treatments (*left panel*, arrows) using GN and Golgi MII (glycan symbols are according to ProGlycAn) and a space-filled three-dimensional model of minimal *hgl1* *N*-glycan M4XF (*right panel*, compare Table 1 and Fig. 3*B*) with proposed shielding of core fucose by untrimmed mannoses persisting on the α 1,6-arm (*blocking symbol*).

CCD epitopes in *hgl1* (Fig. 6). Complex-glycan patterns obtained with one xylose-specific patient serum closely resembled those obtained with the α -PHA-L rabbit antiserum (compare Figs. 3*A* and 6*A*), whereas most fucose-specific patient sera showed reduced *hgl1* recognition similar to α -Fuc_{HRP} antibodies (compare Figs. 5 and 6*A*). Surprisingly, for two fucose-specific patient sera, CCD recognition of *hgl1* was completely abolished and resembled that of *fucTab* (Fig. 6*A*; compare Fuc2-specific to Fuc1-specific). Similar results were obtained with the *hgl1 xylT* double mutant, indicating that core fucose epitopes in *hgl1* and *hgl1 xylT* may be shielded by untrimmed mannoses on the α 1,6-arm. Glycosidase treatments of *hgl1* and *hgl1 xylT* extracts confirmed this hypothesis after probing immunoblots with the Fuc2-specific patient serum (revealing impaired binding caused by mannoses persisting on the α 1,6-

arm in Fig. 6*A*). MII-specific mannose trimming was able to unmask fucose recognition, independent of whether GlcNac residues on the α 1,3-arm remained or not (Fig. 6*B*). Our interpretation of the results is depicted in Fig. 6*C* (*left panel*) and also reflected by space-filled three-dimensional models of *hgl1* *N*-glycan M4XF (right, side, and top views).

DISCUSSION

In planta analyses of newly established Arabidopsis *hgl1 xylT* double and *hgl1 fucTab* triple mutants revealed increased salt sensitivity when core fucose, but not xylose, is missing in the *hgl1* background. In which way glycoproteins of the plant secretory system are compromised by altered surface properties remains to be shown. In analogy to animal cells (41), tethering into larger complexes (lipid rafts) through interaction with endogenous lectins seems to occur in plant cells, e.g. at the root surface, probably mediating symbiosis with mutualistic bacteria and/or defense of pathogenic bacteria (42). We recently published for Arabidopsis receptor-like kinase EFR that mutation of a single conserved *N*-glycosylation site, in addition to enhancing accumulation in the ER, abolished elf-ligand binding at the plasma membrane (2). However, receptor functionality was not influenced by altered *N*-glycan structure as inferred from testing a large set of Arabidopsis Golgi *N*-glycosylation mutants. Hence, further detailed studies on the occupancy of single *N*-glycan positions, the role of different *N*-glycan structures, and their accessibility in putative target proteins are needed to provide conclusive information about the potential mechanism(s) underlying enhanced salt sensitivity described previously (6–8) and in this study.

As shown earlier for *hgl1-1* (13), mass spectrometry confirmed the presence of xylose and core fucose residues in both *hgl1-1* and *hgl1-2* alleles and verified the lack of specific residues in the newly prepared double and triple mutants. In addition, we obtained evidence that prexylosylation is important for efficient fucosylation, because mutants lacking xylose residues reveal decreased amounts of core fucose residues in their *N*-glycans. This contrasts with the report of Bencúr *et al.* (43), which showed that fucosyltransferase activity is inhibited by presence of a xylose residue in the acceptor *N*-glycans. This apparent inconsistency may have been caused by two GlcNac-terminated antennae used as substrate for their *in vitro* study. On the other hand, a broad survey of *N*-glycans in various edible crop parts revealed that mostly complex *N*-glycans bearing only xylose occur, but those bearing only fucose are rarely found in native plants (9). Hence, prexylosylation seems to be crucial for core fucose addition in various plant species including Arabidopsis. In addition, fucosylation appears to be much more affected in the *hgl1 xylT* mutant compared with *xylT* alone. One possible explanation is that lack of xylose in combination with untrimmed mannoses causes glycoproteins to accumulate in medial Golgi cisternae, so that fucosyltransferase, residing more *trans* (44), encounters less substrate. Such a back-up in the plant Golgi apparatus might also be the reason for elevated complex *N*-glycan levels with terminal GlcNac residues in *hgl1 xylT* (ratio of complex-type with and without terminal GlcNac being 2.9 instead of 0.6–0.9 as compared with wild type or the other mutants; Table 1) that are only released by hexosami-

dases residing in vacuoles or in the cell wall (45, 46). Alternatively, *N*-glycans with untrimmed α 1,6-mannoses might be poor substrates for the hexosaminidases or selectively affected by protein degradation.

Generally, glycoproteins prepared from biological sources are characterized by a heterogeneous mixture of trimmed and untrimmed *N*-glycans. Earlier reports showed that xylose is not always bound equally well by specific antibodies (31), and one study suggested that presence of α 1,3-mannose on M3X(F) structures might interfere sterically with binding (20). In our study, three-dimensional-glycan modeling provided a first correlative and plausible explanation for altered xylose recognition. Incompletely trimmed M(5)4XF structures persisting in *hgl1* mutants and also in *N*-glycans with extended α 1,3- and/or α 1,6-antenna(e), are predicted to similarly affect xylose position and to consistently affect antibody binding. Apparently, in fully trimmed M3XF (MMXF) glycans, xylose can form a stable tetrahedron-like structure with the α 1,3- and α 1,6-linked mannoses (A form). By contrast, in bromelain (M2XF or MUXF) and *hgl1* *N*-glycans (B form) xylose seems to adopt a conformation tilted 30°, hence positioning itself on an extended axis with α 1,6-linked mannose(s). The rigidity of xylose linkages in both M2XF (MUXF) (47) and M3XF (MMXF) (48) structures likely allows discrimination by A form- and B form-specific anti- β 1,2-xylose antibodies. Apart from the sterical specificity of the antibodies proposed here, binding of α 1,3-mannose linked to the mannose core could also be involved in recognition of A form xylose, which is not accessible when a terminal GlcNac residue is attached (49). This would be in agreement with Bencúrová *et al.* (31), who found that dually GlcNac-terminated *N*-glycans prevent or at least impair (50) antibody binding. Although we cannot exclude this involvement, our results demonstrate that additional binding to mannose is not sufficient to explain why α -Xyl_{PHA-L} antibodies do not recognize *hgl1*. Because, according to our and earlier MS data (9, and references cited therein), GlcNac-terminated complex glycans are amply present in most plant sources, our results obtained with the rabbit and a limited number of patient sera (preselected for CCD-specific IgE) suggest that A form xyloses are more immunogenic than B form xyloses.

Similar to the case of xylose recognition, CCD patterns of investigated *Arabidopsis* mutants indicated the presence of at least two types of fucose-recognizing antibodies with different specificities. Although both types are present in Fuc1-specific patient sera (and also in the α -HRP rabbit antiserum), only those obstructed by untrimmed mannoses on the α 1,6-arm are present in Fuc2-specific patient sera. Interestingly, the three-dimensional-glycan models predicted no conformational change for core fucoses in *hgl1* *N*-glycans. In some respect, earlier studies also denoted that terminal mannoses can affect recognition of core fucose residues by anti-complex glycan antibodies of different origins. Ramirez-Soto and Poretz (25) reported lower affinity for a fucose-specific rabbit antiserum when terminal mannoses are lost. Similarly, Kurosaka *et al.* (30) proposed that binding of fucose-specific HRP antibodies involves α 1,6-linked mannose. Consequently, additional sugar linkages on the α 1,6-arm, including terminal GlcNac residues, could interfere with binding to core fucose epitopes. This was

also found by Jin *et al.* (50) with a rabbit antiserum raised against a neoglycoprotein with M3F (MMF), and to lower extent with α -HRP. However, the impact of terminal GlcNac residues could not be conclusively assessed in our experiments, because complex glycans decorated with terminal GlcNac on the α 1,6-arm are not highly abundant in wild type (compare Table 1), and treatment of wild type extracts with *N*-acetylglucosaminidase did not affect fucose recognition. On the other hand, treatment of *hgl1* *N*-glycans with recombinant Golgi α -mannosidase II increased immunoreactivity of core fucoses. Based on the bulkiness of α 1,6-linked mannoses persisting in *hgl1*, sterical hindrance would be a plausible explanation for the reduced antibody binding to core fucoses observed in our experiments.

In summary, *N*-glycans of *Arabidopsis hgl1* mutants differ from those of wild type mainly by untrimmed mannoses on the α 1,6-arm. In-depth analyses of newly prepared *Arabidopsis hgl1 xylT* and *hgl1 fucTab* mutants demonstrated that xyloses do not actively contribute to salt tolerance of the *hgl1* host plants. Altered *in planta* accessibility of β 1,2-xylose residues at the glycoprotein surface is supported by impaired recognition of xylose-specific antibodies. Based on the obtained findings, we propose that *hgl1* mutants are hypoallergenic. Thus, considering that the loss of *N*-acetylglucosaminyltransferase I (GnTI) results in elevated pathogen susceptibility of both *Arabidopsis cgl1* mutants (8, 12) and gene-silenced tomato plants (36), it should be interesting to silence Golgi α -mannosidase II in important crop species intended for the production of heterologous glycoproteins with low immunogenic potential. Reduced reactivity of *Arabidopsis hgl1* *N*-glycans probably applies to most kinds of CCD-specific human IgE antibodies but seems to be best in the *hgl1 fucTab* combination. Another approach, likely based on conformational principles reported in this study, would be the down-regulation of β -*N*-acetylhexosaminidase activities recently suggested by Liebminger *et al.* (46). How in these respects modified crop plants perform in the field remains to be investigated, especially concerning the enhanced salt sensitivity observed for *Arabidopsis hgl1 fucTab* null mutants described in this study. Preliminary results obtained with tobacco and tomato lines grown under standard conditions in the greenhouse indicate that stable reduction of Golgi α -mannosidase II alone is compatible with normal growth and development.

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SUPPLEMENTAL DATA

Supplemental Experimental Procedures

Arabidopsis N-glycosylation mutants

Arabidopsis T-DNA insertion lines *fucTa* (SALK_087481), *fucTb* (SALK_063355), *hgl1-1* (SALK_052443), *hgl1-2* (SALK_141821), and *xylT* (SALK_042226) were obtained from the *Arabidopsis* Biological Resource Center (ABRC). Mutant combinations were prepared by genetic crosses of previously characterized lines (Kang et al., 2008). For verification of T-DNA insertions see supplemental Fig. S1. PCR fragments were amplified from genomic *Arabidopsis* DNA using Taq-DNA Polymerase (Segetec, Borken, Germany) and gene-specific primer combinations (10 μ M each, listed in supplemental Table S1).

Isolation of genomic DNA

Leaf material (60 mg) was crushed in liquid nitrogen, extracted with pre-warmed (65°C) TES buffer (100 mM Tris-HCl pH 8, 50 mM EDTA, 700 mM NaCl), and incubated for 15 min at 65°C. Upon chloroform/isoamylalcohol purification, genomic DNA was precipitated from the aqueous phase with an equal volume of isopropanol, pelleted by centrifugation, and washed with ethanol (70%). The air-dried pellet was dissolved in 100 μ l distilled water and 2- μ l aliquots were used for PCR (adopted from Souza-Canada, Lab Times 3, 2007).

Supplemental References

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- Strasser, R., Altmann, F., Mach, L., Glössl, J., and Steinkellner, H. (2004). Generation of *Arabidopsis thaliana* plants with complex N-glycans lacking beta1,2-linked xylose and core alpha1,3-linked fucose. *FEBS Lett.* **561**, 132-136
- Strasser R, Schoberer J, Jin C, Glössl J, Mach L, and Steinkellner H. (2006) Molecular cloning and characterization of *Arabidopsis thaliana* Golgi alpha-mannosidase II, a key enzyme in the formation of complex N-glycans in plants. *Plant J.* **45**, 789-803
- Tomiya, N., Lee, Y.C., Yoshida, T., Wada, Y., Awaya, J., Kurono, M., and Takahashi, N. (1991) Calculated two-dimensional sugar map of pyridylaminated oligosaccharides: elucidation of the jack bean alpha-mannosidase digestion pathway of Man₉GlcNAc₂. *Anal. Biochem.* **193**, 90-100

Supplemental Figure Legends

Supplemental Figure S1. **Test of *hgl1* mutant combinations obtained by crossing of previously established lines.** *A*, Schematic defining primer binding sites on Arabidopsis genomic DNA. Black boxes, exons; open boxes, 5' or 3' untranslated regions (UTR); lines, introns or intergenic sequences. T-DNA insertions are depicted as triangles and primers as arrows (Lba1 in blue, LP primers in green and RP primers in red). Distances between LP and RP primers in wild-type are indicated in bp (double headed arrows between dotted lines) for the relevant loci. Note that the T-DNA-specific Lba1 primer binds ~200 bp inside of the left border (T-DNA insert 4.5 kbp total). *B*, Agarose-gel electrophoreses of PCR primed with genomic DNA of *hgl1 fucTa fucTb* triple mutant plants H12, E3, G6, G4, B1, and H4 verifies homozygous T-DNA insertions in *FUCTa*, *FUCTb*, and *HGL1* loci, respectively. *C*, Verification of *hgl1 xylT* double mutant candidate #5 carrying single homozygous T-DNA insertions in *HGL1* and *XYLT* loci, both located on chromosome 5 (At5g.....). Water (H₂O) and wild-type (wt) DNA-primed samples served as controls. PCR products were obtained only with T-DNA-specific Lba1 + LP combination of the corresponding gene-specific primers (see supplemental Table S1), indicative of homozygous insertions. PstI-digested Lambda DNA (Fermentas, St. Leon-Roth, Germany) was used as size marker (M).

Supplemental Figure S2. **Peptide:N-Glycosidase F digests indicate that xylose residues in *hgl1* mutants are hardly accessible.** *Top*, Schematic of immuno-reactive MMXF *N*-glycan structures depicting that Peptide:N-Glycosidase F (PNGase F, scissors symbol) is sensitive to *core* fucosylation of wild-type (wt) *N*-glycans. Glycan symbols according to (www.proglycan.com). *Bottom*, Arabidopsis leaf extracts were incubated without (-) or with (+) PNGase F as described in Frank et al. (2008) and analyzed by immunoblotting with the antibodies indicated or ConA affino blotting (Faye and Chrispeels, 1985). The same blot was used for sequential development (ECL Advance Western Blot Detection Kit Manual, Amersham/GE Healthcare) after stripping. Abbreviations: α -Fuc_{HRP}, fucose-specific fraction of the HRP antiserum (α -HRP); *fucTab*, *fucTa fucTb* mutant; wt, Arabidopsis wild-type (Col); RbcL, Ribulose-bisphosphate carboxylase/ oxygenase, large subunit (loading reference).

Supplemental Figure S3. ***N*-glycan structures detected by Mass Spectrometry.**

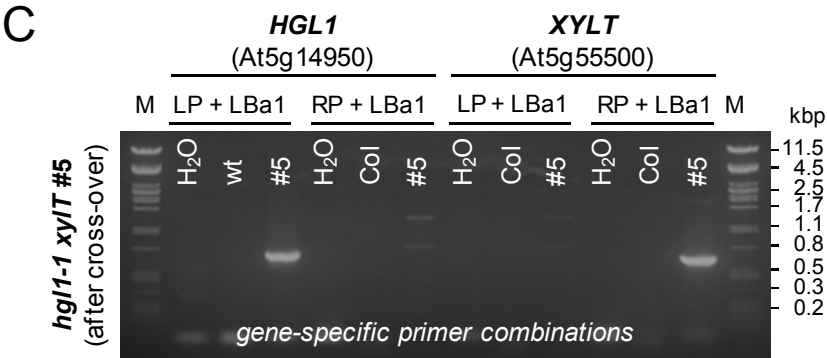
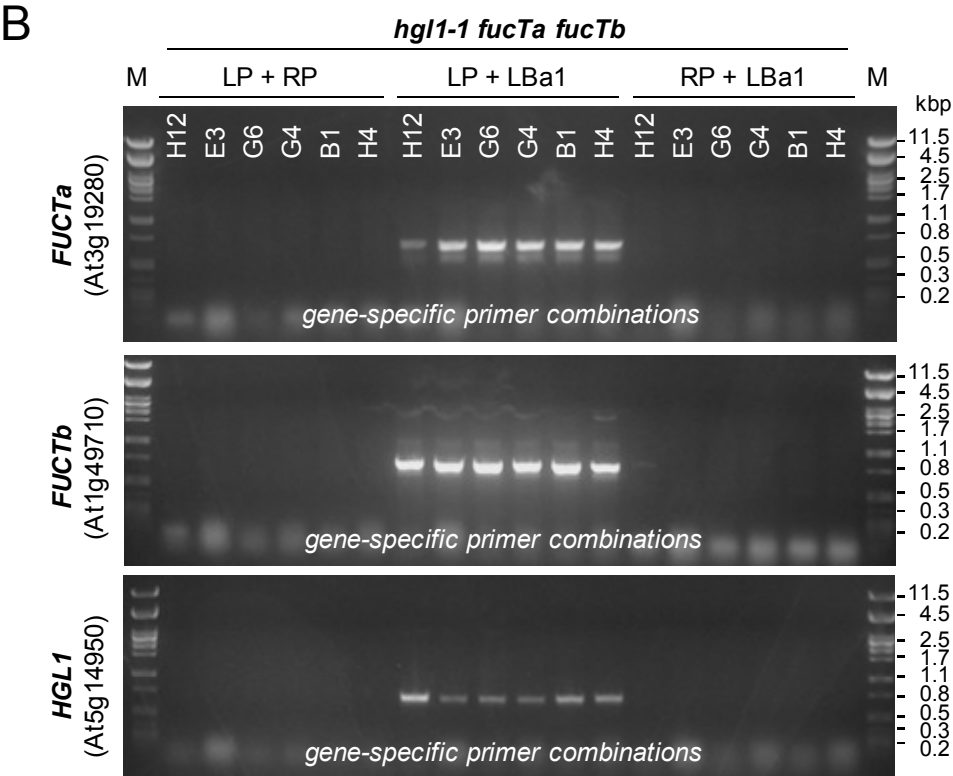
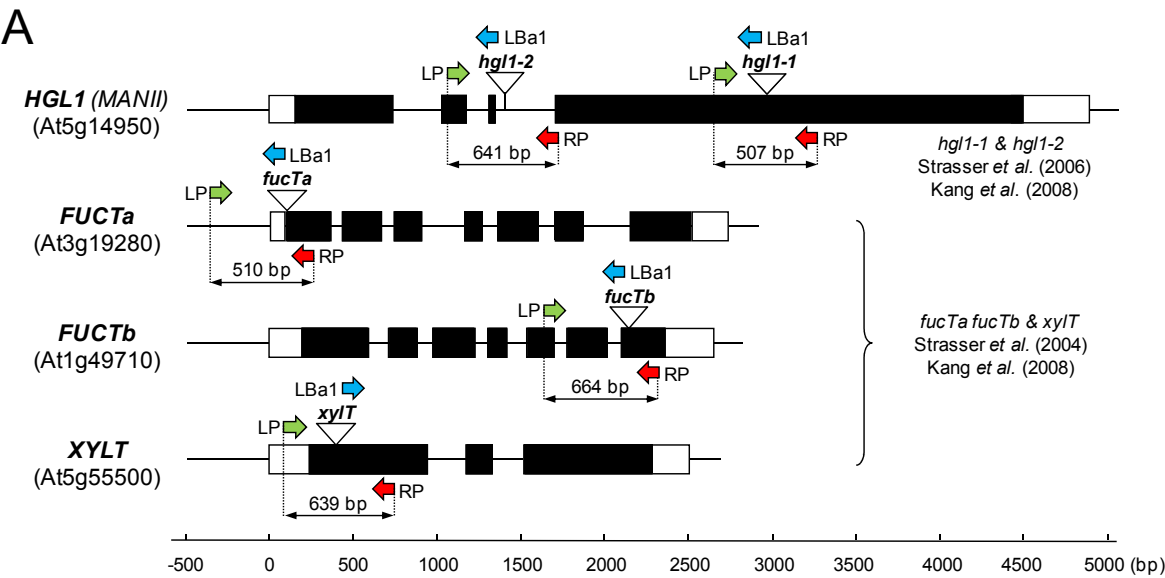
Supplemental Figure S4. ***N*-glycan analysis of PA-sugar chain fractions of Arabidopsis wild-type and indicated mutant lines.** Total *N*-glycans were released from the indicated plant glycoproteins and labeled with pyridylamine (PA, Tomiya et al., 1991) prior to analysis by reverse phase (RP)-HPLC with a C₁₈ column. Glycan symbols according to www.proglycan.com.

Supplemental Tables

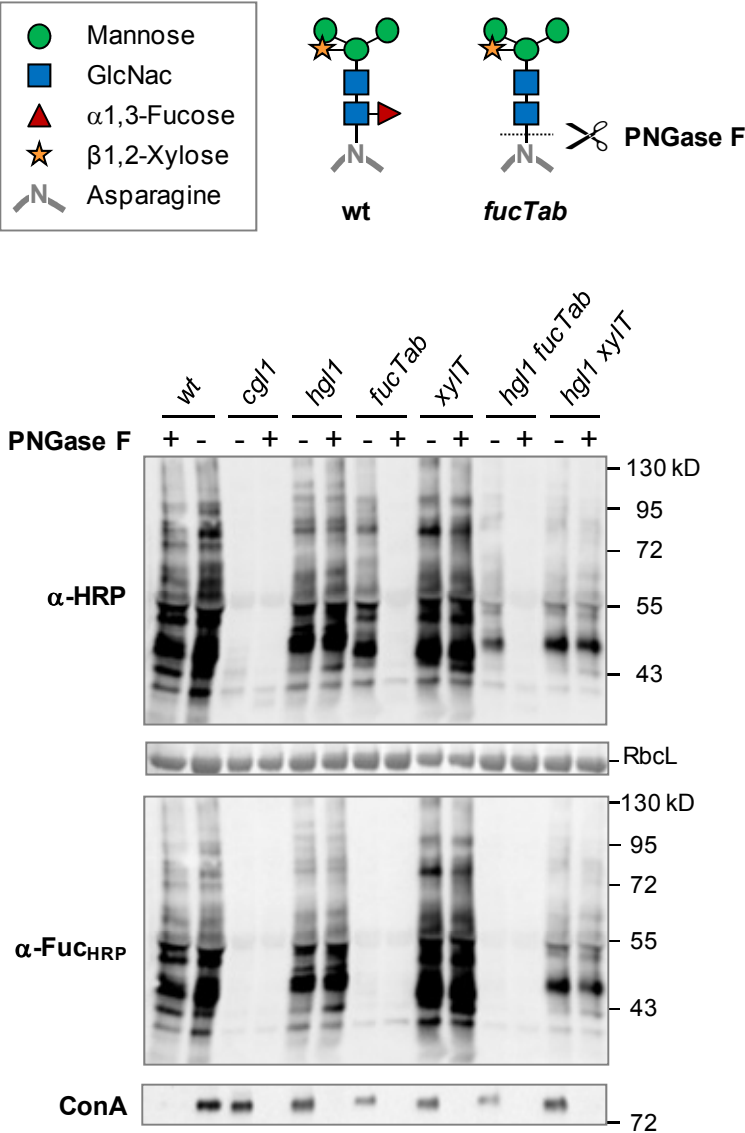
TABLE S1. Genomic PCR primers used in this study.

Name	Sequence
FUCTa LP	TGCCACAACCTTAGCATCTCCT
FUCTa RP	TAGGACCTCGAAGATTGGAGA
FUCTb LP	ATGAAGTATCTCGCAGCTAAC
FUCTb RP	AATGTGACTACTTAGACTCGA
HGL1-1 LP	CGTGGAGCTGAGATCATGATGT
HGL1-1 RP	GTCATGCTGCACTTCAGGAGAA
HGL1-2 LP	AGAGACGCTTCACCTAATAAACAA (Strasser et al. 2006)
HGL1-2 RP	GAAGCTTTAATGCCCTCTCC (Strasser et al. 2006)
XYLT LP	GATGAAGTCTCGCCTGAATCT
XYLT RP	TTGTCGAAACGCAGGAAGCT
Lba1 (left border of T-DNA)	TGTTTACGCTAGTGGGCCATCG

Supplemental Figure S1



Supplemental Figure S2



Supplemental Figure S3

N-glycan structure		Abbreviation	N-glycan structure		Abbreviation
Mannose-type		M3		M3F	
		M4		M4F	
		M5		M5F	
		M6		M3X	
		M7A	Complex-type (fucose- and/or xylose-linked)		M4X
		M7B			M5X
		M8A			M3XF
		M8B		M4XF	
		M9		M5XF	
	GlcNAc-terminated		GnM3		GnM3F
		GnM5		Gn2M3F	
		Gn2M3		GnM5F	
				GnM3X	
Complex-type (GlcNAc-terminated, fucose- and/or xylose-linked)				GnM3X	
				Gn2M3X	
				GnM4X	
				GnM5X	
				GnM3XF	
				Gn2M3XF	
				GnM5XF	

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